Paraoxonase 1 activity and genotyping in systemic lupus erythematosus and their relationships with cardiovascular complications

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ABSTRACT

Introduction: Systemic lupus erythematosus (SLE) is characterized by an enhanced risk of atherosclerosis and cardiovascular diseases (CVD). Human serum paraoxonase 1 (PON1), an antioxidant enzyme closely associated with high density lipoprotein (HDL), has been implicated in the prevention of low density lipoprotein (LDL) oxidation, and these may provide HDL-associated protection against atherosclerosis.

The Aim: Our objective was to evaluate PON1 activity and genotypes in SLE patients and their relationships to cardiovascular complications and some other risk factors of cardiovascular diseases in those patients.

Patient and Methods: Thirty SLE patients, subdivided into patients with CVD and without CVD, and fifteen matched healthy control subjects were studied. Laboratory investigations included lipid profile, lupus anticoagulants (LA), anticardiolipin antibodies (aCL). PON1 activity was determined by paraoxon substrate. PON1 genotyping was conducted by PCR amplification, followed by polymorphism-specific restriction enzyme digestion and gel electrophoresis.

Results: Our study revealed that PON1 activity was significantly decreased in SLE patients groups compared to controls and in SLE patients with CVD compared to those without CVD ($p<0.001$). PON1 activity was significantly negatively correlated with total cholesterol, LDL-C and LDL-C/HDL-C ratio, positively correlated with HDL-C but not significantly correlated with triglycerides, disease activity, LA or aCL antibodies. As regard PON1 192 gene polymorphism, there was significant increase in B allele frequency in SLE patients with CVD compared to those without CVD and control groups, while no significant difference was found between SLE patients without CVD and control group. As regard PON1 55 gene polymorphism, there was no significant difference in genotype distribution or allele frequency between the three groups. The Odds ratio of development of CVD in SLE patients who carry PON1 192B allele was 6 (95% CI 1.2-30.7, $p<0.05$). PON1 activity determined by paraoxon substrate was significantly higher in BB and LL, intermediates in AB and LM, and lower in AA and MM genotypes.

Conclusion: The decreased PON1 activity and the increased PON1 192B allele frequencies in SLE patients may have a role in development of cardiovascular complications. Further studies on large number of patients of PON1 gene polymorphisms of are needed to elucidate this relationship.

Key Words:

Systemic lupus erythematosus, cardiovascular disease, paraoxonase enzyme, paraoxonase genotypes.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease characterized by the production of autoantibodies against multiple antigens[1]. Premature atherosclerosis has been recognized as an important cause of morbidity and mortality in SLE[2], and coronary artery disease (CAD) accounts for up to 30% of all deaths in some reported series[3]. Established risk factors for CAD in the general population are insufficient to explain the increased risk in SLE[4]. Specific factors such as steroid treatment, chronic inflammation, and renal disease could account for enhanced atheroma formation[5]. Previous studies have reported that SLE is characterized by atherogenic lipid profile[6,7]. Furthermore, several studies have demonstrated a relationship between low density lipoprotein (LDL) oxidation and aPL in SLE[8,9] and it has been proposed that an immune response of aPL to oxidized LDL may play a role in the pathogenesis of atherosclerosis in SLE.[10]

Paraoxonase 1 (PON1) is an enzyme with antioxidant activity, which circulates in plasma attached to high density lipoproteins (HDL)[11]. Although the natural substrate(s) of this enzyme are unknown, it is capable of the hydrolysis of a variety of xenobiotics, including organophosphates and aryl esters that are common components of insecticides and nerve agents[12]. Paraoxonase has been shown to metabolize lipid-soluble radicals and contribute to the HDL mediated prevention of LDL oxidation[13], suggesting that it plays an important role in the anti-inflammatory effect of HDL. Because oxidized LDL contributes to the development of atherosclerosis, studies have examined whether paraoxonase activity is associated with cardiovascular disease risk. Reduced enzyme activity was shown in patients with cardiovascular disease (CVD).[14,15]

The PON1 gene maps to human chromosome 7q21.3 at 94.6 Mb, which is in close proximity to a linkage peak on 7q21.1 at 77.5 Mb for SLE[16]. Thus, PON1 is both a potential positional and a potential biologic candidate gene for SLE[17]. PON1 gene contains 2 polymorphisms, both of which are due to amino acid substitutions; PON1 Q192R, the Gln (Q) to Arg (R) substitution at amino acid 192, which are also termed the A and B isoforms, respectively, and PON1 L55M, the Leu (L) to Met (M) substitution at amino acid 55[18,19]. The rates of different substrate hydrolysis are quite variable within PON1 genotypes (at least 13-fold) and represent phenotypes

that can add information about PON1 status beyond genotyping alone[20]. It has been shown that the PON1 genetic polymorphisms may be an independent risk factor for coronary artery disease; both the 55L and 192B genotype have been shown to be associated with increased susceptibility to CAD.[14,21]

Our objective was to evaluate PON1 activity and genotypes in SLE patients and study their relationships to cardiovascular complications and some other risk factors of cardiovascular diseases in those patients.

PATIENT AND METHODS

Thirty SLE patients attending outpatients' clinic and inpatients' department of Rheumatology and Rehabilitation, Zagazig University Hospitals and fifteen age, sex and body mass index (BMI) matched healthy control subjects were enrolled in the study. All of the SLE patients met the American College of Rheumatology revised criteria for classification of the disease[22,23]. Lupus disease activity was assessed using SLE disease activity index (SLEDAI) score[24]. All parameters that are known to influence PON1 activity, such as smoking, chronic renal failure, nephrotic syndrome, diabetes, uncontrolled hypertension, and lipid lowering drug intake, were excluded[25]. All patients were on no more than 8 mg maintenance prednisone (or equivalent steroid), as larger doses cause dyslipidaemia both on short and long term bases. Patients were subdivided according to presence of cardiovascular diseases (CVD) into two groups; 18 SLE patients without CVD and 12 SLE patients with CVD (4 with history of myocardial infarction at least 2 months ago, 6 with angina and 2 with cerebral stroke).

Blood samples were obtained after 12 hours fasting. Sera were separated for lipid profile, PON1 activity and anti-cardiolipin antibodies (aCL) determination. Citrated plasma was used for lupus anticoagulants (LA) detection and EDTA blood were used for PON1 genotyping.

Laboratory investigations: Total cholesterol, triglycerides were determined on ADVIA 1650 autoanalyzer (Siemens Medical Solutions Diagnostic, USA). HDL-C was determined using Eli-tech kit[26] (ELI-TECH Diagnostics, France). LDL-C was calculated according to Friedewald's equation.[27]

Lupus anticoagulant was detected by dilute russell's viper venom time (DRVVT) based lupus anticoagulant detection system (LADS) kit (Tulip dianostics).[28]

Anticardiolipin antibodies were quantitated using indirect solid phase enzyme immunoassay (ELISA) for the measurement of IgG and IgM class autoantibodies (orgentec Diagnostika GmbH, Germany). aCL IgG $\geq$10 GPL U/ml and aCL IgM $\geq$7 MPL U/ml were considered positive.

PON1 activity assay was performed using paraoxon (O,O-diethyl-O-p-nitrophenyl phosphate; Sigma-Aldrich, Germany) as a substrate according to the method described by Mackness[29]. The assay mixture included 2.0 mM/L paraoxon and 2.0 mM/L CaCl2 in 0.1 M/L Tris-HCl buffer (pH 8.0). To 350 µL of reagent mixture, 10µL of serum was added and incubated for 17 seconds. The hydrolysis rates of paraoxon were determined by measuring the released p-nitrophenol at 405 nm at 37°C for 3 minutes on Microlab 300 (Vital

Scientific, the Netherlands). Blank was included to correct for the spontaneous hydrolysis of paraoxon. Enzyme activities were expressed in international units (U) per litre of sera. An international unit is the amount of hydrolyzed substrate in µmol per minute. Extinction coefficient of p-nitrophenol at 405 nm is17, 000/M/cm.

PON1 genotyping: Total genomic DNA was purified from buffy coat samples using the E.Z.N.A. ™ Blood DNA Kit (Omega – biotek. Inc). Genomic DNA samples were stored at -20ºC until genotyping analysis. PON1 genotypes were determined by PCR according to previously published protocols[18,19]. For the polymorphism at position 192, sense primer 5′ ATTGTTGCTGTGG 5′GACCTGAG 3′ and antisense primer CACGCTAAACCCAAATACATCT-C 3′, which encompass the 192 polymorphic region of the human PON1 gene, were used. For the polymorphism at position 55, sense primer 5′ GAAGAGTGATGTATAGCCCCAG 3′ and antisense primer ′TTTAATC-CAGAGCTAATGAAAG-CC 3′ were used.

Amplification of DNA was performed by puReTaq Ready- To- Go PCR Beads (Amersham Biosciences). The PCR reaction mixture contained 5 µl of template DNA, 3 µl of each primer (100 uM/L) [Operon] in a tube containing one PCR bead [200 uM of each dNTP in 10 mM Tris - HCL (pH 9.0), 50 mM HCL and 1.5 mM $MgCl_2$ and 2.5 U Taq DNA polymerase].The following temperature scheme was performed for the amplification using thermal cycler (Gene Amp, PCR system 9700). After denaturing the DNA for 5 min at 95°C, the reaction mixture was subjected to 46 cycles of 1 min of denaturation at 94°C, 30 s of annealing at 61°C and 1 min of extension at 72°C for the polymorphism at position 192. The 99 bp PCR product was digested with 8 units of *AlwI* restriction endonuclease (New England Biolabs, Cambridge, MA, U.S.A.) overnight at 37°C. The digested products were separated by electrophoresis on a 3% (w/v) agarose gel and visualized using ethidium bromide. The B genotype (arginine) contains a unique *AlwI* restriction site which results in products of 66 and 33 bp, whereas the A genotype (Glutamine) will not be cut, allowing the PON1 192 genotype to be determined (Figure1).

For the polymorphism at position 55, the PCR reaction and cycling were the same as described above, except that 30 cycles were carried out. The PCR product (170 bp) was digested with *NlaIII* (New England Biolabs) in the presence of bovine serum albumin (overnight at 37°C).The digested products were separated and identified as above. The L genotype (leucine) does not contain an *NlaIII* site, whereas the M genotpye (methionine) does contain an *NlaIII* site, giving rise to products of 126 and 44 bp (Figure 2).

Statistical Analysis: Data were expressed as mean ± SD for quantitative variables, number and percentage for qualitative ones. ANOVA, t test, χ^2, and Pearson correlation were used for analysis of results .Odds ratio (OR) and 95% confidence interval (CI) were performed to predict the effect of PON1 genotypes on development of SLE and CVD. A P-value < 0.05 was considered significant. Analysis was performed with the SPSS statistical package version 10 (SPSS Inc., Chicago, IL).

RESULTS

The demographic and laboratory characteristics of control group and SLE patients are presented in table 1. No significant differences were detected in age, sex, BMI and disease activity in the studied groups. Total cholesterol, LDL-C, LDL-C/HDL-C ratio and triglycerides were significantly increased and HDL-C was significantly decreased in patients groups compared to controls ($p<0.001$). LA and the number of patients with positive LA and aCL IgG were significantly increased in patients with CVD compared to those without CVD ($p<0.001$, <0.05 respectively) while no significant difference was found in aCL IgM in patients groups ($p>0.05$). As regard PON1 activity, it was significantly decreased in patients groups compared to controls and in patients with CVD compared to those without CVD ($p<0.001$).

On studying the correlation between PON1 activity and other studied clinical and laboratory parameters in SLE patients, total cholesterol, LDL-C and LDL-C/HDL-C ratio were significantly negatively correlated while HDL-C was significantly positively correlated with PON1 activity ($p<0.05$, 0.01,<0.05,<0.01 respectively). Disease activity, LA, and aCL antibodies were not significantly correlated with PON1 activity ($p>0.05$) Table 2.

The distribution of PON1 55 and 192 genotypes and allele frequency in controls and patients groups are reported in table 3. PON1 55 genotype, allele frequency and the percentage of patients carrying L allele were not significantly different among the studied groups ($p>0.05$). As regard PON1 192 genotype distribution, there was significant increase in AB, BB and decrease in AA genotypes in SLE patients with CVD compared to those without CVD and control groups ($p<0.05$). Patients with CVD had higher frequency of PON1 192B allele compared to patients without CVD and control groups ($p<0.01$). The percentage of carriers of PON1 192B allele was significantly increased in patients with CVD compared to patients without CVD and controls ($p<0.05$). While, no significant difference was detected between controls and patients without CVD in PON1 192 genotype distribution or allele frequency.

There was no significant association between PON1 gene polymorphism and the risk of SLE. The odds ratio of development of SLE in PON1 55L allele carriers was 1.4 (95% CI 0.2-9.3, $P>0.05$). Although there was increased risk of development of SLE in PON1 192B allele carriers, this increase did not reach the statistical significance (OR 4, 95% CI 0.9-17.1, $P>0.05$). There was significant increase in the risk of development of CVD among SLE patients who carry PON1 192B allele (OR 6, 95%CI 1.2-30.7, $P<0.05$).While no significant increase in the risk of CVD in SLE patients who carry PON1 55L allele (OR 0.3, 95% CI 0.02-3.7, $P>0.05$).

The relationship between PON1 activity and different PON1 genotypes is presented in table 4. As regard PON1 55 genotypes, PON1 activity using paraoxon as substrate was significantly

higher in LL, intermediate in LM, and lower in MM genotypes in control and patients groups ($p<0.05$). As regard PON1 192 genotype, PON1 activity was significantly higher in AB than in AA genotype in control group ($p<0.05$), and higher in BB, intermediate in AB, and lower in AA genotype in patients group ($p<0.01$).

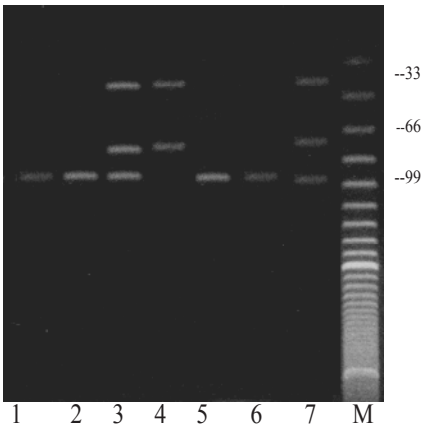

Fig. 1: The electrophoresis of the PCR products of the PON1 genotype 192. Lanes 1,2,5,6 represent AA genotype, lanes 3, 7 represent AB genotype, lane 4 represents BB genotype, lane 8(M) represents 20 bp DNA marker.

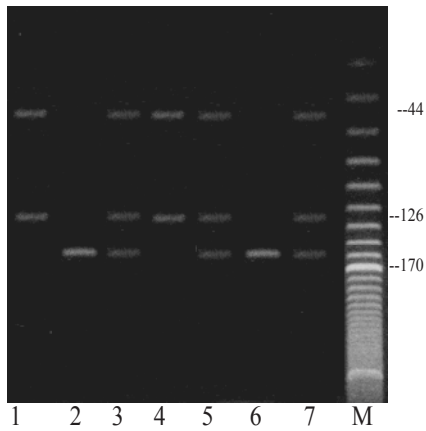

Fig. 2: The electrophoresis of the PCR products of the PON1 genotype 55. Lanes 2, 6 represent LL genotype; lanes 1, 4 represent MM genotype, lanes 3, 5, 7 represent LM genotype, and lane 8(M) represents 20 bp DNA marker.

Table 1: Demographic and laboratory findings in control and systemic lupus erythematosus groups.

Variables	Control (n=15)	SLE without CVD (n=18)	SLE with CVD (n=12)	Test of significance	P
Age (years)	43.2±4.8	40.5±8.7	39.9±5	F=1.1	> 0.05
¶Males	5(33)	5(28)	3(25)	χ^2=0.2	> 0.05
BMI (kg/m^2)	22.5±2.9	23.4±3.8	24.7±2.5	F=1.59	> 0.05
SLEDAI	NA	6.2±2.1	7.1±1.8	t=-1.2	> 0.05
Total Cholesterol (mg/dl)	171.6±16.3	192.9±22[a]	209.2±28.7[a]	F=9.6	< 0.001
HDL-C (mg/dl)	52.6±4.3	45.7±9.7[b]	38.7±5.2[b]	F=12.6	< 0.001
LDL-C (mg/dl)	93.9±15.6	107.6±25.7[b]	133.5±26.5[b]	F=9.9	< 0.001
LDL-C/HDL-C ratio	1.79±0.34	2.56±1.1[b]	3.5±0.8[b]	F=13.4	< 0.001
Triglycerides (mg/dl)	125.5±11.5	194.5±24[a]	196.8±28.8[a]	F=49.5	< 0.001
LA (sec)	34.2±5.7	43.3±7.5[b]	50.2±11.7[b]	F=6.3	< 0.001
aCL IgG (GPL U/ml)	5.29±1.6	10.4±8.2[a]	14.3±13.1[a]	F=3.8	< 0.05
¶No of positive cases	NA	5(28)	5(42)	χ^2=7.2	< 0.05
aCL IgM (MPL U/ml)	3.9±1.4	6.3±6.8[a]	8.4±6.3[a]	F=2.3	> 0.05
¶No of positive cases	NA	3(17)	4(33)	χ^2=5.6	> 0.05
PON1 activity (U/l)	217.3±55.2	118.6±64.5[b]	71.8±36.1[b]	F=9.5	< 0.001

Values are represented means± SD, (n) the number of observations.

¶Results are expressed as frequency (%).

NA: not applicable.

a : Significant difference with control group b : Significant difference with all groups.

Table 2: Correlation between paraoxonase 1 activity, and other clinical and laboratory parameters in SLE patients.

Variables	r	P
SLEDAI	-0.23	>0.05
Total Cholesterol (mg/dl)	-0.46	<0.05*
HDL-C (mg/dl)	0.52	<0.01**
LDL-C (mg/dl)	-0.53	<0.01**
LDL-C/HDL-C ratio	-0.43	<0.05*
Triglycerides (mg/dl)	0.11	> 0.05
LA (sec)	-0.25	>0.05
ACL IgG (GPL U/l)	-0.25	>0.05
ACL IgM (MPL U/l)	-0.19	>0.05

* Significant ** Highly significant

Table 3: Distribution of PON 55 and 192 genotypes and allele frequencies in the studied groups.

Variables	Control (n=15)	SLE without CVD (n=18)	SLE with CVD (n=12)	χ^2	P
PON 55 Genotypes No (%)					
LL	8 (53.3)	10(55.5)	7(58.3)	1.35	> 0.05
LM	5 (33.3)	7(38.9)	3(25)		
MM	2(13.4)	1(5.6)	2(16.7)		
Allele frequencies (%)					
L allele	70	75	71	0.24	> 0.05
M allele	30	25	29		
L alleles Carriers No (%)	13(86.7)	17(94.4)	10(83.3)	1.01	> 0.05
Non L alleles carriers No (%)	2(13.3)	1(5.6)	2(16.7)		
PON 192 Genotypes No (%)					
AA	12 (80)	12(66.7)	3(25)*	9.63	< 0.05
AB	3 (20)	5(27.8)	7(58.3)*		
BB	0(0)	1(5.6)	2(16.7)*		
Allele frequencies (%)					
A allele	90	81	54*	10.14	< 0.01
B allele	10	19	46*		
B alleles Carriers No (%)	3(20)	6(33.3)	9(75)*	8.95	< 0.05
Non B alleles carriers No (%)	12(80)	12(66.7)	3(25)*		

* Significant difference with other groups.

Table 4: Relationships between PON1 genotypes and PON1 activity (U/L) in the studied groups.

Genotypes	Control		SLE patients	
	n	PON1 levels (U/l)	n	PON1 levels (U/l)
PON1 55 Genotype				
LL	8	210.9±54	17	120.1±59.2
LM	5	171.5±25.2	10	68.9±55.1
MM	2	95±7.1†	3	29±10.4†
Test of significance		F = 5.8		F = 4.93
P		< 0.05		< 0.05
PON1 192 Genotype				
AA	11	165.7±43.6	16	71.7±42.8
AB	4	247.7±64.3	11	106.6±63.2
BB	0	--	3	199±46.1§
Test of significance		t= -2.68		F = 7.9
P		< 0.05		< 0.01

Values are represented as means± SD, (n) the number of observations.

† Significant difference with LL genotype.

§ Significant difference with other PON1 192 genotypes.

DISCUSSION

Previous studies have shown that low serum PON1 activity is associated with CAD[14,15,30,31], and that PON1- knock-out mice are susceptible to atherosclerosis[32]. Since SLE patients have up to a 50% increased risk of developing CAD[7], it is reasonable to postulate that PON1 activity may also be associated with SLE risk.

Our study revealed significantly lower PON1 activity in SLE patients compared to controls. Similar results were reported by other studies[6,17,33,34]. Reduced PON1 activity can be an independent risk for atherosclerosis in SLE as there were no correlations between disease activity, LA, aCL antibodies and low PON1 in SLE patients. Similar results were observed by Delgado Alves et al.[6], Kiss et al.[33] and Tripi et al.[17]. However, a decrease in PON1 activity in patients with different autoimmune conditions and with aCL antibodies has been described[35]. The mechanism of decreased PON1 activity in SLE and whether it is a cause or a consequence of the disease is unclear. Since inflammation and proinflammatory cytokines are associated with low PON1 activity[36-38], reduced PON1 activity could be due to inflammation in SLE patients. Negative correlations between PON1 and C-reactive protein and fibrinogen and SLE patients have been described[17]. Inhibition of PON1 by increased oxidative stress which has been implicated in the pathogenesis of SLE has been also considered.[39]

The last two decades have seen accumulation of evidence suggesting the role of PON1 in atherogenesis and

coronary heart disease[14,21]. Our current findings were that low serum PON1 activity in SLE patients with CVD compared to those without CVD and controls. This result confirms the previous observation of low PON1 activity in SLE patients with atherothrombotic events compared to those without such complication.[33]

A variety of physiologic roles for PON1 have been proposed, primarily related to its anti-atherosclerotic activity. It is believed that PON1 protects against the action of oxidized LDL by protecting LDL from oxidation, participating in the destruction of oxidized phospholipids, and decreasing macrophage uptake of oxidized LDL. Secondary effects of this activity include reduction of monocyte adhesion to endothelial cells and macrophage chemotaxis that would normally occur due to oxidized phospholipids.[10,21]

In this study, we found no significant difference in distribution of PON1 55 genotypes and allele frequencies between patients and control groups. No significant differences in PON1 192 genotypes and allele frequencies were found between controls and patients without CVD. The results of our study indicate that the PON1 gene polymorphism is not related to genetic susceptibility to SLE. In contrast to our results tripi et al.[17] reported significant association between PON1 55L but not PON1 192 genotypes and SLE risk.

Our data revealed significant increase in PON1 192B allele in SLE who had cardiovascular complication compared to those without cardiovascular complication and control groups. Odds ratio of development of CVD in SLE patients who carry PON1 192B allele was 6.The associations between PON1 192B allele and CVD have been previously reported[13,14,21]. PON1, being exclusively associated with HDL and apolipoprotein A-1, could synergistically contribute towards cardiovascular disease risk in SLE.[34]

The PON1 polymorphism has substantial influence on PON1 activity determined with paraoxon, which has been replicated in almost all published studies. PON1 192 and 55 polymorphisms explained 46% and 16% of the variations in PON1 activity toward paraoxon[40]. Our data showed that levels of PON1 activity tested with paroxon were highest in subjects with BB and LL genotypes, intermediates in those with AB and LM genotypes and lowest in those with AA and MM genotypes. Our data is consistent with the previous reports.[17, 40]

Since the natural substrate of PON1 is still unknown and the PON1 isoforms can have opposite effects with different substrates, it is possible that these variants behave differently with the physiologic substrate[17]. HDL isolated from BB homozygotes, although most active in the hydrolysis of paraoxon, is much less effective at protecting LDL against lipid peroxidation than HDL from either AA homozygotes or AB heterozygotes[13,41]. Mackness et al.[42] and Aviram et al.[43] demonstrated that purified PON1 A (low activity phenotype) was more effective than PON1 B in protection against LDL peroxidation.

Our results revealed that PON1 activity was significantly negatively correlated with total cholesterol, LDL-C, LDL-C/HDL-C ratio and positively correlated

with HDL-C in SLE patients. In contrast to these results Kiss et al.[33] reported no correlation between PON1 activity and lipid parameters in SLE patients. Lipid and lipoprotein levels have been associated with PON1 activity or genotypes in some[44,45] but not all[40,46] studies. PON1 genotype was significantly associated with variation in the plasma concentration of HDL-C, LDL-C, and triglycerides[47]. Homozygotes for the low-activity allele of PON1 had a less atherogenic lipoprotein profile than did heterozygotes and homozygotes for the high-activity allele.[48]

There are nearly 200 single nucleotide polymorphisms (SNPs) in PON1[17], and this study focused on two of that genetic variation. Other SNPs in PON1 may play a role, or perhaps less common or yet undiscovered DNA sequence variation in PON1 may affect SLE risk. Gene – gene and gene–environment interactions, not investigated in this study, are thought to play an important role in the development of complex diseases such as SLE and CVD, and examination of these relationships may reveal additional information.

CONCLUSION

PON1 activity was significantly decreased in SLE patients and more significantly decreased in SLE patient with cardiovascular complications. The decrease in PON1 activity may have significant effect on development of cardiovascular complications in those patients as it is correlated with atherogenic lipid profile. PON1 192B allele is associated with SLE related cardiovascular complications but not with SLE. Further studies with large number of patients are needed to identify the role of PON1 gene polymorphisms in the development of CVD in those patients.

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