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Lipoprotein-Associated Phospholipase A_2 Activity Is Associated with Large-Artery Atherosclerotic Etiology and Recurrent Stroke in TIA Patients

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Key Words
Stroke, recurrent · Transient ischemic attack, outcome · Phospholipase · Atherosclerosis

Abstract
Background: Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) has emerged as a novel biomarker in cardiovascular diseases due to its ability to predict stroke in population-based studies. We aimed to investigate Lp-PLA_2 levels in transient ischemic attack (TIA) patients and to study their relationship with stroke recurrence. ***Methods:*** Lp-PLA_2 mass and activity were measured by means of the PLAC test with an automated Olympus analyzer and by a colorimetric activity method (diaDexus) in 166 TIA patients and 144 healthy controls. Vascular risk factors and stroke etiology were assessed. Outcome was defined as the presence of recurrent stroke/TIA within 7 and 30 days after the index TIA. Multivariate analyses were performed to identify potential predictors of recurrence. ***Results:*** Both Lp-PLA_2 mass and activity ($p < 0.05$) were higher in TIA than in controls. Several risk factors or previous treatments were associated with Lp-PLA_2 mass and activity level. During follow-up, 20 strokes/TIA (12%) occurred within the first 30 days and the presence of a large-artery atherosclerosis etiology of stroke (HR 3.28, $p = 0.011$), together with the past medical history of hyperlipidemia (HR 3.68, $p = 0.008$) and Lp-PLA_2 activity of >207 nmol/ml/min (HR 2.7, $p = 0.042$) were all significant predictors for recurrent stroke/TIA. ***Conclusions:*** Lp-PLA_2 activity might add significant prognostic information in the early evaluation of TIA patients.

Introduction

Despite improvements in current treatments for the management of vascular risk factors, recurrent vascular events still occur in a high proportion of patients after stroke. Among all strokes, the prognosis of transient ischemic attacks (TIAs), historically considered a benign entity, is not favorable at all. Several studies have assessed the early risk of stroke recurrence after a TIA, and found a variable but higher risk than previously thought. A systematic review and meta-analysis of 18 studies including 10,126 patients revealed a pooled early risk (within 7

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days) of stroke of 5.2% [1]. Studies with longer follow-up computed 8% risk of stroke after 30 days and 9.2% risk after 90 days [2].

In addition to the high recurrence rates, the prediction of recurrent events after a TIA still remains a great challenge. Several clinical factors have been associated with prognosis after a TIA, such as the presence of focal motor deficit or speech disturbance and the duration of symptoms [3]. Likewise, some classification systems have been developed, although they have not been completely validated across different cohorts [4]. For example, one recent multicenter prospective study found the ABCD2 score to be inaccurate as a predictor of imminent stroke at any cutoff point [5].

Other more complex diagnostic tests may help with risk stratification, such as diffusion-weighted MRI [6] by detecting acute ischemic lesions and carotid or transcranial ultrasonography by detecting extracranial and intracranial large-artery stenoses, enabling clinicians to start a specific treatment. However, and particularly in those clinical settings where complex diagnostic tests are not immediately available, biomarkers might improve the prediction of recurrent events, and therefore constitute useful tools for risk stratification [7–10]. Some of these biomarkers have been tested by independent groups with variable success, one of the most promising ones being lipoprotein-associated phospholipase A_2 (Lp-PLA_2) [11].

Lp-PLA_2 is a serine lipase which circulates mainly linked to low-density lipoproteins and, in a small fraction, to high-density lipoproteins. Lp-PLA_2 is known to be a good predictor of both first-ever and recurrent strokes in population-based studies [11]. Also, Lp-PLA_2 has been proposed as marker and involved in the development of atherosclerotic disease. At the time of carotid surgery one study found higher expression of this protein and its products, together with other proinflammatory and oxidative stress markers, in carotid plaques from symptomatic patients as compared with those from asymptomatic patients [12]. Interestingly, the differences observed in the symptomatic group (which comprised ischemic stroke and TIA patients) came mainly from the group of TIA patients who showed the highest Lp-PLA_2 expression.

One previous study on TIA patients identified Lp-PLA_2 activity as a significant predictor of the combined endpoint of recurrent stroke or death within the first 90 days after a TIA and/or the identification of a high-risk mechanism of TIA, requiring immediate treatment [7]. However, to our knowledge, these results have yet not been replicated in other independent cohorts. In our study, we aimed to investigate Lp-PLA_2 mass and activity in patients with acute TIA and to determine their relationship with the risk of early stroke recurrence and with the stroke mechanism.

Patients and Methods

Study Population

We prospectively studied 166 consecutive patients with transient neurological deficit attended by a neurologist in the emergency department. TIA was defined according to the classical definition as acute onset of focal cerebral or monocular symptoms lasting <24 h and thought to be attributable to a vascular cause [13].

Clinical Protocol and Diagnostic Tests

Demographics and classical vascular risk factors were recorded, as well as the clinical characteristics. Clinical symptoms and neurological signs at the examination were assessed and the vascular territory involved in each episode was recorded as carotid, vertebrobasilar or undetermined territory.

Other examinations during admission included medical history, physical examination, routine blood biochemistry and cell blood count, electrocardiogram, chest X-ray, CT scan, cervical carotid and transcranial Doppler (TCD) ultrasonography, and transthoracic echocardiography and Holter ECG. TCD recordings were performed on admission, with the use of a Multi-Dop-X/TCD device (DWL Elektronische Systeme GmbH; Compumedics Germany GmbH, Lindau, Germany). We used a standard method of insonation through the temporal, occipital, and orbital windows without compression testing. Intracranial stenoses were diagnosed if the mean blood flow velocity at a circumscribed insonation depth was >80 cm/s, with side-to-side differences of >30 cm/s and signs of disturbed flow, according to validated criteria [14].

Baseline cervical internal carotid artery (ICA) atherosclerosis was categorized by carotid ultrasonographic examination as follows: absent; mild, when one or both ICAs had <50% stenoses; moderate, when any of the ICA presented 50–69% stenoses, and severe, if any ICA had ≥70% stenoses or there was a history of carotid surgery.

Once all the diagnostic tests had been performed, TIAs were classified etiologically according to the Trial of ORG 10172 [15] as due to large-artery atherosclerosis, small-vessel disease, cardioembolism, stroke of other determined cause or undetermined cause. Patients were followed up for 30 days and clinical interviews were also performed on the 7th day. Endpoints included in this study were the presence of recurrent stroke or TIA within 7 and 30 days. After the index event, secondary prevention treatments were administered, which included antiplatelets, anticoagulants and lipid-lowering therapy, according to our Institutional protocol.

This study was approved by the Ethics Committee of Vall d'Hebron Hospital and all patients or relatives gave informed, written consent.

$ABCD^2$ Scores

In a subgroup of 93 patients, available clinical data were retrospectively extracted to calculate the $ABCD^2$ score, as reported in

the original articles [9, 16]. Briefly, $ABCD^2$ classification consists in a 7-point score, including age, blood pressure, diabetes, clinical features and duration of symptoms. Patients were categorized into 2 groups depending on whether they were at the highest risk (score of ≥4 points) or at low or moderate risk (score of <4 points).

Blood Sampling and Lp-PLA_2 Mass and Activity Determinations

Peripheral venous samples were obtained within the first 24 h from symptom onset in the emergency department. Samples were collected in tubes with no anticoagulant and serum was extracted after 15-min centrifugation (3,500 rpm) at 4°C and then frozen at –80°C until testing was performed. Lp-PLA_2 mass and activity were determined in all TIA patients and in 144 stroke-free healthy controls who were selected from among relatives of stroke patients treated at the hospital and matched by age and gender with them. Healthy controls had a mean age of 63 years (slightly younger than our TIA cases) and were 61% female.

Lp-PLA_2 mass (PLAC test, diaDexus Inc.) was assayed using a turbidimetric immunoassay on an automated clinical chemistry analyzer (Olympus AU 2700). Intra-assay variation was determined (diaDexus Inc.) testing 20 replicates of 1 human serum sample (coefficient of variation (CV) 1.6%) and 20 replicates of 2 controls with Lp-PLA_2 at the lowest and highest concentrations (CV 1.8% for both). Inter-assay variation was performed during 5 consecutive days in duplicates for the controls with values of <6.9%. Since our intra- and inter-assay variation was low, samples were analyzed by single measurements.

Lp-PLA_2 activity was measured with a colorimetric method in a 96-well microplate format (Lp-PLA2 CAM, diaDexus Inc.), which measures a linear kinetic rate over time. All samples were performed blinded to clinical data and tested in duplicates (CV <20%).

Statistical Analyses

Statistical analysis was conducted using the Statistical Products and Service Solution (SPSS) version 15.0. Kolmogorov-Smirnov tests were used to assess Lp-PLA_2 mass and activity distributions. Statistical significance for intergroup differences was assessed by Pearson's χ^2 or the Fisher's exact test for categorical variables and the Student's t, Mann-Whitney U and Kruskal-Wallis test for continuous variables. After the examination of the distribution was analyzed, Lp-PLA_2 mass and activity were categorized by quartiles for further analysis. Cumulative event-free rates for the time to a stroke/TIA were estimated by the Kaplan-Meier product limit method, and patients with Lp-PLA_2 mass and activity at the top and bottom quartiles were compared by the log-rank test. Time to recurrent stroke/TIA was analyzed by censoring at the time to either non-vascular death or last follow-up. Also, a receiver-operating characteristic (ROC) curve was performed to identify an optimal cutoff point of Lp-PLA_2 activity that best discriminates between the presence or absence of a new recurrent event.

Cox proportional hazard models were constructed to estimate hazard ratios (HR) and 95% confidence intervals (95% CI) of the potential role of Lp-PLA_2 as predictor of recurrent stroke/TIA after adjustment for age and classical vascular risk factors in the first week and the first month. A p value of <0.05 was considered significant.

All potential predictors were included in a model and the global predictive capacity was calculated according to the C-statistic.

Table 1. Characteristics of the study TIA participants (n = 166)

Characteristic	Value
Demographic characteristics	
Age, years	72 ± 12
Males	86 (52%)
Risk factors	
Hypertension	88 (53%)
Diabetes mellitus	41 (25%)
Hyperlipidemia	44 (27%)
Current smoking	25 (15%)
Coronary artery disease	25 (15%)
Peripheral artery disease	10 (6%)
Stroke etiology	
Large-artery atherosclerosis	38 (23%)
Cardioembolism	50 (30%)
Small-vessel disease	7 (4%)
Undetermined	71 (43%)
Previous treatments	
Antiplatelet treatment	53 (32%)
Statins	12 (7%)
Oral anticoagulation	18 (11%)

Data are expressed as mean ± SD or n (%) when appropriate.

Results

Baseline Characteristics and Lp-PLA_2 Mass and Activity

Among the 166 TIA patients studied, 70% of them were first-ever TIA patients, whereas the remainder had had one or more previous strokes before the index event. The distribution of demographic factors, classical vascular risk factors and vascular diseases is given in table 1. Lp-PLA_2 mass and activity were not normally distributed in our population and both were significantly higher in TIA cases than in healthy controls (347 vs. 199, $p < 0.001$ for Lp-PLA_2 mass, and 187 vs. 160, $p < 0.001$ for Lp-PLA_2 activity). Table 2 displays the distribution across quartiles in TIA patients and controls.

Among baseline characteristics, men showed a trend towards higher Lp-PLA_2 activity than women ($p = 0.067$) and, regarding past medical history, several factors were found to be associated with Lp-PLA_2 mass and/or activity (table 3), such as hyperlipidemia and the presence of a large-artery atherosclerosis as stroke etiology (Lp-PLA_2 activity 207 nmol/ml/min for those with large artery atherosclerosis vs. 184 nmol/ml/min for those without it, $p = 0.044$).

Table 2. Lp-PLA_2 mass and activity levels by quartile between TIA and control subjects

	Lp-PLA_2 mass, ng/ml		Lp-PLA_2 activity, nmol/ml/min	
	TIA	controls	TIA	controls
First quartile	<273	<167	<151	<130
Second quartile	273–347	167–199	151–187	130–160
Third quartile	347–414	199–243	187–228	160–195
Fourth quartile	>414	>243	>228	>195

Regarding treatments, patients who were taking antiplatelets before the TIA also had lower Lp-PLA_2 activity than patients who were not (180 vs. 213 nmol/ml/min, p = 0.039). We did not find any differences in either Lp-PLA_2 mass or activity in patients taking statins, as has been reported before. However, the proportion of patients under statin treatment before the index event in our cohort was very small (7%).

Follow-Up and Outcome Events

During follow-up, 9 (5.4%) patients presented a recurrent stroke/TIA (8 strokes and 1 TIA) within the first 7 days and 20 (12 strokes and 8 TIA) within the first 30 days (12%).

Univariate analyses were performed to identify all factors associated with the presence of recurrent stroke/TIA at 7 and 30 days, and among all baseline clinical variables, the presence of large-artery atherosclerosis stroke etiology was the most important factor associated with the presence of a 7- or 30-day recurrent event ($p < 0.01$ for all outcomes).

Other associated baseline factors which showed $p < 0.1$ in the univariate analyses were the past medical history of peripheral artery disease for recurrence within the first 7 days, and the past medical history of coronary artery disease, hyperlipidemia or peripheral artery disease, when the event occurred within the first 30 days.

Moreover, data collection was retrospectively collected from emergency department records to calculate the $ABCD^2$ scores in a subgroup of 93 patients with available information for the index TIA event. Stroke recurrence rates for each category were as follows: 0–1, 0%; 2, 12.5%; 3, 14.3%; 4, 7.7%; 5, 5%; 6, 31%, and 7, 16.7%. No significant differences were observed in stroke/TIA recurrence rates regarding the $ABCD^2$ classification into high ($ABCD^2 \geq 4$) versus low and moderate groups ($ABCD^2 < 4$) within the first 7 days (p = 0.59) or the 30 days (p = 0.89).

Regarding Lp-PLA_2 and outcome events, survival analyses were performed to assess the relationship between Lp-PLA_2 mass and activity with the presence of recurrent stroke/TIA within 7 and 30 days. Patients at the lowest quartile of Lp-PLA_2 mass and activity versus those at the highest quartile were compared using the log-rank test, as it is shown in figure 1A. Patients at the top quartile of Lp-PLA_2 activity (values >228 nmol/ml/min) had a significantly higher recurrence rate of stroke/TIA at 7 and 30 days (p = 0.041 for 7-day and p = 0.024 for 30-day recurrence) than patients at the lowest quartile (values <151 nmol/ml/min).

No significant differences were found in the rate of new events considering Lp-PLA_2 mass quartiles (data not shown).

Finally, a ROC curve was performed to identify an optimal cutoff point for Lp-PLA_2 activity discriminating the presence of a recurrent 30-day stroke/TIA with the highest sensitivity and specificity (activity of 207 nmol/ml/min, sensitivity 78%, specificity 66%). The same cutoff point was used for 7-day recurrence. A total of 58 patients (35% of the TIA patients) had Lp-PLA_2 activity levels over this cutoff. Figure 1B shows Kaplan-Meier curves for the 7- and 30-day stroke/TIA comparing groups above or below Lp-PLA_2 activity of 207 nmol/ml/min, which showed similar results.

Independent Predictors of Recurrent Stroke/TIA

Cox proportional hazards multivariate analyses were performed to identify potential predictors for the appearance of 7- and 30-day recurrent stroke/TIA adjusted by age, gender, vascular risk factors and all those factors showing $p < 0.1$ in the univariate analyses. As shown in table 4, large-artery atherosclerotic disease was the only independent predictor for 7-day stroke recurrence (HR 9.3, CI 95% 1.81–48, p = 0.008). However, for 30-day stroke/TIA, the past medical history of hyperlipidemia (HR 3.68; 95% CI 1.04–7.07) together with the presence of large-artery atherosclerotic disease (HR 3.28; 95% CI 1.32–8.15)

Table 3. Demographic characteristics and vascular risk factors according to Lp-PLA_2 mass and activity

Risk factor	Lp-PLA_2 mass ng/ml	p value	Lp-PLA_2 activity ng/ml/min	p value
Overall (n = 166)	347 (273–414)	–	187 (151–228)	–
Age, median				
<74 years (n = 79)	390 (289–482)	0.35	187 (149–220)	0.54
≥74 years (n = 87)	350 (264–416)		186 (160–231)	
Gender				
Male (n = 86)	352 (274–407)	0.71	193 (161–232)	0.067
Female (n = 80)	341 (272–433)		176 (142–221)	
Hypertension				
No (n = 78)	353 (275–419)	0.57	187 (162–225)	0.85
Yes (n = 88)	347 (266–434)		187 (143–235)	
Diabetes mellitus				
No (n = 125)	354 (277–434)	0.47	188 (160–228)	0.78
Yes (n = 41)	333 (264–392)		186 (145–236)	
Hyperlipidemia				
No (n = 122)	360 (277–446)	0.03	197 (277–446)	0.007
Yes (n = 44)	316 (238–364)		167 (149–197)	
Smoking				
No (n = 141)	344 (269–411)	0.67	187 (150–229)	0.87
Yes (n = 25)	378 (294–448)		194 (158–215)	
Coronary artery disease				
No (n = 141)	354 (276–435)	0.095	191 (152–230)	0.19
Yes (n = 25)	340 (264–361)		174 (149–206)	
Peripheral vascular disease				
No (n = 156)	344 (272–437)	0.94	186 (151–227)	0.17
Yes (n = 10)	361 (313–406)		230 (149–254)	
Antiplatelet				
No (n = 113)	381 (277–482)	0.18	213 (173–241)	0.039
Yes (n = 53)	347 (259–398)		180 (148–233)	
Statins				
No (n = 154)	362 (277–454)	0.78	203 (167–240)	0.62
Yes (n =12)	338 (266–543)		189 (150–235)	
Large-artery atherosclerosis (TOAST)				
Present (n = 38)	365 (297–419)	0.75	207 (166–245)	0.044
Absent (n = 128)	346 (270–415)		184 (148–218)	
$ABCD^2$ score				
<4 (n = 30)	399 (311–494)	0.21	202 (164–230)	0.75
≥4 (n = 63)	257 (204–361)		197 (156–238)	
7-day stroke/TIA				
Present (n = 9)	356 (282–426)	0.96	225 (193–240)	0.036
Absent (n = 157)	346 (273–415)		186 (149–226)	
30-day stroke/TIA				
Present (n = 20)	349 (294–490)	0.94	214 (180–236)	0.10
Absent (n = 146)	346 (272–411)		185 (149–225)	

Data are expressed as medians (interquartile range).

and Lp-PLA_2 activity of >207 nmol/ml/min (HR 2.7; 95% CI 1.04–7.07) emerged as significant predictors. Combining clinical predictors (past medical history of hyperlipidemia and large-artery atherosclerotic disease) in a model, the global predictive capacity for 30-day stroke/TIA was 0.683, which improved to 0.70 when Lp-PLA_2 activity was added to the model. Interestingly, the global predictive capacity of the model was further improved (from 0.73 to 0.83) selecting the subgroup of patients (n = 38) with large-artery atherosclerotic stroke etiology.

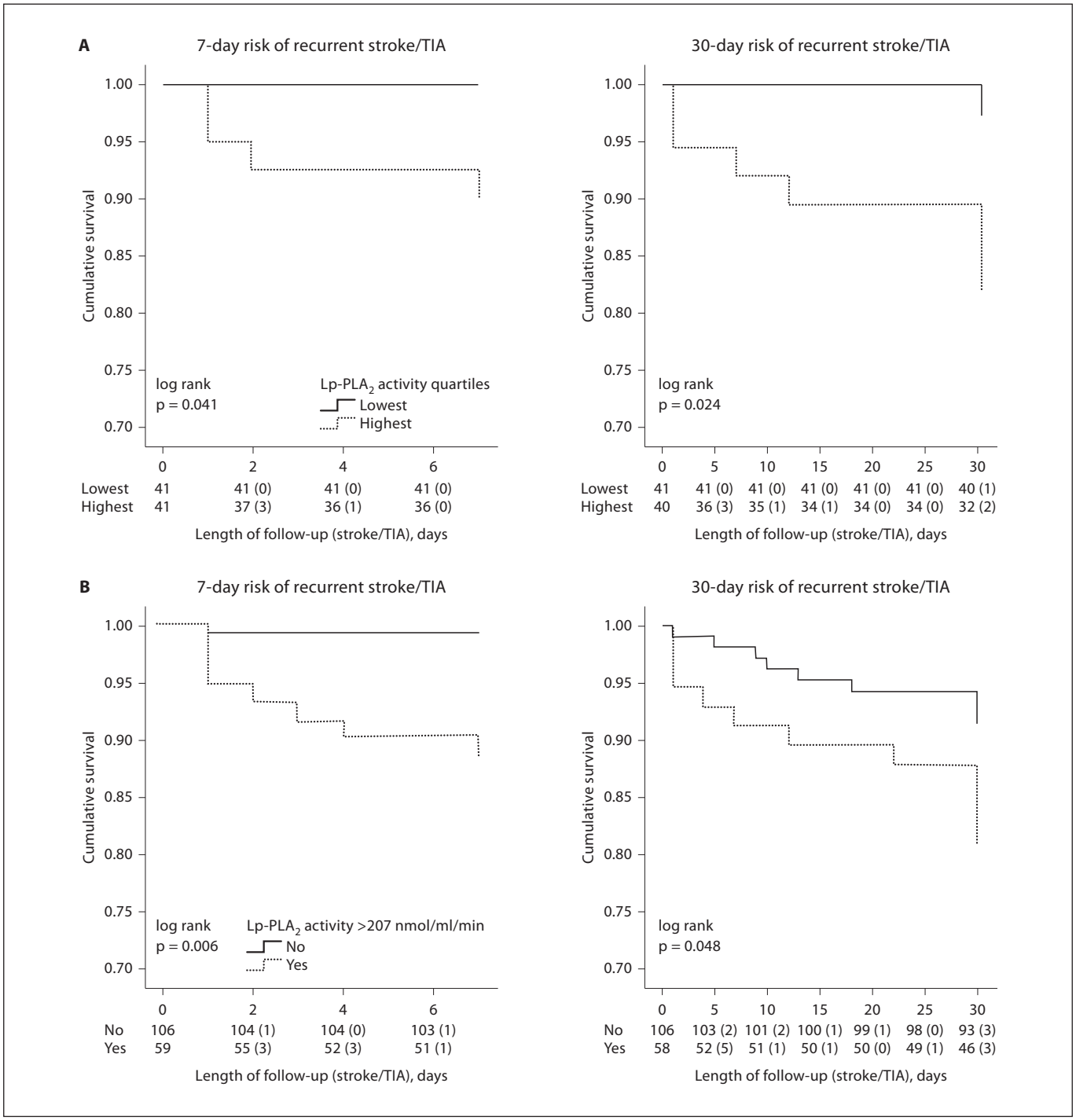

Fig. 1. Kaplan-Meier curves showing survival analyses for the presence of 7- and 30-day risk of recurrent stroke/TIA considering Lp-PLA_2 activity. Analyses were performed comparing cases in the highest versus the lowest quartile of Lp-PLA_2 activity (**A**) and comparing the cases above or below an optimal cutoff point of Lp-PLA_2 activity (**B**).

Table 4. Potential predictors of 7- and 30-day recurrent stroke/TIA after multivariate analyses

	HR (CI 95%)	p value
7-day stroke/TIA		
Large-artery atherosclerosis stroke etiology	9.3 (1.81–48.2)	0.008
30-day stroke/TIA		
Hyperlipidemia	3.68 (1.04–7.07)	0.008
Large-artery atherosclerosis stroke etiology	3.28 (1.32–8.15)	0.011
Lp-PLA_2 activity >207 nmol/ml/min	2.7 (1.04–7.07)	0.042

Discussion

The present study suggests that Lp-PLA_2 activity measured within the first 24 h after a TIA might be used for risk prediction of cerebrovascular recurrent events. Our results also support the use of emergent ultrasonographic vascular studies in the diagnosis of TIA patients, as large-artery atherosclerotic disease was the most important predictor of early stroke recurrence.

Lp-PLA_2 hydrolyzes oxidized phospholipids, releasing compounds with proinflammatory properties, which are involved in the development of atherosclerosis and plaque rupture [11]; therefore, they could be reflecting not only presence of atherosclerotic disease but plaque instability.

In our case, Lp-PLA_2 activity was found to be increased when large-artery atherosclerotic disease was the most likely mechanism for the TIA. However, both large-artery atherosclerosis and elevated Lp-PLA_2 activity are independent predictors of recurrent stroke/TIA, with the highest risk of recurrence achieved in patients with both of them present. Assessment of Lp-PLA_2 activity could be important in cases with limited availability of vascular diagnostic tests within the first 24 h from onset, guiding the selection of high-risk patients for referral to stroke units and preventive treatment intensification.

Blood testing of Lp-PLA_2 for the assessment of patients at risk of ischemic stroke was approved by the US Food and Drug Administration in 2005, since several important studies showed that patients at the top quartile of Lp-PLA_2 had a doubled risk of incident stroke, independent of classical vascular risk factors [17–21]. Moreover, a recent collaborative analysis of 32 prospective studies supports the role of Lp-PLA_2 in risk prediction [22].

The prediction of recurrent events in TIA patients could sometimes be challenging. In our case, a clinical risk stratification system, such as $ABCD^2$ score, does not accurately predict risk in this cohort, since the rate of recurrent events at 30 days was almost equal between patients with the highest versus the lowest or moderate risk according to $ABCD^2$. Several reasons have been proposed regarding the variability in risk prediction among different studies. For example, the predictive ability of this system can be influenced by the attending physician at the initial evaluation (stroke neurologist vs. emergency departments physicians) [23] or by the methodology in clinical data collection (greater predictive value when information included in the $ABCD^2$ score is collected prospectively) [24].

Our results are similar to those reported in a previous study by Cucchiara et al. [7], who found both Lp-PLA_2 mass and activity associated with the composite endpoint of stroke or death within 90 days, or with the identification of a high-risk stroke mechanism requiring specific early evaluation (such as 50% or more stenosis in a vessel referable to symptoms or a cardioembolic source warranting anticoagulation). As in our case, Lp-PLA_2 activity was an independent predictor of the outcome, but also added significant prognostic information to that achieved with only clinical parameters. It is important to emphasize, however, that the rate of stroke recurrence in both our study and that of Cucchiara et al. [7] differs greatly (3% strokes after 90 days of follow-up [7] vs. 12% of stroke or recurrent TIA after 30 days in our study); therefore, Lp-PLA_2 activity seems a promising biomarker of stroke recurrence after a TIA, applicable in populations at varying risk.

Interestingly, we observed that patients on antiplatelet treatments had lower Lp-PLA_2 activity, although the effect of some other concomitant treatments (i.e. statins), traditional risk factors and low-density lipoprotein-cholesterol, among other potential confounders, has not been ruled out [25]. Also, as opposed to Elkind et al. [26], we found higher Lp-PLA_2 activity levels in men than in women [26]. As there are differences between studies (i.e. acute vs. chronic stroke), and data from other reports on the effect of antiplatelet medications on Lp-PLA_2 levels are conflicting [21, 25, 27], further research is needed to determine the nature of these relations.

Also, as Lp-PLA$_2$ has a pro-atherogenic role, the inhibition of this enzyme may constitute a therapeutic target. In one recent study, 330 patients with coronary artery disease received darapladib (an oral direct Lp-PLA$_2$ inhibitor) during 12 months, showing decreased expansion of the lipid-rich necrotic core in coronary plaques in the patients receiving this drug, whereas patients under placebo presented significant progression despite standard-of-care treatment [28]. As far as we know, the same has not been tested in stroke patients.

Study Limitations

Although Lp-PLA$_2$ activity is stable over time, it may change after an acute ischemic and/or coronary event [29], and therefore, acute-phase levels may not be reflective of pre-stroke levels. If so, our results can only be applied when blood is obtained in the acute phase. To better explore this issue, further studies with serial determinations in TIA patients and appropriately matched control subjects are warranted. Also, multicenter replication of these results with larger sample sizes than ours and more recurrent events is of interest.

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