

Carotid Intima-media Thickness, Paraoxonase-1 Gene Polymorphism, Inflammation and Oxidation Status in Children with Family History of Premature Cardiovascular Diseases

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Authors' contributions

This work was carried out in collaboration between all authors. Author AI is the PhD researcher, with major contributed in the collection of samples, assessments of the study and writing of the paper. Author KM performed the biochemical assessments, the statistical analysis of data and contributed in the writing of the paper. Author AA performed the DNA analysis and contributed in the writing of the paper; Authors ED and AP made the ultrasound measurements of cIMT and revised the manuscript. Author AZ performed the collection of samples and revised the paper. Author AH is the team leader, had the initial concept of the study, supervised sample collection and performance of biochemical assessments and revised the paper. All authors approved the final version of the paper to be sent for publication.

Article Information

DOI: 10.9734/CA/2016/25664

Editor(s):

(1) Eirin Massat Alfonso, College of Medicine, Mayo Clinic, USA and Renovascular Research Laboratory, Mayo Clinic, Rochester, Minnesota, USA.

Reviewers:

(1) Sharaf Eldeen Shazly Abdalla, Sohag Univ Hospital, Egypt.

(2) Francis W. Hombhanje, Divine Word University, Papua New Guinea.

(3) Michelle Trindade, State University of Rio de Janeiro, Brazil.

Complete Peer review History: <http://sciencedomain.org/review-history/14227>

Original Research Article

Received 14th March 2016

Accepted 8th April 2016

Published 18th April 2016

ABSTRACT

Aims: Paraoxonase-1 (PON-1) gene polymorphisms and carotid intima-media thickness (cIMT) in adults have been associated to increased incidence of cardiovascular diseases (CVD). Possible

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relation of cIMT to PON-1 polymorphisms and to markers of inflammation and oxidation, in children with family history of premature CVD, was investigated.

Place of the Study: Laboratory for Lipids and Cardiovascular Disease Prevention, 2nd Pediatric Department, AHEPA University Hospital, Thessaloniki, Greece.

Methodology: Eighty four healthy children of normal BMI, were recruited; 42 with positive family history of premature CVD, median age 10 (7-15) years old (study group) and 42 age- and gender-matched controls. Levels of lipid profile parameters, high sensitivity CRP (hsCRP) and oxidized LDL (oxLDL) were determined. cIMT was measured by ultrasounds and PON-1 gene polymorphisms, Q192R and L55M, were investigated using standard PCR-RFLP.

Results: Median cIMT was higher in study group than in controls [0.45 (0.30-0.65) vs 0.4 (0.30-0.60)]. Only ApoA-I presented significant difference between the three subgroups with different PON-1 SNP of the L55M polymorphism ($P=.03$). Regression analysis showed that there was no statistically significant dependence of cIMT on age, lipid profile parameters or markers of inflammation and oxidation.

Conclusion: Family history of premature CVD and PON-1 gene polymorphism are not related with significant differences in cIMT in children. Inflammation and oxidation do not markedly affect cIMT in children with family history of premature CVD.

Keywords: Paraoxonase-1; IMT; gene polymorphisms; C-reactive protein; oxidized LDL; family history.

1. INTRODUCTION

Paraoxonase 1 (PON-1) is a calcium-dependent enzyme exhibiting esterase, lactonase and peroxidase activity. PON-1 accepts a broad range of substrates including organophosphates, diverse lactones and lipid peroxides and has been widely studied for its ability to breakdown pesticides and nerve gases [1].

PON-1 is predominantly synthesized by the liver and it is distributed to other tissues, but mainly found in serum [2]. In serum, PON-1 is attached to high density lipoprotein (HDL) particles and seems to contribute to the anti-atherogenic and anti-inflammatory properties of HDL, as it degrades lipid peroxides, decreases HDL susceptibility to peroxidation, glycation, and homocysteinylolation, and increases cholesterol efflux from macrophages. Therefore, it is thought to protect against the development of atherosclerosis [3].

The human PON-1 gene is located on the long arm of chromosome 7 between q21 and q22 [4]. Two common coding region polymorphisms occur: a glutamine to arginine substitution at a.a position 192 (Q192R, rs662) and a leucine to methionine substitution at a.a position 55 (L55M, rs854560) [5]. These single nucleotide polymorphisms (SNP) impair PON-1 activity [6]; L55M seems to impair PON-1 bioavailability and Q192R SNP determines PON-1 hydrolytic activity towards paraoxon [7]. Studies have shown that carriers of the R and the L allele

seem to have a greater risk of developing Cardiovascular Disease (CVD) [4].

The pathogenetic mechanisms of CVD development involve arterial damage, including both media and intima of large- and medium-sized arteries [8]. In adults, arteriosclerosis and atherosclerosis are closely related, share common risk factors and they increase arterial stiffening and the risk of atherothrombosis, respectively [9]. Among the noninvasive methods for the evaluation of arterial structure, ultrasound imaging of arterial walls allows measurement of intima-media thickness (IMT) [8,9]. The standard sites of IMT measurement in adults are the common carotid artery (CCA), carotid bulb and internal carotid artery (ICA). The basic mode includes evaluation in B-mode presentation, and results are usually shown as the average value of IMT measurement from the right and left CCA. Studies in adult populations have shown that there is a steady increase in the diameter of CCA with age, followed by increased IMT, and that increased IMT is in part a physiological reaction in elevated blood pressure [10]. In the general pediatric population, IMT also increases with age and is related to blood pressure, even in the normal range [10]. Moreover, increased IMT has been observed in CCA of children with metabolic diseases, such as familial hyperlipidemia, and in obese children [11,12].

Low density lipoprotein (LDL) oxidation is considered to play a triggering role in the atherosclerotic plaque formation. This process is

possibly inhibited by HDL, by PON-1 in particular [3]. Moreover, low-grade systemic and sub-endothelial inflammation seems to be implicated in this chronic and complex procedure, as well. Markers of inflammation, such as C-reactive protein (CRP), have been related to the presence of active atherosclerotic disease and to the risk for CVD [13].

PON-1 polymorphisms have not yet been adequately investigated in children population, especially in Greece. The aim of the present study was to investigate the possible relation of IMT to the above-mentioned PON-1 polymorphisms and to markers of inflammation and oxidation, such as CRP and levels of oxidized LDL (oxLDL), respectively, in children with family history of premature CVD, a well-known risk factor for atherosclerosis development.

2. METHODOLOGY

Eighty four healthy children, with normal BMI, participated in the study and they were divided in two groups; 42 children with positive family history of premature CVD, median age 10 (7-15) years old (study group), 23 (55%) boys and 19 (45%) girls, and 42 age- and gender-matched children without family history of premature CVD (controls). The family history of premature CVD was not used for risk estimation, but as a risk factor which might or might not charge each of the two groups negatively, in reference to cardiovascular disease, as long as the hypothesis set in this study aimed in investigating whether family history plays a role in the differences in cIMT among children and its relation to PON-1 gene expression, oxidation and inflammation status. The children were recruited from the Outpatient Clinic for Lipids and Cardiovascular Disease Prevention from Childhood of our Department. Written consent for the participation in the study was obtained from the parents of the children.

Blood was collected after a fasting period of at least 8-hours. Renal, liver and thyroid function, i.e. creatinine, SGOT, SGPT and TSH, as well as blood pressure and fasting blood glucose were determined and used as exclusion criteria. Only healthy children, with normal BMI were included in the study. Blood in EDTA was kept in -80°Celsius for the PON-1 polymorphism study, and serum was isolated and frozen in -80°C. Levels of lipid profile parameters, such as total cholesterol (TC), HDL-cholesterol (HDL), LDL-cholesterol (LDL), triglycerides (TG),

apolipoprotein A-I (ApoA-I), apolipoprotein B-100 (ApoB-100), lipoprotein (α) [Lp(α)], and high sensitivity CRP (hsCRP) were determined in biochemical analyzer COBAS INTEGRA 4000 (ROCHE Diagnostics) and levels of oxLDL with ELISA (Immunodiagnostik, Bernsheim, Germany).

Genomic DNA was extracted with the QIAamp DNA Minikit (QIAGEN,) following manufactures' instructions. A fragment of the PON-1 gene, including PON1 -55 and PON1-192 polymorphisms, was amplified by classic polymerase chain reaction (PCR) in a thermocycler (MJ PTC200) for 35 cycles (95°C for 1 min, 56°C 30 sec, 72°C 1 min) and a final extension of 5 min at 72°C. The following primers (INVITROGEN, Carlsbad, CA, USA) were used (F: Forward, R: Reverse):

PON1-192F: 5' - TTG AAT GAT ATT GTT GCT GTG GGA CCT GAG-3'

PON1-192R: 5'- CGA CCA CGC TAA ACC CAA ATA CAT CTC CCA GAA-3'

PON1-55F: 5'- GAG TGATGT ATA GCC CCA GTT TC - 3'

PON1-55R: 5' - AGT CCATTA GGC AGTATC TCC G - 3'

The presence of the two polymorphisms was assessed by restriction fragment length polymorphism (RFLP). Hinf1 was used to digest 5 μ l of the PCR reaction in a 20 μ l final reaction. The result of the enzymatic cut with the Hinf1 was visualized, after electrophoresis, in a 3% agarose gel stained with Et-Br.

Carotid IMT (cIMT) in both carotid arteries was measured by Doppler ultrasound. The IMT was measured as the distance between the leading edge of the lumen-intima interface and the media-adventitia interface on the far wall of the artery. A mean IMT (mCIMT) was calculated.

2.1 Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics 20, for Windows. In each group of children the normality of distribution of values was investigated with Kolmogorov-Smirnov test. Due to significant difference from normal distribution for most of the variables, median and range of values were used for the description of the sample. For comparison between study group and controls non-parametric Mann-

Whitney and median comparison tests of independent samples were performed. Studying the children as a whole (N=84), we divided them in three subgroups, for each one of the genotypes of the PON-1 polymorphisms, i.e. QQ, QR, RR for Q192R polymorphism and MM, ML, LL for L55M polymorphism of PON-1. For comparisons between these subgroups of the children, non-parametric Kruskal-Wallis test for independent samples was used. Finally, multiple regression analysis was performed in order to investigate possible dependence of cIMT to other parameters under study. Statistical significance was set for $P < .05$.

3. RESULTS

Values of mcIMT and other biochemical parameters assayed, as well as of hsCRP and oxLDL, in both groups as shown in Table 1. Between the two groups only cIMT presented marginally significant difference between the two groups, with those children with positive family history of premature CVD having higher median cIMT than controls (Fig. 1).

Considering all of the 84 children, values of the parameters assayed in the three subgroups of children created with different PON-1 SNP are shown in Table 2. Apart from ApoA-I, no other parameter presented any difference between these subgroups. Genotype distribution for Q192R and L55M was in agreement with frequencies expected under Hardy-Weinberg

equilibrium. The relation between different SNPs of PON-1 and mcIMT is also shown in Table 2.

Results of multiple regression analysis between mcIMT and parameters of lipid profile, markers of inflammation and oxidation are shown in Table 3. The dependency of cIMT on the different variables investigated, i.e. age, lipid parameters, inflammatory and oxidative stress markers, seemed to be non-significant. Family history contributed in mcIMT only 1.7%, and all the other parameters studied contributed below 5%. The least contribution was by TG and Lp(α), with 0.5% and 0.3% respectively.

4. DISCUSSION

Family history of premature CVD is considered one of the major risk factors for the onset of CVD, even from childhood. The antioxidant capacity of HDL, due to PON-1, is considered one of the causes for the inverse relation of HDL serum levels to cardiovascular risk [14]. One of the markers for the estimation of the cardiovascular risk is cIMT, in adults and children as well. Various studies have been conducted in children populations investigating cIMT in children in relation to inherited metabolic diseases, obesity, hypertension, biochemical risk factors for CVD, etc. [15-18]. Nevertheless, results in healthy children population are absent, and in Greece in particular, are absent and, to our knowledge, there are no reports on cIMT in relation to family history of premature CVD.

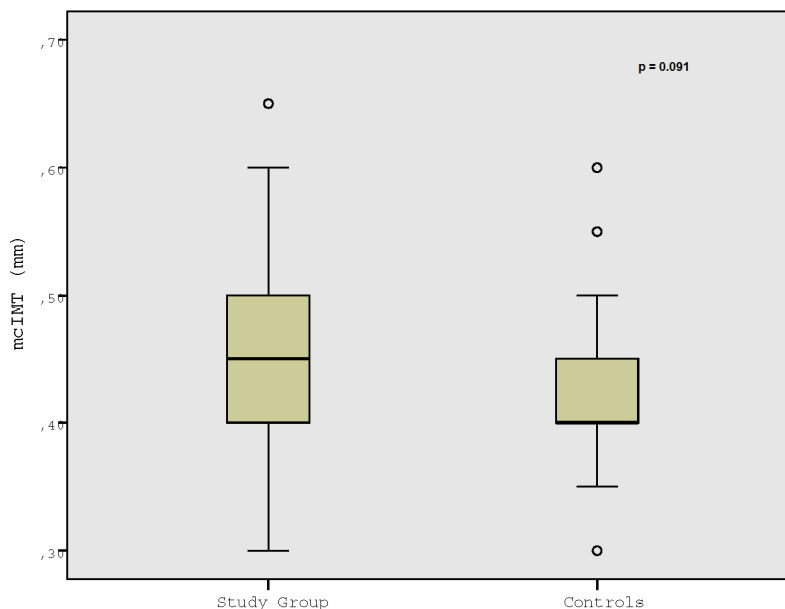


Fig. 1. Difference in cIMT (mm) in study group and controls ($P=.09$)

Table 1. Values of mcIMT, hsCRP and other biochemical parameters assayed, as well as oxLDL, in study group and controls

	Study group (N=42)	Controls (N=42)	P value
	Median (range)		
Age (years)	10 (7-15)	9 (7-15)	
mcIMT (mm)	0.45 (0.30-0.65)	0.4 (0.30-0.60)	0.09
TC (mg/dl)	188.5 (140-378)	182.5 (107-296)	0.38
TG (mg/dl)	63 (32-310)	65.5 (42-288)	0.38
HDL (mg/dl)	48.5 (28-80)	50.5 (18-90)	0.24
LDL (mg/dl)	116 (31-301)	100 (23-222)	0.16
ApoA-I (g/L)	1.41 (0.99-1.95)	1.38 (1.05-2.45)	0.63
ApoB-100 (g/L)	0.8 (0.47-1.56)	0.81 (0.24-1.5)	0.64
LP(α) (mg/dl)	29.85 (1-157)	23.8 (2-217)	0.70
hsCRP (mg/L)	0.07 (0-1.02)	0.08 (0.01-1.58)	0.29
oxLDL (U/L)	160.5 (8-1785)	236.5 (14-2847)	0.44

mcIMT, mean carotid intima-media thickness; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; ApoA-I, apolipoprotein A-I; ApoB-100, apolipoprotein B-100; LP(α), lipoprotein (α); hsCRP, high-sensitivity C-reactive protein; oxLDL, oxidized low density lipoprotein

Table 2. Parameters assayed in the three subgroups of children with Q192R and L55M SNPs

	Groups of Q192R polymorphism			Groups of L55M polymorphism		
	QQ n=48	QR n=31	RR n=5	MM n=54	ML n=14	LL n=16
Age (years)	9 (7-15)	9 (7-15)	14 (7-15)	8 (7-15)	11 (7-15)	11 (7-15)
mcIMT (mm)	0.45 (0.30-0.65)	0.40 (0.30-0.65)	0.40 (0.30-0.60)	0.45 (0.30-0.65)	0.40 (0.30-0.65)	0.45 (0.30-0.60)
TC (mg/dl)	187 (125-378)	195 (107-296)	174 (133-244)	187 (127-378)	192.5 (107-254)	181 (131-244)
TG (mg/dl)	65.5 (32-288)	61 (38-202)	117 (44-310)	66 (32-263)	77.5 (38-288)	61 (39-310)
HDL (mg/dl)	48 (18-90)	51 (33-80)	51 (28-64)	48 (18-80)	49.5 (41-90)	52.5 (28-73)
LDL (mg/dl)	107.5 (31-301)	120 (23-219)	92 (82-164)	114 (31-301)	100 (23-188)	106.5 (73-164)
ApoA-I (g/L)	1.37 (0.99-1.97)	1.38 (1.15-2.45)	1.42 (1.08-1.51)	1.31 (0.99-1.92)*	1.45 (1.15-1.97) *	1.49 (1.08-2.45)*
ApoB-100 (g/L)	0.77 (0.43-1.56)	0.83 (0.24-1.5)	0.92 (0.5-1.06)	0.78 (0.43-1.56)	0.8 (0.24-1.5)	0.91 (0.43-1.08)
LP(α) (mg/dl)	17.95 (2-150)	40.4 (1-217)	15.9 (5-40)	23.7 (2-217)	39.65 (3-131)	20 (1-78)
hsCRP (mg/L)	0.08 (0-1.58)	0.07 (0-1.02)	0.06 (0.01-0.21)	0.07 (0-1.58)	0.14 (0.01-1.01)	0.06 (0.01-0.33)
oxLDL (U/L)	160.5 (8-2201)	244 (23-974)	58 (27-2847)	281 (8-2201)	149 (25-672)	209.5 (27-2847)

* P = .03; mcIMT, mean carotid intima-media thickness; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; ApoA-I, apolipoprotein A-I; ApoB-100, apolipoprotein B-100; LP(α), lipoprotein (α); hsCRP, high-sensitivity C-reactive protein; oxLDL, oxidized low density lipoprotein. Values are expressed as median (range). QQ, QR, RR: genotypes of Q192R polymorphism; MM, ML, LL: genotypes of L55M polymorphism

In our study there was no statistical significant difference in cIMT between children with positive family history of premature CVD and controls, as shown in Table 1, with the study group having higher median values than controls (Fig. 1). Nevertheless, there was no child in either group with cIMT > 0.9 mm, which is considered to have negative prognostic value for future CVD. There was no difference in the lipid profile parameters, hsCRP and oxLDL, markers of inflammation and oxidation, respectively, in the two groups of children. Therefore, children with family history of premature CVD do not seem to have any difference in levels of inflammatory or oxidative indices, in the present study, which are considered secondary markers of increased risk for CVD. Those results are in contrast to previous studies [19,20] showing significant elevated lipids and inflammation markers in children with positive family history of premature CVD.

Moreover, the investigation of lipid profile parameters, hsCRP and oxLDL between children with different Q192R SNP and L55M SNP (Table 2), showed significant difference only in ApoA-I in the latter. The M-variant, which has been related to lower PON-1 concentration and bioactivity [21], seemed to be related to lower levels of ApoA-I in the children of this study. Non-significant was the difference of mcIMT between the subgroups of children with different genotypes of PON-1 (Table 2). This is in contrast to other studies which have shown that decreased PON-1 activity was related to more severe atherosclerosis in adults [22] and that there was significant difference in cIMT in children with familial hypercholesterolemia and different L55M polymorphism of PON-1 gene [23]. Our results, in refer to oxidative stress marker, are in accordance with the study of Sampson MJ et al., who found that circulating oxLDL concentrations are unrelated to PON-1 genotypes [23]. Moreover, the study of Krzystek-Korpaczka M, et al. [24] has shown that oxidative stress affects PON-1 arylesterase activity in girls and inflammation affects its activity in boys. Consequently, PON-1 polymorphism does not seem to be an index to be used for the estimation of CVD risk in children.

In addition, multiple regression analysis (Table 3) showed that there was no significant dependence of cIMT on age, lipid profile parameters or markers of inflammation and

oxidation, in contrast to adults who present higher cIMT with age, according to the study of Eikendal AL, et al. [25]. Therefore, in the children of the present study cIMT does not seem to be dependent on other risk factors for CVD, such as age and dyslipidemia.

Table 3. Results of multiple regression analysis between mcIMT and lipid profile parameters, hsCRP and oxLDL

Independent variables	Dependent Variable mcIMT P value
Age	.53
Family history	.37
TC	.16
TG	.60
HDL	.85
LDL	.29
ApoA-I	.99
ApoB-100	.23
Lp(α)	.69
hsCRP	.77
oxLDL	.17

mcIMT, mean carotid intima-media thickness; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; ApoA-I, apolipoprotein A-I; ApoB-100, apolipoprotein B-100; LP(α), lipoprotein (α); hsCRP, high-sensitivity C-reactive protein; oxLDL, oxidized low density lipoprotein

5. CONCLUSIONS

In the present study, neither positive family history for premature CVD nor the polymorphism of PON-1 gene seems to be related with significant differences in cIMT. Inflammation and oxidation do not markedly affect cIMT in children with family history of premature CVD. This is the first time, to our knowledge, that such results combining cIMT, PON-1 polymorphism, inflammatory and oxidation indices are presented in a population of children. The weakness of our study is the small number of children that participated and the results can only provide indications on the certain hypothesis. Therefore, larger studies are needed to ascertain these results and provide more information on the relation of vascular integrity indices, such as cIMT, and PON-1 polymorphisms, as well as markers of inflammation and oxidation, in order to establish whether the investigation of such parameters can be used as early biomarkers of atherosclerosis progression beginning from childhood.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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