ORIGINAL RESEARCH

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Glucagon-like peptide-1 levels and dipeptidyl peptidase-4 activity in type 2 diabetes

Abstract

Purpose: Hyperglycemia is the major risk factor for microvascular complications in type 2 diabetes mellitus (T2DM) patients. This randomized controlled clinical trial aimed to investigate T2DM patients with microvascular complications with regard to possible relations among serum clusterin (CLU), amylin, secreted frizzled-related protein-4 (SFRP-4), glucagon-like peptide-1 (GLP-1) and dipeptidyl peptidase-4 (DPP-4) activities.

Methods: Subject groups were defined as follows: T2DM without complications (n=25, F/M=9/16, age 53.9±11.1 years); T2DM+Retinopathy (n=25, F/M=13/12, age 63.8±7.1 years); T2DM+Nephropathy (n=25, F/M=13/12, age 58.7±14.4 years); T2DM+Neuropathy (n=25, F/M=15/10, age 63.2±9.6 years); and healthy control subjects (HC) (n=25). CLU, amylin, SFRP-4, DPP-4 and GLP-1 (total and active) activities were measured and compared in blood samples from type 2 diabetic patients with and without microvascular complications.

Results: Significantly lower levels of DPP-4 and GLP-1_{total} ($P<0.005$ and $P<0.001$, respectively) and higher levels of SFRP-4 were measured in subjects with T2DM in comparison with HC ($P<0.05$). Serum CLU, amylin and GLP-1_{active} levels were similar between HC and T2DM patients. Patients with T2DM+microvascular complications had significantly higher DPP-4 and GLP-1_{total} levels when compared with T2DM patients without complications ($P<0.05$ and $P<0.001$, respectively). Regardless of the other features, in all patients with T2DM-associated microvascular complications, a positive correlation was evident between DPP-4 activity and GLP-1_{total} ($r=0.290$; $P<0.01$).

Conclusions: DPP-4 activity and GLP-1_{total} levels were higher in patients with microvascular complications associated with T2DM. Contrary to expectations, no negative correlation was seen between GLP-1 and DPP-4 levels. This result suggests the possible inefficacy of DPP-4 activity as a marker to predict *in vivo* degradation of endogenous GLP-1.

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As a chronic metabolic disorder, type 2 diabetes mellitus (T2DM) not only affects glucose, lipid and protein metabolism but, if left untreated, is also associated with severe comorbidities, most of which are associated with vascular complications. It is estimated that 25% of the patients admitted with microvascular complications at the time of T2DM diagnosis had the disease for longer than five years [1].

Incretins are hormones that are released from the gut into the bloodstream in response to the ingestion of food; they then modulate the insulin secretory response to the products within the food. Two incretins, glucose-dependent insulintropic peptide (GIP) and glucagon-like peptide-1 (GLP-1), share many common actions in the pancreas but have distinct actions outside the pancreas as well. Both incretins are rapidly deactivated by an enzyme called dipeptidyl peptidase 4 (DPP-4) [2]. The proglucagon gene encodes two glucagon-like peptides that have approximately 50% amino acid homology to glucagon; these are designated GLP-1 and glucagon-like peptide-2 (GLP-2, which is not insulintropic, has no glucose-lowering properties and is, therefore, not an incretin) [2]. DPP-4 inhibitors are widely used when managing T2DM, mainly due to their tolerable adverse event profile, with low risk of weight gain and hypoglycemia [3]. DPP-4 inhibitors are orally active and increase endogenous blood levels of active incretins, thus leading to prolonged incretin action. Elevated levels of GLP-1 are thought to be the mechanism underlying their blood glucose-lowering effects [2].

Secreted frizzled-related protein-4 (SFRP-4) is an extracellular regulator that plays a role in an endogenous pathway called the wingless-type mouse mammary tumor virus integration site family (Wnt). Many of the actors in this pathway are known for their regulatory effects on lipid and glucose metabolism; thus, the probable role of this pathway in the pathogenesis of diabetes has drawn attention [4]. The actors in this pathway are overexpressed in the islets of a diabetic case and their effect is to reduce glucose tolerance [5,6].

Amylin is a neuro-endocrine hormone, also known as islet amyloid polypeptide (IAPP), that possess similar properties to hormones secreted from pancreatic β cells with regard to localization, secretion and packaging. Like the other two pancreatic islet hormones, insulin and glucagon, amylin also contributes to the homeostasis of glucose [7,8].

The clusterin/apolipoprotein J (CLU) gene has a ubiquitous expression pattern in human tissues, taking part in a variety of processes. CLU has been linked with cancer progression as well as diabetes and vascular damage in kidneys [9].

The prevailing and clinically relevant action of DPP-4 is the degradation of endogenous GLP-1 [10]. The role of DPP-4 in the regulation of GLP-1 degradation is particularly significant, but no previous studies have addressed this process in patients with microvascular complications of T2DM or in the diabetic population as a whole. In this study, serum GLP-1, DPP-4, SFRP-4, amylin and CLU levels were measured and compared in type 2 diabetic patients with and without microvascular complications. Possible interactions between these parameters and microvascular complications were also highlighted.

Materials and Methods

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki. Ethical approval was received from the University Ethical Committee of Clinical Research (No. 83045809). Informed consent was obtained from all patients.

All patients recruited in this study were diagnosed with T2DM according to guidelines of the American Diabetes Association (ADA) [11]. Subjects were classified based on evidence of microvascular complications: (1) Diabetic Group (DM) (n=25), comprising patients who did not present with microvascular complications (mean age: 53.9 ± 11.1 , nine females and 16 males); (2) Retinopathy Group (n=25), comprising patients with retinopathy (mean age: 63.8 ± 7.1 , 13 females and 12 males); (3) Nephropathy Group (n=25), comprising patients with nephropathy (mean age: 58.7 ± 14.4 , 13 females and 12 males); (4) Neuropathy Group (n=25), comprising patients with neuropathy (63.2 ± 9.6 , 15 females and 10 males) and (5) Healthy Controls (HC) (n=25), comprising subjects with no demonstrated endocrine, cardiovascular or inflammatory diseases (mean age: 58.3 ± 10.6 , 10 females and 15 males).

Diagnostic criteria included having at least one of the following complications: retinopathy, nephropathy, peripheral neuropathy or microangiopathic CVD. Exclusion criteria included having other acute or chronic metabolic, systemic, endocrine or autoimmune inflammatory diseases, cancer, history of chronic alcohol consumption or use of DPP-4 inhibitors, GLP-1 analogues or hepatotoxic drugs (antituberculous, antiepileptic) or oral contraceptive pills.

All recruited T2DM patients were receiving suitable treatment for diabetes: insulin (35%); metformin (68%); and/or sulphonylureas (22%). Patients with accompanying hypertension constituted 86% of the population and were taking beta blockers (31%), thiazide (27%) and/or ACE

inhibitors (21%). Patients with dyslipidemia (83%) were taking antihyperlipidemic medications: statins (48%); glitazone (12%); acarbose (4%); and/or fibrates (7%).

Venous blood samples were drawn between 8–10 a.m. after overnight fasting (10–12 h). The samples were drawn via brachial veins in brachial fossa into plain tubes or anticoagulant (ethylenediaminetetraacetic acid, EDTA)-containing tubes. Biochemical and hormonal parameters were measured on the same day. Protease inhibitor (aprotinin, Sigma-Aldrich, USA) was added immediately into the serum for the stabilization and measurement of GLP-1. For measurement of other parameters, serum and plasma aliquots were immediately frozen and stored at -80°C until further analysis.

Laboratory analyses

Blood samples were obtained at least 24 hours prior to the administration of the drugs, and standardized procedures were followed.

GLP-1_{total} and GLP-1_{active} serum levels were assayed by the antibody sandwich ELISA kit (Cat. EZGLP1T-36K and Cat. EGLP-35K, EMP Millipore, USA). Results were expressed as pM. The sensitivity of GLP-1_{total} ELISA kit was 1.5 pM. Intra- and inter-variation of the coefficient (CV) for GLP-1_{total} were 3% and 11%, respectively. The lowest level of GLP-1_{active} that could be detected by this assay was 2 pM. Intra- and inter-CV for GLP-1 levels were 6.5% and 10%, respectively.

Levels of serum DPP-4 were assayed by the antibody sandwich ELISA kit (Human DPP-4 Kit, Cat. No. YHB1023 Hu, ARP American Research Products, USA) and the results were expressed as pg per mL of serum. The lowest level of DPP-4 that could be detected by this assay was 25 pg/mL. Intra- and inter-CV were 8.5% and 11.5%, respectively.

Levels of serum CLU were determined by the antibody sandwich ELISA kit (Human CLU Kit, Cat. No. YHB0754 Hu, ARP American Research Products, USA), and the results were expressed as µg per mL of serum (µg/mL). The lowest level of CLU that could be detected by this assay was 0.24 µg/mL. Intra- and inter-CV were 6.7% and 8.9%, respectively.

Levels of serum amylin were assayed by the ELISA kit (Human Amylin, Cat. No. YHB0161 Hu, ARP American Research Products, USA). Results were expressed as pg per mL of serum (pg/mL). The sensitivity of this kit was 1.36 pg/mL. Intra- and inter-CV were 7.3% and 9.5%, respectively.

Levels of serum SFRP-4 were determined by the antibody sandwich ELISA kit (Human SFRP-4 Kit, Cat. No. E2327 Hu, Bioassay Technology Laboratory, USA), and the results were expressed as ng per mL of serum (ng/mL). The lowest level

of SFRP-4 that could be detected by this assay was 1.5 pM. Intra- and inter-CV were 3% and 11%, respectively.

Glucose, total cholesterol (TC), triglycerides (TG), HDL-cholesterol (HDL-C), and LDL-cholesterol (LDL-C) concentrations were determined by enzymatic methods (Roche Cobas Integra 400, Germany). Insulin concentrations were measured by the electrochemiluminescence immunoassay (ECLIA) method from Roche-Hitachi E170. Analysis of C-reactive proteins (CRPs) was carried out by nephelometric means (IMAG-Bechman Coulter, Germany). HbA1c concentrations were determined using high performance liquid chromatography (Bio-RAD, Variant Turbo 2, USA).

Albumin excretion in 24-hour urine samples was measured using Roche Hitachi P800 (Roche, Germany) with the ALBT2 microalbumin kit, and the mean value was calculated as daily albumin excretion. Fibrinogen levels were assayed using the traditional chemiluminescence microparticle immunoassay (CMIA) (Abbott Architect i2000, USA). Glomerular filtration rate (GFR) values were estimated by using the chronic kidney disease epidemiology (CKD-EPI) formula.

HOMA-IR was calculated using the formula $HOMA-IR = (\text{glucose [mg/dL]} * \text{insulin [}\mu\text{U/mL]}) / 405$ with the fasting values.

Statistical analysis

Statistical analyses were performed using SPSS 11.0 (SPSS, USA). Data were expressed as means $\pm$ standard deviation (SD). Descriptive statistics were also obtained. The Kolmogorov-Smirnov test of Gaussian distribution was performed for all data. For normally distributed parameters parametric and for abnormally distributed parameters nonparametric tests were used; for this purpose, ANOVA, unpaired Student's *t*, Mann-Whitney *U* and Wilcoxon signed-rank tests were used. CLU, amylin, DPP-4, SFRP-4, GLP-1_{total}, GLP-1_{active}, CRP and HOMA-IR showed non-parametric distribution. To illustrate the relationships between the variables, Pearson's or Spearman's correlation coefficients were used. The value of $P \leq 0.05$ was considered statistically significant.

Results

In Table 1, the characteristics of the given groups are summarized. HC and diabetic patients were significantly different with regard to age ($P < 0.005$). Levels of plasma glucose and serum TG ($P < 0.001$ and $P < 0.05$, respectively), as well as HOMA-IR ($P < 0.01$), were significantly higher in the

TABLE 1. General demographic and biochemical characteristics of control and diabetic groups with or without microvascular complications

	Controls	DM	Retinopathy	Nephropathy	Neuropathy
Ages (years)	58.3±10.6	53.9±11.1	63.8±7.1	58.7±14.4	63.2±9.6
Sex (female/male)	10/15	9/16	13/12	13/12	15/10
Duration of diabetes (years)	-	4.8±4.6	14.6±6.6	6.1±6.0	13.6±7.2
BMI (kg/m ²)	29.5± 4.7	33.1± 6.4	30.9±5.47	32.2±6.3	32.9±5.6
Fasting glucose (mg/dL)	92.5±7.6	136.3±55.2 ^{a3}	179.2±63.8 ^{b4}	155.8±85.9	161.7±78.8
HbA1c (%)	5.59±0.30	6.94±1.95 ^{a3}	8.67±2.13 ^{b4}	7.38±1.82	8.01±2.20 ^{b1}
HOMA-IR	3.32±1.84	5.36±3.85 ^{a2}	6.42±3.75	9.02±7.75	5.13±4.15
Creatinine (mg/dL)	0.75±0.17	0.78±0.20	0.87±0.33	0.89±0.33	0.93±0.53
Urea (mg/dL)	15.5±4.5	14.3±3.8	16.9±9.7	17.5±8.9	19.1±10.1
Total cholesterol (mg/dL)	196.5±39.2	202.9±44.1	174.8±45.5 ^{b1}	186.9±50.4	186.3±62.1
Triglyceride (mg/dL)	129.5±77.8	211.7±39.7 ^{a1}	139.4±93.6 ^{b2}	189.1±76.5	152.8±72.1
HDL-Cholesterol (mg/dL)	49.9±16.9	42.2±15.4 ^{a1}	42.7±12.5	39.8±9.5	43.2±12.4
LDL-Cholesterol (mg/dL)	121.3±34.9	124.5±31.1	104.2±34.9 ^{b1}	110.4±43.7	116.9±53.2
CRP (mg/dL)	2.53±2.22	4.50±5.30	11.6±31.09	18.58±28.02	11.17±13.21 ^{b2}
Fibrinogen (mg/dL)	310±59	319±57	350±70	381±68 ^{b4}	356±90
Homocysteine (μmol/L)	9.77±3.88	10.36±2.53	10.80±3.49	11.96±5.04	11.46±5.80
GFR (mL/min)	148.7±29.5	139.9±37.5	100.4±33.1 ^{b4}	108.9±54.5 ^{b4}	102.4±42.3 ^{b4}
Microalbuminuria (mg/L)	6.33±15.9	5.70±4.5	29.9±54.4 ^{b4}	172.4±216.5 ^{b4}	59.6±101.2 ^{b2}
Urine creatinine (mg/dL)	117.4±82.2	113.9±63.1	91.8±66.2	114.9±76.2	92.1±75.8 ^{b1}
Presence of hypertension (%)	-	47	67	74	90
Presence of dyslipidemia (%)	8	89	77	80	87
Smoking (%)	4%	4%	7%	4%	3%
Therapy with insulin /metformine/sulfonylurea (%)	-	6/69/19	10/60/30	53/60/13	76/33/27
Therapy with ACE inhibitors /β-blockers/thiazides (%)	-	2.8/19/14	67/33/33	33/47/27	33/27/37
Therapy with statins / fibrates/ acarbose/gliclasone	-	28/3/0/9	52/17/9/17	58/4/4/17	73/7/8/12

Statistical difference from healthy controls: a¹, $P<0.05$; a², $P<0.01$; a³, $P<0.001$ Statistical difference from diabetic patients without complications (DM): b¹, $P<0.05$; b², $P<0.01$; b³, $P<0.005$; b⁴, $P<0.001$

diabetics than in the controls. HDL-C levels were significantly lower ($P<0.05$) in the DM group when compared with the HC group. The DM group also had higher HbA1c levels in comparison with the HC group ($P<0.001$).

When compared with the DM group, the nephropathy group had increased fibrinogen ($P<0.001$), CRP ($P<0.01$) and HbA1c levels ($P<0.05$). Plasma glucose levels were also significantly higher ($P<0.001$), while HbA1c, total and LDL-C and TG levels were significantly lower in the retinopathy group than in the DM group ($P<0.001$, $P<0.05$, $P<0.05$ and $P<0.01$, respectively).

Serum concentrations of CLU, amylin, DPP-4, SFRP-4, GLP-1_{total} and GLP-1_{active} are given in Table 2. The DM group had significantly lower DPP-4 and GLP-1_{total} levels ($P<0.005$ and $P<0.001$) and significantly higher SFRP-4 levels ($P<0.05$) in comparison with the HC group. CLU, amylin and GLP-1_{active} levels were not significantly different between the HC and DM groups. In diabetic patients with microvascular complications (i.e., the retinopathy, nephropathy and neuropathy groups), significantly higher DPP-4 and GLP-1_{total} levels were measured compared to those of diabetic patients without complications ($P<0.05$ and $P<0.001$, respectively).

None of the studied parameters were shown to be significantly different between the DM and nephropathy groups. In contrast, serum DPP-4, SFRP-4, GLP-1_{total} and GLP-1_{active} levels were significantly higher in the neuropathy group than in the DM group ($P<0.005$, $P<0.005$, $P<0.001$ and $P<0.01$, respectively), while DPP-4 and GLP-1_{total} levels were significantly higher in the retinopathy group than in the DM group ($P<0.05$ and $P<0.001$, respectively).

In the DM group, CLU levels were significantly correlated with amylin, DPP-4 and SFRP-4 levels ($r=0.892$, $P<0.001$; $r=0.888$, $P<0.001$; and $r=0.712$, $P<0.001$, respectively). Amylin was significantly correlated not only with DPP-4 ($r=0.902$, $P<0.001$) but also with SFRP-4 ($r=0.729$, $P<0.001$). DPP-4 was correlated with SFRP-4 ($r=0.722$, $P<0.001$). The duration of diabetes (given in means of years $\pm$) was correlated with both DPP-4 ($r=0.478$, $P<0.005$) and GLP-1_{total} levels ($r=0.559$, $P<0.005$). GLP-1_{total} levels were negatively correlated with total cholesterol ($r=-0.416$, $P<0.01$), LDL-C ($r=-0.605$, $P<0.01$) and GFR ($r=-0.402$, $P<0.05$). Total cholesterol and fibrinogen were negatively correlated with serum amylin levels ($r=-0.416$, $P<0.01$; and $r=-0.459$, $P<0.01$, respectively). There was also a significant correlation between fibrinogen and DPP-4 levels ($r=-0.565$, $P<0.01$).

In the DM group, CLU levels were significantly correlated with amylin, DPP-4 and SFRP-4 levels ($r=0.892$, $P<0.001$; $r=0.888$, $P<0.001$; and $r=0.712$, $P<0.001$, respectively). A

significant correlation was found between amylin and both DPP-4 ($r=0.902$, $P<0.001$) and SFRP-4 ($r=0.729$, $P<0.001$) levels (Figure 1). DPP-4 levels were correlated with SFRP-4 ($r=0.722$, $P<0.001$). The levels of total cholesterol and fibrinogen were negatively correlated with serum amylin levels ($r=-0.416$, $P<0.01$; and $r=-0.459$, $P<0.01$, respectively). There was also a significant correlation between fibrinogen and DPP-4 levels ($r=-0.565$, $P<0.01$). GLP-1_{total} levels were negatively correlated with total cholesterol ($r=-0.416$, $P<0.01$), LDL-C ($r=-0.595$, $P<0.01$) and GFR ($r=-0.455$, $P<0.05$).

Regression analysis between GLP-1_{total} and GFR, CRP and duration of diabetes are given in Table 3. In a multivariate regression analysis in diabetic patients without microvascular complications, GLP-1_{total} and GLP-1_{active} were negatively correlated with GFR ($P<0.001$ and $P<0.05$, respectively) (Table 4).

In all diabetic patients with microvascular complications (Figure 2), CLU levels were significantly correlated with amylin, DPP-4 and SFRP-4 levels ($r=0.851$, $P<0.001$; $r=0.760$, $P<0.001$; and $r=0.784$, $P<0.001$, respectively). There were significant correlations between amylin and both DPP-4 ($r=0.783$, $P<0.001$) and SFRP-4 ($r=0.796$, $P<0.001$) levels. DPP-4 levels were correlated with SFRP-4 ($r=0.684$, $P<0.001$) and GLP-1_{total} ($r=0.290$, $P<0.01$). A significant relationship was also found between GLP-1_{total} and GFR levels ($r=0.291$, $P<0.001$). The duration of diabetes was correlated with serum SFRP-4 ($r=0.376$, $P<0.01$) and serum GLP-1_{active} levels ($r=0.322$, $P<0.01$).

In the HC group, CLU levels were significantly correlated with amylin, DPP-4 and SFRP-4 levels ($r=0.822$, $P<0.001$; $r=0.918$, $P<0.001$; and $r=0.706$, $P<0.001$, respectively). There was a significant correlation between amylin and both DPP-4 ($r=0.796$, $P<0.001$) and SFRP-4 ($r=0.699$, $P<0.001$) levels. DPP-4 activity was correlated with SFRP-4 ($r=0.722$, $P<0.001$).

Discussion

Glucose homeostasis depends on a complex interplay of multiple substances, including GLP-1, DPP-4, SFRP-4, CLU and amylin. Abnormal regulation of these substances may contribute to complications of diabetes. With this study, we have shown for the first time that diabetic patients with microvascular complications have higher DPP-4 activity and GLP-1_{total} levels than diabetic patients without such complications. Diabetic patients with retinopathy have higher DPP-4 and GLP-1_{total} levels than patients in the DM group. GLP-1_{total} levels were correlated with GFR levels and DPP-4 activity in the diabetic patients with microvascular

TABLE 2. Serum concentrations of clusterin (CLU), amylin, secreted frizzled-related protein-4 (SFRP-4), total and active GLP-1 levels, DPP-4 activity in controls, and diabetic patients with or without complications

	CLU ($\mu\text{g/mL}$)	Amylin (pg/mL)	DPP-4 (ng/mL)	SFRP-4 (ng/mL)	GLP-1 _{total} (pM)	GLP-1 _{active} (pM)
Controls (n=26)	32.2 $\pm$ 20.1	508.9 $\pm$ 232.5	4.78 $\pm$ 2.38	1.54 $\pm$ 0.40	86.4 $\pm$ 32.7	4.45 $\pm$ 1.41
DM (n=25)	33.1 $\pm$ 17.4	491.4 $\pm$ 206.8	3.93 $\pm$ 2.71 ^{a2}	1.94 $\pm$ 0.62 ^{a1}	43.9 $\pm$ 16.6 ^{a3}	4.93 $\pm$ 1.72
Retinopathy (n=25)	37.4 $\pm$ 22.6	538.3 $\pm$ 256.8	4.90 $\pm$ 2.86 ^{b1}	2.18 $\pm$ 1.29	73.6 $\pm$ 28.8 ^{b5}	5.51 $\pm$ 2.68
Neuropathy (n=25)	39.5 $\pm$ 22.2	589.5 $\pm$ 260.1	5.17 $\pm$ 2.78 ^{b3}	3.01 $\pm$ 1.79 ^{b3}	83.3 $\pm$ 22.2 ^{b4}	6.71 $\pm$ 2.82 ^{b2}
Nephropathy(n=25)	30.8 $\pm$ 18.3	457.1 $\pm$ 169.8	3.19 $\pm$ 1.04	1.97 $\pm$ 0.77	43.6 $\pm$ 19.9	5.33 $\pm$ 1.61
Microvascular complications (n=75)	35.9 $\pm$ 21.8	528.3 $\pm$ 235.9	4.42 $\pm$ 2.34 ^{b1}	2.32 $\pm$ 1.43	66.8 $\pm$ 29.1 ^{b4}	5.85 $\pm$ 2.48

Statistical difference from healthy controls: a¹, $P<0.05$; a², $P<0.005$; a³, $P<0.001$ Statistical difference from diabetic patients without complications (DM): b¹, $P<0.05$; b², $P<0.01$; b³, $P<0.005$; b⁴, $P<0.001$ TABLE 3. Regression analysis between GLP-1_{total} and GFR, duration of diabetes and CRP

coefficients*						
Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	92.249	7.960		11.589	0.000
	GFR	-0.269	0.060	-0.444	-4.460	0.000
2	(Constant)	91.694	9.811		9.346	0.000
	GFR	-0.265	0.065	-0.437	-4.061	0.000
	Duration of diabetes	-0.062	0.448	-0.015	-0.139	0.890
	CRP	0.046	0.188	0.027	0.248	0.805

* Dependent variable: GLP-1_{total}TABLE 4. Regression analysis between GLP-1_{active} and GFR, duration of diabetes and CRP

coefficients*						
Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	7.061	0.699		10.107	0.000
	GFR	-0.012	0.005	-0.244	-2.261	0.026
2	(Constant)	7.589	0.855		8.882	0.000
	GFR	-0.014	0.006	-0.275	-2.384	0.020
	Duration of diabetes	-0.036	0.039	-0.105	-0.928	0.356
	CRP	-0.007	0.016	-0.050	-0.421	0.675

* Dependent variable: GLP-1_{active} pm

FIGURE 1. Correlation analysis in diabetic patients without complications.

complications. None of the patients was taking either DPP-4 inhibitory drugs or GLP-1 receptor agonists. This result can be explained via the protective effects of endogenous GLP-1 against the DPP-4 enzyme, as GLP-1 contributes to antidiabetic effects [12].

Studies on GLP-1 have focused on its role in the development and treatment of T2DM [13]. Researchers have shown that ~25% of newly secreted GLP-1 leaves the gut in an intact, active form as ~ 40–50% of GLP-1 degradation takes place in liver. It is also estimated that only ~10–15% of newly secreted, intact GLP-1 reaches the systemic circulation [14]. In the current study, T2DM patients had significantly lower GLP-1_{total} levels than healthy controls, while no difference was

FIGURE 2. Correlation analysis in diabetic patients with microvascular complications.

observed in serum GLP-1_{active} levels between healthy controls and the T2DM group. Metformin treatment did not induce any significant variation in active and total GLP-1 levels. Low GLP-1_{total} levels could be due to either decreased secretion from L-cells or more rapid degradation by DPP-4 in this study's T2DM patients. It is still unclear whether endogenous GLP-1_{total} and GLP-1_{active} levels are related to T2DM and its complications. In this study, in the diabetic patients with microvascular complications (i.e., the retinopathy, nephropathy and neuropathy groups), higher GLP-1_{total} levels were observed than in diabetic patients without complications. Serum GLP-1_{total} and GLP-1_{active} levels were also higher in the

neuropathy group than in the DM group. Diabetic patients with retinopathy had significantly higher GLP-1_{total} levels than those in the DM group. The duration of diabetes was correlated with GLP-1_{total} levels. Furthermore, in a multivariate regression analysis in diabetic patients without microvascular complications, GLP-1_{total} and GLP-1_{active} were negatively correlated with GFR. Results of GLP-1 levels in DM remain controversial: GLP-1 levels have been reported to increase (raising the possibility that beta-cell insensitivity to GLP-1 exists) [15-18], remain unchanged [19-20] or decrease [21-22]. These contradictory findings likely result from different assay specificities, such as measuring only GLP-1-containing moieties or intact GLP-1 and its inactive metabolites together [23]. In another study, GLP-1 was found to have no effect on insulin sensitivity in T2DM patients [24]. There is no evidence that the GLP-1 receptor undergoes desensitization *in vivo* studies. The more the mechanisms that underlie endogenous GLP-1 secretion are understood, the better existing GLP-1-based drugs and further GLP-1-based drug development will become [25]. Furthermore, in a multivariate regression analysis in diabetic patients without microvascular complications, GLP-1_{total} and GLP-1_{active} were negatively correlated with GFR. These results support the importance of screening and early intervention in T2DM [26].

DM patients had lower DPP-4 levels than healthy controls in this study, whereas diabetic patients with microvascular complications showed higher DPP-4 activity than diabetic patients without such complications. Serum DPP-4 activity was higher in the neuropathy and retinopathy groups than in the DM group. There were no significant differences in the studied parameters between the DM and nephropathy groups. Zheng *et al.* [27] showed that increased DPP-4 activity is strongly and independently associated with diabetic nephropathy in T2DM patients, and also that increased plasma DPP-4 activity in T2DM is independent of lifestyle factors, family history of diabetes, BMI, systolic blood pressure, blood lipids and, remarkably, blood glucose, insulin resistance, oxidative stress and inflammation. Ryskjaer *et al.* [28] suggested that plasma DPP-4 activity in the fasting state of T2DM increases regardless of the decrease in active incretin hormones, and that plasma DPP-4 activity cannot be used as a predictive index of endogenous incretin hormone degradation. On the other hand, the positive correlation between DPP-4 activity and HbA1c suggests that metabolic control may influence the level of DPP-4 activity. In the same manner, GLP-1, resulting from DPP-4 activity in DM, remains controversial. Plasma DPP-4 activity in patients with T2DM has been reported to increase [28-30], remain unchanged

[31,32] or decrease [33] in various clinical studies. Urinary DPP-4 activity as a serine protease has been shown to correlate positively with worsening glucose tolerance [34,35]. Although we cannot comment on the causal relationship between DPP-4 activity and complications from diabetes, we nonetheless attempted to explain this association by asking whether increased DPP-4 activity leads to an increased risk of neuropathy and retinopathy.

Active compounds in *Urena lobata* (*U. lobata*), including mangiferin, stigmasterol and β -sitosterol have been shown to prevent degradation of GLP-1 by inhibiting DPP-4 [36]. Purnomo *et al.* [37] observed a significant, eight-fold decrease of serum GLP-1 levels in diabetic rats compared with the control group. An aqueous extract of the leaves of *U. lobata*, at doses of 250 mg/kg bw, 500 mg/kg bw and 1000 mg/kg bw, can prevent degradation of GLP-1 by three-fold, five-fold and seven-fold, respectively, compared with a diabetic group. Increased doses of *U. lobata* extract prolong and enhance GLP-1 bioavailability.

Altenhofen *et al.* [38] showed that the acute *in vivo* effect of the major compound isolated from the aerial parts of *Polygala molluginifolia*, bis-pyrano prenyl isoflavone, exhibits an antihyperglycemic effect by improving glucose tolerance, stimulating GLP-1 secretion (increased serum GLP-1 levels) and inhibiting DPP-4 activity (decreased serum DPP-4 activity) beyond the increase of insulin secretion in hyperglycemic rats. Furuhashi *et al.* [39] reported on a study where sitagliptin (50 mg/day), a dipeptidyl peptidase 4 (DPP-4) inhibitor that increases GLP-1, was administered to patients with type 2 diabetes ($n = 24$) for 12 weeks. Eto *et al.* [40] reported that teneligliptin increased postprandial plasma GLP-1_{active} concentrations relative to a placebo. Likewise, Tsuchimochi *et al.* [41] showed that teneligliptin significantly increased both fasting and postprandial plasma GLP-1_{active} concentrations. Teneligliptin's long half-life and high DPP-4 inhibitory activity might be responsible for these effects.

Cuthbertson *et al.* [42] investigated the acute effects of metformin and GLP-1 alone or in combination with plasma DPP-4 activity, GLP-1_{active} concentrations, and glucose lowering in T2DM. DPP-4 activity after treatment with metformin and GLP-1 as well as after metformin alone was significantly lower than with GLP-1 alone. In patients with T2DM, metformin inhibits DPP-4 activity and thus increases GLP-1_{active} concentrations after subcutaneous injection. In combination with GLP-1, metformin significantly lowers plasma glucose concentrations in T2DM subjects compared with GLP-1 alone, whereas insulin responses are similar. Metformin enhances serum concentrations of injected

GLP-1_{active} (7-36) amide and the combination results in added glucose-lowering potency. Because of its short half-life, GLP-1 cannot currently be employed for clinical treatment of T2DM.

Cuthbertson *et al.* [43] also investigated the acute effects of metformin with and without standard mixed meal (SMM) on plasma DPP-4 activity in T2DM patients. Following SMM, plasma DPP-4 activity was not suppressed by metformin compared with a placebo. Plasma glucose, insulin and GLP-1_{active} were not different; however, DPP-4 activity was suppressed with metformin following fasting compared with an SMM. Metformin inhibits DPP-4 activity in T2DM patients in the fasting state, but Cuthbertson *et al.* failed to show this effect when metformin was taken with an SMM.

Increased CLU levels are associated with T2DM, metabolic syndrome and cardiovascular diseases [44,45]. Kujiraoka *et al.* [46] reported higher serum CLU levels in diabetes patients than in patients with myocardial infarction, chronic coronary artery disease, or diabetes combined with chronic coronary artery disease. In this study, T2DM patients showed CLU concentrations that were positively related to blood glucose; but after adjusting for its relation to blood glucose, mean CLU concentrations were similar in both diabetic and healthy subjects. Trougakos *et al.* [47] demonstrated that the amount of serum CLU increases significantly in patients with T2DM, which is a well-recognized risk factor for atherosclerosis, while no significant correlation was found between serum CLU levels and HbA1c. In the current study, no difference was found in serum CLU levels between healthy controls and the DM group. Differences in CLU levels may be due to medications used by patients. Nevertheless, its main function remains undefined. It is possible that CLU has either multiple, different and independent functions in the body or, conversely, has some universal regulatory function, such as acting as a chaperone by protecting cells in different tissues from stress [48]. Thus, whether increased CLU levels have beneficial or adverse effects on the cardiovascular system remains inconclusive [49].

Amylin, as a neuroendocrine hormone, can aggregate in the pancreas of T2DM patients, which could lead to a low level of soluble amylin in plasma [50]; however, such results are controversial. Plasma amylin concentrations have been reported to be higher, similar, and lower in different studies on individuals with T2DM [51-55]. In the current study, no difference in serum amylin levels was observed between HC, the DM group and the diabetic microvascular complications group. Amylin levels were significantly correlated with CLU, SFRP-4 and DPP-4 activity in T2DM patients both with and

without microvascular complications. As the results are similar and show no difference from prediabetic to diabetic stages, the only explanation is that amylin plays a significant role in neuroendocrine contribution to glucose homeostasis. Different treatments may improve glycemic control and insulin secretion without the adverse effects of severe hypoglycemia in T2DM [7].

Study limitations

SFRP-4 acts by decreasing insulin secretion from pancreatic beta cells; therefore, SFRP-4 could play an important role in the pathogenesis of T2DM [56]. In this study, DM patients had higher SFRP-4 levels than healthy controls, while serum SFRP-4 levels were higher in the neuropathy group than in the DM group. At the same time, SFRP-4 levels were correlated with the duration of diabetes, DPP-4, CLU and amylin levels in the DM group. Garufi *et al.* [57] showed that T2DM subjects had elevated circulating SFRP-4 when compared to lean subjects, but not when compared to obese patients without T2DM. They found a strong correlation between circulating SFRP-4 and insulin resistance, concluding that circulating SFRP-4 may play a role as a biomarker for T2DM. Our results are in accordance with other studies that have found serum SFRP-4 levels in diabetics to be associated with elevated fasting glucose and reduced dispositional index [56-58]. Circulating SFRP-4 may participate in the development of T2DM and seems to be related to deteriorating glucose metabolism as well.

The number of the patients evaluated in this study may be inadequate for generalizable conclusions. Additionally, biomarkers were measured only once, so the data obtained might have been stronger had the measures been verified. Results of longitudinal variations in our subjects were unavailable, as was information about the drugs they used and their effects.

Summary

In summary, our study revealed that levels of some serum biomarkers were abnormal (increased GLP-1_{total} and DPP-4 activity, decreased SFRP-4 levels) in patients with T2DM. Complications of diabetes were not strongly related to the dysregulation of GLP-1 production or secretion. DPP-4 activity and GLP-1_{total} secretion were significantly impaired in diabetic patients with retinopathy and neuropathy, most likely as a consequence of the disease. SFRP-4 may play an important role in the pathogenesis of T2DM. Further prospective studies with larger populations and randomized clinical trials are

needed to clarify the impact of DPP-4 and GLP-1 on the complications of diabetes and, eventually, disturbances related to glucose metabolism in T2DM.

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