Influence of age and sex on levels of anti-oxidized LDL antibodies and anti-LDL immune complexes in the general population

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Abstract **Most studies of antibodies to oxidized LDL have been undertaken in patients with different diseases and cardiovascular risk factors. However, very few studies have researched the distribution and determining factors of antibodies to oxidized LDL in the general population. A total of 1,354 persons (817 females and 537 males) aged 5–65 years were included in this study. They were selected randomly from the population census of Málaga, in southern Spain. The females had lower levels of total cholesterol and triglycerides and higher levels of HDL-cholesterol and a very significant increase ($P < 0.0001$) in levels of anti-oxidized LDL [low density lipoprotein modified by malondialdehyde (MDA-LDL)] antibodies but no difference in levels of immune complexes consisting of LDL and IgG antibodies (anti-LDL immune complex). Younger persons (16–35 years) had higher levels of anti-oxidized LDL (MDA-LDL) antibodies than persons older than 35 years ($P = 0.05$). Levels of immune complexes were significantly higher ($P = 0.05$) in persons aged 5–15 years than in persons older than 40 years. A very weak association was found between levels of anti-oxidized LDL (MDA-LDL) antibodies and anti-LDL immune complexes. The higher prevalence of anti-oxidized LDL (MDA-LDL) antibodies in females and young persons is in agreement with studies that found an inverse association between atherosclerosis and the level of these antibodies.**—Tinahones, F. J., J. M. Gómez-Zumaquero, L. Garrido-Sánchez, E. García-Fuentes, G. Rojo-Martínez, I. Esteva, M. S. Ruiz de Adana, F. Cardona, and F. Soriguer. **Influence of age and sex on levels of anti-oxidized LDL antibodies and anti-LDL immune complexes in the general population.** ***J. Lipid Res.*** **2005.** 46: **452–457.**

Supplementary key words low density lipoprotein • oxidized low density lipoproteins • atherosclerosis

Several lines of evidence have determined that the oxidized products of LDLs are involved in atherogenesis (1, 2). Oxidative modification of LDLs may be a prerequisite for the rapid accumulation of LDLs within macrophages to form foam cells; indeed, oxidized LDL has been found in extracts from atherosclerotic lesions (3). Oxidative modification of LDLs induces the formation of immunogenic epitopes in the LDL molecule, which leads to the formation of antibodies against oxidized LDLs that can be detected in serum (4). These antibodies have been detected in patients with advanced atherosclerotic lesions (5). Levels of anti-oxidized LDL antibodies are increased in patients with coronary atherosclerosis (6, 7), acute myocardial infarction (8), and cerebral or peripheral vascular disease (9), and they have been shown to predict the progression of carotid atherosclerotic lesions (10).

Nevertheless, the clinical importance of these autoantibodies is still under discussion. For example, in patients with diabetes, no association has been found between anti-oxidized LDL antibodies and microvascular complications (11), nor has an association been found between their levels and levels of cholesterol in patients with heterozygous hypercholesterolemia (12) or with the degree of oxidizability in serum (13). In fact, our group found an inverse relation between levels of cholesterol and levels of anti-oxidized LDL antibodies in the general population (14). Recent studies have found no association between the levels of anti-oxidized LDL antibodies and coronary artery disease (15), and others have detected an inverse relation between IgM autoantibodies to oxidized LDL and carotid artery atherosclerosis (16).

The methodological approach for the detection of anti-oxidized LDL antibodies is subject to much variation. Moreover, oxidized LDL autoantibodies have been found both free and forming immune complexes (17). Thus, dif-

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Abbreviations: MDA, malondialdehyde; MDA-LDL, low density lipoprotein modified by malondialdehyde.

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TABLE 1. Differences between males and females in levels of anti-oxidized LDL antibodies, anti-LDL immune complex, age, BMI, and lipid parameters

Variable	Females (n = 817)	Males (n = 547)	*P*
Age (years)	33.30 ± 17.06	31.58 ± 18.38	0.087
BMI (kg/m^2)	24.74 ± 6.34	23.65 ± 5.66	0.000
Cholesterol (mmol/l)	4.96 ± 1.11	5.12 ± 1.11	0.005
HDL-cholesterol (mmol/l)	1.31 ± 0.34	1.17 ± 0.33	0.000
Triglycerides (mmol/l)	1.02 ± 0.58	1.29 ± 0.96	0.000
Ox LDL Ab (optical density)	0.299 ± 0.130	0.273 ± 0.122	0.000
Anti-LDL immune complex (optical density)	0.267 ± 0.096	0.263 ± 0.090	0.484

Data are expressed as means ± SD. BMI, body mass index; Ox LDL Ab, anti-oxidized LDL antibody.

ferent biological roles could be suggested for antibodies, depending on whether they circulate freely or as immune complexes.

Most studies of antibodies to oxidized LDL have been undertaken in patients with different diseases and cardiovascular risk factors. However, very few studies have researched the distribution and determining factors of antibodies to oxidized LDL in the general population. We investigated the possible association between antibodies to oxidized LDL and such population variables as age, sex, and plasma lipid levels.

MATERIALS AND METHODS

Population and measurements

This study was undertaken in the province at Málaga, in southern Spain. A total of 1,354 persons (817 females and 537 males) between the ages of 5 and 65 years were included.

The children were selected randomly from the census of schoolchildren in the area called the Axarquía (province of Málaga). Sampling was carried out in different stages to guarantee the representativity of the whole geographic area, selecting area, village, and children as the sampling units. The study was carried out in state schools. Education in Spain is universal, compulsory, and free for the age group studied, thereby ensuring that the selection of a school unit was fully representative of the whole population.

The adults were selected from the census of the general population of the town of Pizarra (province of Málaga). All institutionalized persons, for whatever reason, were excluded from the study, as were pregnant women and persons with severe clinical problems or psychological disorders. The subjects were requested by mail to attend their local health center for a medical examination. Those who failed to attend their first appointment were sent a second letter giving them another appointment, and all those still not attending were visited at home to ascertain the reason. The final sample distribution by age and sex was not significantly different from the population distribution. The rates of participation were greater than 95% for the children and 75% for the adults.

The study was also approved by the Ethics and Investigation Committee of Carlos Haya Regional University Hospital. After obtaining written informed consent from all subjects or their parents, clinical and anthropometric data were taken, as was a sample of blood, which was extracted after a minimum 10 h fast. Measurements were made of total cholesterol and triglycerides by the enzymatic method (Ecoline 2S; Merck, Darmstadt, Germany), HDL-cholesterol by phosphotungstic acid precipitation (Boehringer Mannheim, Mannheim, Germany), and uric acid by the enzymatic method (Boehringer Mannheim).

LDL isolation

LDL was isolated from a pool of fasting plasma from human blood donors by density gradient ultracentrifugation at 65,000 rpm (Beckman Optima XL-100K ultracentrifuge, vertical rotor NVT65.2) for 35 min at 4°C. This was then further purified with a second ultracentrifugation at 49,000 rpm (fixed angle rotor 70.1) for 18 h at 4°C. The LDL was then dialyzed against PBS (0.14 M NaCl and 0.01 M phosphate buffer) at 4°C for 30 h.

LDL oxidation

Oxidized LDL was prepared by incubating the LDL for 3 h at 37°C with 0.5 M malondialdehyde (MDA) at a constant ratio of 100 μl per milligram of LDL. MDA was prepared fresh by acid hydrolysis of MDA-bis-dimethyl acetal; 88 μl of MDA-bis-dimethyl acetal was incubated with 12 μl of 4 M HCl and 400 μl of distilled water at 37°C for 10 min. The reaction was stopped by adjusting the pH to 7.4 with 1 M NaOH. After conjugation, low density lipoprotein modified by malondialdehyde (MDA-LDL) was extensively dialyzed against PBS.

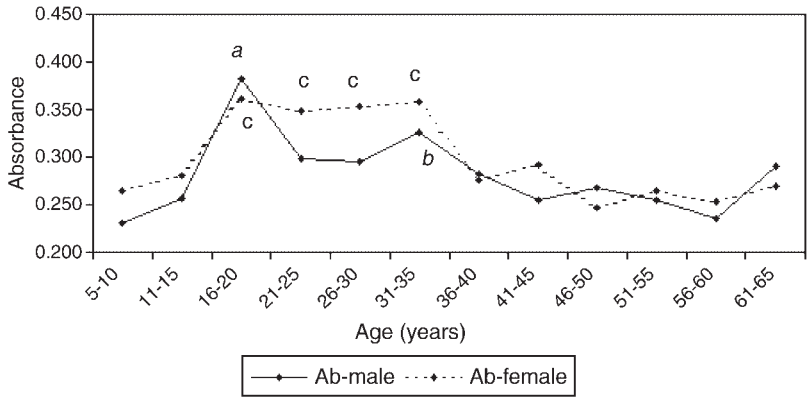

Fig. 1. Mean levels of anti-oxidized LDL antibodies (Ab) in each of the 5 year groups in males and females. [a] Significant differences ($P < 0.05$) between this group and the other 5 year groups in males. [b] Significant differences ($P < 0.05$) between this group and the groups 56–60, 51–55, 41–45, 11–15, and 5–10 in males. [c] Significant differences ($P < 0.05$) between these groups and the other groups in females.

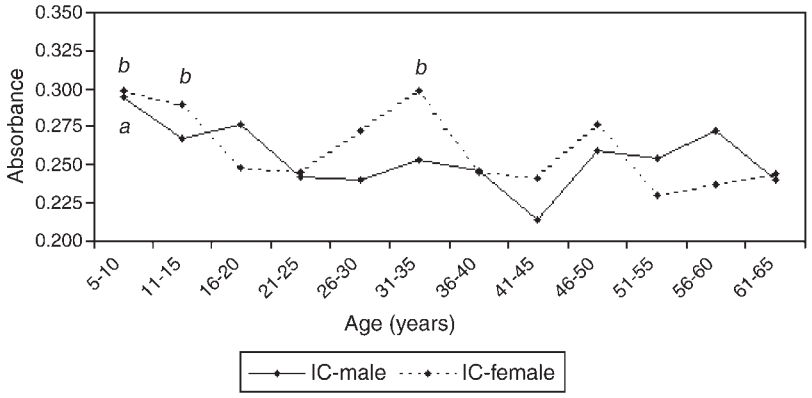

Fig. 2. Mean levels of anti-LDL immune complexes (IC) in each of the 5 year groups in males and females. [a]Significant differences ($P < 0.05$) between this group and the groups 61–65, 41–45, and 35–40 in males. [b]Significant differences ($P < 0.05$) between these groups and the groups 61–65, 56–60, 51–55, 41–45, and 36–40 in females.

Anti-oxidized LDL antibodies

Microtiter plates for the determination of anti-oxidized LDL antibodies were coated with either native LDL or MDA-LDL, both at 10 μg/ml in PBS. The plates were incubated for 2 h at 37°C and overnight at 4°C. After washing four times with PBS, the plates were blocked with 1% BSA/PBS for 2 h at room temperature. Serum samples were diluted 1:100 in 1% BSA/PBS and incubated for 3 h at room temperature. After washing, an alkaline phosphatase-conjugated anti-human IgG (Sigma Chemical, St. Louis, MO) was diluted 1:1,000 in 1% BSA/PBS and added. It was then left for 3 h at room temperature. *p*-Nitrophenyl-phosphate (1 mg/ml; Sigma) in 500 mM carbonate buffer containing 1 mM $MgCl_2$ (pH 9.8) was used as a substrate. The reaction was stopped after 60 min with 1 M NaOH. The absorbance was read in an ELISA reader (Labsystem Multiskan, Helsinki, Finland). The binding of antibodies to oxidized LDL was calculated by subtracting the binding of native LDL from the binding of MDA-LDL. The results were expressed as an optical density.

Detection of immune complexes consisting of LDL and IgG antibodies (anti-LDL immune complexes)

IMMULOM4 microtiter plates (Cultek, Roskilde, Denmark) for ELISA were coated with 100 μl/well at a concentration of 10 μg/ml anti-apolipoprotein B-100 (Calbiochem) in TBS overnight at 4°C. After four washes with TBS, the plates were blocked with 1% BSA/PBS for 2 h at room temperature. The serum samples were diluted 1:100 in 1% BSA/PBS and incubated for 3 h at room temperature. After washing, human anti-IgG conjugated with alkaline phosphatase (Sigma Chemical) was diluted 1:1,000 in 1% BSA/PBS and added. This was left to stand at room temperature for 3 h. *p*-Nitrophenol (1 mg/ml; Sigma) in a diethanolamine buffer (pH 9.8) was used as a substrate. As with the anti-oxidized LDL antibodies, the absorbance was read after 60 min in the ELISA reader at 405 nm. The results were expressed as an optical density.

Statistical analysis

The hypothesis contrast between the means of the continuous variables was analyzed by Student's *t*-test or ANOVA, and differences between groups were detected by Duncan's test. The tendency between variables was measured by Spearman's correlation coefficient. In all cases, the rejection level for a null hypothesis was $\alpha = 0.05$ for two tails. A multiple regression test was performed, considering the levels of anti-oxidized LDL antibodies and anti-LDL immune complexes as dependent variables, with the independent variables consisting of the levels of cholesterol, high density lipoprotein-cholesterol, triglycerides, body mass index, age, and sex.

RESULTS

Table 1 shows the differences according to sex for the lipid parameters and levels of anti-oxidized LDL antibodies. The lipid pattern of the females showed a lower car-

TABLE 2. Simple linear correlations between lipids, BMI, age, Ox LDL Ab, and anti-LDL immune complexes

	Age	BMI	Cholesterol	Triglycerides	HDL-Cholesterol	Ox LDL Ab
BMI	0.7236					
	$P = 0.000$					
Cholesterol	0.4436	0.2995				
	$P = 0.000$	$P = 0.000$				
Triglycerides	0.2629	0.2620	0.4267			
	$P = 0.000$	$P = 0.000$	$P = 0.000$			
HDL-cholesterol	−0.0648	−0.2047	0.1693	−0.3236		
	$P = 0.017$	$P = 0.000$	$P = 0.000$	$P = 0.000$		
Ox LDL Ab	−0.0308	−0.0065	−0.1802	−0.1291	−0.0577	
	$P = 0.258$	$P = 0.815$	$P = 0.000$	$P = 0.000$	$P = 0.037$	
Anti-LDL immune complex	−0.2189	−0.1849	−0.1041	−0.0487	−0.0315	0.0720
	$P = 0.000$	$P = 0.000$	$P = 0.002$	$P = 0.141$	$P = 0.341$	$P = 0.029$

The data represent means ± SD. *P* values were determined by Spearman's test.

TABLE 3. Multiple regression model with the overall sample using anti-oxidized LDL antibodies

Variable	Beta	SE β	*P*
Age	0.00053	0.00032	0.0971
BMI	0.00045	0.00100	0.6512
Sex	0.01919	0.00863	0.0266
Cholesterol	−0.00031	0.00012	0.0094
HDL-cholesterol	−0.00077	0.00036	0.0327
Anti-LDL immune complex	0.09053	0.04571	0.0480
Triglycerides	−0.00013	0.00007	0.0556
Constant	0.32509	0.03515	0.0000

Dependent variable: Ox LDL Ab. Independent variables: age, BMI, sex, cholesterol, HDL-cholesterol, anti-LDL immune complexes, and triglycerides.

TABLE 4. Multiple regression model with the overall sample using anti-LDL immune complexes

Variable	Beta	SE β	*P*
Age	−0.00091	0.00024	0.0002
BMI	−0.00092	0.00075	0.2250
Sex	0.01013	0.00655	0.1227
Cholesterol	0.00009	0.00009	0.3255
HDL-cholesterol	−0.00051	0.00027	0.0599
Ox LDL Ab	0.05201	0.02626	0.0480
Triglycerides	−0.00001	0.00009	0.8711
Constant	0.29715	0.02601	0.0000

Dependent variable: anti-LDL immune complexes. Independent variables: age, BMI, sex, cholesterol, HDL-cholesterol, Ox LDL Ab, and triglycerides.

diovascular risk profile (lower levels of total cholesterol and triglycerides and higher levels of HDL-cholesterol) and higher levels of anti-oxidized LDL antibodies ($P < 0.0001$). No difference was seen in the levels of anti-LDL immune complexes.

The levels of anti-oxidized LDL antibodies were significantly higher in persons aged 16–35 years ($P < 0.05$), with a significant decrease after 36 years (**Fig. 1**). These differences were more marked in the females of this age range than in the males, in whom the increase in antibody titers was only significant in those from 16–20 and 31–35 years.

The levels of anti-LDL immune complexes were significantly higher in the boys aged 5–10 years and in the girls aged 5–15 years ($P < 0.05$) compared with subjects 40–65 years old (**Fig. 2**).

The levels of anti-oxidized LDL antibodies correlated negatively with cholesterol ($P < 0.0001$) and triglycerides ($P < 0.0001$) and positively with anti-LDL immune complexes ($P = 0.029$), with a negative correlation with HDL ($P < 0.05$) (**Table 2**).

The levels of anti-LDL immune complexes correlated negatively with age ($P < 0.0001$), body mass index ($P < 0.0001$), and cholesterol ($P = 0.002$) and positively with the levels of anti-oxidized LDL antibodies ($P = 0.029$) (Table 2).

Multiple regression analysis showed those variable accounting significantly for the variation in levels of anti-oxidized LDL antibodies to be cholesterol ($P = 0.0094$), sex ($P = 0.0266$), HDL-cholesterol ($P = 0.0327$), and the levels of anti-LDL immune complexes ($P = 0.0480$) (**Table 3**). **Figure 3** shows the levels of anti-oxidized LDL antibodies and plasma cholesterol according to age. The increase in levels of anti-oxidized LDL antibodies was related significantly ($P = 0.0094$) to lower cholesterol levels.

Multiple regression analysis showed that the variation in anti-LDL immune complexes was only explained by age ($P = 0.0002$) and the levels of anti-oxidized LDL antibodies ($P = 0.0480$) (**Table 4**).

DISCUSSION

The main findings of this study are that levels of anti-oxidized LDL antibodies are higher in females and young persons and that they correlate negatively with other cardiovascular risk factors. Lower levels of anti-oxidized LDL antibodies have also been reported in elderly persons with high cardiovascular risk (18).

It is not clear, though, whether the anti-LDL immune complexes have a different clinical relevance to the free antibodies. As suggested by some (19), a close association between these two biological variables could be expected; we found a very discrete association between them, with age

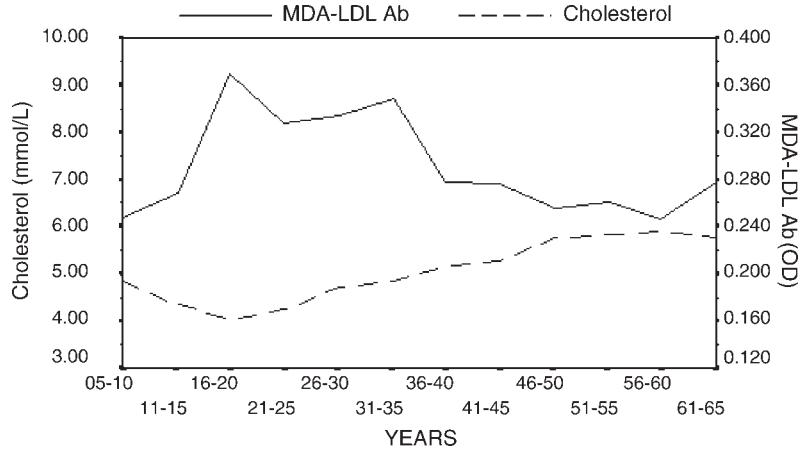

Fig. 3. Mean anti-oxidized LDL antibody and cholesterol levels for each 5 year group in the overall study group. MDA-LDL, low density lipoprotein modified by malondialdehyde; OD, optical density.

having a more direct influence on levels of anti-LDL immune complexes. The lipid variables, however, such as cholesterol, HDL-cholesterol, and triglycerides, accounted better for the variance in antibodies.

The clinical importance of these autoantibodies is controversial. Unlike early results and those of other studies that found high levels of antibodies in patients with atherosclerosis (6–10), our results support the finding of an inverse association between the level of these autoantibodies and the presence of atherosclerosis (11, 16). Furthermore, the inverse association between levels of cholesterol and antibodies to oxidized LDL has already been reported, both in the general population (14) and in patients with heterozygous familial hypercholesterolemia (12). Once again, we too found this same association, but this time in a group that included a large sample of children. The association was seen not only in bivariate correlations but also after multiple regression analysis, in which sex and cholesterol levels were the independent variables that most influenced the levels of anti-oxidized LDL antibodies.

In experimental studies with animal models, the production of antibodies after immunization with oxidized LDLs was associated with a marked reduction in the formation of atheromatous plaques (20–23). Anti-oxidized LDL antibodies in atherosclerotic lesions have also been shown to block the uptake of oxidized LDLs by macrophages, suggesting a possible role in the prevention of the formation of foam cells (24, 25). These results, however, should be interpreted with caution, because normal antibody levels in the general population cannot be compared with immunization studies, which induce a drastic increase in these antibodies.

Several explanations may account for the different associations found between anti-oxidized LDL antibodies and atherosclerosis. The methodological approach to detecting antibodies to modified LDLs is very heterogeneous. For instance, the measurement of antibodies is influenced by the source of LDLs used in the ELISA, with the results varying depending on whether the LDLs come from persons with normal or high cholesterol levels (26). Another important source of variation is the subject's immune status, as levels of anti-oxidized LDL antibodies in patients with lupus erythematosus are very closely related to the degree of disease activity (27). Differences in preexisting clinical conditions and selection criteria are another obstacle.

In summary, the results of our study support the hypothesis that antibodies to oxidized LDL may be inversely associated with the presence of atherosclerosis.

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