Antioxidative and Antiatherosclerotic Effects of Human Apolipoprotein A-IV in Apolipoprotein E–Deficient Mice

Maria A. Ostos, Mariangela Conconi, Laurent Vergnes, Nadine Baroukh, Josep Ribalta, Josefa Girona, Jean-Michel Caillaud, Alberto Ochoa, Mario M. Zakin

Abstract—Mice expressing human apolipoprotein A-IV (apoA-IV) mainly in the intestine were obtained in an apolipoprotein E–deficient (apoE0) background (apoA-IV/E0 mice). Quantification of aortic lesions and plasma lipid determination showed that compared with their control apoE0 counterparts, the apoA-IV/E0 mice are protected against atherosclerosis without an increase in HDL cholesterol. Because oxidized lipoproteins play an important role in atherogenesis, we tested whether the protection observed in these animals is accompanied by an in vivo reduction of the oxidation parameters. The lag time in the formation of conjugated dienes during copper-mediated oxidation, the aggregation state of LDL, and the presence of anti–oxidized LDL antibodies were measured. The presence of oxidized proteins in tissues and the presence of oxidation-specific epitopes in heart sections of atherosclerotic lesions were also analyzed. Except for lag time, the results showed that the oxidation parameters were reduced in the apoA-IV/E0 mice compared with the apoE0 mice. This suggests that human apoA-IV acts in vivo as an antioxidant. In addition, human apoA-IV accumulation was detected in the atherosclerotic lesions of apoA-IV/E0 mice, suggesting that apoA-IV may inhibit oxidative damage to local tissues, thus decreasing the progression of atherosclerosis.

Key Words: antioxidants | apolipoprotein A-IV | atherosclerosis | transgenic mice

Oxidized lipoproteins play an important role in the early stages of atherogenesis. Many studies have documented their occurrence in atherosclerotic lesions, leading to cholesterol ester accumulation and foam cell formation. Numerous mechanisms have been proposed by which the oxidation of lipoproteins could increase the progression of atherosclerosis. These include their enhanced uptake by macrophages, their chemotactic and cytotoxic properties, their immunogenic properties leading to the formation of autoantibodies, and their ability to alter the gene expression of neighboring cells.

Recently, studies to determine the antiatherogenic effect of different substances have demonstrated that lipid-soluble antioxidants reduce lesion formation by mechanisms independent of lipid lowering.

ApoE-deficient (apoE0) mice, which spontaneously develop severe atherosclerosis, represent an appropriate model for testing the oxidation theory of atherosclerosis. In these animals, studies using antisera specific for malondialdehyde (MAD)-lysine and 4-hydroxynonenal (4-HNE)–lysine have revealed the existence of oxidation-specific epitopes in atherosclerotic lesions and have detected important levels of autoantibodies directed against MAD-lysine in their plasma.

ApoA-IV is a 46-kDa plasma protein primarily synthesized in the enterocytes of the small intestine during fat absorption. Although apoA-IV is a major circulating apolipoprotein, its role in lipid transport and lipoprotein metabolism is still not clear. ApoA-IV has been shown to participate in the absorption of dietary fat, in triglyceride transport, and in reverse cholesterol transport. Moreover, apoA-IV appears to modulate the activity of lecithin-cholesterol acyltransferase, cholesterol ester transfer protein, and lipoprotein lipase.

Previous studies have shown that overexpression of apoA-IV in transgenic mice confers significant protection against atherosclerosis. In animals overexpressing mouse apoA-IV, the protection was observed after an atherogenic diet and was accompanied by enhanced HDL cholesterol levels. Mice overexpressing human apoA-IV only in liver were also protected after a cholesterol-rich diet and presented a higher HDL cholesterol concentration and cholesterol efflux in plasma. However, in an apoE-deficient background, the protection was accompanied by an increase in non-HDL cholesterol levels only. These results indicated that apoA-IV may protect against atherosclerosis by mechanisms that are not always related directly to the levels of HDL cholesterol.

Recent in vitro and ex vivo studies have demonstrated that purified rat apoA-IV presented antioxidant properties by protecting LDLs against copper-induced oxidation and fasting intestinal lymph of C57Bl/6 mice against macrophage...
Plasma Concentration of Lipid and Human ApoA-IV in Transgenic Mice and in Control Counterparts

<table>
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<tr>
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<th>C57B1/6</th>
<th>ApoA-IV</th>
<th>ApoE0</th>
<th>ApoA-IV/E0</th>
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<td>(n=9)</td>
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<td>Non-HDL cholesterol, mg/dL</td>
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<td>528±45†</td>
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<tr>
<td>Human ApoA-IV, mg/dL</td>
<td>...</td>
<td>34±6</td>
<td>...</td>
<td>106±6‡</td>
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Values are mean±SD. The numbers of animals examined in parentheses; only data obtained from males are shown. Similar results were observed in females.

*P<0.05 apoE0 vs C57B1/6; †P<0.05 apoA-IV/E0 vs apoE0; ‡P<0.01 apoA-IV/E0 vs apoA-IV.

mediated oxidation.14 These results led to the hypothesis that apoA-IV might be a potent inhibitor of lipid oxidation.

At present, no information is available about the protective role of intestinal human apoA-IV. Similarly, no studies have been undertaken to investigate the protective effect of human apoA-IV against oxidation under in vivo pathological conditions of oxidative stress. We have recently generated transgenic mice expressing human apoA-IV mainly in the intestine.15 In the present study, we have crossed these animals with apoE0 mice to obtain apoA-IV/E0 hybrid mice, and we have first quantified atherosclerotic lesions to measure the protective effect of the human protein. We then studied plasma and lesion oxidation parameters to analyze whether apoA-IV acts in vivo as an inhibitor of lipid oxidation.

Methods

Experimental Animals

Studies were performed in the human apoA-IV transgenic line 8021,15 in which plasma human apoA-IV levels are close to those normally found in humans (34±6 mg/dL, Table). Hybrid mice were produced by crossing animals of line 8021 with apoE0 mice. Both lines have a C57BL/6 genetic background. Double heterozygotes were backcrossed to apoE0 to produce apoA-IV heterozygous transgenic animals homozygous for apoE0 (apoA-IV/E0). Mice were housed in a temperature-controlled room with an alternating light and dark period. They were fed a mouse chow diet and had access to water ad libitum. Because blood collection, food was removed from the cages at 09:00 AM, and 8 hours later, samples were collected from the retro-orbital sinus under isoflurane anesthesia. Different sets of animals from the same colony were used.

All procedures involving animal handling and their care were in accordance with French Pasteur Institut Guidelines for Husbandry of Laboratory Mice.

Plasma Lipid and ApoA-IV Quantification

Mice aged 5 to 6 months were used. Total plasma triglycerides, total cholesterol, and HDL cholesterol were determined enzymatically by using commercial kits (Boehringer-Mannheim).

Human apoA-IV in plasma was quantified by immunonelectrophoresis (Hydragels SEBIA); the antibodies used did not recognize mouse apoA-IV.

Evaluation of Aortic Lesions

Atherosclerotic lesions were quantified by evaluating cross-sectional lesion sizes in the aortic sinus.16 Female mice were used because they are more susceptible to the development of atherosclerotic lesions. Randomly chosen subsets of 10 apoA-IV/E0 and 10 apoE0 mice were euthanized at 5 months of age. Hearts and proximal aortas were removed and fixed. Hearts were cut directly under and parallel to the leaflet, and the upper portions were embedded in OCT medium (Miles Laboratories) and frozen. Ten-micron-thick sections were cut through the aortic sinus. Eighty sections per animal were stained for lipids with oil red O and counterstained with hematoxylin. Cross-sectional areas were analyzed by computerized planimetry. Statistical analysis was performed on 4 different cross-sectional lesion areas per animal, separated by 160 μm. The first section analyzed for each animal corresponded to the origin of the aortic sinus.

Lipoprotein Separation

Lipoproteins from a pool of plasma from apoA-IV/E0 and apoE0 male mice were separated into density (d) fractions (d<1.006, d=1.006 to 1.019, and d=1.019 to 1.063 g/mL) by sequential isopycnic ultracentrifugation, with KBr used to adjust densities.17

Susceptibility of Lipoproteins to Oxidation

The in vitro oxidation susceptibility was assessed in LDL and remnants. Three lipoprotein preparations were examined in each animal group. Lipoproteins were filtered over a G25 Sephadex column equilibrated with 50 mmol/L Tris-HCl buffer, pH 7.4, to remove EDTA and salts. Lipoprotein aliquots (equivalent to 60 mg cholesterol/L) were incubated at 37°C with CuSO4 (5 μmol/L final concentration). The appearance of conjugated dienes was monitored at 234 nm every 10 minutes for 12 hours. The lag phase was defined by the intercept of the tangent drawn to the steepest segment of the propagation phase to the horizontal axis.

LDL Aggregation

The aggregation state of LDL was analyzed by measuring the turbidity generated by aggregates.18 LDL cholesterol (0.5 mg per milliliter of PBS) was homogenized, and absorbance at 680 nm was measured against a PBS blank solution.

Immunodetection by Western Blot of Oxidatively Modified Proteins

Liver and heart from 8-month-old male apoA-IV/E0 and apoE0 mice were used. Mice were killed, and the abdomen and thorax were opened. After vascular perfusion with 50 mmol/L Tris-HCl buffer (pH 7.5), the liver and heart were excised. The organs were then washed and homogenized with a Potter-Elvehjem device in 3 vol of 50 mmol/L Tris-HCl buffer (pH 7.5). The homogenate was centrifuged at 17 600g for 40 minutes, and the supernatant fraction was retained for study.

Immunodetection was performed as described,19 with use of the oxidized protein detection kit Oxyblot (Oncor). The kit provides reagents for immunodetection of carbonyl groups introduced into protein side chains. These carbonyl groups were derivatized to 2- to 4-dinitrophenyl (DNP) hydrazone by reaction with 2,4-dinitrophenylhydrazine. The DNP-derivatized protein samples were separated by SDS-PAGE (10 μg of protein per lane), followed by blotting. After transferring the proteins to nitrocellulose, the membranes were incubated with primary antibody specifically recognizing the DNP moiety of the proteins and with horseradish peroxidase–conjugated antibody directed against the primary antibody. The protein was detected by using a chemiluminescent reagent (ECL, Amersham, Pharmacia Biotech). To assess the distribution and selectivity of 4-HNE modifications in proteins, Western blot analyses were performed by using an anti–4-HNE serum.20

To control the sample load, an additional SDS-PAGE was stained with Coomassie blue.

Immunohistochemistry

Three- and 6-month-old apoA-IV/E0 and apoE0 male mice were used. The thorax and abdomen were opened, and the heart was perfused with PBS containing 20 μmol/L butylated hydroxytoluene and 2 μmol/L EDTA, pH 7.4. The heart was removed and fixed overnight with PBS-formal-sucrose (PBS, 4% paraformaldehyde, 5% sucrose, 20 μmol/L butylated hydroxytoluene, and 2 μmol/L EDTA, pH 7.4). Tissue sections were prepared in a cryostat as described above. The following antibodies were used: MDA2 (monoclonal antibody against MAD-modified LDL) and NA59 (monoclonal antibody against 4-HNE–modified LDL)21 with both conjugated to biotin. Human apoA-IV was detected with a rabbit antiserum specific for
the human protein that does not cross-react with the mouse apoA-IV. Tissue sections were pretreated with acetone and incubated with 2% gelatin in PBS at 37°C for 45 minutes in a humidified chamber. After they were washed with 0.1% Triton X-100 in PBS, the sections were incubated with primary antibodies (1:50 and 1:20 dilution for MAD2 and NA59, respectively, and 1:20 dilution for apoA-IV antiserum) for 1 hour. Sections were washed and incubated for another hour with streptavidin conjugated with alkaline phosphatase or with fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (Sigma Chemical Co) and washed 3 times. In the case of biotinylated antibodies, color was developed with Fast Red TR/Naphthol AS-MX (Sigma). As a negative control, the tissue sections were incubated without primary antibody, and in all cases, they were devoid of specific staining.

**Determination of Anti–Oxidized LDL Antibodies**

Human LDL was isolated as described.22 Oxidized LDL was prepared by incubating LDL (1 mg/mL) with 5 μmol/L CuSO4 in PBS overnight at 37°C. Before each assay, 96-well microtiter plates were freshly coated with 100 μL of oxidized LDL (5 μg/mL) in PBS overnight at 4°C. The wells were blocked with 1% BSA for 2 hours at room temperature. A 100 μL aliquot of diluted sera (1:40) from apoA-IV/E0 and apoE0 male mice was added in duplicated wells and incubated for 2 hours at room temperature. After 3 washes with PBS containing 0.1% Tween 20, goat anti-mouse IgM conjugated with alkaline phosphatase (1:1000 dilution, Sigma) was added to each well, and incubation continued for 2 hours at room temperature. Plates were washed again, and the alkaline phosphatase activity was determined by using p-nitrophenyl phosphate as a substrate and detected at 405 nm.

**Statistical Analysis**

Data were expressed as mean±SD. Statistical analyses were performed by ANOVA. Differences were considered significant at P<0.05. All analyzed variables except triglycerides were normally distributed, as assessed by the Kolmogorov-Smirnov test. Triglyceride values were logarithmically transformed to achieve an approximately normal distribution, and ANOVA was then applied to the transformed values.

**Results**

**Human ApoA-IV Expressed in Intestine Protects Against Atherosclerosis Without an Increase in HDL Cholesterol Concentration**

Plasma lipid levels were determined in male (Table) and female (data not shown) mice after 8 hours of fasting conditions. Intestinal human apoA-IV expression in mice did not significantly affect the fasting levels of triglycerides, total cholesterol, HDL cholesterol, and non-HDL cholesterol in a normal genetic background. In contrast, in apoA-IV/E0 mice, a significant increase in triglyceride and total cholesterol concentration was detected, resulting in a more severe atherogenic lipoprotein profile than that of apoE0 mice. Moreover, the increase in cholesterol was exclusively observed in the non-HDL fraction. In addition, apoE deficiency caused an increase in human apoA-IV plasma levels in apoA-IV/E0 mice. Similar results were observed in male and female mice.

To evaluate the effect of human apoA-IV overexpression on the progression of atherosclerosis, we performed a morphometric quantification of the cross-sectional area of lesions. We carried out the study in 10 apoA-IV/E0 and 10 apoE0 female counterparts and found that overexpression of apoA-IV significantly reduced atherosclerotic lesions in the aortic root by 41% (260.9±26.3 versus 155.2±20.9, respectively; P<0.02).

**Human ApoA-IV Expression Decreases Plasma and Lesion Oxidation Parameters in ApoE0 Mice**

The degree of protection of remnant and LDL against in vitro oxidation can be assessed by determining the lag time in the formation of conjugated dienes during copper-mediated oxidation.23 In the present study, we found no significant differences in the lag times of either remnants or LDL between apoA-IV/E0 and apoE0 mice (105±20 versus 118.3±17.6 minutes, respectively, for remnants and 98.3±22.6 versus 115.7±19.3 minutes, respectively, for LDL).

Aggregation of LDL represents another lipoprotein modification with atherogenic properties, because aggregated LDL is taken up by macrophages at an increased rate, which causes foam cell formation.24,25 In addition, extensive oxidation of LDL leads to its aggregation,26 and these modified forms of LDL are present in the atherosclerotic lesion.27 Figure 1A shows that plasma LDL aggregation decreased twice as much in apoA-IV/E0 mice compared with apoE0 mice. To detect the presence of oxidized proteins in the heart, we performed Western blot experiments with the use of antibodies directed against 4-HNE or DNP. Experiments were performed in the liver and heart. The liver was used as a control, because in apoE0 mice, there is a reduced uptake of particles susceptible to oxidation by this organ. A prominent oxidized protein of 29 kDa was detected in the soluble fraction of heart homogenate (Figure 2). However, compared
with apoE0 heart extracts, apoA-IV/E0 heart extracts have shown less immunoreactivity toward the 4-HNE antiserum. When antibodies directed against DNP are used, the 29-kDa protein strongly interacts in only the apoE0 heart extracts.

The presence of oxidation-specific epitopes in heart sections of atherosclerotic lesions in 3- and 6-month-old mice was observed by using antibodies directed against 4-HNE–modified LDL (representative sections are shown in Figure 3A and 3B [3-month-old mice] and 3C and 3D [6-month-old mice]) and MAD–modified LDL (data not shown). In both cases, the sections from apoE0 mice were stained stronger than were similar sections from apoA-IV/E0 mice. The presence of apoA-IV in the atherosclerotic lesions of transgenic animals was examined also by immunohistochemistry. Figure 3E illustrates the presence of the human protein in the intima, localized extracellularly in the connective tissue matrix. We observed no deposit of the protein in the media, probably because the internal elastic lamina acts as an effective barrier against the deeper intrusion of lipoproteins.28 In parallel with the analyses of oxidative modification of LDL in lesions, the titer of autoantibodies to oxidized LDL was also measured and was found to be significantly lower in the sera of the apoA-IV/E0 than in the sera of the apoE0 counterparts (Figure 1B).

### Discussion

We have recently generated transgenic mice expressing human apoA-IV mainly in the intestine.15 In the present study, we have shown that these animals, in an apoE0 background, are protected against atherosclerosis by a mechanism that does not increase HDL cholesterol concentration. This was also described in apoE0 transgenic mice overexpressing human apoA-IV only in the liver.8 In vitro and ex vivo studies have indicated apoA-IV to be a potent inhibitor of lipid oxidation, and oxidatively modified LDLs have been shown to play an important role in the pathogenesis of atherosclerosis.

![Figure 2. Detection of oxidatively modified proteins in liver and heart extracts. Extracts from 8-month-old male mice were analyzed by SDS-PAGE. Anti–4-HNE and anti-DNP immunostaining and Coomassie blue protein staining are shown.](image)

![Figure 3. Photomicrographs showing oxidation-specific epitopes and human apoA-IV localization in male mouse atherosclerotic lesions. A thorough D. Heart sections of atherosclerotic lesions analyzed with a monoclonal antibody against 4-HNE–modified LDL (NA59) in 3-month-old (A and B) and 6-month-old (C and D), apoE0 (A and C), and apoA-IV/E0 (B and D) mice. Arrows indicate representative areas of the fatty streak lesions in which oxidation-specific epitope staining is different in both groups of mice. E. Section of aortic sinus from 6-month-old apoA-IV/E0 mouse analyzed with antiserum against human apoA-IV. The presence of the protein is seen in green, as indicated by an arrow. Autofluorescence in the elastic lamina, appearing in yellow, is also present in controls (not shown). Original magnification ×400 (A and B) and ×200 (C through E).](image)
atherosclerosis. These arguments prompted us to investigate whether the protective properties of the human apoA-IV expression observed in the apoA-IV/E\textsubscript{0} mice could be correlated with an increase in the in vivo antioxidative activities. Indeed, apoA-IV/E\textsubscript{0} hybrid animals are an excellent model for analyzing this situation, inasmuch as the importance of apoE\textsubscript{0} mice in studying the oxidation theory of atherosclerosis has already been demonstrated.\textsuperscript{4}

We have analyzed plasma and lesion parameters of oxidation, and we have found no differences in the oxidation susceptibility by copper of remnants and LDL in the apoA-IV/E\textsubscript{0} and apoE\textsubscript{0} mice. This suggests that the susceptibility of plasma lipoproteins to oxidation after the addition of copper does not necessarily reflect the extent of lipid peroxidation in the arterial wall, where other more complex oxidative processes are involved. Accordingly, in studies with different antioxidant treatments, a similar antioxidant protection of plasma LDL (similar lag time) was not accompanied by comparable levels of reduction of the atherosclerotic lesion formation.\textsuperscript{29} One could hypothesize that the similar lag time of remnants and LDL that we observed in both strains of mice is due to the absence of apoA-IV known to be stripped off during lipoprotein separation by ultracentrifugation.

Unlike the results for the oxidation susceptibility by copper of lipoproteins, we found that overexpression of human apoA-IV reduces other parameters associated with oxidation, such as the aggregation state; indeed, higher oxidation levels were associated with higher LDL aggregation.\textsuperscript{26} In the present study, the plasma LDL aggregation of apoA-IV/E\textsubscript{0} mice decreased 2 times relative to that of apoE\textsubscript{0} mice.

After conjugated diene decomposition, carbonyl compounds such as MDA and 4-HNE are formed, and these compounds react with matrix and cellular proteins as well as with apoB. These apoB-modified LDLs are considered to be biologically modified LDLs because they are recognized by the scavenger receptor. In contrast to the lag-time measurements, immunoreactivity assays using antibodies specific for such adducts are considered a good in vivo test of modified LDL. We have observed that the expression of human apoA-IV in apoE\textsubscript{0} mice reduced the amount of carbonyl LDL. We have also observed that overexpression of apoA-IV reduces the presence of MAD-modified and 4-HNE–modified LDL in the atherosclerotic plaques. This was observed in mice at 3 months of age, when the atherosclerotic plaque is being formed. A similar result was obtained in 6-month-old animals. This result supports the idea that apoA-IV expression prevents the oxidation in the arterial wall. According to these results, the plasma concentration of anti–oxidized LDL antibodies was significantly lower in apoA-IV/E\textsubscript{0} mice than in apoE\textsubscript{0} mice. This is consistent with the hypothesis that apoA-IV/E\textsubscript{0} transgenic animals are less exposed to oxidized LDL, producing lower levels of antibodies directed against this modified lipoprotein.

Finally, we observed an accumulation of the human apoA-IV in the atherosclerotic lesion of apoA-IV/E\textsubscript{0} mice. Immunohistochemical analysis shows an extracellular localization of apoA-IV, which suggests that the protein is not synthesized in situ but is probably transported there by the blood fluid. This is consistent with the fact that no apoA-IV mRNAs were detected in total RNA obtained from the heart of apoA-IV transgenic mice.\textsuperscript{15} It has been suggested that paraoxonase, apoA-I, and clusterin accumulate in the artery wall of the diseased vessels as a response to increased oxidative stress and that they may help to protect against damage caused by lipid peroxidation.\textsuperscript{32} Our results indicate that apoA-IV can also accumulate in the damaged arterial wall and may therefore act as an antioxidant in situ. The protein might act in the first stages of the fatty streak formation, inasmuch as LDL oxidation is a prerequisite for their uptake by macrophages and the cellular accumulation of cholesterol.

The antioxidant properties of apoA-IV may then be a contributing factor to protection against lesions, participating in the complex protective mechanism against atherosclerosis. Of course, we cannot rule out the possibility that other properties of apoA-IV are also involved in this protection. At least part of the resistance to atherosclerosis induced by apoA-IV expression could be attributable to its participation in reverse cholesterol transport. Previous studies have shown that transgenic mice overexpressing human apoA-IV in the liver are protected against atherosclerosis after an atherogenic diet. These animals exhibited significantly higher levels of HDL cholesterol than did C57Bl/6 mice. Therefore, it has been hypothesized that this protection is related to these enhanced levels and to a higher cholesterol efflux measured in vitro.\textsuperscript{8} However, Stein et al.\textsuperscript{33} have demonstrated in vivo in the same animal model that overexpression of hepatic apoA-IV does not exhibit increased levels of HDL cholesterol and does not enhance cholesterol mobilization under normal dietary conditions. Nevertheless, more recently, it has been reported that in these animals the human apoA-IV overexpression increases cAMP-stimulated cholesterol efflux from J774 macrophages to whole serum or to lipoprotein-deficient serum. These results suggest that apoA-IV, unassociated with HDL particles, might be responsible for the enhanced cholesterol efflux.\textsuperscript{9}

In conclusion, the present report shows that transgenic mice with intestinal expression of human apoA-IV in an apoE\textsubscript{0} background are protected against atherosclerosis in spite of a severe atherogenic lipoprotein profile. It also shows that compared with the apoE\textsubscript{0} mice, these mice present a marked reduction of the oxidation parameters, in the lesion and in the plasma.

Acknowledgments

This work was supported by the European Union (BMH4-CT-97/2597), by the CNRS (URA 1773), by a Marie Curie Research Training Grant (BMH4 98-5125 to M.A.O.), by the CANAM (to L.V.), and by the FRM (to N.B.). We thank B. Friguet for providing the antiserum directed against 4-HNE and J. Witztum for providing the NA59 and MAD2 antibodies.
References


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doi: 10.1161/01.ATV.21.6.1023

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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