Antioxidized LDL Antibodies Are Associated With Different Metabolic Pathways in Patients With Atherosclerotic Plaque and Type 2 Diabetes

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OBJECTIVE—Oxidized lipoproteins and antioxidized LDL antibodies (antioxLDL abs) have been detected in human plasma and atherosclerotic lesions. The principle aim of this study was to analyze the possible relationship between IgG and IgM antioxLDL abs and factors involved in different metabolic pathways (inflammation, lipid metabolism, apoptosis, and cell cycle arrest profile) in the occluded popliteal artery (OPA) compared with the femoral vein (FV).

RESEARCH DESIGN AND METHODS—Fifteen patients with advanced atherosclerosis and type 2 diabetes undergoing lower limb amputation participated in this study. Each patient had OPA and FV biopsy specimens and peripheral arterial occlusive disease. By real-time PCR, gene expression was analyzed from the OPA and FV specimens, and antioxLDL ab levels were measured by specific enzyme-linked immunosorbent assay.

RESULTS—The OPA and FV showed a positive correlation between only IgM antioxLDL ab levels and the expression of genes involved in different metabolic pathways, including inflammation (IL-1β, TNFα, NFκB), lipid metabolism (SCARB1), apoptosis (BAX, caspase 3, AKT1), plaque disruption (MMP2 and MMP10), lipid metabolism (SCARB1, PPARγ), and cell turnover (CDKN1A), and genes for transcription and growth factors (NFκB and VEGFA, respectively).

CONCLUSIONS—The results show that gene expression in the metabolic pathways (apoptosis, lipid metabolism, and inflammation) in the OPA and FV are directly related to the levels of IgM antioxLDL abs.

Oxidized LDL (oxLDL) is the most atherogenic LDL and participates in various processes that lead to plaque formation, such as induction of endothelial injury, accumulation of monocytes/macrophages, and foam cell formation (1,2). Oxidative modification of LDLs induces the formation of immunogenic epitopes in the LDL cholesterol particle, which leads to the formation of antibodies against oxLDLs that can be detected in serum (3). Therefore, LDL cholesterol is associated with thrombosis and atherosclerosis, although the pathophysiology of arterial thrombosis is different from venous thrombosis. The function of the antioxidized LDL antibodies (antioxLDL abs) in the development of atherosclerosis is complex and remains to be determined. These antibodies have been detected in patients with advanced atherosclerotic lesions (4). AntioxLDL ab levels have been shown to predict the progression of carotid atherosclerotic lesions (5) and are increased in patients with coronary disease (6,7) and peripheral vascular disease (8). However, the clinical importance of these antibodies in different cardiovascular pathologies is still under discussion. LDL composition varies by individual, and the degree of oxidative modification is difficult to control and evaluate and cannot determine the levels of antibodies against different epitopes in oxLDL particles. In this sense, relevant studies found important controversies, including an inverse association between cholesterol levels (9) and hypertension (10) with antioxLDL ab levels and improved carbohydrate metabolism as a result of rises in antioxLDL ab levels (11). Simply put, no associations have been found between hypercholesterolemia (12) and microvascular complications in patients with diabetes (13) and the degree of oxidizability in serum (14) with antioxLDL ab levels. Age and sex also may influence these antibody levels (15).

It has been demonstrated that the atherosclerotic process is associated with some signaling pathways, mainly inflammation (16) and lipid metabolism (17), and with other processes such as apoptosis (18) and cell cycle arrest profile (19). However, the association of antioxLDL abs and the cited signaling pathways has not been extensively investigated. In the present study, we aimed to check whether different biomarkers, such as tF; TFPI; TLR2/4; CD34; AGER; CRP; von Willebrand factor; SREBF1/2; Sp1; HIF1A;
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TP53; NKIRAS2; CDKN1A; BCL2; BAX; Casp3; CD83; MMP2, -3, -9, -10, -12, and -13; TIMP1/3; PPARg; Cox1/2; LDLR; LP1; NDUFA2; SCARBl; MCP1; VEGFA; and AKT1, have a correlation with antioLDL abs (IgG and IgM) in different atherosclerotic blood vessels (artery vs. vein).

RESEARCH DESIGN AND METHODS—Patients hospitalized in the Cardiovascular Surgery Department of Carlos Haya Hospital (Málaga, Spain) were recruited between February 2007 and June 2008 (N = 15). All patients had an advanced atherosclerotic process and type 2 diabetes for a mean ± SD of 12 ± 7 years. From patients with clinical stage IV peripheral arterial occlusive disease (PAOD) and amputation of inferior limbs, two types of vascular biopsy specimens were collected: occlusive popliteal artery (OPA) with atherosclerotic plaque and femoral vein (FV). As much of the OPA as the FV was obtained from the vascular bundle of each patient.

The inclusion criteria were age 18–80 years (all patients were >60 and written informed consent). Exclusion criteria were alcoholism, drug addiction, and positive test for HIV.

The presence of atherosclerotic risk factors was evaluated using the European Society of Cardiology and Hypertension definition for hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg), the American Diabetes Association 2010 definition for type 2 diabetes (repeated fasting glucose levels ≥126 mg/dL, if being treated with oral antidiabetic agents or insulin at the time of the study or if HbA1c >6.5%), the National Cholesterol Education Program–Adult Treatment Panel III criteria for triglyceride (≥150 mg/dL) and HDL cholesterol (men <40 mg/dL, women <50 mg/dL) levels, a BMI >30 kg/m² for obesity, and a smoking habit up to 6 months before the hospital admission. Anthropometric and biochemical parameters were sex, age, waist circumference, systolic and diastolic blood pressure, glucose, HbA1c, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides. Additionally, we measured the homeostasis model assessment index, which is used to quantify insulin resistance and β-cell function. The approximating equation for insulin resistance used a fasting blood sample and was derived by (glucose x insulin)/405, where glucose is given in milligrams per deciliter and insulin is given in microunits per milliliter.

The patients were admitted to the hospital 72 h before surgery, and their treatment often was modified to prepare them for surgery. For all patients, there was a drug washout period of 12 h before blood collection. The therapeutic characteristics of the patients were oral antidiabetic drugs (20.0%), including metformin, gliclazide, or repaglinide (6.7% each), insulin (86.7%); antihypertensive drugs (80.0%), including ACE inhibitors (26.7%), angiotensin receptor blockers (20%), calcium channel blockers (20%), β-blockers (20.0%), and diuretics (46.7%); hypolipemiant drugs, including statins (26.7%); antiaggregant drugs (80.0%); and anticoagulant drugs (53.3%). Each patient had a stable treatment regimen in the 6 months before the study, which also was an inclusion criterion for this study.

The study was approved by the hospital ethics committee, and all patients gave written informed consent. The study protocol complied with the principles of the Helsinki Declaration.

Histological analysis

The vascular biopsy specimens, removed immediately after surgery, were immersed in 2-methylbutane and then in liquid nitrogen. Histological studies were performed on 4-mm-thick sections of the vessels, and atheromatous plaque cut in a cryostat at −20°C was thaw mounted onto poly-l-lysine–treated slides. Tissues were then stained with Masson trichrome and photographed under routine light microscopy (Leica Microsystems Ltd.).

IgG and IgM antioLDL abs

IgG and IgM antioLDL abs were measured in duplicate as previously described (9,10). In brief, the LDL was isolated from fasting plasma from human blood donors by density gradient ultracentrifugation. OxLDL was prepared by incubating this native LDL with malonyldialdehyde (MDA). Microtiter plates for determination of IgG and IgM anti-MDA-LDL antibodies were coated with either native LDL or MDA-LDL and the serum of each patient. The binding to native LDL was considered nonspecific. The absorbance was read, and the binding of antibodies to MDA-LDL (antioLDL abs) was calculated by subtracting the binding of native LDL from the binding of MDA-LDL. The results were expressed as an optical density. The intra- and interassay coefficients of variation were 5.0 and 10.1%, respectively.

Real-time PCR

Pieces of OPA and FV vessels were homogenized using the Tripure Isolation Reagent (Roche Molecular Biochemicals, Barcelona, Spain) according to the manufacturer’s instructions in ice and a laboratory batch mixer (T25-Ultra3-Turrax Basic; Ika Equipment Laboratory). cDNA was obtained from 1 μg RNA using the High Capacity cDNA Reverse Transcription Kit (formerly the High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA). RNA purity was determined by measuring the A260/A280 ratio. RNAs with ratios between 1.7 and 2 were not used.

Table 1—Studied genes classified by metabolic pathway involved

<table>
<thead>
<tr>
<th>Genes</th>
<th>Inflammatory molecules</th>
<th>Apoptosis</th>
<th>Lipid metabolism</th>
<th>Translation-transcription regulators</th>
<th>Immune response</th>
<th>Cytoskeleton regulator</th>
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</thead>
<tbody>
<tr>
<td>CRP</td>
<td>F3 (f)</td>
<td>CRD51</td>
<td>PPARγ</td>
<td>SREBF1</td>
<td>CD86</td>
<td>Endoglin TIMP3</td>
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<td>CD83</td>
<td>TFPI</td>
<td>BCL2</td>
<td>LP1</td>
<td>TP53</td>
<td>TLR2</td>
<td>Actin A1</td>
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<td>MMP2</td>
<td>CD34</td>
<td>MMP2</td>
<td>PTGS1 (Cox1)</td>
<td>SREBF2</td>
<td>TLR4</td>
<td>MPP12 TIMP1</td>
</tr>
<tr>
<td></td>
<td>von Willebrand factor</td>
<td>MMP3</td>
<td>PTGS2 (Cox2)</td>
<td>ABCA1</td>
<td></td>
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<tr>
<td></td>
<td>AGER</td>
<td>AKT1</td>
<td>CD36</td>
<td>TFP1</td>
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<td></td>
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<tr>
<td></td>
<td>CCL2 (MCP1)</td>
<td>MMP9</td>
<td>OLR1</td>
<td>Sp1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAX</td>
<td></td>
<td>USP9Y</td>
<td>HIF1A</td>
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</table>

Housekeeping genes were 18S rRNA and GAPDH.
Considered adequate for quantification of mRNA expression. Recombinant RNasin Ribonuclease Inhibitor (Applied Biosystems) was added to prevent RNase-mediated degradation. cDNA was stored at −20°C.

By real-time PCR, 46 genes were studied and classified as shown in Table 1. Gene expression analyses were performed at the mRNA level by TaqMan Low Density Array (TLDA). Predesigned TaqMan probe and primer sets for target genes were chosen from an online catalog (Applied Biosystems). These were factory-loaded into the 384 wells of the TLDA card, which was configured into 8 identical 24-gene sets in duplicate. Twenty-two genes were chosen based on literature reviews of key molecules in inflammation and immunology. Each gene set also contained two housekeeping genes, GAPDH and 18S rRNA.

Five microliters of single-stranded cDNA (equivalent to 100 ng total RNA) were mixed with 45 μL nuclease-free water and 50 μL TaqMan Universal PCR Master Mix. After gentle mixing and centrifugation, the 100-μL mixture was transferred into a loading port on a TLDA card. The card was centrifuged twice for 1 min at 1,100 rpm to distribute the samples from the loading port into each well. It was then sealed and placed in the Micro Fluidic Card Sample Block of an Applied Biosystems 7900HT PCR system. The thermal cycling condition was 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 30 s at 97°C and 1 min at 59.7°C. Expression levels were measured in duplicate. Only the genes with reproducible amplification curves of both duplicates were analyzed and presented. TLDA cards were analyzed with relative quantitation (RQ) documents and the RQ Manager Software for automated data analysis. Gene expression values (RQ) were calculated based on the ΔΔ cycle threshold method. ΔCycle threshold values, defined as the point at which the fluorescence rises above the background fluorescence, were calculated with SDS 2.3 software (Applied Biosystems). A mixture of DNA from OPA used as a calibrator, and 18S rRNA was the reference for normalization.

Statistical analysis
Results are expressed as mean ± SD. Relationships between cellular biomarkers and antioxLDL ab (plasma IgG/IgM antioxLDL ab levels) were examined by means of the Pearson correlation test. Statistical analyses were performed with SPSS for Windows version 17.0 (IBM Corporation Inc., Somers, NY).

The aim of this study was to assess the correlation between plasma IgG/IgM antioxLDL ab levels with specific inflammatory, apoptotic, and lipid metabolism biomarkers in both OPA with atheromatous plaque and FV without plaque. The sample size calculation was realized, considering that a correlation coefficient <0.65 could lead to a type 1 error or a false-positive correlation. Thus, based on this aim, the sample size was calculated to have >70% power (α = 0.05) and an expected correlation coefficient of 0.65. Following this statistical approach, the minimal sample size required was 14 subjects.

RESULTS—To characterize histologically the vascular biopsy specimens from patients with atherosclerosis, sections from the vessels were stained with Masson trichrome and photographed under light microscopy (Fig. 1). The sections corresponding to the OPA had soft plaques with a lipid nucleus and calcium deposits. The capsule had few collagen fibers and vascular smooth muscle cells, although with an excess of macrophages and lymphocytes (Fig. 1A). In the FV biopsy specimens, we found a normal histology, with the adventitial layer.
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comprising connective tissue as well as collagen and elastic fibers, the media layer comprising smooth muscle and elastic fibers, and the inner layer comprising an elastic membrane lining and smooth endothelium where endothelial cells were located (Fig. 1B).

On the other hand, in the patients with PAOD, different anthropometric parameters (sex, age, BMI, and blood pressure) were not correlated with IgG and IgM antioxLDL ab (Table 2). Biochemical parameters showed a negative correlation between IgM antioxLDL ab and c-LDL plasma levels ($r = -0.562$, $P = 0.05$) in OPA, but this significance disappeared in FV.

In addition, we investigated the possible correlation of plasma IgG and IgM antioxLDL ab levels with gene expression of different biomarkers (mRNA levels) in patients with PAOD. The expression was obtained from 46 genes that have an important relevance in all the studied metabolic pathways, and their expression in vessels with a highly atherogenic environment (i.e., OPA, FV) were analyzed. Only 11 genes were significantly correlated with antioxLDL ab in the biopsy specimens (Table 3). The genes altered were those involved in inflammation, apoptosis, cell cycle, cell turnover, transcription factor, and lipid metabolism compared with the housekeeping genes 18S rRNA and GAPDH. In FV, IgG antioxLDL ab showed a negative correlation with MMP10 and a positive correlation with MMP2. On the other hand, there was a positive correlation between IgM antioxLDL ab and AKT1, Casp3, PPARg, TFP1, NFkB, and VEGFA (Table 4). In OPA, IgM antioxLDL ab showed a positive correlation with MMP10, AKTI, BAX, CDKN1A, SCARB1, and VEGFA, and IgG antioxLDL ab had no correlation with the studied genes (Table 5).

To assess the association between IgG and IgM antioxLDL ab and drugs, multiple regression models were used to correct for confounding factors. The association analysis did not fall within any of the proposed models (data not shown). None of analyzed treatments were shown to have correlation with the antibodies studied.

**CONCLUSIONS**—The most relevant finding in this study is that IgM antioxLDL ab showed a significant correlation with different signaling pathways (inflammation, apoptosis, and lipid metabolism) in a highly atherosclerotic environment. To study signaling pathways, there are some important points to consider. The atherosclerotic process is the lipid accumulation in blood vessels, which triggers different responses leading to the plaque development. It has been demonstrated that the atherosclerotic process is accentuated in large arteries where hemodynamic forces are exerted (20). We demonstrate that in both a vein and an artery, plasma levels of IgM antioxLDL ab correlate with these signaling pathways, indicating that this antibody was expressed in a determined environment (atherosclerotic medium) independently of the analyzed vessel. This event does not occur with IgG antioxLDL ab. Of all biochemical and anthropometric characteristics of these patients with diabetes, in accord with other studies (9, 11), only plasma c-LDL levels showed a negative correlation with IgM antioxLDL ab.

Numerous studies have linked both IgG and IgM antioxLDL ab with atherosclerosis, although it seems that there are more consistent data about the negative association of IgM levels with the atherosclerotic process (21). In occluded arteries, only IgM levels are positively correlated with the signaling pathways, as argued by many authors (22). In the present study, we analyzed the biomarkers of the different metabolic pathways that have significant correlation with antioxLDL ab.

With respect to inflammatory biomarkers, it is known that during plaque formation, muscle cells migrate from the media to the intimal layer, where they proliferate and generate growth factors, such as VEGFA. In the past decade, VEGFA was believed to be a proinflammatory biomarker in endothelial cells involved in the atherosclerotic process (23). In the present study, expression of VEGFA is associated with IgM antioxLDL.

### Table 3—Genes with significant correlation with antioxLDL ab in the OPA and FV biopsy specimens

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Reference sequence</th>
<th>Assay ID</th>
<th>Amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPPO</td>
<td>Tissue factor pathway inhibitor</td>
<td>NM_001032281.2</td>
<td>Hs00196731_m1</td>
<td>122</td>
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<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
<td>NM_003376.4</td>
<td>Hs00900054_m1</td>
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<tr>
<td>Apoptosis</td>
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<td></td>
</tr>
<tr>
<td>MMP10</td>
<td>Metalloproteinase 10</td>
<td>NM_002425.2</td>
<td>Hs00233987_m1</td>
<td>82</td>
</tr>
<tr>
<td>MMP2</td>
<td>Metalloproteinase 2</td>
<td>NM_004530.2</td>
<td>Hs00234422_m1</td>
<td>83</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
<td>NM_138761.2</td>
<td>Hs00180269_m1</td>
<td>62</td>
</tr>
<tr>
<td>Casp3</td>
<td>Caspase 3</td>
<td>NM_032991.2</td>
<td>Hs00234387_m1</td>
<td>100</td>
</tr>
<tr>
<td>AKT1</td>
<td>v-akt murine thymoma viral oncogene homolog 1</td>
<td>NM_005163.2</td>
<td>Hs00178289_m1</td>
<td>66</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor 1A</td>
<td>NM_078467.1</td>
<td>Hs00355782_m1</td>
<td>66</td>
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<tr>
<td>Lipid metabolism</td>
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<tr>
<td>SCARB1</td>
<td>Scavenger receptor class B, member 1</td>
<td>NM_00108299.1</td>
<td>Hs00969827_m1</td>
<td>75</td>
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<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator–activated receptor γ</td>
<td>NM_138711.3</td>
<td>Hs00243592_m1</td>
<td>77</td>
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<tr>
<td>Transcription factor</td>
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<tr>
<td>NKIRAS2</td>
<td>NFκB inhibitor interacting Ras-like 2</td>
<td>NM_003998.2</td>
<td>Hs00765730_m1</td>
<td>66</td>
</tr>
<tr>
<td>Housekeeping gene</td>
<td></td>
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</tr>
<tr>
<td>18S rRNA</td>
<td>18S rRNA</td>
<td>X03205.1</td>
<td>Hs99999901_s1</td>
<td>187</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>NM_002046.3</td>
<td>Hs99999905_m1</td>
<td>122</td>
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</table>
Abs in both FV and OPA, demonstrating this relationship. Another inflammatory biomarker, TPPI, is a potent regulator of the tissue factor pathway that together with LDL modulates tissue factor function (24). As expected, it is related positively with IgM antioxLDL ab. Other proinflammatory biomarkers are the metalloproteinases, which are a family of zinc-dependent endopeptidases, and they are a major contributor to plaque rupture (25). MMP10 is a key link between inflammation and thrombosis, particularly in situations of increased thrombotic risk (26). In the present study, we found that in OPA, MMP10 is correlated with IgM antioxLDL ab. All these findings suggest that IgM antioxLDL ab is related to the inflammatory process inside an atherosclerotic environment.

In relation to lipid metabolism pathway, two biomarkers correlated with antioxLDL ab. On the one hand, little is known about the association of SCARB1 with antioxLDL ab. SCARB1 is a multifunctional receptor that mediates bidirectional lipid transport in the macrophage. As such, it depends on the amount of cholesterol in lipid-laden macrophages (27) inside atheroma plaque. In peripheral cells, the lipid uptake by scavenger receptor initiates chronic proinflammatory cascades linked to atherosclerosis. In this sense, the present study demonstrates that SCARB1 is positively correlated with IgM antioxLDL ab levels in OPA. In addition, another lipid biomarker is a nuclear hormone receptor, PPARg, which also is positively correlated with IgM antioxLDL ab levels in FV. It has been demonstrated that PPARg agonists inhibited the progression of atherosclerosis in humans. PPARg agonist-mediated inhibition of the renin-angiotensin-aldosterone system and the thromboxane A2 system as well as endothelial protection may possibly be involved in the inhibitory effects on blood pressure and atherosclerosis (28) in this vessel; thus, the atherosclerotic process is not developed in this environment. Activation of PPARg by its ligands could modulate gene transcription, such as NFκB, thereby leading to multiple antiatherogenic and fibrinolytic effects (29). In fact, NFκB also shows a positive correlation with IgM antioxLDL ab levels in FV.

When IgM antioxLDL ab levels were analyzed in relation with some biomarkers of the apoptosis pathway, we found that AKT1, Casp3, and BAX in FV and only AKT1 and BAX in OPA showed a positive correlation with IgM antioxLDL ab. Recently, it was demonstrated that in human umbilical vein endothelial cells, oxLDL-induced apoptosis was associated with upregulation of proapoptotic BAX and Casp3 and inhibition of antiapoptotic AKT1 accompanied by reciprocal changes in the methylation of promoter regions of these genes (30). In this sense, it could be hypothesized that because of this correlation between these biomarkers and IgM antioxLDL ab, this antibody has a protective function in atheroma plaque. On the other hand, it was demonstrated that Casp3 cleaves specifically with CDKN1A and may trigger the apoptosis pathway (31). CDKN1A is a regulator of cell cycle progression at G1 (32). The expression of this gene is modulated by the tumor suppressor protein p53 (33). In this study, a positive correlation between CDKN1A and IgM antioxLDL ab was found in OPA.

In conclusion, the results of this study show that the gene expression of the apoptosis, lipid metabolism, and inflammation metabolic pathways in the FV and OPA vessels is directly related to the levels of IgM antioxLDL ab. Because this is a cross-sectional study, we could not determine whether IgM antioxLDL ab has a proinflammatory effect in plaques, whether it exerts a protective compensatory response, or whether it is merely a correlational marker for the presence of inflammatory cells in patients with diabetes.

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No potential conflicts of interest relevant to this article were reported.

M.R.B.-L. researched data, contributed to the discussion, wrote the manuscript, and reviewed and edited the manuscript. L.S.-G. and J.L.G.-P., V.L.-C., and F.C. researched data and contributed to the discussion. V.G.-C. researched data and wrote the manuscript. R.G.-H. contributed to discussion and reviewed and edited the manuscript. M.R.B.-L. and F.J.T. are the guarantors of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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