ORIGINAL ARTICLE

Associations between high density lipoprotein mean particle size and serum paraoxonase-1 activity

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Background: High density lipoprotein (HDL) particles are heterogeneous in composition, structure, size, and may differ in conferring protection against cardiovascular disease. HDL associated enzyme, paraoxonase-1 (PON1), has an important role in attenuation of atherogenic low density lipoprotein (LDL) oxidation. The aim of this study was to investigate the associations between HDL particle size and PON1 activity in relation to serum HDL cholesterol (HDL-C) levels. **Materials And Methods:** One hundred and forty healthy subjects contributed to this study. HDL was separated by sequential ultracentrifugation and its size was estimated by dynamic light scattering. Paraoxonase activity was measured spectrophotometrically using paraoxon as substrate. **Results:** Results of this study showed that PON1 activity had negative correlations with HDL mean particle size ($r = -0.22$, $P < 01$), HDL_2/HDL_3 ratio, and serum HDL-C levels ($r = -0.25$, $P < 0.01$). HDL mean particle size and HDL_2/HDL_3 ratio had negative correlation with body mass index (BMI), waist hip ratio (WHR), and serum triglyceride (TG) levels, and positive correlation with serum HDL-C levels. Serum HDL-C levels had significant positive correlations with age, total cholesterol (TC), and apolipoprotein A-I (apo A-I) and significant negative correlation with BMI, WHR, and TG. **Conclusion:** Based on the results of this study, determination of HDL mean particle size beside the serum PON1 activity may help to better understand the CAD risks, pathogenesis, and prognosis, and may also help to design therapeutic protocols toward beneficial modifications of HDL characteristics.

Key words: Dynamic light scattering, high density lipoprotein size, HDL-C, paraoxonase-1 activity, zetasizer

INTRODUCTION

There is a strong inverse relationship between plasma high density lipoprotein (HDL) level and the risk of developing coronary artery disease (CAD).[1,2] The major role of HDL is the reverse cholesterol transport, which leads to cholesterol movement from peripheral tissue and vessel wall to the liver.[3] Anti-inflammatory, antioxidant, anticoagulant, and profibrinolytic actions of HDL are among other functions that may contribute further to its ability to protect against CAD.[3] HDL particles are heterogeneous in their composition, structure, and size. Several methods such as immunoaffinity chromatography, ultracentrifugation.[4] gradient gel electrophoresis.[5] electron microscopy.[6] and nuclear magnetic resonance (NMR) have been used to isolate and characterize HDL subfractions and at least five subfractions of HDL with sizes of 7.3-13 nm have been identified.[7] One of the best known anti-inflammatory and antioxidant functions of HDL is its ability to inhibit the oxidation of low-density lipoprotein (LDL).[8] HDL exerts its inhibitory effects on oxidative modification of LDL, in part, by its related enzyme paraoxonase-1 (PON1) which can hydrolyze lactones, and several non-physiological substrates, such as aryl esters and organophosphates.[9] PON1 is transported in plasma as a component of HDL, and many studies have also shown that PON1 inhibits LDL oxidation *in vitro*.[10] There is a close physiological association between PON1 and HDL in plasma. HDL facilitates the secretion of the PON1 by the liver, stabilizes the enzyme,[11] and provides a hydrophobic environment which is needed for PON1 function.[12] In return, PON1 prevents the oxidation of HDL.[13] There is a wide inter-individual variation in PON1 activity and concentrations due to variation in *PON1* gene, and also, because of the influences of lifestyle factors such as smoking, alcohol consumption, etc.[14,15] A study of populations with high prevalence of atherosclerosis suggested that PON1 could influence HDL concentrations in patients with familial hypercholesterolemia.[16] It has been demonstrated that PON1 is present in small dense subclasses of HDL particles including apolipoprotein A-I (apo A-I) and

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apolipoprotein J.[17] Investigations have also indicated that there is a relationship between PON1 activity and HDL size with CAD risk.[18,19] However, this relationship is not demonstrated in all studies.[20,21]

With these in mind, the protective role of HDL against CAD seems to be a complicated and multi-factorial procedure that depends on the HDL particle size, number, composition, distribution, and its related enzymes such as PON1. The aim of this study was to investigate the associations between HDL mean particle size and serum PON1 activity in relation to serum HDL-C levels in healthy subjects.

MATERIALS AND METHODS

Subjects

For this study, 140 healthy men between 20 and 50 years of age from Isfahan area were recruited to Isfahan Cardiovascular Research Center between June and September 2010. All subjects were healthy, non-smoking, non-obese [body mass index (BMI) ≤30], and non-diabetic [fasting blood sugar (FBS) ≤110 mg/dl] volunteers, without familiar hypercholesterolemia and any sign of cardiovascular or inflammatory diseases, and no one was taking lipid-modifying medication. All participants were interviewed and a checklist relating to their age, medical history, and lifestyle information was completed. All participants signed an informed consent document approved by the Isfahan University of Medical Sciences Medical Ethics Committee. This study was funded by grant number 188140 from Isfahan University of Medical Sciences, Isfahan, Iran.

Anthropometric measurements

Weight was measured while subjects were minimally clothed without shoes and height was measured in a standing position. BMI was calculated as weight in kilograms divided by height in meters squared. Waist circumference (WC) was measured at the narrowest level and that of hip at the maximal level over light clothing, using a tape measure. Waist hip ratio (WHR) was calculated as WC divided by hip circumference.[22] To avoid subjective error, all measurements were taken by the same person. Blood pressure was measured by a qualified physician after a rest for 15 min.

Biochemical analysis

Blood samples (20 ml) were collected by venopuncture after 12-14 h fasting. Serum was separated by centrifugation at 3000 rpm for 15 min. A 100 μl aliquot of serum was stored at −80°C until the measurement of serum PON1 activity. Biochemical measurements and HDL separation were carried out on the day of sampling. HDL-C and LDL-C were determined by direct enzymatic procedures; FBS, total cholesterol (TC), and triglyceride (TG) were measured by enzymatic methods; and apo A-I and apolipoprotein B-100 (apo B-100) were measured by immunoturbidometric methods using an automated analyzer (Hitachi 902, Kyoto, Japan) and commercial kits (Diasys Diagnosis, Holzheim, Germany).

Serum PON1 activity

PON1 activity was measured using 2 mM paraoxon (diethyl *p*-nitrophenyl phosphate, Sigma chemical Co., St. Louis, MO, USA) as the substrate in 100 mM tris buffer (pH 8.0) containing 2 mM of $CaCl_2$.[23] Aliquots of 40 μl of serum were added to 500 μl of substrate medium and the initial rate of hydrolysis (generation of *p*-nitrophenol) was monitored at 412 nm, using a continuously recording spectrophotometer (UV 3100, Shimadzu, Kyoto, Japan) over a period of 2 min. A PON1 activity of 1 unit/L was defined as 1 μmol of *p*-nitrophenol formed per minute. The molar extinction coefficient of *p*-nitrophenol was taken at 17,000 M/cm.[23] All assays were performed in duplicate at 25°C. The within-assay coefficient of variation (CV) for 10 separate measurements was 2.9% and between-assay CV for 10 separate measurements during 20 days was 3.6%.

HDL separation

For HDL separation ($1.063\ g/cm^3 < \varrho < 1.21\ g/cm^3$), polyallomer ultracentrifuge tubes with 8.9-ml capacity (Optiseal, Beckman/Coulter, Fullerton, CA, USA) were first taken with 5.9 ml of serum sample and the sample was overlaid with 3 ml of solution A [containing 0.195 mmol NaCl, 0.62 mmol NaOH, 0.01% (w/v) ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2), d = 1.006 g/ml] in order to make discontinuous density gradient. The tubes were centrifuged in a Beckman Coulter Optima L-100 XP ultracentrifuge equipped with a type 90Ti fixed angle rotor at 60,000 rpm for 6 h at 16°C, acceleration:"5" and deceleration:"7". After centrifugation, the very low density lipoprotein (VLDL) fraction (the white layer at the supernatant) accompanied by 3 ml of the upper layer solution in tubes was removed and residual content of tubes was mixed with 3 ml of solution B (containing 24.8 g NaBr in 100 ml of solution A). The tubes were centrifuged at 60,000 rpm for 12 h at 16°C, acceleration:"9" and deceleration:"7". After centrifugation, the LDL fraction (appearing as a yellow-orange band at the supernatant) accompanied by 3 ml of the upper solution was removed. The residual content of tubes was mixed with 3 ml of solution C (containing 78.32 g of NaBr in 100 ml of solution A) and centrifuged for another 12 h at 16°C, acceleration:"9" and deceleration:"7". After centrifugation, the HDL fraction appearing as a yellow band at the supernatant was collected carefully and stored at −80°C until further analysis.[24] The protein concentration of the HDL fractions was determined according to a modified Lowry method.[25]

HDL mean size determination

A zetasizer nano ZS instrument (Malvern, Worcestershire, UK) with a 532-nm green laser beam was used for the study. The scattered light was collected by detector at an angle of 173° using Non-Invasive Back-Scatter (NIBS) technology and directed to a correlator. The data were analyzed by zetasizer software (DTS, nano series, version 5.02, Malvern) and size information was reported as the Z-average by intensity.[26] All measurements were performed at 25°C in duplicate with automatic duration measurements. In order to determine the suitable solvent with the best reproducibility prior to commencing the measurements, six different solvents [including distilled water, normal saline, and 0.2 M phosphate buffer containing 0.1% (w/v) EDTA-Na_2 at three different pH values of 5.6, 7.4, and 8.3] were used as the HDL dispersant. Hourly measurements of size were made over a period of 5 h and the results were compared. Consequently, 0.2 M phosphate buffer at pH of 7.4 containing 0.1% (w/v) EDTA-Na_2 was selected as the most repeatable dispersant with the lowest aggregation index.

In order to measure HDL mean particle size by dynamic light scattering (DLS) methodology, isolated HDL samples were mixed gently with 1 ml of dispersant (at a final protein concentration of 250 μg/ml) and passed through a syringe filter (Millipore cellulose acetate membrane, 30 mm, 0.2 μm pore size) prior to injection into a disposable polystyrene cell (Malvern) in order to remove dust particles and were then subjected to size determination. Viscosity and refractive index (RI) of water as the dispersant were applied to standard operating protocol (SOP) prior to size determination. The accuracy of size measurements was examined using standard size nanoparticles [Gold Nanoparticles, 20 nm, 0.01% (w/v) aqueous solution, Nanocs Inc., New York, NY, USA] under the same experimental conditions and the results were matched with the diameter quoted by the manufacturer. The within-assay CV for 10 measurements was 1.2% and between-assay CV for 8 measurements was 3.1%. The detection limit of the assay for zetasizer instrument used in the present study was 0.3 nm to 10 μm.

HDL_2/HDL_3 ratio

In order to calculate the ratio of HDL_2 to HDL_3, the accumulative percentage of particles with sizes larger than 8.2 nm was divided to the accumulative percentage of particles with sizes smaller than 8.2 nm.

Statistical analysis

Statistical analyses were performed using SPSS 16.0 (SPSS, Chicago, IL, USA). Before analyses, subjects were divided into three groups based on their serum HDL-C levels[27] [group 1 (n = 49): HDL ≤39 mg/dl; group 2 (n = 46): 39> HDL ≤49 mg/dl; group 3 (n = 45): HDL >49 mg/dl], and variables that were not normally distributed (i.e. TG, apo A-I and apo B-100) were subjected to natural log transformation. Quantitative parameters were compared between groups by analysis of variance (ANOVA) except for PON1 and HDL_2/HDL_3 that were analyzed by a nonparametric Kruskal–Wallis test and data were expressed as means ± SD. Correlations between variables with normal distribution were calculated by Pearson correlation coefficient analysis. Spearman's rho correlation coefficient analysis was used to calculate the correlation between variables that were not normally distributed (i.e. serum PON1 activity).

RESULTS

Clinical characteristic data of all subjects are summarized in Table 1. All subjects in the three groups were age matched. As seen in Table 1, anthropometric indices were significantly different among the three groups. The mean BMI value in group 3 subjects was lower compared with that in group 1 (P = 0.002). WHR in groups 2 and 3 was lower compared with that in group 1 (P <0.05 and P = 0.001, respectively). There was a significant difference in the mean TG values among the three groups (groups 1 and 2, P < 0.001; groups 2 and 3, P = 0.002; and groups 1 and 3, P < 0.001), with group 3 having the lowest values. TC was higher in group 3 compared with those in groups 1 and 2 (P = 0.003 and P = 0.002, respectively). Apo A-I was also significantly higher in groups 2 and 3 in parallel with the increasing serum HDL-C levels. The serum PON1 activity level was lower in group 3 compared with those in groups 2 and 1 (P = 0.023 and P = 0.004, respectively), whereas it was comparable in groups 1 and 2. PON1 activity adjusted for the HDL level (PON1/HDL-C) was also significantly lower in group 3 compared with those in groups 1 and 2 of our study (P < 0.001) and lower in group 2 compared with that in group 1 (P = 0.01). The mean HDL particle size and HDL_2/HDL_3 ratio was higher in the high HDL-C group compared with those in group 2 (P = 0.003 and P = 0.001, respectively) and group 1 (P < 0.001 and P < 0.001, respectively). The HDL_2/HDL_3 ratio also correlated with HDL-C levels in groups 1 and 2. There were no significant differences in blood pressure, FBS, LDL-C, and apo B-100 levels among the three groups.

Correlations between HDL-C, HDL mean particle size, HDL_2/HDL_3 ratio, and serum PON1 activity with anthropometric indices and clinical biomarkers are shown in Table 2. Serum HDL-C levels were positively correlated with TC, apo A-I, and LDL-C levels, and were negatively correlated with BMI, WHR, and TG (P < 0.01). HDL mean particle size and HDL_2/HDL_3 ratio were negatively correlated with BMI (P < 0.01), WHR, and serum TG levels (P <0.05 and P <0.01, respectively), and were positively correlated with serum HDL-C levels (P < 0.01). Results

Table 1: Clinical characteristics of the study participants

Variable	Group 1 (HDL ≤ 39)	Group 2 (39 <HDL ≤ 49)	Group 3 (49 ≤ HDL)	*P* value (G1 and G2)	*P* value (G1 and G3)	*P* value (G2 and G3)
Age (years)	31.35 ± 6.9	31.41 ± 8.4	35.16 ± 8.8	0.999	0.062	0.074
BMI (kg/m^2)	24.5 ± 3	23.2 ± 2.7	22.2 ± 3.2	0.125	0.002	0.281
WHR	0.90 ± 0.05	0.87 ± 0.05	0.86 ± 0.05	0.031	0.001	0.576
SP (mm Hg)	115.5 ± 8.9	115.9 ± 10.8	116.6 ± 11.4	0.986	0.860	0.930
DP (mm Hg)	75.1 ± 5.7	77.2 ± 8.6	76.4 ± 11.4	0.317	0.632	0.850
FBS (mg/dl)	85.3 ± 7.5	84 ± 7.3	83 ± 8.4	0.718	0.376	0.843
TG (mg/dl)	2.28 ± 0.19	2.07 ± 0.17	1.94 ± 0.17	<0.001	<0.001	0.002
TC (mg/dl)	176 ± 34	175 ± 33	203 ± 47	0.979	0.003	0.002
HDL-C (mg/dl)	32.3 ± 4	43.5 ± 2.9	61.9 ± 7.2	<0.001	<0.001	<0.001
LDL-C (mg/dl)	102 ± 20	101 ± 14	109 ± 34	0.995	0.246	0.218
apo A-I (g/L)	2.02 ± 0.04	2.06 ± 0.05	2.10 ± 0.08	0.010	<0.001	0.005
apo B-100 (g/L)	1.98 ± 0.10	1.93 ± 0.15	1.96 ± 0.14	0.213	0.776	0.588
PON1 (U/L)	118.0 (39–162)	115 (41–162)	49 (38–68)	0.858	0.004	0.023
PON1/HDL-C	3.6 (1.2–5.9)	2.7 (0.9–3.6)	0.8 (0.5–1.1)	0.01	<0.001	0.001
HDL mean size (nm)	8.54 ± 0.80	8.89 ± 0.86	9.52 ± 0.98	0.128	<0.001	0.003
HDL_2/HDL_3 ratio	0.84 ± 0.69	1.07 ± 0.82	1.72 ± 1.2	0.031	<0.001	0.001

Table 2: Bivariate correlations between clinical biomarkers and HDL mean size, HDL_2/HDL_3 ratio, and PON1 activity in all subjects

Variable	Serum HDL-C	*P* value	HDLmean size	*P* value	HDL_2/HDL_3 ratio	*P* value	PON1 activity	*P* value
Age (years)	0.198	0.19	0.51	0.546	0.022	0.797	−0.118	0.166
BMI (kg/m^2)	−0.313	<0.001	−0.262	0.002	−0.277	0.001	0.161	0.058
WHR	−0.279	0.001	−0.194	0.026	−0.186	0.032	0.178	0.042
SP (mm Hg)	0.064	0.464	−0.036	0.684	−0.043	0.622	−0.159	0.068
DP (mm Hg)	0.079	0.363	0.111	0.204	−0.071	0.416	−0.166	0.056
FBS (mg/dl)	−0.067	0.431	−0.185	0.029	−0.107	0.109	−0.049	0.570
TG (mg/dl)	−0.625	<0.001	−0.204	0.015	−0.205	0.015	0.113	0.117
TC (mg/dl)	0.381	<0.001	0.036	0.674	0.003	0.975	−0.116	0.173
HDL-C (mg/dl)	1	-	0.448	<0.001	0.414	<0.001	−0.247	0.003
LDL-C (mg/dl)	0.236	0.005	−0.038	0.652	−0.036	0.670	−0.051	0.554
apo A-I (g/L)	0.507	<0.001	0.081	0.344	0.126	0.137	−0.215	0.011
apo B-100 (g/L)	0.014	0.872	−0.121	0.156	−0.082	0.336	−0.035	0.630

in Table 2 also indicate a significant negative correlation between serum PON1 activity and serum HDL-C levels ($P < 0.01$) and apo A-I ($P < 0.05$), and a significant positive correlation with WHR ($P < 0.05$).

Figure 1 shows individual correlations of serum PON1 activity with serum HDL-C level, HDL mean particle size, and HDL_2/HDL_3 ratio. As indicated, serum PON1 activity was negatively correlated with serum HDL-C level ($r = -0.25$, $P = 0.003$), HDL mean particle size ($r = -0.22$, P 0.008), and HDL_2/HDL_3 ratio ($r = -0.23$, $P = 0.007$). There was also a strong positive correlation between serum HDL-C level and HDL mean particle size ($r = 0.45$, $P < 0.001$) [Figure 2].

DISCUSSION

The present study describes the correlation between HDL mean particle size and serum PON1 activity and their relationship with anthropometric indices and lipid profile in healthy male subjects. We found a significant negative correlation between serum PON1 activity and HDL mean particle size in all subjects. Similar negative correlation was observed between serum PON1 activity and HDL_2/HDL_3 ratio. These findings are in accordance with Kontush's investigation which demonstrated that small HDL_3 particles posse higher PON1 activity than large HDL_2 particles.[17] Nishio's investigation on the susceptibility of HDL subfractions to copper-mediated oxidation indicated that HDL_2 is more susceptible to oxidation than HDL_3.[14] It is conceivable that this protective effect is mediated by PON1 activity being higher in small HDL_3 particles compared with large HDL_2 particles. There is increasing evidence suggesting that PON1 makes a central contribution to the antioxidant capacity of HDL.[9,28,29] However, Hasselwander's investigation has indicated that reduction in arylesterase activity of paraoxonase had no effect on the HDL ability to inhibit LDL oxidation in patients on regular hemodialysis.[30] Moreover, it has been demonstrated that HDL also exerts inhibitory effects on LDL oxidation, which are independent of paraoxonase activity.[31] Epidemiological

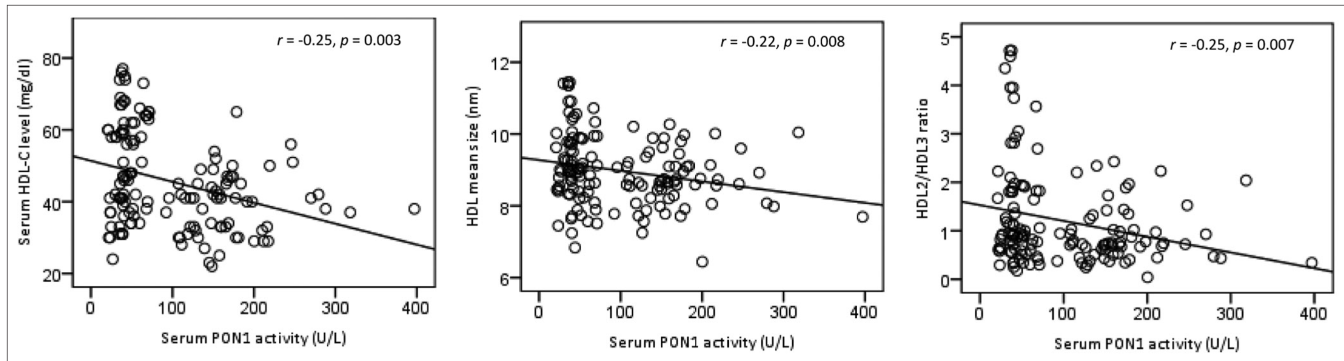

Figure 1: Correlations between serum PON1 activities with serum HDL-C levels, HDL mean particle size, and HDL_2/HDL_3 ratio

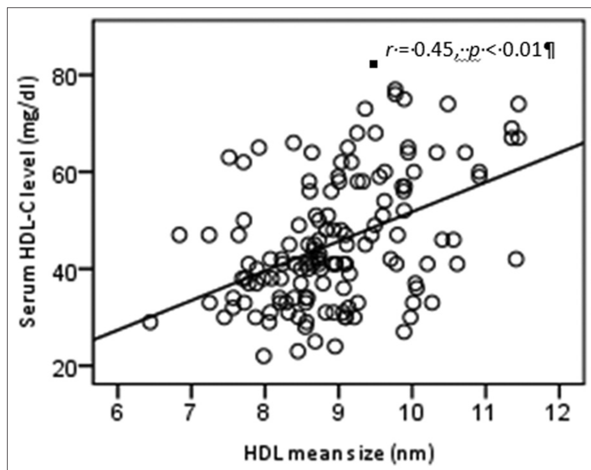

Figure 2: Correlations between HDL mean particle size and serum HDL-C levels

studies on the relationship between PON1 and CAD have reported conflicting results.[32] Several independent studies have indicated that the concentration and activity of PON1 are decreased in CAD patients.[20,33,33] Mackness *et al.* in a prospective study reported a reverse relationship between PON1 activity and CAD risk in men,[18] whereas in another prospective investigation on healthy middle-aged men, Troughton *et al.* did not find any effect of PON1 paraoxonase activity on coronary heart disease (CHD) outcomes.[19] Furthermore, van Himbergen *et al.* have reported that in a general population of Dutch women, increased PON1 paraoxonase activity was a risk factor for cardiovascular outcomes instead of being protective.[21] Our findings have indicated that serum PON1 activity in subjects with high HDL-C levels (group 3 of our study) is significantly decreased in comparison with subjects with medium and low HDL-C levels. This finding is in contradiction with a majority of investigations demonstrating that high PON1 levels and high PON1 activity contribute to increased levels of HDL-C.[17,35] In many examples of HDL deficiency, such as Tangier disease and fish eye disease, serum PON1 is significantly diminished,[36,37] but in other HDL deficiencies, this is not always the case.[38] Blatter *et al.* have reported a positive correlation between PON1 activity and HDL-C levels in control subjects and CAD patients.[39] They also demonstrated a significant decrease in PON1 concentration and its arylesterase and paraoxonase activities in CAD patients in comparison with control subjects. *PON1* expression is partly controlled by its genetic variation.[40] In addition, PON1 levels and activity could be modified by lifestyle determinants such as smoking, vitamin C and E consumption, and alcohol intake.[15] The contribution of PON1 genotypes to HDL-C levels was described by van Himbergen *et al.* who investigated on a population with familial hypercholesterolemia.[16] They described that 55 L, 192 R, −107C, and 907 G variants of the L55M, Q192R, −107C/T, and −907 G/C polymorphisms predicted increased HDL-C levels. They also indicated that like the PON1 genotypes, PON1 levels and paraoxonase, diazoxonase, and arylesterase activities also contributed to increased HDL-C levels. In our study, a strong positive correlation was found between serum HDL-C levels with HDL mean particle size and HDL_2/HDL_3 ratio. A similar direct relationship between HDL particle size and serum HDL-C Levels has been reported by others.[41,42] Decreased serum PON1 activity parallel to increased serum HDL-C levels observed in the present study may be a result of increased HDL particle size and the ratio of HDL_2/HDL_3, and consequently, decrease in the small dense HDL particles. In other words, changes in HDL subspecies distribution could explain the reduced activity of serum PON1. Shiavon *et al.* indicated that serum PON1 activity was decreased in chronic renal failure patients under maintenance hemodialysis.[43] They explained that reduction in PON1 activity was the consequence of the change in HDL subspecies distribution. They found that the PON1 enzyme activity on HDL was almost totally expressed by the HDL_3 subspecies fraction.[44]

Investigations have demonstrated a relationship between HDL particle size and CAD risk. The levels of small HDL_3b and HDL_3c (7.3-8.2 nm) were directly correlated with CAD severity, whereas the levels of intermediate HDL_3a

and HDL_2a (8.2-10 nm) and large HDL_2b (10-13 nm) were inversely correlated with CAD severity.[45] However, the protective role of HDL_2 has not been demonstrated in all studies.[21] Small dense HDL_3 has been shown to exert more powerful antioxidative, protective effects against LDL oxidation compared with the large HDL_2 subfraction.[17,46,47] As ox-LDL is the most atherogenic form of modified LDL, this protective role of HDL_3 particles is very important. Results of the anthropometric determinations in our study have indicated a reverse relationship between HDL mean particle size with BMI and WHR and a direct relationship between PON1 activity and these anthropometric indices. There are, however, a number of limitations of the present study. Because of the effects of female sex hormones such as estrogen and estradiol on lipid profile and PON1 activity and alterations of these hormones during menstrual cycles in women, in the present study we investigated only healthy male subjects; our results need to be verified in women. It is also recommended to determine the relationship between HDL mean particle size and PON1 activity in CAD patients in comparison with healthy subjects.

CONCLUSION

In conclusion, our results indicate that there is negative correlation between HDL mean particle size and serum PON1 activity in healthy Iranian male subjects. An inverse correlation also exists between HDL mean particle size and anthropometric indices, whereas plasma PON1 activity is directly correlated with them. Considering the complicated role of HDL in CAD prevention and its heterogeneous nature, further characterization of HDL subclasses, including particle size and its associated enzymes such as PON1, may be helpful for better understanding of CAD risks and prognosis and also may help to design therapeutic protocols toward beneficial modifications of HDL characteristics.

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