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Arylesterase activity of paraoxonase 1 (PON1) on HDL_3 and HDL_2: Relationship with Q192R, C-108T, and L55M polymorphisms

Sandra Y. Valencia C [a,b,c,*], Carlos A. Isaza M [d], Julieta Henao B [d], Leonardo Beltrán A [d,f], Nelsy Loango [c,e], Patricia Landázuri [c]

[a] Faculty of Health Sciences, Program of Nutrition, Universidad Libre, Colombia
[b] Faculty of Medicine, Fundación Universitaria Autónoma de las Américas, Colombia
[c] Faculty of Health Sciences, Universidad del Quindío, Colombia
[d] Faculty of Health Sciences, Universidad Tecnológica de Pereira, Colombia
[e] Faculty of Basic Sciences and Technologies, Universidad del Quindío, Colombia
[f] Faculty of Health Sciences, Unidad Central del Valle del Cauca, Colombia

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ABSTRACT

Background: Controversy exists regarding the role of the subfractions of high-density lipoproteins (HDL_2 and HDL_3) in cardiovascular disease. The functionality of these particles, and their protective role, is due in part to the paraoxonase 1 (PON1) presence in them. The polymorphisms rs662 (Q192R, A/G), rs854560 (L55 M, T/A), and rs705379 (C-108T) of the PON1 gene have been related to enzyme activity and, with the anti-oxidative capacity of the HDL. The objective was to determine the arylesterase PON1 activity in HDL_3 and HDL_2 and its relationship with the polymorphisms mentioned, in a young population.

Methods: The polymorphisms were determined through mini-sequencing (SnaPshot). The HDL subpopulations were separated via ionic precipitation, cholesterol was measured with enzymatic methods, and PON1 activity was measured through spectrophotometry.

Results: The results show that the PON1 polymorphisms do not influence the cholesterol in the HDL. A variation between 40.02 and 43.9 mg/dL was in all the polymorphisms without significant differences. Additionally, PON1 activity in the HDL_3 subfractions was greater (62.83 ± 20 kU/L) than with HDL_2 (35.8 ± 20.8 kU/L) in the whole population and in all the polymorphisms ($p < 0.001$), and it was independent of the polymorphism and differential arylesterase activity in the Q192R polymorphism (QQ > QR > RR). Thus, 115.90 ± 30.7, 88.78 ± 21.3, 65.29 ± 10.2, respectively, for total HDL, with identical behavior for HDL_3 and HDL_2.

Conclusions: PON1 polymorphisms do not influence the HDL_{-c}, and the PON activity is greater in the HDL_3 than in the HDL_2, independent of the polymorphism, but it is necessary to delve into the functionality of these findings in different populations.

1. Introduction

The HDL exert potent anti-atherogenic activities, which include anti-oxidative and anti-inflammatory actions, which are relevant in attenuating the progression of atherosclerosis [1–3]. The anti-atherogenic functions of HDL lie directly in their composition and structure; however, the impact and mechanisms through which they exert these functions has not been clearly established.

The HDL are a heterogeneous group of particles that differ in their composition of lipids, apolipoproteins, size, density, and charge, which in turn gives them different properties [4]. Epidemiological studies have shown that low concentrations of cholesterol in HDL (HDL_{-c}) constitute an important and independent risk factor of cardiovascular disease; however, recent evidence indicates that changes in the distribution of the HDL subclasses and of their component proteins, such as the PON1, are of greater utility than the concentrations of HDL_{-c} in predicting coronary events [5].

Diverse subHDL populations are generated in the plasma. Initially, HDL are synthesized and secreted from the liver and intestine as nascent particles of pre-ß HDL, formed predominantly by the apoprotein A-I (ApoA-I) and phospholipids. In circulation, more phospholipids, cholesterol, and other minority apoproteins, like the PON1, are added,

* Corresponding author. Faculty of Health Sciences, Program of Nutrition, Universidad Libre, Colombia.
E-mail addresses: sandra5yol@hotmail.com, syvalenciac@uqvirtual.edu.co (S.Y. Valencia C).

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thus forming the diverse subHDL populations. Various systems are used to classify and separate the different populations or subfractions of plasma HDL [6]; one of them is the precipitation method that separates HDL into two principal subfractions, HDL_2 and HDL_3. The HDL_2 is a less-dense particle (d 1.063–1.125 g/ml), which is bigger and enriched with cholesterol esters; the HDL_3, or helical, is a more-dense particle (d 1.1251.21 g/ml), which is small and relatively rich in proteins [7].

The PON1, classified as an aryldialkylphosphatase enzyme (EC 3.1.8.1) [8], is a glycoprotein with anti-atherogenic and anti-oxidant properties and is strongly bound to HDL [9]; it is a promiscuous enzyme that can hydrolyze various substrates, among them paraoxon (diethyl p-nitrophenyl phosphate) from which is derived its name, but which also has lactonase and arylesterase activities. With this last activity, it hydrolyzes oxidized lipids from low-density lipoproteins (LDL) [10,11]. Additionally, strong evidence indicates that PON1 can exert its atheroprotective effect by regulating cholesterol flow from the macrophages and maintaining this lipid's homeostasis [12].

The PON1 activity may be affected by diverse factors, including lifestyle, diseases such as diabetes and obesity, and its polymorphisms. In this last aspect, various polymorphisms have been described for this enzyme, among which are C-108T and L55 M that seem to modify the enzyme concentration and Q192R, which modifies its activity [13,14].

These polymorphisms have been broadly studied and related with cardiovascular disease, but few studies describe the relationship of these genetic variants with enzyme activity in the HDL_2 and HDL_3 subfractions in a healthy, young population; hence, the objective of this study was to determine, in a population of young individuals without diagnosed disease, arylesterase PON1 activity in the HDL subfractions and its relationship with the enzyme's polymorphisms.

2. Methods

Type of study: Descriptive non-experimental.

Population: Males between 18 and 35 years of age who were students and administrative staff from universities in the Colombian coffee region, without diagnosed disease, and with no blood relation to each other. The study excluded smokers and extreme and elite athletes, as well as individuals taking any medication or with diabetes mellitus, cardiovascular disease, liver or kidney damage, or nutritional or collagen disorders.

Sample: The blood sample was taken by venipuncture after 12 h of fasting. The serum was obtained by centrifuging at 1.500 g for 8 min and stored at -80 °C until its analysis.

DNA isolation and genotyping: Three polymorphisms were studied: rs662 (Q192R), rs854560 (L55 M), and rs705379 (C-108T). Genomic DNA was extracted by using the Kit by Promega; amplification was done on three fragments of the PON1 gene. An initial polymerase chain reaction (PCR) was conducted in a total volume of 10 μL, which contained 1–10 ng of DNA, 1X Qiagen Multiplex PCR Master Mix, and 0.2 μM of each of the primers (Table 1). Temperature conditions for the PCR were 95 °C for 15 min, followed by 35 cycles of amplification at 94 °C for 1 min, 60 °C for 90 s, and 72 °C for 50 s, with a final extension at 72 °C for 7 min. Excess primers and nucleotides were removed by adding 1 μL of ExoSAP-IT (Affimetrix) to 2.5 μL of the PCR product. Thereafter, SNP detection was conducted with the mini-sequencing method. By using the PCR product as a template, multiplex reactions were carried out to detect the SNPs, in a 6-μL volume, which contained 1.5 μL of PCR product, 1 μL of Snapshot Reaction Mix (Applied Biosystems), and 3.5 μL of the mixture of SBE (Single Base Extension), primers with 0.2 μM concentration (Table 1). The amplification was conducted with 35 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. For detection, 2 μL of this product were mixed with 9 μL of HiDi-formamide and 0.5 μL of GeneScan-120Liz Internal Size Standard (Applied Biosystems) and were read in the ABI sequencer Prims 3100-Avant Genetic Analyzer (Applied Biosystems). Data were analyzed according to the color of the peaks and size of fragments, through Genemapper software v3.2 (Applied Biosystems). The presence of polymorphisms was confirmed by resequencing selected samples.

HDL_2 and HDL_3 subfractions separation: Total HDL (HDL_t) was isolated by using the procedure of the Human Kit for HDL-cholesterol. That is, 1 mL of diluted Human-HDL-cholesterol reagent (4 ml of HDL reagent and 1 ml of water) was added to 500 μL of plasma; this was incubated for 10 min and centrifuged for 7 min at 3.000 g. The very low density lipoproteins (VLDL) and LDL chylomicrons remained in the precipitate and the HDL_{-t} (HDL_2 and HDL_3) in the supernatant. To separate these from the supernatant, dextran sulfate-Mg^{2+} was added, according to the methods described by Warnick et al. [15], with some modifications made by García-Cardona et al., [16]. To briefly explain, 50 μL of dextran sulfate-Mg^{+2} (2% dextran + 9.49% $MgCl_2$ 50:50 proportion) were added to a volume of 450 μL of the previous supernatant; this was agitated in a vortex and left to rest for 20 min and centrifuged at 13000 rpm; the precipitate corresponds to the HDL_2 lipoproteins, and the supernatant to the HDL_3.

2.1. Total cholesterol (HDL_{-ct}) HDL_2 cholesterol (HDL_{-c2}), and HDL_3 cholesterol (HDL_{-c3})

An aliquot was taken from the two previous supernatants, and HDL_{-c} was determined with the commercial kit, following manufacturer's instructions (Human). The HDL_2 cholesterol was calculated by subtracting HDL_{-c3} from HDL_{ct}.

2.2. PON1 activity

The arylesterase activity in each of the HDL subfractions was determined by using phenyl acetate as substrate with the method reported by Carranza et al. [17], with modifications. To 5 μlμ of the sample was added 995 μl of Buffer 12 mM-Tris-HCl, pH 8, with phenyl acetate 1 mM as substrate, 1.6 mM $CaCl_2$ and Ethylenediaminetetraacetic acid (EDTA) 6 μM at 37 °C. Enzyme activity was calculated by using the phenol molar extinction coefficient (1.3×103 $L*mol^{-1}*cm^{-1}$). Absorbance was measured at 270 nm. The arylesterase activity was expressed as mMol of hydrolyzed phenyl acetate per min per L of serum (kU/L) [18,19].

2.3. Statistical analysis

The allele frequencies were calculated from the number observed from each allele. The Hardy-Weinberg equilibrium was established through the Chi-squared test, which was also used to compare

Table 1
Sequences of the primers and probes.

SNP	Forward (5′-3′)	Reverse (5′-3′)	SBE (5′-3′)
rs662 Gln192Arg	GACATTTTACATTTTTCCTAA	TTCCTCATGTCTATTCC	CTAAACCCAAATACATCTCccAGGAT
rs854560 Leu55Met	TTGAAAGTGGGCATGGGTAT	TGGATCCACATCCTgCAATA	GTCTTCAGAGcCAGTTTC
rs705379 C-108T	GCCGCAGCCCTGCTGGGG	GGAAGGAGCAAAATGGGACT	TGCTGGGGCAGCGCCGATTGGCCCGCCCC

qualitative variables. The analysis of variance (ANOVA) was used for the correlations among the genotypes and the HDL or the enzymatic activity, while the correlations between lipid fractions and enzymatic activity were established through the Pearson correlation. Statistical analyses were performed with the SPSS 19 software for Windows, using 95% confidence intervals and considering values of $p < 0.05$ as significant.

3. Results

3.1. Anthropometric characteristics and HDL-c description of the study population

This part of the study included 133 individuals, whose anthropometric characteristics and concentration of total HDL-c are described in Table 2.

3.2. Genotypic and allelic distribution

Table 3 describes the genotypic and allelic distribution of the three polymorphisms studied. The three were in Hardy-Weinberg equilibrium and no ligament imbalance was evident.

3.3. HDL cholesterol

Table 4 shows the cholesterol distribution in total HDL in the distinct polymorphisms.

The data indicate no significant variation of the HDL_{-c} in the different polymorphisms, which is corroborated with the correlation indicated in Table 5 that shows data of statistical significance among the genotypes, diplotypes, and haplotypes, and the HDL_{-c} concentration. These values indicate that no correlation was found among them.

3.4. PON1 activity in the HDL subfractions and genetic polymorphisms

This study related the activity of the PON1 enzyme from only 78 of the 133 individuals in the HDL subfractions, with the genetic polymorphisms from the same enzyme; the results are evidenced in Table 6.

First, the results show that, in the three polymorphisms, the arylesterase PON1 activity is greater in the HDL_3 than in the HDL_2; secondly, the data indicate that only in the Q192R polymorphism, the enzyme activity in the three HDL populations behave; thus, PON1 activity in QQ > QR > RR with significant differences (Fig. 1).

The study also evaluated the correlation of the combination of polymorphisms and their effect on the PON1 activity in each HDL subfraction; the results are shown in Table 7. These indicate that significance was only evident in the combinations with the Q192R polymorphism present.

4. Discussion

The PON1 is a promiscuous enzyme that can hydrolyze various substrates, like organophosphate compounds, arylesters, cyclic thiolactone carbonates, nerve gas, glucuronides, and estrogen esters [14]. With respect to cardiovascular disease, however, it seems that its principal substrate are peroxides and lipophilic lactones associated with lipoproteins, especially LDL, thus preventing the oxidation of this lipoprotein, given that these oxidized lipids are pro-atherosclerotic and pro-inflammatory [10–12].

In addition, the PON1 is a polymorphic enzyme, and the enzyme's genetic variations can modify its properties in serum or plasma. The gene has various polymorphisms in the coding region, with the principal and most studied being the rs662 (Q192R) and the rs854560 (L55 M). The enzyme also has diverse polymorphisms in the promotor region, among them, the rs705379 (-108C/T) [20–22].

This work researched the polymorphisms mentioned (Q192R, L55 M, and C-108T). The results show that the allelic and gene frequencies of these polymorphisms (Table 3) do not differ much from those found in the North American [23], Latin [24,25], and even Colombian populations [26].

It was interesting to find that although the PON1 is an enzyme strongly bound to HDL, none of its three polymorphisms influenced the cholesterol concentration in these lipoproteins (Tables 4 and 5), given that HDL-c was similar in all the genotypes of the three polymorphisms studied.

The literature shows that the metabolism of HDL involves a complex network of factors that regulate their synthesis, intravascular remodeling, and catabolism; their components in lipids and apoproteins assembled after their secretion (through liver and intestine) are frequently interchanged or transferred to other lipoproteins, giving way to a family of HDL subfractions with similar components (lipids, proteins), but not in the same amount and composition and, hence, do not seem to have the same properties [21,27–29]. Among the protein components of the HDL subpopulations, there are the APOA (its principal apoprotein) and the PON1. This latter enzyme has been more associated with the anti-oxidative and anti-inflammatory capacity of the HDL subclasses than with its cholesterol content [9,13,21,22].

This work also evaluated the arylesterase PON1 activity in the subpopulations' HDL in each of the genotypes of the three polymorphisms (Table 6). The results provide evidence for three important findings: first, the arylesterase activity of the PON1 enzyme was similar in the three polymorphisms; second, when comparing the HDL_3 and HDL_2, the activity was greater in the HDL_3 in all the polymorphisms; and third, only in the Q192R polymorphism was the arylesterase enzyme activity distributed; thus, QQ > QR > RR, with significant differences in the three subfractions studied (HDL_t, HDL_3, and HDL_2). But, besides, when combining the polymorphisms and relating them with the enzyme activity, only the presence of the Q192R polymorphism showed significance with the PON1 activity (Table 7).

These last results confirm data described by other authors showing that enzyme activity depends more on the Q195R polymorphism than on the other two polymorphisms studied [8,14,31]. The literature also describes that the Q192R polymorphism in the PON1 is the principal determining factor in enzyme activity (especially paraoxonase activity), with greatest activity in the QQ genotype, with a marked difference in comparison with the QR and RR [8,14]. These results are similar to those found in our work on the PON's arylesterase activity; however, it must be noted that some authors have found influence from the L55 M and C-108T polymorphisms in PON1 activity and expression [13,23].

This study was relevant in that it demonstrated that, in the three polymorphisms studied, the HDL_3 subfraction has greater PON activity than the HDL_2, given that few works describe this finding in the three polymorphisms simultaneously. Also, data from this work suggest that the greater PON1 activity in the HDL_3 is independent of the polymorphism. Similar results were found by Schiavon et al. [32] in patients with chronic kidney disease, showing that greater PON1 activity was present in the HDL_3 in both patients and controls, but that in the patients this activity was significantly lower, independent of the polymorphism. Our data and that of this and other authors suggest there is greater PON

Table 2
Anthropometric characteristics and HDL-c of the study population.

Characteristic	Value
Age (years)	20.5 ± 2.2
Weight (kg)	71.40 ± 15.38
Height (m)	1.8 ± 0.1
Body mass index (Kg/m^2)	23.4 ± 4.6
HDL_{-ct} (mg/dL)	42.85 ± 9.20
HDL-t PON1(kU/L)	96.9 ± 30
PON1-HDL_3 (kU/L)	62.8 ± 20.2
PON1-HDL_2 (kU/L)	35.8 ± 20.2

HDL-c: high-density lipoprotein cholesterol; PON1 = Paraoxonase 1.

Table 3
Polymorphism in the Paraoxonase gene in the study individuals.

Polymorphism	Genotype	n	Freq. (95%CI)[a]	Allele	n	Freq. (95%CI)[a]	HWE[b]
rs662 (Q192R, 575A/G)	QQ	56	0.42 (0.32–0.47)	Q	179	0.67 (0.61–0.72)	0.29
	QR	67	0.50 (0.46–0.62)	R	87	0.33 (0.28–0.40)	
	RR	10	0.08 (0.03–0.11)				
rs854560 (L55 M, 163T/A)	LL	66	0.50 (0.41–0.58)	L	189	0.71 (0.66–0.77)	0.92
	LM	57	0.43 (0.35–0.51)	M	77	0.29 (0.23–0.34)	
	MM	10	0.07 (0.03–0.12)				
rs705379(C-108T)	TT	45	0.34 (0.26–0.42)	T	150	0.56 (0.50–0.62)	0.69
	CT	60	0.45 (0.37–0.53)	C	116	0.44 (0.38–0.50)	
	CC	18	0.21 (0.14–0.29)				

[a] Frequency (95% confidence interval).
[b] Hardy-Weinberg Equilibrium, p value.

Table 4
Cholesterol concentration in total HDL in the different polymorphisms.

QR192/rs662 (mg/dL)		LM55/rs854560 (mg/dL)		CT108/rs705379 (mg/dL)	
QQ (56)	41.8 ± 9.3	LL(10)	41.0 ± 12.9	CC (56)	42.0 ± 9.3
QR (67)	43.9 ± 8.9	LM (57)	42.9 ± 8.5	CT (67)	43.2 ± 8.9
RR (10)	41.6 ± 11.4	MM (66)	43.1 ± 9.3	TT (10)	42.9 ± 11.4
Total (133)	42.8 ± 9.2	Total (133)	42.8 ± 9.2	Total (133)	42.8 ± 9.2

(n) = number of individuals.

Table 5
Statistical significance of the genotype-HDL_c correlations.

SNP	HDL_c	diplotypes	HDL_c	Haplotype	HDL_c
rs662	0.40	rs662+ rs854560	0.56	rs662 + rs854560 + rs705379	0.59
rs854560	0.80	rs662 + rs705379	0.63		
rs705379	0.86	rs854560 + rs705379	0.87		

HDL_c = cholesterol in high-density lipoproteins.

activity in the HDL_3 and that this is independent of the polymorphism and depends more on the normal or abnormal physiology of the individuals [18].

Greater PON1 activity in the HDL_3 agrees with its normal physiology, given that once the enzyme is synthesized in the liver and secreted to the blood stream, it binds to nascent HDL_3; during HDL maturation, the PON1 is activated and shows a flow toward smaller and larger HDL particles, as well as toward VLDL and to the densest and smallest LDL (LDLs), as reported by Gugliucci et al. in *ex vivo* experiments [21,22].

According to the theory of formation of HDL subfractions, in which HDL_2 are formed from HDL_3 by exchange of lipids and proteins (including PON1), it is suggested that in healthy subjects, PON1 remains in HDL_3 to meet an antioxidant function and does not remain in the HDL_2 to be recycled and carry them to the liver by the reverse transport of cholesterol (TRC) [18,33,34], a theory that can be supported by our data and that of other authors who have found greater activity in HDL_3 than in HDL_2 [32,35,36].

Although the function of HDL_3 in cardiovascular disease, sedentary habits, and some metabolic disorders is controversial, given that many authors have assigned it a protective role and others have not [16,37,38], the evidence shown in this work and in others of greater enzyme activity in this subfraction would agree with its athero-protective function because the PON1 activity has been assigned functions, like the activation of the endothelial nitric oxide synthase, prevention of

Table 6
PON1 activity in polymorphisms.

PON1 polymorphisms	PON1 activity kU/L			
	(n)	HDL_t	HDL_3	HDL_2
Total population	(78)	96.84 ± 29.8*+	62.83 ± 20.2	35.8 ± 20.8
rs662 (Q192R/575A/G)	QQ (31)	115.90 ± 30.7*+	72.37 ± 18.8	46.16 ± 23.9
	QR (38)	88.78 ± 21.3*+	59.32 ± 18.8	31.04 ± 15.7
	RR (9)	65.29 ± 10.2*+	44.85 ± 14.6	20.44 ± 8.8
rs854560 (L55 M/ 163T/A)	LL (6)	86.89 ± 17.8	58.93 ± 16.1	27.96 ± 17.4
	LM (30)	105.54 ± 32.1	69.62 ± 21.2	38.64 ± 21.9
	MM (42)	92.05 ± 28.5	58.93 ± 19.1	34.93 ± 20.5
rs705379(C-108T)	CC (19)	95.39 ± 31.8	60.34 ± 20.2	38.22 ± 20.3
	CT (34)	93.57 ± 20.6	62.75 ± 14.7	33.22 ± 21.9
	TT (25)	102.41 ± 38.3	64.86 ± 26.5	37.56 ± 20.1

HDL = High-density lipoproteins. * = $p < 0.0001$ with respect to the PON1 activity in each genotype within the polymorphism. + = $p < 0.0001$ with respect to the PON1 activity in each subfraction.

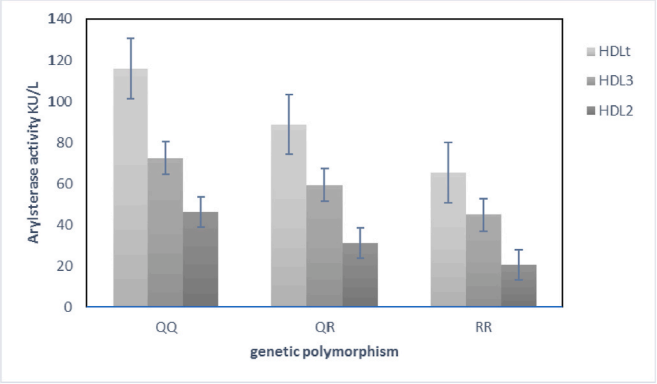

Fig. 1. Arylesterase activity of the PON1 in the HDL_t, HDL_3, and HDL_2, according to the rs662 genotype (Q192R, 575A/G). The vertical lines indicate the 95% confidence interval.

lipid oxidation and hydrolysis of oxidized lipids in the LDL, and reduction of foam-cell formation among other functions related with the ECV [8,20].

Hence, we can suppose that if the HDL_3 have the greatest PON1

Table 7
Significance value of the correlation of polymorphisms on the enzyme activity.

HDL subfraction	L55 M/Q192R	C-108T/Q192R	C-108T/L55 M
HDL_t	<0.0001	< 0.0001	< 0.522
HDL_3	<0.003	< 0.018	< 0.768
HDL_2	<0.001	< 0.018	< 0.625

HDL: High-density lipoproteins. significance level $p<0.05$.

activity, they are protective against the oxidative damage of LDL; hence, they serve as an anti-atherosclerotic factor, although their clinical relevance is still under debate. But HDL_2 also could have a protective role, due to their role in TRC, but the balance between one and another will depend on the state of the organism. Whether or not the protective function of HDL_3, given their anti-oxidative capacity, is complementary or contrary to the function of HDL_2 due to their role in cholesterol transport needs further research in healthy individuals with diverse pathologies and in follow-up studies.

A great limitation of this study is that the paraoxonase activity and the salt-stimulated paraoxonase activity were not measured, which implies that an antioxidant capacity of the enzyme was not measured, however, several authors have not found significant differences between the activity paraoxonase and arylesterase activity in various populations including in the Harangi et al. study in dyslipidemic patients and controls (38).

5. Conclusion

Our data show that although the PON is an enzyme closely associated with HDL, the cholesterol concentration in them is independent of the polymorphisms. Moreover, it is confirmed that the Q192R polymorphism determines – in part – the PON1 activity and that its effect is mediated by the Q allele, given that this allele seems to have greater activity than the R. Likewise, this work demonstrates that the arylesterase activity of the PON1 is higher in the HDL_3 subfractions than in the HDL_2 and that this activity in the subfractions is independent of the polymorphisms.

Ethics approval and consent to participate

The research was approved by the institutional Bioethics Committee (Acta No. 31 Punto 03 Numeral 3.2.2–2016). All participants signed an informed consent, prior to blood sample.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

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Credit author statement

Sandra Y. Valencia C: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Visualization, Supervision, Project administration, Funding acquisition.

Carlos A. Isaza M.: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Supervision, Project administration, Funding acquisition

Julieta Henao: Methodology**,** Validation, Investigation, Resources, Supervision, Project administration, Funding acquisition

Leonardo Beltrán: Methodology, Validation, Investigation, Funding acquisition

Nelsy Loango: Methodology, Validation, Investigation, Writing - Original Draft, Project administration, Funding acquisition

Patricia Landazuri: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no competing financial interests.

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