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Circulating dipeptidyl peptidase-4 activity is associated with insulin resistance in type 1 diabetic patients

Short title: DPP4 activity and insulin resistance

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Abstract

Aim: The pathophysiology of insulin resistance (IR) comprises a complex adipokine mediated cross-talk between white adipose tissue and other organs. Dipeptidyl peptidase-4 (DPP4) is protease recently proposed as a novel adipokine linked to IR. We aimed to assess the relationship between fasting serum DPP4 activityand IR in type 1 diabetic (T1DM) patients.

Methods: A cross-sectional study comprised 44 T1DM patients aged >18 and <65 years. IR was esimated using the equation for insulin sensitivity derived from euglycemic-hyperinsulinemic clamp studies-estimated glucose disposal rate (eGDR). DPP4 serum activity was determined spectrophotometrically as a rate of cleavage of 7-Amino-4-Methyl Coumarin (AMC) from H-Gly-Pro-AMC.

Results: Patients were divided according to DPP4 activity tertiles (<25.40; ≥36.54 U/L). Fasting serum DPP4 activity was related to disease duration (p=0.012), systolic (p=0.009) and diastolic (p=0.047) blood pressure, waist circumference (p=0.037), urine albumin excretion (p=0.022) and conversely related to eGDR (p=0.004). The linear regression has shown that eGDR decreases for 0.203 $mgkg^{-1}min^{-1}$ by each increase of serum DPP4 activity of 1 U/L (p<0.001) after adjustment for adjusted for age, gender, disease duration, albuminuria and the use of antihypertensives and statins.

Conclusion: Serum DPP4 activity is associated with IR in T1DM patients and it might play an important role in its pathophysiology.

Key words: dipeptidyl peptidase-4, insulin resistance, type 1 diabetes

1. Introduction

Insulin resistance (IR) is an inability of insulin to produce its actions in peripheral tissue derived either from interference of insulin binding to its surface receptor or by impairment of

insulin signalization distal from the cell surface [1-3]. Although IR typically characterise type 2 diabetes mellitus (T2DM), while the insulin deficiency is considered as a primary defect in type 1 diabetes mellitus (T1DM), a consistent body of literature suggests that there is a certain degree of IR in patients with T1DM [4, 5]. The mechanisms of IR in T1DM is likely due to a combination of supraphysiologic levels of exogenous insulin and obesity. In the past, it was thought that IR in T1D was primarily related to hyperglycemia [6]. It was recently proposed that adults with T1DM have both impaired glucose utilization and impaired insulin-induced non-esterified fatty acid suppression, independent of glycemic control [7]. Skeletal muscle IR is a known feature of T1DM and is due to decreased glucose transport into myocytes from impaired insulin-stimulated upregulation of GLUT4 transporter [8]. White adipose tissue has been recognized as major endocrine organ producing a huge diversity of adipokines which build a complex inter- and intra- cellular feedback loops that could link obesity to IR [9, 10]. Lamers et al. (2011) [11] have performed a comprehensive proteomic profiling of the media derived from primary human adipocytes and proposed dipeptidyl peptidase-4 (DPP4) as a novel adipokine linking adipose tissue to IR.

DPP4 is a serine exopeptidase also known as adenosine desaminase complexing protein 2 (ADCP 2) or T-cell activation antigen CD26 which cleaves X-proline dipeptides from the N-terminus polypeptides such as chemokines, neuropeptides and peptide hormones [12]. It exists in two forms: as an integral membrane glycoprotein expressed ubiquitously on the cell surface and in a soluble form in the circulation. A fraction of soluble DPP4 originates from the immune system cells which explains its altered abundance and the circulating activity in various immune mediated conditions [13] although the major source of soluble DPP4 fraction remains unknown. Since T1DM is an immune mediated condition, the reports on elevated DPP4 activity in serum is not suprising [14]. The data from two independent studies suggest that DPP4 activity is higher in patients with T1DM compared to healthy controls but

independently of islet-cell antibody status, C-peptide level, disease duration or glycated haemoglobin (HbA1c) level [14, 15] However, they do report an inverse correlation with body mass index and insulin sensitivity [15]. Serum DPP4 activity is also higher in T2DM individuals with IR compared to those without [16]. DPP4 is highly expressed on kidney cell surface and data are available to suggest that DPP4 levels may associated with of renal function [17]. Moreover, insulin sensitivity, assessed by hyperinsulinemic-euglycemic clamp, is continuously associated with a greater risk of increasing albuminuria [18]. Accordingly, the aim of this study was to investigate relationship between fasting serum DPP4 activity, insulin resistance and renal function in T1DM patients.

2. Subjects

This cross-sectional was study undertaken at the University Clinic for diabetes, endocrinology and metabolic diseases Vuk Vrhovac (Zagreb, Croatia). We recruited 44 T1DM C-peptide negative (C-peptide <0.3 ng/mL) patients aged >18 and <65 years comming for their comprehensive annual review. The sample size was in accordance with G power 3.1.7 calculation for correlations (two tailed t test, total sample size=44, α=0.05, 1-β=0.8, ρ=0.4). The diagnosis of T1DM was defined as suggested by American Diabetes Association guidlines from the year 2010 [19]. The inclusion criteria were: age at onset of diabetes younger than 40 years, positive autoantibodies and time to definite insulin therapy less then a year. Non inclusion criteria were: medical history of cardiovascular diseases or electrocardiogram (ECG) evidence of ischemic heart disease, any systemic disease and any infection in the previous month, thyroid hormone therapy, medications that might affect glucose metabolism and insulin sensitivity such as glucocorticoids or oral contraceptives. The study subjects could be using antihypertensive or lipid lowering drugs (i.e., statins:

atorvastatin and simvastatin). The study was conducted according to the guidelines laid down in the Declaration of Helsinki. Written informed consent was obtained from and signed by all patients.

2.1 *Subjects and Methods*

Insulin sensitivity was calculated using the equation derived from euglycemic-hyperinsulinemic clamp studies, estimated glucose disposal rate (eGDR): 24.31–12.2X(WHR)–3.29X(AHT)–0.57X(HbA1c), where the units are $mgkg^{-1}min^{-1}$, WHR indicates the waist to hip ratio, AHT indicates blood pressure, and is expressed as: 0-no, 1-yes. Those on blood pressure medications or with blood pressure >140/90 mmHg were considered to have hypertension the equation was derived from a substudy of 24 EDC (Epidemiology of Diabetes Complications) participants who underwent euglycemic-hyperinsulinemic clamp studies [20]. Lower eGDR levels indicate greater insulin resistance.

2.2 *Laboratory analysis*

The detailed description of the methods concerning anthropometric measurement and standard laboratory procedurs was conducted as previously described [21]. Fasting venous blood samples were collected for the determination of biochemistry panel, lipid profile status, glycated haemoglobin A1c (HbA1c) and serum DPP4 activity. After clotting, the sera were separated and kept at −70°C until the determination of enzymatic activity. Urin albumin excretion (UAE) was measured from at least two 24-h urine samples and determined as the mean of 24-h urine collections and expressed as mg/24h. Patients performed collections on two consecutive days to minimize variability. Data on serum creatinine levels, age, sex and race were used to calculate the estimated GFR (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula, which has been shown to be accurate in

determining renal function in diabetic patients with normal renal function [22, 23]. DPP4 activity was measured by a colorimetric assay procured from Sigma, St. Louis, MO, USA in a microplate reader (Cary Eclipse Varian, Agilent Technologies) at 460 nm, 37^0C in a continuous monitoring for 35 minutes. In this assay, DPP4 cleaves H-Gly-Pro-AMC to release a florecent product, 7-Amino-4-Methyl Coumarin (AMC) which can be measured spectrophotometrically. All the DPP4 assays were run in duplicates. Briefly, 50 µL of serum sample was added to 96-well plates, followed by the addition of 10 µL assay buffer. After 10 min of pre-incubation at 37^0, the enzymatic reaction was started with the addition of 40 µL of Master Reaction Mix containing 2 µL substrate and 38 µL of the assay buffer. Liberation of AMC was monitored continuously at excitation 360 nm and emission 460 nm every 5 min for up to 35 min in a 96-well black flat bottom plate. Fluorometric catalysis rates were determined from the linear portion of the curve of the increase in fluorescence and were calculated as the slope of the regression line determined from the line. DPP4 was expressed as pmole/min/mL (U/L). One unit of activity was defined as the amount of enzyme which will hydrolize the DPP4 substrate to yiled 1.0 µmole of AMC per minute at 37^0C.

2.3 *Data analysis and Statistics*

The data distribution was assessed by Shapiro-Wilk test. All the continuous variables were log-transfrormed and reported as mean values and 95%CI of means, whereas categorical variables were reported as numbers and percentages. Because we found normal distribution of the data, the differences between three study groups were tested by one-way ANOVA followed by Bonferroni's correction for multiple comparisons while the categorical variables were analysed by the χ^2 test. Correlations between fasting serum DPP4 activity with anthropometric and metabolic variables were determined using Pearson's correlation

coefficient. All the tests were two-sided. The association between fasting serum DPP4 activity and eGDR value was further evaluated in multivariate linear regression. Adjustments were performed for age, gender, disease duration, eGFR, UAE, the use of statins and antihypertensive agents since it is yet to be clarified whether they affect serum DPP4 activity. Level of statistical significance was chosen to be 0.05. Statistical analysis was performed by Statistical Package for the Social Sciences (SPSS) ver. 17.0 and MedCalc 11.0 for Windows.

3. Results

The clinical and biochemical characteristics of all 44 T1DM patients are given in Table 1. Out of 44 study participants, 28 (63.6%) were male, mean age approximately 45 years and 21 years of diabetes duration. Thirty patients (68.2%) were using statins and 22 (50%) antihypertensive agents, i.e. angiotensin-converting enzyme inhibitors (ACEI). Patients were divided into three groups according to the tertiles (25^{th}, 50^{th} and 75^{th}) of fasting serum DPP4 activity. The detailed clinical and laboratory findings and the difference between them are given in Table 2. The group of patients in the 1^{st} tertile of fasting serum DPP4 activity had significantly shorter disease duration (p=0.012), lowest systolic (p=0.009) and diastolic (p=0.047) blood pressure, waist circumference (p=0.037) and UAE (p=0.022)compared to second and third tertile. The eGDR was significantly higher in the group with lowest serum DPP4 activity compared to tertiles with higher DPP4 activity (p=0.004). The groups also showed significant difference in the statins use (7 (46.6%) *vs* 10 (66.6%) *vs* 13 (92.8%), p<0.001). Fasting serum DPP4 activity showed positive correlation with age (r=0.321, p=0.034), systolic blood pressure (r=0.423, p=0.004), UAE (r=0.279, p=0.014) anddisease duration (r=0.474, p=0.001) while negative correlation with eGDR (r=-0.612, p<0.001). The simple linear regression with eGDR as dependent variable has shown that eGDR significantly

decreases for 0.221 $mgkg^{-1}min^{-1}$ for each increase of serum DPP4 activity of 1 U/L ($p<0.001$) in the unadjusted model and for 0.155 $mgkg^{-1}min^{-1}$ after adjustment for the possible confounders (age, gender, disease duration, UAE and eGFR and the use of ACEI agents as well as statins) (Table 3.).

4. Discussion

We assessed metabolic variables, insulin sensitivity and renal function parameters in three groups of T1DM patients according to the mean values of fasting serum DPP4 activity. The group of patients with highest DPP4 activity had the lowest insulin sensitivity after controlling for all possible factors affecting DPP4 serum activity [15]. There are only several reports on serum DPP4 activity in T1DM, with one study reporting a significant correlation between serum DPP4 activity and HbA1c [24] and another suggesting the opposite [14]. We could neither confirm or reject the correlation between serum DPP4 activity and HbA1c since the group of patients in the second tertile had highest HbA1c but the difference did not reach statistical significance. Furthermore, Varga et al. (2010.) [16] determined serum DPP4 activity at fasting sate and after test meal in 41 T1DM, 87 T2DM patients and in 25 healthy volunteers. Serum DPP4 activity was significantly higher both in fasting and postprandial state in patients with T1DM than in T2DM and control subjects irrespective of HbA1c or fasting serum glucose. Those results suggest that the type of diabetes might be an important factor in DPP4 activity determination.

Since T1DM is an autoimmune disease, we can hypothesise that elevated serum DPP4 activity might be attributable to variety of inflammatory reactions leading to pancreatic β cell destruction. Despite, two independent studies indicate that serum enzyme activity is not affected by islet-cell or glutamic acid decarboxylase antibodies [14, 15]. Here we found

significantly higher DPP4 activity in subjects with longer disease duration. We speculate that DPP4 activity is correlated with long term exposure to a high glucose concentration.

Similar to Iwabuchi et al. (2013) [15] we found no correlation between enzyme activity in serum and BMI, although the BMI was slightly higher in the third tertile. The measurements of central obesity, i.e. waist circumference and waist-to-hip ratio were significantly higher in the same group. That is in accordance with the well established fact that visceral adipose tissue is the key player in cytokine dysregulation leading to IR [5, 25]. Lamers et al. (2011) [11] suggested that both cell surface resident and soluble DPP4 provide multiple autocrine and paracrine functions resulting in IR and MS since although they did not assess the DPP4 enzymatic activity but its concentration. They showed that DPP4 treatment of primarily human adypocites, skeletal and smooth muscle cells results in a dose-dependent decrease in insulin-stimulated Akt phosphorylation, one of the key players in intracellular insulin signalling pathways **[**26] which clearly referees to DPP4 enzymatic activity and not concentration. In addition, they also demonstrated negative correlation of DPP4 serum concentration with adiponectin levels which was further confirmed regarding the DPP4 serum activity [15]. Adiponectin is already described as a factor that might play a protective role on IR development in general [27], as well as in T1DM population [21]. Furthermore, we also demonstrated that systolic and diastolic blood pressure are lowest in the first tertile and that systolic blood pressure correlates positively with enzymatic activity. With respect to this and the recent data on high expression of this enzyme in the kidney [17] there is a possibility that kidney function may play a role in DPP4 serum activity. Following the data interpretation in the light of indexes of kidney function, DPP4 serum activity remained independently associated with lower insulin sensitivity. In support to our study results, Yang et al. (2014) [28] have recently demonstrated that DPP4 activity is significantly higher in healthy subjects with higher blood pressure and insulin resistance determinated by HOMA-IR index in

apparently healthy Chinese man and woman. Recent experimental and clinical studies suggest that DPP4 inhibition reduces blood pressure [29] although the underlying mechanism remains poorly understood. Currently, there are evidence that DPP4 inhibition leads to increased nitric-oxide (NO) bioavailability but the question whether the it occurs in a direct or indirect pathway indicating the potential role of DPP4 substrates, especially *via* glucagon-like peptide-1 (GLP-1) receptor [30, 31].

Our study has several limitations that should be pointed out: the sample size was too small to derivate any general conclusions, the insulin sensitivity was not assessed by a euglycemic clamp which is a gold standard in insulin sensitivity determination and we did not measure any DPP4 substrates in the circulation. It is expected that if the serum DPP4 activity is increased there should be changes in the circulating levels of a number of substrates of DPP4. However, we recently found that the circulating GLP-1 is lower in T1DM population with higher IR compared to those without as well as when compared to healthy population [32] which could partially explain this study results since the inclusion criteria were the same. Despite, we can conclude that serum DPP4 activity may be a surrogate marker of IR in T1DM patients or that it might play an important role in its pathophysiology. However, it is possible that DPP4 might activate inflammatory response resulting in IR or that serum DPP4 activity may be secondary to diabetic nephropathy rather than IR *per se* which cannot be accessed from s single, small-group cross-sectional study. Further investigation with comprehensive evaluation of insulin sensitivity in a larger sample are warranted to elucidate the role soluble DPP4 activity in IR development, based on the present study results, DPP4 inhibition might offer an alternative therapeutical approach in T1DM offering a potential protective effect in metabolic deterioration in T1DM population.

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Table 1. Clinical characteristics and the fasting serum DPP-4 activities of type 1 diabetic patients with mean values indicated in bold letters and (95% Confidence Intervals of means indicated in parenthesis) or absolute numbers in bold letters (and percentages in parenthesis)

Age (years)	**45,36**	(41,47 - 49,26)
Gender (male, n (%))	**28**	(63.6%)
Diabetes duration (years)	**23,71**	(20,42 - 26,99)
HbA1c (%)	**7,4**	(7,1 - 7,7)
BMI (kg/m^2)	**25,93**	(24,98 - 26,88)
Waist circumference (cm)	**89,29**	(86,02 - 92,57)
WHR	**0,883**	(0,859 - 0,907)
Systolic blood pressure (mmHg)	**128,97**	(124,09 - 133,86)
Dyastolic blood pressure (mmHg)	**80,91**	(78,59 - 83,24)
Total cholesterol (mmol/L)	**4,9**	(4,7 - 5,2)
HDL cholesterol (mmol/L)	**1,59**	(1,51 - 1,69)
HDL2 (mmol/L)	**0,49**	(0,44 - 0,54)
HDL3 (mmol/L)	**1,11**	(1,03 - 1,18)
LDL cholesterol (mmol/L)	**2,85**	(2,61 - 3,09)
TG (mmol/L)	**1,09**	(0,97 - 1,23)
VLDL cholesterol (mmol/L)	**0,51**	(0,45 - 0,56)
DPP4 activity (U/L)	**31,42**	(29,68 - 33,15)
eGDR ($mgkg^{-1}min^{-1}$)	**7,11**	(6,47 - 7,73)
UAE (mg/24h)	**63,41**	(4,97-121,85)

Legend: eGFR:estimated glomerular filtration rate; eGDR:estimated glucose disposal rate; UAE:urian albumin excretion

Table 2. Clinical and laboratory characteristics according to the fasting serum DPP-4 activity tertiles (<25.40 U/L; ≥36.54 U/L) of type 1 diabetic patients with mean values indicated in bold letters and (95% Confidence Intervals of means indicated in parenthesis) or absolute numbers in bold letters (and percentages in parenthesis)

	Percentiles of DPP4 activity (U/L)					
	25th n=15		50th n=15		75th n=14	
	Mean	95% CI	Mean	95% CI	Mean	95% CI
DPP4 activity (U/L)*	**24.66**	23.52-25.82	**32.38**	31.05-33.69	**37.62**	36.87-38.86
Age (years)*	**42,13**	35,09 - 49,17	**40,87**	33,35 - 48,38	**53,64**	49,09 - 58,19
Gender (male, N (%))	**11**	(73.3%)	**6**	(40%)	**11**	(78.6%)
Diabetes duration (years)*	**18,53**	13,44 - 23,63	**22,87**	17,45 - 28,28	**30,14**	23,88 - 36,41
Fasting plasma glucose (mmol/L)	**6,4**	5,2 - 7,5	**6,7**	5,6 - 7,8	**9,3**	**6,8 - 11,7**
HbA1c (%)	**6,9**	6,5 - 7,5	**8,1**	7,1 - 9,1	**7,4**	6,9 - 7,9
BMI (kg/m^2)	**25,53**	24,39 - 26,68	**25,93**	23,69 - 28,17	**26,36**	24,55 - 28,16
Total daily insulin requirement (U/kg)	**0,615**	0,508 - 0,722	**0,651**	0,563 - 0,740	**0,657**	0,564 - 0,749
Waist circumference (cm)*	**86,47**	79,37 - 93,57	**90,43**	84,53 - 96,33	**91,07**	86,08 - 96,05
Waist-to-height-ratio*	**0,855**	0,814 - 0,896	**0,888**	0,850 - 0,925	**0,908**	0,858 - 0,958
Systolic blood pressure* (mmHg)	**119,67**	114,28 - 125,05	**131,67**	122,33 - 141,01	**136,07**	126,39 - 145,79
Diastolic blood pressure (mmHg)*	**77,33**	74,59 - 80,08	**82,50**	77,34 - 87,66	**83,00**	78,71 - 87,29
Total cholesterol (mmol/L)	**4,73**	4,34 - 5,14	**4,99**	4,59 - 5,37	**5,24**	4,64 - 5,83
HDL cholesterol (mmol/L)*	**1,75**	1,54 - 1,96	**1,52**	1,38 - 1,65	**1,51**	1,41 - 1,62
LDL cholesterol (mmol/L)	**2,53**	2,16 - 2,91	**2,96**	2,57 - 3,35	**3,07**	2,51 - 3,63
VLDL cholesterol (mmol/L)	**0,47**	0,39 - 0,58	**0,52**	0,40 - 0,63	**0,56**	0,46 - 0,68
Triglycerides (mmol/L)	**1,04**	0,84 - 1,29	**1,13**	0,87 - 1,38	**1,21**	0,99 - 1,48
eGDR (mgkg^{-1}min^{-1})*	**8,487**	7,228 - 9,747	**6,932**	5,995 - 7,868	**5,796**	5,129 - 6,463
UAE (mg/24h)*	**8,15**	4,92-13,41	**11,99**	5,23-27,14	**20,36**	7,35-56,39

Legend: *p<0.05; eGFR:estimated glomerular filtration rate; eGDR:estimated glucose disposal rate; UAE:urian albumin excretion

Table 3. Linear regression analysis for the serum DPP4 activity (U/L) and the eGDR ($mgkg^{-1}min^{-1}$) derived from five separate models

Model	B	p value	95,0% Confidence Interval for B	
			Lower Bound	Upper Bound
Serum Dpp4 activity (U/L)	-0.221	<0.001	-0.331	-0.132
Serum Dpp4 activity (U/L) adjusted for age and gender	-0.219	<0.001	-0.303	-0.135
Serum Dpp4 activity (U/L) adjusted for age, gender and disease duration	-0.223	<0.001	-0.313	-0.133
Serum Dpp4 activity (U/L) adjusted for age, gender, disease duration and the use of statins	-0.156	0.005	-0.265	-0.047
Serum Dpp4 activity (U/L) adjusted for age, gender, disease duration, the the use of statins and the use of antihypertensive agents (i.e. ACEI)	-0.155	0.007	-0.264	-0.046
Serum Dpp4 activity (U/L) adjusted for age, gender, disease duration, the the use of statins, UAE, eGFR and the use of antihypertensive agents (i.e. ACEI)	-0,203	<0.001	-0,298	-0,108

Legend: UAE-urine albumin excretion; eGFR-estimated glomerular filtration rate