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# Assessment of progranulin in egyptian type 2 diabetic patients as a novel biomarker for diabetic nephropathy

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## Abstract

Progranulin (PGRN) is a cysteine rich secreted protein, expressed in epithelial cells, immune cells, neurons, and adipocytes. The present study aimed to determine serum progranulin level in Egyptian type 2 diabetic patients and the association between its level with diabetic and renal biomarkers to evaluate it as a predictor marker of diabetic nephropathy. This study included 60 subjects classified as: 20 normal control, 20 diabetic patients with normal kidney function (DM) and 20 diabetic patients with nephropathy (DN). Concentrations of serum PGRN, Cystatin-C and IL-6 were analyzed by enzyme linked immune sorbent assay (ELISA). Diabetic and renal biomarkers were measured in all subjects. Serum levels of PGRN were markedly higher in type 2 diabetic patients with significantly higher values detectable in clinical diabetic nephropathy. The elevations ins-PGRN parallel the degree of hyperglycemia, hyperinsulinemia, and insulin resistance in type 2 diabetic patients. Renal biomarkers a slight significant change in DM group, this change was augmented in DN group. Cystatin-C and IL-6 were slightly increased in DM group while, e-GFR was non-significantly changed. In DN group, the increase in serum Cystatin-C and IL-6 were highly significant, while e-GFR was dramatically decreased. Our results suggest that PGRN may be considered as a highly sensitive and specific biomarker for diabetic nephropathy.

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## Introduction

Diabetes mellitus (DM) is currently one of the most rapidly growing "epidemics" around the world. According to the International Diabetes Federation, 415 million people are currently affected by this disease worldwide. By the year 2040, the patient number is expected to rise up to 642 million, reaching a global prevalence of 10% (Bi *et al.,* 2012; International Diabetes Federation, 2015).Diabetic nephropathy is one of the most important complications of diabetes mellitus. Currently, around 30% of diabetic patients develop nephropathy, but the incidence is rapidly rising, as a result of the growing number of patients suffering from type 2 diabetes, in combination with the earlier onset of the disease (Zimmet *et al.*,2001).

To prevent the dreadful consequence, development of new assays for diagnostic of DKD has always been the priority in the research field of diabetic complications (Chih-Hung *et al.,* 2016). At present, urinary albumin-to-creatinine ratio and estimated glomerular filtration rate (e-GFR) are the standard methods for assessing glomerular damage and renal function changes in clinical practice. However, clinical trials have demonstrated that this dogma may be incorrect (Mauer *et al.,* 2009; Jerums *et al.,* 2008; Mann *et al.,* 2008). First, renal function as measured by glomerular filtration rate (GFR) declines before the development of proteinuria, demonstrating that there is an earlier phase of kidney damage that could be detected and targeted with interventions. Second, kidney damage can progress even when microalbuminuria has regressed (Steinke and Mauer, 2008). On the other hand, the precision of creatinine-based GFR estimates is limited in hyper-filtration status. These facts make albuminuria and e-GFR less reliable indicators for early-stage DKD (Chih-Hung *et al.,* 2016). Due to the limitations of e-GFR and albuminuria in the early diagnosis of DKD, enormous efforts have been made to investigate and validate alternative biomarkers in recent decades.

Progranulin (PGRN) is a cysteine rich secreted protein, known as granulin/epithelin precursor (GEP), acrogranin, and PC cell-derived growth factor (PCDGF), is a 593amino acid growth factor (He and Bateman, 2003).

The widespread expression of PGRN can be found in adipose tissue, epithelial tissue, gastrointestinal tract, reproductive organs, and so forth, which is involved in cell growth and survival and inflammatory response (Lu *et al.,* 2014). It has been suggested that the full length form of the protein (PGRN) has anti-inflammatory action, while released granulins have the opposite effect, increasing the production of pro-inflammatory cytokines, (He and Bateman, 2003). Its Proteolyticaly cleavage by elastase and generates granulin peptides (GRNs) in tissue, some of which enhance inflammation process (Youn *et al.,* 2009).

Several clinical investigations also demonstrated that serum PGRN was associated with the parameters of adiposity, glucose tolerance, insulin resistance, and inflammatory factors (Richter *et al.,* 2013). Furthermore, progranulin serum concentrations significantly increase with deteriorating renal function assessed as CKD stage independent of age, sex, and BMI (Judit *et al.*, 2013). The aim of the present study was to determine serum progranulin in Egyptian type 2 diabetic patients and the association between progranulin level with diabetic and renal biomarkers to evaluate it as an early predictor of diabetic nephropathy.

## Materials and methods

*Subjects*

A total of 80subject, 20 normal healthy person and 60 patients with type 2 diabetes mellitus from Ain Shams Specialized Hospital were enrolled in the study. The subjects included 46 males and 34 females. The diagnosis of type 2 diabetes mellitus was performed according to the World Health Organization (WHO) criteria. Diabetic nephropathy was diagnosed by measuring the estimated glomerular filtration rate (e-GFR).The diabetic patients were subdivided into two groups: diabetics with normal kidney function (30patients) and diabetics with nephropathy (30patients). The duration of the disease was 6-8 years in diabetics with normal kidney function and >10years in diabetics with nephropathy. The patients in the diabetic groups were uncontrolled type 2 diabetic patients treated with oral hypoglycemic agent with a dose adjusted according to the state of each patient.

Before starting, informed consent was obtained from all participants. Approval was taken from the research committee of General Organization of Teaching Hospitals and Institutions. Exclusion criteria of the subjects were as follows: past history of malignancy, diabetic macrovascular complications, hypertension, other endocrine diseases or taking drugs which affect glucose and lipid metabolism, chronic hepatitis, recent inflammatory disease, acute trauma, pregnancy, and history of drug abuse.

*Methods*

Blood samples were collected in the morning after 12h overnight fasting into plain vacutainer tubes. Blood was then centrifuged at 3000 rpm for 10 min at 4 °C. Serum samples were rapidly separated, aliquoted, and stored at 80 °C until the measurements of PGRN, IL-6, total protein, albumin, and creatinine concentrations. Another part of blood was taken on EDTA for determination of insulin and HbA1c levels. For glucose estimation, potassium fluoride was added to tubes. Hemolysed samples were excluded. Concentrations of serum PGRN, Cystatin-C and IL-6 were analyzed by enzyme linked immunosorbent assay (ELISA) using the commercially available ELISA kits (Quantikine, R&D Systems) and followed the manufacturer's recommendations. Plasma glucose concentrations were assayed at once by glucose oxidase method according to Trinder, 1969. Serum total protein and albumin were determined colorimetrically according to the method of Yatzidis, 1987 and Doumas *et al.*, 1972, respectively. Serum creatinine was determined colorimetrically according to the method of Jaffe, 1986. Plasma insulin concentration was determined using a commercially available ELISA kit (Biosource Europe SA, Nivelles, Belgium) based on the method of Flier *et al,*. 1997. Insulin resistance was defined by homeostasis model assessment for insulin resistance (HOMA-IR). HOMA-IR was calculated by dividing the product of fasting plasma glucose (mmol/L) and fasting plasma insulin (mU/L) by 22.5 (Matthews *et al.,* 1985).

HbA1c % was measured according to the method of Grey *et al.*, 1996, using an immunoturbidimetric assay on Dimension RxL Max (Dade Behring). e-GFR was calculated from the Modification of Diet in Renal Disease (MDRD) study equation: (mL/min/1.73m$^2$)= 186 × (Scr)−1.154 × (Age)−0.203 × (0.742if female). Serum lipid profiles, including total cholesterol (TC), triacylglycerol (TAG), high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c),and kidney functions, including blood urea nitrogen (BUN) and s-creatinine (CREA), were detected by biochemical autoanalyzer.

*Statistical analysis*

Statistical analysis was performed using Statistical Package for the Social Science (SPSS) for Windows (version 16.0, Chicago, IL, USA).Data are presented as means ±SD. The data were analyzed by one-way analysis of variance (ANOVA). A $P$ <0.05 was considered statistically significant. The difference among the groups was compared using post hoc testing performed by the Bonferroni test. Receiver operating characteristics (ROC) analysis was employed to calculate the area under the curve (AUC) for serum progranulin to find the best cutoff value for identifying its sensitivity and specificity.

**Results**

Diabetic biomarkers in Table 1 demonstrate that fasting plasma glucose, postprandial plasma glucose, plasma insulin, HbA1c% and HOMA-IR showed significant increases (P<0.001) in type 2 diabetic patients (105.5, 156.05, 55.99, 61.4 and 228.57%, respectively) and diabetic nephropathy patients (151.58, 216.91, 119.55, 150.87 and 464.28 % respectively), compared to normal control subjects.

Type2 diabetic nephropathy patients showed significant increases (P<0.05) in FG and PPG, while a high significant increase (P<0.001) in plasma insulin, HbA1c% and HOMA-IR values, compared to diabetic patients.

**Table 1.** Levels of fasting and postprandial plasma glucose, insulin glycosylated hemoglobin, and HOMA-IR in all studied groups.

| Groups | Parameters | | | | |
|---|---|---|---|---|---|
| | Fasting glucose (FG) m mole/l | Postprandial glucose (PPG) m mole/l | Insulin µIU/ml | HbA1C % | HOMA-IR |
| NC | 5.04± 0.97[a] | 6.03± 1.22[a] | 6.34±0.36[a] | 5.7± 0.4[a] | 1.4± 0.43[a] |
| DM | 10.36± 2.25[b] | 15.44± 1.91[b] | 9.89±0.94[b] | 9.2± 1.6[b] | 4.6± 0.62[b] |
| % change from CP< | 105.5 | 156.05 | 55.99 | 61.40 | 228.57 |
| DN | 12.68± 2.71[c] | 19.11± 3.82[b] | 13.92±1.80 | 14.3± 1.2[b] | 7.9±0.76[c] |
| % change from C | 151.58 | 216.91 | 119.55[c] | 150.87 | 464.28 |
| % change from D | 22.39 | 23.76 | 40.74 | 55.43 | 71.74 |

Values are given as means ±SD for groups of 20 individuals. Groups sharing the same lowercase letters are not statistically different. NC: Normal control DM: Diabetics without nephropathy DN: Diabetics with clinical nephropathy.

DM group showed a slightly significant increase in TAG and V-LDL (21.1 and 22.4 % respectively) compared to NC group ($P<$ 0.05). Dyslipidemia (including high serum TC, TAG, LDL-c,VLDL-C and low HDL-c), were observed in DN group( 89, 154.2, 130.6, 136.3 and -32.2% respectively) compared to NC group ($P<$ 0.001) (Table 2).

**Table 2.** Levels of different parameters of lipid profile in all studied groups.

| Groups | Parameters | | | | |
|---|---|---|---|---|---|
| | TC mg/dl | TAG mg/dl | HDL-c mg/dl | LDL-c mg/dl | V-LDL mg/dl |
| NC | 181.9± 29.6[a] | 111.8±42.6[a] | 50.9± 11.2[a] | 108.8± 25.1[a] | 22.3± 4.6[a] |
| DM P< | 188.6± 40.6[a] | 135.4±28.2[b] | 47.6± 10.9[a] | 113.4± 34.1[a] | 27.3± 2.1[a b] |
| | 3.68 | 21.1 | 6.48 | 4.2 | 22.4 |
| DN | 343.9± 71.2[b] | 284.3± 25.4[c] | 34.5± 6.3[b] | 250.9±29.7[b] | 52.7± 9.4[b] |
| % change from C | 89.0 | 154.2 | 32.2 | 130.6 | 136.3 |
| % change from D | 82.3 | 109.9 | 27.5 | 121.2 | 93.04 |

Values are given as means ±SD for groups of 20 individuals. Groups sharing the same lowercase letters are not statistically different. NC: Normal control, DM: Diabetics without nephropathy, DN: Diabetics with clinical nephropathy.

Table 3 showed that renal biomarkers (s-creatinine, u-creatinine, s-BUN and A/C ratio) a slight significant change in DM group (20.5, -16.5, 22.6and 48.5% respectively). this change was augmented (P< 0.001) in DN group( 258.9, -50.48, 254.1and 5938% respectively.

Serum total protein and albumin were not significantly changed in both diabetic groups, on the other hand urinary albumin was non-significantly changed in DM group while, it was synergistically increased (P< 0.001) in DN group compared to NC group.

Table 4 illustrates that serum progranulin, cystatin-c and IL-6 were slightly increased in DM group(39.01, 24.8 and 55.9% respectively) while e-GFR was non-significantly changed.

In DN group, the increase in serum progranulin, cystatin-c and IL-6 were augmented (121.2, 69.4 and 120.4% respectively), while e-GFR was dramatically decreased (68.6%) compared to NC group ($P<$ 0.001).

Results of the Receiver Operating Characteristic (ROC) curve displaying that progranulin provided the highest diagnostic information with area under the

curve (AUC) of 1.0, and cut-off value of 105 ng/ml followed by s-cystatin-c with an AUC of 0.98 and a cutoff value of 675 ng/ml (Fig. 1) (table 5).

**Discussion**

DN is a common microvascular complication in patients with poorly controlled diabetes (Moon *et al.,* 2014), greatly affecting the life quality and survival of the patients. DN is now considered to be the major cause of ESRD (Gregg *et al.,* 2014).The prevention of the disease or at least the postponement of its progression has emerged as a key issue. Adverse outcomes of kidney failure can be prevented or delayed through early detection and treatment (Ying *et al.,* 2016).

**Table 3.** Levels of serum total protein, serum and urinary albumin, serum and urinary creatinine, serum BUN and A/C ratio in all studied groups.

| Groups | Parameters | | | | | | |
|---|---|---|---|---|---|---|---|
| | Serum T. protein g/dl | Albumin | | creatinine | | Serum BUN mg/dl | A/C ratio |
| | | Serum g/dl | Urine mg/L | Serum mg/dl | Urine g/L | | |
| NC | 7.07± 0.5[a] | 4.2±0.4 [a] | 15.7±2.4 [a] | 0.78± 0.2 [a] | 145.6±23 [a] | 14.6±4.1 [a] | 10.3±1.4 [a] |
| DM | 7.45± 0.4 [a] | 4.3±0.3 [a] | 17.6±4.8 [a] | 0.94±0.2 [a] | 121.5± 42.8 [a] | 17.9±3.8 [a] | 15.3±3.1 [a] |
| % change from C | 5.38 | 2.3 | 12.1 | 20.5 | 16.5 | 22.6 | 48.5 |
| DN | 7.04±0.7 [a] | 4.08±0.4[b] | 449± 114[b] | 2.8±0.34[b] | 72.1± 19.9[b] | 51.7±6.9[b] | 622±35.9[b] |
| % change from C | 0.42 | 2.8 | 2759 | 258.9 | 50.48 | 254.1 | 5938 |
| % change from D | 5.5 | 5.1 | 2451 | 197.8 | 40.6 | 188.8 | 3965 |

Values are given as means ±SD for groups of 20 individuals. Groups sharing the same lowercase letters are not statistically different. NC: Normal control, DM: Diabetics without nephropathy , DN: Diabetics with clinical nephropathy.

Progranulin (PGRN) is a fascinating multifunctional protein, which has been implicated in cell growth, wound repair, tumorigenesis, neurodevelopment, neurodegeneration. In addition, it is a kind of adipocytokines with important functions in modulation of inflammatory events. Studies in the last decade have shown that inflammation is a key process in the development of diabetes mellitus and diabetic nephropathy. Therefore, progranulin caught our attention because it is a novel adipokine marker of chronic inflammatory response in type2 diabetes capable of directly affecting the insulin signaling pathway (Lin *et al.,* 2015). In addition, the role of progranulin in Egyptian type 2 diabetic nephropathy has not been fully investigated.

Our study demonstrated that serum PGRN concentrations were significantly higher in type 2 diabetes patients (39.01%), compared to those of healthy subjects, this increase was augmented (121.2%) in patients with nephropathy. Additionally, the elevations in serum PGRN parallel the degree of hyperglycemia, hyperinsulinemia, and insulin resistance in type 2 diabetic patients. This observation seems to suggest that PGRN is associated with diabetic nephropathy and may be involved in the pathogenesis of diabetic nephropathy.

There is evidence that PGRN levels are increased in T2DM when compared to non-diabetic subjects. PGRN is closely related to glucose metabolism. There is a positive correlation between PGRN and HbA1C, fasting plasma glucose and 2 h post-challenge plasma glucose (Qu *et al.,* 2013). Elevated PGRN concentrations are also observed in impaired glucose tolerance subjects, revealing its role in prediabetic states (Toenjes *et al.,* 2010). The association of PGRN with T2DM is mainly explained by its role in adipose tissue and insulin resistance.

Insulin resistance is key feature of type 2 diabetes and can directly result in hyperinsulinemia. Recently, a report shows that PGRN could induce insulin resistance through stimulating IL-6 expression in adipocytes (Matsubara *et al.,* 2012).

Meanwhile, many studies find that IL-6 could increase the expression of cytokine signaling-3 (SOCS3) via activation of janus-activated kinase-signal transducer and activator transcription (JAK-STAT) signaling pathway in adipocytes to inhibit tyrosine phosphorylation of insulin receptor substrate (IRS-1), leading to impaired insulin signaling (Shi *et al.,* 2004). Type 2 diabetes is associated with a state of chronic low-grade inflammation which is characterized by increased pro-inflammatory factors and decreased anti-inflammatory factors.

**Table 4.** Levels of serum progranulin, IL-6, s. cystatin-c and e-GFR in all studied groups.

| Groups | Parameters | | | |
|---|---|---|---|---|
| | s. progranulin ng/ml | IL-6 pg/ml | s. cystatin-c ng/ml | e-GFR (mL/min/1.73 m²) |
| NC | 66.13± 18.4 [a] | 1.86±0.45 | 540 ± 23.5 [a] | 105.5±18 [a] |
| DM | 91.93±22.7[b] | 2.9±0.38[b] | 674± 20.1[b] | 91.6± 21[b] |
| %change from C | 39.01 | 55.9 | 24.8 | -13.17 |
| DN | 146.3±21.4[c] | 4.1±0.56[c] | 915± 32.6[c] | 33.12±10[c] |
| % change from C | 121.2 | 120.4 | 69.4 | -68.6 |
| % change from D | 59.14 | 41.3 | 35.7 | -63.84 |

Values are given as means ±SD for groups of 60 individuals. Groups sharing the same lowercase letters are not statistically different. NC: Normal control, DM: Diabetics without nephropathy, DN: Diabetics with clinical nephropathy.

PGRN promotes IL-6 expression, impairing insulin signaling. Moreover, it is a chemo-attractant protein that recruits monocytes into adipose tissue, promoting inflammatory response with increased cytokines levels (Matsubara *et al.,* 2012).

In this study it was demonstrates that inflammatory marker IL-6 was elevated in sera of type 2 diabetic patients and markedly elevated in sera of type 2 diabetic patients with nephropathy. This observation could be explained that the physiological function of PGRN is complex, with the full-length form of the protein having anti-inflammatory activity, whereas proteolytic cleavage generates granulin peptides that promote inflammatory activity (Eriksenand and Mackenzie, 2008). During the inflammatory process, progranulin is digested into smaller peptides, called granulins, which are proinflammatory and neutralize the anti-inflammatory effect of intact progranulin (Liu and Bosch, 2012).This suggests that PGRN is associated with diabetic nephropathy and may be involved in its pathogenesis.

**Table 5.** Area under the curve and cut-off value of s- progranulin and s-cystatin-c in DN patients group.

| Variables | Area Under the curve (AUC) | Cut-off value |
|---|---|---|
| Progranulin (ng/ml) | 1.00 | 105 |
| s-cystatin- c (ng/ml) | 0.98 | 675 |

In agreement with Matsubara *et al.,* 2012 who identified PGRN for the first time as a novel pro-inflammatory adipokine by differential proteome analysis of cellular models of insulin resistance. They showed that PGRN expression was induced by TNF*α* or dexamethasone and decreased with differentiation of adipocytes, and ablation of PGRN prevented mice from high fat diet-induced insulin resistance and blocked elevation of an inflammatory cytokine, IL-6, in adipose tissue. Combined with the afore mentioned relationship between PGRN and IL-6 in our study, we speculated that one of the mechanisms responsible for PGRN-induced insulin resistance may be associated with increased IL-6 levels.

Hyperlipidemia is an associated complication of T2DM (Kakadiya *et al.,* 2010). In this study, it was demonstrated that diabetic patients with nephropathy

showed evidence of hyperlipidemia represented by significant increase in TC, TAG, LDL-c and VLDL-c, in addition to significant decrease in HDL-c. Our results suggest that dyslipidemia can be a risk factor for kidney damage in diabetic patients. This result is consistent with those of previous studies demonstrating that lipid metabolism may participate in the development of glomerular and tubular alterations, leading to nephron destruction (Yun *et al.,* 2011).

Dyslipidemia, including increased serum levels of TAGs and decreased HDL-c, is a characteristic feature of the metabolic syndrome and T2DM. Consistent with reports that PGRN can affect lipid metabolism by inhibiting free fatty acid uptake and lipogenesis and/or by enhancing intracellular lipolysis (Kojima *et al.,* 2009).

Also, Okura *et al., 2010* showed that high density lipoprotein binds to progranulin and suppresses its conversion into pro-inflammatory granulins.

In agreement with our results, it was reported that other metabolic disorders associated with T2DM have also been linked to PGRN. A positive correlation observed between total cholesterol (Youn *et al.,* 2009), triglycerides and PGRN suggests a role in dyslipidemia. Patients with metabolic syndrome present higher serum PGRN concentration (Li *et al.,* 2014).In this study, increased progranulin in diabetic patients with nephropathy parallels the significant decreases in urinary creatinine and e-GFR, compared to normal subject. On the other hand, it parallels the significant increase in urinary albumin and s-cystatin c.

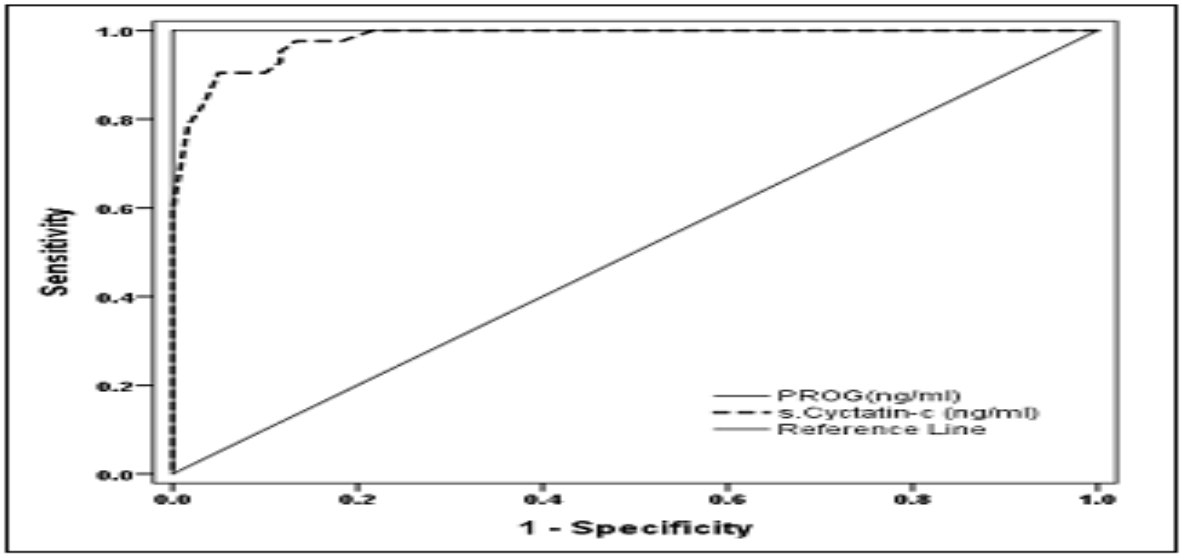


**Fig. 1.** Receiver operating characteristic (ROC) curves of progranulin and cystatin-c in DN patients group.

There is little evidence regarding the association of PGRN and DKD in T2DM patients. The pro-inflammatory effects of this adipokine could be involved in the pathway of renal damage, decreasing GFR and increasing albuminuria. When CKD is established, PGRN clearance is reduced and its effects could be potentiated.

Recent study reported that serum progranulin levels increased with deteriorating renal function, and the renal elimination was a major route for circulation PGRN.

Epidemiological studies have shown that nephropathy is closely associated (Richter *et al.,* 2013). Therefore, reduced renal elimination may be one of the reasons for the elevated circulating progranulin in diabetic nephropathy.

On the other hand, increased progranulin in diabetic patients with diabetic nephropathy parallels the significant decreases in urinary creatinine and eGFR, compared to normal control. In contrast, in diabetic patients,

although progranulin was significantly increased, the kidney function biomarkers were insignificantly changed with the exception of the significant decrease in urinary creatinine, compared to normal control.

The routine classical evaluation of diabetic nephropathy includes the appearance of microalbuminuria, decreased creatinine clearance and increased serum creatinine. But, it has been reported that a decline in the renal function of patients with diabetes was not always accompanied by an increased A/C ratio. About 20%-30% of patients with type 2 diabetes, accompanied by renal insufficiency, showed normoalbuminuria (Kramer *et al.,* 2007). To overcome these limitations, many clinicians additionally used creatinine in evaluating such patients. However, serum creatinine also depends on creatinine production, extra-renal elimination and tubular handling. Moreover, tubular involvement may precede glomerular involvement because several tubular proteins and enzymes are detectable even before the appearance of microalbuminuria and a rise in serum creatinine (Uslu *et al.,* 2005). Therefore, other biomarkers for estimation of renal function have been searched for and one of them was Cystatin- c.

Yun *et al.*, 2011, confirmed that cystatin-c could be one of the additional tubular factors which represent kidney state of diabetic patients therefore; it is a useful biomarker for the early detection of diabetic nephropathy.

In the present study ROC curve displaying that progranulin is more sensitive and specific than cystatin- c, thus, PGRN may be considered as a highly accurate and sensitive marker for early detection of diabetic nephropathy.

**Conclusion**

The results of our study showed for the first time that serum PGRN concentrations increased in Egyptian patients with nephropathy. The increased serum progranulin levels were closely related to the progress of diabetic nephropathy, suggesting that PGRN may be considered as a marker for diabetic nephropathy and its severity.

Thus, the level of PGRN in patients with type 2 diabetes should be paid high attention and it could be a potential therapeutic target for the management of type 2 diabetes and diabetic nephropathy. Further studies using larger populations will be needed to confirm our observations and to validate the current findings.

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