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PHARMACOGENETIC STUDY OF FENOFIBRATE IN TUNISIAN TYPE 2 DIABETIC PATIENTS "A PRELIMINARY STUDY"

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ABSTRACT

Fibrates act to attenuate atherogenic dyslipidemia in type 2 diabetic patients. However an increase of serum homocysteine (tHcy) after fenofibrate treatment has been reported, compromising its cardiovascular benefit. The effect of polymorphisms in cholesteryl ester transfer protein (CETP), apolipoprotein A5 (apo A 5), and methylenetetrahydrofolate reductase (MTHFR) genes on fenofibrate treatment in type 2 diabetic patients. Patients are taking lipid lowering drugs for the first time. Polymorphisms are studied by RFLP-PCR (Restriction Fragment Length Polymorphism-Polymerase Chain Reaction). Biochemical parameters are measured by enzymatic methods. CETP activity is determined by exogenous way. Total plasma homocysteine levels (tHcy) were assessed by capillary gas chromatography-mass spectrometry method. After fenofibrate use, a significant decrease of Triglyceride (TG) level (29 %) and a decrease of total cholesterol (TC) ($p = 0.081$) and CETP activity ($p = 0.089$) were noted. However, the High Density Lipoprotein-Cholesterol (HDL-C) concentration has increased ($p= 0.081$) while Low Density Lipoprotein-Cholesterol (LDL-C) levels did not vary. Moreover, the prevalence of hyperhomocysteinemia rose to 100 %. Both apo A5 TT and TC carriers showed significant decrease of TG levels. Whereas the HDL-C variation is better in TT genotype (23.5 % vs -1.3 % for TT and TC respectively; $p = 0.062$). The decrease of TG levels after fenofibrate treatment is more important in B1B1 than in B2B2 genotype of CETP polymorphism. Only B1B1 homozygous showed a decrease of CETP activity and an increase of HDL-C. After fenofibrate use, the increase of tHcy levels was more important in MTHFR T carriers than in CC homozygous (39.97 ± 14.77 vs. 28.02 ± 8.59 µmol/l, respectively). The TT apo A5, B1B1 CETP and CC MTHFR carriers benefit the most from lipid lowering fenofibrate treatment. Pharmacogenomic studies have a great economic and health interest for a better treatment of type 2 diabetic patients.

Keywords: Apo A5, CETP, Fenofibrate, Homocysteinemia, Lipid profile, MTHFR, Pharmacogenetics.

INTRODUCTION

Type 2 diabetes is characterized by an atherogenic lipid and lipoprotein profile: plasmatic high triglyceride (TG) level, low HDL-C concentration and small dense LDL.[1] Such profile contributes to the increased risk of macrovascular disease.[1] Also, high levels of total plasma homocysteine (tHcy) have been found in patients with diabetes and coronary heart disease (CHD) and were considered as a strong and independent predictor of Coronary Artery Disease

(CHD) events.[2] These levels are influenced by potential covariates as vitamins B6, B12 folate, creatinine, and the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism. Hypertriglyceridemia is associated with atherosclerosis because i) it is a marker for insulin resistance and atherogenic metabolic abnormalities; and ii) the small size of TG-enriched lipoprotein enables them to infiltrate the blood vessel wall where they are oxidized, bind to receptors on macrophages, and ingested, leading to the development of the atherosclerotic lesion.[1] Whereas, the HDL-C particles have important antiatherogenic effects, including reverse cholesterol transport, inhibition of LDL-C oxidation and antiplatelet and anti-inflammatory actions.[3] In order to attenuate the cardiovascular risk in type 2 diabetic patients, correction of the atherogenic dyslipidemia was the aim of several studies. In fact many drugs are used to ameliorate dyslipidemia such as statins and fibrates. However treatment with fibrates has been shown to be the most effective in type 2 diabetes.[4] Besides their effects on blood lipid levels, lipid-lowering drugs have the ability to influence several recently identified factors associated with the increased risk of coronary artery disease (CAD). It has been found that long-term treatment with pravastatin reduces the C-reactive protein (CRP) level in plasma independent of lipid-lowering effects.[5] Significant increase of plasma homocysteine levels after fenofibrate therapy has been reported[6], whereas statins appear to have no effect on homocysteine levels.[7] In fact, fibrates are peroxisome proliferator-activated receptors (PPAR) agonists and were shown to be particularly suited for atherogenic dyslipidemia.[8] However, the response to fibrates was shown to be heterogenous among patients. This variation could be a result of genetic and/or nutritional factors. Among candidate genes suspected to affect the response to fibrates are genes implicated in lipid and lipoprotein metabolism: 3-hydroxy-3-methyl-glutaryl-CoA (HMG-Co A) reductase[9], apolipoprotein E[10], lipoprotein lipase.[11] As fibrates are not known to alter the principal determinants of total plasma homocysteine (tHcy): vitamins B6, B12, or folate status; or have an effect on renal function that would explain the observed increase in tHcy; intervention of genetic polymorphisms of key enzymes implicated in Hcy metabolism, essentially the C677T polymorphism of the MTHFR gene should be explored. Cholesteryl ester transfer protein (CETP) is a key protein in reverse cholesterol transport.[12] The Taq IB polymorphism was its most studied polymorphism. In type 2 diabetes, B1 allele was associated with decreased HDL-C concentration and increased CETP activity.[13,14] Increased CETP activity was also shown to be associated with coronary artery disease extend.[13] Apolipoprotein A5 (apo A 5) is an apolipoprotein associated with HDL, VLDL and chylomicron particles.[15] It modulates the hepatic Very Low Density Lipoprotein (VLDL) synthesis and/or secretion and it has a positive effect on LPL activity.[16] Thus apo A5 was considered as a TG modulating gene. Many Single Nucleotide Polymorphisms (SNPs) were identified in this gene.[17] The SNP3 was associated with TG level variation.[18-20] In this issue, we studied the effect of fenofibrate on lipid, lipoprotein and CETP activity levels in type 2 diabetic patients. Furthermore, we aimed to see the influence of Taq IB (of CETP) and SNP3 (of apo A 5) polymorphisms on this relationship. More else, we studied the effect of fibrate on homocysteine according to C677T MTHFR polymorphism.

SUBJECTS AND METHODS

Subjects

A total of twenty one type 2 diabetic patients were recruited from the department of Cardiology of the teaching Hospital of Monastir. The Institution's Ethics Committee for studies on human subjects approved the study and each patient consented to participate in the research. Clinical examination was performed for all the participants. Obesity was defined as body mass index (BMI) $\leq$ 30 kg/m2 (BMI calculated as weight divided by height 2) and waist-to-hip ratio (WHR) calculated from measurements of the waist circumference taken at the midpoint between umbilicus and xiphoid and hip

circumference, at the widest point around the hips. Diabetes were diagnosed and classified according to American Diabetes Association criteria (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997). Diabetic patients were either treated by diet and/or given antidiabetic drug (biguanidine and/or sulfamide or metformin). The recruitment of the diabetic patients was restricted by the following criteria: a body mass index (BMI) more than 35 kg/m^2, glycosylated hemoglobin (Hb A1C) more than 12%, taking insulin or lipid lowering drug; presence of renal, liver failure or thyroid disease; use of antioxidant therapy, vitamin supplementation or hormonal replacement therapy for the post menopausal women. None of the patients consumed alcohol 3 days prior to the study or less. Diagnosis of cardiovascular disease was based on a history of angina with a positive exercise test or an abnormal coronary angiogram or on a history of myocardial infarction. Hypertension (HTA) was diagnosed according to the JNC criteria (Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure). The duration of treatment with lipanthyl® 200 M (fenofibrates) was 4 weeks. It's for the first time that the patients use lipid lowering drugs. Along this period all participants abstained strictly from alcohol, and were asked not to take high-fat diet. Blood samples were collected before and after drug use. They were collected after overnight fasting (12 h) into tubes containing EDTA or heparine.

Laboratory Procedures

Plasma glucose, glycosylated hemoglobin (HbA1c), plasma creatinine, lipid, lipoproteins and CETP activity were determined as described in our previous studies.[13] Total plasma homocysteine levels (tHcy) were assessed by capillary gas chromatography-mass spectrometry method as described elsewhere.[21] After DNA extraction, Taq IB polymorphism of CETP and SNP3 of apo A5 were studied using RFLP-PCR technique as done in our previous works.[13,18] Three genotypes characterized each polymorphism: B1B1, B1B2 and B2B2 for CETP ; TT, TC and CC for apo A5. MTHFR polymorphism was conducted as previously described.[22] *MTHFR* genotypes were classified as native (CC), heterozygous (CT) and homozygous (TT).

Statistical Analyses

Data management and statistical analysis were performed using SPSS 10.0 software. Results are summarized as mean ± SD. Logarithmic transformation of CETP activity and TG concentrations were performed. Paired student's t-test was performed for lipid concentration, homocysteine concentration and CETP activity before and after fenofibrates treatment. An unpaired Student's t-test was used to compare the differences in the degree of reduction in plasma concentrations between the CETP, the apo A5 and the MTHFR genotypic groups. A two-tailed P-value < 0.05 was considered to be statistically significant for all analyses.

RESULTS

Lipid, lipoprotein CETP activity and tHcy levels of the type 2 diabetic patients at baseline are presented in Table 1. The main characteristics of type 2 diabetic patients before treatment with fenofibrates are the high level of triglyceride and the decreased concentration of HDL-C. An increased CETP activity and tHcy levels were also shown. After fenofibrate treatment, we noted a significant decrease of TG levels (29 %) and a decrease of total cholesterol (p = 0.081). Also the CETP activity decreased but the difference did not reach statistical significance (p = 0.089). However, the HDL-C concentration has increased but the increase is almost significant (0.081). The LDL-C did not differ before and after treatment (Table 1). Plasma tHcy levels determined in the fasting state are shown in Table 1. Fibrates use was associated with a mean 193.38% increase in tHcy levels (ranging from 17.84 ± 7.82 to 34.50 ± 12.15 µmol/l;p= 0.013). No decrease in tHcy was noted in any of the patients. The prevalence of hyperhomocysteinemia, defined here as tHcy levels > 15 µmol/l was 50 % before fenofibrate use and rose to 100 %. To determine how genetic factors influence the fenofibrate treatment

response on lipid metabolism, we examined differences in fasting plasma lipid levels and CETP activity at baseline and after drug intervention among different apo A5 and CETP genotypic groups (Tables 2 and 3). According to apo A5 polymorphism, both the TT and TC genotypic groups of apo A5 showed a significant decrease of TG concentrations. Whereas the HDL-C variation is better in TT of apo A5 genotype than in TC one (23.5 % vs -1.3 % for TT and TC respectively; p = 0.06 2). However, the degree of change in plasma total cholesterol, LDL-C and CETP activity did not differ between the two genotypic groups (Table 2). According to CETP polymorphism, the decrease of TG levels after fenofibrate treatment is more important in B1B1 genotype than in B2B2. The total cholesterol concentration and the CETP activity decreased only in B1B1 genotype. However the increase of HDL-C concentration was shown only in B1B1 genotype. This increase is almost significant (p = 0.058) (Table 3).

The MTHFR (C677T) genotype was determined. Mutation effects were evaluated by examination of the change in tHcy levels and lipid parameters after fenofibrate use in different genotypic groups. At the beginning of the treatment period, T allele carriers exhibited lower tHcy levels than CC homozygous. After fenofibrate use the increase of tHcy levels was more important in T carriers than in CC homozygous (39.97 ± 14.77 vs 28.02 ± 8.59 µmol/l, respectively) (Table 4). As lipid parameters were concerned we observed a significant decrease in triglyceride levels after fenofibrate use in both groups. Also, we showed that HDL-C increased, almost significantly, in CC homozygous without any variation in TT homozygous.

DISCUSSION

Type 2 diabetes is associated with an increased risk of cardiovascular disease. Lipid lowering drugs are usually used to prevent such disease. They aim to ameliorate the lipid and lipoprotein profiles. The effect of fibrates on TG levels and HDL-C concentrations were well established.[23,24] We showed that after fenofibrate treatment, TG level decreased and HDL-C concentration increased. In fact, fibrates decreased TG level by stimulating TG rich lipoprotein catabolism and inhibition of VLDL secretion.[24] Whereas it increased HDL-C concentration by influencing the expression of HDL apolipoprotein such as the apo A-I[25], then stimulating the reverse cholesterol way.[26] CETP is a key protein in the reverse cholesterol transport. We showed that CETP activity decreased after fenofibrate therapy. The same result was shown by Guerin et al. in 9 patients with combined hyperlipidemia.[27] The mechanism by which fenofibrate influences CETP activity is not yet established. In fact the decreased CETP activity is explained by a decreased CETP level as we used an exogenous way to measure this activity. In addition, a decreased CETP mass and activity were shown in patients with type 2diabetes and dyslipidemia after treatment with atorvastatin.[28] As CETP activity was associated with coronary artery disease (CAD) extent[13], the decreased CETP activity after fenofibrate treatment strengthens the idea that fenofibrate therapy is a good strategy for reducing CAD and its extent in type 2 diabetic patients. However, the use of an inhibitor of CETP (torcetrapid) increased risk of death and ischemic cerebrovascular disease despite improving the lipid profile.[29] But, The findings of Johannsen *et al.*[30] together with our results provide reassurance that pharmacological inhibition of CETP may reduce risk of ischemic vascular events and total mortality, when not accompanied by the off-target effects of torcetrapib. The correction of lipid and lipoprotein profiles is not necessary achieved in all the patients treated with fenofibrate. Pharmacogenomic variability is an important determinant of drug response. Many genes are associated with the degree of lipid lowering effect of Fibrates.[9-11] In our work we studied the effect of apo A5, CETP and MTHFR genotypes on the lipid lowering efficiency of fenofibrate in tyoe 2 diabetics. Patients who carry different apo A5 variants responded differentially to fenofibrate treatment: Patients having TT genotype displayed higher increase in HDL-C concentration and higher decrease in triglyceride level (4.6 % vs.

26.6 %) relative to their values before the treatment when compared to TC genotype. The SNP3 (6 1131 T>C) is located in the promoter region and fenofibrate highly upregulates apo A5 expression through PPAR receptor.[25] This can be one of the explanations of the interaction between response to fenofibrate treatment and SNP3 polymorphism. Another study reported that the SNP3 polymorphism was not associated with lipid variation in response to the fenofibrate intervention in contrast to 56 C>G polymorphism.[31] Type 2 diabetic patients having the B1B1 genotype of CETP are more predisposed to CAD than B2 carriers.[13] They are characterized by decreased HDL-C concentrations.[13] When treated with fenofibrate, patients carrying B1B1 genotype, have significant decreased triglyceride and total cholesterol concentrations and elevated HDL-C concentrations compared to baseline data. However those parameters did not differ after fenofibrate treatment in B2B2 genotype. This result showed that B1 B1 genotype carriers benefit most from fenofibrate therapy as it was shown for atorvastatin[31] and contrary to those shown for simvastatin.[32] In another study, the CC genotype of CETP gene promoter polymorphism at position -629 C/A offers a better benefit of statin therapy associated with lowered level of LDL-C and LP (a).[31] More studies and larger studied populations are needed to better conclude and understand the genetic effect on the drug response. After fenofibrate treatment, the CETP activity decreased in B1B1 but not significantly whereas it increased in B2B2 genotype. Although the difference between variation of lipid, lipoprotein and CETP activity before and after fenofibrate treatment in both genotypic groups are not significant, we can see the beneficial effect of fenofibrate in B1B1 compared to B2B2. In fact the number of individuals having B2B2 genotype is little because the frequency of this genotype in our Tunisian population is very low.[13] In the present analysis, fenofibrate was found to double plasma homocysteine levels in the fasted states. Furthermore, it have been reported in a study by R. Bissonnette *et al.*[34] that there was a significant increase in total homocysteine concentration in the postprandial state (14% for placebo and 21% for fenofibrate), compared with fasting tHcy levels, in the absence of a significant amount of dietary methionine in the fat meal. Dierkes *et al.*[6] examined the effect of fenofibrate and bezafibrate on plasma tHcy levels. Fenofibrate was associated with a 44% increase in tHcy levels, from 13.1 µmol/l at baseline to 20.0 µmol/l, P = 0.009. Similarly, Land-ray *et al.*[35] examined patients with chronic renal failure treated with fenofibrate. Compared with baseline levels, fenofibrate use was associated with an increase in tHcy from 15.1 to 21.8 µmol/l. In addition, Bissonnette *et al.* showed that plasma levels of vitamins B6, B12 and folate did not change on fenofibrate. No changes therefore during the study occurred in factors known to affect tHcy levels (i.e. age, male gender, levels of vitamins B12, folate or creatinine levels).[34] There was no significant relationship between percentage change in homocysteine levels and MTHFR genotype. The mechanism by which fenofibrate increases plasma total homocysteine levels is unknown. An increase in methionine may have an effect on the levels of S-adenosylmethionine, a key regulatory molecule for the transsulfuration and reméthylation pathways.[36] One of the actions of fibrates is through their non-covalent association with peroxisome proliferator activator receptors (PPAR), which are known to form heterodimers with nuclear binding proteins of the 9-cis retinoic acid receptor (RxR) family. The Fibrate-PPAR-RxR heterodimer is thought to act at the level of the promoter region of specific genes involved in lipoprotein metabolism at a consensus sequence TGCCCTTTCCCCC[37,38] and to modulate transcriptional regulation.[39] The possibility that fenofibrate interferes with the remethylation cycle or the transsulfuration pathways at the transcriptional level remains to be explored. Table 4 showed that plasma homocysteine level increase is more important in T carriers than in CC homozygous. Moreelse, in T carriers, we did not see any variation in HDL-C concentration. So, in thisgroup (T carriers), fenofibrate may be replaced by other lipid lowering drugs.

CONCLUSION

In summary, fenofibrate is a good therapy to prevent CAD in type 2 diabetes by decreasing TG level and increasing HDL-C concentration. Moreover fenofibrate decreased the CETP activity, which is associated with CAD extent. Such result let us consider CETP as a targeting protein for the prevention and management of cardiovascular disease. We reported also that the lipid lowering efficacy of fenofibrate is influenced by apo A5, CETP and MTHFR polymorphisms. The TT apo A5, B1B1 CETP and CC MTHFR carriers benefit the most from lipid lowering fenofibrate treatment. In the case of the other genotypic groups, fenofibrate should be replaced by another lipid lowering drug. For a better treatment of type 2 diabetes, pharmacogenomic studies are with great economic and health interest.

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Table 1: Lipid parameters, CETP activity and tHcy levels in type 2 diabetic patients before and after treatment with fenofibrate

Group	TG (mmol/L)	TC (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	CETP activity (nmol /mL/2 h)	tHcy (µmol/)
Before fenofibrates	2.75 ± 1.17	4.9 ± 1.43	0.60 ± 0.17	3.00 ± 1.32	92.08 ± 93.41	17.84 ± 7.82
After fenofibrates	1.76 ± 0.79	4.62 ± 0.98	0.67 ± 0.16	3.11 ± 0.96	71.59 ± 81.81	34.50 ± 12.15
P value	0.001	0.085	0.081	0.405	0.089	0.013

TG, Triglyceride; TC, Total Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; CETPA, Cholesterol Ester Transfer Protein Activity (nmol Cholesteryl Ester/mL/2 h) defined as the quantity (nmol) of total tritiated cholesteryl ester (3SH-CE) transferred from HDL (donor lipoproteins) to LDL and VLDL (acceptor lipoproteins) in the presence of a small volume of the patient's plasma (10 AL). ; tHcy total plasma Homocysteine.

Table 2: Effect of apo A5 polymorphism on CETP activity and lipid lowering effect of fenofibrate

	Fenofibrate treatment	apo A5 polymorphism TT n = 8	TC n = 7	*P* value of variation
TG (mmol/L)	Before	3.36 ± 1.40	2.33 ± 0.90	
	After	1.61 ± 0.74	1.65 ± 0.73	0.205
	P value	0.018	0.028	
TC (mmol/L)	Before	5.65 ± 1.48	4.42 ± 1.28	
	After	5.10 ± 0.76	4.21 ± 1.00	0.620
	P value	0.167	0.340	
HDL-C (mmol/L)	Before	0.60 ± 0.17	0.64 ± 0.15	
	After	0.72 ± 0.18	0.62 ± 0.14	0.062
	P value	0.077	0.657	
LDL-C (mmol/L)	Before	3.44 ± 1.49	2.72 ± 1.33	
	After	3.61 ± 0.88	2.84 ± 0.95	0.93
	P value	0.553	0.618	
CETP activity (nmol /mL/2 h)	Before	92.8 ± 106.7	88.7 ± 107.6	
	After	56.7 ± 77.9	84.7 ± 97.3	0.911
	P value	0.323	0.889	

TG, Triglyceride; TC, Total Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; CETPA, Cholesterol Ester Transfer Protein Activity (nmol Cholesteryl Ester/mL/2 h) defined as the quantity (nmol) of total tritiated cholesteryl ester (3SH-CE) transferred from HDL (donor lipoproteins) to LDL and VLDL (acceptor lipoproteins) in the presence of a small volume of the patient's plasma; Hcy Homocysteine. p value of variation, TT versus TC.

Table 3: Effect of CETP polymorphism on CETP activity and lipid lowering effect of fenofibrate

		CETP polymorphism B1B1 n = 14	B2B2 n = 4	*P* value of variation
TG mmol/l	Before	3.02 ± 1.29	1.88 ± 0.33	
	After	1.78 ± 0.74	1.33 ± 0.47	0.605
	P value	0.004	0.235	
TC mmol/l	Before	5.52 ± 1.37	3.73 ± 0.44	
	After	5.00 ± 0.90	3.90 ± 0.78	0.154
	P value	0.02	0.581	
HDL-C mmol/l	Before	0.61 ± 0.14	0.70 ± 0.18	
	After	0.69 ± 0.15	0.70 ± 0.25	0.330
	P value	0.058	0.991	
LDL-C mmol/L	Before	3.50 ± 1.37	2.17 ± 0.51	
	After	3.48 ± 0.96	2.59 ± 0.48	0.286
	P value	0.914	0.150	
CETP activity (nmol /mL/2 h)	Before	116.0 ± 103.2	17.9 ± 35.2	
	After	86.8 ± 89.1	29.0 ± 80.5	0.740
	P value	0.102	0.858	

TG, Triglyceride; TC, Total Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol; CETPA, Cholesterol Ester Transfer Protein Activity (nmol Cholesteryl Ester/mL/2 h) defined as the quantity (nmol) of total tritiated cholesteryl ester (3H-CE) transferred from HDL (donor lipoproteins) to LDL and VLDL (acceptor lipoproteins) in the presence of a small volume of the patient's plasma; Hcy Homocysteine. *P* value of variation, B1B1 versus B2B2.

Table 4: Effect of fenofibrate on tHcy according to MTHFR polymorphism

		MTHFR polymorphism CC n = 4	T carriers n = 4	*P* value of variation
tHcy µmol/l	Before	20.82 ± 5.24	16.92 ± 10.00	
	After	28.02 ± 8.59	39.97 ± 14.77	NS
	P value	0.214	0.092	
TG mmol/l	Before	3.6 ± 1.4	2.3 ± 0.82	
	After	1.7 ± 0.9	1.4 ± 0.47	NS
	P value	0.04	0.001	
TC mmol/l	Before	5.05 ± 1.7	4.64 ± 1.1	
	After	4.52 ± 1	4.46 ± 0.85	NS
	P value	0.23	0.43	
HDL-C mmol/l	Before	0.56 ± 0.1	0.62 ± 0.2	
	After	0.75 ± 0.21	0.64 ± 0.17	NS
	P value	0.096	0.77	
LDL-C mmol/L	Before	3.50 ± 1.37	2.95 ± 1.2	
	After	3.48 ± 0.96	3.1 ± 0.81	NS
	P value	0.914	0.361	

TG, Triglyceride; TC, Total Cholesterol; HDL-C, High Density Lipoprotein Cholesterol; LDL-C, Low Density Lipoprotein Cholesterol, tHcy total plasma Homocysteine. p value of variation, CC versus T carriers.

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