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Assessment of Serum Progranulin Association with Autophagy in Diabetic Patients

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Assessment of the association of serum progranulin with autophagy in diabetic patients

Running title: Progranulin and autophagy in diabetes

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Abstract

Introduction: Progranulin (PGRN) has newly arisen as an important regulatory protein of glucose metabolism and insulin sensitivity. Progranulin expression is interrelated with lysosomal function strongly linked to autophagy pathway. We aimed to evaluate the correlation between PGRN protein and microtubule-associated protein light chain 3B (LC3B) expression level in diabetic patients.

Material and methods: Blood samples of 70 type 2 diabetic Egyptian patients were provided for analysis of concentrations of serum progranulin and interleukin 6 (IL-6) using ELISA, and quantifying expression of LC3B RNA level using qPCR. A group of 20 healthy volunteers were also enrolled.

Results: Serum levels of PGRN and IL-6 as well as LC3B gene expression levels were markedly higher in type 2 diabetic patients. Additionally, our study revealed a cut-off value of 18.14 ng/mL for progranulin serum level and 3.233 for LC3B expression level, with sensitivities of 83.6% and

75.4% and specificities of 83.8% and 58.3%, respectively. Circulating PGRN levels are positively correlated with body mass index (BMI), glucose concentration, and IL-6.

Conclusion: Our results support the hypothesis that progranulin is introduced as a novel marker of chronic inflammatory response in type 2 diabetes that aggravates insulin resistance via activated autophagy, indicating the importance of this novel adipokine in the regulation of glucose metabolism and as a promising therapeutic target in the treatment of diabetes.

Key words: diabetes; progranulin; autophagy; microtubule-associated proteins light chain 3B; interleukin 6

Introduction

Progranulin (PGRN) is a 68–88 kDa cysteine-rich secreted protein, also known as granulin-epithelin precursor, proepithelin, or PC-cell-derived growth factor [1, 2]. It is encoded by GRN (PGRN gene) and expressed in many cell types, including epithelial cells, immune cells, neurons, and adipocytes. Progranulin is secreted in an intact form and can be cleaved into granulins by proteases [3]. Granulins are small proteins of approximately 6 kDa characterised by a conserved motif of 12 cysteines, and they play a role in the extracellular regulation of cell function and growth [2].

Progranulin, as an autocrine growth factor, has recently been found to play a role in a variety of physiological and pathological processes, including inflammation, and glucose and lipid metabolism [4]. Recent studies found that administration of progranulin caused glucose intolerance and insulin insensitivity through the triggering of autophagy in the adipose tissue of mice [5], suggesting that progranulin could be a critical adipokine regulating glucose and lipid metabolism.

Progranulin is emerging as an important regulatory adipokine of glucose metabolism and insulin sensitivity. Individuals with visceral obesity and type 2 diabetes (T2D) were shown to have increased PGRN serum levels [6]. Additionally, circulating PGRN levels are positively correlated

with body mass index (BMI), fat mass, fasting glucose, insulin levels, and insulin resistance, the hallmark of T2D [6, 7].

Moreover, it has been found that PGRN plays an important role in inflammation as anti-inflammatory protein affecting the tumour necrosis factor receptor (TNFR) signalling. Additionally, TNFR have been identified as receptors of PGRN. PGRN binds and antagonises both TNFR1 and TNFR2, inhibiting TNF α signalling pathway in a dose-dependent manner. The TNF α signalling pathway is a major mediator of inflammation, and it is involved in different human diseases including diabetes and rheumatoid arthritis [8].

Several studies have been performed in order to elucidate the association between PGRN and metabolic diseases. Elevated levels of PGRN were found in the white adipose tissue of a well characterised mouse model of obesity. The administration of murine recombinant PGRN to wild-type mice induced an increase of serum insulin level, increased fat mass, insulin resistance, and elevated levels of interleukin 6 (IL-6), which is strongly involved in the development of insulin resistance [9]. Therefore, PGRN mediates insulin resistance *in vivo*, inducing IL-6 expression in adipose tissue [10].

Likewise, it has been found that short-term treatment with recombinant PGRN induces insulin resistance both in murine liver and hepatocytes, and this was associated with defective autophagy [11]. The relationship between autophagy and PGRN was attributed to the involvement of a variety of adipocytokines, including IL-6 and TNF α [12]. Interestingly, PGRN-induced insulin resistance and defective autophagy in liver was found to be reverted by the administration of a TNFR blocking peptide, thus indicating that PGRN mediates insulin resistance and impaired autophagy via TNFR signalling in the liver [11].

Furthermore, experimental studies reported that PGRN increases autophagic activity and triggers endoplasmic reticulum (ER) stress in cultured human adipocytes, having an impact on insulin signalling [9].

Autophagy is the major mechanism involved in the degradation and recycling of intracellular components, and its alterations have been proposed to cause beta cell dysfunction [13]. However, autophagy may be enhanced under a variety of atrophic conditions, as shown by the expression of several proteins involved in lysosomal degradation, including LC3 [14].

Microtubule-associated protein 1A/1B light chain 3B (hereafter referred to as LC3) is a protein that in humans is encoded by the MAP1LC3B gene, and it is a member of the highly conserved ATG8 protein family. LC3 is a central protein in the autophagy pathway, where it functions in autophagy substrate selection and autophagosome biogenesis. LC3 is the most widely used marker of autophagosomes [15].

To the best of our knowledge, we were the first to investigate the correlation between PGRN and autophagy with reference to inflammatory response in diabetic patients.

Material and Methods

Subjects

This cross-sectional study was approved by the Ethical Committee of Al-Azhar University and was conducted in accordance with the Helsinki Declaration. All subjects signed written, informed consent, and this consent procedure was approved by the Ethics Boards of Al-Hussein hospital.

The analysis was performed on 70 Egyptian patients who were diagnosed with diabetes mellitus type 2 and attended the Intensive Care Unit (ICU) of Al-Hussein hospital, Al-Azhar University, and 20 healthy normal volunteers age- and sex-matched with the patient group.

The participant inclusion criteria included being in the age range 35–70 years, having DM and hypertension for a minimum of five years, systolic blood pressure > 140 mmHg or diastolic blood pressure > 90 mmHg under antihypertensive treatment, and the absence of medication that might interfere with urinary albumin excretion. Patients with renal tract pathological conditions, cardiovascular disease, non-diabetic kidney diseases, malignancy diagnosis, obstructive uropathy, hepatitis, HIV, who had begun dialysis, or had received a renal transplant were excluded from the study.

Samples

A total of 70 blood samples were collected from T2DM patients (41 males and 29 female) in addition to 20 samples from healthy persons as control samples (12 males and eight females). Sera were collected following a standardised protocol at least 6–8 h after the last meal, from samples in sterile falcon tubes, and prepared for RNA isolation and the indicated biochemical analysis then stored within 15 min at -80°C .

Laboratory and biochemical parameters

Samples of all participants were labelled with a unique identifier to protect subject confidentiality. Blood sugar was measured using the glucose oxidase method, serum glycosylated haemoglobin (HbA_{1c}) was tested on an Olympus AU640 (Japan), and cholesterol, high-density lipoprotein-cholesterol (HDL-C), as well as low-density lipoprotein-cholesterol (LDL-C) were detected on a Hitachi 7180 analyser (Japan) with commercial reagents.

Body mass index (BMI) was determined as weight (in kilograms) divided by height (in metres) squared. Systolic and diastolic blood pressure were measured from the subjects' right hand using a standard mercury manometer, with subjects in a sitting position.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of serum PGRN were analysed using a commercially available ELISA kit (Wuhan Fine Biotech Co., Ltd, China) and that of serum IL-6 using Quantikine (R&D Systems, Inc., USA) according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA for LC3B and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was isolated using TriZol (Invetrogen) and chloroform then dissolved in RNase free water. The RNA concentration and purity were confirmed using an Ultraspec 1000, UV/visible spectrophotometer (Amersham Pharmacia Biotech, Cambridge, England) and NanoDrop 2000 (Thermo Scientific, USA). 100 ng of total RNA was reverse transcribed into single-stranded complementary DNA by using a miScript Reverse Transcription Kit (Qiagen, MD) following the manufacturer's protocol. The synthesised complementary DNA was used for quantitative polymerase chain reaction using the qPCR tubes then run on a StepOnePlus™ System (Applied Biosystems, Inc., Foster, CA) in the presence of QuantiTect SYBR Green PCR Kit (Qiagen, MD), and the results were computerised. The following gene-specific primers were used: LC3B-forward-5'-ATACBCCTCTCAGGAGACTTT-3', LC3B-reverse-5'- CCAAATGAGAACTCTGGAACG-

3', and GAPDH-forward, 5'- GTC TCC TCT GAC TTC AAC AGC G- 3' and GAPDH-reverse, 5'- ACC ACC CTG TTG CTG TAG CCA A- 3'.

Target sequences were amplified at 95°C for 15 minutes, followed by 40 cycles at 94°C for 15 seconds and 55°C for 30 seconds, then for 30 seconds at 70°C. GAPDH was used as an endogenous normalisation control. All assays were performed in triplicate. The fold change in messenger RNA expression was determined in DM patients compared to controls according to the $2^{-\Delta\Delta Ct}$ method, where $\Delta Ct = Ct \text{ target gene} - Ct \text{ reference gene}$ and $\Delta\Delta Ct = \Delta Ct (\text{sample 2}) - \Delta Ct (\text{sample 1})$, and where sample 1 is the control sample and sample 2 is the experimental sample [16].

Statistical analysis

Statistical analysis was performed using SPSS version 21.0 (SPSS, Chicago, IL, USA). Data are presented as mean \pm standard deviation. $P < 0.05$ was considered statistically significant. Differences in the measured expression of MAP1LC3B mRNA between healthy individuals and diabetic patient groups were analysed by one-way analysis of variance (ANOVA). Correlations were evaluated by Spearman correlation coefficient. The diagnostic accuracy of the progranulin, IL-6, and LC3B gene for DM were evaluated by receiver operating characteristic (ROC) analysis.

Results

The baseline demographic and biochemical characteristics of the study population are summarised in Table I. Progranulin and IL-6 showed a significant increase in the diabetic patients compared to normal subjects. The expression level of LC3B mRNA was measured, and its level was significantly increased in the diabetic patients when compared to the control subjects (Fig. 1).

The receiver operating characteristic (ROC) curve was designed to distinguish diabetic patients from the control group (Fig. 2), and the results revealed that the best cut-off values of progranulin, IL-6, and LC3 are 18.14, 1.33, and 3.23, respectively, with certain sensitivities and specificities (Tab. II). Combined sensitivities and specificities of PGRN, IL-6, and LC3 are represented in Table III.

Regarding correlations, progranulin levels were positively correlated with BMI, glucose concentration, and IL-6. LC3 expression levels were negatively correlated with glucose concentration, LDL, CHL, and systolic pressure, and positively with diastolic pressure. Also,

levels of IL-6 were positively correlated with glucose concentration and systolic pressure (Tab. IV).

Discussion

Progranulin is a protein with growth factor-like properties regulating several biological functions. Interestingly, PGRN has been found to be strongly involved in metabolism [17].

New markers have been studied in the pathogenesis of diabetes, involving many adipokines [18, 19], such as PGRN [7]. There is evidence that PGRN levels are increased in T2DM when compared to non-diabetic subjects. PGRN is closely related to glucose metabolism. Furthermore, there is a positive correlation between PGRN and plasma glucose A_{1c} [6, 20]. Elevated PGRN concentrations are also observed in subjects with impaired glucose tolerance, revealing its role in prediabetic states [20].

In agreement with these findings, the present study showed significantly elevated PGRN protein concentration in the diabetic group when compared to the controls; furthermore, HbA_{1c} and BMI were significantly increased in the diabetic group.

Obesity and visceral fat are probably the main risk factors for T2D [9] and are involved in its pathophysiology as well as inflammation and insulin resistance [21]. Moreover, a study evaluating T2DM patients reported that obese subjects present higher levels of PGRN [13]. Taking in consideration the fact that BMI is a simple index of weight-for-height that is commonly used to classify overweight and obesity in adults, this study agreed with our finding revealing a significant difference of BMI between diabetic and control groups with significant positive correlation with PGRN.

Further studies in the last decade have shown that inflammation is a key process in the development of diabetes mellitus [22]. The association of PGRN with T2DM is mainly explained by its role in adipose tissue and insulin resistance. Progranulin promotes IL-6 expression, impairing insulin signalling [10]. Moreover, it is a chemoattractant protein that recruits monocytes into adipose tissue, promoting inflammatory response with increased cytokines levels [6].

The physiological function of PGRN is complex, with the full-length form of the protein having trophic and anti-inflammatory activity, whereas proteolytic cleavage generates granulin

peptides that promote inflammatory activity [23]. During the inflammatory process, progranulin is digested into smaller peptides, called granulins, which are proinflammatory and neutralise the anti-inflammatory effect of intact progranulin [24].

In the current study PGRN concentrations and the inflammatory marker IL-6 are markedly elevated in the sera of T2DM. Furthermore, serum PGRN levels had remarkable positive correlations with IL-6. Kloeting et al. [25] present evidence that progranulin induces proinflammatory IL-6. Therefore, it is speculated that serum PGRN could stimulate the adipocytes to release more IL-6.

Experimental studies reported that PGRN promotes IL-6 expression in adipose cells, and its elevation enhances cytokine signaling-3 (SOCS3) expression via activation of JAK-STAT signalling. This mechanism can inhibit tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), leading to insulin resistance [10].

Furthermore, Guo et al. [26] explored the mechanism of progranulin action and revealed that progranulin treatment activated oxidative stress and endoplasmic reticulum (ER) stress, elevated autophagy, and induced insulin insensitivity in adipocytes and adipose tissue of mice.

Autophagy is a highly conserved pathway. Impairment of autophagy is implicated in the pathogenesis of diabetes. Furthermore, autophagy has been closely linked to inflammation in part by regulation of adipokine production. Experimental study showed that mice with a conditional deletion of autophagy gene (*Atg7*) in the intestinal epithelium showed enhanced mRNA expression of interleukin 1 β (IL-1 β) [27]. Additional studies in vivo demonstrated that obesity-induced endoplasmic reticulum stress causes chronic inflammation in adipose tissue, as shown by up-regulation of tumour necrosis factor alpha (TNF α), IL-1 β , and IL-6 [28]. These observations raised the possibility that PGRN might be a promising new link among obesity, autophagy and insulin resistance (i.e. diabetes).

It was revealed that administration of PGRN attenuated insulin signalling and triggered autophagy in in vivo and in vitro studies, suggesting a causative role of tumour necrosis factor receptor 1 (TNFR1) in PGRN-induced impaired insulin sensitivity, implicating that decreasing PGRN level, by influencing its turnover or production, is consequently a promising therapeutic approach applied to metabolic disorders [5].

Linnemann et al. [29] reported that IL-6 robustly activates signal transducer and activator of transcription 3 (STAT3), a transcription factor that is involved in autophagy. The authors also reported that IL-6 stimulates LC3 conversion and autophagosome formation in cultured pancreatic β cells. In the same study, both mRNA and protein expression of ATG7 (the enzyme responsible for the fatty acid conjugation that converts LC3-I to LC3-II) were stimulated by IL-6. IL-6-mediated regulation of autophagy occurs through multiple complementary mechanisms involving both repression of mammalian target of rapamycin complex 1 (mTORC1) and stimulation of mammalian target of rapamycin complex 2 (mTORC2).

Direct stimulation of autophagy is a novel mechanism for IL-6-mediated protection of pancreatic β cells from stress-induced apoptosis [29]. This is consistent with previous observations that exercise (which stimulates IL-6) activates autophagy in muscle cells and is critical for cellular homeostasis [30]. Because the IL-6 receptor and its downstream signalling target are both down-regulated in human islets in type 2 diabetes [29], IL-6-mediated autophagy stimulation appears to be impaired in type 2 diabetes. It is hypothesised that the inability of the diabetic islet to respond to increased circulating IL-6 in diabetes may contribute to β -cell dysfunction and death.

Indeed, the expression level of LC3II is highly correlated with the number of autophagosomes [15]. Paula-Gomes et al. [31] reported that LC3 mRNA and, consequently, LC3II protein levels were enhanced in the heart tissue of diabetic animals and in fasted cardiomyocytes, suggesting that the autophagic flux is increased, which concurred with our findings. In the present study, the expression level of LC3B mRNA indicated a significant elevation in the diabetic group compared to the normal group.

On the other hand, insulin treatment of the patient in another study was suggested to be responsible for the reduction in the level of LC3B. Also, Su et al. [32] reported that the expressions of LC3, ATG5, BECN1, Cathepsin B, and FOXO3A proteins in ovaries from insulin-treated mice were significantly reduced compared to those from control mice.

In addition, in the present study the ROC curve showed that progranulin had higher specificity than IL-6 and LC3 but lower sensitivity than IL-6 and higher sensitivity than LC3. The combined use of the three parameters increased the sensitivity, reaching 100%.

Although the potential role of progranulin in activating autophagy and inducing insulin resistance has been identified, the intracellular events responsible for progranulin-mediated effects in autophagy and diabetes remain not fully understood.

Conclusion

In conclusion, this body of evidence, combined with our findings, strongly suggests that PGRN is a novel marker that plays role in inflammatory response in type 2 diabetic patients via activated autophagy. Moreover, this novel adipokine, which is involved in regulation of glucose metabolism, could be a promising therapeutic target in the treatment of diabetes. Additionally, LC3 has great clinical value as a promising biomarker in diabetic assessment.

Ethical approval and consent to participate

This study was approved by the Ethical Committee of Al-Azhar University and was conducted in accordance with the Helsinki Declaration.

Consent for publication

All subjects signed written, informed consent, and this consent procedure was approved by the Ethics Boards of Al-Hussein hospital.

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due to patients' privacy and ethical restrictions but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

NSH and MGAH conceived of the presented idea, NAM collected samples, NSH, MGAH, and NAM analysed and interpreted the patient data, NSH and MGAH carried out the practical experiments, NSH and MGAH drafted the manuscript, and all authors read and approved the final manuscript.

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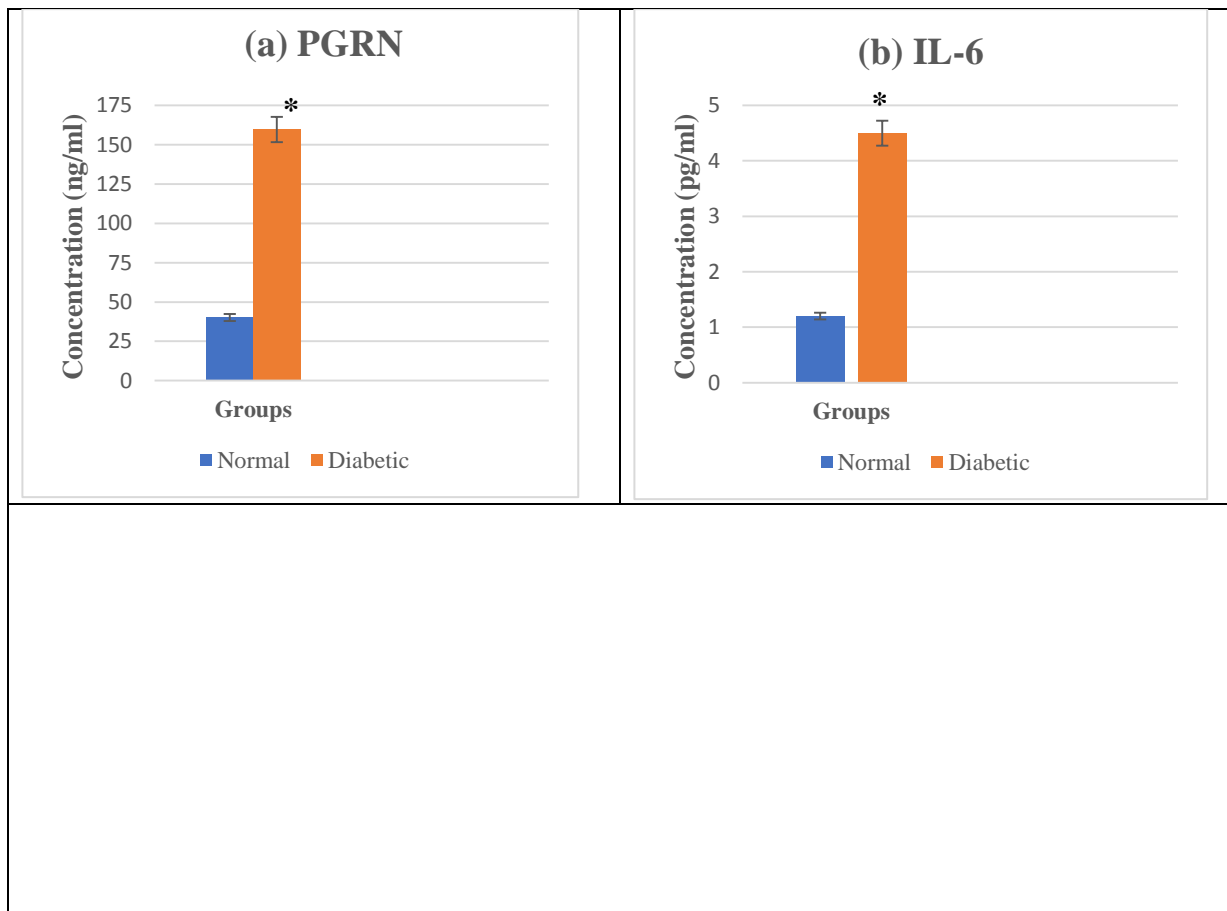
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Figure 1. Differential expression of LC3, protein level of progranulin and IL-6 among different groups (n = 90). Values expressed as mean \pm SE. *p < 0.05 is significant when compared to controls



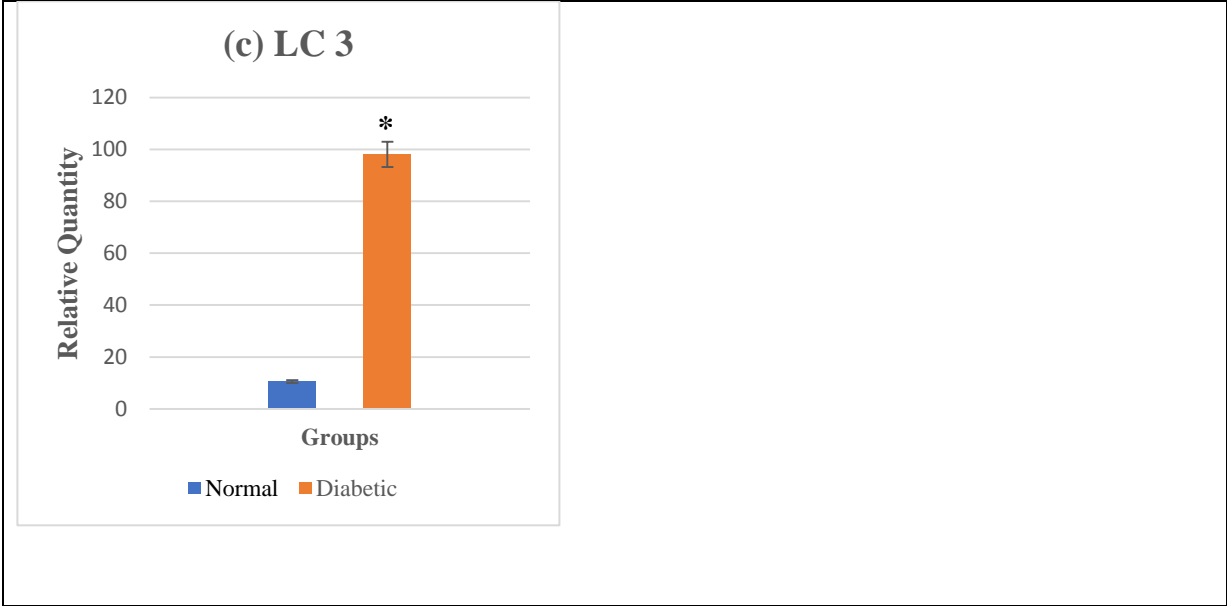
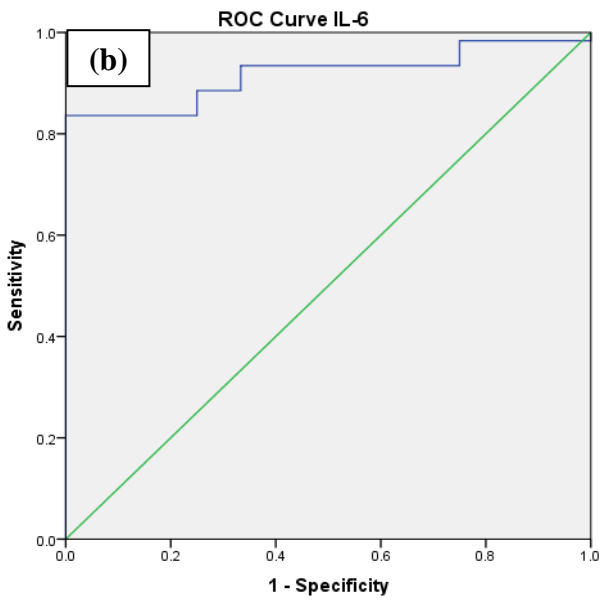
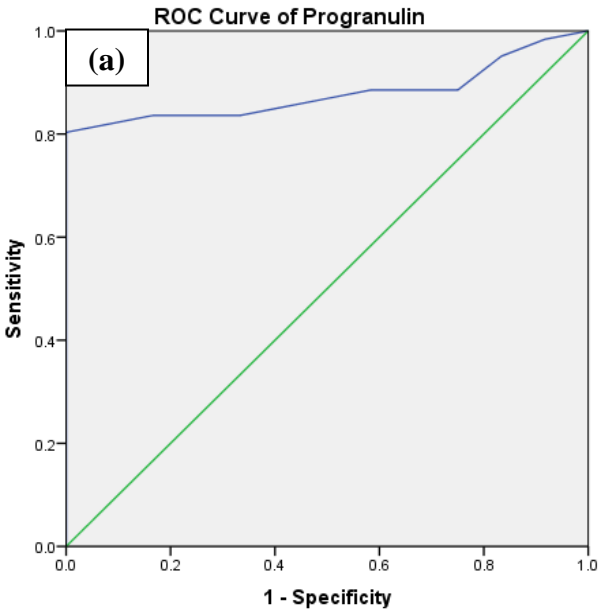


Figure 2. Receiver operating characteristic (ROC) curves of (a) progranulin, (b) interleukin 6 (IL-6), (c) light chain 3B (LC3)



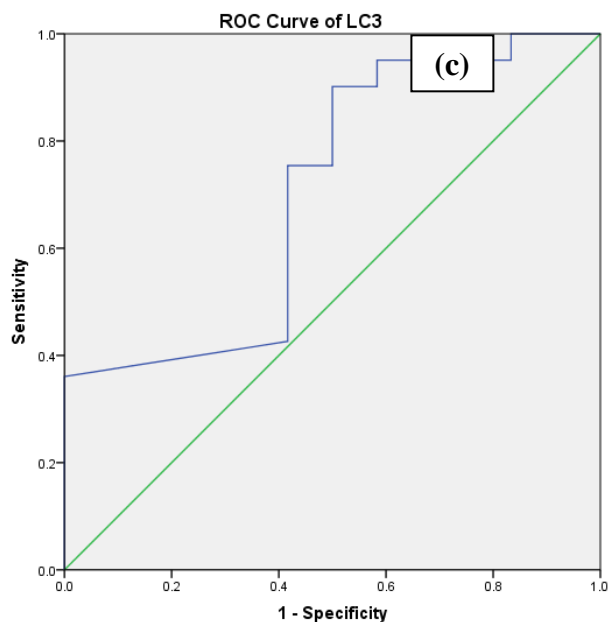


Table I. Clinical characteristics of the participants among the different groups in the study

	Groups						P	F ^(a)
	Diabetic patients			Healthy volunteers				
	Mean	Median	± SD	Mean	Median	± SD		
Age [years]	54.75	55.00	9.24	54.25	54.50	5.69	0.856	0.33
BMI [kg/m ²]	34.99	35	3.42	28.67	29.00	2.39	0.002*	10.265
HbA _{1c} (%)	5.80	5.50	0.91	4.87	5.000	0.74	0.001*	11.215
Glucose [mg/dl]	280.70	262.00	114.35	111.17	111.50	5.32	0.000*	26.072
SBP [mmHg]	123.60	125.0	9.88	114.17	120.00	9.73	0.003*	9.197
DBP [mmHg]	80.73	80.00	5.76	79.16	80.00	9.96	0.570	.453
HDL [mmol/l]	33.72	31.00	8.12	33.67	31.50	8.80	0.983	.000
LDL [mmol/l]	127.65	118.00	30.15	119.16	110.00	23.25	0.360	0.848
Cholesterol [mmol/l]	207.24	200.00	33.16	204.16	200.00	29.37	0.766	0.089
Duration [years]	11.64	12.00	4.66	00	00	00	0.000*	73.961

BMI — body mass index; HbA_{1c} — glycosylated haemoglobin; SBP — systolic blood pressure; DBP — diastolic blood pressure; HDL — high-density lipoprotein; LDL — low-density lipoprotein; SD — standard deviation; *one-way ANOVA test; * p < 0.05: significantly different

Table II. Area under curve, cut-off value, and performance characteristics of progranulin, interleukin 6 (IL-6), and light chain 3B (LC3) to discriminate diabetic patients from the control group

Parameter	AUC	Cut-off value	Sensitivity	Specificity
progranulin	0.878	18.141	83.6%	83.3%
IL-6	0.918	1.33	88.5%	75.8%
LC3	0.706	3.233	75.4%	58.3%

Table III. Combined sensitivity and specificity of progranulin (PGRN), interleukin 6 (IL-6), and light chain 3B (LC3)

Combined parameters	Combined Sensitivity	Combined Specificity
PGRN + IL-6	96.7%	83.3%
PGRN + LC3	96.7%	83.3%
IL-6+ LC3	100%	75.0%
PGRN+ IL-6 +LC3	100%	58.3%

Table IV, Correlation between progranulin (PGRN), light chain 3B LC3 (LC3), and interleukin 6 (IL-6) and clinical parameters among groups of study.

Parameter		PGRN	LC3	IL6
Glucose	Correlation coefficient (r)	.231	-.308	.424
	Sig. (2-tailed) (P)	.049*	.008*	.000*
HbA _{1c}	Correlation coefficient (r)	.090	-.100	-.098
	Sig. (2-tailed) (P)	.451	.400	.411
BMI	Correlation coefficient (r)	.351	.135	.169
	Sig. (2-tailed) (P)	.002*	.257	.152

SBP	Correlation coefficient (r)	.108	-.353	.382
	Sig. (2-tailed) (P)	.362	.002*	.001*
DBP	Correlation Coefficient (r)	.075	.257	.055
	Sig. (2-tailed) (P)	.527	.028*	.643
HDL	Correlation Coefficient (r)	-.159	-.018	-.013
	Sig. (2-tailed) (P)	.179	.878	.916
LDL	Correlation Coefficient (r)	.165	-.305	.064
	Sig. (2-tailed) (P)	.164	.009*	.593
CHL	Correlation Coefficient (r)	.087	-.313	.054
	Sig. (2-tailed) (P)	.467	.007*	.649
Duration	Correlation Coefficient (r)	.362	-.042	.394
	Sig. (2-tailed) (P)	.002*	.725	.001*
Progranulin	Correlation Coefficient (r)	1.000	.020	.397
	Sig. (2-tailed) (P)	.	.865	.000*
LC3	Correlation Coefficient (r)	.020	1.000	-.122
	Sig. (2-tailed) (P)	.865	.	.302
IL-6	Correlation Coefficient (r)	.397	-.122	1.000
	Sig. (2-tailed) (P)	.000*	.302	.

BMI — body mass index; HbA_{1c} — glycosylated haemoglobin; SBP — systolic blood pressure; DBP — diastolic blood pressure; HDL — high-density lipoprotein; LDL — low-density lipoprotein; Spearman's correlation p: not significant (> 0.05), * p < 0.05: significant