

Endothelial Microparticle Levels Are Similar in Acute Ischemic Stroke and Stroke Mimics Due to Activation and Not Apoptosis/Necrosis

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Abstract

Background: Endothelial microparticles (EMPs) are <2- μ m membranous blebs from endothelial cell membranes that have been demonstrated to be elevated in vasculopathic conditions. One study has demonstrated elevated EMPs in acute ischemic stroke (AIS) versus age- and comorbidity-matched controls.

Objectives: To determine the level of EMPs in stroke mimics and AIS and determine if EMPs are released as a result of activation or apoptosis/necrosis in AIS.

Methods: EMP levels in plasma of patients with AIS and stroke mimic patients were quantified by flow cytometry. Stroke status was verified in all patients by magnetic resonance imaging. Patients were matched for age and comorbidities. Markers for apoptosis/necrosis (platelet/endothelial cell adhesion molecule-1 [PECAM-1]/CD31 antigen) and activation (E-selectin/CD62e antigen) were compared. A PECAM-1/E-selectin ratio of >4.0 was used to determine whether EMPs were generated via activation or apoptosis/necrosis. Data were compared between groups using the Mann-Whitney U test.

Results: EMP levels were similar in stroke mimic patients when compared with AIS; there was no difference between groups (PECAM-1, $p = 0.393$; E-selectin, $p = 0.579$). The PECAM-1/E-selectin ratio was also similar for AIS and stroke mimics, and all were >4.0.

Conclusions: EMP levels were similar in patients with AIS and stroke mimic patients. The PECAM-1/E-selectin ratio demonstrated that EMPs were generated via activation and not apoptosis/necrosis. This suggests that EMPs may not be a good marker for AIS, given the inability to discriminate between stroke mimics and AIS.

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The diagnosis of acute ischemic stroke (AIS) remains largely a clinical diagnosis, with radiographic confirmation after permanent injury has occurred to the brain. Many conditions such as seizures, hypoglycemia, and infection may cause symptoms and signs similar to stroke without the same underlying disease process. The availability of thrombolytic therapy, which is most useful in the first three hours after onset of AIS, has made the need for a confirmatory test for AIS more pressing. Despite this need, no test, such as the cardiac biomarkers in routine use for early identification of acute coronary syndrome, is yet available for AIS. Endothelial microparticles (EMPs) present a potential biomarker.

Endothelial microparticles are <2- μ m membranous blebs from endothelial cell membranes¹ and are characteristic of the endothelial cell metabolic state before cell fragmentation. EMPs have been demonstrated to be elevated in vasculopathic conditions such as acute myocardial infarction,^{2–4} coronary artery disease,² hypertension,⁵

hyperlipidemia,⁶ and diabetes.⁷ EMPs are elevated in inflammatory disorders⁸ and thrombotic disorders.⁹ Several studies suggest that EMPs may be the mediators of disease, through thrombogenesis, vascular insult, and inflammation, rather than just markers of disease. In addition, EMPs carry receptors from their parent cells that allow their cell type identification. EMPs are distinctive in their phenotypic markers, as well as procoagulant ability, and thus distinguishing between their subtypes is useful.

Frequently, the type of receptor present also allows investigators to derive what incited the cell to fragment, for instance, cellular activation or apoptosis/necrosis. In the case of EMPs, receptors have been identified by other investigators as reliable indicators of cellular activation or apoptosis.¹⁰ The ratio of platelet/endothelial cell adhesion molecule-1 (PECAM-1)/E-selectin has been demonstrated to distinguish between EMPs generated secondary to activation versus apoptosis.¹⁰ The ratio of PECAM-1/E-selectin antigen binding to the receptor on the surface of the EMP is what allows this discrimination. A ratio of >4.0 indicates activation, while a ratio of <0.4 indicates apoptosis/necrosis.¹⁰

There is evidence to suggest that regional brain ischemia results from endothelial apoptosis/necrosis of atherosclerotic lesions, leading to thrombogenic endothelial lesions.¹¹⁻¹³ The issue of EMP release in AIS was examined by Simak et al., who demonstrated a significant difference in EMP levels between patients with magnetic resonance imaging (MRI)-verified AIS versus age- and comorbidity-matched controls without AIS but with similar comorbidities.¹⁴ The comparison of EMP levels in AIS to EMP levels in stroke mimic patients has not, to our knowledge, been explored. In addition, whether EMP elevations in AIS are a result of activation or apoptosis/necrosis is unknown.

In this study, we hypothesized that AIS is a cerebrovascular condition with significant apoptosis/necrosis of endothelial cells occurring in the cerebral vasculature at the site of and distal to the vascular occlusion, with EMP elevations in AIS relative to stroke mimics. Furthermore, we hypothesized these elevations are secondary to apoptotic/necrotic changes in the endothelium.

METHODS

Study Design

This was a prospective blood banking project. Institutional review board approval was obtained, and informed consent was obtained from all patients before collection of the samples. Separate institutional review board approval for analysis of samples for levels and types of EMPs was obtained. Informed consent and sample collection were performed by clinical research associates specifically trained in the consent process and sample processing.

Study Setting and Population

Blood samples were collected on a convenience basis from a large tertiary care hospital emergency department (average yearly census, 85,000 visits) or two community hospital emergency departments (average

yearly census, 35,000 visits each). Study participants were selected from among patients whose blood samples were banked as a component of an ongoing suspected stroke blood specimen banking project. Inclusion criteria for the banking project included clinical suspicion of and hospital admission for AIS or hemorrhagic stroke. No further explicit inclusion criteria such as stroke severity, measured by the National Institutes of Health Stroke Scale, or specific focal neurologic findings were required. Noncontrasted computed tomography (CT) of the head was performed in all subjects before admission to the hospital. Specific exclusion criteria included onset of symptoms more than 24 hours before blood collection; any central nervous system infection within the past 30 days; any form of head trauma or injury within the past 30 days; known primary or metastatic cancer involving the brain; any form of underlying medical condition that, in the opinion of the investigator, would prohibit the patient from completing the study; and blood must have been drawn for tests performed as a part of routine care. Only patients admitted to the hospital with conditions consistent with transient ischemic attack or cerebrovascular accident were included in this study.

Study Protocol

The emergency diagnosis was made or corroborated by the attending emergency physician. After collection of the samples, the patient's course was followed up through review of the medical record, and determination of AIS was made by review of the medical record as well as review of diffusion-weighted MRI (DW-MRI) reports demonstrating acute cerebral ischemia. Additionally, stroke mimic patients were followed up through medical record review of the 365 days after discharge to verify no subsequent diagnosis of cerebral ischemia was made. All discharge diagnoses were determined by either attending neurologists or attending internists.

Patients subsequently demonstrated not to have experienced AIS, but who had a clinical presentation consistent with AIS meriting admission to the hospital, were deemed to be stroke mimics. Patients with stroke mimics did not demonstrate evidence of diffusion restriction on MRI suggestive of cerebral ischemia and had a discharge diagnosis that was not consistent with AIS.

Mimic samples were matched to AIS samples based on demographics and comorbidities. Diagnosis of hypertension was made either by preexisting diagnosis of hypertension based on medical record review or by a measurement of systolic blood pressure >180 mm Hg in the emergency department with concurrent evidence of end-organ damage, including possible AIS.

The outcome measure for this study was absolute count of EMPs per milliliter of plasma. Previous literature suggested the magnitude of the difference between stroke and comorbidity-matched controls would be on the order of 7,000 particles/ μ L and that the standard deviation would be of the order of 2,000 particles/ μ L.⁴ Such a large effect size would require six samples per group in a Mann-Whitney U test with a power of 80% and the significance level set to 5%. Assuming increased variability within the mimic group compared with controls used in other studies, we elected to use a sample size of ten per group.

Sample Collection and Blood Processing. Blood from patients with suspected AIS was collected within 24 hours of symptom onset (mean, 10.2 hours; range, 3.0–23.2 hours). Samples from patients ultimately diagnosed with AIS were collected an average of 12.0 hours (range, 5.5–23.2 hours) after symptom onset. Samples were collected in Becton-Dickinson (Franklin Lakes, NJ) buffered sodium citrate (blue top) plasma tubes and centrifuged at 180g for 10 minutes at 4°C. The plasma was then stored in cryotubes at –70°C for approximately 1.5 years. Data from several investigators suggest no substantial degradation of EMPs in samples stored at –70 to –80°C.^{15,16}

Quantification of Microparticles. Fifty-microliter aliquots of undiluted plasma samples were centrifuged at 20,000g for 10 minutes at 10°C. Forty microliters of supernatant was removed. The pellet was resuspended in 0.1 µm filtered 50 µL of phosphate-buffered saline (Gibco, Invitrogen Corp., Carlsbad, CA) containing 5 mmol/L CaCl₂. Four microliters of each of the following antibodies was added: phycoerythrin-labeled α-human CD31 (BD Pharmingen, San Jose, CA), phycoerythrin-Cy5-labeled α-human CD62e (BD Pharmingen), and fluorescein isothiocyanate-labeled α-human CD42b (BD Pharmingen). APC-labeled α-human CD45 (BD Pharmingen) was used to exclude leukocyte microparticles as a significant contributor to microparticle levels and was never elevated above trigger for detection in experimental conditions. Fluorescein isothiocyanate-conjugated α-mouse immunoglobulin G (H+L) (goat) (ICN Biomedicals, Irvine, CA) was used as a negative control and was never elevated above trigger for detection in experimental conditions. EMP markers for apoptosis (CD31) and for activation (CD62e) were evaluated. Platelet marker CD42b was used to exclude particles that were CD31⁺ and/or CD62e⁺, as well as CD42b⁺. CD42b is a platelet membrane glycoprotein complex essential for normal platelet adhesion and clot formation at sites of vascular injury. It is found solely on platelets and thus is used as an exclusionary marker for platelet microparticles.

Reactions were incubated at 4°C on a 120 rpm horizontal shaker for 30 minutes. PBS containing 5 mmol/L CaCl₂ was added to bring the total volume to 500 µL. Samples were transferred to TruCount tubes (Becton-Dickinson) to allow quantification of particle amount by comparing number of particle events with bead events.

Three-color flow cytometry was performed on a BD Biosciences FACS Calibur platform (BD Biosciences, San Jose, CA) with a medium flow rate setting and a 120-second stop time. Negative control fluorescein isothiocyanate-labeled mouse α-mouse immunoglobulin G did not demonstrate an appreciable signal within the plasma sample and was used to discriminate between signal range and baseline fluorescence within the sample.

A region of interest on the flow cytometry histogram was gated utilizing fluorescent latex beads of known size (Sigma-Aldrich, St. Louis, MO), and the trigger for detection of fluorescent signal was set to screen out background fluorescence. Fluorescence channels and light scatter were set at logarithmic gain. EMPs were defined as particles bearing antigens (CD31⁺/CD42b[–] or CD62e⁺/CD42b[–]) and less than 2 µm in size (forward light

scatter). Size estimation was performed by performing flow cytometry on initial samples with standard-sized beads from 0.5 to 10 µm in size (Sigma-Aldrich). Data were collected and analyzed on BD CellQuest (BD Biosciences Immunocytometry Systems). The number of EMPs per microliter was calculated using equation 1 abstracted directly from the Becton-Dickinson TruCount tubes package insert. The number of beads per test was obtained from the manufacturer's data per lot of tubes, and the test volume per 120-second run was calculated using equation 2.

$$\text{EMP}/\mu\text{mol/L} = \frac{\text{Number of events in region of interest}}{\text{Number of events in absolute bead count region}} \times \frac{\text{Number of beads per test}}{\text{Test volume}} \quad (1)$$

$$\mu\text{L}/120 \text{ seconds} = \frac{\text{Number of bead events in 120 seconds}}{\text{Number of beads per test}} \times \text{diluent volume} \quad (2)$$

Data Analysis

Data are described using medians and ranges or frequencies and proportions as appropriate. Comparisons of counts between groups used the Mann-Whitney U test; counts were not normally distributed. Analyses were conducted using SPSS version 13.0 (SPSS Inc., Chicago, IL).

RESULTS

The demographics of the AIS and stroke mimic patients are given in Table 1. For patients with confirmed infarcts, the mean National Institutes of Health Stroke Scale score was 2.7 (range, 0–5). Admitting and final diagnoses of the stroke mimic patients are presented in Table 2. Figure 1 shows the CD31 and CD62e counts for AIS and stroke mimics. Comparison of AIS with mimic samples revealed no significant difference between CD31 ($p = 0.393$) and CD62e ($p = 0.579$). The EMP ratio of CD62e/CD31 was used to differentiate activation (>4.0) from apoptosis (Figure 1). The ratios were similar among all groups, and all were >4.0. (Figure 2).

DISCUSSION

Most studies examining the presence or absence of EMPs in clinical subjects compare disease state with healthy controls. In fact, Simak et al. have done this with age- and comorbidity-matched normal controls and nicely demonstrated elevated circulating EMPs in the setting of AIS.¹⁴ In clinical practice, differentiation between normal and disease state is often obvious.

However, distinguishing between a diagnosis of AIS and a stroke mimic is frequently difficult. This forces the clinician to make challenging therapeutic decisions without a confirmatory diagnostic study for AIS. Patients

Table 1
Subject Demographics

	AIS (n = 10)	Mimics (n = 10)
Age, yr (range)	66.7 (52–90)	66.1 (50–86)
Male/female	5/5	5/5
Time since onset to blood draw (min)	713	510
No. of patients with comorbidities		
Prior AIS	1	2
Hypertension	8	8
History of coronary artery disease or acute myocardial infarction	2	2
Recent coronary revascularization	2	2
Diabetes	3	3
Hyperlipidemia	5	5
Atrial fibrillation	1	0

AIS = acute ischemic stroke.

were selected as candidates for the stroke banking project based on clinical suspicion of AIS after neurologic examination, laboratory evaluation, and performance of noncontrast CT of the head. With persistent concern for possible AIS either by history or physical examination, the patient was enrolled in the stroke banking project. No explicit inclusion criteria, such as National Institutes of Health Stroke Scale or focal neurologic findings, were required.

Considering that the emergency department diagnosis of AIS remains nearly completely clinical in nature, without biomarker confirmation or readily available radiologic confirmation at the time patients are considered for possible therapeutic intervention (and thus were included in our banking project), we sought to replicate clinically realistic study conditions to allow for results that could be generalized. All patients were admitted with a clinical diagnosis that was consistent with AIS and negative findings on noncontrast CT of the head. The groups were then stratified by presence or absence of DW-MRI restriction consistent with AIS or not. There was no evidence of diffusion restriction on MRI of the brain in any patient in the stroke mimic group. The samples were

Table 2
Stroke Mimic Diagnoses

ED Diagnosis	Discharge Diagnoses
Diplopia	Diplopia
Syncope	Syncope
Transient ischemic attack	Chronic bronchitis NOS, dizziness
Possible seizure	Convulsions
CVA	Hearing loss
Seizure	Seizure
CVA	TIA
Palpitations, possible CVA	Open wound/cellulitis of head
TIA, pneumonia	Pneumonia
CVA	Cerebral artery occlusion NOS without infarction

NOS = not otherwise specified; CVA = cerebrovascular accident; TIA = transient ischemic attack.

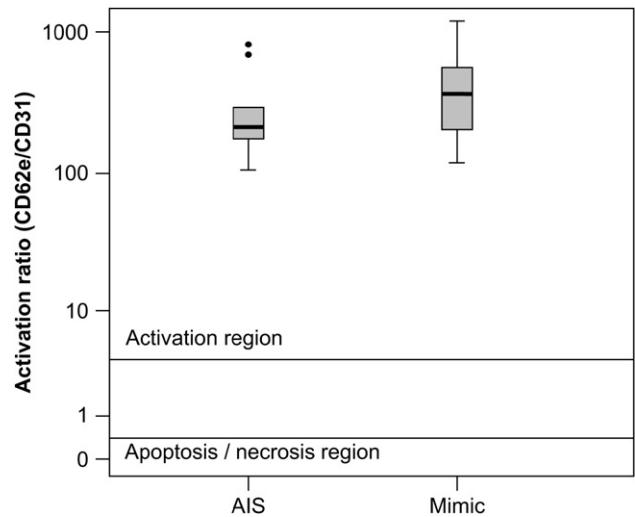


Figure 1. Activation ratio for acute ischemic stroke (AIS) and stroke mimics. Ratio of total counts of endothelial microparticle $\times 10^3/\mu\text{L}$ for $[\text{CD62e}^+/\text{CD42b}^-]/[\text{CD31}^+/\text{CD42b}^-]$.

then matched by age and comorbidities, and EMP levels were assessed. Patients with a clinical diagnosis of AIS are by default a heterogeneous group with differences in infarct location and size, clinical presentation,

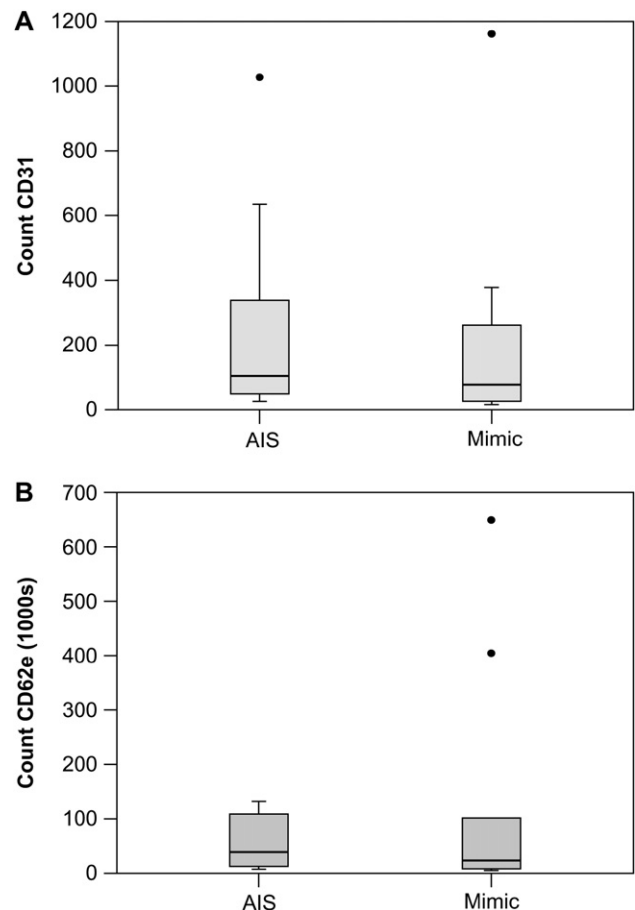


Figure 2. (A) Total endothelial microparticle (EMP) counts for platelet/endothelial cell adhesion molecule-1 ($\text{CD31}^+/\text{CD42b}^-$) and (B) E-selectin ($\text{CD62e}^+/\text{CD42b}^-$) for acute ischemic stroke (AIS) and stroke mimics. Counts in $\text{EMP} \times 10^3/\mu\text{L}$.

thrombotic or embolic etiology, risk factors, comorbidities, and patient age. Consideration of matching on other factors was made, but given the innate heterogeneity of this group, we matched only on age and comorbidities. An alternative would be to limit patient selection to a single gender, comorbidity, and age range, but this would have limited the ability to generalize the results.

No difference in EMP levels between AIS and stroke mimics was observed; comorbid conditions potentially confound the ability of EMPs to be used as a biochemical marker of AIS. EMPs have been found to be elevated in inflammatory disorders⁸ as well as thrombotic disorders.⁹ EMPs have been found to be able to bind to and activate monocytes, as well as carry von Willebrand factor, which functions differently than in its soluble form.¹ EMPs have been demonstrated to be elevated in vasculopathic conditions such as acute myocardial infarction,²⁻⁴ coronary artery disease,² hypertension,⁵ hyperlipidemia,⁶ and diabetes.⁷

Why no difference was found between AIS and stroke mimics, when previous work demonstrated a difference in EMP levels between AIS and normal age- and comorbidity-matched controls, remains unclear. We hypothesize that this may be related to acute illness (AIS) superimposed on their chronic illnesses, resulting in similar EMP level elevations between groups; Table 1 lists emergency department and discharge diagnoses of the mimics, describing the acute disease. It is also possible that the low National Institutes of Health Stroke Scale of the AIS patients in this study contributed to these results, suggesting that the infarcts were small in size and thus had lower levels of EMPs than previously demonstrated. Other potential biomarkers of stroke, such as S100, demonstrate release kinetics dependent on stroke size and peak levels at 72 hours.¹⁷ The possibility also exists that the EMP levels are variable, resultant from the release time course, but there are no data currently to substantiate this. The sole paper examining EMPs in AIS had a longer mean time to blood draw of 37 hours (range, 18.5–51.8 hours).¹⁴ Our time course for sampling was on average 10.2 hours from symptom onset (range, 3–23 hours). We are not aware of data describing the complete time course of release of EMPs in vivo. Ferreira et al.¹⁸ examined EMP levels in vivo after postprandial hypertriglyceridemia. EMP levels were elevated after a high-fat meal as early as three hours, but the complete time course of release was not addressed. In vitro studies suggest release within six hours following activation with tumor necrosis factor α or mitomycin c.¹⁹ In human umbilical vein endothelial cells in vitro, when activated with thrombin, EMPs were released within 12 hours.²⁰ Our time window of sampling should have captured elevated EMPs, considering radiographically apparent necrosis occurs within 12 hours of infarct,²¹ but this cannot be confirmed. We are also unaware of data that address clearance of EMPs from the circulation. Given the relatively early sampling times in this study, and the likely continuing endothelial injury in the penumbra, it is unlikely that the release and clearance of EMPs were completed at the time of sample acquisition. The lack of EMP level difference has important implications in the development of EMPs as markers of AIS. From these results, EMPs lack sufficient specificity to rule out AIS

in stroke mimic patients, which reduces their suitability as a biomarker for AIS.

In addition to increased EMPs being observed in both AIS and mimics, we found EMPs resulted from endothelial cell activation as opposed to apoptosis. Classically, AIS has been seen as a disease occurring as a result of the apoptotic core of an endothelial plaque rupturing. We therefore hypothesized the CD31 counts in the AIS patients would be high, resulting in an activation ratio <0.4. Our data suggest EMPs present in circulation following AIS do not result from apoptosis or necrosis. This is suggested by the lack of CD31⁺ particles found in our samples, and EMPs in AIS are likely generated as a result of activation. The implications of this finding are that EMPs are not generated as a result of apoptosis/necrosis of endothelium from lesions distal to vascular occlusion but are more likely a result of a general inflammatory condition secondary to illness and not specifically related to AIS. This supports the lack of difference between the levels of EMPs between the AIS and stroke mimic groups.

Several outliers of elevated EMP levels exist in both the AIS and stroke mimic groups. In the AIS group, the patient with elevated EMP levels was the only one with known hepatitis C infection in either group and also had sphenoid sinus opacification on neuroimaging, consistent with sinusitis. These two conditions may have resulted in extraneous elevations in the EMP levels. In the stroke mimic group, two outliers were found. The first patient demonstrated a significant history of chronic obstructive pulmonary disease/chronic bronchitis, which was the final discharge diagnosis, and the second patient was the only patient with active cancer, bladder cancer in this case. These inflammatory/neoplastic processes may account for the outlier values in this study. One stroke mimic patient was assigned a diagnosis of “cerebral artery occlusion not otherwise specified without infarction” on discharge. This diagnosis was based on the magnetic resonance angiography imaging of the cerebral vasculature demonstrating narrowing of the left internal and external carotid. This said, there was no evidence of infarct or ischemia on the diffusion-weighted sequence, making it unlikely that the patient had AIS.

LIMITATIONS

The antigenic markers used in this study, while utilized in previous investigations, may not be optimal in the setting of AIS. In the investigation by Simak et al.,¹⁴ CD105 (endoglin), CD144 (VE-cadherin), CD54 (ICAM-1), and phosphatidylserine were used as markers for EMP, with significantly elevated phosphatidylserine-positive EMP levels found in AIS. As a result of the use of these different markers, differences may exist between the data found in our study and the investigation by Simak et al.,¹⁴ although there is no a priori reason to believe so. Indeed, several investigations into EMP levels in other pathologic conditions have used CD31 and CD62e, as we have done.^{7,10} The only paper investigating differential markers in activation versus apoptosis has utilized CD31 and CD62e and their relative index.¹⁰

Flow cytometry is a technique that was originally developed to differentiate cell types and may not be optimal

for exact quantification of cell or particle numbers in a given sample. The use of the TruCount tubes in this protocol provided a validated method of quantification, and thus we believe that this provides a reliable reproducible method of quantifying EMP levels by flow cytometry.

Patient samples were stored at -70°C for approximately 1.5 years. While data from several investigators suggest no substantial degradation of EMPs in samples stored at -70 to -80°C , some degradation may still be possible despite undergoing only one freeze-thaw cycle.^{14–16} All plasma samples were handled identically, and thus this should minimize intraexperimental variability.

Insufficient centrifugation potentially results in inaccurate EMP level measurement in flow cytometry. Our protocol utilized an initial centrifugation of the plasma specimen at $180g$ for 10 minutes to obtain platelet-poor plasma. Subsequently, the platelet-poor plasma was centrifuged at $20,000g$ for 10 minutes, with the resultant sedimented MP resuspended in buffer. This protocol closely follows previously described experimental methods isolating EMPs.¹⁴

CONCLUSIONS

Plasma samples from patients with DW-MRI-verified AIS demonstrated no differences in EMP counts from stroke mimics; elevated EMPs may not be specific to AIS. Elevated EMP counts observed in AIS likely result from endothelial cell activation and not from apoptosis or necrosis, as was hypothesized from the classic AIS model of apoptotic plaque rupture and subsequent thrombosis.

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