

Endothelial injury in childhood stroke with cerebral arteriopathy

A cross-sectional study

Despina Eleftheriou,
MBBS
Vijeya Ganesan, MD
Ying Hong, PhD
Nigel J. Klein, PhD
Paul A. Brogan, PhD

Correspondence & reprint requests to Dr. Eleftheriou: d.eleftheriou@ich.ucl.ac.uk

ABSTRACT

Objective: Circulating endothelial cells (CECs) and microparticles (MPs) have been reported to reflect endothelial injury, cellular activation, and MP-mediated thrombin generation. We tested the hypothesis that these indices differ between children with cerebral arteriopathy and arterial ischemic stroke (AIS) recurrence, and those with a single event.

Methods: This was a single-center cross-sectional study of 46 children with AIS and cerebral arteriopathy matched with pediatric controls. AIS recurrence was defined as new acute neurologic deficit with radiologic evidence of further cerebral infarction. CECs and MPs were identified with immunomagnetic bead extraction and flow cytometry, respectively. MP function as assessed by thrombin generation was determined using a fluorogenic assay.

Results: Ten children had AIS recurrence while 36 had a single AIS event. CECs were raised in children with recurrent AIS, compared to those with no recurrence ($p = 0.0001$), and in controls ($p = 0.0001$). Total circulating annexin V+ MPs were significantly greater in children with recurrence than in those with no recurrence ($p = 0.0020$). These MPs were of endothelial or platelet origin, and a subpopulation expressed tissue factor. Finally, MP-mediated thrombin generation was enhanced in children with recurrent AIS compared to those with no recurrence ($p = 0.0001$), providing a link between inflammation, endothelial injury, and increased thrombotic tendency.

Conclusion: Despite the wide spectrum of clinical and radiologic presentation of childhood AIS, indices of endothelial injury and cellular activation are different in patients with single and recurrent events. This novel approach has potential for furthering understanding of AIS pathophysiology and prognosis. *Neurology*® 2012;79:2089-2096

GLOSSARY

AIS = arterial ischemic stroke; **AVM** = arteriovenous malformation; **CA** = catheter arteriography; **CEC** = circulating endothelial cell; **CI** = confidence interval; **EMP** = endothelial microparticle; **ETP** = endogenous thrombin potential; **FCA** = focal cerebral arteriopathy; **FITC** = fluorescein isothiocyanate; **FLAIR** = fluid-attenuated inversion recovery; **GOSH** = Great Ormond Street Hospital; **MP** = microparticle; **MPFP** = microparticle-free plasma; **MR** = magnetic resonance; **MRA** = magnetic resonance angiography; **OR** = odds ratio; **PACNS** = primary angiitis of the CNS; **PE** = phycoerythrin; **PPP** = platelet-poor plasma; **PSGL-1** = P-selectin glycoprotein ligand 1; **TF** = tissue factor; **TGA** = thrombin generation assay; **vWF** = von Willebrand factor.

Childhood arterial ischemic stroke (AIS) results in neurologic morbidity in over two-thirds of survivors and recurrence in up to 20%.¹⁻³ Current treatment approaches primarily focus on preventing recurrence.⁴ Most affected children have cerebral arteriopathies, which although radiologically similar, are likely to represent distinct pathologic entities.^{1,5-8} For example, both focal cerebral arteriopathy (FCA) and primary angiitis of the CNS (PACNS) manifest focal proximal occlusive disease radiologically. However, FCA is generally monophasic, while PACNS typically has a recurrent course, possibly due to persistence of an aberrant inflammatory response.⁹ Previous studies have proposed radiologic predictors of arteriopathy progression and AIS recurrence but neither these, nor any currently described biomarker, robustly distinguish between patients with a likely monophasic or recurrent course.^{7,8}

Endothelial microparticles (MPs) and whole circulating endothelial cells (CECs) allow tracking of the endothelial events associated with vascular injury in patients with systemic vasculitides.¹⁰⁻¹⁵

From the Paediatric Rheumatology Department (D.E., Y.H., P.A.B.), Neurosciences Unit (V.G.), and Infectious Diseases and Microbiology Unit (N.J.K.), Institute of Child Health and Great Ormond St Hospital for Children, London, UK.

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Supplemental data at
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Supplemental Data



MPs are membrane vesicles $<1 \mu\text{m}$ in size, released during cell activation and rich in aminophospholipids.^{10,12–14} The procoagulant activity of MPs can be assessed by determining the MP-mediated thrombin generation *in vitro*.^{13,16,17} We have previously shown that children with systemic vasculitis and thrombosis have an enhanced MP-mediated thrombin generation thus linking inflammation, endothelial injury, and prothrombotic propensity.¹³

In this study we examined whether children with recurrent AIS have evidence of increased endothelial injury (increased CEC and endothelial MP) and excessive MP-mediated thrombin generation compared with those with a single AIS event.

METHODS Study design and patient population. This was a cross-sectional study of children aged >28 days with AIS and imaging evidence of cerebral/cervical arteriopathy presenting or under follow-up at Great Ormond Street Hospital (GOSH) from September 2007 to January 2011. AIS was defined as an acute focal neurologic deficit attributable to cerebral infarction in a corresponding arterial distribution. Cerebral/cervical arteriopathy was defined as focal or segmental stenosis or occlusion, with regular or irregular abnormalities of the arterial wall⁷ and categorized according to current consensus definitions.¹⁸ The category of transient cerebral arteriopathy in this classification has more recently been superseded by the term FCA, which was used in this study.¹⁹ Exclusion criteria were systemic conditions that cause endothelial injury (sickle cell disease, systemic vasculitis, other autoimmune diseases); histopathologic confirmation of cerebral vasculitis; cardiac disease; and other potentially confounding syndromic diagnoses. Patients with arterial occlusion but no other arterial wall changes were also excluded as these radiologic appearances could result from cardioembolic or paradoxical embolism.⁷ In addition to the cross-sectional study, children with a new presentation of AIS and cerebral/cervical arteriopathy were evaluated prospectively. Blood samples were obtained from healthy controls comprising children undergoing minor surgical procedures (such as hernia repair, preoperative blood samples obtained) with no identifiable medical history of chronic illnesses or syndromic diagnosis. A disease control group was included comprising of children with cerebral arteriovenous malformations (AVM) since these patients have noninflammatory vascular pathologies.

Standard protocol approvals, registration, and patient consent. The study was approved by the Institutional Ethics Committee (COREC 07/Q0508/58). Informed consent was obtained from all parents/guardians and participants/child healthy controls.

Clinical, laboratory, and radiologic data. All children with AIS routinely underwent magnetic resonance imaging (MRI) and magnetic resonance angiography (MRA) within 48 hours of presentation for confirmation of the diagnosis and identification of possible cerebral/cervical arteriopathy. MRI and MRA were performed on a 1.5-T MR scanner using a standard imaging protocol, including T2-weighted turbo spin-echo imaging in the axial plane, fluid-attenuated inversion recovery (FLAIR) sequence in the coronal plane, T1-weighted spin-echo imaging in the sagittal plane, and 3-dimensional short-echo time-of-flight MRA of the circle of Willis.

Catheter arteriography (CA) was performed at the clinician's discretion, as this may provide adjunctive yield for detection of arteriopathy.²⁰ Blood was drawn before CA was performed to exclude procedure-related endothelial injury. Cerebral/cervical arteriopathy was identified on MRA, or CA where available, and categorized using currently recommended definitions.^{6,7,18,19,21} All children underwent comprehensive investigation for AIS risk factors (see supplemental material on the *Neurology*[®] Web site at www.neurology.org). Treatment was based on current international management guidelines.^{21–23} Patients were then re-imaged with MRI/MRA at 6 and 12 months and annually thereafter for clinical monitoring.

Patients were categorized into 2 groups according to AIS recurrence (>1 week after initial clinical event) and identified at re-presentation to our institution. Where clinical recurrence occurred, children underwent additional MRI/MRA imaging to identify new areas of arterial territory infarction or progression of arteriopathy, defined as progression of previously identified arterial disease (more extensive segment involved, more severe occlusive disease) or involvement of previously unaffected vessels or new arterial occlusion.

Circulating endothelial cells. CECs were identified with immunomagnetic bead extraction based on an international consensus protocol.^{11,24} CECs were defined as *Ulex europaeus* lectin (Sigma-Aldrich) bright cells $>10 \mu\text{m}$ in size, with >5 magnetic beads attached (figure e-1A).^{11,15,24}

Cell-derived microparticles. MPs were identified by flow cytometry as previously described (figure e-1, B–D).^{12,13} Blood was collected in 3.2% buffered citrate and centrifuged at 5,000 *g* for 5 minutes twice to obtain platelet-poor plasma (PPP). MPs were sedimented from 200 μL of PPP after centrifugation at 17,000 *g* for 60 minutes and resuspended in An V binding buffer (BD PharMingen, Oxford, UK) prior to incubating with fluorescent monoclonal antibodies: fluorescein isothiocyanate (FITC), phycoerythrin (PE) or PERCP-Cy5.5 labeled annexin V (BD PharMingen), mouse PE-labeled antihuman CD62e (clone 68-5H11, BD PharMingen), mouse PE-labeled antihuman CD31 (clone L133.1, BD PharMingen), mouse PE-labeled antihuman CD11b activation epitope (clone CBRM1/5, Biolegend), mouse Cy5-labeled antihuman CD14 (clone 61D3, AbD Serotec), mouse FITC-labeled antihuman TF (clone VD8, American Diagnostica), and mouse PE-labeled antihuman P-selectin glycoprotein ligand 1, PSGL-1 (clone KPL1, BD PharMingen). Additional labeling with mouse antihuman CD42a-PERCP (BD PharMingen) to exclude platelet origin was conducted. Samples were analyzed with a FACS Calibur flow cytometer (BD PharMingen).

Thrombin generation assay and von Willebrand factor antigen levels. The thrombin generation assay (TGA) measures the amount of active thrombin produced in plasma or whole blood after re-calcification by monitoring the conversion of a fluorogenic substrate cleaved by thrombin as the latter is generated.^{13,16,17} Direct calculation of molecular concentrations of thrombin are possible by comparison to a concurrently run calibrator.^{13,16,17} For the previously described MP-mediated TGA,^{13,17} MPs were sedimented from PPP and resuspended in 200 μL of control microparticle-free plasma (MPFP) containing 30 $\mu\text{g}/\text{mL}$ of corn trypsin inhibitor (Sigma) to inhibit contact activation. The MPFP was prepared from approximately 50 mL plasma obtained from healthy volunteers. Subsequently 40 μL of MPs resuspended in control MPFP was added to the plate well, followed by 50 μL of calcium-fluorogenic substrate (0.5 mmol/L of Z-G-G-R-AMC and 7.5 mmol/L of calcium final concentrations, Pathway Diagnostics). No exogenous TF or phospholipids were added. The thrombin generated was measured by fluorogenic excitation/emission at 360/460 nm at 1-minute time intervals for 90 minutes in an Optima fluorescence plate reader

(BMG). Indices recorded were 1) height of peak thrombin (nM); 2) lag time (time to onset of thrombin generation); 3) velocity index; and 4) endogenous thrombin potential (ETP), equivalent to the area under the curve (figure e-1E). Levels of von Willebrand factor (vWF) antigen were measured in PPP using a commercially available enzyme-linked immunoassay (American Diagnostica).

Statistical analysis. Numeric results were summarized as median and range. The Kruskal-Wallis test was used to examine overall differences in experimental laboratory markers between the study groups followed by the Mann-Whitney *U* test. Fisher exact test was used to compare categorical data between groups. Associations between biomarkers were assessed using Spearman rank correlation coefficient. The independent association of CECs, total annexin V+ MPs, and peak thrombin on the primary outcome of AIS recurrence were assessed using a multivariable logistic regression model, unadjusted and adjusted for age, gender, and time from stroke event to blood test sampling. Results were expressed as odds ratio (OR) with 95% confidence intervals (CIs) and *p* values. Sensitivity, specificity, and likelihood ratios were then calculated to examine the potential test characteristics of CECs for identification of AIS recurrence. *p* Values of less than 0.05 (2-sided) were regarded as significant. Statistical analysis was performed using SPSS version 17.

RESULTS Characteristics of the study population. A total of 116 children with AIS were identified during the study period. Of those, 54 met the inclusion criteria; however, 8 patients/families refused participation. Of the 46 participants (age 8.8 years, range 0.9–17.4), 10 had AIS recurrence and 36 had a single event. The children who refused consent had an age of 6.2 years (4.2–8.4) and none had recurrent events. None of the children with a single AIS event had evidence of clinically silent infarction on repeat imaging at 6 and 12 months after index AIS. The disease control group included 10 children, median age 9 years (3–16), with cerebral AVM, 4 of whom had a history of intracranial hemorrhage. The demographics of the study population, the routine clinical laboratory parameters, imaging features, and arteriopathy classification are summarized in table 1. None of the children had clinical signs or symptoms of systemic inflammatory disease or evidence of a cardiac abnormality or intracardiac shunt. Evaluation took place at a median of 7 months (6–13) following recent AIS for those children with AIS recurrence compared to 11 months (6–24) for those with no recurrence (*p* = 0.1000). Those with recurrent AIS more commonly had diffuse neurologic deficits, headaches, multifocal and bilateral lesions, and lesions in both anterior and posterior circulation territories on MRI. They were also more likely to have been treated with steroids. Treatment with either steroids or immunosuppression was initiated following recurrence. All children with AIS recurrence had evidence of new brain infarction with progression of previously identified arterial disease in 6 children (60%), new arterial lesions in 8 children (80%), and new arterial occlusion in the presence of other arterial wall changes in 2 patients (20%).

Eight children, aged 6.5 years (5–13), were studied prospectively at initial presentation with AIS and at

latest follow up of 12 months (9–18). Six had no AIS recurrence following the initial event, while 2 children (treated with aspirin and warfarin but no immunosuppression) re-presented with AIS recurrence during follow-up (see table e-1 for presenting features).

Circulating endothelial cells. Levels of peripheral blood CEC were significantly higher in children with AIS compared with healthy child controls (*p* = 0.03). As depicted in figure 1A, the CEC count in children with recurrent AIS was significantly higher at 120/mL (64–732) compared to 32/mL (0–64) in the 36 children with no recurrence (*p* = 0.0001), 16/mL (8–40) in children with cerebral AVM (*p* = 0.0002), and 24/mL (0–60) in 20 child controls (*p* = 0.0001).

Figure 1B summarizes the CEC changes for the 8 children studied prospectively. The 6 children with a monophasic disease course had no significant changes in CEC counts (*p* = 0.545) while the 2 children with AIS recurrence showed a sustained raised CEC count at 184 (136–232) cells/mL during follow-up.

Circulating microparticles. Children with AIS recurrence had significantly higher total AnV+ MPs compared to those with no recurrence, *p* = 0.0020; children with cerebral AVM, *p* = 0.0001; and controls, *p* = 0.0001 (table 2). Children with recurrent AIS had higher platelet MPs expressing P-selectin, endothelial MPs (EMPs) expressing either the endothelial activation marker CD62E (E-selectin) or CD31+, and neutrophil-derived MPs expressing the activation marker aCD11b compared to children with single AIS (table 2). Of note, circulating EMPs expressing CD62E correlated significantly with CECs, *r* = 0.67, *p* = 0.0001, suggesting a significant association between both biomarkers of endothelial injury (figure 1C).

We then examined tissue factor (TF) expression on MP of monocytic (CD14) origin between the study groups since these MPs are known to be highly prothrombotic.^{13,25} TF+CD14+AnV+ MPs were elevated in those with AIS recurrence compared to those with no recurrence and healthy controls (table 2). As TF activity at the thrombus edge depends on interactions between platelet P-selectin and PSGL-1 on monocyte MPs,²⁵ we determined whether there were any differences in PSGL-1+CD14+AnV+ MPs between the patient groups. Those with AIS recurrence had significantly higher levels compared to those with no recurrence.

In the 8 children studied prospectively, AnV+ MPs remained low in 6 children with no AIS recurrence; in contrast, AnV+ MPs were elevated and remained high at follow-up in 2 children with AIS recurrence (figure 1D).

TGA and vWF antigen. Having demonstrated higher AnV+ MPs (confirming high procoagulant phosphatidylserine content), we then investigated their capacity to generate thrombin. TGA parameters for all studied groups are summarized in table 3. Children

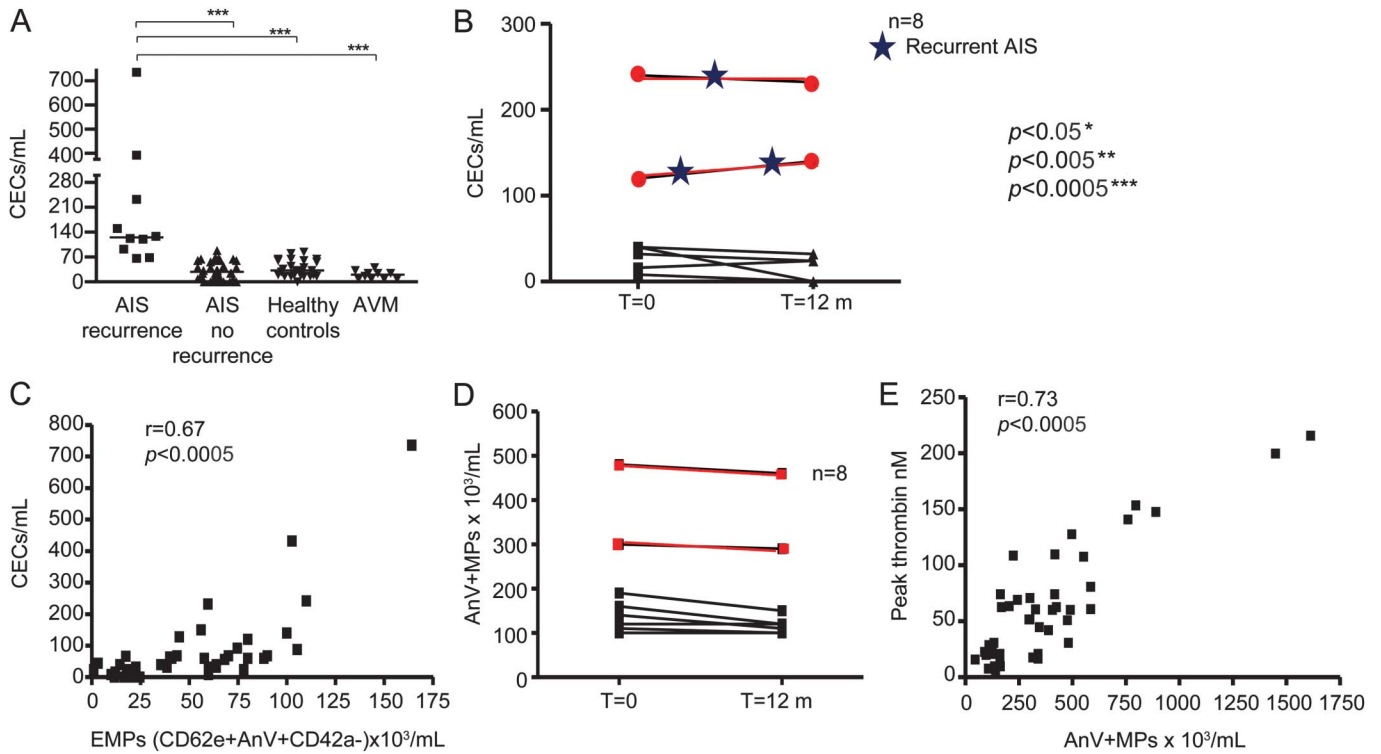
Table 1 Study population characteristics^a

Demographics	AIS recurrence (n = 10)	AIS no recurrence (n = 36)	p Value
M:F	6:4	16:20	0.4839
Age, y	8.2 (0.9–15.4)	9.4 (2.7–17.4)	0.681
Time from AIS to evaluation, mo	7 (6–13)	11 (6–24)	0.1
Clinical features			
Focal neurologic deficit	10 (100)	35 (97)	1
Diffuse neurologic deficits	7 (70)	4 (11)	0.0006
Headache	5 (50)	2 (5)	0.0031
Seizures	1 (10)	1 (2.7)	0.3913
Classification of cerebral arteriopathy ¹⁸	Arterial dissection, 3 (30)	TCA/FCA, 19 (52)	NA
	PACNS, 1 (10)	Arterial dissection, 4 (11)	
	PVA, 1 (10)	PVA, 3 (8)	
	Moyamoya, 1 (10)	Moyamoya, 5 (14)	
	Unclassified, 4 (40)	Unclassified, 5 (14.5)	
ESR, mm/h (normal range 0–10 mm/h)	5.4 (1–12)	6.6 (1–28)	0.561
CRP, mg/L (normal range <10 mg/L)	5 (3–16)	5 (3–6)	0.873
CSF analysis	Normal (6/6 studies)	Normal (5/5 studies)	1
Thrombophilia screen	Any abnormality, 2 (20)	Any abnormality, 7 (19)	1
	MTHFR heterozygous, 1 (10)	Protein S deficiency, 2 (5)	
	Low protein S, 1 (10)	MTHFR homozygous, 1 (3)	
		MTHFR heterozygous, 3 (8)	
		Factor V Leiden heterozygous, 1 (3)	
MRI			
Multifocal and bilateral lesions	9 (90)	8 (22)	0.0002
MRA			
Multifocal and bilateral lesions	9 (90)	8 (22)	0.0002
Anterior and posterior circulation	7 (70)	4 (11)	0.0006
Collaterals	4 (40)	3 (8)	0.0311
Catheter angiography (total patients)	10	14	
Multifocal and bilateral lesions	9 (90)	8 (22)	0.0875
Anterior and posterior circulation	7 (70)	6 (16)	0.6968
Previously described predictors of AIS recurrence ⁷	10 (100)	4 (11)	0.0001
Treatment			
Aspirin	10 (100)	36 (100)	1
Anticoagulation (heparin/warfarin)	3 (30)	5 (14)	0.0149
Corticosteroids	4 (40)	1 (3)	0.0057
Cyclophosphamide	2 (20)	1 (3)	0.1147
Surgical revascularization	2 (20)	4 (11)	0.5975

Abbreviations: AIS = arterial ischemic stroke; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; FCA = focal cerebral arteriopathy; MRA = magnetic resonance angiography; MTHFR = methylene-tetrahydrofolate reductase; NA = not applicable; PACNS = primary angiitis of the CNS; PVA = post varicella arteriopathy; TCA = transient cerebral arteriopathy.

^aFocal neurologic deficits included hemiparesis, facial weakness, and hemisensory loss. Diffuse neurologic deficits included neurocognitive dysfunction, personality changes, and concentration difficulties. Arterial dissection (diagnosed both on MRA and catheter arteriography) involved the posterior circulation in all patients. Syndromic cases of moyamoya were excluded from the study. Previously described recurrence predictors include occlusion at any time or progressive arteriopathy.⁷ Values are median (range) or n (%). p Values were calculated using Fisher exact test for categorical data and Mann-Whitney U for continuous variables. p Values <0.05 (2-sided) were regarded as significant.

Figure 1 Circulating endothelial cells (CECs) and cellular microparticles (MP) in children with recurrent arterial ischaemic stroke (AIS)



A) CEC count in children with recurrent AIS was significantly higher compared to those children with no recurrence ($p = 0.0001$), children with cerebral arteriovenous malformation (AVM) ($p = 0.0002$), and child controls ($p = 0.0001$). (B) Prospective changes in CEC count in 8 children with arteriopathy and AIS. CECs at presentation for 6 children with a monophasic disease course were 36 (8-40) cells/mL and at follow-up 24 (0-32) cells/mL. For 2 children there was clinical AIS recurrence associated with cerebral arteriopathy progression (shown in red) associated with a sustained increase in CEC count at latest follow-up. (C) Circulating endothelial-derived MPs expressing CD62E correlated significantly with CECs, $r = 0.67$, $p = 0.0001$. (D) For the 6 children with a stable course with no AIS recurrence, total AnV+ MPs were at 130 (120-190) $\times 10^3$ /mL at initial assessment to 115 (120-150) $\times 10^3$ /mL at follow-up, and in 2 children with recurrence (marked in red), 390 (300-480) $\times 10^3$ /mL at presentation to 375 (290-440) $\times 10^3$ /mL at follow-up. (E) The total circulating AnV+ MPs correlated significantly with MP-mediated peak thrombin nM, $r = 0.73$, $p = 0.0001$. Spearman rank correlation was employed to examine the association between studied parameters. Kruskal-Wallis test followed by the Mann-Whitney U test was used to examine differences between the study groups. p Values of less than 0.05 (2-sided) were regarded as significant. EMP = endothelial microparticles.

with AIS recurrence had significantly enhanced MP-mediated peak thrombin generation, ETP, lag time, and velocity index compared to those children with nonrecurrent AIS. The total circulating AnV+ MPs correlated significantly with peak thrombin nM

(figure 1E), consistent with our hypothesis that the hypercoagulability in patients with AIS was directly related to the increased total number of PS-expressing (AnV+) MPs. Finally, levels of plasma vWF antigen (previously reported to relate to cerebrovascular

Table 2 Circulating microparticle profiles in children with arterial ischemic stroke^a

	AIS recurrence (n = 10)	AIS no recurrence (n = 36)	Healthy controls (n = 10)	Cerebral AVM (n = 10)
Total AnV+MPs	659 (136-1616)	226 (49-893); $p = 0.002$	89 (25-236); $p = 0.0001$	90 (20-160); $p = 0.0001$
EMP AnV+CD62E+CD42a-	85 (44-164)	38 (1-105); $p = 0.0001$	0.5 (0.2-10); $p = 0.0001$	15 (2-35); $p = 0.002$
EMP AnV+CD31+CD42a-	80 (55-130)	40 (15-90); $p = 0.002$	2 (1-9); $p = 0.0001$	5 (2-10); $p = 0.003$
PMP AnV+CD42a+CD62P+	93 (18-220)	44 (2-120); $p = 0.0100$	11 (5-20); $p = 0.0002$	12 (5-15); $p = 0.0001$
NMP AnV+aCD11b+CD42a-	60 (8-125)	21 (1-40); $p = 0.0200$	4 (0.5-17); $p = 0.0040$	19 (1-40); $p = 0.0030$
TF+CD14+AnV+	70 (22-190)	29 (1-146); $p = 0.0040$	2 (0.2-6.7); $p = 0.0001$	10 (2-30); $p = 0.0030$
PSGL-1+ CD14+AnV+	35 (15-98)	12 (5-58); $p = 0.0200$	1.3 (1.1-1.5); $p = 0.0200$	5(1.2-8.2); $p = 0.003$

Abbreviations: AIS = arterial ischemic stroke; AnV = annexin V; AVM = arteriovenous malformation; EMP = endothelial microparticles; MP = microparticle; NMP = neutrophil microparticles; PMP = platelet microparticles; PSGL-1 = P-selectin ligand glycoprotein-1; TF = tissue factor.

^aValues are microparticles (median, range) $\times 10^3$ /mL. The Kruskal-Wallis test followed by the Mann-Whitney U test was used to examine differences between the study groups. p Values reported refer to comparison with the group of children with recurrent AIS. p Values of less than 0.05 (2-sided) were regarded as significant.

Table 3 Thrombin-generation parameters and von Willebrand factor antigen in children with arterial ischemic stroke^a

	AIS recurrence (n = 10)	AIS no recurrence (n = 36)	Healthy controls (n = 10)	Cerebral AVM (n = 10)
Peak thrombin nM	127.3 (30-215)	28 (10-73.40); <i>p</i> = 0.0001	38.75 (17.70-61); <i>p</i> = 0.0020	35 (17-65); <i>p</i> = 0.0030
ETP nM × min	2,620 (800-4,671)	1,118 (451-2,218); <i>p</i> = 0.0002	1,100 (501-1,808); <i>p</i> = 0.0030	1,200 (600-1,900); <i>p</i> = 0.0040
Lag time min	18 (12-22)	26 (22-31); <i>p</i> = 0.0100	32 (28-36); <i>p</i> = 0.0010	31 (25-38); <i>p</i> = 0.0020
Velocity index nM/min	15.40 (5.7-21)	3.2 (2-8.6); <i>p</i> = 0.0010	1.5 (0.6-2.25); <i>p</i> = 0.0040	2.5 (0.8-3.2); <i>p</i> = 0.0040
vWF antigen IU/mL	0.850 (0.330-2.250)	0.743 (0.370-1.890); <i>p</i> = 0.8700	0.34 (0.31-0.7); <i>p</i> = 0.0100	0.51 (0.4-0.632); <i>p</i> = 0.040

Abbreviations: AIS = arterial ischemic stroke; AVM = arteriovenous malformation; ETP = endogenous thrombin potential (corresponding to the area under the curve for thrombin generation assay curves); vWF = von Willebrand factor.

^aValues are median (range). The Kruskal-Wallis test followed by the Mann-Whitney *U* test was used to examine differences between the study groups. *p* Values reported refer to comparison with the group of children with recurrent AIS. *p* Values of less than 0.05 (2-sided) were regarded as significant.

inflammation)²⁶ were not significantly different between patient groups, but were significantly higher in the group of children with AIS recurrence compared to controls (table 3).

Logistic regression analysis of biomarkers and AIS recurrence.

Logistic regression analysis was used to examine the relationship of CECs, total AnV+MPs, and peak thrombin to AIS recurrence (table 4). AIS recurrence was associated with high CECs, high total AnV+MPs, and elevated peak thrombin in both nonadjusted and adjusted analyses. In addition, CECs together with the previously described predictors of AIS recurrence (occlusion or progressive arteriopathy)⁷ resulted in an increased post-test probability of identifying children at risk of AIS recurrence from 67 (95% CI 44%–79%) to 86% (95% CI 55%–94%) (table e-2).

DISCUSSION In this cross-sectional study of a group of children with AIS and cerebral arteriopathy, we have identified biomarkers that differ significantly between children with recurrent AIS and those with a monophasic course. Our observations suggest that a state of chronic endothelial activation and injury (increased CECs and EMPs), platelet activation (increased platelet MPs), and increased MP-mediated thrombin generation could be important determinants of AIS recurrence.

The morphology and course of cerebral/cervical arteriopathies are the most important determinants of recurrence thus far identified in childhood AIS.^{1,5} Current classifications are radiologic,^{6,7} although it is well-recognized that MRA and CA have limitations in

identifying and differentiating between distinct pathologic entities. In this study we chose to classify patients based solely on AIS recurrence (corroborated both clinically and radiologically) as this is a robust, verifiable, and clinically important event.

CECs have been used as markers of endothelial damage across an ever-widening variety of diseases, including systemic vasculitis.^{11,12,15} Importantly, CEC levels are predominantly affected by disease activity and endothelial injury and not treatment per se.^{11,12,15} Our study now suggests that elevated CECs, and therefore persistence of endothelial injury, vary between children with single and recurrent AIS. CECs could therefore be an attractive candidate biomarker to detect those at risk of recurrent AIS, a finding that is consistent with the strong correlation between CECs and disease activity in systemic vasculitis.¹²

We also examined levels and functions of circulating MPs in our patient groups. Circulating MPs possess a plethora of potent proinflammatory effects including endothelial binding and activation by upregulation of oxidative stress.^{14,27} MPs also have important prothrombotic properties.^{13,14,25} This is largely due to PS exposure on the MP outer surface directly contributing to activation of the extrinsic coagulation pathway and indirectly enhancing the TF capability as well as delivering TF to the thrombus edge.^{13,14,25} Further support for this latter mechanism comes from our observation that these TF+ MP also express PSGL-1 responsible for binding of TF+MP to activated platelets expressing P-selectin.^{25,28}

Table 4 Unadjusted and adjusted odds ratios by multivariable logistic regression analysis for arterial ischemic stroke recurrence^a

Biomarker	Unadjusted			Adjusted (age/gender/time from AIS to evaluation)		
	OR	95% CI	<i>p</i>	OR	95% CI	<i>p</i>
Circulating endothelial cells	2.438	2.060-2.645	0.005	2.021	2.004-2.038	0.016
Annexin V+ microparticles	1.410	1.100-1.545	0.004	1.220	1.039-1.317	0.020
Peak thrombin nM	1.515	1.173-1.705	0.003	1.319	1.040-1.429	0.010

Abbreviations: AIS = arterial ischemic stroke; CI = confidence interval; OR = odds ratio.

^aAdjusted odd ratios for age/gender/time from AIS to evaluation. *p* < 0.05 was considered significant.

In combination, the biomarker data presented here implicate persistence of endothelial inflammation and injury and excess MP-mediated thrombin generation as potentially important mechanisms determining AIS recurrence in children. These data provide a scientific rationale for currently used strategies to prevent AIS recurrence with antiplatelet therapy (such as aspirin), but if combined with radiologic and histopathologic features could provide a rationale for corticosteroids, or more aggressive immunosuppression (cyclophosphamide or mycophenolate mofetil) to suppress vascular inflammation in select cases.^{9,29}

Our study has limitations. It will be of considerable interest to establish the specificity of our findings by comparison with patients with AIS without arteriopathy (who may have small vessel CNS vasculitis, as suggested by some authors),³⁰ and in relation to other well-established risk factors such as cardiac disease. We chose to initially focus on children with AIS and cerebral arteriopathy specifically in order to inform the controversy surrounding the role of persistent vascular inflammation contributing to progression of arteriopathy in this group. Secondly, we did not perform cerebrovascular biopsies to confirm persistent cerebral vasculitis, but this is reflective of current practice, where cerebroarterial or other CNS biopsy is rarely undertaken. In addition, the AIS recurrence rate in our study was higher (22%) than that reported in other cohorts,¹ reflecting probable referral bias to our tertiary center. Even though several lines of evidence suggest that CEC and MP are important pathogenic mediators that can amplify endothelial injury and inflammation,^{27,31} the cross-sectional nature of this study precludes any causal effect to be determined. Finally, the sample size was small and the patients did not all receive the same treatments, although they were treated in line with published clinical guidelines.^{21–23} Ultimately the goal would be to prevent AIS recurrence by identifying the high-risk profile patients based on these biomarkers and initiate therapy after first AIS. Further prospective studies to confirm or refute our findings and form the basis for secondary stroke prevention risk-stratified approaches will require multicenter collaboration.

Our data suggest that persistent endothelial injury, platelet activation, and excessive thrombin generation could be important mechanisms underlying recurrent childhood AIS. Prospective validation of our findings is now required to firmly establish the role of these biomarkers as prognostic markers in childhood AIS and cerebral arteriopathy.

AUTHOR CONTRIBUTIONS

Despina Eleftheriou: study design, data collection, laboratory measurements, statistics, writing of the manuscript. Vijeya Ganesan: study design and data acquisition, writing of the manuscript. Ying Hong: data collection, laboratory measurements and review of the manuscript. Nigel J.

Klein: study design, review of the manuscript. Paul A. Brogan: study design, data collection, statistics, writing of the manuscript.

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DISCLOSURE

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***PRRT2* gene mutations: From paroxysmal dyskinesia to episodic ataxia and hemiplegic migraine (See p. 2115)**

This podcast begins and closes with Dr. Robert Gross, Editor-in-Chief, briefly discussing highlighted articles from the November 20, 2012, issue of *Neurology*. In the second segment, Dr. Jeff Waugh talks with Drs. Renzo Guerrini and Alice Gardiner about their papers on paroxysmal dyskinesia and hemiplegic migraine.

Dr. Stacey Clardy reads our e-Pearl of the week about plus-minus lid syndrome. In the next part of the podcast, Dr. Ted Burns focuses his interview with Steven Lewis and Allison Weathers on encephalopathy caused by liver and kidney dysfunction. Disclosures can be found at www.neurology.org.

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