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

Short title: Diabetic retinopathy and DPP-4 activity-is there a loop?

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Introduction

Diabetic retinopathy (DR) is an important complication of diabetes which progresses from mild to moderate nonproliferative change in retinal microvascular architecture and arterial blood flow to severe proliferative vascular abnormalities [1, 2]. It is widely recognized as the most frequent cause of visual impairment and legal blindness among adults aged 20-74 [3], especially in type 1 diabetic (T1DM) patients [4]. According to the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) nearly all T1DM patients develop retinopathy during the first two decades of the disease [5]. Hyperglycaemia has been proposed as a major risk factor of DR development and progression in The Diabetes Control and Complications Trial (DCCT) [6]. Numerous studies suggested that hyperglycaemia induces hemodynamic pathways leading to endothelial dysfunction which plays a crucial role in intimal denudation, DR induction and deterioration [7, 8]. Interestingly, there is also evidence that oscillating hyperglycaemia may cause more damage than persistent hyperglycaemia, probably due to increase in oxidative stress [9]. The retinal endothelium has a limited capacity of self-reparation since it is made of terminally differentiated cells with a low proliferative potential. That is why the reparation process is accomplished through the endothelial progenitor cells (EPCs) located in the bone marrow and mobilized into circulation mostly via the up regulation of the stromal derived factor α (SDFα) or its receptor [10]. The impairment in this process is demonstrated in experimental diabetes models [11]. SDFα is a pro-angiogenic peptide cleaved by enzymatic activity of dipeptidyl peptidase-4 (DPP4), a protease with elevated ubiquitously expression and activity in T1DM subjects [12, 13]. DPP4 inhibitors are successfully implemented in treatment of type 2 diabetes mellitus (T2DM) as a group of oral hypoglycaemic agents preventing the degradation of

endogenous incretin hormones: glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) which seem to have an effect beyond glycaemic control [14]. There are several studies indicating that DPP4 inhibitors might have beneficial effect on nonproliferative retinopathy (NPR) development as well as on its progression to proliferative retinopathy (PR) [15- 17]. Thus, we aimed to explore the relationship between serum DPP4 activity and DR in T1DM patients.

Materials and methods

This cross-sectional study undertaken at the University Clinic for diabetes, endocrinology and metabolic diseases Vuk Vrhovac (Zagreb, Croatia) recruited 44 T1DM C-peptide negative (C-peptide <0.5 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 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 detailed description of the methods concerning anthropometric measurement and standard laboratory procedurs was conducted as previously described [19]. 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. 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 activity 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.

Diabetic retinopathy was tested in specialized clinical ambulance by ophthalmologist-retinal specialist. Retinopathy was diagnosed by binocular indirect slit lamp fundoscopy and fundus photography after mydriasis with eye drops containing 0.5 % tropicamide and 5 % phenylephrine. Color fundus photographs of two fields (macular field, disc/nasal field; macular field: positioned in such a way that the exact center of the optic disc laid at the nasal end of the horizontal meridian of the field view; disc/nasal field: such that the optic disc was positioned one disc-diameter in from the

temporal edge of the field, on the horizontal meridian) of both eyes were taken with a suitable 45° fundus camera (VISUCAM, Zeiss) according to the EURODIAB retinal photography methodology [19]. EURODIAB classification scheme was used because it uses two-field 45° fundus photography and standard photographs to grade retinal lesions. In each patient the "worse" eye was graded for retinopathy using fundus photographs. Patients were classified in 2 groups: absence of DR and DR (both PR+NPR) presence since NPR may progress to PR and therefore, patients with DR may have possibly belong to the second group at a certain time point.

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 differences between two study groups were tested by Student's t-test while the categorical variables were analysed by the $\chi 2$ test. Binary logistic regression analysis was used to predict the probabilities of the associations of variables that reached significance in the univariate analysis with DR groups including the non-significant possible confounding factors (age and gender). 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.

Results

The clinical characteristics and the fasting serum DPP4 activity of total 44 T1DM patients included in this cross-sectional study are detailed described in Table 1. Briefly, there were 28 (63.6%) male patients, mean age 45.36 years, diabetes duration 23.71 years, glycated haemoglobin A1c (HbA1c) of 7.4%. Thirty patients (68.2%) were using statins, i.e. atorvastatin and 22 (50%) antihypertensive agents. Twelve (27.3%) patients have had previous laser photocoagulation due to PR. They were stratified into two groups according to retinopathy prevalence. Group 1 comprised 14 (31.85%) with DR absence and Group 2 30 (68.15%) patients with both NPR (N=21, 47.70%) and PR (N=9, 20.45%) prevalence. The group 1 had significantly shorter diabetes duration (16.43 *vs* 27.1 years, p=0.002), lower HbA1c level (6,9 *vs* 7,9 *vs* 7,7 %, p=0.019) as well as lower fasting serum DPP4 activity (25,85 *vs* 33,39 *vs* 34,91 U/L, p<0.001) when compared to second group (Table 2.).History of previous laser photocoagulation was positive only in the second group (12 vs 0; p<0.00001). There were no patients with the history of laser photocoagulation due to macular oedema. The between group difference in the statins of antihypertensive therapy use did not reach statistical difference (data not shown). In the binary logistic regression model adjusted for age, gender, diabetes duration, HbA1c level and history of laser photocoagulation DPP4 activity remained associated with DR (1.637 (1.129-2.373); p=0.009)) (Table 3.).

Discussion

Since DPP-4 inhibitors have been successfully introduced in T2DM treatment showing beneficial pleiotropic effect beyond glycaemic control for this class of drugs [14, 20] and simultaneously growing body of evidence about increased serum DPP4 activity in T1DM patients [12, 13], this cross-sectional study was designed primarily in order to elucidate the possible association of serum DPP4 activity and DR as most

frequent microvascular complication in diabetes [3, 4]. Metabolic and anthropometric variables and fasting serum DPP4 activity were assessed in two groups of T1DM patients according to the DR prevalence. Beyond already well-established factors associated with DR (diabetes duration and HbA1c), we showed that serum DPP4 activity is significantly higher in T1DM patients with DR and that the association remained significant in the binary regression analysis after appropriate adjustments. To the best of our knowledge there is no data about DPP4 activity and DR in T1DM at the time. Although the effect of DPP4 inhibition on diabetic vasculature is still being discussed, their clinical application in T2DM glycaemia management is reported to provide beneficial effects regarding oxidation, apoptosis and vasoprotection, suggesting that it might be a useful tool in DR improvement [15-17].

It is well established that EPCs are nowdays considered a mirror of cardiovascular health [21] and their circulating levels are decreased in diabetic subjects compared to healthy controls [10].Tissue ischemia is the strongest stimulus for EPCs mobilization from bone marrow [11]. Avogardo et al. (2011) [8] proposed that the majority of cytokines which mediate EPCs mobilization do so via modulation SDFα or its receptor, CXCR4. Moreover, in order to heal damaged endothelium, EPCs are required to migrate from bloodstream to target tissues which is again mediated by high local levels of SDFα. All those pathways are attenuated by inceased DPP4 activity since it degradates SDFα. That is why they consider pharmacological inhibition of circulating DPP4 activity might restaurate hyperglycaemia induced endothelial dysfunction, i.e. mediate endothelial repairment. In theory, that could explain our finding regarding increased DPP4 activity and the DR prevalence, especially when concerning NPR. However, since NPR may progress to PR, the increased DPP4 activity association with a DR prevalence might seem paradoxically.

It is expected that higher DPP4 activity leads to SDFα cleavage and the decreased neovascularization process which exists in PR. However, this DPP4 mediated retinopathy angiogenic paradox can currently be explained by two theories. First, there are evidence that peripheral EPCs from human T1DM patients with PR display higher clonogenic potential and enhaced endothelial differentiation [22, 23] so we may speculate that those EPCs overcome the process of endothelial reparation and lead to pathological endothelial proliferation. Second, hyperglycaemia induced retinal ischemia induces pericyte loss, an early event leading to endothelial activation and the release of local pro angiogenic factors resulting in endothelial proliferation and PR development [8, 25]. In addition, DPP4 inhibition reduces microvascular tone through direct nitric oxide (NO) system preventing acute retinal ischemia events [26]. Therefore, we can generate the conclusion that increased serum DPP4 activity might increase vascular tonus resulting in pericyte loss and retinal endothelial proliferation. Finally, but not less important to emphasise once again, hyperglycaemia expressed as HbA1c is recognized as the most important factor for DR development and progression in T1DM [6]. Increased systemic DPP4 activity degradates incretine hormones GLP-1 and GIP which analogues are primarily used in T2DM [24] glucoregulation, but might also benefit glycaemic control in patients with T1DM as recently suggested [27]. The use of GLP-1 analogues provides blood lipid improvements, lowers blood pressure and improves markers of renal function, in particularly, albuminuria which are all shown to be associated with DR [6, 7], in addition it has been observed the more homogenecity with human GLP-1 the observed effect seem to provide a better effect [14, 24, 28, 29]. The increased serum DPP4 activity might increase the degradation process of endogenous GLP-1

resulting in worsening of the mentioned parameters which has also been observed in our study results although the difference did not reach statistical difference.

This study has several limitations that should be pointed out: the sample size was too small and we accessed only systemic DPP4 activity, did not measure EPCs nor GLP-1 so any general conclusion about its relationship with DR cannot be made. Despite, we can conclude that serum DPP4 activity may be associated with both DR types in T1DM patients independently of HbA1c and diabetes duration or that it might play an important role in pathophysiology of its development. However, further study investigation in a larger sample are warranted to elucidate the question whether there is a strong association between DPP4 activity and DR severity and/or progression and if so, what is the exact underlying mechanism.

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Tables

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)
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)
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)
Urine albumine excretion (mg/24h)	**12,41**	(8,022 – 19,18)
CKD-EPI ($mL\ min^{-1}\ 1.73m^{-2}$)	**100,71**	(94,27-107,13)
Nonproliferative retinopathy (%)	**21**	(47.7 %)
Proliferative retinopathy (%)	**9**	(20.45 %)

Table 2. Clinical and laboratory characteristics according to the diabetic retinopathy prevalence among groups 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)

	Without retinopathy (N=14)		With retinopathy (N=30)	
	Mean	95% CI	Mean	95% CI
Age (years)	44,64	37,24 - 52,05	45,7	40,84 - 50,56
Gender (male, N (%)	11 (78,57%)		17 (56,67%)	
Diabetes duration (years)*	16,43	12,44 - 20,42	27,1	23,09 - 31,12
HbA1c (%)*	6,9	6,4 - 7,4	7,7	7,19 - 8,16
BMI (kg/m^2)	26,43	24,62 - 28,24	25,7	24,52 - 26,87
Systolic blood pressure (mmHg)	125,94	117,65 - 134,81	130	123,92 - 136,09
Dyastolic blood pressure (mmHg)	78,69	75,16 - 82,39	76.7	70,31 - 83,09
Resting hearth rate (beats per minute)	71,79	62,92 - 80,66	78,38	69,93 - 86,84
Total cholesterol (mmol/L)	4,97	4,54 - 5,42	4,94	4,64 - 5,25

HDL cholesterol (mmol/L)	1,52	1,41 - 1,63	1,63	1,51 - 1,75
LDL cholesterol (mmol/L)	2,96	2,53 - 3,34	2,80	2,48 - 3,11
TG (mmol/L)	1,04	0,89 - 1,23	1,21	1,02 - 1,41
VLDL cholesterol (mmol/L)	0,47	0,41 - 0,56	0,56	0,47 - 0,65
DPP4 activity (U/L)*+	25,85	23,45 28,46	33,84	32,24 - 35,45
Urine albumine excretion (mg/24h)	8,71	3,73 - 20,29	14,54	8,56 - 24,70
CKD-EPI (mL min^{-1} $1.73m^{-2}$)	102,57	91,54 - 113,61	99,83	91,51 - 108,16

Legend: *p<0.05; +standard deviation of the DPP4 activity for the group without DR is ±4.83 and for the group with DR is ±4.31 U/L.

Table 3. Logistic regression analysis of DPP4 activity with presence of DR. Data are OR (95% CI) from separate models.

Independent variable	Model A	Model B	Model C	Model D
DPP4 activity (U/L)	1.384(1.151- 1.664)*	1,403(1.154-1.705)*	1,391(1.139-1.698)*	1.887 (1.073-3.321)*
	MODEL E			
	1.637 (1.129-2.737)			

*p < 0.05.

Legend: Model A-crude model; Model B-crude model adjusted bx age and gender; Model C-crude model adjusted by age, gender and disease duration; Model D-crude model adjusted by age, gender, disease duration and HbA1c level; MODEL E- crude model adjusted by age, gender, disease duration, HbA1c level and previous laser photocoagulation surgery.