Metabolomics predicts stroke recurrence after transient ischemic attack

Mariona Jové, PhD*

Gerard Mauri-Capdevila, MD*

Idalmis Suárez

Serafi Cambray, PhD

Jordi Sanahuja, MD

Alejandro Quílez, MD

Joan Farré, MD

Ikram Benabdelhak

Reinald Pamplona, MD, PhD

Manuel Portero-Otín, MD, PhD

Francisco Purroy, MD, PhD

Correspondence to
Dr. Purroy:
fpurroygarcia@gmail.com

ABSTRACT

Objective: To discover, by using metabolomics, novel candidate biomarkers for stroke recurrence (SR) with a higher prediction power than present ones.

Methods: Metabolomic analysis was performed by liquid chromatography coupled to mass spectrometry in plasma samples from an initial cohort of 131 TIA patients recruited <24 hours after the onset of symptoms. Pattern analysis and metabolomic profiling, performed by multivariate statistics, disclosed specific SR and large-artery atherosclerosis (LAA) biomarkers. The use of these methods in an independent cohort (162 subjects) confirmed the results obtained in the first cohort.

Results: Metabolomics analyses could predict SR using pattern recognition methods. Low concentrations of a specific lysophosphatidylcholine (LysoPC[16:0]) were significantly associated with SR. Moreover, LysoPC(20:4) also arose as a potential SR biomarker, increasing the prediction power of age, blood pressure, clinical features, duration of symptoms, and diabetes scale (ABCD2) and LAA. Individuals who present early (<3 months) recurrence have a specific metabolomic pattern, differing from non-SR and late SR subjects. Finally, a potential LAA biomarker, LysoPC(22:6), was also described.

Conclusions: The use of metabolomics in SR biomarker research improves the predictive power of conventional predictors such as ABCD2 and LAA. Moreover, pattern recognition methods allow us to discriminate not only SR patients but also early and late SR cases. *Neurology® 2015;84:36–45*

GLOSSARY

ABCD2 = age, blood pressure, clinical features, duration of symptoms, and diabetes scale; **IDI** = integrated discrimination improvement; **LAA** = large-artery atherosclerosis; **Lp-PLA** = lipoprotein-associated phospholipase A; **NRI** = net reclassification improvement; **LysoPC** = lysophosphatidylcholine; **MS/MS** = tandem mass spectrometry; **PLS-DA** = partial least squares discriminant analysis; **ROC** = receiver operating characteristic; **SLC** = solute carrier; **SR** = stroke recurrence.

Stroke is the leading cause of acquired neurologic incapacity.[1] In almost 20% of cases, stroke is preceded by TIA, providing a great opportunity for prevention.[2] Thus, the risk of stroke is particularly high during the few days after the onset of TIA symptoms. However, TIA patients are a heterogeneous group in terms of symptoms, risk factors, underlying pathology, and early prognosis.[3] The presence of large-artery atherosclerosis (LAA) has been the main established predictor of subsequent stroke[4–6] so far, but a definitive prognostic tool for stroke recurrence (SR) is not defined.

Recently, in stroke research, there has been significant interest in biomarker development for predicting SR. Candidate approaches have rendered few biomarkers to be useful in the prognosis of TIA patients such as C-reactive protein,[7] copeptin,[8] and lipoprotein-associated phospholipase A (Lp-PLA),[9] but the validity of the proposed biomarkers is debated.[10] Regarding biomarker discovery for etiology classification, only the activity of Lp-PLA2 has been shown effective for the detection of LAA,[9,11] and very recently pro–brain natriuretic peptide levels have been associated with the diagnosis of atrial fibrillation.[12]

New techniques such as metabolomics provide the opportunity to identify new biomarkers.[13] This has been applied in stroke research[14,15] but no description of SR after early recovery such as that present in TIA patients appeared.

The aim of the present study was to perform a metabolomic analysis of plasma from a cohort of TIA patients in order to find (1) new candidate biomarkers associated with SR, (2) temporal patterns of recurrence, and (3) the potential impact of LAA in the plasma metabolomic profile. Results were then validated in an independent cohort, thus ensuring robustness of the resulting candidate biomarker.

METHODS **Standard protocol approvals, registrations, and patient consents.** The study was approved by the ethics committee of the Arnau de Vilanova University Hospital. Patient consent was obtained from all the donors.

Subjects. We prospectively recruited consecutive patients with transient neurologic deficit who were attended by a neurologist in the emergency department during the first 24 hours after the onset of symptoms (REGITELL registry).[16] These patients were aleatorized into 2 cohorts (e-Methods on the *Neurology*® Web site at Neurology.org). TIA was defined as acute onset of focal cerebral or monocular symptoms lasting <24 hours attributable to brain ischemia. The number of TIA patients attended in our hospital during the study period (January 2008 to January 2012) determined the sample size. Risk factor profile; clinical characteristics; age, blood pressure, clinical features, duration of symptoms, and diabetes score (ABCD2)[17]; ultrasound; and neuroimaging protocols were recorded as described in e-Methods.

Follow-up and clinical endpoints. Endpoint event was SR. A stroke physician performed clinical visits during the follow-up at 7 days, 3 months, and every 6 months. Imaging data were required to confirm brain ischemia in all SR patients considered. Recurrence of a TIA was not considered an endpoint.

Metabolomic analysis. For nontargeted metabolomics analysis, metabolites were extracted from plasma samples and analyzed using liquid chromatography coupled to mass spectrometry as described in e-Methods.

Statistical analysis. Statistics calculations were performed using SPSS software for Macintosh, version 20 (SPSS, Chicago, IL), *R* software, or the Stata 11 statistics package (StataCorp, College Station, TX). Further information for tests used appears in e-Methods.

RESULTS **Clinical characteristics of the study population.** Table 1 shows baseline characteristics of both cohorts, comprising 293 patients. As confounders for the metabolomic analyses, we excluded significant differences in characteristics for vascular risk factors, clinical symptoms, ABCD2 score, etiologic subtypes, or discharge treatments between the 2 cohorts. Previous anticoagulation tended to be more frequent among those patients from the first cohort. The cohorts did not differ significantly in terms of SR.

A total of 35 patients had SR. Two patients died due to recurrent ischemia. In the univariate analysis (table 2), only LAA and ABCD2 score were associated with SR. Clopidogrel and anticoagulation at discharge were associated with SR.

Subsequent stroke risk exhibits a specific metabolomic profile. Untargeted metabolomic analysis in cohort 1 indicated plasma biomarker potentially indicating future SR. Molecular features detected (present in at least 50% of samples in the same group) are represented in the heat map (figure 1B). TIA patients with SR had a specific metabolomic signature, as shown by partial least squares discriminant analysis (PLS-DA) (figure 1C), reaching an accuracy ranging from 66% (in positive ionization, specificity = 0.63, sensitivity = 0.95) to 73% (in negative ionization, specificity = 0.68, sensitivity = 1). The individual importance of molecules in the model is presented in the supplemental data (dataset 1).

Univariate statistics revealed that 94 ions differentiated SR from non-SR patients ($p < 0.05$) (dataset 2, supplemental data). By using orthogonal approaches (exact mass, isotope distribution, tandem mass spectrometry [MS/MS], and retention time database), 6 molecules were identified: 1-monopalmitin, dodecanoic acid, meso-erythritol, threonate, and lysophosphatidylcholine (LysoPC[16:0]) downregulated and myristoyl-ethanolamine upregulated ($p < 0.05$) in SR patients. After pathway analysis, these molecules clustered into free fatty acid metabolism, energy metabolism, and solute carrier (SLC)–mediated transmembrane transport pathways (dataset 3, supplemental data).

Temporal patterns of stroke recurrence define specific metabolomic profiles. The results (figure 1D) show that PLS-DA of metabolomic profiles has a high accuracy in differentiating early SR from later SR groups (dataset 1). A total of 325 ions differentiated the groups by analysis of variance ($p < 0.05$, dataset 2). These molecules (14 metabolites identified among all the differential ions), indicating early recurrence, clustered into different metabolic pathways with 13 common between recurrence and temporal patterns of recurrence, including fatty acid metabolism (biosynthesis, transport, and receptors) and regulation of insulin secretion (table e-1, dataset 3).

Patients with LAA have a specific metabolomic pattern. We focused on LAA etiology because of its importance as a SR predictor.[4–6] We evaluated metabolomic profiles of patients with symptomatic carotid or intracranial stenosis of at least 50%. The results (figure 1E) revealed that metabolomic profiles are able to offer a high accuracy predicting presence of LAA (PLS-DA accuracy 91%–98%, positive: specificity = 0.79,

Table 1 Comparative characteristics, treatments, and stroke recurrence

Variable	Cohort 1 (n = 131)	Cohort 2 (n = 162)	Total (n = 293)
Vascular risk factors			
Age, y, mean (SD)	70.5 (11.4)	72.1 (11.2)	71.7 (10.8)
Male	85 (64.9)	94 (58.0)	179 (61.1)
Previous stroke	28 (21.4)	35 (21.6)	63 (21.5)
Hypertension	91 (69.5)	111 (68.5)	201 (68.8)
Coronary disease	18 (13.7)	21 (13.0)	39 (13.3)
Diabetes mellitus	41 (31.3)	47 (29.0)	88 (30.0)
Smoking	18 (13.7)	19 (11.7)	37 (12.6)
Hypercholesterolemia	49 (37.4)	61 (37.7)	110 (37.5)
Previous atrial fibrillation	17 (13.0)	17 (10.5)	34 (11.6)
Basal treatments			
Aspirin	30 (22.9)	36 (22.4)	66 (22.6)
Clopidogrel	9 (6.9)	18 (11.2)	27 (9.2)
Anticoagulation	19 (14.5)	12 (7.5)	31 (10.6)
Statins	43 (32.8)	46 (28.6)	89 (30.6)
Renin-angiotensin system blockers	65 (49.6)	78 (48.1)	144 (49.1)
Clinical symptoms			
Duration			
<10 min	12 (9.3)	17 (10.8)	29 (9.9)
10–59 min	50 (38.2)	61 (37.7)	111 (37.9)
1 h	69 (52.7)	84 (51.9)	153 (52.2)
Cluster TIA	36 (27.5)	33 (20.4)	65 (22.2)
Weakness	70 (53.4)	85 (52.4)	155 (52.9)
Isolated sensory symptoms	12 (9.2)	13 (8.0)	25 (8.5)
Speech impairment	78 (59.5)	103 (64.0)	181 (62.0)
Vertebrobasilar	10 (7.6)	12 (7.5)	22 (7.5)
ABCD2	5.2 (4.0-6.0)	5.1 (4.0-6.0)	5.1 (4.0-6.0)
Etiologic subtypes			
Large artery atherosclerosis	33 (25.2)	32 (19.9)	65 (22.3)
Cardioembolism	23 (17.6)	37 (23.0)	60 (20.5)
Small vessel disease	28 (21.4)	30 (18.6)	58 (19.9)
Undetermined cause	47 (35.9)	62 (38.5)	109 (37.3)
DWI (n = 251)	59 (45.7)	56 (36.8)	115 (40.9)
Time points			
Stroke recurrence at 7 days follow-up	6 (4.6)	5 (3.1)	11 (3.8)
Stroke recurrence at 90-day follow-up	8 (6.1)	7 (4.3)	15 (5.1)
Stroke recurrence at 1-year follow-up	13 (9.9)	10 (7.2)	23 (8.5)
Stroke recurrence during the follow-up	20 (15.3)	15 (9.3)	35 (11.9)

Abbreviations: ABCD2 = age, blood pressure, clinical features, duration of symptoms, and diabetes scale; DWI = diffusion-weighted imaging.

sensitivity = 1; negative: specificity = 0.86, sensitivity = 1; dataset 1). A total of 73 metabolites were significantly different (Student t test) between LAA and non-LAA (dataset 2), with the resulting molecules identified after MS/MS analyses being androsterone, stearic acid, ascorbic acid, and LysoPC(22:6), all upregulated in patients with LAA ($p < 0.05$).

Table 2 Variables associated with stroke recurrence in both cohorts of patients

Variable	Stroke recurrence at 7-day follow-up			Stroke recurrence at 90-day follow-up			Stroke recurrence at 1 year follow-up			Stroke recurrence during the follow-up		
	No (n = 282)	Yes (n = 11)	p	No (n = 278)	Yes (n = 15)	p	No (n = 247)	Yes (n = 23)	p	No (n = 258)	Yes (n = 35)	p
Vascular risk factors												
Age, y, mean (SD)	71.6 (10.9)	75.5 (8.6)	0.247	71.6 (10.9)	74.5 (8.2)	0.318	71.3 (11.01)	75.6 (7.5)	0.073[a]	71.7 (11.6)	76.1 (6.5)	0.148
Male	175 (62.1)	4 (36.4)	0.115	172 (61.9)	7 (46.7)	0.239	152 (61.5)	11 (47.8)	0.198	157 (60.9)	22 (62.9)	0.819
Previous stroke	63 (22.4)	1 (9.1)	0.295	63 (1)	1 (6.7)	0.204	51 (20.7)	6 (26.1)	0.548	55 (21.4)	9 (25.7)	0.627
Hypertension	193 (68.4)	9 (81.8)	0.347	191 (68.7)	11 (73.3)	0.706	165 (66.8)	19 (82.6)	0.120	174 (67.7)	28 (80.0)	0.307
Coronary disease	39 (13.8)	0 (0)	0.370	39 (14.0)	0 (0)	0.234	30 (12.1)	4 (17.4)	0.468	30 (11.7)	8 (22.8)	0.403
Diabetes mellitus	83 (29.4)	5 (45.5)	0.255	81 (29.1)	7 (46.7)	0.158	71 (28.7)	9 (39.1)	0.341	69 (26.8)	19 (54.3)	0.103
Smoking	37 (13.1)	0 (0)	0.370	37 (13.3)	0 (0)	0.231	35 (0)	0 (0)	0.053[a]	35 (13.6)	2 (5.7)	0.220
Hypercholesterolemia	105 (37.2)	5 (45.5)	0.752	105 (37.8)	5 (33.3)	0.792	88 (35.6)	10 (43.5)	0.500	97 (37.7)	13 (37.1)	0.859
Previous atrial fibrillation[a]	32 (11.3)	2 (18.2)	0.487	32 (11.5)	2 (13.3)	0.688	25 (10.1)	6 (26.1)	0.034[a]	25 (9.7)	9 (25.7)	0.211
Basal treatment												
Aspirin	63 (22.4)	3 (27.3)	0.706	63 (22.7)	3 (20.0)	1	52 (21.1)	6 (26.1)	0.581	57 (22.2)	9 (25.7)	0.637
Clopidogrel	27 (9.6)	0 (0)	0.607	27 (9.7)	0 (0)	0.376	19 (7.7)	4 (17.4)	0.119	23 (8.9)	4 (11.4)	0.635
Anticoagulation	29 (10.3)	2 (18.2)	31 (10.6)	29 (10.5)	2 (13.3)	0.726	24 (9.8)	6 (26.1)	0.030	22 (8.5)	9 (25.7)	0.016
Statins	84 (30.0)	5 (45.5)	0.321	84 (30.4)	5 (33.3)	0.164	72 (29.4)	10 (43.5)	0.161	77 (30.0)	12 (34.3)	0.637
Renin-angiotensin system blockers	134 (48.0)	9 (81.8)	0.033	132 (48.0)	11 (73.3)	0.066	116 (47.5)	14 (60.9)	0.221	121 (46.9)	22 (62.9)	0.105
Clinical symptoms												
Duration												
<10 min	29 (10.2)	0 (0)	0.482	30 (10.8)	3 (20.0)	0.175	23 (9.3)	0 (0)	0.297	28 (10.9)	1 (2.9)	0.913
10–59 min	107 (37.9)	4 (36.4)		107 (38.4)	0 (0)		93 (37.7)	10 (43.5)		100 (38.9)	11 (31.4)	
1 h	146 (51.9)	7 (63.6)		141 (50.7)	4 (26.7)		131 (53.0)	13 (56.5)		126 (49.0)	27 (77.1)	
Cluster TIA	60 (21.3)	5 (45.5)	0.071	60 (21.6)	5 (33.3)	0.336	55 (22.3)	5 (21.7)	0.954	60 (23.3)	5 (14.3)	0.738
Weakness	147 (52.1)	8 (72.7)	0.227	145 (52.2)	10 (66.7)	0.302	145 (52.2)	10 (66.7)	0.907	132 (51.4)	23 (65.7)	0.290
Isolated sensory symptoms	25 (8.9)	0 (0)	0.607	23 (8.3)	2 (13.3)	0.372	23 (9.3)	2 (8.7)	0.922	22 (62.9)	3 (8.6)	0.834
Speech impairment	171 (60.9)	10 (90.9)	0.057	169 (6.0)	12 (80.0)	0.140	148 (60.2)	17 (73.9)	0.264	155 (60.3)	26 (74.3)	0.138
Vertebrobasilar	21 (7.5)	1 (9.1)	0.842	21 (7.6)	1 (6.7)	0.896	18 (7.3)	1 (4.3)	1	21 (7.6)	1 (0.0)	0.954

Continued

Table 2 Continued

Variable	Stroke recurrence at 7-day follow-up			Stroke recurrence at 90-day follow-up			Stroke recurrence at 1 year follow-up			Stroke recurrence during the follow-up		
	No (n = 282)	Yes (n = 11)	p	No (n = 278)	Yes (n = 15)	p	No (n = 247)	Yes (n = 23)	p	No (n = 258)	Yes (n = 35)	p
ABCD2	5.0 (4.0–6.0)	6.0 (4.0–7.0)	0.058[a]	5.0 (4.0–6.0)	6.0 (4.0–7.0)	0.056[a]	5.0 (4.0–6.0)	6.0 (4.0–7.0)	0.335	5.0 (4.0–6.0)	6.0 (4.0–7.0)	0.005
Etiology												
Large artery atherosclerosis	57 (20.3)	87 (72.7)	0.002	56 (20.2)	9 (60.0)	0.007[a]	47 (19.1)	12 (52.2)	0.002	49 (19.1)	16 (45.7)	0.008
Cardioembolism	59 (21.0)	1 (9.1)		59 (21.3)	1 (6.7)		47 (19.1)	6 (26.1)		52 (20.2)	8 (22.9)	
Small vessel disease	57 (20.3)	1 (9.1)		55 (19.9)	3 (20.0)		51 (20.7)	3 (13.0)		53 (20.6)	5 (14.3)	
Undetermined cause	96 (34.2)	1 (9.1)		95 (34.3)	2 (13.3)		89 (36.2)	2 (8.7)		103 (40.1)	6 (17.1)	
DWI abnormalities	113 (41.4)	2 (25.0)	0.478	110 (40.9)	5 (41.7)	0.957	101 (42.3)	10 (50.0)	0.502	99 (39.8)	16 (50.0)	0.267
LysoPC 20:4 <1,14,000 (MS counts)	135 (48.0)	7 (63.6)	0.310	132 (47.7)	10 (66.7)	0.189	118 (47.8)	15 (65.2)	0.109	116 (45.1)	26 (74.3)	0.001
LysoPC 16:0 >0.710 (MS counts)	206 (72.0)	10 (90.1)	0.57	202 (72.7)	14 (93.3)	0.172	175 (70.9)	20 (87.0)	0.313	183 (70.9)	32 (91.4)	0.107

Abbreviations: ABCD2 = age, blood pressure, clinical features, duration of symptoms, and diabetes scale; DWI = diffusion-weighted imaging; LysoPC = lysophosphatidylcholine; MS = mass spectrometry.
[a] Not significant after Bonferroni adjustment.

Results validation using an independent cohort. The multivariate analysis of an independent cohort (cohort 2) confirmed (1) metabolomic signature of SR; (2) metabolomics-based differentiation of SR temporal patterns; and (3) differences between LAA and non-LAA (figure e-1, A–C). Common pathways affected by temporal patterns of recurrence between the 2 independent cohorts included biosynthesis of unsaturated fatty acids and SLC-transport-related pathways (dataset 3). In both cohorts, LysoPC(16:0) was significantly decreased in early SR patients (figure 2, A and B), whereas LysoPC(22:6) was upregulated in LAA patients (figure 2, C and D).

Predictive potential of SR biomarkers described. In order to define the capacity of these potential biomarkers, receiver operating characteristic (ROC) curves were performed using metabolites present in at least 70% of the samples in the same group (SR and non-SR), pooling both cohorts, due to low number of SR. The results (figure 2E, table 3, and table e-2) support the role of plasma LysoPC(20:4) as a potential biomarker, increasing significantly the predictive power of ABCD2 or LAA score from 64%–67% to 71%, as assessed by net reclassification improvement (NRI) or integrated discrimination improvement (IDI) tests (see supplemental data). The Hosmer-Lemeshow test for calibration of the risk of prediction models shows that while the ABCD2 model was well-calibrated (χ^2 7.05, p = 0.069), the ABCD2 + LAA model was not (χ^2 13.79, p = 0.003). The ABCD2 + LAA + LysoPC(20:4) model showed the highest calibration (χ^2 3.58, p = 0.309), whereas the ABCD2 + LAA + LysoPC(16:0) model showed the lowest calibration values (χ^2 17.33, p = 6e-04).

When only early SR was considered, an unidentified metabolite (figure e-1D) arose as a potential biomarker also increasing the predictive power of ABCD2 or LAA score (from 62%–67% to 71%).

DISCUSSION The results demonstrate the feasibility and utility of the metabolomic approach to reveal potential biomarkers for SR after TIA, and for its most frequent independent predictor, LAA. We identified candidate biomarkers for both SR prediction and LAA detection, both belonging to LysoPC family. These biomarkers arose after analysis in 2 independent cohorts (n = 131; n = 162), reinforcing potential study generalizability.

We initially identified that a small set of metabolites are different between SR and no SR. Considering only this small set of metabolites, we reach an accuracy of >60% in segregating these 2 populations, suggesting that a combination of relatively low number of metabolites is, at least, as good as previous

Figure 1   Study design and metabolomics profile of TIA patients
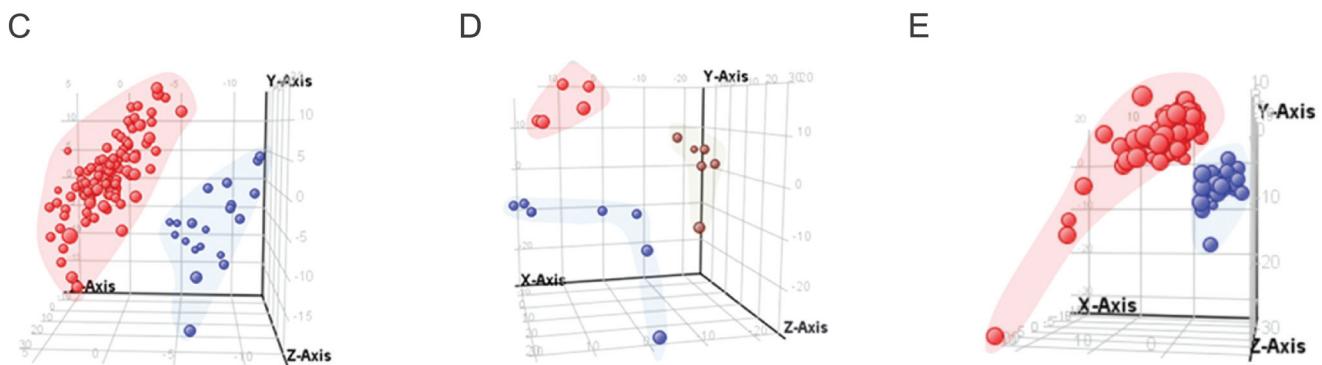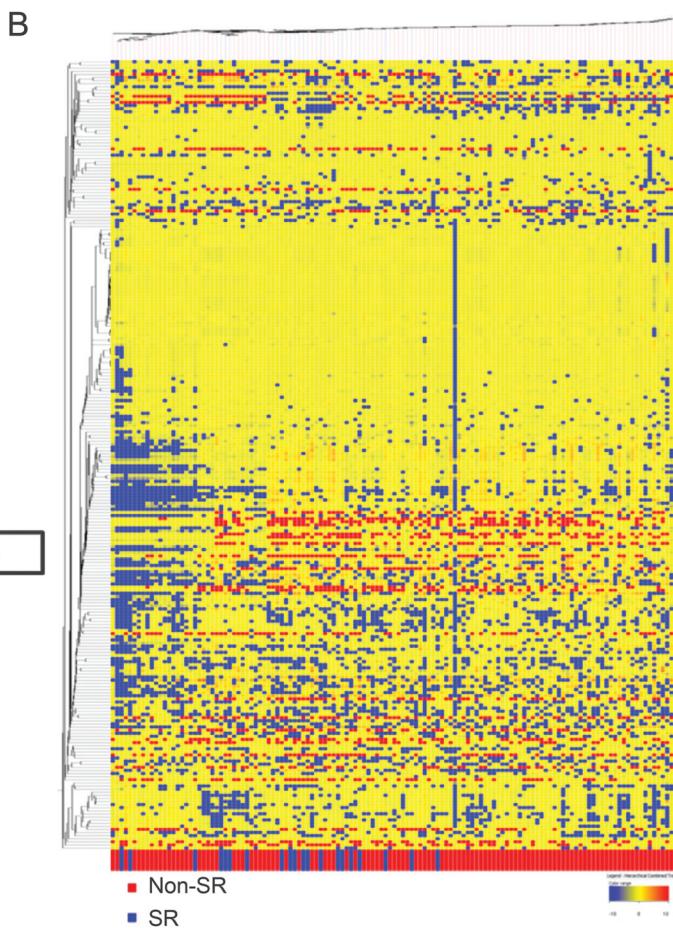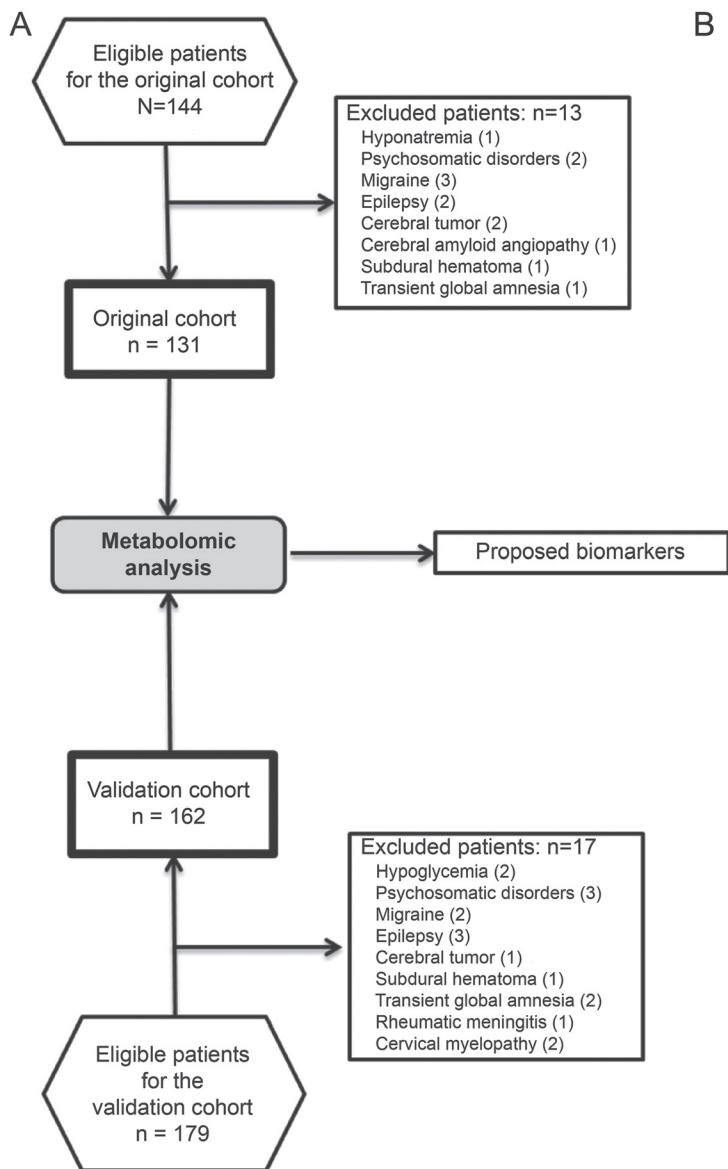
(A) Flow diagram of experiment design. Metabolomic profile of stroke recurrence (SR) and large-artery atherosclerosis (LAA) in TIA patients in cohort 1. (B) Heat map representation of hierarchical clustering of molecular features found in each sample. Each line of this graphic represents an accurate mass ordered by retention time, colored by its abundance intensity and baselining to median/mean across the samples. The scale from −10 blue (low abundance) to +10 red (high abundance) represents this normalized abundance in arbitrary units. Tridimensional partial least squares discriminant analysis (PLS-DA) graphs demonstrate that SR ([C] blue spots represent SR plasma samples; red ones represent non-SR samples) and TIA temporal patterns recurrence ([D] early recurrence [<90 days] is represented in blue spots, medium [>90 days and <1 any] in red, and late [>1 year] in brown) determine a plasma metabolome. (E) Tridimensional PLS-DA graphs show differences between patients with LAA. Blue spots represent LAA and red ones non-LAA plasma samples.

Figure 2   Plasma lysophosphatidilcholines as TIA recurrence biomarkers
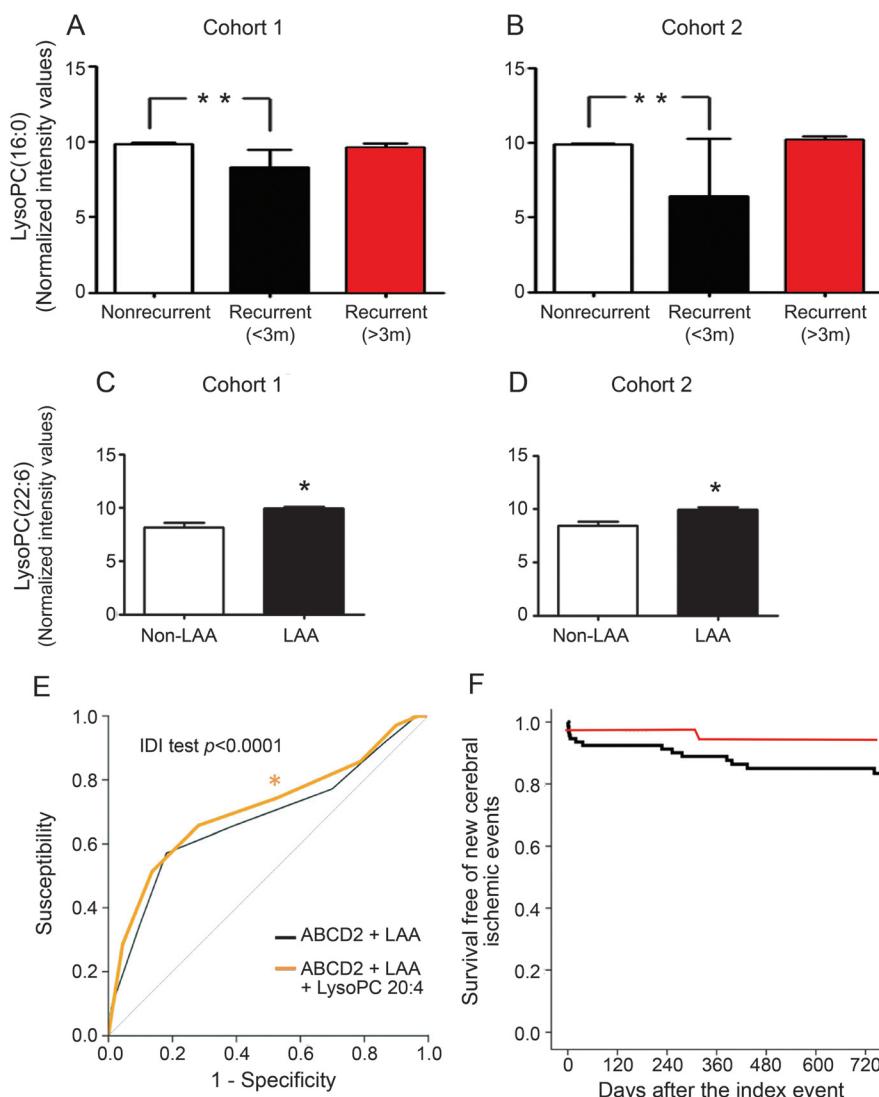
(A) Lysophosphatidylcholine (LysoPC) (16:0) arose as a potential blood biomarker of stroke recurrence ([A] cohort 1; [B] cohort 2). **Indicates significant differences ($p < 0.0001$) by analysis of variance test with Tukey multiple comparison test. LysoPC(22:6) as a blood biomarker of large-artery atherosclerosis (LAA) ([C] cohort 1; [D] cohort 2). *Indicates significant differences by Student t test (at least $p < 0.05$). (E) The inclusion of LysoPC (20:4) levels to age, blood pressure, clinical features, duration of symptoms, and diabetes scale (ABCD2) and LAA score to receiver operating characteristic curve increase the predictive power of stroke recurrence (areas: ABCD2 = 0.646, $p = 0.0.05$; ABCD2 + LAA = 0.678, $p = 0.001$; ABCD2 + LAA + LysoPC(20:4) = 0.711, $p < 0.001$; integrated discrimination improvement (IDI) test for comparison of prediction models: $p < 0.0001$). (F) Kaplan-Meier estimates of the proportion of patients remaining free from any cerebral ischemic event. Red line indicates LysoPC(20:4) >1,14,000 MS counts; black line indicates LysoPC(20:4) <1,14,000 MS counts. MS = mass spectrometry.

clinical, imaging, or etiologic variables in predicting SR. Future work devoted to identify these and new metabolites will help to increase the accuracy of the test and ultimately develop a more scalable tool for patients to assess their risk of SR.

Low plasma levels of LysoPC(16:0) arose as a potential predictor of recurrence, especially in patients with early SR. This compound is one of the products of Lp-PLA2 whose levels have been shown to be increased in adverse prognosis after stroke.[11,18] Moreover, high levels of Lp-PLA2 and LysoPC(16:0)

are associated with symptomatic carotid plaques.[19] LysoPC(16:0) by itself increases on brain after stroke[20,21] where it mediates phagocyte recruiting[22] that can contribute to ischemic brain injury[23] and also produces neuroinflammatory effects when applied on CNS.[24] All these results could pinpoint a beneficial effect of reduced LysoPC(16:0) levels that argue against our claim that it marks an early SR risk, but although high levels of plaque LysoPC(16:0) are correlated with high plasma and plaque levels of Lp-PLA2, plasma LysoPC(16:0) levels are not.[25] It

Table 3   Predictive accuracy of clinical variables and biomarkers derived from the metabolomic study for recurrent stroke at the 7-day, 90-day, and 1-year follow-up, and during all follow-up

Biomarker	Cohort	Stroke recurrence at 7-day follow-up		Stroke recurrence at 90-day follow-up		Stroke recurrence at 1-year follow-up		Stroke recurrence during the follow-up	
		AUC (CI 95%)	p	AUC (CI 95%)	p	AUC (CI 95%)	p	AUC (CI 95%)	p
ABCD2	Both cohorts	0.65 (0.47–0.84)	0.085	0.64 (0.47–0.80)	0.075	0.56 (0.42–0.69)	0.359	0.65 (0.54–0.76)	0.005
	Validation cohort	0.70 (0.42–0.98)	0.126	0.68 (0.43–0.93)	0.110	0.55 (0.34–0.76)	0.608	0.68 (0.51–0.85)	0.023
ABCD2 plus LAA	Both cohorts	0.72 (0.55–0.89)	0.013	0.68 (0.52–0.84)	0.019	0.60 (0.46–0.74)	0.103	0.68 (0.57–0.79)	<0.001
	Validation cohort	0.77 (0.53–1.00)	0.038	0.73 (0.49–0.97)	0.039	0.61 (0.39–0.83)	0.257	0.72 (0.55–0.89)	0.004
ABCD2 plus LAA plus LysoPC 20:4 <1,14,000	Both cohorts	0.72 (0.54–0.89)	0.014	0.70 (0.54–0.85)	0.011	0.62 (0.49–0.75)	0.048	0.71 (0.60–0.82)	<0.001
	Validation cohort	0.75 (0.49–1.00)	0.054	0.74 (0.51–0.96)	0.035	0.61 (0.40–0.83)	0.228	0.74 (0.57–0.91)	0.002
ABCD2 plus LAA plus LysoPC 16:0 ≥0.710	Both cohorts	0.64 (0.45–0.82)	0.110	0.64 (0.45–0.81)	0.111	0.57 (0.43–0.72)	0.293	0.67 (0.56–0.79)	0.002
	Validation cohort	0.72 (0.37–1.00)	0.192	0.67 (0.56–0.79)	0.002	0.56 (0.31–0.81)	0.590	0.72 (0.53–0.90)	0.010

Abbreviations: ABCD2 = age, blood pressure, clinical features, duration of symptoms, and diabetes scale; AUC = area under the receiver operating characteristic curve; CI = confidence interval; LAA = large-artery atherosclerosis; LysoPC = lysophosphatidylcholine.

has been demonstrated that plasma LysoPC(16:0) can inhibit Lp-PLA2 activity both in vitro and in vivo,[26] thereby suppressing their neuroinflammative properties.[27] Apart from suppressing Lp-PLA2 activity, plasmatic LysoPC(16:0) is neuroprotective on brain ischemia models,[28] mainly through activation of TREK-1 potassium channels.[29] Finally, it is known that LysoPC(16:0) is a potent inducer of superoxide ion production in endothelial cells[30] and controlled levels of superoxide serve as angiogenic factors in ischemic angiogenesis.[31] Due to all these neuroprotective properties, decreased plasma levels of LysoPC (16:0) could contribute to an early SR risk. Nonetheless, when we evaluated potential risk models accounting LysoPC(16:0), it did not add a significant value over the clinical parameters explained by ABCD2 and LAA presence. This fits with the fact that ABCD2 and LAA are closely related to pathophysiologic events previously invoked in the homeostasis of LysoPC16:0 (enumerated above).

The potential implication of LysoPC metabolism in SR was further reinforced by the finding of LysoPC(20:4) as a potential biomarker of SR based on ROC curves of aggregated cohorts. Interestingly, after the use of NRI, IDI, and Hosmer-Lemeshow test, the statistics indicated that LysoPC(20:4) significantly improves prediction capacities of combined ABCD2 and LAA scores. These data are in line with other LysoPC species containing fatty acids of 20C length, decreased in plasma in experimental models of ischemia.[32] This molecule exhibits anti-inflammatory potential in preclinical models,[33] reinforcing the role of inflammation in ischemic recurrence or tolerance. Interestingly, as with other biomarkers,[8,11] LysoPCs exhibit the potential to increase the prognostic accuracy of clinical scores or LAA and could help management decisions, a hypothesis to be validated in further studies.

Finally, we identified LysoPC(22:6) as a potential LAA biomarker. As far as we know, no previous studies have focused on the role of LysoPC(22:6) on carotid plaque formation or stability but increased levels in symptomatic LAA patients could point to a role in this processes. N-3 polyunsaturated fatty acids, such as docosahexaenoic acid (22:6), are precursors of molecules implicated in the resolving phase of inflammation. Therefore, the higher levels of LysoPC(22:6) in LAA could be an indicator of reactive response to inflammation associated with atheroma plaque development.

As limitations of the present work, we recognize the significant heterogeneity and small sample size for SR among the studied population. The metabolome, even in healthy phenotypically similar patients, exhibits a considerable variance across the time.[34–36] Furthermore, the samples were taken in the emergency

room, without controlling for previous food intake. Nevertheless, the results provided were strengthened by the use of 2 independent cohorts. Further, we are not able to identify an important percentage of metabolites present in samples, due to the major bottleneck in metabolomics: the lack of comprehensive metabolite databases.[37] However, the use of databases repositories with accurate mass and retention time in reproducible chromatographic systems will ease the future identification of these metabolites.

In the future, metabolomics could be useful to detect new biomarkers related to other interesting phenomena related to transient ischemia, the ischemic tolerance. Nonlacunar ischemic stroke patients with recent previous episodes of TIA have a favorable outcome, suggesting a neuroprotective effect of TIA by inducing a phenomenon of precondition ischemia.[38]

AUTHOR CONTRIBUTIONS

Mariona Jové: design and conceptualization of the study, analysis and interpretation of the data, drafting and revising the manuscript for intellectual content. Gerard Mauri-Capdevila: design and conceptualization of the study, analysis and interpretation of the data, drafting and revising the manuscript for intellectual content. Idalmis Suárez: analysis of the data, revising the manuscript for intellectual content. Serafí Cambray: design and conceptualization of the study, interpretation of the data, revising the manuscript for intellectual content. Jordi Sanahuja: analysis and interpretation of the data, revising the manuscript for intellectual content. Alejandro Quílez: analysis and interpretation of the data, revising the manuscript for intellectual content. Joan Farré: analysis and interpretation of the data, revising the manuscript for intellectual content. Ikram Benabdelhak: analysis and interpretation of the data, revising the manuscript for intellectual content. Reinald Pamplona: design and conceptualization of the study, revising the manuscript for intellectual content. Manuel Portero-Otín: design and conceptualization of the study, analysis and interpretation of the data, drafting and revising the manuscript for intellectual content. Francisco Purroy: design and conceptualization of the study, analysis and interpretation of the data, drafting and revising the manuscript for intellectual content.

DISCLOSURE

The authors report no disclosures relevant to the manuscript. Go to Neurology.org for full disclosures.

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