INFLUENCE OF AGE AND SEX ON LEVELS OF ANTI-OXIDIZED LDL ANTIBODIES AND ANTI-LDL IMMUNE COMPLEXES IN THE GENERAL POPULATION.

First author's surname: Tinahones

Short title: Age, sex and anti-oxidized LDL antibodies.

Francisco J Tinahones*, MD, PhD; Juan Miguel Gómez-Zumaquero†, B.Sc, PhD; Lourdes Garrido-Sánchez‡, B.Sc; Eduardo García-Fuentes‡, B.Sc, PhD; Gemma Rojo-Martínez‡, B.Sc, PhD; Isabel Esteva*, MD, PhD; Maria Soledad Ruiz de Adana*, MD; Fernando Cardona†, B.Sc, PhD; Federico Soriguer*, MD, PhD.

Servicio de Endocrinología y Nutrición*, Laboratorio de Investigación†, Carlos Haya Regional University Hospital, Málaga, Spain.

Address for correspondence:

Francisco J. Tinahones Madueño

E-mail: fjtinahones@terra.es
Tel: +34 952408749 Fax: +34 952286704

Abbreviations: oxidized LDL (low density lipoprotein oxidized), HDL-C (high density lipoprotein cholesterol), MDA (malondialdehyde), MDA-LDL (low density lipoprotein modified by malondialdehyde), BMI (body mass index ), Ab (antibodies), IC (immune complexes), Ox LDL Ab ( Anti-oxidized LDL antibodies), anti-LDL IC ( anti-LDL immune complexes).
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 1354 persons (817 female and 537 male) aged 5-65 years were included in the study. They were selected randomly from the population census of Malaga, 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 (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 over 35 years of age ($P=0.05$). Levels of immune complexes were significantly higher ($P=0.05$) in persons aged 5-15 years than persons over 40 years of age. 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 line with those who have found an inverse association between atherosclerosis and the level of these antibodies.

Supplementary key words: LDL • oxidized LDL • antibodies • immune complexes • atherosclerosis.
INTRODUCTION

Several lines of evidence have determined that the oxidized products of low density lipoproteins (LDL) are involved in atherogenesis (1-2). Oxidative modification of LDL may be a prerequisite for the rapid accumulation of LDL within macrophages to form foam cells; indeed, oxidized LDL has been found in extracts from atherosclerotic lesions (3). Oxidative modification of LDL induces the formation of immunogenic epitopes in the LDL molecule, which leads to the formation of antibodies against oxidized LDL 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 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 between their levels and levels of cholesterol in patients with heterozygous hypercholesterolemia (12), nor with the degree of oxidability 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, different 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, or plasma lipid levels.

MATERIALS AND METHODS

Population and measurements

The study was undertaken in the province at Malaga, situated in southern Spain. A total of 1354 people (817 female and 537 male) between the ages of 5 and 65 years were included in the study.

The children were selected randomly from the census of schoolchildren in the area called the Axarquía (province of Malaga). Sampling was carried out in different stages to guarantee 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 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 Malaga). All institutionalized persons, for whatever reason, were excluded from the study, as were pregnant women, and those persons with a severe clinical problem or psychological disorder. 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 in order to ascertain the reason. The final sample distribution by age and sex was not significantly 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 the subjects or their parents, clinical and anthropometric data were taken, as well as a sample of blood, which was extracted after a minimum 10 hours fast. Measurements were made of total cholesterol and triglycerides by enzymatic method (Ecoline 2S, Merck, Darmstadt, Germany), high-density lipoprotein (HDL) cholesterol by phosphotungstic acid precipitation (HDL-C, Boehringer Mannheim, Mannheim, Germany) and uric acid by enzymatic method (Boehringer Mannheim).

**LDL isolation**

LDL was isolated from a pool of fasting plasma from human blood donors by density gradient ultracentrifugation at 65000 rpm (BECKMAN Optima XL-100K ultracentrifuge. USA, vertical rotor NVT65.2) for 35 minutes at 4°C. This was then further purified with a second ultracentrifugation at 49000 rpm (fixed angle rotor 70.1) for 18 hours at 4°C. The LDL was then dialyzed against PBS (4°C for 30 hours) (0.14 M NaCl/0.01 M phosphate buffer).

**LDL oxidation**

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

**Anti-oxidized LDL antibodies**

Microtiter plates for determination of anti-oxidized LDL antibodies were coated with either native- or MDA-LDL, both at 10 μg/ml in PBS. The plates were incubated for 2 hours at 37°C and overnight at 4°C. After washing 4 times with PBS, the plates were blocked with 1% BSA/PBS for 2 hours at room temperature. Serum samples were diluted 1:100 in 1% BSA/PBS and incubated for 3 hours at room temperature. After washing, an alkaline phosphatase-conjugated anti-human IgG (Sigma Immuno Chemical, St. Louis, MO) was diluted 1:1000 in 1% BSA/PBS and added. It was then left for 3 hours at room temperature. One mg/ml p-nitrophenyl-phosphate (Sigma) in 500 mM carbonate buffer containing 1mM MgCl2 (pH 9.8) was used as substrate. The reaction was stopped after 60 min with 1M NaOH. The absorbance was read in an ELISA reader (Labsystem Multiskan, MS, 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 (OD).

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

IMMULOM4 microtiter plates (Cultek, Roskilde, Denmark, Europe) for ELISA were coated with 100μl/well at a concentration of 10μg/ml of anti-apoB100 (Calbiochen) in TBS overnight at 4°C. After four washes with TBS, the plates were blocked with 1% BSA/PBS for two hours at room temperature. The serum samples were diluted 1:100 in 1% BSA/PBS and
incubated for three hours at room temperature. After washing, human anti-IgG conjugated with alkaline phosphatase (Sigma Immuno Chemicals) was diluted 1:1000 in 1% BSA/PBS and added. This was left to stand at room temperature for three hours. One mg/ml of p-nitrofenol (Sigma) in a diethanolamine buffer (pH 9.8) was used as substrate. As with the anti-oxidized LDL antibodies, the absorbance was read after 60 minutes in the ELISA reader at 405nm. The results were expressed as an optical density (OD).

**Statistical analysis**

The hypothesis contrast between the means of the continuous variables was analyzed by the Student 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 an alpha=0.05 for two tails. A multiple regression test was made, considering the levels of anti-oxidized LDL antibodies and anti-LDL immune complexes as dependant 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 cardiovascular 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 drop after the age of 36 years (Figure 1). These differences
were more marked in the females of this age range than the males, in whom the rise in antibody titers was only significant in those between 16-20 and 31-35 years.

The levels of anti-LDL immune complexes were significantly higher in the boys aged 5-10 and in the girls aged 5-15 years \((P<0.05)\) compared with the age groups of 40-65 years (Figure 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 \((P<0.0001)\), BMI \((P<0.0001)\) 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 \((P=0.0094)\) with 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 a 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, a close association between these two biological variables could be expected (25); we found a very discrete association between them, with age 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 which found high levels of antibodies in patients with atherosclerosis (6-10), our results support those of others who found 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 have found this same association, but this time in a group of persons which included a large sample of children. The association was seen not only in bivariate correlations but also after multiple regression analysis, where sex and cholesterol levels were the independent variables which most influenced the levels of anti-oxidized LDL antibodies.
In experimental studies with animal models the production of antibodies after immunization with oxidized LDL was associated with a marked reduction in the formation of atheromatous plaques (19-22). Anti-oxidized LDL antibodies in atherosclerotic lesions have also been shown to block the uptake of oxidized LDL by macrophages, suggesting a possible role in the prevention of the formation of foam cells (23-24). These results should, however, be interpreted with caution, because normal antibody levels in the general population cannot be compared with immunization studies, which induce a drastic rise 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 LDL is very heterogeneous. For instance, the measurement of antibodies is influenced by the source of LDL used in the ELISA, with the results varying depending on whether the LDL comes 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 with the degree of disease activity (27). Differences in pre-existing 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.
ACKNOWLEDGEMENTS

The study was partly funded by the Red de Centros de Metabolismo y Nutrición (RCMN, C03/08) of the Instituto de Salud Carlos III.

The authors thank Ian Johnstone for help with the English language version of the manuscript.
REFERENCES


**Figure 1.** Mean levels of anti-oxidized LDL antibodies 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, 5-10 in males.

\(^c\)Significant differences (P<0.05) between these groups and the other groups in females.

**Figure 2.** Mean levels of anti-LDL immune complexes 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, 35-40 in males.

\(^b\)Significant differences (P<0.05) between these groups and the groups 61-65, 56-60, 51-55, 41-45, 36-40 in females.

**Figure 3.** Mean anti-oxidized LDL antibody and cholesterol levels for each 5-year group in the overall study group.
TABLE 1. Differences between males and females in levels of anti-oxidized LDL antibodies, anti-LDL immune complex, age, BMI and lipid parameters.

<table>
<thead>
<tr>
<th></th>
<th>Females</th>
<th>Males</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=817)</td>
<td>(n=547)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>33.30±17.06</td>
<td>31.58±18.38</td>
<td>0.087</td>
</tr>
<tr>
<td>BMI (Kg/m2)</td>
<td>24.74±6.34</td>
<td>23.65±5.66</td>
<td>0.000</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.96±1.11</td>
<td>5.12±1.11</td>
<td>0.005</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.31±0.34</td>
<td>1.17±0.33</td>
<td>0.000</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.02±0.58</td>
<td>1.29±0.96</td>
<td>0.000</td>
</tr>
<tr>
<td>Ox LDL Ab (OD)</td>
<td>0.299±0.130</td>
<td>0.273±0.122</td>
<td>0.000</td>
</tr>
<tr>
<td>Anti-LDL IC (OD)</td>
<td>0.267±0.096</td>
<td>0.263±0.090</td>
<td>0.484</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation.
Ox LDL Ab: Anti-oxidized LDL antibodies
Anti-LDL IC: Anti-LDL immune complexes
TABLE 2. Simple linear correlations between lipids, BMI, age, anti-oxidized LDL antibodies and anti-LDL immune complexes.

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>BMI</th>
<th>Cholesterol</th>
<th>Triglycerides</th>
<th>HDL chol</th>
<th>Ox LDL Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>0.7236</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.4436</td>
<td>0.2995</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.2629</td>
<td>0.2620</td>
<td>0.4267</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL chol</td>
<td>-0.0648</td>
<td>-0.2047</td>
<td>0.1693</td>
<td>-0.3236</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ox LDL Ab</td>
<td>-0.0308</td>
<td>-0.0065</td>
<td>-0.1802</td>
<td>-0.1291</td>
<td>-0.0577</td>
<td></td>
</tr>
<tr>
<td>Anti-LDL IC</td>
<td>-0.2189</td>
<td>-0.1849</td>
<td>-0.1041</td>
<td>-0.0487</td>
<td>-0.0315</td>
<td>0.0720</td>
</tr>
</tbody>
</table>

Ox LDL Ab: Anti-oxidized LDL antibodies. Anti-LDL IC: Anti-LDL immune complexes. The data represent mean ± SD. P values were determined by Spearman’s test.
TABLE 3. Multiple regression model with the overall sample. Dependent variable: anti-oxidized LDL antibodies. Independent variables: age, BMI, sex, cholesterol, HDL cholesterol, anti-LDL immune complexes, triglycerides.

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>SE beta</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.00053</td>
<td>0.00032</td>
<td>0.0971</td>
</tr>
<tr>
<td>BMI</td>
<td>0.00045</td>
<td>0.00100</td>
<td>0.6512</td>
</tr>
<tr>
<td>Sex</td>
<td>0.01919</td>
<td>0.00863</td>
<td>0.0266</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-0.00031</td>
<td>0.00012</td>
<td>0.0094</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>-0.00077</td>
<td>0.00036</td>
<td>0.0327</td>
</tr>
<tr>
<td>Anti-LDL IC</td>
<td>0.09053</td>
<td>0.04571</td>
<td>0.0480</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>-0.00013</td>
<td>0.00007</td>
<td>0.0556</td>
</tr>
<tr>
<td>Constant</td>
<td>0.32509</td>
<td>0.03515</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

Anti-LDL IC: Anti-LDL immune complexes.

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>SE beta</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.00091</td>
<td>0.00024</td>
<td>0.0002</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.00092</td>
<td>0.00075</td>
<td>0.2250</td>
</tr>
<tr>
<td>Sex</td>
<td>0.01013</td>
<td>0.00655</td>
<td>0.1227</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.00009</td>
<td>0.00009</td>
<td>0.3255</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>-0.00051</td>
<td>0.00027</td>
<td>0.0599</td>
</tr>
<tr>
<td>Ox LDL Ab</td>
<td>0.05201</td>
<td>0.02626</td>
<td>0.0480</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>-0.00001</td>
<td>0.00009</td>
<td>0.8711</td>
</tr>
<tr>
<td>Constant</td>
<td>0.29715</td>
<td>0.02601</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

Ox LDL Ab: Anti-oxidized LDL antibodies
Figure 1
Figure 2

Absorbance

Age (years)

IC–male
IC–female

0.200
0.225
0.250
0.275
0.300
0.325
0.350

5−10 11−15 16−20 21−25 26−30 31−35 36−40 41−45 46−50 51−55 56−60 61−65
Figure 3

The graph shows the relationship between years and cholesterol (mmol/L) along with MDA-LDL Ab (OD). The x-axis represents the years, and the y-axis represents cholesterol levels and MDA-LDL Ab (OD) values. The graph indicates a trend where cholesterol levels peak around the ages of 31-35 and then decrease, while MDA-LDL Ab (OD) values seem to increase with age.