Title
Association of carotid plaque Lp-PLA(2) with macrophages and Chlamydia pneumoniae infection among patients at risk for stroke.

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Authors
Atik, Berna
Johnston, S Claiborne
Dean, Deborah

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Abstract

**Background:** We previously showed that the burden of *Chlamydia pneumoniae* in carotid plaques was significantly associated with plaque interleukin (IL)-6, and serum IL-6 and C-reactive protein (CRP), suggesting that infected plaques contribute to systemic inflammatory markers in patients with stroke risk. Since lipoprotein-associated phospholipase A2 (Lp-PLA2) mediates inflammation in atherosclerosis, we hypothesized that serum Lp-PLA2 mass and activity levels and plaque Lp-PLA2 may be influenced by plaque *C. pneumoniae* infection.

**Methodology/Principal Findings:** Forty-two patients underwent elective carotid endarterectomy. Tissue obtained at surgery was stained by immunohistochemistry for Lp-PLA2 grade, macrophages, IL-6, *C. pneumoniae* and CD4+ and CD8+ cells. Serum Lp-PLA2 activity and mass were measured using the colorimetric activity method (CAM™) and ELISA, respectively. Serum homocysteine levels were measured by HPLC. Eleven (26.2%) patients were symptomatic with transient ischemic attacks. There was no correlation between patient risk factors (smoking, coronary artery disease, elevated cholesterol, diabetes, obesity, hypertension and family history of genetic disorders) for atherosclerosis and serum levels or plaque grade for Lp-PLA2. Plaque Lp-PLA2 correlated with serum homocysteine levels (*p* = 0.013), plaque macrophages (*p* = 0.001), and Lp-PLA2 (*p* = 0.001), which predominantly infected macrophages, co-localizing with Lp-PLA2.

**Conclusions:** The significant association of plaque Lp-PLA2 with plaque macrophages and *C. pneumoniae* suggests an interactive role in accelerating inflammation in atherosclerosis. A possible mechanism for *C. pneumoniae* in the atherogenic process may involve infection of macrophages that induce Lp-PLA2 production leading to upregulation of inflammatory mediators in plaque tissue. Additional *in vitro* and *in vivo* research will be needed to advance our understanding of specific *C. pneumoniae* and Lp-PLA2 interactions in atherosclerosis.

Introduction

Carotid atherosclerosis is a major risk factor for an ischemic stroke [1]. While lipid metabolism and inflammation have been the major focus of atherosclerosis research for many years, there has been a growing interest in lipoprotein-associated phospholipase A2 (Lp-PLA2) because it is a key enzyme both in lipid metabolism and in stimulating inflammation [2].

Lp-PLA2 is a calcium-independent member of the phospholipase A2 enzyme family. Monocytes, macrophages, T-lymphocytes, mast cells and liver cells are the main sources for Lp-PLA2 [3,4]. It is carried primarily by low-density lipoprotein (LDL). Lp-PLA2 catalyzes the hydrolysis of oxidized LDL, which produces proinflammatory mediators lysophosphatidylcholine (LysoPC) and oxidized fatty acid (oxFA) [5].

Many clinical studies have found an association between increasing serum levels of Lp-PLA2 mass and/or activity at the time of a cardiovascular incident in addition to an elevated risk of mortality and morbidity over time [6,7,8]. One study showed that Lp-PLA2 mRNA and protein levels were six times higher in atherosclerotic lesions compared to normal tissue samples [9]. Cumulative evidence suggests that *C. pneumoniae* also plays an important role in atherosclerosis [10,11,12,13,14]. The organism is thought to infect pulmonary monocytes that are then transported via the vasculature to localize in arteries where infection can spread [15]. *C. pneumoniae* is a ubiquitous pathogen that frequently causes upper and lower respiratory tract infections worldwide [16]. More than half of the patients with atherosclerosis have evidence for *C. pneumoniae* infection based on a variety of studies using detection methods such as immunohistochecmistry.

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**Conclusions:** The significant association of plaque Lp-PLA2 with plaque macrophages and *C. pneumoniae* suggests an interactive role in accelerating inflammation in atherosclerosis. A possible mechanism for *C. pneumoniae* in the atherogenic process may involve infection of macrophages that induce Lp-PLA2 production leading to upregulation of inflammatory mediators in plaque tissue. Additional *in vitro* and *in vivo* research will be needed to advance our understanding of specific *C. pneumoniae* and Lp-PLA2 interactions in atherosclerosis.

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interaction in atherosclerosis.

infection, suggesting an interactive role in accelerating inflamma-
tion in the presence of plaque Lp-PLA2 or infection was significantly associated with up-regulation of plaque Lp-PLA2
mass and activity levels as well as plaque Lp-PLA2 would be
significantly elevated in the presence of plaque C. pneumoniae
infection, suggesting an interactive role in accelerating inflamma-
tion in atherosclerosis.

Materials and Methods

Ethics Statement

The University of California at San Francisco (UCSF) and
Children's Hospital Oakland Research Institute (CHRCO)
Institutional Review Board committees approved the study.
Informed written consent was obtained for all study subjects.
The study was conducted according to the principles of
the Declaration of Helsinki.

Study subjects

In this cross-sectional study, subjects underwent elective carotid
endarterectomy at UCSF, as described previously [19]. The
treated carotid artery was associated with signs and/or symptoms
of neurologic disease.

Lipoprotein-associated phospholipase A2 (Lp-PLA2)
detection by immunohistochemistry (IHC) in carotid
artery tissue

Lp-PLA2 was detected by IHC using three, five-micron sections
per carotid plaque in optimal cutting temperature (OCT) medium.
The carotid plaque tissue was stored at −80°C in OCT until
sectioning. Briefly, each section was blocked with casein (Biocare
Medical, Concord, CA) and stained with anti-Lp-PLA2 monoclo-
nal antibody (diaDexus, Inc., South San Francisco, CA) diluted
1:400 in diluent (Biocare). Samples were washed with TBS,
blocked with avidin (Biocare), washed again and blocked with
biotin (Biocare) prior to applying goat, anti-mouse IgG antibody
(Biocare). Streptavidin was applied followed by alkaline phospha-
tase chromagen-fast red (Biocare). The section was counterstained
with hematoxylin to detect each cell. In independent experiments,
excess primary antibody and, separately, excess secondary
antibody was used on adjacent sections to ensure no non-specific
staining of either antibody for Lp-PLA2. In addition, a mouse non-
immune IgG (Biocare) was used as a final negative control.

Using light microscopy at 400×, samples were read independ-
ently by two individuals who were blinded to all patient data.
Samples were graded based on percentage of the tissue staining for
Lp-PLA2 for the entire plaque section. We used 1, 2 or 3+ for
the entire carotid section for each patient sample (3 sections per
patient carotid sample) where a grade of ≥1 was considered
positive for Lp-PLA2: 1, 1-25% of the tissue; 2, 26-50% of the
sample were used to determine the within-sample variation, and
the average of the three was used for analysis. Because of the ease
of visualization of the staining for Lp-PLA2 (see Fig 1A), software
was not required for quantitation.

All sections that stained positive for Lp-PLA2 were probed with
chlamydial-specific heat shock protein 60 (CHsp60) MAb (Affinity
Bioreagents) to determine the precise co-localization of Lp-PLA2
and infection using the methods as described above except that a
horseradish peroxidase-conjugated secondary antibody with
chromogen diaminobenzidine (DAB; Biocare) was used to detect
chlamydial. Positive and negative controls were as described
previously [18,19]. All three sections for each patient were read in
entirety and analyzed as described for Lp-PLA2 except that the
cells were counted to determine the number with Lp-PLA2 alone,
C. pneumoniae alone, and the number with both for each section as
quantitative measures.

Two additional adjacent sections for each patient sample were
used to co-localize C. pneumoniae with macrophages. The sections
were stained for C. pneumoniae as above, and macrophages were
stained with fast red as for Lp-PLA2 except that the primary
monoclonal antibody was macrophage specific (CD68; Biocare).

While other infectious agents may also be involved in
atherosclerosis, the evaluation of these pathogens was beyond
the scope of this study.

Prior data on the patient population used for analyses

In our prior studies, plaque tissues were noted to have a high
grade of atherosclerosis [18,19]. Data on the same population
from our previous publications were also used for analyses [18,19].
These included IHC to detect macrophages, CD4+ cells, CD8+
cells, IL-6, and C. pneumoniae in the adjacent sections of the same
block of carotid tissue used for Lp-PLA2 IHC above. In addition,
we previously determined plaque IL-6 gene expression by
quantitative (q)RT-PCR, plaque C. pneumoniae burden by qRT-
PCR, serum C-reactive protein (CRP) levels, and IL-6 serum
protein levels, the methods of which are described in detail in our
references [18,19].

Measurement of serum mass and activity levels for
lipoprotein-associated phospholipase A2 (Lp-PLA2) and
homocysteine levels

All serum biochemical analyses were performed on serum from
blood or blood plasma obtained at the time of carotid
endarterectomy.

Lp-PLA2 mass was determined by ELISA (PLAC® Test;
diaDexus) in serum according to the manufacturers instructions
using two specific monoclonal antibodies in a 96-well format.
Quantitation was calibrated to a recombinant Lp-PLA2 antigen
standard. The lower detection limit was 2 ng/mL; interassay
coefficient of variation (CV) was between 6% and 7%. Lp-PLA2
activity levels were measured by CAMIM assay (diaDexus) in
serum according to the manufacturers instructions. Samples were analyzed in a 96-well microplate with a colorimetric substrate converted on hydrolysis by phospholipase enzyme. Briefly, 25 μL of sample, standard, or control was added per well, followed by addition of assay buffer plus substrate. Change in absorbance was measured at 405 nm. Lp-PLA2 activity in nmol/min/mL was calculated from the slope, based on a standard conversion factor from a p-Nitrophenol calibration curve. Activity levels between 13.5–46.1 were considered as quartile-1, 46.2–69 as quartile-2, 69.1–89.2 as quartile-3, and 89.3–143.2 as quartile-4.

Homocysteine levels in serum (the preferred sample type) were measured by fluorometric high-performance liquid chromatography (HPLC; Quest Diagnostics).

**Statistical analysis**

Clinical and laboratory characteristics of patients were compared by serum Lp-PLA2 mass and activity levels and Lp-PLA2 plaque grade. Before comparing continuous variables for Lp-PLA2 plaque grades, the normality assumption was checked by Shapiro-Wilk test and the distributional diagnostic plots for these variables: age, Lp-PLA2 serum activity, and homocysteine levels. All except homocysteine had a normal distribution. Square root transformation of homocysteine levels was performed to achieve the normality assumption.

Student t-test was used to compare plaque Lp-PLA2 positive vs. negative groups for continuous variables with normal distribution. Pearson chi-square test was used to compare plaque Lp-PLA2 positive vs. negative groups for binomial variables: history of smoking, coronary artery disease, elevated cholesterol, diabetes, obesity, hypertension and family history of genetic disorders, elevated serum CRP, serum IL-6, C. pneumoniae by qRT-PCR and plaque IL-6, CD4+ macrophages, and C. pneumoniae. Multiple logistic regression was also performed for these comparisons, including all variables associated with Lp-PLA2 (at p<0.20) with a stepwise removal of any that did not contribute (at p>0.10). Since Lp-PLA2 serum activity results were categorized into four quartiles, Kruskal-Wallis test was used for comparing serum Lp-PLA2 quartiles for clinical characteristics. Nonparametric Spearman Rank test was used to calculate correlation coefficients between variables with Bonferroni adjustment. A P
Results

Patient characteristics and association with serum Lp-PLA2 activity and plaque Lp-PLA2

Characteristics of the 42 study patients are shown in Tables 1 and 2 stratified by Lp-PLA2 plaque status and Lp-PLA2 serum activity, respectively. We considered the 42 patients to be a representative cohort of patients with neurologic signs and/or symptoms consistent with carotid vascular disease in addition to the fact that they were enrolled consecutively after informed consent from the pre-operative evaluation clinic at UCSF as previously described [18,19]. None of the patients had stroke but all had neurologic symptoms, indicating carotid ischemia: 30 (71.4%) had symptoms on the left side, 11 (26.2%) on the right, and 1 (2.4%) bilaterally. It should be noted that the treated carotid artery was associated with symptoms on the ipsilateral side. There were no significant correlations of risk factors (smoking, coronary artery disease, elevated cholesterol, diabetes, obesity, hypertension and family history of genetic disorders) or clinical characteristics with Lp-PLA2 serum levels or tissue Lp-PLA2 grade >1 (Tables 1 and 2) or with C. pneumoniae infection as defined by qRT-PCR or IHC as described previously [18,19] (Tables 3 and 4).

Correlation among carotid plaque characteristics and serum levels of inflammatory markers

Serum Lp-PLA2 mass and activity levels were significantly correlated (r = 0.76, p = 0.001, Table 3). High Lp-PLA2 activity was also correlated with Lp-PLA2 mass (r2 = 0.37 and r4 = 0.48, p1 = 0.015 and p2 = 0.001, respectively, Table 4).

Interestingly, 94.7% (18/19) of patients who had plaque Lp-PLA2 also had plaque C. pneumoniae. Plaque Lp-PLA2 presence (for all quantitative grades above 1) was significantly correlated with C. pneumoniae (r = 0.39, p = 0.001) and macrophages (r = 0.37, p = 0.01, Table 3), and with higher serum homocysteine levels (r = 0.38, p = 0.013, Table 3). Plaque Lp-PLA2 co-localized with C. pneumoniae, macrophages and CD4+ lymphocytes by IHC in the shoulder and necrotic core of the plaques as has been noted by others [9]. We found that 32% of cells showed evidence for Lp-PLA2 protein and infection with C. pneumoniae (Figure 1A). In addition, 39% of macrophages were infected with C. pneumoniae (Figure 1B).

In Table 3, the correlation between carotid plaque Lp-PLA2 and plaque IL-6 expression, IL-6 detected by IHC, serum IL-6, and CRP was statistically insignificant for all plaque Lp-PLA2 grades. Serum Lp-PLA2 mass levels were negatively correlated with plaque IL-6 expression and IL-6 detected by IHC (r = −0.31, p = 0.048; r = −0.34, p = 0.03, respectively), and not correlated with serum IL-6 or CRP. Serum Lp-PLA2 activity levels were negatively correlated with IL-6 detected by IHC (r = −0.32, p = 0.04) and not correlated with plaque IL-6 expression, serum IL-6 or CRP.

Figure 2A shows staining of Lp-PLA2 (red) in the perivascular necrotic area of carotid plaque. This region was rich in macrophages in addition to C. pneumoniae infected macrophages. Figure 2B shows the adjacent section stained with secondary antibody and omission of primary antibody against Lp-PLA2 as a control for specificity. There were similar results for staining with the control mouse non-immune IgG antibody (data not shown).

Discussion

This is the first study, to our knowledge, that evaluates the correlation between Lp-PLA2 serum mass and activity levels with presence of Lp-PLA2 in carotid plaques, and the association of these indicators with plaque C. pneumoniae and other inflammatory mediators. Lp-PLA2 serum mass and activity levels correlated well with one another but not with plaque Lp-PLA2. However, plaque Lp-PLA2 was significantly correlated with plaque C. pneumoniae infection, macrophages and serum homocysteine levels. A high percentage of macrophages were infected, and many cells showed co-localization of Lp-PLA2 with C. pneumoniae. Thus, a possible mechanism for C. pneumoniae in the atherogenic process may involve infection of macrophages that induce Lp-PLA2 production.

Table 1. Clinical characteristics of study population by plaque Lp-PLA2.

| Characteristics | Total (n = 42) | Plaque Lp-PLA2 negative (n = 23) | Plaque Lp-PLA2 positive (n = 19) | P Valueb |
|-----------------|---------------|---------------------------------|---------------------------------|----------|
| Age (mean ± s.d.) | 72 (9.4) | 72.4 (+/− 10.6) | 72.3 (+/− 8) | 0.987 |
| Sex: Male | 30 (71.4%) | 15 (65.2%) | 15 (78.9%) | 0.327 |
| Female | 12 (28.6%) | 8 (34.8%) | 4 (21.1%) | |
| Smoker | 33 (78.6%) | 17 (73.9%) | 16 (84.2%) | 0.418 |
| CAD | 23 (54.8%) | 11 (47.8%) | 12 (63.2%) | 0.320 |
| Hypertension history | 36 (85.7%) | 20 (87%) | 16 (84.2%) | 0.800 |
| High cholesterol | 27 (64.3%) | 14 (60.8%) | 13 (68.4%) | 0.611 |
| Diabetes mellitus | 9 (21.4%) | 4 (17.4%) | 5 (26.3%) | 0.483 |
| Symptomatic | | | | 0.264 |
| Left side | 30 (71.4%) | 18 (78.3%) | 12 (63.2%) | |
| Right side | 11 (26.2%) | 4 (17.4%) | 7 (36.7%) | 0.611 |
| Both | 1 (2.4%) | 1 (4.3%) | 0 | |

Abbreviations: Lp-PLA2, Lipoprotein-associated phospholipase A2; CAD, Coronary Artery Disease.

Values expressed are for plaque Lp-PLA2 grade of ≥1 as the results were the same for any grade ≥1.

P values were generated by chi-square test except for age, where t-test was used for comparison. doi:10.1371/journal.pone.0011026.t001
leading to upregulation of inflammatory mediators in plaque tissue.

We found no significant correlation between patient risk factors for atherosclerosis and serum levels or plaque grade for Lp-PLA2 in our study. Our findings are similar to those of others [29] but in contrast to some publications that reported a correlation between clinical characteristics or risk factors and serum Lp-PLA2 mass or activity levels [30,31]. Earlier publications initially found strong correlations between serum Lp-PLA2 levels and clinical characteristics, which decreased significantly after adjustment for

| Characteristics       | Lp-PLA2 Activity 1st quartile (n = 10) | Lp-PLA2 Activity 2nd quartile (n = 10) | Lp-PLA2 Activity 3rd quartile (n = 10) | Lp-PLA2 Activity 4th quartile (n = 11) | P Valueb |
|-----------------------|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|----------|
| Age (mean +/- s.d.)   | 67.9 (+/- 10.1)                       | 71.0 (+/- 10.1)                       | 75.9 (+/- 7.8)                        | 73.7 (+/- 8.5)                       | 0.519    |
| Sex:                  |                                       |                                       |                                       |                                       | 0.403    |
| Male (n = 29b)        | 8 (27.6%)                             | 5 (17.2%)                             | 8 (27.6%)                             | 8 (27.6%)                            |          |
| Female (n = 12b)      | 2 (17%)                               | 5 (41%)                               | 2 (17%)                               | 3 (25%)                              |          |
| Smoker                | 8 (25%)                               | 7 (21.9%)                             | 6 (25%)                               | 11 (28.1%)                           | 0.916    |
| CAD                   | 2 (9.1%)                              | 7 (31.8%)                             | 6 (27.3%)                             | 7 (31.8%)                            | 0.101    |
| Hypertension          | 7 (20%)                               | 9 (25.7%)                             | 8 (22.9%)                             | 11 (31.4%)                           | 0.243    |
| High cholesterol      | 4 (15.4%)                             | 8 (30.8%)                             | 5 (19.2%)                             | 9 (34.6%)                            | 0.115    |
| Diabetes mellitus     | 3 (33.3%)                             | 1 (11.1%)                             | 1 (11.1%)                             | 4 (44.5%)                            | 0.337    |

Symptomatic: 0.095

Right side 9 (31%) 7 (24.2%) 4 (13.8%) 9 (31%)
Left side 1 (9.3%) 2 (18.2%) 6 (54.6%) 2 (18.2%)
Both sides 0 1 (100%) 0 0

Abbreviations: Lp-PLA2, Lipoprotein-associated phospholipase A2; Lp-PLA2, Activity, range measured in nmol/min/mL; CAD, Coronary Artery Disease.

Table 3. Correlations among plaque characteristics and serum levels of inflammatory markers.

| Carotid Plaque | Serum Lp-PLA2 mass (ng/mL) | Serum Lp-PLA2 activity |
|----------------|---------------------------|------------------------|
| Cpn by qRT-PCR | 0.21                      | -0.28                  | -0.23                                |
| Cpn by IHC     | 0.39                      | -0.24                  | -0.08                                |
| IL-6 expression| 0.23                      | -0.31                  | -0.22                                |
| IL-6 by IHC    | 0.11                      | -0.34                  | -0.32                                |
| Macrophages    | 0.37                      | 0.06                   | 0.11                                 |
| CD4+           | 0.17                      | -0.04                  | -0.13                                |
| CD8+           | -0.02                     | -0.10                  | -0.13                                |
| B-cell         | -0.11                     | -0.22                  | -0.32                                |
| Lp-PLA2        | 1                         | 0.14                   | 0.19                                 |

| Serum | Serum Lp-PLA2 mass (ng/mL) | Serum Lp-PLA2 activity |
|-------|---------------------------|------------------------|
| Lp-PLA2 mass (ng/mL) | 0.14                      | 1                      | 0.76b                                |
| Lp-PLA2 activity | 0.19                      | 0.76b                  | 1                                    |
| CRP   | 0.18                      | -0.25                  | -0.21                                |
| IL-6  | 0.08                      | -0.27                  | -0.19                                |
| Homocysteine | 0.38                      | -0.006                 | 0.12                                 |

Abbreviations: Lp-PLA2, Lipoprotein-associated phospholipase A2; Cpn, C. pneumoniae; qRT-PCR, quantitative real-time reverse transcription PCR; IL-6, interleukin-6; IHC, immunohistochemistry; CRP, C-reactive protein.

*p < 0.001.
**p < 0.01.
***p < 0.013.
****p < 0.05.
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measures of atherosclerosis [32,33]. In our study, the lack of correlation between either clinical characteristics or risk factors and Lp-PLA2 might be explained by the small sample size. However, serum Lp-PLA2 mass and activity levels may not be consistently reliable risk markers for atherosclerosis.

There is prior evidence that both serum Lp-PLA2 mass and activity levels are influenced by infection such as hepatitis C, malaria and influenza [34,35,36]. For example, malaria researchers have shown a positive correlation between circulating levels of Lp-PLA2, parasitemia and severity of disease [35]. Studies of the interrelationship of influenza with inflammatory responses and atherosclerosis were initiated based on the observation of a strong association between acute respiratory infections, acute MI and sudden death in winter [36]. In a murine model of influenza, Lp-PLA2 activity in high density lipoproteins (HDL) was found to decrease two days after inoculation of influenza, reaching the lowest levels within a week, while Lp-PLA2 modification of LDL and lipid peroxide products increased as monocyte migration was induced [36].

In our study, only plaque Lp-PLA2, but not serum Lp-PLA2 mass or activity levels, was significantly associated with the presence of *C. pneumoniae* [18]. Prior studies have shown that persistent *C. pneumoniae* infection, characterized by up-regulation of chlamydial heat shock protein 60 expression, induced LDL oxidation that leads to macrophage activation [37]. It is well known that oxidized LDL is also a substrate of Lp-PLA2 catalyzed reactions, resulting in LysoPC and oxFA [5]. LysoPC induces proinflammatory cytokines and chemokines, such as IL-1β, IL-6, TNF-α, and monocyte chemoattractant protein 1 (MCP-1) [38]. IL-1β, IL-6 and TNF-α trigger atherogenesis by activating vascular smooth muscle cells [39] and inducing secretion of cellular adhesion molecules [40] and matrix metalloproteinase (MMP) by monocytes during later stages of atherosclerosis [41]. MCP-1 recruits T cells and monocytes, inhibits endothelial nitric oxide (causing endothelial dysfunction), induces monocyte-macrophage colony-stimulating factor (M-CSF) secretion by smooth muscle cells and stimulates macrophage proliferation [42,43,44]. In our study, we found that plaque Lp-PLA2 was significantly correlated with plaque macrophages, which is consistent with these studies.

Several studies have shown that *C. pneumoniae* activated macrophages induce pro-inflammatory cytokine/chemokines, such as IL-6, IL-8 and MCP-1 [18,43,46]. In our previous evaluation of the same tissue samples as in the present study, we found that macrophages in the carotid plaques co-localized with CD4+ lymphocytes [18], which can secrete pro-inflammatory cytokines and further fuel the atherogenic process. Both CD4+ cells and macrophages release interferon gamma (IFN-γ), which can resolve chlamydial infection or stimulate a non-replicative persistent state that can result in chronic infection that is likely resistant to antimicrobial treatment.

IL-6 is an acute phase reactant secreted by activated macrophages, Th2 cells and B cells. We previously showed that

### Table 4. Correlations between serum Lp-PLA2 activity, and plaque and serum Lp-PLA2 mass.

| Serum Lp-PLA2 activity (range in nmol/min/mL) | Plaque Lp-PLA2* | Serum Lp-PLA2 mass (ng/mL) |
|---------------------------------------------|----------------|--------------------------|
| 1st quartile (13–46.1)                      | 0.04 (p = 0.822) | –0.47 (p < 0.001)        |
| 2nd quartile (46.2–69)                      | –0.14 (p = 0.375) | –0.31 (p = 0.043)        |
| 3rd quartile (69.1–89.2)                    | 0.02 (p = 0.987) | 0.37 (p = 0.015)         |
| 4th quartile (89.3–143.2)                   | 0.13 (p = 0.417) | 0.48 (p = 0.001)         |

*Abbreviations: Lp-PLA2, Lipoprotein-associated phospholipase A2.*

*Values expressed are for plaque Lp-PLA2 grade of ≥1 as there were no significant correlations with grades >1.*

doi:10.1371/journal.pone.0011026.t004

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Several studies have shown that *C. pneumoniae* activated macrophages induce pro-inflammatory cytokine/chemokines, such as IL-6, IL-8 and MCP-1 [18,43,46]. In our previous evaluation of the same tissue samples as in the present study, we found that macrophages in the carotid plaques co-localized with CD4+ lymphocytes [18], which can secrete pro-inflammatory cytokines and further fuel the atherogenic process. Both CD4+ cells and macrophages release interferon gamma (IFN-γ), which can resolve chlamydial infection or stimulate a non-replicative persistent state that can result in chronic infection that is likely resistant to antimicrobial treatment.

IL-6 is an acute phase reactant secreted by activated macrophages, Th2 cells and B cells. We previously showed that
quantitatively higher levels of carotid plaque C. pneumoniae measured by qRT-PCR and semi-quantitative IHC was associated with higher IL-6 expression in both plaques and serum [18]. Subsequent studies have also shown that atherosclerotic progression, based on intima-media wall thickness, was associated with higher serum IL-6 levels among C. pneumoniae patients [47]. In vitro studies, C. pneumoniae induces the production of IL-6 in peripheral monocytes and smooth muscle cells [45]. Neither serum nor plaque IL-6 correlated with serum Lp-PLA2 activity or mass levels or with plaque Lp-PLA2 grade in our study. However, one other study also failed to show a correlation between serum IL-6 and Lp-PLA2 activity [48]. This might be due to the indirect pathways induced by Lp-PLA2 where the temporal influence of Lp-PLA2 and up-regulation of serum IL-6 are missed because only a single serum sample is obtained at the time of endarterectomy. Similarly, given that we found a lack of association of serum Lp-PLA2 mass or activity levels with plaque Lp-PLA2 and with plaque C. pneumoniae, it is possible that either the timing of sample collection yields a false negative result or that what is occurring locally in the tissue does not always reflect the circulating systemic data whether there is a direct interaction between homocysteine and serum homocysteine levels. Homocysteine exerts an independent effect on vascular smooth muscle cell proliferation, although the mechanism[s] is not well understood [49]. It is unclear from our data whether there is a direct interaction between homocysteine and plaque Lp-PLA2 that may accelerate atherosclerotic progression.

Overall, we found that macrophages, many of which were infected with C. pneumoniae, co-localized with Lp-PLA2. A high percentage of cells demonstrated co-localization of Lp-PLA2 and C. pneumoniae. These findings suggest that macrophages may be activated by C. pneumoniae infection, inducing Lp-PLA2 production and subsequent proinflammatory mediators, and, under the influence of Lp-PLA2 byproducts, result in macrophage proliferation that in turn release inflammatory mediators. This scenario indicates a possible indirect mechanism for C. pneumoniae involvement in the atherogenic process. However, additional research focused on in vitro cell and in vivo animal models will be needed to advance our understanding of the interaction of C. pneumoniae infection with Lp-PLA2 in inflammation and atherosclerotic disease.

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Author Contributions

Conceived and designed the experiments: SCJ DD. Performed the experiments: BA. Analyzed the data: BA SCJ DD. Contributed reagents/materials/analysis tools: DD. Wrote the paper: BA SCJ DD.

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