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Paraoxonase-1 activities in children and adolescents with type 1 diabetes mellitus

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Background: Paraoxonase-1 is an HDL-associated esterase that acts as an anti-atherogenic agent by protecting LDL from oxidation. This study investigates paraoxonase-1 activities in children and adolescents with type 1 diabetes mellitus and possible associations with other biochemical markers. Patients and methods: The study enrolled 82 children and adolescents with type 1 diabetes mellitus and 41 controls with similar age and gender distribution. Serum paraoxonase-1 arylesterase and salt-stimulated paraoxonase activities were assessed by measuring the rates of phenyl acetate and paraoxon hydrolysis, respectively; paraoxonase-1 lactonase activity and oxidized LDL were assessed by a pH-sensitive colorimetric assay and ELISA, respectively. Glycated haemoglobin HbA_{1c} and lipid profile were assayed with an immunoturbidimetric method and commercially available kits, respectively. Results: We found lower paraoxonase-1 activities in diabetics when compared to controls. The decrease was statistically significant only for the lactonase activity, the difference being higher when referring to the subgroup with poor glycaemic control. The lactonase activity/HDL ratio was also lower in diabetics *vs.* controls, but without statistical significance. Both lactonase and arylesterase activities were negatively correlated with HbA_{1c} in diabetics, but only the latter was statistically significant ($\rho = -0.21$, $P = 0.055$; $\rho = -0.24$, $P = 0.03$, respectively). A correlation coefficient of $\rho = 0.196$ ($P = 0.078$) was found between oxidized LDL and HbA_{1c}. Conclusion: All paraoxonase-1 activities were lower in diabetic children and adolescents, but only the decrease in the lactonase activity was statistically significant. Although lipid profile and glycaemic control were altered in diabetics, no differences were observed between groups regarding oxidized LDL level.

Key words: paraoxonase-1, lactonase, arylesterase, children, type 1 diabetes mellitus

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INTRODUCTION

The human serum paraoxonase-1 (PON1) enzyme – best known for its paraoxonase (Pon-ase) and arylesterase (Ar-ase) activities – is the most investigated member of the paraoxonase family due to its antioxidant and antiatherogenic activities as a component of high density lipoproteins (HDL) (Harel *et al.*, 2004; She *et al.*, 2012). Structure reactivity studies suggest that the physiological function of PON1 is that of lactonase (Lact-ase), being able to hydrolyze various lactones including δ-valerolactone and homocysteine thiolactone (Khersonsky *et al.*, 2005; James *et al.*, 2006). Clinical implications of this finding are of a particular interest. Due to lactone structure similarities, compounds arising from the hydrolysis of oxidized phospholipids have been proposed as PON1 physiological substrates (James *et al.*, 2006). Growing evidence proved that PON1 is a key mediator in the limitation of both low density lipoproteins (LDL) and HDL oxidation (Aviram *et al.*, 1998; Ng *et al.*, 2005), in the stimulation of macrophage cholesterol efflux (Rosenblat *et al.*, 2006), and therefore in atheroprotection (Mackness *et al.,* 2013).

Type 1 diabetes mellitus (T1DM), with or without vascular complications, is characterized by increased levels of oxidative stress, glycation and glycoxidation. As a result, diabetic patients are predisposed to a higher degree of micro- and macro-vascular complications and are more prone to develop cardiovascular events than healthy subjects (Baynes *et al.,* 1999). In children and adolescents with T1DM, data about PON1 activities, in particular Lact-ase, are either missing or insufficient. Several studies reported low Ar-ase and/or Pon-ase activity in adults with T1DM that was associated with poor metabolic control and vascular complications (Abbott *et al.*, 1995; Boem *et al.*, 2001; Mackness *et al.*, 2002; Ferretti *et al.*, 2004; Flekac *et al.*, 2008). In contrast, Valabhji *et al.* (2001) did not observe reduced Ar-ase activity in subjects with good glycaemic control and without dyslipidaemia.

Because atherosclerosis – which begins at an early age (Ross *et al.,* 1986) – increases the morbidity and mortality through cardiovascular disease in the developed countries, it has become mandatory to investigate and comprehend the factors that impede or promote the progression of fatty streaks.

In this study we evaluated for the first time the profile of PON1 activities (Pon-ase, Ar-ase and Lact-ase) along with oxidized LDL (oxLDL), glycated haemoglobin (HbA_{1c}) and other biochemical markers in a group

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Abbreviations: Ar-ase, arylesterase; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; HbA_{1c}, glycated hemoglobin; HDL, high density lipoproteins; IQR, interquartile range; Lact-ase, lactonase activity; LDL, low density lipoproteins; LPDS, lipoprotein-deficient serum; oxLDL, oxidized LDL; PON1, paraoxonase-1; Pon-ase, paraoxonase activity; S.D., standard deviation; ssPon-ase, salt-stimulated paraoxonase activity; T1DM, type 1 diabetes mellitus; TC, total cholesterol; TG, triglycerides

of children and adolescents with T1DM *vs.* healthy subjects, and the possible associations with other biochemical markers.

PATIENTS AND METHODS

Study groups. The study was conducted in accordance with the Declaration of Helsinki and approved by the University Ethics Committee. Written informed consent was obtained from the parents of all the participants before enrolling in the study.

The diabetic group consisted of 82 children and adolescents receiving insulin treatment and free of other acute or chronic conditions. We divided the diabetic subjects in two subgroups, one with good glycaemic control (GG; n = 45; $HbA_{1c} < 9\%$) and the other one with poor glycaemic control (PG, n = 37; $HbA_{1c} \geq 9\%$).

The control group consisted of 41 healthy volunteers with similar age and gender distribution, without inflammatory or other pathologic conditions.

Venous blood samples were collected after overnight fasting on a clot activator and were centrifuged at $590 \times g$ for 10 minutes; serum samples were stored at –80ºC until assayed.

PON1 lactonase activity (kU/L). Lact-ase activity, expressed as kU/L, was measured using a pH-sensitive colorimetric assay (Khersonsky *et al.*, 2005), with minor variations. Briefly, 3 μl of serum were incubated with 1 mM δ-valerolactone in 1.2 ml of 2.5 mM bicine buffer (pH 8.3), containing 0.15 M NaCl, 1 mM $CaCl_2$ and 0.2 mM m-cresol purple. The bleaching rate, resulting from the carboxylic acid formation, was monitored at 577 nm for 90 seconds. For the calculation of the rate factor, a standard calibration curve was performed with 10 mM acetic acid.

PON1 arylesterase activity (kU/L). Ar-ase activity was measured spectrophotometrically according to Eckerson *et al.* (1983), with minor variations. Briefly, 3 μl of serum were incubated in 1.2 ml of 20 mM Tris-HCl (pH 8.0) containing 1 mM phenyl acetate and 1 mM $CaCl_2$. The rate of phenyl acetate hydrolysis was monitored for 90 seconds at 270 nm. Blank samples without serum were analyzed at the same time to correct for spontaneous hydrolysis of the substrate. For activity calculation, expressed as kU/L, a molar extinction coefficient (1310 $M^{-1}cm^{-1}$) was used.

Salt-stimulated PON1 paraoxonase (ssPon-ase) activity (U/L). ssPon-ase activity was measured spectrophotometrically according to Eckerson *et al.* (1983), with minor variations. Briefly, 25 μl of serum were incubated with 1 mM paraoxon in a total volume of 1.2 ml of 50 mM glycine-NaOH buffer (pH 10.5) containing 2 M NaCl and 1 mM $CaCl_2$. The rate of p-nitrophenol hydrolysis was monitored for 90 seconds at 412 nm. Blank samples without serum were assayed simultaneously to correct for spontaneous hydrolysis of the substrate. For activity calculation, a molar extinction coefficient (18290 $M^{-1}cm^{-1}$) was used.

All enzymatic activities were measured at 25°C, with a UV-VIS double-beam spectrophotometer (V-530 Jasco) equipped with temperature controller (ETC 505T Jasco).

Glycated haemoglobin (HbA_{1c}). HbA_{1c} – expressed as percentage (%) – was measured by an immunoturbidimetric method with Architect *c*Systems using the Multigent Reagent kit (Abbott Laboratories, USA).

Lipid profile. Serum triacylglycerols (TG), HDL and total cholesterol (TC) were assayed with an Architect C4000 analyzer (Abbott, USA) using commercially available kits. LDL was calculated using the Friedewald formula (LDL = TC – HDL – TG/5) (Friedewald *et al.*, 1972).

Oxidized LDL (oxLDL). Serum oxLDL levels were assessed with a Sunrise Tecan microplate reader (Tecan Group Ltd, Switzerland) using the Oxidized LDL Competitive ELISA assay kit (Mercodia AB, Sweden).

Statistical analysis. Qualitative data are presented as counts and percentages. The association between qualitative variables was assessed with Pearson's chi-square test. Quantitative data are presented as mean ± standard deviation (SD) for normally distributed data or otherwise as median and interquartile range (IQR). Normality of the data was checked with strip-chart, quantile-quantile plot and Shapiro-Wilk test. Student's *t*-test for independent samples was used to check for differences between two independent groups of normally distributed data and otherwise by the Mann-Whitney U test. Pairwise comparisons between multiple groups regarding non-normally distributed quantitative variables were performed with Tukey-Kramer test.

Spearman correlation coefficient was used to assess the relations between quantitative variables not following the normal distribution.

Two multiple linear regression analyses for the Lact-ase activity were performed: one on subjects with diabetes, including HbA_{1c} and diabetes duration as covariates; the other on all the subjects, including diabetes as a grouping variable (yes *vs.* no) instead of HbA_{1c}. Beside these variables, the full model contained: age, gender, TG, TC, LDL, HDL, TC/HDL ratio and oxLDL. Then, a best subset variable selection procedure was employed using Bayesian information criterion to identify the best model. Regression diagnostics procedures were employed verifying linearity, residuals' normality, multicolinearity and heteroskedasticity. The regression coefficients along with 95% confidence intervals (CI) were presented. For all statistical tests used, the significance level α was 0.05 and the two-tailed *P* value was computed. The statistical analysis was made in R environment for statistical computing and graphics, version 1.15.1 (R Development Core Team, 2010).

RESULTS

The main characteristics of the studied groups are summarized in Table 1.

Age and gender distributions were similar in the diabetic and control groups. As expected, both the glycaemic control (reflected by the HbA_{1c} level) and the lipid profile were significantly altered in diabetic patients. Compared to controls, TC and LDL levels were significantly increased and the HDL level was significantly decreased in diabetics.

Regarding PON1 activities, we found lower values for diabetics when compared to controls. The decrease was statistically significant only for the Lact-ase activity, the difference being higher when referring to the subgroup with poor glycaemic control. Also, the Lact-ase/HDL ratio was lower in diabetics *vs.* controls, but not statistically significant.

We found that both, the Lact-ase and Ar-ase, were negatively correlated with HbA_{1c} in the diabetic subjects, but only the latter was statistically significant [ρ = –0.21 (95% CI –0.37 – –0.01), $P = 0.055$; $\rho = -0.24$ (95% CI –0.43 – –0.001), $P = 0.03$, respectively]. A positive correlation was observed between oxLDL and HbA_{1c}

Table 1. Demographic features and biochemical data of the diabetic and control groups

Parameters	Controls	Type I diabetics			*P*
		HbA1c < 9%	HbA1c ≥ 9%	Overall diabetics	
Number	41	45	37	82	
Age (years)	11±3.7	10.8±4	13.1±3.3‡	11.8±3.8	0.284
Gender (females, %)	22 (54)	22 (49)	14 (38)	36 (44)	0.307
Duration of diabetes (years)	–	3.5 (2–6)	3.5 (1–6)	3.5 (1.6–6)	–
HbA1c (%)	–	8.02 (6.98–8.59)	10.41 (9.35–11.86)	8.83 (7.92–10.09)	<0.001*
Lipid profile					
TC (mg/dl)	136 (123–149)	166 (136–83)†	165 (154–180)‡	165 (143–182)	<0.001
HDL (mg/dl)	59 (51–65)	55 (47–62)	52 (46–58)‡	54 (46–59)	0.030
LDL (mg/dl)	86 (81–94)	100 (77–112)	100 (86–108)‡	100 (81–110)	0.012
TC/HDL	2.3 (1.9–3.1)	3.1 (2.6–3.3)†	3.2 (2.7–3.5)‡	3.1 (2.6–3.5)	<0.001
PON1 activities					
Lact-ase (kU/L)	35 (30–45)	31 (27–37)	28 (25–32)‡	29 (26–35)	<0.001
Ar-ase (kU/L)	80 (67–96)	79 (66–90)	72 (65–80)	74 (65–85)	0.080
ssPon-ase (U/L)	215 (134–469)	174 (127–324)	187 (121–374)	181 (121–354)	0.450
PON1 activity/HDL					
Lact-ase/HDL	0.66 (0.48–0.71)	0.57 (0.48–0.69)	0.54 (0.47–0.66)	0.56 (0.48–0.69)	0.08
Ar-ase/HDL	1.38 (1.1–1.78)	1.42 (1.24–1.67)	1.38 (1.16–1.71)	1.39 (1.24–1.69)	0.8
ssPon-ase/HDL	4.08 (2.18–7.79)	3.5 (2.28–6.17)	4.52 (2.16–7.14)	3.54 (2.21–6.68)	0.65
LDL/Ar-ase	1.11±0.33	1.25±0.39	1.38±0.38	1.31±0.39	0.002
Other markers					
oxLDL (U/L)	46 (40–54)	46 (32–60)	47 (36–55)	46 (34–59)	0.940

HbA_{1c}, glycated haemoglobin; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Lact-ase, lactonase activity; Ar-ase, arylesterase activity; ssPon-ase, salt stimulated paraoxonase activity; oxLDL, oxidized low-density lipoprotein. Values are mean ±S.D. for normally distributed data, median (interquartile range) for non-normally distributed data or counts (percentages). *P* value between controls *vs.* overall diabetics; **P* value between diabetic subgroups; †$P < 0.05$ controls *vs.* subgroup with $HbA_{1c} < 9\%$; ‡$P < 0.05$ controls *vs.* subgroup with $HbA_{1c} > 9\%$.

[$Q = 0.196$ (95% CI –0.06 – 0.35), $P = 0.078$], but the association failed to achieve the significance level.

The linear regression analysis performed on all the subjects gave a final model predicting lactonase that contained the presence of diabetes as the only variable. The coefficient for diabetes presence was –6.50 (95% CI –9.75 – –3.25), $P < 0.001$ and the determination coefficient was 0.11. The presence of diabetes was associated with a low Lact-ase level. The final linear regression model predicting lactonase, performed only on diabetic subjects after the selection procedure, contained only the HbA_{1c} variable and although not statistically significant, it was close to the significance level. The coefficient for HbA_{1c} presence was –0.83 (–1.68 – 0.03), $P = 0.063$ and the determination coefficient was 0.04.

DISCUSSION

Data about PON1 in diabetic subjects are controversial. Several studies reported low Ar-ase and/or Pon-ase activity in adults with T1DM that had poor metabolic control and vascular complications (Abbott *et al.*, 1995; Boem *et al.*, 2001; Mackness *et al.*, 2002; Ferretti *et al.*, 2004; Flekac *et al.*, 2008), but Valabhji *et al.* (2001) did not observe reduced Ar-ase activity in subjects with good glycaemic control and without dyslipidaemia. In diabetic children and adolescents (duration of diabetes 8.8 years), Kordonouri *et al.* (2001) found low Ar-ase only in patients with blood glucose > 10 mmol/l. However, the results reported by Nair *et al.* (2011) showed normal Ar-ase and Pon-ase activities in diabetic children (approximately 10 years of age). We also did not find significantly differences regarding Ar-ase and Pon-ase in diabetics *vs.* controls.

Some of the above PON1-related studies did not provide data about the distribution of PON1 genotypes, so we could not exclude the hypothesis that the controversial results might be due to significant differences in PON1 genotypes distribution between the diabetic and control groups. We have identified the individual genotype of our subjects, according to Eckerson *et al.* (1983) (results not included) and we have observed a similar *PON1* Q192R genotype distribution in both diabetic and control groups.

Growing evidence suggests that Lact-ase is the physiological activity of PON1, having at least three antiatherogenic effects: protection of LDL, HDL and macrophages against peroxidation, promotion of macrophage cholesterol efflux and reduction of macrophage-mediated oxLDL uptake (Shih *et al.*, 2000; Rozenberg *et al.*, 2003; Rozenberg *et al.*, 2008; Aharoni *et al.*, 2013). The study presented here shows for the first time that the Lact-ase activity is significantly reduced in children and

adolescents with T1DM, but when dividing Lact-ase by HDL, the difference between groups became statistically not significant ($p = 0.08$ instead of $p < 0.001$). This might suggest that the low PON1 carrier concentration (observed in the diabetic children) could explain, at least partially, the decrease in Lact-ase activity. If the low level of HDL would be the only source of this decrease, there ought to exist a good correlation among these parameters, which we did not notice (Spearman correlation coefficient = 0.11 (95% CI 0.08–0.32, $p = 0.214$). As a result, we thought that other causes have to be taken into account, such as quantitative and/or qualitative alterations in HDL and thus in PON1. Rosenblat *et al.* (2006) reported that, in diabetic subjects, significant amount of serum PON1 is dissociated from HDL to the lipoprotein-deficient serum (LPDS). Therefore, PON1 conformation is changed and consequently the Pon-ase activity is stimulated, but Lact-ase and Ar-ase activities drastically decrease. Furthermore, they observed that PON1 in LPDS is not able to protect against lipid peroxidation and to stimulate macrophage cholesterol efflux and concluded that abnormal serum PON1 distribution could be responsible for the accelerated atherosclerosis in these patients. Consistent with this data, purified Lact-ase activity – but not HDL-bound Lact-ase – showed an increased susceptibility to be inactivated under oxidative stress conditions (ascorbate/Cu^{2+} oxidation), when compared to the Ar-ase and Pon-ase activities (Nguyen *et al.,* 2009). More recent findings suggest that PON1 has a catalytic promiscuity with different active sites configurations for Pon-ase *vs.* Ar-ase and Lact-ase activities, which could be responsible for the preserved Pon-ase activity *vs.* altered Lact-ase and Ar-ase activities in diabetic patients (Ben-David *et al.,* 2012). Besides that, in diabetic patients, HDL and/or PON1 could be glycated and glycoxidated, leading to impaired PON1 stability and/or activities (Kordonouri *et al.*, 2001; Mastorikou *et al.*, 2008).

The results presented here also showed that the diabetic subjects were dyslipidaemic and had poor glycaemic control, but had normal levels of oxLDL. A higher level of LDL was associated with a lower Ar-ase activity in the diabetic group *vs.* control group. LDL is considered to be a substrate for the antioxidant activity of PON1, and the Ar-ase activity (which is not influenced by Q192R polymorphism) is a substitute of PON1 serum concentration. We have observed statistically higher values for the LDL/Ar-ase ratio in diabetic subjects, a result that could suggest a reduced antioxidant protection of LDL. However, the level of oxLDL was similar in the investigated groups. Compared to our results, higher levels, but not statistically significant, of oxLDL have been reported in T1DM children (9–16 years of age) with short duration of the disease (Hamad *et al.*, 2010). Since the oxidized phospholipids from LDL are the physiological substrates of PON1 Lact-ase, we expected that reduced levels of Lact-ase activity must be associated with high levels of serum oxLDL in the diabetic subjects. Conversely, we did not observe any differences when compared to the control group, probably due to either an efficient antioxidant mechanism (involving other enzymes) or due to rapid removal of oxLDL from the blood (due to immune complexes and macrophage scavenger receptors) (Li *et al.,* 2011).

In our study, both Lact-ase and Ar-ase activities were inversely correlated with HbA_{1c}, although only the latter was statistically significant. The correlation between Lact-ase and HbA_{1c} and also the regression between the two were very close to the statistical significance level. Previous studies reported low Pon-ase and Ar-ase activities related to high blood glucose levels, but not to HbA_{1c} levels (Kordonouri *et al.*, 2001). A possible explanation for the significant correlation of Ar-ase with HbA_{1c} could be a higher HbA_{1c} concentration in our diabetic group (almost half of the subjects had $HbA_{1c} > 9\%$) suggesting they constantly had elevated blood glucose levels that could directly or indirectly affect PON1 by glycation and/or glycoxidation.

The small sample size was a limitation of this study, however our results underline the importance of further analyzing the relationship between PON1 and childhood diabetes from the perspective of the Lact-ase activity.

CONCLUSIONS

All PON1 activities were lower in children and adolescents with T1DM, but only the decrease in the Lact-ase activity was statistically significant. The observed negative correlation between HbA_{1c} and Lact-ase or Ar-ase was close to the statistical significance level for the first one and significant for the second one. Although the Lact-ase activity, lipid profile and the glycaemic control have been altered in diabetic subjects, we did not observe any differences between groups regarding the oxLDL level.

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Disclosure

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