

# Evidence Supporting a Key Role of Lp-PLA2-Generated Lysophosphatidylcholine in Human Atherosclerotic Plaque Inflammation

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**Objective**—To determine whether the level of lysophosphatidylcholine (lysoPC) generated by lipoprotein-associated phospholipase A2 (Lp-PLA2) is associated with severity of inflammation in human atherosclerotic plaques. Elevated plasma Lp-PLA2 is associated with increased cardiovascular risk. Lp-PLA2 inhibition reduces atherosclerosis. Lp-PLA2 hydrolyzes low-density lipoprotein-oxidized phospholipids generating lysoPCs. According to in vitro studies, lysoPCs are proinflammatory but the association between their generation and plaque inflammation remains unknown.

**Methods and Results**—Inflammatory activity in carotid plaques (162 patients) was determined immunohistochemically and by analyzing cytokines in homogenates (multiplex immunoassay). LysoPCs were quantified using mass spectrometry and Lp-PLA2 and the lysoPC metabolite lysophosphatidic acid (LPA) by ELISA. There was a strong correlation among lysoPC 16:0, 18:0, 18:1, LPA, and Lp-PLA2 in plaques. LysoPC 16:0, 18:0, 18:1, LPA, and Lp-PLA2 correlated with interleukin-1 $\beta$ , interleukin-6, monocyte chemoattractant protein-1, macrophage inflammatory protein-1 $\beta$ , regulated on activation normal T-cell expressed and secreted, and tumor necrosis factor- $\alpha$  in plaques. High lysoPC and Lp-PLA2 correlated with increased plaque macrophages and lipids and with low content of smooth muscle cells, whereas LPA only correlated with plaque macrophages. Lp-PLA2, lysoPC 16:0, 18:0, and 18:1, but not LPA were higher in symptomatic than in asymptomatic plaques.

**Conclusion**—The associations among Lp-PLA2, lysoPCs, LPA, and proinflammatory cytokines in human plaques suggest that lysoPCs play a key role in plaque inflammation and vulnerability. Our findings support Lp-PLA2 inhibition as a possible strategy for the prevention of cardiovascular disease. (*Arterioscler Thromb Vasc Biol.* 2012;32:1505-1512.)

**Key Words:** atherosclerosis ■ carotid plaque ■ inflammation ■ lipoprotein-associated phospholipase A2 ■ lysophosphatidylcholine

Arterial inflammation is the principal driving force responsible for atherosclerotic plaque development and destabilization. There is strong evidence that this inflammation is induced by the retention and oxidation of low-density lipoprotein (LDL) in the subendothelial space.<sup>1,2</sup> Oxidized LDL is cytotoxic and may induce inflammation by causing arterial cell injury and by recruiting oxidized LDL-specific proinflammatory T cells to the artery wall.<sup>3</sup> It has also been proposed that oxidized LDL may activate inflammation directly through interaction with macrophage Toll-like receptors.<sup>4</sup> An alternative pathway through which oxidized LDL may initiate inflammation is through the release of bioactive metabolites. Oxidized phospholipids in LDL are hydrolyzed by lipoprotein-associated phospholipase A2 (Lp-PLA2) to generate

lysophosphatidylcholines (lysoPCs) and oxidized nonesterified fatty acids.<sup>5</sup> LysoPCs have been shown to be a potent chemoattractant for T-cells and monocytes,<sup>6,7</sup> promote endothelial dysfunction,<sup>8</sup> induce release of arachidonic acid,<sup>9</sup> and induce apoptosis of endothelial and vascular smooth muscle cells.<sup>10,11</sup> These in vitro observations imply that lysoPCs may contribute to the development of atherosclerotic plaques as well as to plaque vulnerability and rupture. This hypothesis is also supported by studies showing that the Lp-PLA2 and lysoPCs content of human carotid plaques predict future cardiovascular events and that Lp-PLA2 and lysoPC's plaque expression are increased in symptomatic human carotid plaques.<sup>12-14</sup> Plasma levels of Lp-PLA2 and its activity have also been shown to be independent predictors of cardiovascular risk in several

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**Table 1. Clinical Characteristics of the Patients (n=162)**

|                                            |                     |
|--------------------------------------------|---------------------|
| Age, years                                 | 68.9 (SD 8.7)       |
| Body mass index                            | 26.8 (SD 3.9)       |
| Gender                                     | 106 male/ 56 female |
| Degree of stenosis, %                      | 90 (IQR 80–95)      |
| Diabetes mellitus, %                       | 44 (n=71)           |
| Hypertension, %                            | 74 (n=120)          |
| Smoking (in the past or currently), %      | 78 (n=127)          |
| Dyslipidemia, %                            | 94 (n=153)          |
| Fasting lipoproteins, mmol/L               |                     |
| Total cholesterol                          | 4.3 (IQR 3.6–5.1)   |
| LDL cholesterol                            | 2.4 (IQR 1.9–3.2)   |
| HDL cholesterol                            | 1.1 (IQR 0.9–1.3)   |
| Triglycerides cholesterol                  | 1.3 (IQR 0.9–1.8)   |
| Hemoglobin, g/L                            | 141 (IQR 130–149)   |
| Creatinin, mmol/L                          | 86 (IQR 73–100)     |
| High-sensitive CRP                         | 4.1 (IQR 2.1–6.8)   |
| White blood cell count, 10 <sup>9</sup> /L | 8 (SD 6.4–9.2)      |
| HbA1c, mmol/L                              | 6.4 (IQR 5.6–7.4)   |
| Statins, %                                 | 88 (n=143)          |

CRP indicates C-reactive protein; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein.

epidemiological studies.<sup>15–18</sup> These observations have focused attention on Lp-PLA2 as a possible target for the treatment of atherosclerosis. Darapladib, a reversible oral Lp-PLA2 inhibitor, has recently been shown to reduce the development of atherosclerosis in hypercholesterolemic swine and to halt necrotic core expansion and development of coronary plaques in humans.<sup>19,20</sup> It seems reasonable to assume that darapladib acts by inhibiting the generation of lysoPCs in atherosclerotic lesions, but the possible associations among Lp-PLA2, lysoPCs, and atherosclerotic plaque inflammation in humans remain to be determined. In the present study, we have analyzed the associations of Lp-PLA2 and lysoPC's levels with the degree of inflammation in 162 human carotid plaques.

## Methods

### Patients

One hundred sixty-two human carotid plaques were collected at carotid endarterectomies. The indications for surgery were plaques associated with ipsilateral symptoms (transient ischemic attack, stroke, or amaurosis fugax) and stenosis, measured by duplex, >70% (n=91) or plaques not associated with symptoms and stenosis >80% (n=71). All patients were preoperatively assessed by a neurologist. One day before surgery, a venous blood sample was collected (EDTA-plasma) from each patient and stored for further analysis. Informed consent was given by each patient. The study was approved by the local ethical committee. The clinical characteristics of the patients are summarized in Table 1.

### Sample Preparation and Histology

After surgical removal, plaques were snap-frozen in liquid nitrogen in the operating room. Fragments of 1 mm, from the most stenotic region, were taken for histology. Transversal sections from the fragment were stained with CD68, Oil Red O,  $\alpha$ -actin, and Masson. Plaque homogenates were prepared as previously described.<sup>21</sup>

## LysoPC, Lp-PLA2, LPA, Phosphate, and Cytokine Assessment

Lp-PLA2 in plaque homogenate and plasma were analyzed using ELISA. Lp-PLA2 in LDL was determined with Western blotting. LysoPC's levels in plaque homogenate and plasma were analyzed using mass spectrometry,<sup>22</sup> and cytokines were analyzed with Luminex technology (Human Cytokine/chemokine immunoassay, Millipore Corporation, MA). Monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-10, and IL-6 release from cultured human mononuclear leukocytes macrophages and endothelial cells were also assessed by ELISA. Lysophosphatidic acid (LPA) levels in plaque homogenate and plasma as well as plaque levels of phosphate were analyzed using ELISA (Echelon Biosciences Inc, Salt Lake City, UT). A more detailed description of the LPA ELISA is provided in the online-only Data Supplement. All values were normalized to plaque total phosphate.

## Statistics

Non-normally distributed variables were presented as median and interquartile range (IQR) and normally distributed variables were presented as mean and SD or SEM. Two-group comparisons were performed with Mann-Whitney tests as appropriate. Spearman  $\rho$  was used for correlation analysis. Differences were considered statistically significant at  $P<0.05$ . Adjustments for multiple comparisons were not performed. More detailed description of methods is available in the online-only Data Supplement.

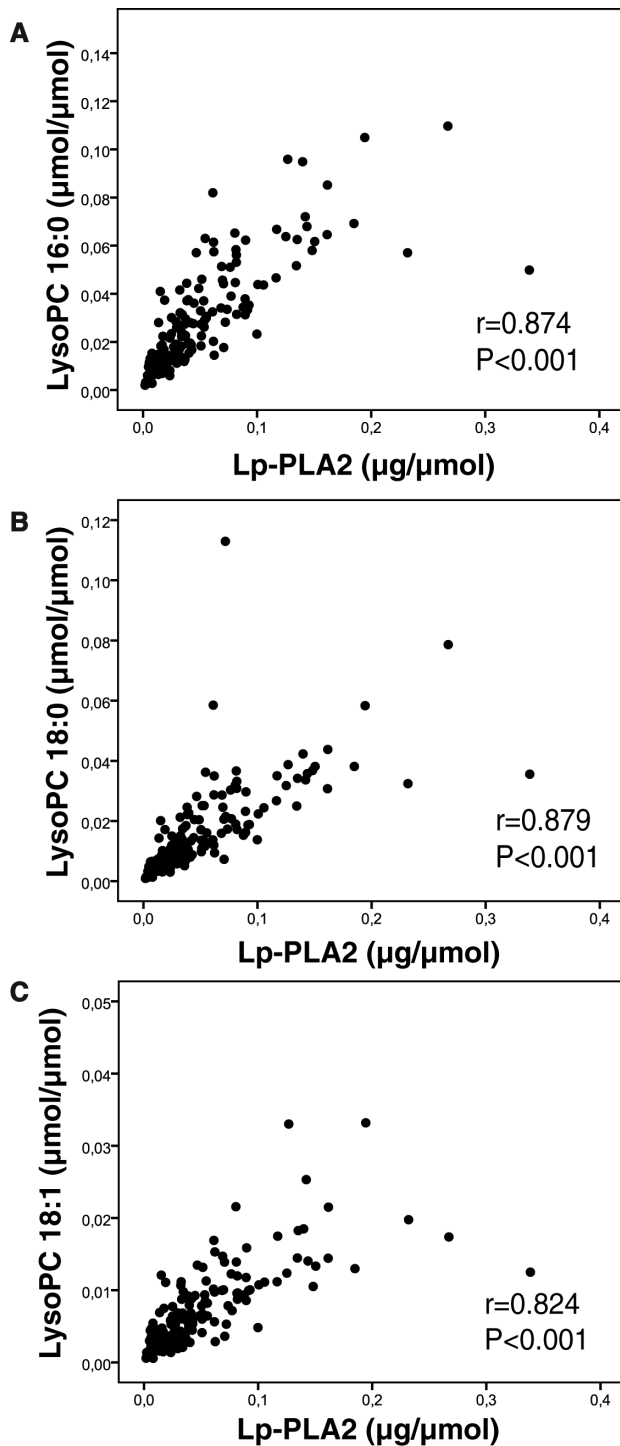
## Results

### Associations Between Lp-PLA2 and LysoPC

There was a strong association between the amounts of lysoPC 16:0, 18:0, 18:1 (together representing 70% of the 15 assessed lysoPC subspecies), and Lp-PLA2 in the plaques ( $r=0.874$ ,  $P<0.001$ ;  $r=0.879$ ,  $P<0.001$ ;  $r=0.824$ ,  $P<0.001$ ; Figure 1). There was also an association between plaque levels of the lysoPC metabolite lysophosphatidic acid and plaque lysoPC 16:0, 18:0, 18:1, and Lp-PLA2 levels ( $r=0.442$ ,  $P<0.001$ ;  $r=0.422$ ,  $P<0.001$ ;  $r=0.441$ ,  $P<0.001$ ;  $r=0.444$ ,  $P<0.001$ , respectively). A significant association was also observed between the plasma levels of Lp-PLA2 and the amount of Lp-PLA2 present in plaque tissue ( $r=0.297$ ,  $P<0.001$ ), as well as between plaque levels of lysoPC 16:0, 18:0, and plasma Lp-PLA2 ( $r=0.173$ ,  $P<0.05$ ;  $r=0.167$ ,  $P<0.05$ ). There was no significant association between plasma Lp-PLA2 and plaque lysoPC 18:1 or LPA levels.

### LysoPC, LPA, Lp-PLA2, and Plaque Inflammation

Plaque levels of lysoPC 16:0, 18:0, 18:1, and LPA demonstrated significant associations with the level of several proinflammatory cytokines and chemokines in the plaque including IL-1 $\beta$ , IL-6, MCP-1, macrophage inflammatory protein-1 $\beta$ , regulated on activation normal T-cell expressed and secreted, and TNF- $\alpha$ , and with the exception of LPA also with the anti-inflammatory cytokine IL-10 (Table 2). In contrast, there were inverse relations between the plaque content of lysoPC 16:0, 18:0, 18:1, LPA, and eotaxin (Table 2), a chemokine that primarily acts on eosinophils. When comparing lysoPC 16:0, 18:0, and 18:1 levels with the histological characteristics of the plaques, associations were observed with the plaque area stained for neutral lipids and macrophages, whereas inverse correlations were found for smooth muscle cell-stained area (Table 3). LPA was only



**Figure 1.** Association among plaque levels of (A) lysoPC 16:0, (B) lysoPC 18:0, (C) lysoPC 18:1 and Lp-PLA2 in human atherosclerotic carotid plaques.

significantly associated with plaque macrophage staining (Table 3). LysoPC 16:0, 18:0, and 18:1 content was higher in plaques associated with symptoms than in asymptomatic plaques (0.030 [IQR 0.015–0.051] versus 0.015 [IQR 0.011–0.029]  $\mu\text{mol}/\mu\text{mol}$  phosphate,  $P<0.001$ ; 0.015 [IQR 0.008–0.029] versus 0.007 [IQR 0.005–0.015]  $\mu\text{mol}/\mu\text{mol}$  phosphate,  $P<0.001$ ; 0.008 [IQR 0.004–0.013] versus 0.004

[IQR 0.002–0.007]  $\mu\text{mol}/\mu\text{mol}$  phosphate,  $P<0.001$ ; Figure 2A–C). There was no significant difference in LPA levels between symptomatic and asymptomatic plaques.

We also determined the plasma levels of lysoPC 16:0, 18:0, 18:1, and LPA. The plasma levels of the 3 lysoPC species demonstrated a high degree of covariation ( $r=0.64$ – $0.79$ ,  $P<0.005$  for all values). Plasma LPA was associated with the plasma level of lysoPC 18:0 ( $r=0.018$ ,  $P<0.05$ ), but not with plasma lysoPC 16:0 and 18:1. Plasma Lp-PLA2 demonstrated an association with plasma LPA ( $r=0.018$ ,  $P<0.05$ ) but not with plasma lysoPC levels. There were no significant correlations between the plasma levels of lysoPC 16:0, 18:0, 18:1, LPA, and the plaque cytokine content.

### Lp-PLA2 and Plaque Inflammation

In addition, Lp-PLA2 levels were higher in symptomatic plaques than in asymptomatic plaques (0.044 [IQR 0.019–0.088] versus 0.027 [IQR 0.01–0.05]  $\mu\text{g}/\mu\text{mol}$  phosphate,  $P<0.001$ ; Figure 2D). In accordance with lysoPC 16:0 and 18:0, Lp-PLA2 levels correlated with IL-1 $\beta$ , IL-6, IL-10, IL-12 (p40), MCP-1, macrophage inflammatory protein-1 $\beta$ , regulated on activation normal T-cell expressed and secreted, TNF- $\alpha$ , and inversely with eotaxin (Table 2). Lp-PLA2 also correlated with the histological staining for neutral lipids and macrophages and inversely with smooth muscle cells (Table 3), but not with collagen as determined with Masson trichrome. There were no significant correlations between plasma Lp-PLA2 and plaque cytokines (data not shown). Lp-PLA2 activity in plaque extracts was also determined. Although these measurements were more difficult to standardize, Lp-PLA2 activity still demonstrated the expected association with Lp-PLA2 mass ( $r=0.24$ ,  $P<0.005$ ) as well as with IL-6, MCP-1, macrophage inflammatory protein-1 $\beta$ , regulated on activation normal T-cell expressed and secreted, and TNF- $\alpha$  (Table I in the online-only Data Supplement). The Lp-PLA2 activity was also significantly associated with plaque macrophage and neutral lipids ( $r=0.28$ ,  $P<0.001$  and  $r=0.28$ ,  $P<0.001$ , respectively), but not with immunostaining for smooth muscle cell  $\alpha$ -actin or collagen as assessed by the Masson trichrome staining.

### Association of Lp-PLA2 and LysoPCs With Cardiovascular Risk Factors and Treatment

There was a significant association between plasma levels of LDL and the concentrations of lysoPC 16:0, 18:0, 18:1, and Lp-PLA2 in plaques ( $r=0.257$ ,  $P<0.005$ ;  $r=0.242$ ,  $P<0.005$ ;  $r=0.252$ ,  $P<0.005$ ;  $r=0.223$ ,  $P=0.007$ , respectively), whereas no significant association was seen with plaque levels of LPA. We did not observe any significant associations among high-sensitive C-reactive protein, total or high-density lipoprotein cholesterol, triglycerides, HbA1c in plasma, and the plaque contents of lysoPC 16:0, 18:0, 18:1, LPA, or Lp-PLA2. Plaque levels of lysoPC 16:0, 18:0, 18:1 and Lp-PLA2 did not differ in patients treated with statins or not. Plaques from patients with diabetes mellitus had lower levels of Lp-PLA2 in plaques (0.027 [IQR 0.011–0.052] versus 0.041 [IQR 0.024–0.081]  $\mu\text{g}/\mu\text{mol}$  phosphate,  $P=0.008$ ). Subjects not treated with antihypertensive medication had higher levels

**Table 2. Correlations Among Plaque LysoPC 16:0, 18:0, 18:1, LPA, Lp-PLA2, and Plaque Cytokines**

| Cytokine      | LysoPC 16:0      | LysoPC 18:0      | LysoPC 18:1      | LPA             | Lp-PLA2          |
|---------------|------------------|------------------|------------------|-----------------|------------------|
| IL-1 $\beta$  | $r=0.296^{***}$  | $r=0.297^{***}$  | $r=0.296^{***}$  | $r=0.25^{***}$  | $r=0.362^{***}$  |
| IL-6          | $r=0.750^{***}$  | $r=0.756^{***}$  | $r=0.718^{***}$  | $r=0.294^{***}$ | $r=0.686^{***}$  |
| MCP-1         | $r=0.790^{***}$  | $r=0.793^{***}$  | $r=0.737^{***}$  | $r=0.411^{***}$ | $r=0.750^{***}$  |
| MIP-1 $\beta$ | $r=0.699^{***}$  | $r=0.693^{***}$  | $r=0.656^{***}$  | $r=0.367^{***}$ | $r=0.716^{***}$  |
| RANTES        | $r=0.580^{***}$  | $r=0.583^{***}$  | $r=0.534^{***}$  | $r=0.283^{***}$ | $r=0.606^{***}$  |
| TNF- $\alpha$ | $r=0.554^{***}$  | $r=0.544^{***}$  | $r=0.519^{***}$  | $r=0.399^{***}$ | $r=0.537^{***}$  |
| IL-10         | $r=0.318^{***}$  | $r=0.307^{***}$  | $r=0.306^{***}$  | $r=0.112$       | $r=0.372^{***}$  |
| IL-12 (p40)   | $r=0.295^{***}$  | $r=0.299^{***}$  | $r=0.273^{***}$  | $r=0.173$       | $r=0.341^{***}$  |
| IL-12 (p70)   | $r=-0.045$       | $r=-0.05$        | $r=-0.003$       | $r=0.22^{**}$   | $r=-0.011$       |
| Eotaxin       | $r=-0.391^{***}$ | $r=-0.393^{***}$ | $r=-0.382^{***}$ | $r=-0.176^*$    | $r=-0.455^{***}$ |

IL indicates interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor; RANTES, regulated on activation normal T-cell expressed and secreted; LPA, lysophosphatidic acid.

Significance marked by \* $P<0.05$ , \*\* $P<0.01$ , and \*\*\* $P<0.005$ .

of Lp-PLA2 and lysoPC 18:0 (0.048 [IQR 0.029–0.093] versus 0.033 [IQR 0.014–0.070]  $\mu\text{g}/\mu\text{mol}$  phosphate,  $P=0.04$  and 0.014 [IQR 0.009–0.025] versus 0.010 [IQR 0.005–0.021]  $\mu\text{mol}/\mu\text{mol}$  phosphate,  $P=0.037$ , respectively). Plaque LPA was lower in patients with diabetes mellitus than in patients without diabetes mellitus (0.0013 [IQR 0.0007–0.0038] versus 0.0030 [IQR 0.0014–0.0053]  $\mu\text{mol}/\mu\text{mol}$  phosphate,  $P=0.002$ ).

### Effect of Lp-PLA2-Generated LysoPC on Release of MCP-1

The observations of strong associations between the plaque contents of Lp-PLA2, lysoPCs, and proinflammatory cytokines are indicative of a chain of events in which lysoPCs, generated by action of Lp-PLA2 on oxidized phospholipids in LDL, activate local inflammation. However, they do not exclude the possibility that this inflammation is the result of a direct effect of oxidized LDL on inflammatory cells rather than being attributable to the action of Lp-PLA2 on oxidized LDL. To investigate the relative contribution of lysoPC on generation of the proinflammatory effects of oxidized LDL, we exposed cultured human mononuclear leukocytes to 10 or 100  $\mu\text{g}/\text{mL}$  of LDL minimally modified by a preincubation with  $\text{FeSO}_4$  (mmLDL) with or without addition of the Lp-PLA2 inhibitor SB-435495 (mmLDL<sup>sb</sup>). The presence of Lp-PLA2 in the LDL preparations was demonstrated using Western blotting (Figure 3A). Exposure of LDL to  $\text{FeSO}_4$  resulted in about doubling of the lysoPC content. Coincubation with SB-435495 inhibited  $\approx 50\%$  of the increase in lysoPC 16:0 and 18:1 and  $\approx 25\%$  of the increase in lysoPC 18:0 (Table

II in the online-only Data Supplement). Addition of mmLDL resulted in a  $>3$ -fold increase in the release of MCP-1 from the cells (Figure 3B). About 40% of this increase was inhibited if the cells were exposed to mmLDL that had been generated in the presence of SB-435495 (Figure 3B), whereas addition of SB-435495 after  $\text{FeSO}_4$  modification of LDL did not affect mmLDL-induced MCP-1 release (data not shown). Similar observations were made for mmLDL-induced release of IL-6 from cultured endothelial cells (Figure 3C), whereas mmLDL did not stimulate the release of MCP-1 from endothelial cells (Figure 3D). In accordance with the effect on mononuclear leukocytes, mmLDL stimulated the release of MCP-1 from cultured human macrophages by a mechanism that was inhibited by SB-435495 (Figure 3E).

### Discussion

The present observations provide strong support for the notion that lysoPCs generated through hydrolysis of oxidized phospholipids in LDL by Lp-PLA2 play an important role in human atherosclerotic plaque inflammation. The concentration of lysoPC 16:0, 18:0, and 18:1 present in plaques correlated with the plaque content of Lp-PLA2 and lipids, but only lysoPC 16:0 and 18:0 correlated weakly with Lp-PLA2 levels in plasma. The most likely explanation to this observation is that the majority of the lysoPC 16:0, 18:0, and 18:1 in atherosclerotic lesions is generated locally by the action of Lp-PLA2 on oxidized LDL. Alternatively, the action of Lp-PLA2 on oxidized LDL makes the latter smaller and denser (sdLDL), thereby increasing its affinity for extracellular matrix and

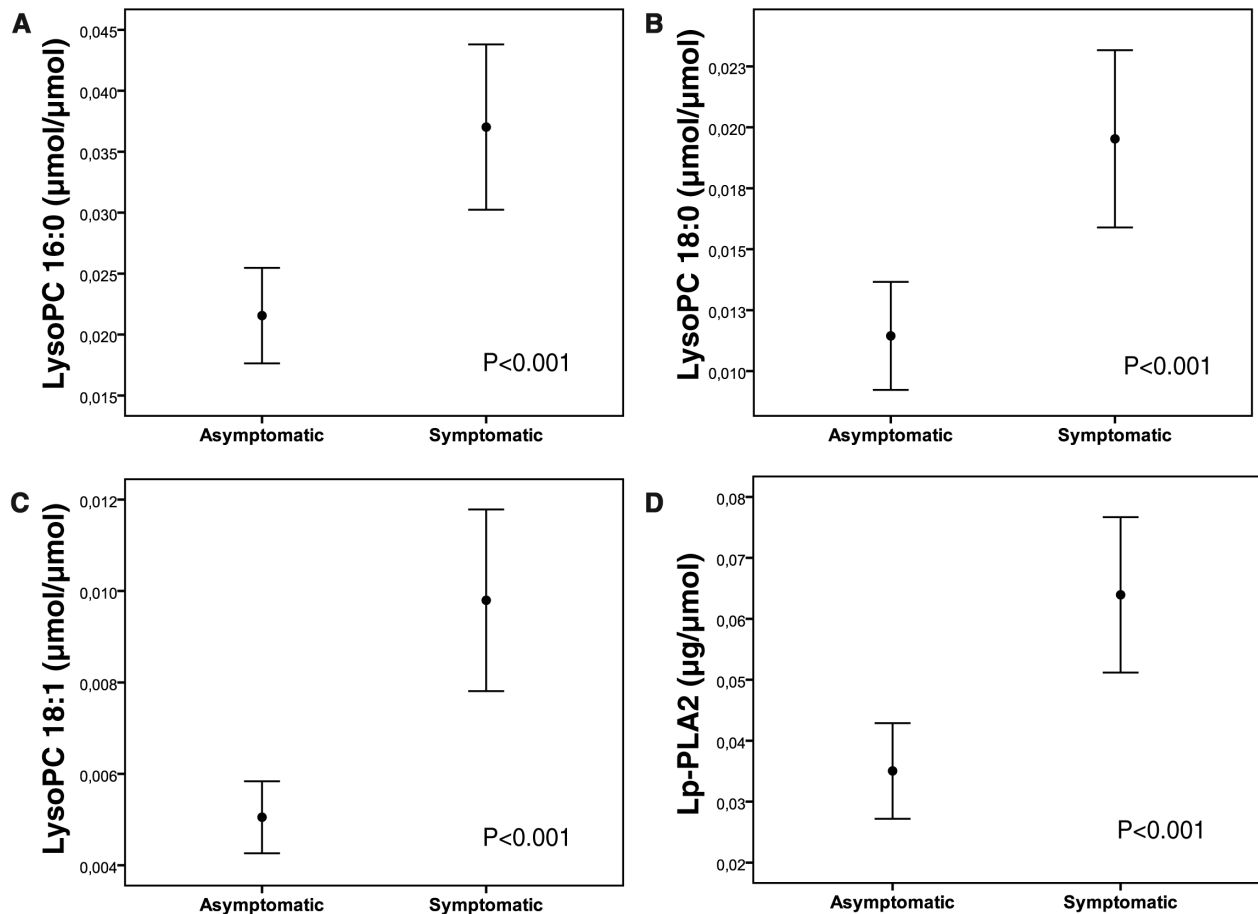
**Table 3. Correlations Among LysoPC 16:0, 18:0, 18:1, Lp-PLA2, and Plaque Histology**

|                 | LysoPC 16:0      | LysoPC 18:0      | LysoPC 18:1      | LPA            | Lp-PLA2          |
|-----------------|------------------|------------------|------------------|----------------|------------------|
| $\alpha$ -actin | $r=-0.281^{***}$ | $r=-0.262^{***}$ | $r=-0.288^{***}$ | $r=-0.079$     | $r=-0.253^{***}$ |
| CD68            | $r=0.288^{***}$  | $r=0.283^{***}$  | $r=0.297^{***}$  | $r=0.225^{**}$ | $r=0.216^{**}$   |
| Oil Red O       | $r=0.476^{***}$  | $r=0.462^{***}$  | $r=0.497^{***}$  | $r=0.145$      | $r=0.352^{***}$  |
| Masson          | $r=-0.06$        | $r=-0.021$       | $r=-0.001$       | $r=-0.021$     | $r=-0.072$       |

LPA indicates lysophosphatidic acid.

Significance marked by \*\* $P<0.01$ , and \*\*\* $P<0.005$ .





**Figure 2.** LysoPC 16:0 (A), lysoPC 18:0 (B), lysoPC 18:1 (C), and Lp-PLA2 (D) levels in plaques associated with symptoms and asymptomatic plaques.

accumulation in the arterial wall. Both Lp-PLA2 and lysoPCs are predominantly found in smaller and denser LDL.<sup>23,24</sup>

### LysoPC, Lp-PLA2, and the Vulnerable Plaque Phenotype

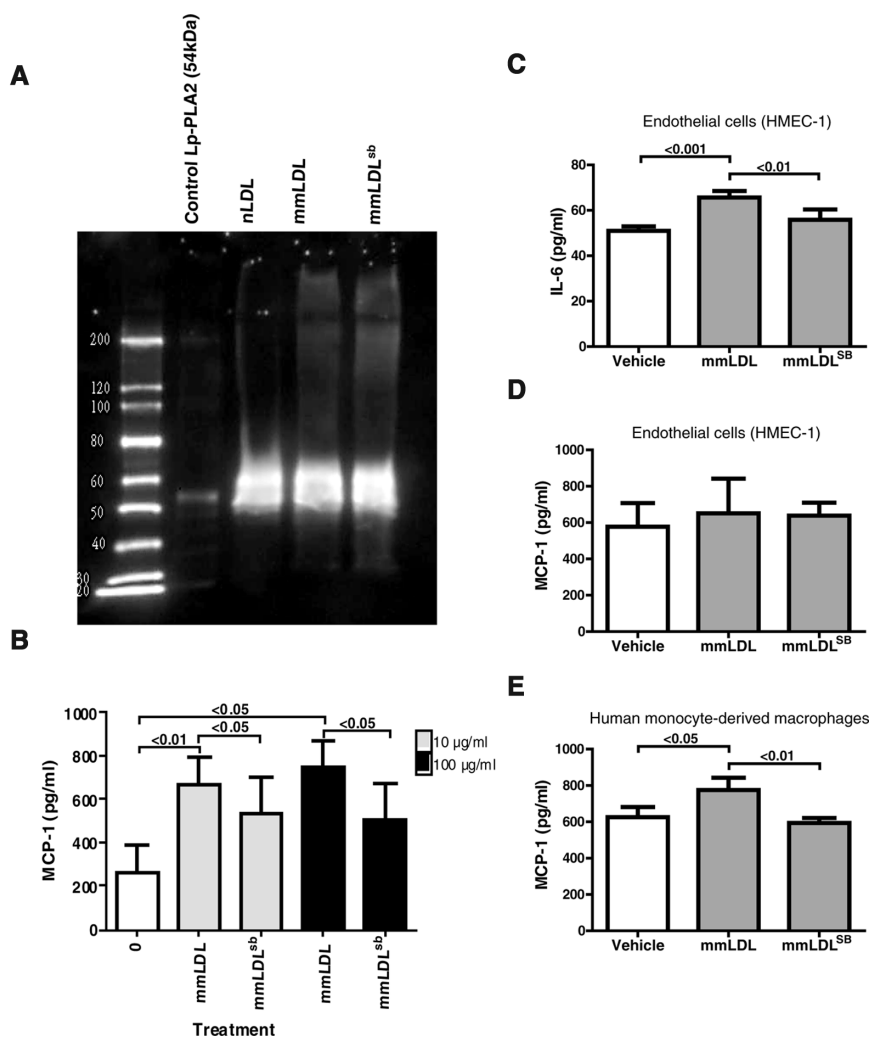
Moreover, we demonstrate associations between the plaque contents of both lysoPCs and Lp-PLA2 with the level of plaque inflammation. The content of lysoPC 16:0, 18:0, 18:1, and Lp-PLA2 was higher in plaques associated with clinical symptoms than in the asymptomatic plaques. LysoPC 16:0, 18:0, and 18:1 levels were also inversely related to the smooth muscle cell content of the plaques, suggesting that lysoPC 16:0, 18:0, and 18:1 contributes not only to plaque inflammation but also to plaque vulnerability.

### LysoPC, Lp-PLA2, and Plaque Inflammation

The concept of an important role of lysoPCs in plaque inflammation is well in line with observations from a number of studies performed on cell culture. LysoPC has been shown to attract monocytes and lymphocytes and to induce chemokine secretion *in vitro*.<sup>6,7,25–27</sup> MCP-1 is a potent chemokine expressed in atherosclerotic lesions and absence of MCP-1 reduces atherosclerosis in mice.<sup>28,29</sup> LysoPC has been shown to induce the gene expression of MCP-1 in human microvascular endothelial cells as well as in rat aortic smooth muscle cells.<sup>26,30</sup> In accordance with these studies, we demonstrate here

that  $\approx 40\%$  of the MCP-1 release from human mononuclear leukocytes caused by exposure to mmLDL can be attributed to products of Lp-PLA2 and that there is a strong correlation between the levels of lysoPC and MCP-1 in atherosclerotic lesions. LysoPC has also been shown to enhance the release of IL-6 from peripheral blood mononuclear cells and human coronary artery smooth muscle cells,<sup>9,25</sup> regulated on activation normal T-cell expressed and secreted from microvascular endothelial cells,<sup>26</sup> and TNF- $\alpha$ <sup>27</sup> as well as IL-1 $\beta$  from human monocytes.<sup>31</sup> The present findings of significant associations between lysoPC and these cytokines in atherosclerotic plaques support the *in vivo* relevance of these *in vitro* studies. In addition, Shi et al<sup>25</sup> showed that TNF- $\alpha$  and IL-1 $\beta$  induced Lp-PLA2 activity, which may result in induction of a vicious cycle of lysoPC generation and inflammation.

We further observed an inverse association among lysoPC 16:0, 18:0, 18:1, LPA, Lp-PLA2, and eotaxin, a chemokine attracting eosinophiles. The role of eotaxin in atherosclerosis and as a possible circulating marker for the disease is controversial.<sup>32,33</sup> Although eotaxin accumulates in atherosclerotic plaques, it is predominantly located in areas rich in vascular smooth muscle cells,<sup>34</sup> but it is not known if it influences the degree of inflammation in the plaque. The negative correlation found in this study among lysoPC 16:0, 18:0, 18:1, LPA, Lp-PLA2, and eotaxin suggests that eotaxin is down-regulated in inflammatory plaques. The anti-inflammatory



**Figure 3.** Induction of cytokine release by minimally modified LDL (mmLDL) oxidized in the presence of the Lp-PLA<sub>2</sub> inhibitor SB-435495. **A**, Western blot visualizing Lp-PLA<sub>2</sub>. **B**, Monocyte chemoattractant protein-1 (MCP-1) release from peripheral blood mononuclear cells treated with 10 or 100 µg/mL mmLDL modified with FeSO<sub>4</sub> with or without SB-435495 (SB). **C**, Interleukin (IL)-6 and MCP-1 release from **(D)** endothelial cells (human microvascular endothelial cell line [HMEC-1]) and from **(E)** human monocyte-derived macrophages treated with 40 µg/mL mmLDL modified with FeSO<sub>4</sub> with or without SB-435495. Vehicle (buffer from second dialysis), 0 (medium only), mmLDL with LP-PLA<sub>2</sub> inhibitor SB-435495 (mm-LDL<sup>SB</sup>).

cytokine IL-10 correlated with lysoPCs and Lp-PLA<sub>2</sub>. IL-10 is expressed in atherosclerotic plaques and is upregulated through the action of ox-LDL.<sup>35,36</sup> The correlation among Lp-PLA<sub>2</sub>, lysoPCs, and IL-10 may reflect that IL-10 often increases in inflammation as a counterbalancing factor.

Lp-PLA<sub>2</sub> activity was also determined in plaque extracts. In our hands, this assay was more difficult to standardize than that for Lp-PLA<sub>2</sub> protein. Lp-PLA<sub>2</sub> activity was significantly but not strongly correlated with Lp-PLA<sub>2</sub> protein levels. It also demonstrated significant association with several proinflammatory cytokines in the plaque as well as with plaque macrophages and lipids. However, these associations were generally weaker than those for Lp-PLA<sub>2</sub> mass possibly because of the difficulties in standardizing the assay.

### LPA and Plaque Inflammation

A novel observation from this study is the association between plaque levels of LPA and plaque inflammation. LPA is a phospholipid that is derived from enzymatic cleavage of lyso-phospholipids such as lysoPC. The biological effect of LPA is mediated through a family of G-protein-coupled LPA-receptors<sup>37</sup> and includes stimulation of endothelial-monocyte adhesion in vitro by inducing the expression of adhesion

molecules and chemokines.<sup>38,39</sup> Zhou and coworkers<sup>40</sup> reported that the endothelial release of the chemokine CXCL1 induced by oxidized LDL is mediated through activation of LPA receptors. Accordingly, it is possible that the association between lysoPC and plaque inflammation described here is the effect of the formation of LPA rather than a direct effect of lysoPC.

### Lp-PLA<sub>2</sub> Inhibition as a Therapeutic Approach

The proinflammatory properties of lysoPC together with epidemiological observations of an association between high plasma levels of Lp-PLA<sub>2</sub> and increased cardiovascular risk have focused attention on Lp-PLA<sub>2</sub> inhibition as a possible novel therapeutic approach.<sup>12,15–18</sup> Indeed, selective inhibition of Lp-PLA<sub>2</sub> with darapladib has been found to reduce the development of coronary atherosclerosis in hypercholesterolemic swine.<sup>19</sup> Treatment with darapladib also inhibited lesion Lp-PLA<sub>2</sub> activity, reduced lesion lysoPC content, and down-regulated the expression of proinflammatory genes in coronary arteries in this animal model. Moreover, in a recent study of 330 patients with angiographically documented coronary disease, darapladib treatment was found to halt the progression of plaque necrotic core expansion.<sup>20</sup> Darapladib is currently being studied in 2 large phase III trials: Stabilization of Atherosclerotic Plaque

by Initiation of Darapladib Therapy Trial, NCT00799903 (STABILITY), a fully enrolled trial involving 15 828 patients with coronary heart disease<sup>41</sup> and the Stabilization of Plaques Using Darapladib–Thrombolysis in Myocardial Infarction 52 Trial, NCT01000727 (SOLID-TIMI 52), which is estimated to include 11 500 patients with acute coronary syndromes. The observations from the present study of a strong association between plaque levels of lysoPC 16:0, 18:0, 18:1, and inflammatory activity suggest that Lp-PLA2 inhibitors, such as darapladib, act by reducing the generation of lysoPC in lesions. The present study also confirms earlier findings that lysoPC and Lp-PLA2 are significantly higher in symptomatic plaques (eg, plaques that are likely to have recent history of rupture) than in asymptomatic plaques,<sup>14</sup> implicating the generation of lysoPCs in the development of plaque vulnerability. In the present study, we also observed an inverse association between the plaque contents of smooth muscle cells and lysoPCs. High concentrations of lysoPC induce apoptosis in endothelial cells and smooth muscle cells, which is detrimental to the formation of a stabilizing cap.<sup>10,11</sup> This represents another mechanism through which lysoPCs may cause plaque vulnerability. The amount of apoptosis in human carotid plaques, measured with terminal deoxynucleotidyl transferase (TdT) end labeling, has been shown to correlate with lysoPC in the plaques.<sup>12</sup>

### Limitations of the Study

There are some limitations of the present study that should be considered. First, the strong association between plaque lysoPC's levels and inflammation does not prove a causal relation. It cannot, for example, be excluded that this association is because of the circumstance that both are linked to the presence of oxidized LDL in the lesions and that the inflammation is caused by a direct action of oxidized LDL rather than through the generation of lysoPCs. However, our finding that inhibition of Lp-PLA2 during LDL modification by FeSO<sub>4</sub> removes much of the MCP-1-releasing effect suggests that lysoPC 16:0 is responsible for a considerable part of the proinflammatory properties of oxidized LDL.

In conclusion, the present study provides support for the notion that lysoPC 16:0, 18:0, and 18:1 generated through hydrolysis of oxidized phospholipids in LDL by Lp-PLA2 plays an important role in human atherosclerotic plaque inflammation and strengthens the scientific rationale for inhibiting Lp-PLA2 to prevent development of acute cardiovascular events.

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### Disclosures

None.

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## **“Supplement Material.”**

## **Methods**

### *Patients*

Patients with ipsilateral carotid artery occlusion or restenosis after previous CEA were excluded from this study. Cardiovascular risk factors, such as hypertension (systolic blood pressure > 140 mm Hg), diabetes, smoking (in the past or currently) and fasting lipoproteins (total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides) were recorded, as well as, the use of medications (anti-hypertensive drugs, diabetes treatment, and statins). Venous blood samples (EDTA-plasma) from the patients were collected one day before endarterectomy.

### *Sample preparation*

Plaques were weighed and homogenised as previously described.<sup>1</sup>

### *Histology*

Sections 8 µm (1mm) were thawed, fixed with Histochoice (Amresco, Ohio, USA), dipped in 60% isopropanol and then in 0.4% Oil Red O in (60%) isopropanol (for 20 min) to stain lipids. Masson's trichrome using Ponceau-acid fuchsin (Chroma-Gesellschaft, Schimdt GmbH, Germany) Phosphomolybdic Acid and Fast Green (Sigma Aldrich Chemie GmbH, Germany) aniline blue (BDH, Dorset, England) was used to assess plaque collagen content. For macrophage assessment primary antibody Monoclonal mouse anti-human CD68, clone KP1 (DakoCytomation, Glostrup, Denmark), diluted in 10% rabbit serum 1:100, and secondary antibody Polyclonal Rabbit anti-mouse, Rabbit F(ab')<sub>2</sub> (DakoCytomation, Glostrup, Denmark), dilution 1:200 in 10% of rabbit serum, were used. When staining for vascular smooth muscle cells (alpha-actin) primary antibody monoclonal mouse anti-human smooth muscle actin clone 1A4 (DakoCytomation, Glostrup, Denmark), diluted in 10% rabbit serum 1:50, and secondary antibody biotin rabbit anti-mouse Ig (DakoCytomation, Glostrup, Denmark), dilution 1:200 in

10% of rabbit serum, were used. To assess the calcified areas, the area of the holes where calcium had been present was measured. Measurements of the area of plaque (% area) for the different components were quantified blindly using Biopix iQ 2.1.8 (Gothenburg, Sweden) after scanning with ScanScope Console Version 8.2 (LRI imaging AB, Vista CA, USA).

#### *Cytokine assessment*

Aliquots of 50  $\mu$ L of plaque homogenate were centrifuged at 13000 x g for 10 minutes. Twenty-five  $\mu$ L of the supernatant was removed and used for measuring fraktalkine, interferon- $\gamma$  (IFN- $\gamma$ ), interleukin (IL)-10, IL-12 (p70), IL 12 (p40) IL-1 $\beta$ , IL-6, monocyte chemoattractant protein (MCP-1), monocytes inflammatory protein-1 $\beta$  (MIP-1 $\beta$ ), platelet-derived growth factor-AB/BB (PDGF-AB/BB), Regulated on Activation Normal T Cell Expressed and Secreted (RANTES), vascular endothelial growth factor (VEGF), sCD40L, eotaxin and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ). The procedure was performed according to the manufacturer's instructions (Human Cytokine/chemokine immunoassay, Millipore Corporation, MA, USA) and analyzed with Luminex 100 IS 2.3 (Austin, Texas, USA).

#### *Lp-PLA2 assessment*

Lp-PLA2 protein levels (also referred to as LP-PLA2 mass) were measured using ELISA. Aliquots of 50  $\mu$ L of plaque homogenate were centrifuged at 13000 x g for 10 minutes. Thereafter, 25  $\mu$ L of the supernatant were removed and used to measure Lp-PLA2 levels. The procedure was performed according to manufacturer's instruction (The PLAC Test ELISA Kit, diaDexus Inc, San Francisco, California, USA). LpPLA2 was measured with Magellan V 6.4 program (absorbance 450 nm) in a Sunrise ELISA reader (Tecan, Austria GmbH). Lp-PLA2 activity was also measured in the same amount of supernatant as described above and the procedure was performed according to manufacturer's instruction (Lp-PLA2 colorimetric

activity method, diaDexus Inc, San Francisco, California, USA). The same ELISA reader was used, as described above, with an absorbance 405 nm.

#### *LysoPC assessment*

We analyzed the homogenates as described previously for other tissues.<sup>2</sup> A volume of 160  $\mu$ L of pre-cooled methanol was added to each tube containing 40  $\mu$ L of frozen plaque homogenate. The samples were homogenized using a Precellys 24 homogenizer (PEQLAB Biotechnology GmbH, Germany) equipped with an integrated cooling unit in combination with tubes containing ceramic beads (50 x 0.5 mL, 1.4 mm beads "small", Bertin-purchase no. 03961-1-203). Homogenization was repeated three times for 20 s at 5500 rpm, with 30 sec cooling intervals between the homogenization steps. The homogenates were centrifuged for 5 min at 10.000 x g at room temperature and 20  $\mu$ L of the supernatants were analyzed for lysoPC 16:0, 18:0, and 18:1 with AbsoluteIDQ<sup>TM</sup> p150 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). The assay comprises 163 metabolites (41 acylcarnitines, 14 amino acids, 1 hexose, 92 glycerophospholipids, 15 sphingolipids) including 15 lysoPCs. Further details on quantification and assays on API 4000 mass spectrometer (AB Sciex, Darmstadt, Germany) have been described by us<sup>2-4</sup>. Since the present study focused exclusively on the role of the Lp-PLA2 pathway in atherosclerotic plaque inflammation only data regarding lysoPC 16:0, 18:0, and 18:1 from the metabolite panel quantified by LC-MS/MS were used. EDTA plasma samples of the same patients have been analysed by direct application of 10  $\mu$ L plasma to the AbsoluteIDQ<sup>TM</sup> p150 kit<sup>2-4</sup>.

#### *LPA assessment*

LPA levels were measured using ELISA. Plaque homogenate supernatants (20  $\mu$ L) were used. The procedure was performed according to manufacturer's instruction (Lysophosphatidic Acid Assay Kit II, K-2800S, Echelon Biosciences Inc., Salt Lake City, USA). LPA was



measured with Magellan V 6.4 program (absorbance 450 nm) in a Sunrise ELISA reader (Tecan, Austria GmbH). The antibody used in this ELISA recognizes the following LPA species (order in high to low): C18:3 > C18:2 > mixed LPA > C20:4 > C14:0 > C18:1 > C16:0 > C18:0. The antibody does not pick up LPA C20:0. The antibody has <5% cross-reactivity with Sphingosine 1 phosphate, phosphatidic acid, lysoPC and platelet activating factor (PAF).

#### *Phosphate assessment*

ELISA technique was used measuring phosphate in 10 µL of plaque homogenate supernatant. Supernatants were prepared as described above and diluted in 990 µL of distilled water. Analyses were performed according to manufacturer's instruction (PiBlue™ Phosphate Assay Kits (POPB-500), BioAssay systems, Hayward, California, USA). LPA was measured with Magellan V 6.4 program (absorbance 620 nm) in a Sunrise ELISA reader (Tecan, Austria GmbH).

#### *Analysis of cytokine release from cultured human mononuclear leukocytes and endothelial cells*

Plasma LDL was isolated from several healthy donors by ultracentrifugation. Minimally modified (mm) LDL was produced by 48 hours incubation with 0.5 µM FeSO<sub>4</sub> in a 0.15 M NaCl buffer with pH 6.8. LDL was desalted on a 10-DG Chromatography column (Bio-Rad laboratories) prior to modification. LDL was dialyzed during 48 hours with 500 mL 0.5 µM FeSO<sub>4</sub> with or without 100 µM of the LP-PLA2 inhibitor SB-435495 (GSK, mmLDL and mmLDL<sup>sb</sup>) in Spectra/Por® CE membranes (Spectrum Laboratories Inc.) with a MWCO 100. To inhibit further oxidation, 1 mM EDTA was added and each tube was overlaid by N<sub>2</sub>. MmLDL was stored at 4 °C and used within 3 week. Protein was measured with BCA Protein Assay Kit (Pierce) according to manufacturer's recommendations.

Human mononuclear leukocytes (MNL) were isolated from buffy coats using Ficoll Paque (GE Healthcare). The cells were centrifuged for 30 minutes at 1200 rpm (4 °C) and the MNL cell layer was removed. Red blood cells were lysed with Red blood Cell Lysing Buffer (Sigma) and the MNLs were washed with PBS before being frozen at -80 °C in 500 µL 20% DMSO (Sigma) in RPMI 1640 (Gibco) and 500 µL human serum, and thereafter kept in liquid nitrogen until used. Thawed MNLs (200.000 per well) were treated over night with different concentrations of mmLDL or mmLDL<sup>sb</sup> diluted in RPMI 1640, supplemented with 5 mL (2 mM) L-glutamine (Gibco), 0.5 mL (50 µM) 2-mercaptoethanol (Gibco), 100 U/mL Penicillin and 100 µg/mL Streptomycin sulphate (Gibco), 5 mL HEPES (Gibco), 5 mL sodium pyruvate (Gibco), and 10% Lipoprotein Deficient Serum from Human Plasma (Sigma) (referred to as cRPMI-10). The cells were differentiated into macrophages in the presence of 20 ng/ml recombinant human M-CSF (rh M-CSF, PeproTech) for 7 days. The medium and cytokines were replaced every 3-4 days. Medium from the wells was carefully removed, pooled and stored in -20 °C before thawed for cytokine measurements.

In order to study additionally endothelial cells, human dermal microvascular cell line (HMEC-1) was obtained from the Centers for Disease Control and Prevention (CDC, USA). The cells were maintained in complete MCDB 131 medium supplemented with 10% FBS, 1% penicillin-streptomycin, L-Glutamine (2 mM), Epidermal growth factor (10 ng/mL), and Hydrocortisone (1 µg/mL, Sigma).

Cytotoxicity of mmLDL and of mmLDL<sup>sb</sup> was measured as lactate dehydrogenase (LDH) released into the medium from cultured cells. MNL (200.000 /well) were treated for 4 hours with mmLDL or with mmLDL<sup>sb</sup> in different concentrations diluted in cRPMI-10. LDH release into the medium was measured with Cytotoxicity Detection Kit (Roche) on a Sunrise

(Tecan) According to manufacturer's protocol. MCP-1 was measured in supernatants from MNL after overnight stimulation with 10 or 100 µg/mL of mmLDL or of mmLDL<sup>sb</sup>. MCP-1 measurement was performed with MCP-1 DuoSet (R&D system) according to manufacturer's instruction, and measured on Sunrise (Tecan).

### *ELISA*

HMEC-1 cells or human macrophages were treated with 40 µg/mL of nLDL, mmLDL or mmLDL with LP-PLA2 inhibitor, or were treated with buffer from second dialysis (vehicle) or only culture medium as control for 24 hours. MCP-1, TNF-α, IL-10 and IL-6 were measured in cell culture supernatants by standard sandwich ELISA kits (R&D system) according to manufacturer's instruction.

### *Western blot*

Western blot was performed on native LDL, mmLDL and mmLDL<sup>sb</sup>. Twenty-five µg of protein was, after boiling (5 minutes, 105 °C) with loading buffer (500 mM Tris, 6% SDS, 30% glycerol, 0.03% bromophenol blue, and 5% β-mercapto ethanol) loaded on a 8% Acrylamide/Bis gel (30%, 37.5:1, Bio-Rad). Electrophoresis was performed at 100 V in Running Buffer (Bio-Rad) supplemented with 0.2% SDS and 2mM EDTA, for 1.5 hours. Blotting was performed on an Immun-Blot PVDF Membrane (0.1µm) (Bio-Rad) at 4 °C, 100V for 1 hour. The PVDF membrane was blocked for 2 hours at room temperature (RT) with TBS supplemented with 0.1% Surfact-Amps 20 (TBS-T) (Thermo Scientific) and 5% non-fat dry milk powder before the primary antibody, PAF Acetylhydrolase (human) Polyclonal Antibody (Cayman Chemical) was added (1:1000) and incubated over night at RT. The membrane was washed and the secondary antibody, Goat Anti-Rabbit Immunoglobulins (1:1000) (Dako Cytomation), added before the membrane was developed with Chemiglow (Alpha Innotech) and photographed with AlphaImager (Alpha Innotech). As a positive control

PAF Acetylhydrolase (human) Western Ready Control was used (Cayman Chemical). MagicMark XP Western protein standard (Invitrogen) was used for comparison of protein size.

**Supplemental table I.** Correlations between plaque Lp-PLA2 activity and plaque cytokines.

| Cytokine      | Lp-PLA2 activity |
|---------------|------------------|
| IL-1 $\beta$  | r=0.126          |
| IL-6          | r=0.254***       |
| MCP-1         | r=0.241 ***      |
| MIP-1 $\beta$ | r=0.218 **       |
| RANTES        | r=0.214 *        |
| TNF- $\alpha$ | r=0.182 *        |
| IL-10         | r=0.128          |
| IL-12 (p40)   | r=0.02           |
| IL-12 (p70)   | r=-0.08          |
| Eotaxin       | r=-0.138         |

Significance marked by \*  $P < 0.05$ , \*\*  $P < 0.01$  and \*\*\*  $P < 0.005$ .



**Supplemental table II.** LysoPC ( $\mu\text{mol/L}$ ) content in LDL, mmLDL and mmLDL<sup>sb</sup>

|                                               | LDL  | mmLDL | mmLDL <sup>sb</sup> |
|-----------------------------------------------|------|-------|---------------------|
| LysoPC 16:0 ( $\mu\text{mol}/\mu\text{mol}$ ) | 30   | 63.6  | 44.9                |
| LysoPC 18:0 ( $\mu\text{mol}/\mu\text{mol}$ ) | 18.8 | 33.9  | 29.0                |
| LysoPC 18:1 ( $\mu\text{mol}/\mu\text{mol}$ ) | 4.7  | 9.3   | 7.3                 |

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