

# Autoantibody titers against OxLDL are correlated with Achilles tendon thickness in patients with familial hypercholesterolemia

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**Abstract** Achilles tendon xanthomas are associated with increased cardiovascular risk in patients with familial hypercholesterolemia (FH). Oxidized low density lipoprotein (OxLDL), the antibodies against OxLDL, and the LDL-associated phospholipase A<sub>2</sub> (Lp-PLA<sub>2</sub>) may play important roles in atherogenesis. We investigated the possible association between plasma levels of OxLDL, Lp-PLA<sub>2</sub> activity, and autoantibody titers against various types of mildly OxLDL with Achilles tendon thickness (ATT). ATT was determined by sonography in 80 unrelated heterozygous FH patients. Three different types of mildly OxLDL were prepared: OxLDL<sub>L</sub>, OxLDL<sub>P</sub>, and OxLDL<sub>D</sub>, at the end of the lag, propagation, and decomposition phases of oxidation, respectively. Similar types of OxLDL were also prepared after inactivation of the LDL-associated Lp-PLA<sub>2</sub>. These types were denoted OxLDL(-)<sub>L</sub>, OxLDL(-)<sub>P</sub>, and OxLDL(-)<sub>D</sub>. FH patients exhibited significantly higher plasma OxLDL levels and serum IgG titers against OxLDL<sub>P</sub> and OxLDL<sub>D</sub> compared with 40 normolipidemic apparently healthy controls. ATT values were positively correlated with autoantibody titers against OxLDL<sub>P</sub> and OxLDL<sub>D</sub>; however, in multiple regression analysis, ATT was independently associated only with the autoantibody titers against OxLDL<sub>D</sub>. We conclude that the IgG autoantibody titers against OxLDL<sub>D</sub> but not OxLDL or Lp-PLA<sub>2</sub> may play an important role in the pathogenesis of Achilles tendon xanthomas in FH patients.—Tsouli, S. G., D. N. Kiortsis, E. S. Lourida, V. Xydis, L. D. Tsironis, M. I. Argyropoulou, M. Elisaf, and A. D. Tselepis. Autoantibody titers against OxLDL are correlated with Achilles tendon thickness in patients with familial hypercholesterolemia. *J. Lipid Res.* 2006. 47: 2208–2214.

**Supplementary key words** antibody • oxidized low density lipoprotein • platelet-activating factor acetylhydrolase • phospholipase A<sub>2</sub>

Oxidative modification of LDL is an important event in the development of atherosclerosis. Oxidized low density

lipoprotein (OxLDL) is present in atherosclerotic lesions and promotes atherosclerosis by several different mechanisms (1–3). During LDL oxidation, the lipids and apolipoprotein B-100 (apoB-100) undergo a variety of chemical changes via radical-mediated reactions as well as modifications by chemically active products formed on OxLDL particles (4).

Oxidation of LDL in vitro occurs in three phases: *i*) the lag phase, during which consumption of the endogenous antioxidants occurs; *ii*) the propagation phase, in which the *sn*-2 unsaturated fatty acids of phosphatidylcholine molecules are rapidly oxidized to their hydroperoxides; and *iii*) the decomposition phase, when these hydroperoxides are cleaved, generating reactive aldehydes, such as malondialdehyde and 4-hydroxynonenal, as well as oxidized phospholipids (OxPL) containing short acyl groups at the *sn*-2 position (4). OxPL can be hydrolyzed into lysophosphatidylcholine (lyso-PC) by the LDL-associated phospholipase A<sub>2</sub> (Lp-PLA<sub>2</sub>), also known as platelet-activating factor (PAF) acetylhydrolase (5). Lp-PLA<sub>2</sub> exhibits a Ca<sup>2+</sup>-independent phospholipase A<sub>2</sub> activity and preferentially hydrolyzes phospholipids containing short acyl groups at the *sn*-2 position, such as PAF and OxPL (5).

OxLDL is immunogenic, and its constituents OxPL, aldehydes, and lyso-PC play important roles in OxLDL antigenicity, participating in the formation of several epitopes (6, 7). These epitopes are recognized by specific autoantibodies, which are present in serum of healthy individuals as well as in various disease states, including atherosclerotic diseases (reviewed in 8). Because the extent of LDL oxidation and the LDL-associated Lp-PLA<sub>2</sub> activity significantly influence the levels of OxPL, lyso-PC, and aldehydes during LDL oxidation (9), these factors could also influence the types of epitopes formed on OxLDL and consequently the specificity and the plasma

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levels of autoantibodies against OxLDL. Indeed, we recently showed that the extent of LDL oxidation and the activity of LDL-associated Lp-PLA<sub>2</sub> significantly affect the antibody titers against OxLDL (10).

Tendon xanthomas are deposits of lipid and connective tissue in tendons and are commonly found in patients with severe hyperlipidemia, such as familial hypercholesterolemia (FH) (11–13). Importantly, it has been shown that the presence of tendon xanthomas in patients with FH is associated with increased cardiovascular risk independently of the underlying LDL receptor gene mutation (14). The composition of tendon xanthomas exhibits many similarities to that of atherosclerotic plaques (13). Indeed, tendon xanthomas are characterized by the extracellular deposition of unesterified and esterified cholesterol as well as connective tissue and by the accumulation of foam cells (15, 16). The underlying mechanisms by which some FH patients develop tendon xanthomas and others do not remain to be established. Only recently, it was demonstrated that macrophages from FH patients with tendon xanthomas are more sensitive to the actions of OxLDL compared with cells from FH patients without tendon xanthomas (17).

Autoantibodies against OxLDL may play important roles in atherosclerosis (8, 18). To the best of our knowledge, there is a paucity of data concerning the autoantibody titers in FH patients with respect to the presence of tendon xanthomas. Therefore, the aim of this study was to evaluate the autoantibody titers against various types of mildly OxLDL as well as the plasma levels of OxLDL and Lp-PLA<sub>2</sub> activity in relation to Achilles tendon thickness (ATT) determined by sonography in heterozygous FH patients.

## METHODS

### Patients

Eighty unrelated patients with heterozygous FH aged 18–75 years participated in the study. The diagnosis of FH was made according to recently proposed criteria (19) and was confirmed by the appropriate genetic analysis (20). Forty normolipidemic apparently healthy volunteers with no history of hypercholesterolemia or coronary artery disease were also included in the study. Exclusion criteria included history or clinical signs of cardiovascular disease, peripheral arterial disease, or stroke, hepatic dysfunction (levels of transaminases  $\geq$  1.5 times the upper limit of normal), renal insufficiency (serum creatinine  $>$  1.6 mg/dl), proteinuria ( $>$ 0.5 g/day), diabetes mellitus (fasting plasma glucose concentration  $\geq$  126 mg/dl or use of antidiabetic medications), hypertension (arterial blood pressure  $>$  140/90 mmHg or use of antihypertensive medications), and thyroid-stimulating hormone levels  $>$  5  $\mu$ U/ml. None of the participants was taking lipid-lowering drugs, including statins, or any other medication known to affect lipid metabolism, including hormonal therapy, during the last 24 weeks. During the study, all patients were on an isocaloric diet, and no significant change in body weight for at least 3 months before entry into the study was reported. Blood samples were obtained after a 12 h overnight fast. Informed consent was obtained from all patients, and the study was approved by the Ethics Committee of the University Hospital of Ioannina.

### Ultrasound scanning procedure

All examinations were performed using a high-resolution B-mode ultrasound machine (Accuson XP 128) and a linear array 7 MHz transducer on the same day as the blood collection. The subjects were examined in the prone position with the foot hanging free over the end of the table. All measurements were made on sagittal scans. The ATT was measured bilaterally, 4 cm above the insertion site to tuber calcanei. Special care was taken to place the transducer perpendicular to the tendon, avoiding an artificially hypoechogenic pattern. A supplementary examination was performed on transverse scans to reveal the presence of xanthomas outside the point of measurements. The same radiologist performed all examinations without knowing the clinical status of the subject. The echogenicity of Achilles tendon was classified into three grades: grade 1, normal (fibrillar structure of the tendon preserved); grade 2, diffuse (heterogeneous echo pattern); and grade 3, xanthomas (focal hypoechoic lesions) (13).

### Analytical methods

All laboratory determinations were performed after patients had fasted for 12 h overnight. Serum levels of total cholesterol, HDL-cholesterol, and triglycerides were determined enzymatically on the Olympus AU600 Clinical Chemistry analyzer (Olympus Diagnostica, Hamburg, Germany). Serum LDL-cholesterol was calculated using the Friedewald formula (provided that triglycerides levels were  $<$ 350 mg/dl) (21). ApoB-100 levels were measured with a Behring Nephelometer BN100 (Liederbach, Germany) (22).

### Preparation of various types of OxLDL

LDL ( $d = 1.019$ – $1.063$  g/ml) was isolated by sequential ultracentrifugation from pooled fresh plasma (23). Before oxidation, a part of purified LDL was incubated with 0.5 mM pefabloc for 1 h at 37°C to irreversibly inactivate the Lp-PLA<sub>2</sub>; then it was dialyzed extensively against 10 mM PBS to remove the excess of pefabloc (10). LDL with active Lp-PLA<sub>2</sub> or pefabloc-treated LDL [containing inactive Lp-PLA<sub>2</sub> and denoted LDL(-)], at a final concentration of 100  $\mu$ g protein/ml, was oxidized in the presence of 5  $\mu$ M CuSO<sub>4</sub> for 3 h at 37°C. The kinetics of oxidation were determined by monitoring the increase in the absorbance at 234 nm every 10 min (10). Oxidation of LDL or LDL(-) was terminated by the addition of 0.01% (v/v) EDTA at the end of the lag phase [OxLDL<sub>L</sub> or OxLDL(-)<sub>L</sub>, respectively], at the end of the propagation phase [OxLDL<sub>P</sub> or OxLDL(-)<sub>P</sub>, respectively], or at 3 h of oxidation during the decomposition phase [OxLDL<sub>D</sub> or OxLDL(-)<sub>D</sub>, respectively]. The cutoff point between the lag and propagation phases was determined as described recently (10). All types of OxLDL were stored at 4°C under nitrogen for up to 4 weeks.

### Measurement of antibody titers

The serum autoantibody titers of IgG class against all forms of OxLDL were determined by an ELISA method, as we described recently (10). Briefly, ELISA microplates were coated with 10  $\mu$ g of protein of each type of OxLDL described above or native LDL and incubated overnight at 4°C. After washing, the microplates were postcoated with 1% gelatin. After an extensive wash, 50  $\mu$ l of the serum samples (diluted 1:50, v/v) was added to each well and incubated for 1 h at room temperature. To estimate the nonspecific binding of antibodies, experiments were also performed in gelatin-coated wells. After incubation, microplates were washed and then 50  $\mu$ l of an HRP-conjugated rabbit anti-human IgG monoclonal antibody (Dako Cytomation) (diluted

1:1,000, v/v) was added to the wells and incubated for 1 h at room temperature. After the removal of the unbound HRP-conjugated secondary antibody, enzyme reaction was performed using *o*-phenylenediamine and H<sub>2</sub>O<sub>2</sub> as substrates, and the optical density was measured at 492 nm (10). The serum autoantibody titers of IgM class against all forms of OxLDL were also determined by the same method using 50  $\mu$ l of the serum samples (diluted 1:10, v/v) and HRP-conjugated rabbit anti-human IgM monoclonal antibody (Dako Cytomation) (diluted 1:2,000, v/v). The results were expressed as the ratio of antibody binding to various types of OxLDL or OxLDL(-) versus native LDL or LDL(-), respectively. It should be noted that nonspecific binding (the binding of serum samples to gelatin-coated wells) was remarkably low, at  $0.021 \pm 0.005$  and  $0.034 \pm 0.008$  for IgG and IgM antibodies, respectively.

### Measurement of OxLDL levels

Plasma levels of OxLDL were measured by a competitive enzyme-linked immunosorbent assay using a specific murine monoclonal antibody (4E6) according to the instructions provided by the manufacturer (Mersodia, Uppsala, Sweden). The specificity of this method was studied by performing the assay in five different plasma samples in which 5 or 15 ng of protein of native LDL or OxLDL<sub>D</sub> was added exogenously. Intra-assay and interassay coefficients of variation of the assay were 6.0% and 7.0%, respectively.

### Determination of Lp-PLA<sub>2</sub> activity

Lp-PLA<sub>2</sub> activity in plasma and in HDL-rich plasma (HDL-Lp-PLA<sub>2</sub> activity) was measured by the trichloroacetic acid precipitation procedure using 1-*O*-hexadecyl-2-[<sup>3</sup>H-acetyl]*sn*-glycero-3-phosphocholine ([<sup>3</sup>H]PAF) as a substrate (100  $\mu$ M final concentration), as we described previously (22). The reaction was performed for 10 min at 37°C, and Lp-PLA<sub>2</sub> activity was expressed as nmol PAF degraded/min/ml plasma.

### Statistical analysis

All results are expressed as means  $\pm$  SD. Continuous variables were tested for lack of normality by the Kolmogorov-Smirnov test. For comparisons between individual groups, statistical analysis was performed by the independent-samples *t*-test for parameters with normal distribution; otherwise, the Mann-Whitney *U*-test was used. To assess the correlations between the various parameters, Pearson and Spearman correlation coefficients were calculated. To compare antibody titers between the different phases of oxidation, we also used one-way ANOVA. Moreover, stepwise multiple regression analysis was performed to examine factors independently associated with ATT in FH patients. Thus, in this model, we used ATT as a dependent variable and all parameters that were significantly correlated with ATT in the univariate analysis as independent variables. All statistical analyses were carried out with SPSS 12.0 (SPSS, Inc., Chicago, IL). *P* < 0.05 was considered statistically significant.

## RESULTS

### Characteristics of the study population

The clinical and biochemical characteristics of the study population are shown in **Table 1**. As expected, FH patients had significantly higher levels of serum total cholesterol, LDL-cholesterol, and triglycerides compared with normolipidemic individuals. Lp-PLA<sub>2</sub> activity in total plasma was

TABLE 1. Clinical and biochemical characteristics of the study population

Parameters	Controls (n = 40)	FH Patients (n = 80)
Age, years	43.0 $\pm$ 9.7	45.0 $\pm$ 14.6
Men/women, n/n	15/25	32/48
Smokers, n (%)	15 (37.5)	24 (30)
Body mass index, kg/m <sup>2</sup>	25.1 $\pm$ 6.5	24.4 $\pm$ 3.2
Total cholesterol, mg/dl	202 $\pm$ 32	322 $\pm$ 40 <sup>a</sup>
LDL-cholesterol, mg/dl	136 $\pm$ 26	235 $\pm$ 34 <sup>a</sup>
HDL-cholesterol, mg/dl	51.4 $\pm$ 9.0	50.5 $\pm$ 11.8
Triglycerides, mg/dl	80.0 $\pm$ 44.1	183 $\pm$ 56.0 <sup>a</sup>
ApoB-100, mg/dl	141 $\pm$ 26	168 $\pm$ 31 <sup>a</sup>
OxLDL, U/l	42.2 $\pm$ 14.8	109.1 $\pm$ 28.1 <sup>a</sup>
LDL-cholesterol/apoB-100	0.99 $\pm$ 0.29	1.27 $\pm$ 0.31
OxLDL/apoB-100, U/mg	0.03 $\pm$ 0.01	0.07 $\pm$ 0.02 <sup>a</sup>
Total plasma Lp-PLA <sub>2</sub> activity, nmol/ml/min	54.8 $\pm$ 14.1	63.2 $\pm$ 22.2 <sup>b</sup>
HDL-Lp-PLA <sub>2</sub> activity, nmol/ml/min	3.3 $\pm$ 1.5	3.4 $\pm$ 1.3
Achilles tendon xanthomas, n (%) <sup>c</sup>	—	15 (18.8)
Echostructure of Achilles tendon		
Grade 1, normal, n (%)	40 (100.0)	42 (52.5)
Grade 2, diffuse, n (%)	—	30 (37.5)
Grade 3, xanthomas, n (%)	—	8 (10.0)

ApoB-100, apolipoprotein B; FH, familial hypercholesterolemia; HDL-Lp-PLA<sub>2</sub>, HDL-associated LDL-associated phospholipase A<sub>2</sub>; Lp-PLA<sub>2</sub>, LDL-associated phospholipase A<sub>2</sub>; OxLDL, oxidized low-density lipoprotein. Values are means  $\pm$  SD unless indicated otherwise.

<sup>a</sup>*P* < 0.001 compared with controls.

<sup>b</sup>*P* < 0.05 compared with controls.

<sup>c</sup>Achilles tendon xanthomas were detected by physical examination.

also significantly higher in FH patients compared with controls, whereas HDL-Lp-PLA<sub>2</sub> activity was similar between the two groups, a finding in accordance with our previously published results (22). Achilles tendon xanthomas were detected by physical examination in only 15 FH patients (18.75%) (Table 1). Furthermore, four patients exhibited xanthomas in other locations (two elbows and two tibia).

### OxLDL levels

FH patients exhibited significantly higher plasma OxLDL levels than controls. The ratio of OxLDL to apoB-100 (i.e., the proportion of oxidized apoB-100 to total apoB-100) was also significantly higher in FH patients compared with controls (Table 1). OxLDL was positively correlated with total cholesterol (*r* = 0.48, *P* = 0.004), LDL-cholesterol (*r* = 0.471, *P* = 0.005), and apoB-100 (*r* = 0.653, *P* = 0.001). Moreover, OxLDL and the ratio of OxLDL to apoB-100 were positively correlated with total plasma Lp-PLA<sub>2</sub> activity (*r* = 0.436, *P* = 0.02 and *r* = 0.354, *P* = 0.04, respectively), whereas a negative correlation was observed between these parameters and HDL-Lp-PLA<sub>2</sub> activity (*r* = -0.343, *P* < 0.05 and *r* = -0.339, *P* < 0.05, respectively). In an effort to investigate the specificity of the assay used to determine OxLDL in plasma, we added two different amounts of either native LDL or OxLDL into five different plasma samples containing  $88 \pm 12$  U/l OxLDL. We found that in plasma enriched with 5 or 15 ng of nonoxidized native LDL, the OxLDL levels determined with this assay were increased to  $141 \pm 21$  or  $399 \pm 44$  U/l, respectively. Similarly, enrichment of the plasma samples with 5 or 15 ng



of OxLDL<sub>D</sub> resulted in an increase of OxLDL levels to  $175 \pm 30$  or  $499 \pm 63$  U/l, respectively.

### Autoantibody titers against all types of OxLDL

We determined the autoantibody titers of IgG and IgM class against three different types of OxLDL (OxLDL<sub>L</sub>, OxLDL<sub>P</sub>, and OxLDL<sub>D</sub>) and three types of OxLDL(-) [OxLDL(-)<sub>L</sub>, OxLDL(-)<sub>P</sub>, and OxLDL(-)<sub>D</sub>] prepared as described in Methods.

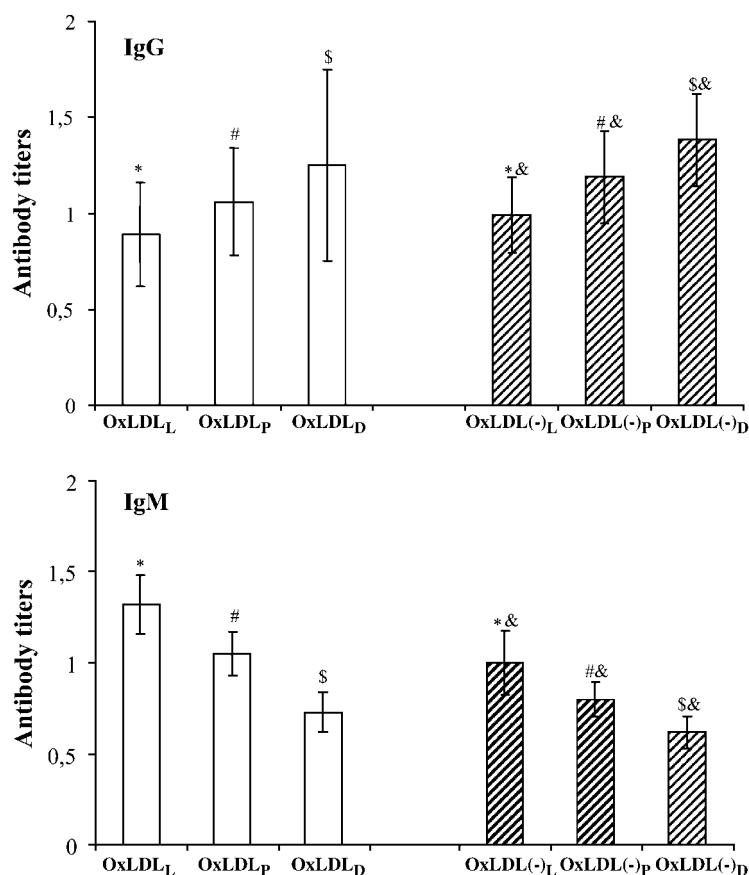
IgG and IgM antibody titers against all types of OxLDL and OxLDL(-) were detected in FH patients as well as in controls. The IgG titers in FH patients were significantly higher when the OxLDL<sub>P</sub> and OxLDL<sub>D</sub> types were used as antigens compared with OxLDL<sub>L</sub>, the titers against OxLDL<sub>D</sub> being higher than those against OxLDL<sub>P</sub> (Fig. 1). By contrast, FH patients exhibited higher IgM titers against OxLDL<sub>L</sub> compared with OxLDL<sub>P</sub> and OxLDL<sub>D</sub> types, the titers against OxLDL<sub>D</sub> being lower than those against OxLDL<sub>P</sub>. The same phenomenon was observed when the OxLDL(-) types were used as antigens (Fig. 1). Furthermore, the IgG titers against each type of OxLDL(-) were higher compared with the respective type of OxLDL, whereas the opposite phenomenon was observed for the IgM titers (Fig. 1). Similar results were obtained for the IgG or IgM titers measured in the control population (data not shown). Interestingly, FH patients exhibited significantly higher IgG autoantibody titers against OxLDL<sub>P</sub> and OxLDL<sub>D</sub> compared with controls

(Table 2), whereas no difference in the IgM titers against any type of OxLDL or OxLDL(-) was observed between the two groups (data not shown). It should be noted that no correlation was found between the IgG or IgM titers against any type of OxLDL and the plasma levels of OxLDL.

### Sonographic findings

Normal echostructure of Achilles tendon, in which the fibrillar structure of the tendon was preserved (grade 1), was observed in all subjects in the control group. Normal echostructure of Achilles tendon was also observed in 42 FH patients, whereas 38 patients (47.5%) exhibited abnormal echostructure. Among the patients with abnormal echostructure, 30 exhibited diffuse heterogeneous echo patterns (grade 2), whereas xanthomas with focal hypoechoic lesions in Achilles tendon (grade 3) were observed in eight patients (Table 1). As shown in Fig. 2, significant differences in ATT values were observed among grades 1, 2, and 3 in FH patients. Importantly, among FH patients with abnormal tendon echostructure (grades 2 and 3), men exhibited significantly higher ATT values compared with women ( $5.89 \pm 1.02$  vs.  $5.00 \pm 0.81$ ;  $P < 0.01$ ).

In univariate analysis, ATT values in the total population of FH patients were positively correlated with sex and serum LDL-cholesterol levels, whereas an inverse correlation was observed between ATT values and serum HDL-cholesterol levels (Table 3). Interestingly, among the



**Fig. 1.** Bar graphs showing the autoantibody titers as a function of the oxidation phase of oxidized low density lipoprotein (OxLDL) and OxLDL(-) in familial hypercholesterolemia (FH) patients. LDL with active LDL-associated phospholipase A<sub>2</sub> (Lp-PLA<sub>2</sub>) or pefabloc-treated LDL [containing inactive Lp-PLA<sub>2</sub> and denoted LDL(-)] at a final concentration of 100 µg protein/ml was oxidized in the presence of 5 µM CuSO<sub>4</sub> for 3 h at 37°C. Oxidation of LDL or LDL(-) was terminated by the addition of 0.01% (v/v) EDTA at the end of the lag phase [OxLDL<sub>L</sub> or OxLDL(-)<sub>L</sub>, respectively], at the end of the propagation phase [OxLDL<sub>P</sub> or OxLDL(-)<sub>P</sub>, respectively], or at 3 h of oxidation during the decomposition phase [OxLDL<sub>D</sub> or OxLDL(-)<sub>D</sub>, respectively]. The serum autoantibody titers of IgG class or IgM class against all types of OxLDL were determined by an ELISA method. \*  $P < 0.001$  compared with OxLDL<sub>P</sub> and OxLDL<sub>D</sub>; #  $P < 0.001$  compared with OxLDL<sub>L</sub> and OxLDL<sub>D</sub>; \$  $P < 0.05$  compared with LDL<sub>P</sub> and OxLDL<sub>L</sub>; &  $P < 0.05$  compared with the respective type of OxLDL. Results are expressed as means  $\pm$  SD.

TABLE 2. Autoantibody titers of IgG class against various types of OxLDL in FH patients and controls

Antigen	Controls (n = 40)	FH Patients (n = 80)
OxLDL <sub>L</sub>	0.86 ± 0.25	0.89 ± 0.27
OxLDL <sub>P</sub>	0.91 ± 0.27	1.06 ± 0.28 <sup>a</sup>
OxLDL <sub>D</sub>	0.95 ± 0.34	1.25 ± 0.5 <sup>b</sup>
OxLDL(-) <sub>L</sub>	1.03 ± 0.23	0.99 ± 0.20
OxLDL(-) <sub>P</sub>	1.14 ± 0.26	1.19 ± 0.24
OxLDL(-) <sub>D</sub>	1.30 ± 0.3	1.38 ± 0.24

OxLDL<sub>L</sub>, -<sub>P</sub>, and -<sub>D</sub>, OxLDL in the lag, propagation, and decomposition phases, respectively; OxLDL(-)<sub>L</sub>, -<sub>P</sub>, and -<sub>D</sub>, OxLDL in which the endogenous Lp-PLA<sub>2</sub> was inactivated before oxidation, at the end of the lag, propagation, and decomposition phases, respectively.

<sup>a</sup>*P* < 0.05 compared with controls.  
<sup>b</sup>*P* < 0.001 compared with controls.

antibody titers measured against several types of OxLDL, ATT was positively correlated only with the IgG titers against OxLDL<sub>P</sub> and OxLDL<sub>D</sub>, whereas there was no correlation between ATT and the plasma levels of OxLDL (Table 3). Subsequently, multiple regression analysis was performed to examine factors independently associated with ATT in the FH patients. In this model, we used ATT as the dependent variable and all parameters that were significantly correlated with ATT in the univariate analysis as independent variables. As shown in Table 4, the parameters independently associated with ATT were sex, LDL-cholesterol, and antibody titers against OxLDL<sub>P</sub>.

## DISCUSSION

This study shows for the first time that autoantibodies against mildly OxLDL are increased in patients with heterozygous FH and, most importantly, that autoantibodies against OxLDL<sub>D</sub> are independently associated with ATT. By contrast, the plasma levels of OxLDL or Lp-PLA<sub>2</sub> activity, although increased significantly in FH patients compared with controls, are not correlated with ATT.

OxLDL is present in human plasma, and several studies have been devoted to detecting OxLDL by ELISA methods using monoclonal antibodies that recognize primarily a

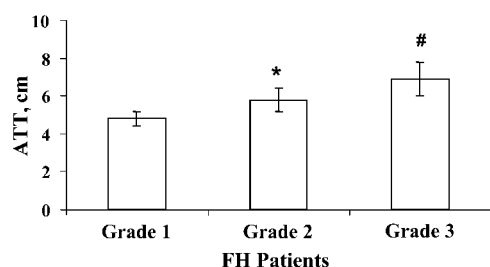


Fig. 2. Bar graph showing the Achilles tendon thickness (ATT) in FH patients as a function of the tendon echostructure grade. Grade 1, normal echostructure of Achilles tendon in which the fibrillar structure of the tendon was preserved; grade 2, diffuse heterogeneous echo pattern; grade 3, xanthomas with focal hypochoic lesions. \* *P* < 0.05 compared with either grade 1 or grade 3; # *P* < 0.01 compared with grade 1. Results are expressed as means ± SD.

TABLE 3. Univariate analysis of factors affecting ATT in FH patients

Independent Variables	<i>r</i>	<i>P</i>
Sex	0.430	0.0001
Age	-0.077	NS
Body mass index	-0.181	NS
Smoking habit	0.110	NS
Total cholesterol	0.123	NS
LDL-cholesterol	0.258	0.028
HDL-cholesterol	-0.253	0.032
Triglycerides	-0.015	NS
Lp-PLA <sub>2</sub> activity	-0.073	NS
HDL-Lp-PLA <sub>2</sub> activity	-0.075	NS
OxLDL	0.126	NS
IgG anti-OxLDL <sub>L</sub>	0.034	NS
IgG anti-OxLDL <sub>P</sub>	0.364	0.005
IgG anti-OxLDL <sub>D</sub>	0.603	0.0001
IgG anti-OxLDL(-) <sub>L</sub>	0.222	NS
IgG anti-OxLDL(-) <sub>P</sub>	0.173	NS
IgG anti-OxLDL(-) <sub>D</sub>	0.105	NS
IgM anti-OxLDL <sub>L</sub>	0.132	NS
IgM anti-OxLDL <sub>P</sub>	0.143	NS
IgM anti-OxLDL <sub>D</sub>	0.098	NS
IgM anti-OxLDL(-) <sub>L</sub>	0.111	NS
IgM anti-OxLDL(-) <sub>P</sub>	0.201	NS
IgM anti-OxLDL(-) <sub>D</sub>	0.124	NS

ATT, Achilles tendon thickness.

specific type of OxLDL or a specific epitope on OxLDL (21, 24–26). One of these methods uses the monoclonal antibody 4E6, which is directed against a conformational epitope generated as a consequence of the substitution of at least 60 lysine residues of apoB-100 with aldehydes (27). Using this assay, we show that FH patients exhibit higher OxLDL plasma levels as well as a higher ratio of OxLDL to apoB-100 levels compared with controls, results that are in accordance with recently published data (28). OxLDL was positively correlated with LDL-cholesterol and apoB-100, suggesting that LDL is an important determinant of the plasma levels of OxLDL as determined with the present assay. However, using different antibodies against OxLDL, other studies failed to show any correlation between OxLDL and LDL levels (25, 29). In this regard, we show that the present assay does not have a high specificity for OxLDL and that it also detects nonoxidized LDL in plasma. However, the ratio of OxLDL to apoB-100 in FH patients is 2-fold higher compared with that in controls, a phenomenon not observed for the ratio of LDL-cholesterol to apoB-100, suggesting that although this method does not have a high specificity for OxLDL, it may give useful information for the OxLDL levels in plasma when they are expressed as a ratio to apoB-100 levels. It is important that further studies be performed before a con-

TABLE 4. Multivariate analysis of factors affecting ATT in FH patients

Independent Variables	Beta	<i>P</i>
Sex	0.261	0.031
LDL-cholesterol	0.284	0.015
HDL-cholesterol	-0.191	NS
Anti-OxLDL <sub>P</sub>	0.135	0.285
Anti-OxLDL <sub>D</sub>	0.280	0.038

clusion is drawn regarding the specificity of the methods used to detect OxLDL in plasma.

This study shows for the first time that OxLDL is positively correlated with total plasma Lp-PLA<sub>2</sub> activity. This correlation could be simply attributed to the fact that plasma Lp-PLA<sub>2</sub> is associated mainly with LDL, which is positively correlated with OxLDL. However, enzyme activity was also positively correlated with the ratio of OxLDL to apoB-100; thus, we may not exclude the possibility that the increased Lp-PLA<sub>2</sub> activity in FH patients leads to the liberation of increased amounts of free aldehydes (through the degradation of OxPL). These react with lysine residues of apoB-100 (30), generating increased amounts of immunogenic epitopes on OxLDL recognized by 4E6. In contrast to total plasma Lp-PLA<sub>2</sub>, HDL-Lp-PLA<sub>2</sub> activity is negatively correlated with plasma OxLDL levels. This phenomenon could be attributed to the HDL-mediated protection of LDL from oxidation, in which HDL-Lp-PLA<sub>2</sub> plays an important role (5).

Despite the increased plasma levels of OxLDL in FH patients, this parameter is not correlated with ATT. Thus, although OxLDL is important for lipid deposition and the formation of foam cells in Achilles tendons (15), its plasma levels may not be a determining factor for the formation of tendon xanthomas. Indeed, macrophages from FH patients with tendon xanthomas exhibit higher predisposition to foam cell formation in the presence of OxLDL and are more sensitive to the inflammatory activities of OxLDL compared with macrophages from FH patients without tendon xanthomas, suggesting that the important determinant for their formation is the genetically determined higher sensitivity of macrophages to OxLDL actions (17).

This study further showed that the autoantibody titers of IgG class against OxLDL<sub>P</sub> and OxLDL<sub>D</sub> are increased in FH patients compared with controls, whereas no difference between these two groups was observed in the IgM titers against all types of OxLDL or OxLDL(-). Contrasting results have been published regarding whether the IgG antibodies against OxLDL have any causal role in atherosclerotic diseases (8, 21, 31–33), whereas most of the studies suggest that IgM OxLDL antibodies may play an antiatherogenic role (reviewed in 18). In this regard, lower plasma IgM OxLDL autoantibodies have been noted in patients with myocardial infarction (21, 30), stable coronary artery disease (30, 34), and borderline hypertension (33) and have been inversely associated with increased carotid intima thickness (34). Because only the IgG titers against OxLDL<sub>P</sub> and OxLDL<sub>D</sub> are increased in our FH patients, we suggest that IgG antibodies against mildly oxidized forms of LDL (up to 3 h of oxidation) may be primarily involved in the premature atherosclerosis observed in FH. By contrast, a previous study did not find any difference in the autoantibody titers between heterozygous FH patients and normolipidemic controls; however, extensively OxLDL (at least 12 h of oxidation) or malondialdehyde-LDL were used as antigens in that study (32). Overall, we suggest that the extent of LDL oxidation significantly influences the plasma autoantibody levels in

FH patients. This is further supported by our recently published data in patients with stable coronary artery disease (10). According to these results, the OxLDL<sub>P</sub> and OxLDL<sub>D</sub> types are enriched in lyso-PC and free aldehydes but contain significantly less OxPL compared with the corresponding OxLDL(-) types, as a result of the Lp-PLA<sub>2</sub>-mediated hydrolysis of OxPL. Thus, we suggest that epitopes formed in the presence of lyso-PC and aldehydes may be primarily responsible for the increased IgG titers observed in FH patients. However, these epitopes need to be characterized in future studies.

Importantly, only the IgG titers against OxLDL<sub>D</sub> are independently associated with ATT, suggesting that these antibodies are involved in the pathogenesis of Achilles tendon xanthomas. The pathogenesis of tendon xanthomas resembles that of atherosclerotic plaque (13). The role of immune mechanisms in atherosclerosis is complicated, and there are contradictory results regarding whether autoantibodies against OxLDL or OxLDL immune complexes may promote or inhibit atherosclerosis (8, 18). Thus, it remains to be established whether the IgG antibodies against OxLDL<sub>D</sub> promote or inhibit the development of Achilles tendon xanthomas. Furthermore, prospective studies are required to demonstrate whether the IgG titers against OxLDL<sub>D</sub> in FH patients may have any prognostic value for the development of Achilles tendon xanthomas, which are associated with cardiovascular risk factors and a higher incidence of coronary artery disease in this population (14).

In conclusion, this study demonstrates for the first time that IgG titers against OxLDL<sub>D</sub> are independently associated with ATT, suggesting that they may play a role in the pathogenesis of ATX in heterozygous FH patients. ■■

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