

***APOA5* GENE VARIANTS, LIPOPROTEIN PARTICLE  
DISTRIBUTION AND PROGRESSION OF CORONARY HEART  
DISEASE: RESULTS FROM THE LOCAT STUDY.**

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progression

## Abstract

Animal and human studies support a role for apoAV in triglyceride (TG) metabolism. We examined the relationship of *APOA5* -1131T>C and S19W with lipid subfractions and progression of atherosclerosis in the LOCAT gemfibrozil study of post by-pass men. Compared to -1131TT men (n=242), carriers of the -1131C allele (n=54) had significantly higher total TG (p=0.03), reflected in significantly increased very low density lipoprotein (VLDL) mass (higher VLDL-TG, VLDL-cholesterol, VLDL-protein and surface lipids (all p<0.05)). Since apoB levels were unaffected by genotype this suggests an increase in VLDL size and not number. Compared to the 19SS men (n=268), 19W carriers (n=44) had higher intermediate density lipoprotein (IDL)-TG, IDL-cholesterol (p=0.04) and IDL-surface components (free cholesterol (p=0.005) and phospholipids (p=0.017)), but not protein content, suggesting an increase in IDL lipid-enrichment resulting in an increase in IDL size. 19W carriers also showed a trend towards increased progression of atherogenesis; change in average diameter of segments (-0.46 ( $\pm$  0.011 mm) compared to -0.016 ( $\pm$ 0.006mm) in 19SS men (p=0.08). There was no effect of genotype on the response of these parameters to gemfibrozil treatment. These results shed new light on the role of *APOA5* variants in TG metabolism and CHD risk.

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## Introduction

Animal and human studies of the newly identified *APOA5* gene are consistent in identifying *APOA5* as a major determinant of plasma TG levels (1). Thus *APOA5*, forming a cluster with *APOA4-C3-AI* on chromosome 11q23, constitutes a locus involved in TG and HDL determination (2, 3). In both transgenic and knockout mouse models, it is clear that apoAV is inversely associated with plasma TG levels. Transgenic mice over-expressing human *APOA5* (1) or adenoviral vector-mediated transfer of *APOA5* into mice (4) produce a 60-70% decrease in TG while *apoA5* knockout mice have 4 fold higher plasma TG than controls (1). The human *APOA5* gene is fairly polymorphic and in Caucasians, three common haplotypes have been identified; wild type haplotype *APOA5*\*1, *APOA5*\*2 (defined by rare alleles of –1131T>C, c-3A>G, IVS3+476G>T and c1259T>C) and *APOA5*\*3 defined by rare allele of S19W (5), and thus genotyping for –1131T>C or S19W, essentially acting as tagging SNPs (6), defines these three haplotypes. The –1131C variants and to a lesser extent 19W, have been associated with raised TG in healthy Caucasians (1, 5, 7, 8, 9), and in African Americans and Hispanics (5), and with higher relative risk of developing dyslipidemias (10, 11). The rare allele of –1131T>C is more common in Japanese compared to Caucasian (0.34 vs. 0.08, respectively) (12) and shows strong association with TG levels, even in young school-going children. However, despite these consistent associations with TG levels, the function and role of apoAV in TG metabolism remains unclear.

In order to examine, in more detail, the relationship between *APOA5* gene variants and the metabolism of TG-rich particles, we determined the association between *APOA5* -1131T>C and S19W with lipids, lipoproteins and apolipoproteins as well as

ultracentrifuged lipoprotein subfractions, in the Finnish LOCAT trial of post-coronary-bypass men (13) . To date, variation in the peroxisome proliferator-activated receptor alpha gene, *PPARA* L162V and intron 7G>C (14), the stromelysin gene, *MMP3* 5A/6A(15), the platelet endothelial cell adhesion molecule-1 gene, *PECAMI* 53G>A (16), and the alpha-1-antitrypsin gene, *AAT* V213A and 11478G>A(17), have all shown significant association with progression of atherosclerosis in LOCAT, with the *AAT* V213A showing a pharmacogenetic interaction with the response to gemfibrozil treatment. In contrast, variation in the hepatic lipase (HL) gene *LIPC* – 514C>T, while showing strong association with HL activity and lipid parameters, showed no association with atherosclerosis progression (18). The strong association between *APOA5* and TG, and the establishment by meta-analysis of TG as an independent CHD risk factor (19), suggests that *APOA5* gene variants might be associated with atherogenesis. LOCAT, with angiographic measures of disease progression over 3.5 years of the study, afforded us the opportunity to examine this.

## Methods

### Study

*Patients:* The entry criteria and baseline characteristics of the study population have been described (13). In essence, the LOCAT study entry criteria included men who had undergone coronary artery bypass grafting with HDL cholesterol equal or less than 1.1 mmol/l. Ethical approval was granted for the study, and all patients provided written consent. Quantitative coronary angiography was performed before randomization and after 2 years of double-blind, randomised, placebo-controlled gemfibrozil treatment (1200 mg/day). A total of 372 (out of 395) patients completed the study with the number of dropouts being equally distributed between the gemfibrozil and placebo groups (20).

*Quantitative coronary angiography:* Two main angiographic outcome variables were used in the present analysis. Detailed description of the angiographic variables and the main angiographic results have been provided elsewhere (13, 20, 21). First, the average diameter of coronary segments (ADS) was used as a parameter describing the extent of diffuse coronary artery disease (CAD). The difference in ADS between baseline and the last angiogram (DADS) provided an estimate of the on-trial effect (ADS). Second, the minimum lumen diameter (MLD) was used as a parameter to characterise the extent of focal CAD. Accordingly, the on-trial increase of focal coronary artery stenosis, or reduction in diameter of stenotic segments, was best described by the change in minimum lumen diameter (DMLD).

*Lipoprotein and lipid determinations:* Blood samples were obtained after an overnight fast at the randomization visit, 1 year after randomization, and 2 years after

randomization. Lipoproteins (VLDL,  $d < 1.006$  g/mL; IDL,  $d = 1.006$  to  $1.019$  g/mL; LDL,  $d = 1.019$  to  $1.063$  g/mL; HDL,  $d = 1.063$  to  $1.210$  g/mL) were separated by preparative ultracentrifugation as described elsewhere (13). Triglyceride, cholesterol, free (non-esterified) cholesterol, and phospholipid were measured in unfractionated serum and in the lipoprotein fractions, and protein was measured in the fractions (13). Cholesteryl ester concentrations were calculated as  $1.67 \times$  (total minus free cholesterol [in mg/dL]) (22). Lipoprotein compositions were calculated as the percentages of triglyceride, esterified cholesterol, free cholesterol, phospholipid, and protein (all in mg/dL) of the sum of these constituents. Serum apolipoprotein B (apoB) concentrations were determined as described (13). Height was measured at baseline; weight, waist and hip circumferences, blood pressure, and heart rate were determined at each visit.

#### *DNA genotyping*

Genotyping for the *APOA5*-1131T>C and S19W was carried out using the protocol reported previously (7).

#### *Statistical analysis*

Deviations from Hardy-Weinberg (H-W) equilibrium were assessed using a  $\chi^2$  test. The linkage disequilibrium coefficient between -1131T>C and S19W was estimated using 'delta' (23).

Lipid, lipoprotein and apolipoprotein values are expressed as mean  $\pm$ SD. Differences in these variables according to genotype were analysed by ANOVA. Those lipid or lipoprotein variables that showed a skewed distribution were log transformed before the analysis.

The influence of genotype on progression of coronary atherosclerosis was analysed by analysis of covariance. The per-patient changes in ADS and MLD from baseline to follow-up (DADS and DMLD, respectively) were entered into the models as dependent variables (14). The genotype (-1131T>C or S19W) was the independent variable. All analyses were adjusted for the randomised treatment group allocation, baseline value of the dependent variable (ADS or MLD), and time between baseline and follow-up angiograms by entering these variables as covariates. Values of DADS and DMLD by genotypes are expressed as adjusted least-squares means  $\pm$  SE.

A p-value less than 0.05 was considered statistically significant.

## Results

The baseline characteristics for the sample are presented in Table 1. Genotype distributions for both variants were in H-W equilibrium, with rare allele frequencies for the -1131T>C and S19W being 0.095 (95%CI 0.07, 0.12) and 0.072 (0.05, 0.09), respectively, which were similar to those reported for other European countries (8). There was no statistically significant allelic association between these two sites; Delta = -0.08 (p=0.77).

### *Association of -1131T>C with baseline lipid and lipoprotein subfractions*

The association of the -1131T>C with baseline lipids in total serum and lipoprotein fractions, separated by ultracentrifugation, are presented in Tables 2a, b, respectively. In agreement with previous studies, *APOA5* -1131T>C displayed a significant effect on plasma TG levels, and carriers of the rare -1131C allele had statistically significantly higher TG levels than common allele homozygotes,  $1.85 \pm 1.00$  mmol/l compared to  $1.56 \pm 0.67$  mmol/l, respectively (p=0.03). The availability of lipoprotein subfractions meant that the distribution of lipid components within these fractions could be determined. Compared to -1131TT men, carriers of the rare -1131C allele, consistently had higher lipid, surface components (with the exception of phospholipids) and protein constituents of VLDL. Thus, in -1131C carriers, VLDL-cholesterol was higher (p=0.03), as was cholesteryl esters in VLDL (p=0.04), VLDL protein content (p=0.02), and surface free cholesterol (p=0.05), compared to -1131TT men (Table 3), which was reflected in a significant higher VLDL mass in -1131C carriers (p=0.03) (Table 2b). ApoB levels, however, were no different in TT vs. C carriers ( $102.74 \pm 18.90$  compared to  $102.67 \pm 16.31$ , respectively, p=0.98) (Table 2a).

There was no statistically significant difference in the change of any of these parameters from baseline to on-trial in the group which was randomised to gemfibrozil (data not shown), thus *APOA5* –1131T>C was not influencing the response to gemfibrozil treatment.

#### *Effect of APOA5 –1131T>C and progression of atherosclerosis*

The effect of –1131T>C genotype on the progression of atherosclerosis over the period of study was examined using two different parameters; the changes in average diameter of segments (DADS), a measure of diffuse atherosclerosis, and minimal lumen diameter (DMLD), a measure of focal progression of disease. The –1131T>C had no significant effect on the change of either parameter; TT –0.025 ( $\pm$ 0.007)mm vs. CT+TT –0.004 ( $\pm$ 0.014)mm  $p=0.16$  for DADS and TT –0.075 ( $\pm$ 0.01)mm vs. CT+TT –0.044 ( $\pm$ 0.022)mm  $p=0.20$  for DMLD.

#### *Association of S19W with baseline lipid and lipoprotein fractions*

The S19W was not associated with differences in total TG or cholesterol levels (Table 2a). However there was an association of this variant with IDL parameters (Table 4). Compared to men homozygous for the 19S allele, carriers of the 19W allele had borderline higher TG content of IDL ( $p=0.087$ ) but statistically significantly higher IDL-cholesterol ( $p=0.04$ ), higher surface free cholesterol ( $p=0.005$ ) and higher phospholipids ( $p=0.017$ ). However, the protein content of IDL was not affected by genotype, suggesting that the lipid composition of the IDL particle was altered. Once again there was no effect of genotype on the response of these parameters to gemfibrozil treatment (data not shown).

*Effect of APOA5 S19W on progression of atherosclerosis*

The S19W variant was associated with a borderline significant change in ADS (DADS) over the course of the study. Men who carried the 19W allele had a trend toward greater progression of disease  $-0.046 (\pm 0.016)$  mm compared to decrease in DADS of  $-0.016 (0.006)$ mm in 19S homozygotes ( $p=0.082$ ). These results together with a similar trend seen in DMLD are presented in Figure 1.

## Discussion

The differential results of the two common *APOA5* variants -1131T>C and S19W, with lipoprotein compositional data available in LOCAT, provides novel insights into the role of apoAV in TG metabolism. As with previous studies, -1131T>C and S19W showed no allelic association (5,7) and therefore were acting independently. These two variants are known to define the two common *APOA5* haplotypes, *APOA5\*2* and *APOA5\*3* (5).

### *-1131T>C and plasma TG levels*

In LOCAT, the rare allele of -1131T>C variant, in agreement with all previous studies, was associated with significantly higher plasma TG levels compared to common allele homozygotes. The lipid compositional data suggests that this is reflecting an increase in VLDL-TG, and the higher levels of VLDL components, with the exception of phospholipids and apoB, are corroborated by the significant increase in the total VLDL mass. Although the protein content of VLDL was associated with genotype, the levels of apoB did not differ among genotypes, and this might reflect a change in apoE and/or apoCIII content.

The potential effect of lower apoAV resulting from the -1131T>C, which acts as a marker for haplotype *APOA5\*2*, would be an increase in plasma TG synthesis leading to an increase in VLDL mass which could reflect either an increase in VLDL secretion or a decrease in catabolism or both. We can only speculate on these options. These results are in agreement with two previous studies which showed that the -1131C carriers displayed significantly higher VLDL mass measures (1, 9).

*-1131T>C and LD with other variants in cluster*

The  $-1131T>C$  is in complete positive LD with a  $-3G>A$  and together these variants help define the *APOA5*\*2 haplotype (5). As detailed elsewhere, both these variants could potentially be functional (24). We have examined the association of these two variants with lipid parameters in a study of Japanese Americans, and this haplotype appears to be a major genetic determinant of LDL particle size and triglyceride levels.

As well as the potential direct functional effects of these variants, we previously showed that there is overall strong LD across the *APOA5-A4-C3* gene cluster, and in order to investigate whether the association of *APOA5* variants with TG levels were acting independently of *APOC3* variants (also associated with differences in TG levels), we examined the association of 9 SNP haplotypes across the *APOA5-A4-C3* genes with lipid levels in a large prospective study of middle aged men (7). Results from that study showed that the five haplotypes that were associated with the highest TG levels, carried either *APOA5* 19W or *APOC3*  $-482T$ , thus demonstrating that these two variants were independently determining TG levels. The *APOA5*  $-1131T>C$  was in strong positive LD with *APOC3*  $-482T>C$ , a functional change disrupting an insulin responsive element (25) and although the haplotype associated with the second highest TG levels carried both the *APOA5*  $-1131C$  and *APOC3*-482C it was not possible to identify the independence of these effects.

We examined the recently published *in vitro* structural analysis of apoAV, to gain some insight into the potential basis of these results (26, 27). ApoAV was identified as a molecule with high lipid affinity (26), low elasticity and slow binding kinetics

with a suggestion that it may retard the second step in VLDL assembly. We previously speculated that if apoAV were to act by limiting the TG content of growing lipoprotein particles, for example if it were to influence MTP function, the resulting VLDL would be TG-enriched (7). Weinberg et al report that a BLAST research showed 55% identity between the C-terminal domain of apoAV and residues 239-260 of mouse MTP, suggesting that apoAV might have MTP-like activity (27). Weinberg et al also showed that apoAV over-expression in COS-1 cells led to poor apoAV secretion, supporting the idea that the primary role of apoAV is in intercellular hepatic metabolism (27). This would explain why plasma apoAV levels are low (4). Thus, the predicted reduced *APOA5* transcription or translation, due to the functional changes resulting from either or both -1131T>C or -3G>A, could explain the resulting increase in VLDL mass, in -1131C/-3A men compared to -1131T/-3G men. Taking into account these structure/function studies of apoAV (26, 27), it would seem that the likely cause of VLDL mass increase is a decrease in apoAV synthesis and an increase in TG incorporation into VLDL.

*-1131T>C has little effect on disease progression.*

In NPHSII, a prospective study of UK men, there was in fact very little similarity in the ranking of the haplotypes by TG or CHD prevalence (28). Men who carried the haplotype defined by *APOA5*-1131C/*APOC3*-482T showed CHD prevalence well below that of men who carried the common haplotype defined by all 9 common alleles (28), supporting the idea that the -1131C is not associated with increased CHD risk. This emphasises that high TGs alone are not a good discriminator of CHD risk (29).

### *S19W and increase in IDL number*

Although we and others have previously reported the association of the S19W with differences in plasma TG levels (1, 5, 7), this association has not been as consistent as the findings with -1131T>C. We reported that in the European Atherosclerosis Study II, a study of University students comparing those whose father had suffered a premature MI, before the age of 55, called the 'cases', with age matched 'controls' drawn from the same University environment, 19W was not associated with significantly higher plasma TG levels in either the 'cases' or 'controls', although there was significant heterogeneity of effect between groups (8). In the 'controls', 19W carriers in fact had 2% lower TG levels than 19SS men. In LOCAT, the difference in total TG levels according to S19W was not statistically significant ( $p=0.34$ ) and represented only a 5% increase in TG. While in NPHSII, TG levels in 19W carriers were 10% higher compared to SS men ( $p<0.006$ ) (unpublished data, PJ Talmud and E Hawe), although when comparing rare homozygotes with common homozygotes, WW men had 52% higher TG than SS men ( $p<0.003$ ) (7). The lack of a significant association of 19W with TGs in LOCAT may reflect the considerably smaller sample size compared to NPHSII (297 vs 2,497, respectively). In LOCAT, men who carried the 19W allele had borderline statistically significantly higher TG in IDL ( $p=0.087$ ), however other IDL constituents, such as cholesterol ( $p=0.04$ ), and particularly the surface lipids, namely free cholesterol ( $p=0.005$ ) and phospholipids ( $p=0.017$ ) were higher in 19W carriers compared to 19S homozygotes. The protein content of IDL remained unaffected by the variant. Taken together, the results suggest that S19W is associated with an increase in IDL particle size with primarily cholesterol enrichment. These cholesterol-enriched remnant particles could provide a good substrate for cholesteryl ester transfer protein resulting in a depletion of cholesteryl ester in

exchange for TG-enrichment. It has been suggested that TG-rich IDL particles, after hydrolysis by hepatic lipase could lead to the increase in small dense pro-atherogenic LDL particles (30) which might explain the increased risk associated with *APOA5* S19W.

As with the other *APOA5* variants the direct functional role of S19W has not been confirmed, although this amino acid change within the apoAV signal sequence has the potential to be functional.

#### *S19W and atherosclerosis progression*

In contrast to the data from the -1131T>C, the S19W variant was associated with differences in atherogenesis over time in LOCAT. Although only showing borderline effects, 19W carriers had increased progression of diffuse atherosclerosis as suggested by the change in DADS ( $p=0.082$ ), but not focal disease (DMLD). Once again comparing this data to results from our previous study, NPHSII, the haplotype defined by the 19W on a background of common alleles was associated with the highest plasma TG levels and men carrying this haplotype had the 4<sup>th</sup> highest prevalence of CHD, suggesting this variant, unlike -1131T>C, promotes atherosclerosis by generating a pro-atherogenic particle.

#### *No effect of APOA5 variants on the response to gemfibrozil treatment.*

LOCAT is a gemfibrozil trial and although, compared to placebo, gemfibrozil was effective in reducing lipid levels and disease progression (13, 20), there was no association between *APOA5* variants and this response. This is not surprising since although *APOA5* has a PPRE and gemfibrozil is a PPAR- $\alpha$  ligand, the PPRE is a

considerable distance from the -1131 site (860bp). Furthermore, we previously examined the effect of functional *PPAR* $\alpha$  variants on disease progression in LOCAT, but also found no association of *PPAR* $\alpha$  variants with lipid levels (14).

In agreement with other studies (1, 9, 24) our data suggests a role for apoAV in the determination of lipid subfraction composition. By considering this lipoprotein subfraction data in the LOCAT study we can speculate on potential different mechanisms for the effect of *APOA5* variants on the secretion of TG-rich particles, and this goes some way to explain their individual roles in atherosclerosis progression. Further confirmation, in additional studies and in-depth molecular experiments, are needed to determine the functional basis for these effects.

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**Figure Legend:**

The changes in average diameter of segments (DADS) and minimum lumen diameter (DMLD) and SEMs in the LOCAT study.

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Table 1 Baseline characteristics of the men participating in LOCAT who completed the trial, mean±SD

	Baseline values
Number of men	372
Mean age (years)	59.1±6.8
BMI (kg/m <sup>2</sup> )	26.4±2.2
Triglycerides (mmol/l)	1.60±0.75
Cholesterol (mmol/l)	5.17±0.71
HDL-C (mmol/l)	1.02±0.17
LDL-C (mmol/l)	3.43±0.57

Table 2 a Baseline total lipid, lipoprotein and apolipoprotein values according to *APOA5* -1131T>C and S19W

	-1131			S19W		
	TT	TC+CC	P-value	SS	SW+WW	P-value
	N: 242	54		N: 268	44	
TG (mmol/l)	1.56±0.67	1.85±1.00	0.03	1.60±0.75	1.68±0.65	0.34
Chol (mmol/l)	5.18±0.73	5.26±0.63	0.44	5.16±0.70	5.32±0.70	0.14
Free Chol (mmol/l)	1.48±0.21	1.51±0.19	0.33	1.48±0.21	1.51±0.20	0.26
ApoB mg/dl	102.74±18.90	102.67±16.31	0.98	102.14±18.61	105.09±17.70	0.33
Cholesteryl ester mg/dl	239.05±34.90	241.76±30.42	0.60	238.03±33.92	245.67±34.94	0.18
Phospholipid mg/dl	194.50±25.31	195.93±24.44	0.70	193.64±24.99	199.33±25.83	0.17

Table 2b Lipoprotein mass (mg/dl) according to *APOA5* -1131T>C and S19W

	-1131			S19W		
	TT	TC+CC	P-value	SS	SW+WW	P-value
	N: 242	54		N: 268	44	
VLDL	163.99±87.97	203.74±121.25	0.03	170.03±97.56	177.30±85.63	0.51
IDL	41.81±17.35	43.82±12.82	0.15	41.54±16.25	45.46±18.81	0.13
LDL	385.66±61.49	381.46±57.55	0.65	382.93±59.51	391.06±67.99	0.43
HDL	295.87±42.99	286.88±40.42	0.16	293.31±42.86	298.38±40.93	0.49

Table 3 Ultracentrifuged Lipoprotein subfractions according to *APOA5* -1131T>C

	VLDL		IDL		LDL		HDL	
	-1131TT	-1131C+	-1131TT	-1131C+	-1131TT	-1131C+	-1131TT	-1131C+
TG (mmol/l)	1.02±0.58	1.29±0.83	0.12±0.05	0.12±0.03	0.26±0.07	0.25±0.08	0.16±0.04	0.16±0.04
P	0.04		0.42		0.61		0.82	
Chol (mmol/l)	0.48±0.30	0.61±0.39	0.23±0.13	0.24±0.12	3.43±0.60	3.41±0.58	1.03±0.18	1.00±0.15
P	0.03		0.16		0.82		0.14	
Free Chol	0.23±0.14	0.29±0.18	0.08±0.04	0.09±0.04	0.94±0.17	0.91±0.18	0.22±0.04	0.21±0.04
P	0.05		0.12		0.29		0.23	
Protein	23.04±8.69	26.82±13.65	11.44±3.31	11.32±3.48	82.47±14.19	81.14±12.90	143.39±26.89	139.87±26.11
P	0.02		0.86		0.52		0.37	
Chol Ester	15.86±10.86	20.09±14.36	9.53±6.15	10.01±5.31	161.06±29.12	161.14±27.79	52.55±9.64	50.69±8.13
P	0.04		0.28		0.98		0.19	
Phospholipid	25.94±14.32	30.37±18.67	7.43±3.98	7.93±3.32	84.00±14.42	82.87±14.98	77.09±12.00	74.59±9.76
P	0.27		0.13		0.60		0.14	

Table 4 Ultracentrifuged Lipoprotein subfractions according to *APOA5* S19W

	VLDL		IDL		LDL		HDL	
	19SS	19W+	19SS	19W+	19SS	19W+	19SS	19W+
TG	1.06±0.65	1.12±0.57	0.12±0.05	0.13±0.04	0.25±0.07	0.26±0.07	0.16±0.04	0.17±0.04
P	0.41		0.09		0.40		0.15	
Chol	0.49±0.32	0.54±0.29	0.22±0.12	0.26±0.15	3.41±0.58	3.48±0.63	1.03±0.18	1.04±0.16
P	0.29		0.04		0.47		0.58	
Free Chol	0.24±0.15	0.26±0.13	0.08±0.04	0.10±0.06	0.93±0.17	0.94±0.18	0.22±0.04	0.22±0.04
P	0.30		0.01		0.97		0.54	
Protein	23.69±10.16	23.86±7.88	11.36±3.39	11.74±2.92	81.98±13.30	82.67±18.09	142.34±26.50	144.58±28.29
P	0.63		0.31		0.77		0.61	
CE	16.37±11.79	17.87±10.88	9.40±5.93	10.75±6.33	160.15±28.38	164.28±31.31	52.09±9.50	52.81±8.66
P	0.37		0.12		0.39		0.65	
Phospholipid	26.45±15.42	28.17±14.42	7.28±3.71	8.79±4.62	83.30±14.27	85.53±15.48	76.53±12.04	76.83±8.93
P	0.47		0.02		0.34		0.87	

