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Low Serum Paraoxonase-1 Lactonase and Arylesterase Activities in Obese Children and Adolescents

Low Serum Paraoxonase-1 Lactonase and Arylesterase Activities in Obese Children and Adolescents

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Abstract

Serum paraoxonase-1 (PON1) binds mainly to high density lipoproteins (HDLs) and protects low density lipoproteins (LDLs) against oxidation. While paraoxonase and arylesterase activities are traditionally assayed, lactonase activity, accounting for protection against LDL oxidation, was less investigated in obese children and adolescents. Therefore, we aimed to measure lactonase, paraoxonase and arylesterase activities, oxidized LDL (ox-LDL) and malondialdehyde (MDA) levels in obese children and adolescents.

Study population included 68 children (35 obese and 33 normal-weight). Arylesterase and paraoxonase activities were assayed spectrophotometrically. Lactonase activity, ox-LDL and MDA levels were measured using a pH-sensitive colorimetric assay, an ELISA technique and a fluorimetric method, respectively. The lipid profile was assessed by common methods.

Lactonase and arylesterase activities were decreased in the presence of obesity. MDA, but not ox-LDL levels, showed significant differences between groups. Multiple regression analysis identified a reciprocal relationship and a possible association between lactonase and arylesterase activities and obesity.

Keywords*: paraoxonase, lactonase, arylesterase, childhood obesity, low density lipoproteins, malondialdehyde*

Rezumat

Paraoxonaza-1 serica (PON1) se leaga in principal de lipoproteinele cu densitate mare (HDL) si protejeaza lipoproteinele cu densitate joasa (LDLs) impotriva oxidarii. In timp ce activitatile paraoxonazica si arilesterazica

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sunt analizate in mod traditional, activitatea lactonazica, cea care confera de fapt protectia impotriva oxidarii LDL, a fost mai putin investigata la copiii si adolescentii obezi. Prin urmare, in acest studiu, ne-am propus sa cuantificam activitatile lactonazica, paraoxonazica si arilesterazica, nivelurile de LDL oxidat (ox-LDL) si malondialdehida (MDA) la copiii si adolescentii cu obezitate.

Lotul de studiu a inclus 68 de copii (35 cu obezitate si 33 cu greutate normala). Activitatile paraoxonazica si arilesterazica au fost analizate spectrofotometric. Activitatea lactonazica, nivelurile de ox-LDL si MDA au fost masurate cu ajutorul unui test colorimetric sensibil la pH, o tehnică ELISA si respectiv, o metoda fluorimetrica. Profilul lipidic a fost evaluat prin metode obisnuite.

Activitatile lactonazica si arilesterazica au prezentat valori scazute in prezența obezitatii. Nivelul MDA, dar nu si cel de ox-LDL, a aratat diferente semnificative intre grupurile analizate. Analiza de regresie multipla a identificat o relatie de reciprocitate si o posibila asociere intre activitatile lactonazica si arilesterazica, si obezitatea la copii si adolescenti

***Cuvinte cheie**: paraoxonaza, lactonaza, arilesteraza, obezitate infantila, lipoproteine cu densitate joasa, malondialdehida*

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Introduction

Paraoxonase-1 (PON1), a member of the paraoxonase family, is synthesized in the liver and transported in the plasma mainly by the high density lipoprotein (HDL) particles from where it can be transferred in the areas of low density lipoprotein (LDL) accumulation, where it prevents its oxidation (1). From a functional perspective, PON1 hydrolyzes a broad spectrum of molecules ranging from organophosphates (OPs) to lactones (2), as it performs multiple tasks depending on its substrate and networking capacity of its active center residues (3). While paraoxonase (Po.ase) and arylesterase (Ar.ase) activities are being traditionally assayed, lactonase (Lct.ase) activity, which accounts for HDL-mediated protection against LDL oxidation (4), has been less investigated. These activities are modulated by single nucleotide polymorphisms in the promoter (mainly c.-108C>T or *rs705379*) and the coding region (p.Leu55Met or *rs854560* and p.Gln192Arg or *rs662*) of the *PON1* gene. While the c.-108C>T and the p.Leu55Met polymorphisms influence enzyme concentration and stability (5), the Gln-to-Arg substitution at position 192 accounts for great variations of the Po.ase activity (6), from the Gln (Q) isoform with the lowest activity to the Arg (R) isoform with the highest activity (7). Furthermore, oxidative stress influences the three activities differently, Lct.ase being the most susceptible (8).

Childhood obesity, defined as a body mass index (BMI) higher than the 95th percentile for the same age and sex, is a major public health issue affecting millions of children worldwide. Excessive adiposity increases the risk for atherosclerosis-related coronary artery disease by promoting inflammation, oxidative stress and high plasma cholesterol levels, which negatively influence *PON1* gene transcription (9) and activity (8). In adults, low PON1 levels are associated with conditions that involve lipid peroxidation and systemic inflammation such as diabetes mellitus or metabolic syndrome and are an independent risk factor for coronary heart disease (10). Furthermore, low levels of Ar.ase or Po.ase have been observed in obese children (11-14), but Lct.ase, considered the physiologic activity of PON1 (2), has scarcely been investigated in this condition (14). Having these in mind, we undertook a complex approach to compare Lct.ase, Po.ase and Ar.ase, along with oxidized LDL (ox-LDL), malondialdehyde (MDA) and lipid profile in a group of children and adolescents with non-genetic obesity *vs.* normal-weight children.

Subjects and Methods

Study population

The study population consisted of 68 children, 35 obese (OB) and 33 normal-weight (Control, C), consecutively recruited from the local Pediatric Hospitals. All participants underwent a complete standard physical examination. BMI was calculated as the ratio of the weight (expressed in kilograms, kg) to the square of height (expressed in meters, m^2). The children were classified as obese or normal-weight according to the WHO-BMI percentiles with the cut-off limit for the study group set at the 95th percentile. The children had no previous dietary interventions, nor were enrolled in any physical education programmes. Children suffering from any chronic diseases (e.g. diabetes mellitus) or presenting any acute inflammatory conditions were excluded from study. An informed written consent was obtained from all the parents or legal tutors of children included in the study. The research protocol was developed in accordance with the WMA Declaration of Helsinki and was approved by the University Ethics Committee.

Blood sample collection

Venous blood samples were drawn in clot-activator vacutainers in the morning, following a 12-hour overnight fast. The serum, isolated by low-speed centrifugation, was used for lipid profile determination and the remaining quantity was immediately divided into aliquotes and stored at -80°C for later assay of PON1 activities, MDA and ox-LDL levels.

PON1 enzymatic activities

PON1 Ar.ase, salt–inhibited arylesterase (*si*Ar.ase) and salt-stimulated paraoxonase (*ss*Po.ase) activities were determined using phenyl acetate and paraoxon as substrates, according to Eckerson *et al.* (7), with slight modifications.

Briefly, *si*Ar.ase and Ar.ase were measured using an incubation mixture containing phenyl acetate (Sigma, USA) in 1mM $CaCl_2$, 20mM Tris-HCl buffer (pH 8) with and without 1M NaCl, to which serum was added in order to initiate the reaction. The rate of substrate hydrolysis was established measuring the increase in absorbance at 270 nm. The enzymatic activity (expressed in kU/L) was calculated using the corresponding molar extinction coefficient for phenol (1,310 $M^{-1}cm^{-1}$).

The *ss*Po.ase working mixture contained 1mM paraoxon (diethyl 4-nitrophenyl phosphate, Sigma-Aldrich Chemie GmbH, Germany), 1mM $CaCl_2$ and 2M NaCl in 50mM glycine-NaOH buffer (pH 10.5). After adding the serum, the hydrolysis of paraoxon was recorded at 412 nm. The molar extinction coefficient of 18,290 $M^{-1}cm^{-1}$ for p-nitrophenol was used to calculate the enzymatic activity, expressed in U/L.

Lct.ase activity (expressed in kU/L) was measured by adapting the pH-sensitive colorimetric assay proposed by Khersonsky and Tawfik (2). The reaction mixture contained 1mM δ-valerolactone (Acros Organics, Belgium) in 0.1mM m-cresol purple (from 60mM stock solution in dimethyl sulfoxide), 1mM $CaCl_2$ and 150mM NaCl in 2.5mM bicine buffer (pH 8.3). After adding serum to the assay mixture, the hydrogen ions release by the 5-hydroxyvaleric acid (the product of Lct.ase action) was recorded spectrophotometrically at 577 nm. The initial rate of hydrolysis was calculated using the slope value (in units of absorbance change per minute) multiplied by a rate factor derived from a standard calibration curve obtained with acetic acid. Units were defined as the number of micromoles of acid produced per time unit.

All PON1 activities were determined at 25^{o}C with a V-530 Spectrophotometer using a Peltier thermostated cell holder, equipped with a magnetic stirrer (Jasco, USA). A blank sample containing incubation mixture without serum was used in order to correct for the spontaneous hydrolysis of the substrate.

PON1 phenotype distribution

The ratio of *ss*Po.ase to *si*Ar.ase activities (double-substrate method applied on the logarithm-transformed data) was used to identify individual phenotypes according to Eckerson *et al*. (7) with slight modifications. Knowing that NaCl differently influences Po.ase and Ar.ase activities, which correspond to a specific phenotype, we have used the *ss*Po.ase activity and the *si*Ar.ase activity to obtain a better phenotype differentiation. The ratios were trimodally distributed on the histogram, corresponding to low activity - AA phenotype (isoform QQ), intermediate activity - AB phenotype (isoform QR) and high activity - BB phenotype (isoform RR).

Oxidized LDL (ox-LDL) levels

Serum ox-LDL levels were measured using an Oxidized LDL Competitive ELISA assay kit (Mercodia AB, Sweden) according to the manufacturer's instructions, on a Sunrise Tecan microplate reader (Tecan Group, Switzerland).

Malondialdehyde (MDA) levels

MDA levels were assayed using the fluorometric method proposed by Conti *et al*. (15), on a Perkin-Elmer UK model LS 45 Fluorescence Spectrometer (Perkin Elmer, UK). MDA was treated with 1,3-diethyl-2-thiobarbituric acid in an acid medium and the resulted fluorescent compound was extracted with butanol and determined by synchronous fluorescence spectroscopy with $\Delta\lambda$=14 nm between the excitation (515 nm) and emission wavelengths.

Other parameters

Serum triacylglycerols (TG), HDL and total cholesterol (TC) were assayed on an Architect C4000 analyzer (Abbott, USA) using commercially available kits. LDL levels were calculated using the Friedewald formula (LDL=TC-HDL-TG/5), all subjects having TG levels below 3.9 mm/l. The atherogenic index of plasma (AIP) was expressed as logarithm of the molar ratio of TG to HDL (16).

Statistical analysis

Qualitative data was presented as counts and percentages. The association between qualitative variables was assessed using the Chi-square test or the Fisher exact test. The normal distribution of data was checked with strip-chart, quantile-quantile plot and Shapiro-Wilk test.

Quantitative data was presented as mean and standard deviation (for normally distributed data) or by median and interquartile range (for non-normally distributed data). To check for differences between two independent groups of quantitative data, the Mann-Whitney U test (for non-normally distributed data) or the Student test (for normally distributed data) were used. The Lct.ase and Ar.ase activities were assessed against several explanatory variables: obesity (coded 1-yes, 0-no), age, gender, HDL, LDL, ox-LDL, TG and MDA. The relationship between obesity and other explanatory variables was assessed with logistic regression.

For all regression analysis we performed the univariate analysis. We built the full models and then using a stepwise backward/forward model selection based on the Akaike information criterion we selected the variables for the final models. Results were presented as coefficients/odds ratios and 95% CI. For all statistical tests the significance level alpha was set at 0.05 and the two-tailed p value was computed. The statistical analysis was performed in R environment for statistical computing and graphics, version 3.0.1 (17).

Results

Baseline clinical and laboratory parameters of the study groups are presented in *Table I*.

Statistical analysis did not show significant differences regarding age, gender distribution, TG and ox-LDL.

Table I. Baseline clinical and laboratory parameters of the study groups

Variable	Control (n=33)	Obese (n=35)	p
Age (years)	11.85 ± 2.83	12.19 ± 3.41	0.658
Female n (%)	24 (66.67)	20 (60.61)	0.601
TC (mmol/L)	3.43 [3.12 - 3.92]	4.12 [3.47 - 4.62]	< 0.001
LDL (mmol/L)	1.48 [1.15 - 2.12]	2.4 [1.92 - 2.99]	< 0.001
HDL (mmol/L)	1.5 ± 0.16	1.16 ± 0.22	< 0.001
TG (mmol/L)	0.79 [0.57 - 1.13]	0.9 [0.69 - 1.13]	0.144
Ox-LDL (U/L)	47.02 ± 14.67	46.08 ± 10.49	0.763
Ox-LDL/HDL	0.8±0.25	1.01±0.24	<0.001
MDA (µmol/L)	1.65 [1.58 - 2.11]	2.62 [2 - 2.97]	< 0.001
PON1 activities			
Ar.ase (kU/L)	66.55 [61 - 85.1]	54.87 [48.42 - 61.09]	< 0.001
ssPo.ase (U/L)	214.1 [154.3 - 470.9]	288 [172.28- 477.55]	0.414
Lct.ase (kU/l)	14.2 [9.39-28.07]	10.4 [8.87-12.11]	0.013
PON1 activities/HDL			
ssPo.ase/HDL	3.45 [2.62-7.38]	5.71 [4.01-10.2]	0.02
Ar.ase/HDL	1.13 [1-1.5]	1.15 [1.02-1.47]	0.82
Lct.ase/HDL	0.26 [0.15-0.51]	0.23 [0.21-0.26]	0.33

n (%) - number (percentage); TC - total cholesterol; LDL - low density lipoproteins; HDL - high desity lipoproteins; TG - triacylglycerols; Ox-LDL - oxidized LDL; MDA – malondialdehyde; Ar.ase - arylesterase; ssPo.ase - salt-stimulated paraoxonase; Lct.ase - lactonase. Non-normally distributed data are presented as median [lower quartile – upper quartile]; Normally distributed data are presented as mean ± standard deviation (SD)

Serum TC, LDL and MDA levels were significantly higher and HDL levels were significantly lower in obese children, while AIP values were in normal limits for both groups (OB: -0.09 ± 0.21 *vs.* C: -0.27 ± 0.20; p<0.001).

There were no statistically significant differences between obese and control groups regarding the PON1 phenotype distribution [AA=19 (54.28%); AB=11 (31.42%); BB=5 (14.28%) *vs.* AA=17 (51.51%); AB=11 (33.33%); BB=5 (15.15%); p=0.98].

Ar.ase and Lct.ase activities were significantly decreased in obese children compared to controls (*Figure 1*), but *ss*Po.ase levels were not

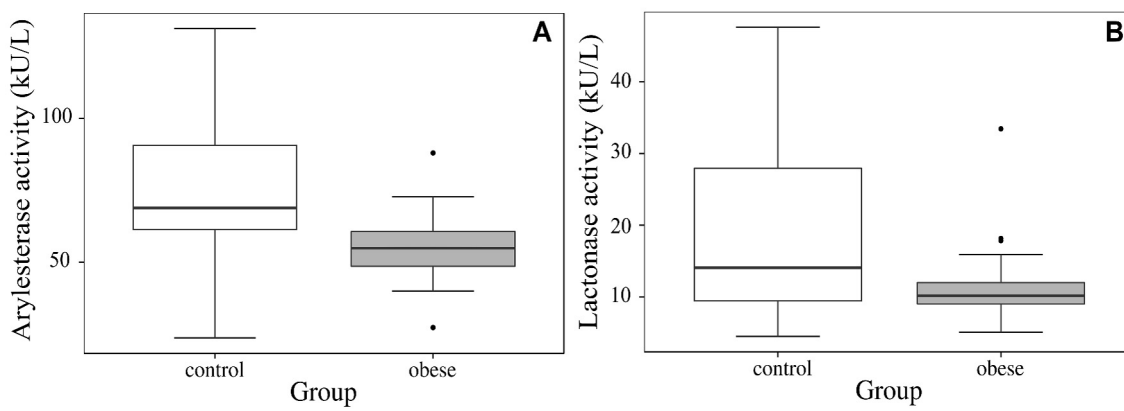

Figure 1. Lct.ase (A) and Ar.ase (B) activities in the investigated groups

significantly different. After dividing each PON1 activity by the HDL level, there were no longer statistically significant differences between subjects and controls for Ar.ase and Lct.ase activities (*Table I*).

In the univariate linear regression, obesity was associated with decreased Lct.ase and Ar.ase (B=-8.53, R^2=0.18; p<0.001; B=-19.29, R^2=0.19; p<0.001, respectively). Decreased HDL was positively associated with Lct.ase and Ar.ase activities (B=0.3; R^2=0.08; p<0.05; B=0.72; R^2= 0.095; p=0.01, respectively).

Multiple regression analysis showed that obesity was associated with decreased Ar.ase and Lct.ase activities even when the parameters were adjusted for the HDL levels (*Tables II, III and IV)*. The stepwise selection procedures used to build all the final regression models removed

Table II. Multiple linear regression analysis for Ar.ase and Lct.ase as dependent variables

Ar.ase*	**B**	**(95% CI)**	**p**
Obese (yes/no)	-19.43	[(-28.1) – (-10.76)]	< 0.001
*ss*Po.ase activity (U/L)	0.0387	0.02 - 0.06	< 0.001
Lct.ase**	**B**	**(95% CI)**	**p**
Obese (yes/no)	-11.14	[(-16.22) – (-6.05)]	< 0.001
Age (years)	-0.6	[(-1.27) - 0.07]	0.077
LDL (mmol/L)	3.83	0.34 - 7.32	0.032

Ar.ase – arylesterase; *ss*Po.ase - salt-stimulated paraoxonase; Lct.ase - lactonase; LDL - low density lipoproteins; B - unstandardized regression coefficient; 95% CI – 95% confidence interval. The adjusted determination coefficients were 0.35* and 0.31** respectively (p<0.001)

Table III. Multiple linear regression analysis adjusted for HDL using Ar.ase, Po.ase and Lct.ase as dependent variables

Activity	**Variable**	**B**	**(95% CI)**	**p**	**P model**
Ar.ase †	Obese	-17.28	[(-30.58)–3.99]	0.012	<0.001
	HDL	5.9	[(-20.29)–32.09]	0.654	
ssPo.ase ††	Obese	66.3	[(-97.48)–230.07]	0.422	0.523
	HDL	184.58	[(-137.98)–507.13]	0.257	
Lct.ase †††	Obese	-8.03	[(-14.17)–1.9]	0.011	0.002
	HDL	1.47	[(-10.61)–13.55]	0.809	

Ar.ase - arylesterase; ssPo.ase - salt-stimulated paraoxonase; Lct.ase - lactonase; HDL - high density lipoproteins; B - unstandardized regression coefficient; 95% CI – 95% confidence interval. The adjusted determination coefficients were 0.16†, 0.01††, 0.15†††respectively

Table IV. Multiple logistic regression analysis for obesity as a dependent variable

Variable	OR adjusted	(95% CI)	p
HDL (mmol/L)	0.72	(0.53 - 0.86)	0.005
LDL (mmol/L)	1.09	(1.03 - 1.2)	0.018
ssPo.ase (U/L)	1.01	(1 - 1.02)	0.02
Ar.ase (kU/L)	0.92	(0.85 - 0.98)	0.02
Lct.ase (kU/L)	0.73	(0.5 - 0.89)	0.026
Ox-LDL (U/L)	1.07	(0.97 - 1.23)	0.224

HDL - high density lipoproteins, LDL - low density lipoproteins, ssPo.ase - salt-stimulated paraoxonase, Ar.ase - arylesterase, Lct.ase - lactonase, ox-LDL - oxidized low density lipoproteins; OR - odds ratio; 95% CI – 95% confidence interval

MDA as a possible explanatory variable candidate.

Discussion

The main finding of our research was that PON1 Lct.ase and Ar.ase activities, but not *ss*Po.ase, are decreased in the presence of excessive adiposity. Until now, relatively little effort has been made to investigate the role of PON1 in obesity, and the few published results are inconsistent. In obese adults, both low (18-21) or unchanged levels of Po.ase and Ar.ase (22) have been reported. Similarly, in obese children and adolescents, either low (11, 23, 24), unaffected levels of Po.ase and Ar.ase (25-27) or incresed levels of Ar.ase (28) have also been observed. Recently, unchanged (25) or reduced (14) levels of Lct.ase activity (assayed with dihydrocoumarin and 5-thiobutyl butyrolactone, respectively) have been reported in obese children.

The discrepancies between the data regarding PON1 activities in obese *vs.* control subjects could be explained by the relatively small size of the investigated groups, the great variability of PON1 activities in the same ethnic population, the complex nature of obesity (which depends on both genetic and environmental factors), and lifestyle, which could influence HDL composition and thus PON1 stability and activities. Furthermore, the differences between the methods used for assaying PON1 activities (e.g. the composition of assaying mixture) could be another cause of discrepancy. The pH-indicator assay which we used to monitor δ-valerolactone hydrolysis permits the measurement of the initial rate but not of the entire reaction course (29). Moreover, while δ-valerolactone and phenyl acetate bind in a similar fashion to the PON1 active site and use the same catalytic mechanism, the hydrolysis of paraoxon (an aromatic phosphotriester) and of dihydrocoumarin (an aromatic lactone) uses distinctive parts of the active site network, therefore the binding and catalytic mechanisms are dissimilar (3). All the above could explain the Ar.ase and Lct.ase behavior observed in our study, and the unchanged Po.ase and Lct.ase reported by Ruperez *et al*. (25) in the obese group.

The association of PON1 with HDL is a prerequisite for maintaining a normal serum enzyme activity. HDL lipidic and proteic components interact in order to promote PON1 secretion as well as maintaining its stability in the blood stream (30). As in our study, reduced HDL levels have been previously reported in obese children (11, 13, 14, 23, 25). These alterations have been attributed to both an enhancement in the uptake of HDL by the adipocytes and an increase in the apoA-I catabolism in HDL particles. In the absence of ApoA-I, the PON1-HDL complex is less stable and PON1 loses its activity (mainly Lct.ase) faster than in normal controls (1, 10, 31, 32). Therefore, due to a reduced level of the carrier and the instability of the PON1–HDL complex, low PON1 levels are to be expected. Accordingly, we observed that the differences between groups regarding the Ar.ase and Lct.ase activities lost their statistical significance after being divided by the HDL levels, suggesting that, in the case of the obese children, low HDL levels account, at least partially, for the diminished Ar.ase and Lct.ase.

Another factor which could affect PON1 levels is oxidative stress, observable not only in obese adults but also in obese children. In this respect, we have observed higher MDA levels (one of the end products of lipid peroxidation) in obese subjects. This increment has also been reported by Codoner-Franch *et al*. (21, 33).

PON1 is involved in the protection of LDL and HDL against oxidative alteration (34). Moreover, the enzyme is time-dependent inactivated by this process, due to an interaction of oxidized phospholipids and cholesterol esters with the free SH-group of cysteine-284 (the inhibition

percent of LDL oxidation being higher than the percent lost in PON1 activity) (35).

These interactions influence the three activities differently, Lct.ase being the most susceptible (8). Current research indicates that the Ar.ase and mainly the Lct.ase activities are related to the protective capacity of PON1 against LDL oxidation, ox-LDL inducing the enzyme's "suicide-like" behavior by impairing the networking of its active site residues (8). Moreover, ox-LDL can induce HDL composition alteration (10) and decrease the *PON1* gene expression (9). As obesity promotes oxidative stress, we expected to find high levels of serum ox-LDL in the obese subjects. Conversely, we did not observe statistically significant differences between the obese and the control groups, similar to Demirel *et al*. (36), Kelishadi *et al*. (37) and Ruperez *et al.* (25). Kelly *et al*. (38) found increased ox-LDL levels in children with excessive adiposity, significantly associated with obesity only in the older subjects (12-18 years of age). However, statistically significant differences were observed between groups regarding the ox-LDL to HDL ratio, a parameter which better evaluates the risk for further developing atherosclerosis-related complications and thus the extent of the LDL alterations induced by oxidation/oxidative stress (39).

The unchanged ox-LDL levels in obese children could be due to a good antioxidant protection and/or an efficient and specific removal from their plasma by the macrophages and macrophage-like cells in the liver (40). In this respect, the AIP values observed in obese children indicated a low risk of atherosclerosis-related complications (16), as ox-LDL certainly plays an important role in atherogenesis.

The small sample size was definitely a limitation of this study, however our results underline the importance of further analyzing the relationship between PON1 and childhood obesity from the perspective of the Lct.ase activity. In order to enrich the results of the present study, large prospective studies are needed and other markers of oxidative stress should be analyzed.

Conclusion

Low PON1 Lct.ase and Ar.ase activities are associated with the presence of obesity in children and adolescents. This may account for a higher susceptibility to atherosclerosis-related diseases with age.

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Conflicts of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Abbreviations

95% CI – 95% confidence interval
AIP - atherogenic index of plasma
apoA-I – apolipoprotein A-I
Ar.ase - arylesterase activity

BMI - body mass index
HDL - high density lipoprotein
Lct.ase - lactonase activity
LDL - low density lipoprotein
MDA - malondialdehyde
n (%) - number (percentage)
OPs - organophosphates
OR - odds ratio
ox-LDL - oxidized LDL
Po.ase - paraoxonase activity
PON1 - Paraoxonase-1
SD - standard deviation
*si*Ar.ase - salt–inhibited arylesterase activity
*ss*Po.ase - salt-stimulated paraoxonase activity
TC - total cholesterol
TG - serum triacylglycerols

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