Decreased Paraoxonase-2 Expression in Human Carotids During the Progression of Atherosclerosis

Giuliana Fortunato, Maria Donata Di Taranto, Umberto Marcello Bracale, Luca Del Guercio, Francesca Carbone, Cristina Mazzacaccara, Alberto Morgante, Francesco Paolo D’Armiento, Maria D’Armiento, Massimo Porcellini, Lucia Sacchetti, Giancarlo Bracale, Francesco Salvatore

Objective—Many gene products involved in oxidation and inflammation are implicated in the pathogenesis of atherosclerosis. We investigated paraoxonase 2 (PON2), 5-lipoxygenase (5-LO), and 5-LO activating protein (FLAP) expression and malondialdehyde (MDA) levels in carotid lesions to assess their involvement in plaque formation.

Methods and Results—We measured gene expression and MDA levels in atherosclerotic plaques from 59 patients undergoing carotid endarterectomy, and in plaque-adjacent tissue from 41/59 patients. Twenty-three fetal carotids and 6 mammary arteries were also investigated. Real-time polymerase chain reaction and immunohistochemistry revealed decreased PON2 expression in plaques versus adjacent regions (P<0.005, P<0.001, respectively), mammary arteries (P<0.031, P<0.001, respectively), and fetal carotids (both P<0.001). mRNA levels of 5-LO and FLAP were higher (P<0.038, P<0.005, respectively) in lesions versus fetal carotids. MDA was higher in plaques versus plaque-adjacent tissue and fetal carotids. PON2 mRNA was downregulated by oxidative stress in 5 ex vivo experiments, thereby indicating its possible atheroprotection role.

Conclusions—We demonstrate that PON2 mRNA and protein are decreased in plaques versus plaque-adjacent tissue, mammary arteries, and fetal carotids. Our data indicate that the protective effect of PON2 could fail during atherosclerosis exacerbation; this was confirmed by the increase of MDA levels. The increase of 5-LO and FLAP mRNA expression confirms their role as inflammatory markers associated to atherosclerosis.

Key Words: atherosclerosis ■ paraoxonase 2 (PON2) ■ carotid plaque ■ oxidative stress ■ fetal carotid and mammary artery

Carotid atherosclerotic plaques are important risk factors for stroke, which is in turn a major cause of morbidity and mortality. Lipid oxidation plays a key role in the pathogenesis of atherosclerosis. The production of oxidized molecules is related to an imbalance between oxidant and antioxidant factors. Accumulation of oxidized low-density lipoprotein (ox-LDL) in artery walls is an initiating event of fatty streak development. Moreover, oxidized products give rise to inflammatory events which, in turn, lead to the progression of plaques. Increasing evidence suggests that genes such as paraoxonases (PONs) and lipoxygenases (LOs), which are involved in oxidative stress and inflammation, play key roles in the onset and progression of atherosclerotic plaques.

The PON family consists of 3 genes (PON1, PON2, and PON3) that have similar antioxidant properties but different expression profiles: PON1 and PON3 are expressed in the liver and circulate in close association with apolipoprotein A-1 in high-density lipoprotein (HDL), whereas PON2 is expressed in many different types of tissues and cells including cells of the artery wall. PON polymorphisms are associated with atherosclerosis, diabetes, prostate cancer, Alzheimer dementia, Parkinson disease, and acquired hearing loss. Interestingly, a study of PON2-deficient mice showed that PON2 protected against atherogenesis in vivo by modulating lipoprotein properties, thereby reducing cellular oxidative stress and attenuating the inflammatory response.
Many inflammatory stimuli originate from the 5-lipoxygenase (5-LO) enzyme and 5-LO activating protein (FLAP) that catalyze the synthesis of leukotrienes. In a mouse model, 5-LO deficiency resulted in a dramatic decrease in the development of aortic lesions. Furthermore, specific variants of genes that encode 5-LO and FLAP confer an enhanced risk of myocardial infarction and stroke.

To gain insight into the roles of PON2, 5-LO, and FLAP in the development and progression of human atherosclerotic lesions, we have measured their expression levels in carotid plaques and in plaque-adjacent regions, together with oxidative stress parameters.

Methods

Subjects and Specimens

Carotid and peripheral blood samples were obtained from 59 consecutive patients undergoing carotid endarterectomy for stenosis \( \geq 70\% \) or stenosis ranging from 50\% to 70\% associated to clinical symptoms according to American Heart Association (AHA) guidelines.[26] The patients were enrolled at the Dipartimento Assistenziale di Chirurgia Generale Toracica, Vascolare e Endovascolare (University of Naples Federico II). Symptomatic patients were defined subjects with a history of transient ischemic attacks, stroke, or amaurosis fugax. The study was performed according to the current version of the Helsinki Declaration. Informed consent was obtained for each patient. The demographic and biochemical features of the population studied are reported in supplemental Table I (available online at http://atvb.ahajournals.org).

The plaque area was excised from all 59 patients. From 41/59 patients we also collected tissue from the region immediately adjacent to the plaque (supplemental Figure IA). Details about tissue specimens are reported in supplemental Methods.

Carotid samples from 23 aborted fetuses were obtained from legally approved therapeutic abortions at the Dipartimento di Scienze Biomorfologiche e Funzionali–Sezione Anatomia Patologica e Citopatologica (University of Naples Federico II). Details about fetal carotid samples are reported in supplemental Methods.

As an additional control of adult vascular tissue we measured PON2 mRNA and PON2-positive cells in 6 nonatherosclerotic mammary arteries obtained from patients undergoing mastectomy surgery.

Lesion Characterization

Atherosclerotic plaques and the adjacent regions were classified based on histology (see Table). Details about classification are reported in supplemental Methods. Other vascular tissues lacked lesions. Supplemental Figure IB through ID shows an example of hematoxylin-eosin stain of the analyzed tissues.

Immunohistochemical Examination

The immunohistochemical procedure we used to analyze malondialdehyde (MDA) and 4-hydroxynonenal-modified lysines and PON2 protein is described in supplemental Methods. We carried out double immunofluorescence staining in plaques, adjacent regions, fetal carotids, and mammary arteries. n indicates number of examined samples. Intima-media layers were used as reported under Materials and Methods.

mRNA Expression Evaluation

RNA Extraction and cDNA Preparation

Total RNA isolation and cDNA preparation was performed as reported in supplemental Methods.

Real-Time Quantitative PCR

cDNA was amplified on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using predesigned assays. Details about assays, choice of endogenous control, validation tests, and PCR reaction conditions are reported in supplemental Methods.

Analysis of Gene Expression

We measured mRNA expression normalized to the GUSB gene (endogenous control) by the comparative \( C \) method using the SDS 2.2.1 software package (Applied Biosystems). A blood leukocyte pool was used as RNA calibrator sample to compare samples

<table>
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<th>Table. Lesion Number and Type According to the American Heart Association Classification</th>
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<td>n (%)</td>
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<td>Plaque</td>
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<tr>
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</tr>
<tr>
<td>Lesion type VII</td>
</tr>
<tr>
<td>Lesion type VIII</td>
</tr>
<tr>
<td>Plaque-adjacent regions</td>
</tr>
<tr>
<td>Lesion type II</td>
</tr>
<tr>
<td>Lesion type III</td>
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<tr>
<td>Lesion type IV</td>
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</table>

*Plaques examined. †Plaques for which adjacent regions were available.
analyzed in different plates. mRNA expression levels are indicated as expression units relative to the endogenous control and calibrator sample used. Relative PON2, 5-LO, and FLAP mRNA levels were expressed as the mean of 2 quantification experiments, each carried out in duplicate.

**Laboratory Analyses**

**Malondialdehyde**
MDA was measured in plasma and in homogenized tissue using a colorimetric assay (Bioxytech MDA-586, Oxis Research) based on reaction with N-methyl-2-phenylindole. Imprecision of the method, evaluated on low and high concentration controls, was <10%.

**Biochemical Markers**
Biochemical markers were measured as described in supplemental Methods.

**Ex Vivo Oxidation Experiments**
The region adjacent to plaques was collected from 5 patients in physiological solution containing penicillin and streptomycin, washed several times, and minced into very small pieces. Tissue fragments were suspended in Dulbecco Modified Eagle Medium (DMEM; Euroclone) with 10% FBS and 1% glutamine, and then divided in several aliquots in a 6-multiwell plate. Tissue fragments were incubated or not with 10 mmol/L N-acetyl-L-cysteine (NAC; Sigma-Aldrich) for 18 hours, and then exposed to 2 mmol/L H₂O₂ for 5 hours. To determine whether prevention of oxidation inhibited the effects of oxidation, we exposed samples to NAC before H₂O₂ incubation. We also treated 2 samples with NAC alone as a control. The plaque-adjacent region used as a control was incubated with culture medium only. All treatments were carried out at 37°C and 5% CO₂. An aliquot of treated culture medium was retained for MDA measurement. Finally, tissue fragments were lysed in GTC/β-mercaptoethanol solution to extract total RNA.

**Statistical Analysis**
Statistical analyses were carried out using the Statistical Package for the Social Sciences version 14.0 (SPSS Inc). More details are reported in supplemental Methods.

**Results**

**PON2 Expression**
We evaluated PON2 expression in plaques, their adjacent regions, fetal carotids, and nonatherosclerotic mammary ar-
teries. PON2 mRNA expression was significantly lower in plaques versus the adjacent regions ($P<0.005$) and versus the control tissues, namely fetal carotids ($P<0.001$) and mammary arteries ($P<0.031$; Figure 1A).

We also evaluated PON2 expression at protein level by immunohistochemical staining (supplemental Figure IH through IK). The percentage of PON2-positive cells in plaques, adjacent regions, fetal carotids, and mammary arteries (Figure 1B) showed the same trend as PON2 mRNA (Figure 1A) decreasing from mammary arteries to plaques. In particular, adult mammary arteries showed an even higher percentage of PON2-positive cells than fetal carotids ($P<0.001$).

No differences were observed between PON2 mRNA levels in the different lesion types. PON2 mRNA expression levels were significantly lower in peripheral leukocytes (mean $\pm$ SEM = 1.01 $\pm$ 0.04) than in plaques (mean $\pm$ SEM = 2.94 $\pm$ 0.23; $P<0.001$) and in their respective adjacent regions (mean $\pm$ SEM = 4.16 $\pm$ 0.38; $P<0.001$). To evaluate whether large amounts of entrapped leukocytes could have affected the lower expression of PON2 mRNA in plaques, we excluded complicated plaques (type VI) from the analysis. We found that after exclusion of complicated plaques, the difference in PON2 mRNA expression between plaques (mean $\pm$ SEM = 3.01 $\pm$ 0.26) and their adjacent regions (mean $\pm$ SEM = 4.34 $\pm$ 0.44) remained significant ($P<0.008$).

Double immunofluorescence staining showed that PON2 was highly expressed in endothelial cells and less expressed in monocytes/macrophages in mammary arteries as well as in fetal carotids (Figures 2C, 2D, 2G, and 2H). PON2 reactivity was almost lacking in SMCs (Figure 2K and 2L). T-lymphocytes were absent in these tissues (Figure 2O and 2P). In plaque-adjacent regions PON2 expression in endothelial cells and macrophages decreased with respect to control tissues, showing also a reduction of the total number of endothelial cells (Figure 2B and 2F). However, in the plaque-adjacent region there was low PON2 reactivity in the SMCs whereas no reactivity was observed in T-lymphocytes (Figure 2J and 2N). Finally, within the plaque tissue a small number of endothelial cells was observed, some of which without PON2 reactivity (Figure 2A).

Some macrophages, SMCs, and T-lymphocytes expressed PON2 (Figure 2E, 2I, and 2M), although the total number of PON2-positive cells was low. There was no reactivity in extracellular regions. Therefore, the general pattern seems to indicate a decreasing gradient of PON2 reactivity from control tissues to plaque-adjacent regions and then to advanced plaques, both attributable to a loss of PON2-expressing cells (mainly endothelial cells) and to a reduced positivity of the remaining cells.

**Oxidative Status Evaluation**

We evaluated MDA concentration as an index of oxidative status in plaques and in their respective adjacent regions. As shown in Figure 3, MDA levels were higher in plaques versus the respective adjacent regions (1.6-fold; $P<0.032$) and versus fetal carotids (3.2-fold; $P<0.002$). However, the concentration of MDA in the plaque-adjacent regions did not differ from that observed in fetal carotids. The immunohistochemical analysis using antibodies against lysine residues modified with MDA or 4-hydroxynonenal confirmed this result (supplemental Figure IE through IG). The levels of oxidized products and PON2 expression had an opposite trend.

**Modulation of PON2 mRNA Expression by Oxidative Stress in Ex Vivo Samples**

The opposite trend between MDA and PON2 expression levels in plaques suggested that oxidative stress could affect PON2 expression. To test this hypothesis, we incubated plaque-adjacent regions from 2 patients with $\text{H}_2\text{O}_2$ in the presence and absence of the antioxidant reagent NAC. We tested the tissue adjacent to plaques because it includes all the carotid cell types involved in atherosclerosis and undergoes such early atheromatous changes as accumulation of lipids that can be oxidized under our experimental conditions. An aliquot of the plaque-adjacent region was incubated with culture medium only as control at baseline. As shown in Figure 4, $\text{H}_2\text{O}_2$ induced a significant increase of MDA (36.0%; $P<0.048$) in the culture medium and a corresponding significant decrease.
of PON2 mRNA expression levels (~26.6%; P<0.036) compared with the nontreated control sample. Pretreatment of samples with 10 mmol/L NAC prevented H₂O₂ oxidation. Treatment with only NAC did not affect PON2 mRNA expression levels (mean PON2 expression evaluated on 2 samples: 103.5%). In fact, MDA in culture medium and PON2 mRNA expression levels did not change compared with the baseline control.

5-Lipooxygenase and FLAP mRNA Expression

5-LO and FLAP mRNA expression was negligible in fetal carotids, and increased in plaques (22.2- and 9.0-fold compared with fetal carotids, respectively) and in their respective adjacent regions (18.0- and 8.5-fold compared with fetal carotids, respectively), which is consistent with increased expression of inflammatory genes in low grade lesions (Figure 5). The mean values of 5-LO and FLAP mRNA expression in plaques and in plaque-adjacent regions were higher in symptomatic patients than in asymptomatic patients although the difference was not significant (data not shown). Moreover, 5-LO and FLAP mRNA expression was significantly higher in peripheral leukocytes versus plaques (~8 fold) and versus their adjacent regions (~8 fold; both P<0.0001). The increased levels of 5-LO and FLAP mRNAs in plaques were not attributable to circulating leukocytes trapped in plaques. In fact, 5-LO and FLAP mRNA levels did not differ between plaques and adjacent regions even after the exclusion of 7 complicated lesions associated to hemorrhage and thrombosis. There was a strong correlation between 5-LO and FLAP mRNA levels in the examined tissues (plaques: correlation coefficient =0.95, P<0.0001; adjacent regions: correlation coefficient =0.90, P<0.0001; peripheral leukocytes: correlation coefficient =0.60, P<0.0001), which indicates that these genes were simultaneously regulated.

A nonlinear correlation was found between PON2 mRNA levels and 5-LO or FLAP mRNA levels (F= 18.4, P=0.00005 and F=9.7, P=0.003 respectively).

Discussion

Here we report the novel finding that PON2 expression progressively decreases in plaque-adjacent regions and plaque tissues, as also compared with the internal layers of fetal carotids and nonatherosclerotic mammary arteries, thereby providing evidence that PON2 protection is lost in these altered tissues as atherosclerosis develops.

Double immunofluorescence staining revealed PON2 expression in endothelial cells and monocytes/macrophages in healthy and atherosclerotic tissues. The total number of PON2-positive cells gradually decreased during lesion progression. The endothelial layer was lost in advanced plaques thereby contributing to the overall decrease in PON2 mRNA expression. Accordingly, whereas PON2 expression decreased in plaques, the oxidative stress marker MDA increased. We also demonstrate that oxidative stress applied to cells of plaque-adjacent regions can downregulate PON2 expression. Therefore, oxidative stress might play a role in reducing endogenous antioxidant property and thereby exacerbating oxidative status.

Rosenblat et al reported an increase of PON2 expression in macrophages of ApoE-deficient mice under oxidative stress. A compensatory effect of increased PON2 expression after oxidative stress has also been reported in a macrophage cell line. However, subsequently, Rosenblat and colleagues reported decreased PON2 expression in macrophages from hypercholesterolemic subjects treated with acetylated-LDL and oxidized-LDL. Very recently, Levy et al demonstrated that PON2 expression in intestinal specimens is downregulated after induction of oxidative stress by iron/ascorbate. This observation which is in line with our results showed that PON2 expression is similarly downregulated by oxidation in different kind of tissues. A study of PON2 function performed on different vascular cell models shows that PON2 decreases the production of intracellular reactive oxygen species. Furthermore, recent in vivo studies carried out with murine models showed that PON2 deficiency aggravates atherosclerosis, whereas adenovirus-mediated PON2 overexpression exerts a protective effect by reducing the plaque area. Therefore our results showing that pronounced oxidative stress causes a decrease of PON2 mRNA expression also in regions adjacent to carotid plaques is in line with the overall general idea that PON2 enzyme activity is impaired in its efficacy to protect against atherosclerosis progression when the lesions are already well advanced and no longer reversible. Our finding that oxidation markers are increased within the lesion site reinforces the concept that oxidative stress is associated to the development and progression of atherosclerotic lesions. The different responses to oxidative stress between macrophages and whole atherosclerotic lesions could be attributable to the fact that the latter contain such diverse cell types as endothelial cells, smooth muscle cells, and inflammatory cells with different PON2 regulatory capability. Our finding that PON2 protein is mainly expressed in endothelial cells lends further support to this hypothesis. The action of an oxidant agent such as lithium supports the very recent finding that oxidative stress decreases the expression of PON2 mRNA, in line with our results.
The effect of antioxidant treatment against atherosclerosis is unclear. Despite numerous trials no beneficial effects of antioxidants have ever been convincingly demonstrated in patient studies. In particular, it has been demonstrated that vitamin E supplementation is not effective in patients with advanced lesions. 34 It may be hypothesized that this lack of effect might be attributable to a severe imbalance of the oxidant/antioxidant status associated to a reduction of PON2, as shown by our results.

Oxidation and inflammation are widely acknowledged to be associated with the atherosclerotic process. 3,7 In this study we also demonstrate that the mRNA expression of the 5-LO and FLAP genes, which are involved in inflammation, are higher in plaques and in plaque-adjacent regions with respect to fetal carotid tissue. This finding is in agreement with a previous study showing enhanced 5-LO and FLAP expression in human atherosclerotic lesions as compared with control iliac arteries, 35 which supports the concept that the 5-LO enzymatic pathway is involved in plaque development. In addition, the strong correlation found between 5-LO and FLAP mRNA expression levels suggests that these genes are simultaneously involved in plaque development and progression of atherosclerosis as compared with control iliac arteries. 35,36 It may be hypothesized that this lack of effect might be attributable to a severe imbalance of the oxidant/antioxidant status associated to a reduction of PON2, as shown by our results.

In a study carried out with carotid endarterectomy specimens, and with aorta and coronary arteries from explanted hearts, the number of 5-LO-expressing cells increased as the lesion progressed. 36 This also appears to be in line with our finding of upregulated 5-LO and FLAP mRNA expression in plaque-adjacent regions. Therefore, taken together, these data suggest that established lesions promote the development and progression of atherosclerosis in adjacent regions probably because of their close proximity to plaques. Consequently, these regions may be considered “preplaque” areas. 5-LO expression, but not FLAP expression, was reported to be higher in plaques from symptomatic patients versus asymptomatic patients. 35, 37 We found a similar pattern showing a definite trend of increase, although the difference between symptomatic and asymptomatic individuals was not significant.

In conclusion, we provide the first demonstration that both PON2 mRNA and protein are decreased in human advanced carotid plaques. Furthermore, we confirm the accumulation of oxidized products and increased mRNA expression of the 5-LO pathway in plaques, as shown by the fact that 5-LO and FLAP increase simultaneously during atherosclerosis development. Lastly, taken together, our findings highlight the importance of the oxidation balance, together with inflammatory processes, in the development and progression of atherosclerosis at the plaque formation level.

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We are grateful to Jean Ann Gilder for editing the text and to Dr Dino Franco Vitale for his help in the statistical analysis.

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Disclosures
None.

References


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Supplemental Data

METHODS

Subjects and specimens

The plaque area was excised from all 59 patients. From 41/59 patients we also collected tissue from the region immediately adjacent to the plaque (Fig. 1A). Each endarterectomy specimen included the intima and media layers at the level of the lesion. Intima-media was manually dissected during surgery, and used for the experiments, also because of the difficulty in separating intima from media in appropriate amounts in all instances. Absence of adventitia contamination was confirmed by histological analysis. Each sample was divided into sections, which were used for: 1) histological examination, 2) RNA extraction and 3) oxidative status evaluation. The section for histological analysis was immediately frozen at -80°C. The other sections were either homogenized in guanidinium buffer containing β-mercaptoethanol (GTC/β-mercaptoethanol) for RNA extraction or in PBS with antioxidant probucol for oxidative status evaluation.

Fetal carotid samples were withdrawn using the Perinatal Autopsy Protocol published by the Armed Forces Institute of Pathology in cooperation with the American Registry of Pathology. The age of fetuses, calculated from anamnestic and ultrasonographic data, ranged between 18 and 23 gestational weeks. The carotids were removed within five hours of the expulsion of fetuses, and tissue samples were dissected manually under the microscope to obtain control tissue as similar as possible to the endarterectomy samples. 12/23 samples were used for RNA extraction and 11/23 for oxidative stress evaluation and PON2 immunohistochemical staining.
**Lesion characterization**

Atherosclerotic plaques and the adjacent region were classified based on histological analysis according to American Heart Association criteria (1, 2). In particular lesion type Vb and Vc were referred as type VII and VIII, respectively because this wording is reported in the 2000 update of the AHA classification (3).

**Immunohistochemical examination**

The immunohistochemical procedure we used to analyze malondialdehyde (MDA) and 4-hydroxynonenal modified lysine is described elsewhere (4). Before PON2 staining, serial sections were cut at 3-5 µm and pretreated for 2 min in a microwave oven at 750 W in 10 mM citrate buffer pH 8.0 for antigen unmasking. PON2 staining was performed with a rabbit polyclonal antibody directed against a human PON2 fragment (amino acids 89-105, Orbigen) used at a concentration of 1:50. The optimum working dilution was empirically determined by testing serial dilutions of antibody to obtain high-intensity specific staining with negligible non specific background. As a negative control, we used tissue slices stained with a non immune serum and secondary antibody. No reactivity was detected in extra-cellular regions. The percentage of PON2-positive cells was calculated as the number of immunoreactive cells/total cells in five different microscope fields. We carried out double immunofluorescence staining in plaques, adjacent regions, fetal carotids and mammary arteries to detect PON2 expression in different cell types using the following specific markers: factor VIII (DAKO) for endothelial cells, CD68 (DAKO) for monocytes/macrophages, actin (DAKO) for smooth muscle cells (SMC) and CD3 (DAKO) for T-cells. Tissue sections were incubated: 1) with PON2 antibody in combination with one of the above-mentioned mouse primary antibodies; 2) with fluoresceine-conjugated anti-rabbit IgG (Sigma); 3) with rhodamine-conjugated goat anti-mouse IgG (Sigma); and 4) with propidium iodide. The images were obtained with confocal microscopy (LSM 510, Zeiss) and evaluated by three independent observers.
mRNA expression evaluation

RNA extraction and cDNA preparation

Total RNA was isolated from GTC/β-mercaptoethanol homogenates of plaques, the adjacent regions, mammary arteries and fetal carotids using the Chomezynski (acid phenol extraction) method (5) proceeded by two steps of chloroform extraction to remove lipids. Peripheral leucocytes were isolated from whole blood and total RNA was extracted with TriPure Isolation Reagent (Roche) according to the manufacturer’s instructions. The integrity of all RNA samples was verified by gel electrophoresis. cDNA was synthesized from 1 µg of total RNA using Random Hexamers (Amersham Bioscience), M-MLV reverse transcriptase (Invitrogen) and RNAguard (RNase inhibitor; Amersham Biosciences).

Real-time quantitative PCR

cDNA was amplified on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using a pre-developed assay containing primers and 5’ FAM-labeled probe spanning the exon junctions (PON2, assay number Hs00165563_m1; 5-LO, assay number Hs00167536_m1; FLAP, assay number Hs00233463_m1; Applied Biosystems). The TaqMan Human Endogenous Control Plate procedure (Applied Biosystems) was used to evaluate the expression of 11 housekeeping genes in samples from various tissues. Beta-glucuronidase (GUSB-Applied Biosystems) had minimal expression differences and was therefore a good endogenous control. We verified that the amplification efficiency of PON2, 5-LO and FLAP was similar to that of the GUSB control gene so that we could use the Comparative C<sub>T</sub> Method for data analysis. Singleplex reactions were performed in duplicate on 384-well plates in a final volume of 15 µl containing 0.8 ng/µl of cDNA.
Laboratory analyses

Biochemical markers

Biochemical markers were measured on serum after an overnight fast. Total serum cholesterol, triglyceride and glucose levels were evaluated by standard methods using an automated analyzer (Modular P3, Roche). The HDL and LDL cholesterol concentrations were measured using a homogeneous enzymatic colorimetric assay and the automated Modular P3 analyzer (Roche). C-reactive protein was measured on serum with the ultra sensitive method (Dade-Behring).

Statistical analysis

Continuous variables were expressed as means ± SD. Variables that did not have a Gaussian distribution are reported as median values and interquartile range. Continuous variables were compared using the T-test. The paired T-test was used to compare data from plaques, their adjacent region and peripheral leucocytes in the same subject. The relationship between different variables was tested with the Spearman correlation. Correlation between PON2 and 5-LO or FLAP mRNA levels were tested with non linear (logarithmic) model. A p value <0.05 was considered significant.
References


Supplemental Table I General characteristics of the studied population (n=59)

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Values are reported as mean ± SD.

* Significantly different between males and females (p<0.005);

† Median value and interquartile range (non parametric distribution).
Supplemental Figure I. Panel A: Carotid endarterectomy sample. Squared areas indicate stenotic plaque (P) and the adjacent region (AR). Panels B to D: Hematoxylin-eosin stain of plaques, the adjacent region and fetal carotid respectively. Panels E to G: Malondialdehyde (MDA) immunohistochemical staining of plaques, the adjacent region and fetal carotid respectively. Panels H to K: PON2 immunohistochemical staining of a mammary artery, a plaque, its adjacent region and a fetal carotid respectively. Bars indicate 10 µm.