European Journal of Vascular and Endovascular Surgery 43 (2012) 154–159

Contents lists available at SciVerse ScienceDirect

European Journal of Vascular and Endovascular Surgery

journal homepage: www.ejves.com

Circulating Lipoprotein-associated Phospholipase A2 in High-grade Carotid Stenosis: A New Biomarker for Predicting Unstable Plaque CME

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WHAT THIS PAPER ADDS

- Our work focussed on a new biomarker for predicting vulnerability of carotid plaque, the plasmatic level of lipoprotein-associated phospholipase A2. We reported that Lp-PLA2 is increased in patients with unstable and high-grade carotid stenosis.
- Lp-PLA2 may thus be a relevant biomarker that could classify a carotid plaque as vulnerable or not, and predict neurologic risk of a carotid stenosis in asymptomatic subjects.

ARTICLE INFO

Article history:
Received 7 June 2011
Accepted 10 October 2011
Available online 9 November 2011

Keywords:
Lp-PLA2
High-grade carotid stenosis
Atherosclerosis
Unstable plaque

ABSTRACT

Objective: To test plasma levels of lipoprotein-associated phospholipase A2 (Lp-PLA2) in patients with high-grade carotid stenosis according to plaque histology.
Methods: This cross-sectional single-centre study included patients with ≥70% North American Symptomatic Carotid Endarterectomy Trial (NASCET) carotid stenosis, who were treated surgically. Serum Lp-PLA2 and high-sensitivity C-reactive protein (hs-CRP) were determined on the day of surgery. Histopathological analysis classified carotid plaque as stable or unstable, according to AHA classification.
Results: Of the 42 patients (mean age 70.4 ± 10.5 years; 67% men), neurological symptoms were present in 16 (38%). Unstable plaques were found in 23 (55%). Median plasma level of Lp-PLA2 was significantly higher in patients with unstable plaque compared to those with stable plaque (222.4 (174.9–437.5) interquartile range (IQR) 63.5 vs. 211.1 (174.9–270.6) IQR 37.2 ng ml^{-1}; $p = 0.02$). Moreover, median Lp-PLA2 level were higher in asymptomatic patients with unstable plaque (226.8 ng ml^{-1} (174.9–437.5) IQR 76.8) vs. stable plaque (206.9 ng ml^{-1} (174.9–270.6) IQR 33.7; $p = 0.16$). Logistic regression showed that only the neurological symptoms (OR = 30.9 (3.7–244.6); $p < 0.001$) and the plasma Lp-PLA2 level (OR = 1.7 (1.1–12.3); $p = 0.03$) were independently associated with unstable carotid plaque as defined by histology.
Conclusions: This study showed that circulating Lp-PLA2 was increased in patients with high-grade carotid stenosis and unstable plaque. Lp-PLA2 may be a relevant biomarker to guide for invasive therapy in asymptomatic patients with carotid artery disease.

Systematic surgery of patients with ≥70% North American Symptomatic Carotid Endarterectomy Trial (NASCET)[1] asymptomatic carotid stenosis is actually debated. This reflects the considerable improvement in medical treatment not only by strict control of

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doi:10.1016/j.ejvs.2011.10.009

cardiovascular (CV) risk factors, but also by the recent provision of anti-platelet therapy, statins and angiotensin-converting enzyme (ACE) inhibitors. This medical approach may reduce the risk of stroke inherent with carotid stenosis to levels below the stroke-risk due to surgery.[2] However, for some patients, despite this best medical treatment, the risk of carotid stenosis is not completely abolished and additional surgery can be useful, especially in patients who would otherwise have >10 years of life expectancy.[3]

In this context, several markers have been tested to select patients who would most benefit from surgery: systemic biological markers (high-sensitivity C-reactive protein (hs-CRP), metalloproteinase-2 MMP-2 and MMP-9)[4] and imaging techniques (contrast ultrasound, high-resolution magnetic resonance imaging (MRI) and nuclear techniques) have been assessed for their ability to detect unstable carotid plaques. Recently, lipoprotein-associated phospholipase A2 (Lp-PLA2) has been considered as an original independent biomarker of both coronary heart disease and ischaemic stroke associated with atherosclerosis.[5] Lp-PLA2 is a calcium-independent member of the phospholipase A2 enzyme family, and can hydrolyse oxidised phospholipids to generate lysophosphatidylcholine and oxidised fatty acids, which have proinflammatory properties. Lp-PLA2 is secreted *de novo* by plaque inflammatory cells[6] and is thus implicated in the genesis of vulnerable plaques. In 2008, an international consensus panel included a level of Lp-PLA2 >200 ng ml^{-1} as a new parameter to improve the stratification of CV-risk patients.[7] Recently, Mannheim et al. showed that local expression of Lp-PLA2 in carotid plaque is increased in patients with clinical vulnerable plaque.[8]

The objective of our study was to compare plasma levels of Lp-PLA2 in patients with high-grade carotid stenosis according to plaque histology.

Materials and Methods

Patients

Patients with high-grade carotid stenosis, who were referred to our vascular surgery department for a carotid endarterectomy, were included from June 2009 to June 2010. All symptomatic patients had a clinical evaluation by a neurologist, a brain imaging and cardiac examinations (trans-oesophageal echocardiography and electrocardiogram (ECG) monitoring). Informed consent was obtained from all patients. All procedures were approved by the local human-ethics committee. High-grade carotid stenosis was defined by a ≥70% diameter reduction using the NASCET method.[1] All the data were collected prospectively. Hypercholesterolaemia was defined as low-density lipoprotein (LDL) cholesterol >160 mg dl^{-1} or long-term statin therapy. Medical therapy was defined as the treatment at the admission in our vascular surgery department. NASCET clinical criteria[1] were used to classify patients as neurologically symptomatic or asymptomatic. Patients with clinical signs of infection were excluded. A carotid endarterectomy was performed using conventional surgical techniques and removed carotid plaque was collected for histopathological analysis.

Laboratory measurements

On the day of surgery, blood samples were taken for subsequent analysis (hs-CRP and Lp-PLA2), and plasma were stored at −70 °C until analysis. Lp-PLA2 was measured using the PLAC™ Test (diaDexus Inc., San Francisco, CA, USA) test, which uses a dual monoclonal antibody immunoassay, which was standardised to recombinant Lp-PLA2.[9] Hs-CRP was measured using the SYNCHRON LX PRO System, which has been validated to the Beckman Coulter method. Biological analyses were performed blinded to the clinical details.

Histological assessment and immunohistochemistry of carotid plaques

The samples were fixed for 24 h in 4% neutral buffered formalin, dehydrated in graded alcohols, cleared in xylene and embedded in paraffin. Serial (5 μm) samples were cut for haematoxylin–eosin staining (HES) and immunohistochemical procedures to identify specific antibodies. Automated immunohistochemistry was performed with an avidin–biotin–peroxidase complex using a Ventana Benchmark XT. The following antibodies were used: anti-CD 3 (polyclonal rabbit anti-human, Dako, pre-diluted 1/2) and anti-CD 68 (monoclonal mouse anti-human clone KP1, Dako, diluted 1/2500).

Morphological characteristics of the carotid plaque were established on HES sections according to the classification of the American Heart Association (AHA)[10] (Fig. 1). Lesions referred to as stable were AHA type V, characterised by fibrous conjunctive tissue, together with extracellular lipids and laminated acellular collagen, without any endothelial disruption (Va), or were only fibrous conjunctive tissue (Vb). Lesions referred to as unstable were type VI, characterised by ulceration of the endothelial surface (VIa) or recent intra-plaque haemorrhage (VIb), or an intra-plaque thrombosis (VIc). Six sections per patient were analysed. The quantification of T lymphocytes and macrophages was established from immunohistochemical sections that were taken from the area around the lipid core. Quantitative evaluation of 10 optical fields was performed at ×400 magnification. Results are expressed as the number of cells per 10 fields.

Statistical analyses

The patients' characteristics were compared at baseline according to the plaque's morphology and the presence of neurological symptoms in the patient. Results were expressed as mean ± standard deviation (SD) or median (min–max) with interquartile range (IQR). Bivariate analyses were conducted with all appropriate continuous variables using Student's *t*-test or a Mann–Whitney test. Categorical variables were analysed by the chi-squared or Fisher's exact test. The statistical analysis was considered to be significant for $p < 0.05$. Multivariate analysis was carried out by elaborating a logistic regression model to estimate the probability of an unstable plaque according to the relevant parameters. The selection of variables was made according to a literature review[11] and the results of bivariate analyses (variables associated with $p < 0.05$): age, sex, the existence of a hypercholesterolaemia, the presence of neurological symptoms, the Lp-PLA2 level and the hs-CRP level. Analysis was performed using *R* for Windows, version 2.11.1.

Results

Population characteristics

A total of 42 patients (28 men and 14 women) were included with a mean age of 70.4 ± 10.6 years. During the inclusion period, 30 patients were not included because of unavailable biological or histological samples or patients' rejection. Neurological symptoms were present in 16 patients (38%): five (31%) had a transient ischaemic attack (three with hemispheric symptoms and two with amaurosis fugax) and 11 (69%) had a stroke. Mean carotid-diameter reduction was 82.8 ± 8.6%. At hospital admission, 41 patients (98.0%) were receiving anti-platelet therapy, 26 (61.9%) were receiving statins and 18 (42.9%) were receiving ACE inhibitors. All patients warranted that they took their treatment. The mean time between neurological symptoms and carotid surgery was 11.4 ± 7.2

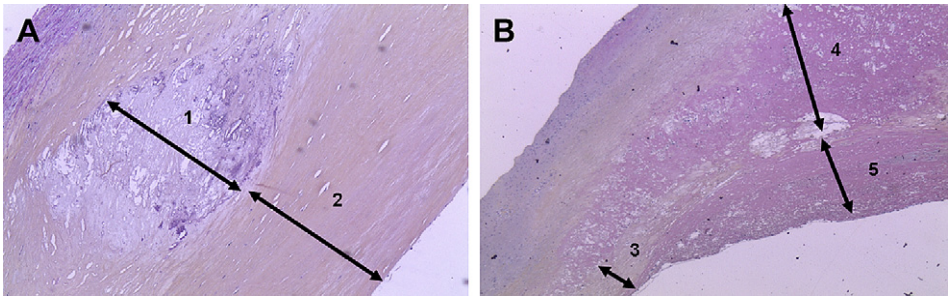

Figure 1. Examples of stable (A) and unstable plaque (B) obtained by histological analysis according to the American Heart Association's classification.[19] A: Plaque defined as stable (AHA type V) are characterised by a small lipid core (1) and a large fibrous cap (2) on haematoxylin–eosin staining (HES). Original magnification ×200. B: Plaque defined as unstable (AHA type VI) are characterised by a thin fibrous cap (3), a large and altered lipid core (4), and an intra-plaque thrombosis with rupture of the cap (5) on HES-staining. Original magnification ×100.

days. After histological analysis, the plaques were classified as unstable (55%, $n = 23$) or stable (45%, $n = 19$). The most frequently encountered cells were macrophages (mean per 10 fields: 33.6 ± 15.3) more than T lymphocytes (mean per 10 fields: 13.5 ± 11.7).

Characteristics for all patients regarding 'neurological symptoms' vs. the 'asymptomatic' patients, and 'unstable plaques' vs. 'stable plaques', are summarised in Table 1. There was no statistical difference regarding age, gender, CV-risk factors, prior history of coronary artery disease or peripheral artery disease, or medical therapy. Hypercholesterolaemia was more frequent in asymptomatic patients and in patients with stable plaques ($p = 0.04$). Unstable plaque was significantly associated with the presence of neurological symptoms ($p = 0.001$) and with the number of macrophages in the plaque ($p = 0.02$). There was no association between high-density lipoprotein (HDL) and Lp-PLA2.

Plasma Lp-PLA2, neurological symptoms and carotid-plaque morphology

Median plasma level of Lp-PLA2 in the total patient population was 214.6 ng ml^{-1} (174.9–437.5). Thirty-one patients (74%) had an Lp-PLA2 level ≥200 ng ml^{-1}, 18 patients had unstable plaque (78%, $n = 23$) and 13 had stable plaque (68%, $n = 19$, $p = 0.18$). Median plasma level of Lp-PLA2 was significantly higher in patients with unstable plaque including ulcerated plaque compared to those with stable plaque (222.4 (174.9–437.5) IQR 63.5 vs. 211.1 (174.9–270.6) IQR 37.2 ng ml^{-1}; $p = 0.02$) (Fig. 2). There was no significant difference between patients with or without neurological symptoms (220.0 (182.2–349.7) IQR 62.6 vs. 213.0 (174.9–437.5) IQR 33.7 ng ml^{-1}; $p = 0.43$) (Fig. 2).

Plasma hs-CRP, neurological symptoms and carotid-plaque morphology

Median plasma level of hs-CRP in the total patient population was 4.7 mg l^{-1} (0.5–83). This was significantly higher in patients with unstable plaque compared to those with stable plaque (11.0 (1–83) IQR 16.8 vs. 2.0 (0.5–25) IQR 3.5 mg l^{-1}; $p = 0.01$) (Fig. 3). Similarly, median plasma level of hs-CRP was significantly higher in patients with neurological symptoms compared to asymptomatic patients (12.0 (2–83) IQR 19.0 vs. 1.9 (0.5–25) IQR 3.0 mg l^{-1}; $p = 0.009$) (Fig. 3).

Lp-PLA2 and hs-CRP plasma levels in asymptomatic patients with unstable carotid plaques vs. stable plaques

In patients without neurological symptoms, eight had unstable plaques and 18 had stable plaques, as assessed by histology. Characteristics were identical whatever the carotid-plaque morphology (Table 2). Plasma Lp-PLA2 was higher in patients with unstable plaques compared to those with stable plaques (226.8 (174.9–437.5) IQR 76.8 vs. 206.9 (174.9–270.6) IQR 33.7 ng ml^{-1}; $p = 0.16$) (Fig. 2). However, hs-CRP levels were similar between patients with unstable or stable plaques (2.8 (1–13.6) IQR 6.5 vs. 1.9 (0.5–25) IQR 3.0 mg l^{-1}; $p = 0.53$) (Fig. 3).

Table 1
Clinical and therapeutic characteristics of all patients and of those with neurological symptoms or a plaque morphology.

	All patients	Neurological symptoms			Plaque morphology		
	$n = 42$ (%)	No	Yes	p	Stable	Unstable	p
		$n = 26$ (%)	$n = 16$ (%)		$n = 19$ (%)	$n = 23$ (%)	
Age (years; mean ± SD)	70.4 ± 10.6	72.2	67.4	0.18	73.1	68.2	0.14
Male gender	28 (66.6)	16 (61.5)	12 (75.0)	0.57	12 (63.2)	16 (69.6)	0.91
Body-mass index (mean)	26	26.1	25.8	0.80	26	25.9	0.95
Hypertension	32 (76.2)	22 (84.6)	10 (62.5)	0.20	17 (89.5)	15 (65.2)	0.14
Diabetes mellitus	7 (16.7)	6 (23.1)	1 (6.2)	0.31	5 (26.3)	2 (8.7)	0.27
Hypercholesterolaemia	30 (71.4)	22 (84.6)	8 (50.0)	**0.04**	17 (89.5)	13 (56.5)	**0.04**
Smoker	13 (31.0)	8 (30.8)	5 (31.2)	0.75	5 (26.3)	8 (34.8)	0.79
CAD or PAD	15 (35.7)	9 (34.6)	6 (37.5)	0.64	6 (31.5)	9 (39.1)	0.61
Antiplatelets	41 (98.0)	26 (100)	15 (93.8)	0.77	19 (100)	22 (95.7)	0.83
Statins	26 (61.9)	18 (69.2)	8 (50.0)	0.20	13 (68.4)	13 (56.5)	0.30
Neurological event	16 (38.1)	0 (0)	16 (100)	–	1 (5.0)	15 (65.0)	**0.001**
Unstable plaque	23 (54.8)	8 (30.8)	15 (93.7)	**0.001**	0 (0)	23 (100)	–
Macrophages CD68 (mean ± SD)	33.6 ± 15.3	32.8	34.8	0.87	27.7	38.4	**0.02**
T lymphocytes CD3 (mean ± SD)	13.5 ± 11.7	13.3	13.9	0.70	11.5	15.0	0.19

CAD: coronary artery disease, PAD: peripheral artery disease.

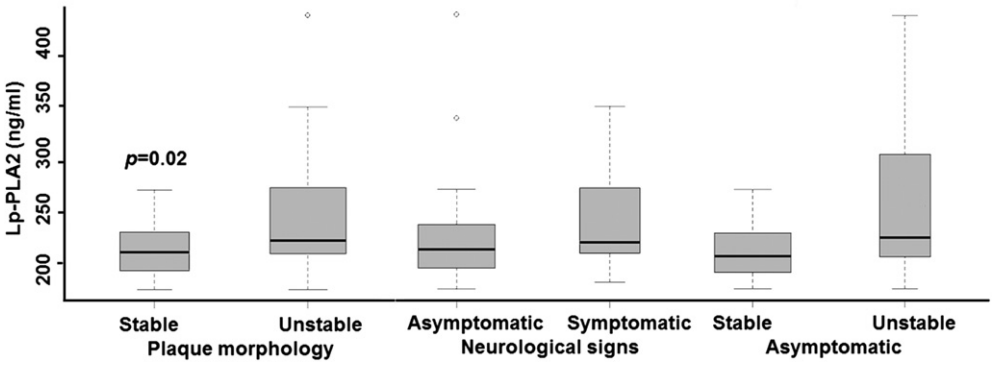

Figure 2. Box and whisker plots representing circulating Lp-PLA2 levels in patients with regard to plaque morphology or/and neurological symptoms. Circulating Lp-PLA2 levels were significantly increased in patients with unstable plaque ($p = 0.02$). The bottom and top of the box represent the 25th and 75th percentile and the band near the middle of the box is the median. The ends of the whiskers represent the lowest datum still within 1.5 interquartile range of the lower quartile, and the highest datum still within 1.5 interquartile range of the upper quartile.

Multivariate analyses

After logistic regression, only the presence of neurological symptoms and elevated plasmatic Lp-PLA2 levels were independently associated with unstable plaque. The risk of unstable carotid plaque was increased 1.7-fold to an Lp-PLA2 level of $\geq$200 ng ml^{-1} (OR = 1.7 (1.1–12.3); $p = 0.03$) and was 30.9 times more with neurologic symptoms (OR = 30.9 (3.7–244.6); $p < 0.001$). This result was not improved when hs-CRP level was associated to Lp-PLA2 level.

Discussion

Our study shows that the patients with high-grade carotid stenosis and unstable plaques have a significantly higher plasmatic Lp-PLA2 level than patients with stable plaques. These findings strongly suggest a role for Lp-PLA2 in the pathophysiology and clinical presentation of unstable carotid stenosis, thus supporting the report of Mannheim et al. in 2008.[8] These authors enrolled 167 patients who had undergone a carotid endarterectomy; they found increased local expression of Lp-PLA2 in symptomatic patients and vulnerable plaque. These results may be interesting to predict neurological risk of a carotid stenosis in asymptomatic patients in whom surgery is actually highly debated given the low rate of stroke with the best medical therapy.[2,3]

Interest in Lp-PLA2 as a biomarker for CV disease emerged 10 years ago after WOSCOPS publication (West of Scotland Coronary Prevention Study, WOSCOPS), which showed a positive association between elevated circulating concentrations of Lp-PLA2 and the risk of coronary events.[12] Since then, several reports have confirmed these results in coronary artery and cerebrovascular diseases.[13] Although some reports inconclusively found this positive association,[14] two recent meta-analyses and one review have confirmed that, after multivariate adjustments, increased levels of Lp-PLA2 remain a reliable marker for increased risk of CV events.[5,15,16]

In this study, we chose to measure the mass of Lp-PLA2 without analysing its activity. Lp-PLA2 is an enzyme that can be measured by either its mass or its activity. In a recent meta-analysis, Madjid et al. found 16 studies that measured mass, 12 studies that measured activity and six that included both mass and activity.[15] Currently, there is no consensus on the best method to estimate Lp-PLA2 level. However, in 2008, a panel's recommendation, which incorporated Lp-PLA2 testing into the CV disease-risk assessment guidelines, used mass to measure Lp-PLA2 when stratifying patients.[7] Recently, the US Food and Drug Administration approved the immunoassay PLAC test, which measures Lp-PLA2 mass to screen patients who are at high risk of CV disease. In this context, we have preferred to assess the mass of Lp-PLA2, and not its activity. However, a large-scale, randomised, multicentre trial is needed to determine which assay method is superior.

Our study reports that patients with neurological symptoms, or with unstable carotid plaque, have hs-CRP levels significantly higher than asymptomatic patients or those with stable plaque. Similarly, Alvarez et al., in 2003, reported this correlation in 62 patients with high-grade carotid stenosis.[4] Moreover, after logistic regression, Lp-PLA2, but not hs-CRP, was independently associated with unstable plaque, as defined by histology. Elkind et al. studied

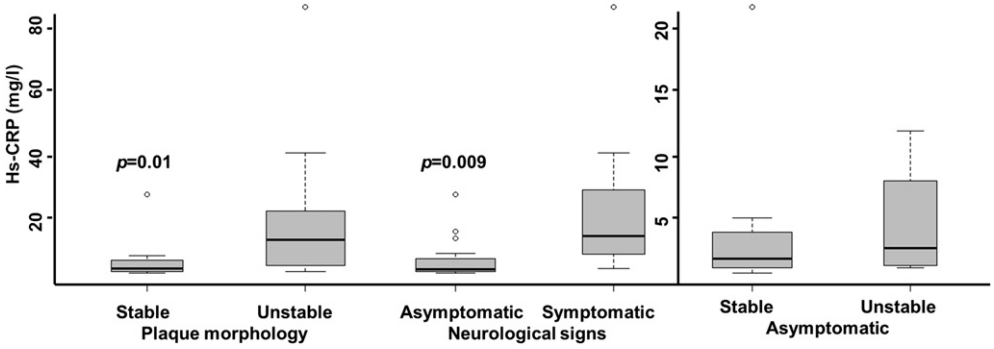

Figure 3. Box and whisker plots representing hs-CRP levels of patients with regard to plaque morphology or/and neurological symptoms. Hs-CRP levels were significantly increased in patients with unstable plaque ($p = 0.01$) and neurological symptoms ($p = 0.009$).

Table 2
Clinical and therapeutic characteristics of asymptomatic patients regarding plaque morphology.

	Plaque morphology		
	Stable	Unstable	*p*
	$n = 18$ (%)	$n = 8$ (%)	
Age (years; mean ± SD)	72.9 ± 10.7	70.6 ± 6.4	0.53
Male gender	11 (61.1)	5 (62.5)	0.71
Body-mass index (mean)	26.3	25.6	0.59
Hypertension	16 (88.9)	6 (75.0)	0.75
Diabetes mellitus	5 (27.8)	1 (12.5)	0.72
Hypercholesterolaemia	16 (88.9)	6 (75.0)	0.75
Smoker	5 (27.8)	3 (37.5)	0.66
CAD or PAD	6 (33.3)	3 (37.5)	0.68
Antiplatelets	18 (100)	8 (100.0)	0.57
Statins	13 (72.2)	5 (62.5)	0.97
Macrophages CD68 (mean ± SD)	28.3 ± 14.2	42.9 ± 13.6	**0.03**
T lymphocytes CD3 (mean ± SD)	11.5 ± 10.5	17.2 ± 12.0	0.26

CAD: coronary artery disease, PAD: peripheral artery disease.

Lp-PLA2 mass and hs-CRP in 467 patients after a first ischaemic stroke and concluded that Lp-PLA2, but not hs-CRP, was associated with the recurrence of ischaemic neurological events.[17] In addition, a biovariability study on samples from 43 non-fasting healthy adults reported greater stability of Lp-PLA2 compared to hs-CRP levels in peripheral blood.[18] All these results suggest that enhancement of hs-CRP may be a consequence of stroke and that Lp-PLA2 is more relevant than hs-CRP to predict vulnerability of carotid plaques.

Pathophysiological mechanisms that explain the association between circulating Lp-PLA2 and unstable atherosclerosis plaques are unclear and complex; however, a pro-atherogenic biological role for Lp-PLA2 has ever been suggested before. Lp-PLA2 has been shown to promote modifcation of LDL, to enhance binding to matrix proteoglycans, and to facilitate their aggregation and oxidation. The accumulation of such modified LDL in the intimal subendothelial space is a key initiation step in endothelial activation and atherosclerotic-plaque rupture.[15,16] Locally, Lp-PLA2 has been detected in human carotid atherosclerotic plaques, but not in areas of the adjacent normal arterial wall.[19] Its expression is mainly confined to plaque areas with massive lipid accumulation and leucocyte infiltration, cellular necrosis and calcification: this suggests that Lp-PLA2 is a marker for unstable plaque. As Lp-PLA2 is produced by macrophages and foam cells within atherosclerotic vulnerable plaques, excessive Lp-PLA2 may be released into peripheral blood and, thus, could indicate these unstable plaques.

Study limitations are the small number of asymptomatic patients who do not allow to obtain a significant association between Lp-PLA2 and unstable plaque, even though the trend is strong. Also, we were not able to determine a cut-off point for Lp-PLA2 to classify patients but we demonstrated that the risk of unstable carotid plaque was increased 1.7-fold to an Lp-PLA2 level of $\geq$200 ng ml^{-1}, which is the cut-off point used by the international consensus on Lp-PLA2 to classify the level of CV risk.[7] Moreover, we used as definition of hypercholesterolaemia: LDL cholesterol >160 mg dl^{-1} or long-term statin therapy. With this definition, we obtained significantly more patients with hypercholesterolaemia in asymptomatic patients and in stable plaque because these patients ever took statin therapy for CV diseases or carotid stenosis. However, logistic regression showed that Lp-PLA2 was correlated to plaque instability whatever level of hypercholesterolaemia. Finally, Lp-PLA2 may reflect a systemic state of vulnerable atherosclerosis without localising the real culprit lesion. Several teams have developed imaging techniques, using contrast ultrasound, high-resolution MRI and nuclear imaging to analyse parameters of instability, such as intra-plaque haemorrhage or a thrombus. A multi-marker strategy that coupled biology and imaging could improve identification of at-risk asymptomatic atherosclerotic lesions to guide effective therapy.

Conclusions

We have shown in this preliminary study that circulating Lp-PLA2 was increased in patients with unstable carotid stenosis. These findings strongly support a role for Lp-PLA2 in the pathophysiology and clinical presentation of carotid artery disease. Lp-PLA2 may be a relevant biomarker that could identify vulnerable carotid stenosis. Further studies are warranted to confirm these preliminary results and to determine a threshold value to predict the neurological risk of a carotid stenosis in asymptomatic subjects.

Acknowledgements

We thank the paramedical teams of the Vascular Surgery Department for their help in conducting this work.

Funding

This study was supported by The French Society for Vascular Surgery (Société de Chirurgie Vasculaire de Langue Française).

Conflict of Interest

None.

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