

**Altered lipid subfraction profile and impaired antioxidant defense of high-density lipoprotein in Smith-Lemli-Opitz syndrome**

**Running title:** Lipid subfractions and PON1 in SLOS

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***Statement of financial support:*** The work is supported by a grant from the Hungarian Scientific Research Fund (OTKA 84196) and by the TÁMOP-4.2.2.A-11/1/KONV-2012-0031 project (University of Debrecen, Debrecen, Hungary). The TÁMOP project is co-financed by the European Union and the European Social Fund.

***Conflict of interest:*** All authors state that they have nothing to declare.

***Category of study:*** Clinical study

## **ABSTRACT**

**BACKGROUND** Smith-Lemli-Opitz syndrome (SLOS) is a rare disease caused by biallelic mutation in the 7-dehydrocholesterol (7DHC) reductase gene. High oxidizability of 7DHC and the appearance of small-sized low-density lipoprotein (LDL) subfractions indicate increased endogenous oxidative stress that is counterbalanced by natural antioxidant defense mechanisms including the high-density lipoprotein (HDL)-associated paraoxonase-1 (PON1) enzyme. PON1 prevents lipoproteins from oxidative modifications; however, PON1 activity and the distribution of lipoprotein subfractions have not been studied in SLOS.

**METHODS** 7DHC levels and PON1 arylesterase activities were measured spectrophotometrically in eleven SLOS patients and ten healthy children. Lipoprotein subfractions were detected by polyacrylamide gel electrophoresis.

**RESULTS** Compared to controls, there was a shift towards the small-dense LDL subfraction and the large HDL subfraction in SLOS. PON1 arylesterase activity was significantly decreased in SLOS patients and correlated negatively with the proportion of small-dense LDL subfraction and the proportion of large HDL subfraction. Significant positive correlations were detected between PON1 arylesterase activity and the ratios of intermediate and small HDL subfractions.

**CONCLUSIONS** Decreased PON1 activity and the deleterious shift in the distribution of lipoprotein subfractions may contribute to the impaired antioxidant status observed in SLOS. Monitoring of serum PON1 arylesterase activity may be a complementary biomarker in SLOS.

## INTRODUCTION

Cholesterol is an important structural component of cell membranes and serves as a precursor of steroid hormones and bile acids. It is a major constituent of the myelin formed in the brain, spinal fluid and in the peripheral nervous system. From all organs, brain is the richest in cholesterol, containing approximately 20% of the whole body cholesterol (1). Cholesterol is synthesized from lanosterol by a series of oxidations, reductions and demethylations (2); therefore it is not surprising that inherited defects of cholesterol biosynthesis may lead to numerous diseases.

Smith-Lemli-Opitz syndrome (SLOS, OMIM 270400) is a rare autosomal recessive genetic disease originating from biallelic mutations in the gene encoding 3 $\beta$ -hydroxysterol- $\Delta^7$ -reductase (DHCR7, EC 1.3.1.21), which is located on chromosome 11q13. This genetic defect is manifested as a large and variable spectrum of phenotypes, including multiple congenital malformations, microcephaly, neurological defects, photosensitivity, syndactyly of the second and third toes, growth and mental retardation, and behavioral problems (3, 4). For phenotypic characterization, the modified Bialer scoring system of Kelley and Hennekam has been used weighting embryologically separate organ systems equally (5). DHCR7 enzyme catalyzes the reduction of 7-dehydrocholesterol (7DHC) to cholesterol in the last step of cholesterol biosynthesis pathway (6). SLOS patients exhibit extremely high levels of 7DHC and reduced levels of cholesterol in the tissues, plasma and other fluids. In accordance with the results of Tint et al. (7), we previously reported that both the initial value of serum cholesterol level and the cholesterol/7DHC ratio had an important prognostic value in the severity, development and life expectancy of SLOS children (8). To date, routine therapy has been cholesterol supplementation in SLOS; while combined cholesterol-simvastatin therapy (9, 10) or cholesterol-antioxidant treatment might also be effective in SLOS patients (11).

However, as it was recently reviewed, simvastatin is not recommended for SLOS treatment due to its potential side effects (12). High oxidizability of 7DHC has been reported to enhance endogenous oxidative stress in cell and animal models of SLOS (13) and in several human studies (14). Additionally, inactivation of the endogenous antioxidant defense mechanisms may also contribute to increased oxidative stress. Oxidation of the predominantly smaller and denser low-density lipoprotein (LDL) particles play a key role in this process (15). Human paraoxonase-1 (PON1) is a high-density lipoprotein (HDL)-associated enzyme with significant antioxidant properties preventing lipoproteins from oxidative modification (16). PON1 activity was found to diminish in several human diseases characterized by enhanced oxidative stress, including coronary artery disease, diabetes mellitus, hyperlipidemia, obesity, and several autoimmune diseases (17-20).

As it was demonstrated recently, high 7DHC level accelerates the formation of oxysterols in SLOS resulting in increased cellular susceptibility for oxidative stress (21-22). To date, however, there is no data on the distribution of lipoprotein subfractions in SLOS, while information about the antioxidant PON1 activity is also lacking in these patients. Therefore, we aimed to study the distributions of LDL and HDL subfractions in SLOS patients and compared their data to healthy children. We also examined PON1 activities in these groups and intended to clarify possible associations between PON1 antioxidant status and various lipoprotein subfractions.

## RESULTS

Clinical and laboratory characteristics of study participants are summarized in Table 1. Mean SLOS clinical severity score was  $30.9 \pm 15.1$ ; one child with severe disease (clinical severity score  $>50$ ) died as a newborn, seven patients exhibited typical symptoms (clinical severity score 20-50), and three patients had mild features of SLOS group (clinical severity score  $<20$ ). 7DHC level in SLOS patients was 205 (85-274) mg/L, while 7DHC levels were below the limit of detection in healthy children ( $<0.15$  mg/L). Mean total cholesterol, LDL-C and HDL-C levels were significantly lower in patients with SLOS ( $p < 0.0001$ ,  $p < 0.0001$  and  $p < 0.01$ ; respectively) compared to healthy children. A strong, significant negative correlation was detected between the clinical severity score and HDL-C levels ( $r = -0.804$ ,  $p = 0.003$ ) (Figure 1a).

Compared to controls, the proportion of large LDL subfraction was significantly lower ( $p < 0.001$ ), while the proportion of small-sized, dense LDL subfraction was significantly higher ( $p < 0.01$ ) in SLOS patients (Table 1). Furthermore, the mean LDL size was significantly lower in patients with SLOS than in healthy children ( $p < 0.001$ ). These data are in accordance with the LDL subfraction pattern in these patients: seven SLOS patients represented an abnormal LDL pattern; one child had intermediate and only three children had normal LDL subfraction pattern. In contrast, none of the control children displayed abnormal LDL subfraction pattern, two of them were found to have an intermediate pattern, while the remaining eight children represented normal LDL subfraction patterns. Figure 2 demonstrates an abnormal subfraction pattern (mean LDL size: 25.34 nm) in a typical SLOS patient (Figure 2a) and a normal LDL subfraction pattern (mean LDL size: 27.31 nm) in a healthy child (Figure 2b). Densitometric scanning of LDL subfractions identified an SLOS patient with

predominantly smaller and denser LDL subfractions (shown in red in Figure 2a) indicating an increased susceptibility for enhanced oxidative stress in SLOS. On the other hand, Figure 2b is a representative sample of lipoprotein subfraction pattern of a healthy child typically with rather larger and less dense LDL subfraction pattern (shown in yellow).

We also detected a shift towards the larger and less dense HDL subfractions in children with SLOS (Table 1). The proportion of the large HDL subfractions was significantly higher ( $p<0.001$ ), while proportions of intermediate and small HDL subfractions were significantly lower in SLOS children compared to healthy controls ( $p<0.001$  and  $p<0.01$ , respectively). In Figure 3, we also demonstrate a densitometric scan of HDL subfractions in a patient with typical SLOS (Figure 3a) representing the dominance of large HDL subfractions and the decreased prevalence of intermediate and small HDL subfractions compared to a healthy control child (Figure 3b).

Serum PON1 paraoxonase activity was lower in the SLOS group compared to controls, SLOS: 37.05 (9.98-93.22) U/L vs. controls: 72.85 (42.04-206.93) U/L;  $p=0.069$ ; although the difference was not significant between the two groups. In turn, serum PON1 arylesterase activity was found to be significantly decreased in SLOS children compared to controls (SLOS:  $64.30\pm 52.69$  U/L vs. controls:  $107.93\pm 26.83$  U/L;  $p=0.022$ ) (Figure 1b).

Examining the correlations between serum PON1 arylesterase activity and lipoprotein parameters (Table 2), we detected a significant positive correlation between PON1 arylesterase activity and total cholesterol level ( $r=0.543$ ,  $p=0.045$ ). In addition, there were positive, although not significant trends of associations between PON1 arylesterase activities and LDL-C concentrations and the proportions of large LDL subfractions, respectively. In turn, a significant negative correlation was found between PON1 arylesterase activity and the proportion of small-dense LDL subfraction ( $r=-0.578$ ,  $p=0.031$ ). Additionally, PON1 arylesterase activity correlated positively with mean LDL size ( $r=0.610$ ,  $p=0.021$ ).

Although there was no significant association between PON1 arylesterase activity and HDL-C levels; PON1 arylesterase activity correlated negatively with the proportion of large HDL subfraction ( $r=-0.798$ ,  $p=0.001$ ). In contrast, significant positive correlations were found between PON1 arylesterase activities and the proportions of intermediate and small HDL subfractions ( $r=0.652$ ,  $p=0.012$  and  $r=0.663$ ,  $p=0.010$ ), respectively.

## DISCUSSION

According to our knowledge, this is first report on the distribution of LDL and HDL subfractions and the status of HDL-associated antioxidant PON1 activity in SLOS patients. Based upon the lipoprotein subfraction analyses, we detected a shift towards the small-dense LDL subfraction and the dominance of the large-sized HDL subfraction, which indicate disturbed cholesterol biosynthesis in SLOS. Decreased PON1 arylesterase activity and elevated levels of 7DHC were also demonstrated in these children, revealing enhanced endogenous oxidative stress and impaired antioxidant status.

Previous studies found altered lipoprotein parameters and impaired cholesterol biosynthesis in SLOS (23, 24). Also, initial cholesterol concentrations were found to be fundamental in determining severity and life expectancy in SLOS (7, 8). Corroborating previous data, significantly decreased total cholesterol, LDL-C and HDL-C levels were measured in our patients and we found a significant negative correlation between HDL-C level and the clinical severity score, emphasizing the putative role of HDL in the pathomechanism of SLOS.

LDL and HDL are heterogeneous particles consisting multiple lipoprotein subpopulations that vary in protein and lipid components, physicochemical properties, particle size and density (25). Although lipoprotein levels are essential in the prognosis of SLOS, the distributions of LDL and HDL subfractions have not been studied yet. Compared to healthy infants, we found an unexpectedly higher proportion of small-dense LDL subfraction and lower mean LDL size in children with SLOS. The prevalence of abnormal LDL subfraction pattern was 63% in SLOS, whereas none of the healthy children displayed LDL abnormality. The latter data are in congruence with the results of the STRIP Study indicating a 1-4% prevalence of the small-dense LDL subfraction in healthy children (26). Since the



predominantly smaller and denser LDL subfractions are extremely prone to undergo oxidative modification (15), their dominance might indicate the presence of oxidative stress in SLOS; however, further studies are needed to clarify such association in this disease.

HDL subpopulations play various roles in lipid metabolism, as large-sized HDL subfractions are primarily involved in reverse cholesterol transport - a mechanism by which HDL takes up cholesterol in the peripheral tissues and transports it to the liver; while small-sized HDL subfractions rather exert anti-inflammatory effects protecting LDL from oxidation (27, 28). In this study, we observed altered distribution of HDL subfractions in SLOS patients who were characterized by a significantly higher proportion of large-sized HDL subfraction and lower proportions of intermediate and small-sized HDL subfractions. Based upon these results, one may hypothesize that altered distribution of HDL subfractions may reflect enhanced reverse cholesterol transport compensating for extremely low cholesterol levels found routinely in SLOS. HDL-associated PON1 is widely recognized as one of the major antioxidant enzymes of HDL and localized primarily on small-sized HDL subfractions (29, 30). Diminished PON1 activity was reported in several disease entities involving altered redox balance and increased oxidative stress (31). Therefore, lower proportion of small-sized HDL subfractions and decreased PON1 arylesterase activity in SLOS patients might be related to their impaired synthesis in the liver and they probably indicate a reduced antioxidant capacity.

It must be noted, that PON1 possesses various enzyme activities with different substrate specificities including paraoxonase and arylesterase activities. PON1 paraoxonase activity is determined by a Q192R polymorphism in the coding region and shows a trimodal distribution; therefore, PON1 paraoxonase activity has a large inter-individual variation (up to 15-40 times) among the general population (31). In contrast, PON1 arylesterase activity is not affected by this polymorphism; therefore, this activity is distributed unimodally and reflects

protein concentration and antioxidant capacity of PON1 (32, 33). Compared to controls, we found a marked but not significant reduction of serum PON1 paraoxonase activity in children with SLOS. Presumably due to the relatively small sample size and the trimodal distribution of the PON1 paraoxonase activity, we did not detect a difference in this variable between the studied groups. In turn, we demonstrated a significant negative correlation between the proportion of oxidizable small-dense LDL subfraction and the antioxidant PON1 arylesterase activity. The proportion of large HDL subfraction correlated negatively, while the proportions of intermediate and small HDL subfractions correlated positively with PON1 arylesterase activity. These correlations support previous observations that small-sized HDL subfraction may have the highest PON1 activity (34). Thus, one might conclude that decreased PON1 arylesterase activity and altered lipoprotein subfractions may serve as novel biomarkers in SLOS.

In line with decreased PON1 arylesterase activity, we also found markedly increased levels of 7DHC further enhancing oxidative stress in SLOS. 7DHC is highly disposed to react with molecular oxygen, producing over a dozen different oxysterols, such as 3 $\beta$ ,5 $\alpha$ -dihydroxycholest-7-en-6-one (DHCEO) *in vitro* and *in vivo* (21, 22, 35). Since 7DHC and 7DHC-derived oxysterols are potent pro-oxidant agents, these molecules may also contribute to enhanced lipid peroxidation and protein degradation resulting in reduced antioxidant capacity and PON1 activity.

## **CONCLUSION**

Decreased PON1 arylesterase activity and an unfavorable shift in the distribution of lipoprotein subfractions may contribute to impaired antioxidant status in SLOS. Monitoring of serum PON1 arylesterase activity may serve as a useful complementary biomarker in these patients. Further studies are needed to determine the clinical benefits of PON1 arylesterase activity measurement in SLOS.

## **PATIENTS AND METHODS**

### **Study population**

11 children with clinical and biochemical diagnosis of SLOS (aged between 0.1-20 years, four girls and seven boys) and 10 healthy control children (age of 0.3-19 years, five girls and five boys) were enrolled in our study. Besides the presence of characteristic clinical symptoms, the diagnosis of SLOS was proven by serum 7DHC level. Anatomical abnormalities of ten embryologically separate organs (brain, oral region, limbs, eye, heart, kidney, liver, lung, bowel and genitals) have been scored (5). On the basis of the clinical severity scores, patients were assigned to three groups: (i) patients with mild SLOS (n=3, clinical severity score<20), (ii) typical SLOS (n=7, clinical severity score 20–50) and (iii) severe SLOS (n=1, clinical severity score>50). After setting up the diagnosis, all patients received cholesterol supplementation (Cholesterol Module, 50–250 mg/kg/day, Nutricia; no 18.012), which was complemented with simvastatin in nine patients (dosage 0.2–0.4 mg/kg/day). None of the studied children received dietary and antioxidant supplements and were free of clinically significant infectious diseases. All parents provided written informed consent. The study protocol was approved by the Ethical Committee of University of Debrecen and the study was carried out in accordance with the Helsinki Declaration.

### **Sample collection and laboratory measurements**

Venous blood samples were taken after an overnight fasting and sera were separated immediately. Routine laboratory parameters were determined from fresh sera with Cobas c501 analyzer (Roche Ltd, Mannheim, Germany). Total cholesterol levels were measured by using enzymatic, colorimetric tests (cholesteroxidase, p-aminophenazone – CHOD-PAP).

HDL-cholesterol (HDL-C) levels were determined by a homogenous, enzymatic, colorimetric assay (Roche HDL-C plus 3<sup>rd</sup> generation). The rapid determination of serum 7DHC was performed by an UV spectrophotometric method as described previously (8). Sera were kept frozen at -70°C for subsequent LDL and HDL subfraction analysis and PON1 activity measurements.

### **LDL subfraction analysis**

LDL subfractions were detected by polyacrylamide gel electrophoresis using the Lipoprint System (Quantimetrix Corp., Redondo Beach, CA) according to the manufacturer's instructions. Briefly, 25 µl of serum samples were added to polyacrylamide gel tubes along with 200 µl loading gel solution containing Sudan Black as lipophilic dye. After 30 min of photopolymerization at room temperature, electrophoresis was performed for one hour with a 3 mA/gel tube.

Lipoprotein fractions (bands) were identified after electrophoresis by their mobility (Rf) using VLDL as the starting reference point (Rf 0.0) and HDL as the ending reference point (Rf 1.0). In between, IDL subfractions (Midband A, B and C) and LDL subfractions were distributed from LDL1 to LDL7 (Rf 0.32, 0.38, 0.45, 0.51, 0.56, 0.6, and 0.64, respectively). LDL1 and LDL2 bands corresponded to the larger, buoyant LDL subclasses (designed as normal "Pattern A"), whereas bands from LDL3 to LDL7 represented the predominantly smaller, denser LDL subclasses (designed as abnormal "Pattern B"). Percentages of the area under the curve (AUC%) were calculated with Lipoware computer software (Quantimetrix Corp., Redondo Beach, CA). Proportion of large LDL (large LDL%) was defined as the sum of the percentage of LDL1 and LDL2, whereas proportion of small LDL (small LDL%) was defined as the sum of LDL3 to LDL7. Cholesterol concentrations of each lipoprotein bands were determined by multiplying the relative AUC of subfractions by

total cholesterol concentration of the sample. LDL-cholesterol (LDL-C) is calculated by an automatic way using the Lipoware software as the sum of the cholesterol concentrations of all the LDL subfractions plus the Midband A, B and C. Mean LDL size (nm) was also calculated by the Lipoware software.

### **HDL subfraction analysis**

HDL subfractions were also detected by polyacrylamide gelelectrophoresis with the Lipoprint System (Quantimetrix Corp.). 25  $\mu$ l of serum samples were added to polyacrylamide gel tubes along with 300  $\mu$ l loading gel solution containing Sudan Black as lipophilic dye. After 30 min of photopolymerization at room temperature, electrophoresis was performed for 50 min with a 3 mA/gel tube.

Stained HDL subfractions (bands) were identified by their mobility after electrophoresis. The LDL/VLDL band was the starting reference point ( $R_f$  0.0) and albumin was the ending reference point ( $R_f$  1.0). AUC% were calculated with the Lipoware computer software (Quantimetrix Corp.). Up to ten HDL subfractions, distributed between the LDL/VLDL and albumin bands were grouped into three major classes: large (HDL1 to HDL3), intermediate (HDL4 to HDL7) and small (HDL8 to HDL10) HDL subfractions.

### **Human paraoxonase-1 measurements**

PON1 paraoxonase activity was analyzed on a microtiter plate by a kinetic, semiautomated method utilizing paraoxon (O,O-diethyl-O-p-nitrophenyl-phosphate, Sigma Aldrich, Hungary) as a substrate. PON1 arylesterase activity was assayed with a phenylacetate substrate (Sigma Aldrich, Hungary) and the hydrolysis of phenylacetate was monitored at 270 nm (36).

### **Statistical analyses**

Statistical analysis was performed by STATISTICA software (ver 8.0; StatSoft Inc., Tulsa, OK). The normality of data distribution was tested by Kolmogorov-Smirnov test. Data are presented by descriptive analysis (mean  $\pm$  SD in case of normal distribution, or median (lower quartile – upper quartile) in case of non-normal distribution). Comparisons between groups were performed by Student's paired t-test in case of normally distributed variables and by Mann-Whitney U-test in case of variables with non-normal distribution. Correlations between continuous variables were assessed by linear regression analysis using Pearson's test. Results were considered significant at the level of  $p < 0.05$ .

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## FIGURE LEGENDS

Figure 1: Correlation between clinical severity scores and HDL-C levels in SLOS patients (n=11;  $r=-0.804$ ;  $p=0.003$ ) (a); Serum PON1 arylesterase activity in SLOS and healthy control children (\* $p=0.022$ ;  $\square$  mean; box: mean  $\pm$  standard deviation; whiskers: mean  $\pm 1.96$ 'standard deviation) (b).

Figure 2: LDL subfraction profile in a typical SLOS patient (a) and in a healthy child (b).

Figure 3: HDL subfraction profile in a typical SLOS patient (a) and in a healthy child (b).

Table 1. Clinical and laboratory characteristics of the study participants

	<b>SLOS (n=11)</b>	<b>Control (n=10)</b>	<b>p</b>
<b>Gender</b>	4 girls / 7 boys	5 girls / 5 boys	
<b>Age (years)</b>	5.71 ± 6.93	5.36 ± 6.02	n.s.
<b>Clinical severity score</b>	30.9 ± 15.1		
<b>7-Dehydrocholesterol (mg/L)</b>	205 (85-274)	n.d.	
<b>Total-Cholesterol (mmol/L)</b>	1.73 ± 0.9	3.52 ± 0.63	<0.0001
<b>LDL-C (mmol/L)</b>	0.9 ± 0.4	1.88 ± 0.46	<0.0001
<b>Large LDL (%)</b>	16.9 (14.5-22.4)	26.1 (23.8-30.4)	<0.001
<b>Small-dense LDL (%)</b>	10.2 (2.3-13.3)	0.8 (0.0-2.7)	<0.01
<b>Mean LDL size (nm)</b>	25.7 ± 0.83	27.16 ± 0.32	<0.001
<b>LDL subfraction pattern</b>			
<b>normal (n)</b>	3	8	
<b>intermediate (n)</b>	1	2	
<b>abnormal (n)</b>	7	0	
<b>HDL-C (mmol/L)</b>	0.55 ± 0.38	1.04 ± 0.27	<0.01
<b>Large HDL (%)</b>	73.5 (47.4-77.2)	34.5 (31.6-41.8)	<0.001
<b>Intermediate HDL (%)</b>	19.8 (18.9-39.6)	49.5 (44.3-50.5)	<0.001
<b>Small HDL (%)</b>	7.1 (2.3-11.0)	16.0 (13.9-18.5)	<0.01

SLOS, Smith-Lemli-Opitz syndrome; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; n.s., non-significant; n.d., non-detectable, <0.15 mg/L.

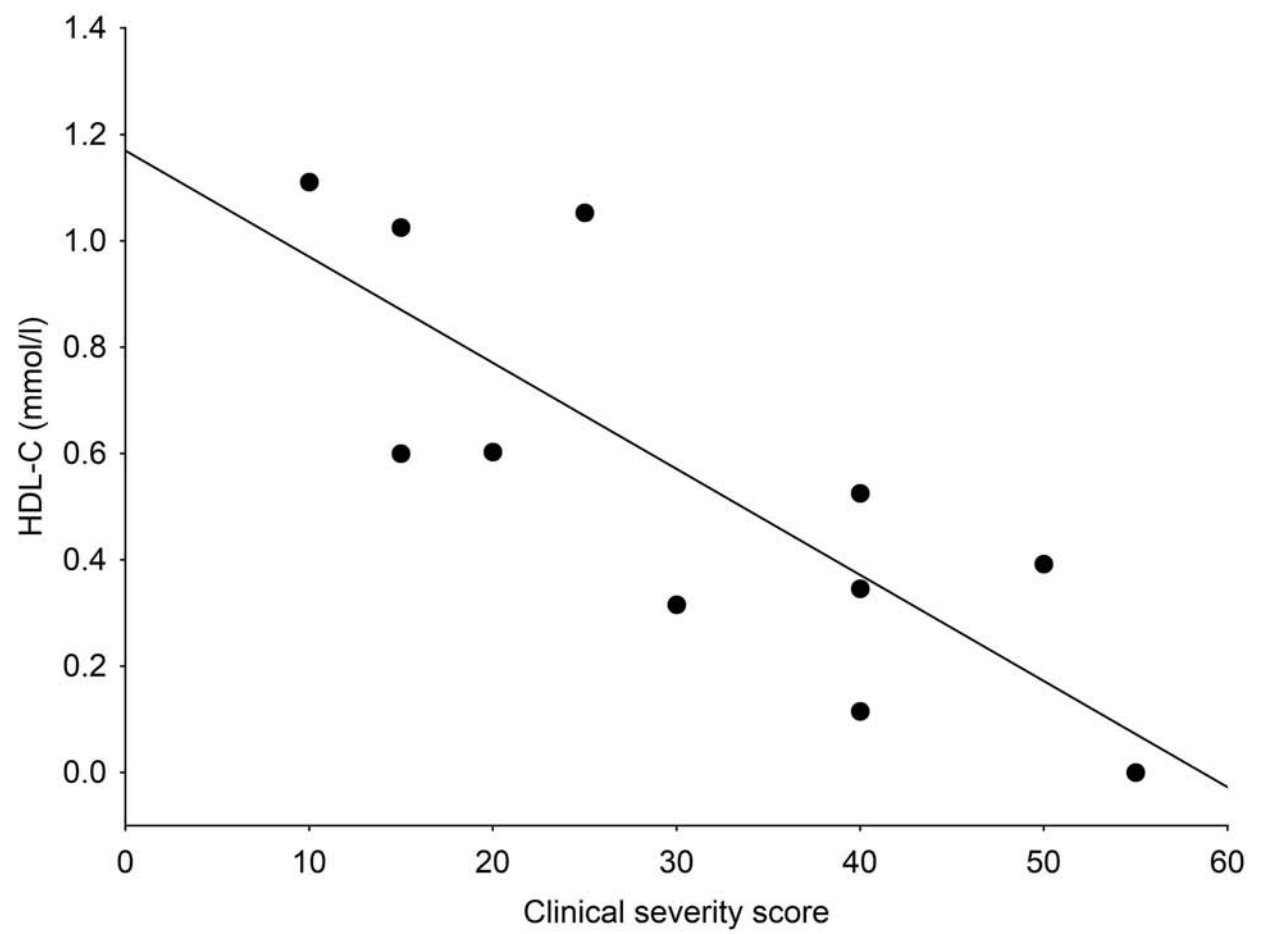
Values are presented as mean ± standard deviation or median (lower quartile – upper quartile).

Table 2. Correlations between serum PON1 arylesterase activity and lipoprotein parameters in all studied participants

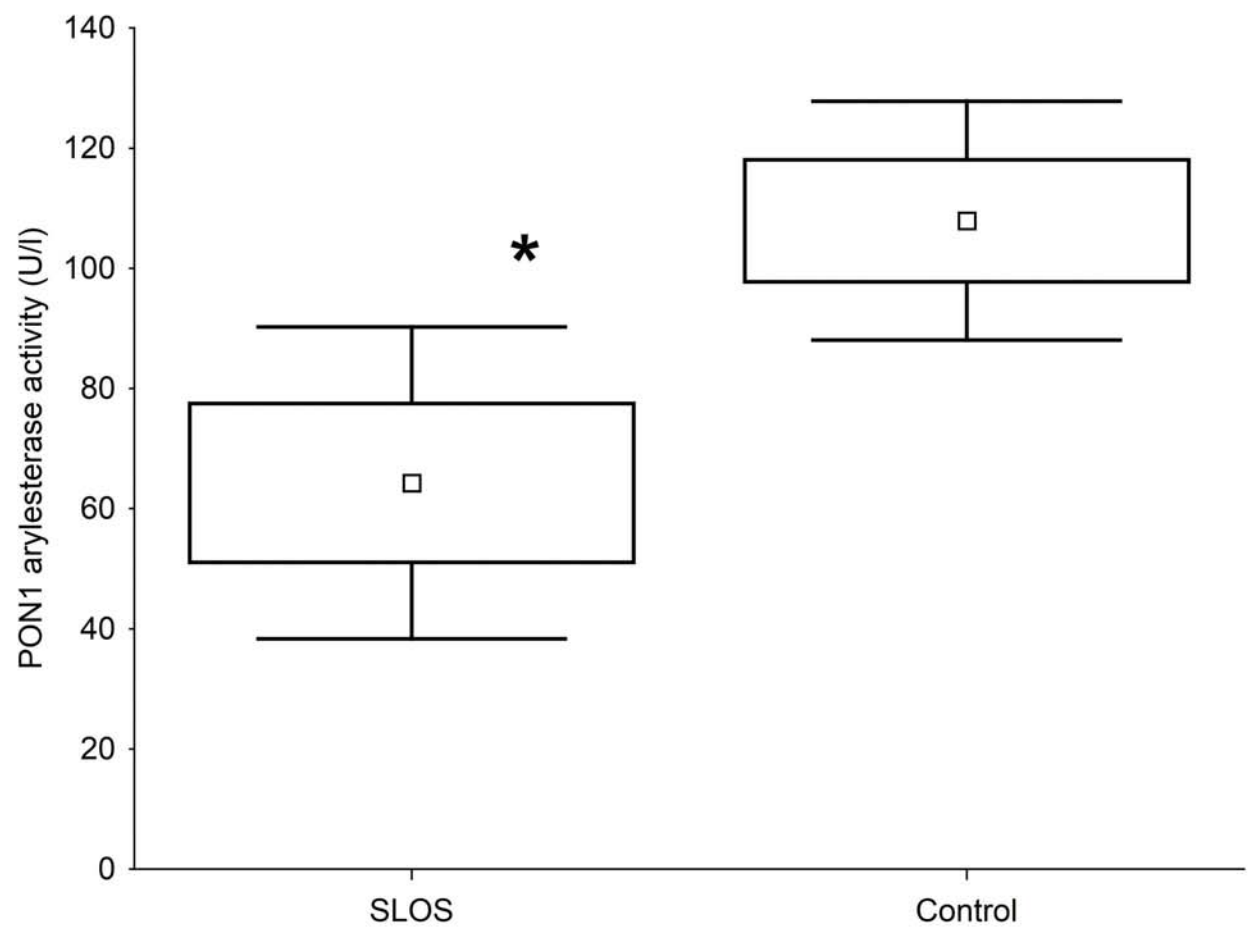
<b>Variables</b>	<b>r</b>	<b>p</b>
<b>Total-Cholesterol (mmol/L)</b>	0.543	0.045
<b>LDL-C (mmol/L)</b>	0.533	n.s.
<b>Large LDL (%)</b>	0.349	n.s.
<b>Small-dense LDL (%)</b>	-0.578	0.031
<b>Mean LDL size (nm)</b>	0.610	0.021
<b>HDL-C (mmol/L)</b>	0.457	n.s.
<b>Large HDL (%)</b>	-0.798	0.001
<b>Intermediate HDL (%)</b>	0.652	0.012
<b>Small HDL (%)</b>	0.663	0.010

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; n.s. non-significant

**(a)**

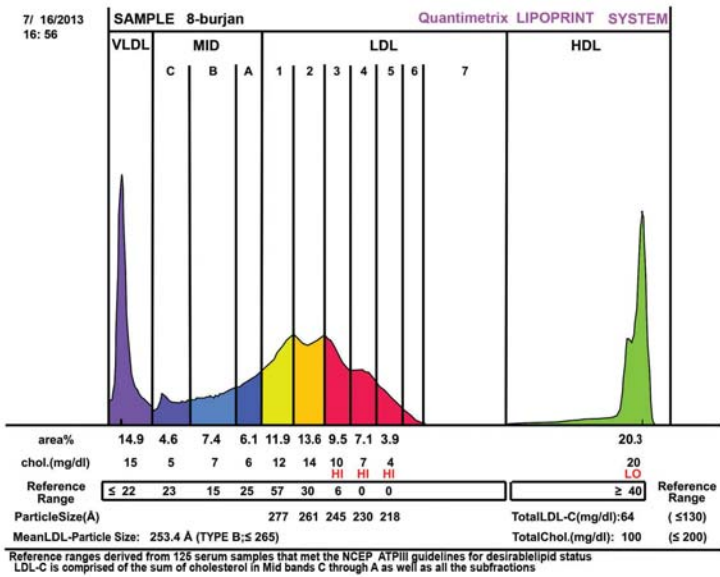


**(b)**

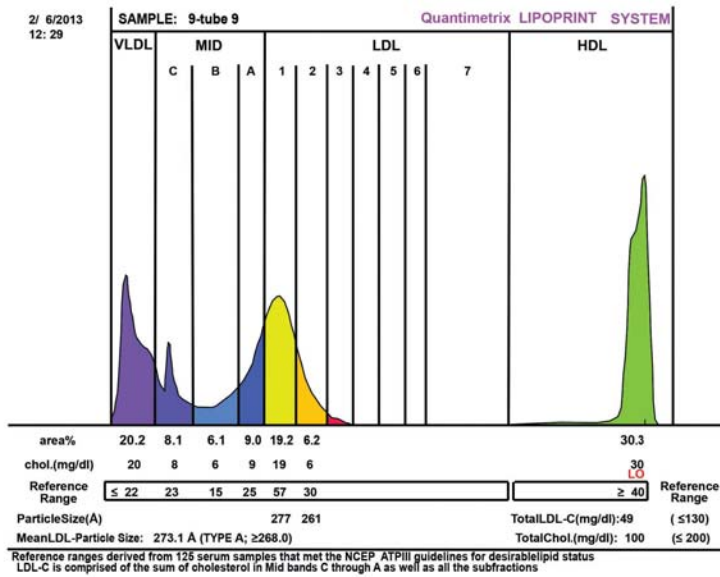




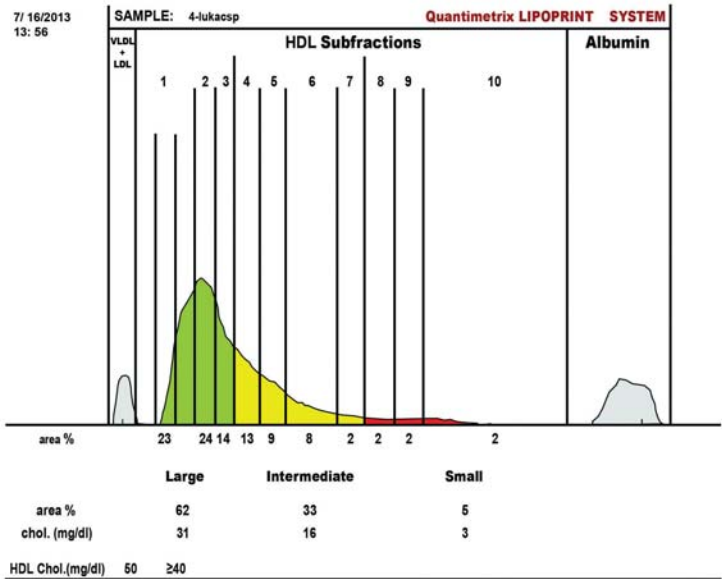
(a)



(b)



(a)



(b)

