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Paraoxonase Genotype, LDL-oxidation and Carotid Atherosclerosis in Male Life-long Smokers

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Paraoxonase (PON-1) is a high-density lipoprotein (HDL) associated enzyme that hydrolyzes lipid peroxides *in vitro*, which may therefore protect against the onset of atherosclerosis. Heavy smokers are more exposed to oxidative stress and hence at high-risk for oxidative modification of LDL.

Our hypothesis is that the anti-oxidative properties of PON-1 inhibit LDL oxidation, especially in populations exposed to high oxidative stress.

We have studied the effects of PON-1 genotype and smoking to variation in oxidative status parameters and intima-media thickness (IMT), a surrogate marker of atherosclerosis. The contribution of two common polymorphisms in the PON-1 gene (Q192R and L55M) to LDL oxidizability, autoantibodies directed against oxLDL and IMT were studied in 207 male life-long smokers. Smokers were classified into average, heavy and excessive smokers based on pack years of cigarettes smoked.

PON-1 genotype was not associated with autoantibodies to oxLDL, LDL oxidizability or IMT. Smoking was associated with IMT in subgroups with the high levels of LDL, but not in the population at large.

The lack of association of PON-1 genotype with oxidative status parameters and IMT suggests that PON-1 is not a major inhibitor of LDL oxidation in a population of life-long smokers.

Keywords: Paraoxonase genotype; Smoking; Atherosclerosis; LDL oxidizability; oxLDL Autoantibodies; Intima-media thickness

INTRODUCTION

Serum paraoxonase (PON-1) is a high-density lipoprotein (HDL) associated enzyme capable of hydrolyzing organophosphates.[1] *In vitro*, PON-1 protects low-density lipoprotein (LDL) from oxidative modification by hydrolyzing lipid peroxides. This property argues for a potential protective role of PON-1 against atherosclerosis.[2] The hypothesis was supported by observations, that PON-1 deficient mice are more susceptible to develop atherosclerosis than wild-type mice when fed a high-fat/high-cholesterol diet.[3]

The coding sequence of the PON-1 gene contains two polymorphic sites: a leucine (L) to methionine (M) transition at position 55 (L55M) and a glutamine (Q) to arginine (R) transition at position 192 (Q192R). The L55M polymorphism affects the enzyme concentration, partly due to linkage with polymorphism in the PON-1 promoter region[4] and possibly via altered ability of PON-1 to form a complex with HDL: the L55M polymorphism is located in the N-terminal side of PON-1 which may play a role in the binding of PON-1 with HDL.[5] The Q192R polymorphism determines the catalytic efficiency towards a number of organophosphate substrates, including paraoxon. The 192R variant hydrolyses paraoxon more efficiently than the 192Q variant, the *in vivo* substrate of PON-1, however, is not known. Furthermore, the Q192R polymorphism is not related to PON-1 levels.[6,7]

Results of studies on the contribution of the L55M and Q192R polymorphism to the risk of cardiovascular disease (CVD) are inconsistent.[8–18]

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Recent findings from a prospective study have demonstrated that the low PON-1 activity is a predictor of CVD.[19]

Previously, our group found a relation between the PON-1 genotype combination LLQQ and increased Intima-media thickness (IMT) in high risk subjects with familial hypercholesterolemia (FH).[20] IMT is an assessment of the combined thickness of the intima-media layer of the common carotid artery (CCA) as measured by B-mode ultrasonography. IMT is a powerful predictor for future cardio vascular events and is often used as a surrogate marker for clinical outcomes.[21]

Smoking is associated with both high oxidative stress and increased risk of CVD.[22] Furthermore, cigarette smoke promotes the oxidation of LDL in the presence of peroxidases.[23] Therefore, the anti-oxidative effects of the PON-1 polymorphisms are more likely to be expressed in a population consisting of heavy smokers than in the general population.

Our hypothesis is that the PON-1 genotype significantly determines the harmful effect of smoking to the oxidative modification of LDL and thus atherosclerosis. For this we determined, the LDL oxidizability, the levels of oxLDL autoantibodies and the IMT among the genetic variants of the L55M and Q192R polymorphisms of the PON-1 gene, in a high risk population of life-long smokers. Additionally, the effects of different smoking gradations on LDL oxidizability, levels of oxLDL autoantibodies and the IMT were investigated.

METHODS

Subjects

The study population consisted of 218 male chronic smokers who had participated in a clinical trial on atherosclerosis progression.[24] Of the 218 participants, 207 participants with complete PON-1 genotype and carotid IMT information were included. Height, weight, blood pressure and the IMT of the common carotid artery (CCA IMT) were measured at baseline (Table I). DNA, plasma and serum samples were stored at -80°C until analysis. All participants gave written informed consent to the use of their blood samples for scientific research. The study was approved by the institutional review boards of University Medical Centre, Nijmegen and Wageningen University.

Ultrasound Measurement of the Carotid IMT

Ultrasound scanning of the carotid arteries was performed with a Biosound Phase-two real time scanner (BiosoundEsaote, Indianapolis, IN, USA) equipped with a 10 MHz transducer, as described in detail elsewhere.[25] IMT measurements were done for both anterior and posterior walls of the distal 1.0 cm straight part of both common carotid arteries. Images were analyzed with a semiautomatic software program (Eurequa; TSA company, Meudon, France). The CCA IMT is expressed as the mean of the anterior and posterior walls of the left and right CCA.

TABLE I General characteristics of 207 male smokers

Characteristics	Values
Age (years)	60 ± 6
Pack years of cigarette smoking	38 ± 21
CCA IMT (mm)	0.96 ± 0.15
BMI (kg/m^2)	26.0 ± 3.3
Systolic blood pressure (mm Hg)	142 ± 17
Diastolic blood pressure (mm Hg)	84 ± 8
Total cholesterol (mmol/l)	6.0 ± 1.0
Triglycerides (mmol/l)	1.7 ± 1.0
HDL cholesterol (mmol/l)	1.2 ± 0.4
LDL cholesterol (mmol/l)	4.1 ± 1.0

Values are means ± standard deviation. CCA IMT, Common carotid artery intima media thickness; BMI, Body mass index; HDL, High-density lipoprotein; LDL, Low-density lipoprotein.

PON-1 Genotyping

The L55M and Q192R mutations were determined by PCR RFLP using primers and restriction enzymes as described by Humbert *et al.*[6] Restriction fragments were separated on a 2% agarose gel and visualized with ethidium bromide. The L-55 allele corresponded to non-digested 170-bp fragments, the M-55 allele to 44-bp and 126-bp fragments (Fig. 1A), the Q-192 allele to non-digested 99-bp fragments, and the R-192 allele to 33-bp and 66-bp fragments (Fig. 1B). Results by two independently working technicians were indistinguishable except for 5 observations, which were reanalyzed until consensus.

Markers of Oxidative Status: LDL Oxidizability

Susceptibility of LDL to *in vitro* oxidation was determined in 165 suitable samples by monitoring formation of conjugated dienes at 234 nm on a PE-lambda 12 spectrophotometer (Perkin Elmer Ltd., Beaconsfield, UK) as described by Esterbauer *et al.*[26] and as modified by Princen *et al.*[27] The time profile of the absorption pattern shows three distinct phases: the lag time, the propagation phase and the decomposition phase. The lag time is defined as the time interval between the intercept of the linear least-square slope of the absorbance curve with the initial absorbance axis and was taken as a measure of LDL resistance to oxidation. The rate of diene formation is calculated as the slope of

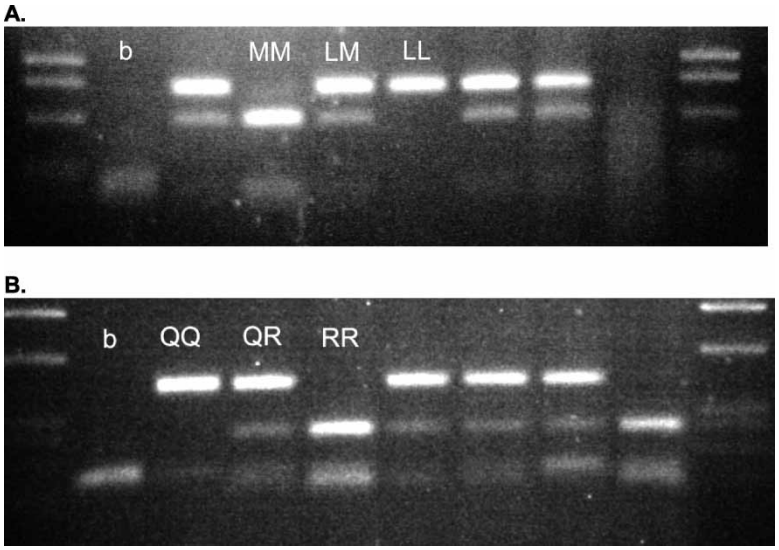

FIGURE 1 Representative figures of RFLP of PON-1 L55M (A) and Q192R (B) polymorphisms. The L-55 allele corresponded to non-digested 170-bp fragments and the M-55 allele to 44- and 126-bp fragments. The Q-192 allele corresponds to non-digested 99-bp fragments and the R-192 allele to 33- and 66-bp fragments. Negative control is indicated by b.

the propagation phase and reflects the autocatalytic chain reaction of the lipid peroxidation process. Finally, the net diene concentration is the difference between the absorbance at time zero and the maximum absorbance and reflects the extent of oxidative modification of the isolated LDL.

Markers of Oxidative Status: Antibodies to Oxidized LDL

IgG and IgM antibodies to oxidized LDL (oxLDL Ab) were assayed by ELISA as described in detail previously.[28] In short, antibodies were captured by using native and copper-oxLDL as antigens and detected with peroxidase-conjugated antibody from goat specific for human IgG or IgM (Sigma–Aldrich). Binding to oxLDL over binding to native LDL was taken as a measure of antibodies to oxLDL.

Lipids and Lipoproteins

Cholesterol and triglyceride concentrations in serum were determined by enzymatic methods (Boehringer-Mannheim, Mannheim, Germany) on a Hitachi 747 analyzer (Hitachi, Tokyo, Japan). HDL cholesterol was determined after precipitation of LDL cholesterol, very low-density lipoprotein and chylomicrons using phosphotungstate/Mg^{2+}. LDL cholesterol in serum was calculated using the Friedewald formula. As reported previously, this includes lipoprotein remnant-associated cholesterol.[29]

Data Analysis and Statistics

Smoking status is expressed in pack years (the number of cigarettes smoked per day multiplied by the years of active smoking divided by twenty) and classified in tertiles (average, heavy and excessive) with cut of points on 23 and 47 pack years.

The age adjusted relation between smoking, L55M genotype and Q192R genotype with CCA IMT, LDL oxidizability and oxLDL antibodies was tested with linear regression analysis. Pack years, L55M genotype and Q192R genotype served as independent variable and CCA IMT, lag time, net diene concentration, rate of diene formation, IgG antibodies and IgM antibodies as dependent variable.

CCA IMT was adjusted for age by the algorithm: β(mean age population − age smoker) + CCA IMT. Where β is the coefficient derived from the linear regression model with age as independent variable and CCA IMT as dependant variable.

The relation between age adjusted CCA IMT and pack years of cigarette smoking, lipoprotein levels, LDL oxidizability and oxLDL antibodies was tested with the Pearson correlation coefficient.

Interaction between smoking status and PON-1 genotype in relation to CCA IMT and the parameters for the oxidative status (lag time, net diene production, rate of diene formation and antibodies to oxLDL) was studied by analyzing subgroups.

For interaction, tertiles were defined for lipid ratio (total cholesterol/HDL cholesterol) and for plasma LDL cholesterol. Lipid ratio levels lower than 4.7, between 4.7 and 6.0, and higher than 6.0 were assigned to the first, second and third tertile, respectively. Plasma LDL cholesterol levels lower than 3.7 mmol/l, between 3.7 and 4.5 mmol/l and higher than 4.5 mmol/l, were assigned to the first, second and third tertile, respectively.

The significance between subgroups was studied using the Independent-Samples *t*-test. All analyses were performed with SPSS version 10.0.

RESULTS

The population consisted of 207 male smokers with a mean age of 60 (Table I). The subjects had smoked for a mean of 38 pack years. BMI and systolic blood pressure were increased, while mean total cholesterol, HDL cholesterol and triglyceride levels were within limits for normal.

The PON-1 L55M genotypes LL, LM and MM occurred in 77 (37.2%), 104 (50.2%) and 26 (12.6%) subjects, respectively. The PON-1 Q192R genotypes QQ, QR and RR were present in 101 (48.8%), 92 (44.4%) and 14 (6.8%) subjects, respectively. The observed genotype distributions did not significantly differ from the calculated expected distributions, assuming a Hardy–Weinberg equilibrium. The Q192R polymorphism was in linkage disequilibrium with the L55M polymorphism: 98% of the carriers of the 192R allele also have an L allele at position 55.

Table II presents the effects of smoking status and PON-1 genotypes on the oxidation parameters (lag time, net diene production, rate of diene formation and antibodies to oxLDL) and CCA IMT. Smoking status was associated with a statistically significant difference in lag time ($p = 0.04$, Fig. 2) and rate of diene formation ($p = 0.03$, Fig. 3). No significant difference was observed between the smoking groups and the levels of IgG and IgM antibodies to oxLDL. LDL oxidation parameters (lag time, net diene production and rate of diene formation) did not correlate with the levels of IgG and IgM antibodies to oxLDL (data not shown). Age adjusted CCA IMT was not associated with smoking status. Individual PON-1 polymorphisms were not associated with the CCA IMT or with oxidative status. However, a nearly significant trend ($p = 0.06$, Table II) for the IgG antibody titers in the L55M genotype was observed in our data.

In Table III, the correlation between age adjusted CCA IMT and pack years of cigarette smoking, lipoprotein levels, LDL oxidizability and oxLDL autoantibodies is presented. There was no correlation between pack years of cigarette smoking and CCA IMT. Plasma HDL cholesterol was associated with decreased CCA IMT values ($r = -0.200$, $p = 0.004$, Fig. 4A), while plasma LDL cholesterol was associated with increased CCA IMT values ($r = 0.208$, $p = 0.003$, Fig. 4B). None of the oxidative status variables were associated with CCA IMT.

TABLE II Mean characteristics for CCA IMT, oxidation parameters and oxLDL autoantibodies by smoking status and PON-1 L55M and Q192R genotype

	Smoking status*						*p*-Value**
	Average smoking		Heavy smoking		Excessive smoking		
CCA IMT (mm)	0.93 ± 0.15	69	0.96 ± 0.16	69	0.98 ± 0.16	69	0.41
Lag time (min)	87 ± 11	58	88 ± 9	56	92 ± 9	51	0.04
Net diene concentration (nmol/mg protein)	627 ± 62	58	622 ± 55	56	602 ± 63	51	0.08
Rate of diene formation (nmol/mg protein/min)	15.2 ± 2.4	58	14.8 ± 1.9	56	14.0 ± 2.3	51	0.03
oxLDL IgG antibodies (OD450)	0.37 ± 0.17	68	0.38 ± 0.20	67	0.37 ± 0.19	69	0.84
oxLDL IgM antibodies (OD450)	0.48 ± 0.29	65	0.49 ± 0.33	64	0.54 ± 0.33	67	0.12
	PON-1 L55M genotype						
	LL		LM		MM		
CCA IMT (mm)	0.96 ± 0.15	77	0.95 ± 0.16	104	0.98 ± 0.16	26	0.93
Lag time (min)	90 ± 10	60	89 ± 11	83	86 ± 8	22	0.16
Net diene concentration (nmol/mg protein)	621 ± 51	60	613 ± 69	83	626 ± 53	22	0.97
Rate of diene formation (nmol/mg protein/min)	14.8 ± 1.9	60	14.6 ± 2.6	83	15.2 ± 1.9	22	0.82
oxLDL IgG antibodies (OD450)	0.34 ± 0.15	76	0.39 ± 0.21	102	0.41 ± 0.18	26	0.06
oxLDL IgM antibodies (OD450)	0.52 ± 0.30	70	0.47 ± 0.32	101	0.59 ± 0.34	25	0.62
	PON-1 Q192R genotype						
	QQ		QR		RR		
CCA IMT (mm)	0.96 ± 0.15	101	0.97 ± 0.16	92	0.91 ± 0.13	14	0.94
Lag time (min)	88 ± 10	80	89 ± 11	74	87 ± 8	11	0.76
Net diene concentration (nmol/mg protein)	616 ± 66	80	617 ± 58	74	628 ± 39	11	0.50
Rate of diene formation (nmol/mg protein/min)	14.8 ± 2.4	80	14.5 ± 2.2	74	15.7 ± 1.4	11	0.74
oxLDL IgG antibodies (OD450)	0.37 ± 0.16	100	0.37 ± 0.21	90	0.38 ± 0.17	14	0.86
oxLDL IgM antibodies (OD450)	0.52 ± 0.32	96	0.49 ± 0.32	86	0.53 ± 0.25	14	0.65

Note: Values represent mean ± standard deviation followed by the number of observations. *Smoking status is defined in "Data analysis and statistics" section in the methods. **p-Values are based on linear regression analysis and are adjusted for age. CCA IMT, Common carotid artery intima media thickness; oxLDL, Oxidized low-density lipoprotein.

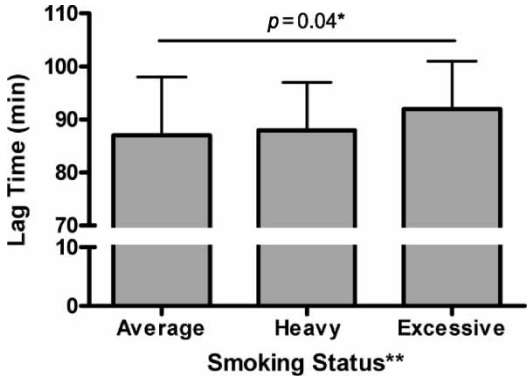

FIGURE 2 Resistance to *in vitro* oxidation (represented by lag time) of LDL of the different smoking status groups. Data presented as means ± standard deviation. *p for trend is based on linear regression and is adjusted for age. **For classification of smoking status see "Analysis and statistics" section.

We observed a trend for the interaction between smoking and LDL levels in relation to age adjusted CCA IMT (Fig. 5). A similar trend was observed for the interaction between smoking and ratio HDL cholesterol/total cholesterol (data not shown). The interaction among smoking status, PON-1 genotype, plasma HDL cholesterol and LDL cholesterol levels in relation to lag time, net diene production, rate of diene formation, antibodies to oxLDL and CCA IMT was not significant (data not shown).

DISCUSSION

We have investigated the effects of PON-1 genotypes, smoking and lipid/lipoprotein profile on parameters of oxidative status and variation of CCA IMT, a surrogate marker of atherosclerosis. Smoking was associated with LDL oxidizability but not with atherosclerosis. There was no relationship between PON-1 genotype and markers of oxidative status or

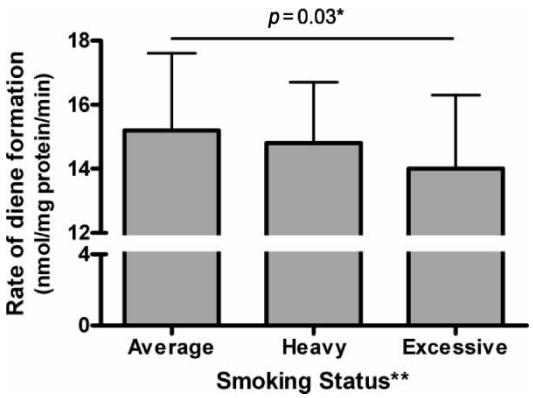

FIGURE 3 Rate of diene formation of the different smoking status groups. Data presented as means ± standard deviation. *p for trend is based on linear regression and is adjusted for age. **For classification of smoking status see "Analysis and statistics" section.

TABLE III Correlation between age adjusted CCA IMT, smoking, lipoproteins, LDL oxidizability and oxLDL autoantibodies

	CCA IMT (mm)*		
	R	*p*-Value	*n*
Pack years of cigarette smoking	0.057	0.413	207
HDL cholesterol (mmol/l)	−0.200	0.004	207
LDL cholesterol (mmol/l)	0.208	0.003	202
Lag time (min)	−0.087	0.266	165
Net diene concentration (nmol/mg protein)	−0.010	0.903	165
Rate of diene formation (nmol/mg protein/min)	0.045	0.569	165
oxLDL IgG antibodies (OD450)	−0.027	0.706	204
oxLDL IgM antibodies (OD450)	−0.061	0.394	196

*Values represent the Pearson correlation coefficient (*R*) followed by the *p*-value (*p*) and the number of observations (*n*). CCA IMT, Common carotid artery intima media thickness; HDL, High-density lipoprotein; LDL, Low-density lipoprotein; oxLDL, Oxidized low-density lipoprotein.

CCA IMT. Furthermore, no relationship was observed between LDL oxidizability and antibodies to oxLDL and CCA IMT.

Smoking is a major pro-oxidative stimulus.[22] Studies on the effects of smoking and LDL oxidation, however, presented controversial results.[30–32] Our findings that smoking was associated with reduced rates of LDL oxidation and prolonged lag times, indicate that profound smoking leads to LDL which is more resistant to oxidation. This apparent anti oxidative property of smoking may be caused by the antioxidative potential of cigarette smoke, as suggested by a previous report,[33] and/or may be related to a continuous oxidative pressure, which results in circulating LDL particles that are less prone to oxidation *in vitro*. The exact biological meanings of these small differences in LDL oxidizability, remain unclear. However, in this population of life-long smokers lag time and the oxidation rate of LDL, do not correlate with CCA IMT and thus may play no role in the development atherosclerosis.

PON-1 protects LDL against oxidative modifications, *in vitro*.[2] The *in vivo* action of PON-1 in healthy non-smoking subjects is reflected by a decreased *ex vivo* oxidizability of LDL.[34] Remarkably, however, PON-1 has no influence on the oxidizability of LDL in a population of smokers (present study) or diabetics.[35,36] The absence of a relation between PON-1 and the oxidizability of LDL suggests that PON-1 does not play a major role in oxidizability of LDL *in vivo*. Alternatively, the absence of such a relationship may be due to reduced PON-1 levels and activity in these populations,[37,38] and/or to a masking effect of smoking. In line with this, Sen-Banerjee *et al.*[39] observed an association of PON-1 Q192R genotype with an increased risk of myocardial infarction in non-smokers but not in smokers. Unfortunately in the present study no serum was collected for PON-1

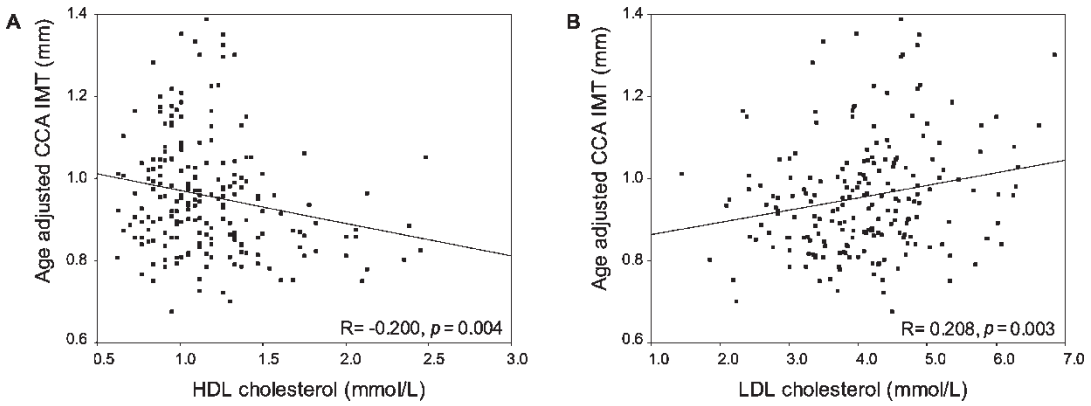

FIGURE 4 Pearson correlations between age-adjusted CCA IMT and HDL cholesterol (panel A) and LDL cholesterol (panel B) of 207 male life-long smokers. CCA IMT, common carotid artery intima-media thickness; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

activity or concentration measurements. More research is therefore needed to elucidate the importance of PON-1 on the oxidizability of LDL and its relevance *in vivo*.

Autoantibodies to oxLDL have been proposed as marker of pro-oxidative state.[40–44] The absence of a relationship between smoking and oxLDL autoantibody titers in our study suggests that autoantibodies to oxLDL do not reflect smoking induced oxidative stress. Furthermore, it remains to be established whether the contribution of these autoantibodies to oxLDL can be used to predict atherosclerosis.[45–47]

To our knowledge, this is the second study examining the effects of PON-1 genotypes on autoantibodies to oxLDL. Recently, Malin *et al.* studied 49 healthy men and observed no differences in oxLDL autoantibody levels between the low- and high-active genotypes of PON-1.[48] We observed a borderline significant trend between IgG autoantibody titers and the L55M polymorphism, the 55MM variant having the highest levels. Since PON-1 55MM homozygotes are more effective than 55LL homozygotes in protecting LDL from oxidation *in vitro*,[49] our finding is in favor of an inverse correlation of *in vivo* LDL oxidation with autoantibodies to oxLDL, as recently shown by Shoji *et al.*[41] Further studies on PON-1 genotypes and antibodies to oxLDL should include measurement of oxLDL to give more insight in the usefulness of autoantibodies as marker for oxidation in relation to PON-1.

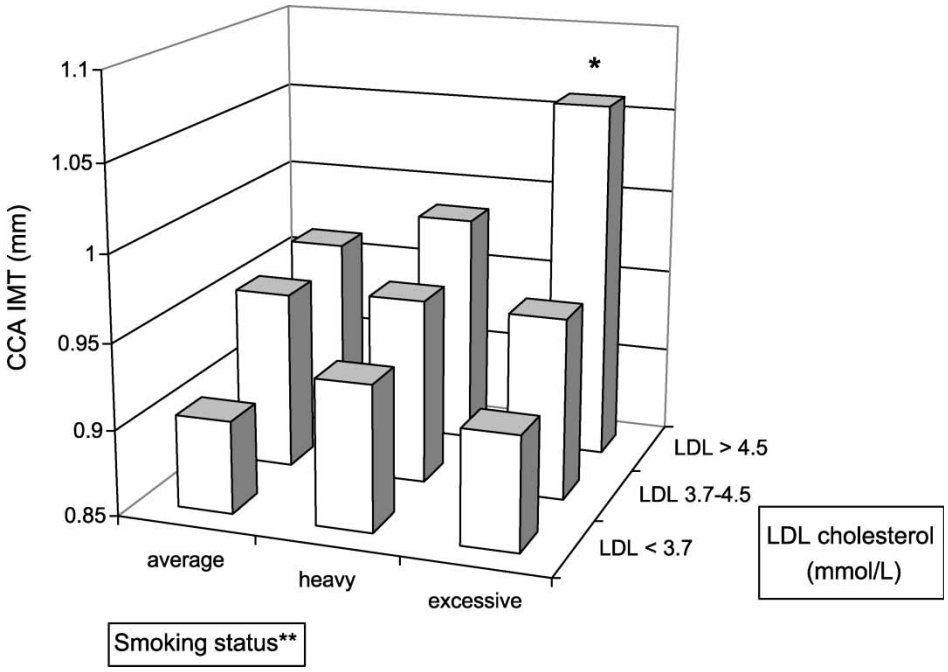

FIGURE 5 The relationship between the smoking-LDL levels interaction and age-adjusted CCA IMT. *p value of 0.002 for excessive smokers with LDL levels higher than 4.5 mmol/l when compared to the remaining smoking statuses and LDL levels combined. **For classification of smoking status see "Data analysis and statistics" section. CCA IMT, common carotid artery intima-media thickness; LDL, low-density lipoprotein.

Large population studies have shown that smoking is a major risk factor for increased CCA IMT.[50,51] In our population this relationship was present in subgroups with high levels of LDL cholesterol, but not in the population at large, suggesting that the effect of smoking on CCA IMT is strongest in high-risk groups for CVD.

The contribution of the L55M and Q192R polymorphism to the risk of CVD has frequently been investigated. If anything, the PON-1 192RR and the 55LL genotype predicted an increased risk for CVD.[8–12] In contrast to smoking, there was no clear relation between PON-1 genotype and CCA IMT, indicating that PON-1 genotype is not a strong risk factor for atherosclerosis in smokers. PON-1 phenotype was not investigated in this population since no serum was available for PON-1 activity and concentration measurements.

In conclusion, smoking was associated with IMT in subgroups with the high levels of LDL, but not in the population at large. PON-1 genotype does not contribute to the susceptibility of LDL oxidation, in this population of life-long smokers. There is no clear relation between PON-1 genotype and autoantibodies to oxLDL and PON-1 genotype has no effect on CCA IMT. These results suggest that PON-1 does not play an important role in atherogenesis in a population of life-long smokers.

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