Antiphospholipid Antibodies and the Risk of Stroke in Urban and Rural Tanzania
A Community-Based Case–Control Study

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Background and Purpose—The burden of stroke is high in sub-Saharan Africa, and improved knowledge of risk factors is needed. Antiphospholipid antibodies are a common acquired stroke risk factor in young individuals. Antiphospholipid antibodies may be induced by infectious diseases. Sub-Saharan Africa has a high infectious burden, and we analyzed the contribution of antiphospholipid antibodies to the risk of stroke in an incident population from rural and urban Tanzania.

Methods—Stroke cases and age- and sex-matched community-acquired controls from the rural Hai district and urban Dar-es-Salaam areas of Tanzania were recruited in a wider study of stroke incidence between June 2003 and June 2006. Lupus anticoagulant, anticardiolipin, anti-β2-glycoprotein I, and antiphosphatidylserine/prothrombin antibodies were determined in stored plasma, as well as IgG antibodies against Treponema pallidum.

Results—Data from 158 stroke cases and 369 controls were analyzed. Thirty cases (19%) and 4 controls (1%) had a lupus anticoagulant (odds ratio, 20.8; 95% confidence interval, 7.2–60.5). Anticardiolipin IgG was the only other antiphospholipid antibody subtype associated with increased stroke risk (odds ratio, 2.1; 95% confidence interval, 1.0–4.3), but this association disappeared when corrected for IgG antibodies against Treponema pallidum results. The prevalence of anti-β2-glycoprotein I IgG antibodies in the Tanzanian healthy population was high when Dutch cutoff values were applied (67%), whereas presence of anti-β2-glycoprotein I IgM was associated with a reduced stroke risk (odds ratio 0.3; 95% confidence interval, 0.1–1.1).

Conclusions—The presence of lupus anticoagulant is a strong, and to date unrecognized, risk factor for stroke in Tanzania, especially in young and middle-aged individuals. (Stroke. 2016;47:2589-2595. DOI: 10.1161/STROKEAHA.116.013760.)

Key Words: Africa ■ antiphospholipid antibodies ■ β2-Glycoprotein I ■ lupus anticoagulant ■ stroke ■ syphilis ■ Tanzania

The burden of stroke is increasing in many low- and middle-income countries.1 In Tanzania, age-standardized stroke incidence rates in a rural population (Hai district) were similar to those seen in high-income countries, whereas incidence rates from urban Dar-es-Salaam were higher than that seen in most other settings.2 A strategy for stroke prevention is urgently needed. This requires knowledge of stroke risk factors, but data on risk factors relevant for sub-Saharan Africa (SSA) are scarce. Recent findings from the international, multicenter, INTERSTROKE study, which included 4 SSA countries, and a prospective study in Tanzania confirmed the importance of well-established stroke risk factors such as high blood pressure (BP), diabetes mellitus, dyslipidemia, smoking, and overweight.3,4 Nonetheless, other unique risk factors may apply to populations in SSA and contribute to the disproportionate burden of stroke. An example is HIV infection, which was recently identified as an important stroke risk factor in Tanzania by our group.4

Antiphospholipid antibodies (aPL) are a common acquired risk factor for stroke. These antibodies comprise a heterogeneous group of antibodies that react with phospholipids or phospholipid-binding proteins of which β-2-glycoprotein-I (β2-GPI) is considered the main antigenic target. The presence of aPL is demonstrated either directly with ELISA (anticardiolipin antibodies [aCL] or anti-β2-GPI antibodies) or functionally by showing the ability of aPL to prolong in vitro coagulation times (lupus anticoagulant [LA]). The importance of aPL, and especially LA, as risk factors for stroke is...
increasingly recognized, particularly in young individuals. For example, a recent systematic review found an aPL prevalence of 17.4% in patients with a cerebrovascular event below the age of 50 with presence of aPL conferring a 5-fold higher risk for stroke.\(^1\)

Induction of aPL may be triggered by infectious diseases.\(^6\) SSA has a high infectious burden, and we hypothesized that aPL are a common risk factor for stroke in this region. We therefore investigated whether aPL were independently associated with the risk for stroke in a rural and urban population in Tanzania.

Methods

Study Design and Participants

For the current study, stored citrate-anticoagulated blood samples of participants of the Tanzania Stroke Incidence Project were used. This study recorded stroke incidence in 2 well-defined, demographic, surveillance sites in Tanzania during a 3-year period from June 2003. These sites were the Hai district in northern Tanzania, a rural area where agriculture is the primary economic activity; and urban Dar-es-Salaam, the largest city in Tanzania. Key results, including details on study design, procedures, and characteristics of study areas and participants, have been previously published.\(^4\) In short, patients with stroke were identified within the community and at health facilities using a system of community-based investigators and liaisons with local hospital and medical center staff.\(^5\) Patients with first-ever and recurrent stroke, who fulfilled the standard World Health Organization definition of stroke criteria, were eligible for enrolment. Controls who were matched to cases for age (±3 years) and sex were recruited from the background census population of the Hai and Dar-es-Salaam demographic surveillance sites.

Ethics approval for the Tanzania Stroke Incidence Project study was obtained from the National Institute of Medical Research, Dar-es-Salaam, and from the Newcastle and North Tyneside Joint Ethics Committee, UK. Written informed consent was provided by each participant or by a close relative when participants were unable to provide consent.

Measurement of Established Stroke Risk Factors

Independent significant risk factors for stroke have been identified in this population previously.\(^4\) All cases and controls were interviewed by members of the study team using the same pro forma, with the exception of sections relating to the stroke itself. Demographic information, social history, and medical history were recorded, and participants underwent a medical assessment and examination. BP was recorded at least 7 days after stroke, to allow for elevation in BP immediately post stroke. Three measurements were taken, and the average of the second and third measurement was used. Hypertension was defined as a mean systolic or diastolic BP >160 and 90 mm Hg, respectively, a history of hypertension, or taking of antihypertensive drugs before stroke. Smoking habits were categorized into current smokers (smoked tobacco in the past 12 months) and former smokers (those who had smoked but not in the past 12 months). The presence or absence of diabetes mellitus was based on self-report of a prestroke diagnosis by a physician. A lipid spectrum was measured at North Tyneside General Hospital with an automated biochemical analyzer. Dyslipidemia was defined as a ratio of total cholesterol to high-density lipoprotein cholesterol of ≥5.0. A computed tomography head scan was performed in stroke cases who survived long immediately post stroke. Three measurements were taken, and the average of the second and third measurement was used. Hypertension was defined as a mean systolic or diastolic BP >160 and 90 mm Hg, respectively, a history of hypertension, or taking of antihypertensive drugs before stroke. Smoking habits were categorized into current smokers (smoked tobacco in the past 12 months) and former smokers (those who had smoked but not in the past 12 months). The presence or absence of diabetes mellitus was based on self-report of a prestroke diagnosis by a physician. A lipid spectrum was measured at North Tyneside General Hospital with an automated biochemical analyzer. Dyslipidemia was defined as a ratio of total cholesterol to high-density lipoprotein cholesterol of ≥5.0. A computed tomography head scan was performed in stroke cases who survived long enough to undergo this.\(^7\) Findings of ischemia, hemorrhagic infarct, or no evidence of stroke were classified as ischemic stroke.

Antiphospholipid Antibodies

Anticardiolipin IgG and IgM antibodies were measured with a commercially available kit (IBL international GmbH, Hamburg, Germany), according to the instructions of the manufacturer. Results are expressed in IgG phospholipid units (GPL) or IgM phospholipid units (MPL). Antibodies against the complex of prothrombin and phosphatidylylsine (IgG and IgM) were measured with the QUANTA Lite aPS/PT kit (Inova diagnostics, San Diego, CA), according to the instructions of the manufacturer. Results are expressed in AU (arbitrary units)/mL. Anti-β2-GPI antibodies were measured with a home-made ELISA as previously described.\(^4\) Cutoff was set at the 90th and 95th percentile of the value recorded for the Tanzanian control group.

The presence of LA in plasma samples was assessed with dilute Russell Viper Venom Time screen and confirm reagents (LA screen and LA confirm; Life Diagnostics, Clarkston, GA), as described previously and in adherence to the guidelines for LA testing.\(^8\) In short, plasma samples were mixed with an equal volume of pooled normal plasma to exclude coagulation factor deficiencies. Coagulation times were recorded on a MC-10 coagulometer (Merlin Medical, Lemgo, Germany). Mixed plasmas were added to cuvettes and allowed to equilibrate at 37 °C for 2 minutes. Coagulation was initiated by addition of an equal volume of dilute Russell Viper Venom Time reagent. When coagulation times obtained with LA screen reagents were prolonged, that is, exceeded the 99th percentile of normal as determined in samples from 120 healthy Dutch controls, coagulation tests were repeated with LA confirm reagents. Normalized LA-ratios were subsequently calculated according to the following equation: (LA screen (sample)/mean LA screen (normal))/(LA confirm (sample)/mean LA confirm (normal)). Samples were deemed LA positive when the normalized LA ratio exceeded the 99th percentile of normal as determined in the samples of the 120 healthy Dutch controls (normalized LA ratio >1.15).

High-Sensitive C-Reactive Protein

Plasma concentrations of high-sensitive C-reactive protein were measured on a TECAN Freedom EVO robot (Tecan, Switzerland) with anti-C-reactive protein (CRP) duoset antibodies from R&D systems (Abingdon, UK), as previously described.\(^10\)

SEROLOGY FOR TREPONEMA PALLIDUM

TREPONEMA PALLIDUM serology was assessed with a commercially available kit (TREPONEMA pallidum IgG ELISA; IBL international GmbH, Hamburg, Germany) according to the instructions of the manufacturer. Samples were considered positive for anti-Treponema pallidum antibodies when the concentration of anti-Treponema IgG exceeded 11 U.

Statistical Analysis

Data were analyzed with SPSS software (version 22.0; SPSS Inc, Chicago, IL). Logistic regression was used to analyze LA and other aPL as stroke risk factors taking the control group as a reference. Odds ratios (OR) and 95% confidence intervals (95% CI) were adjusted for age (continuous), area (Hai or Dar-es-Salaam), and sex. Subsequently, OR for LA was also adjusted for the traditional risk factors, hypertension, smoking, diabetes mellitus, and dyslipidemia. We used 2-tailed tests throughout, and a P value <0.05 signified a statistically significant difference.

Results

Data from 158 stroke cases (102 in Hai and 56 in Dar-es-Salaam) and 369 controls (223 in Hai and 146 Dar-es-Salaam) were included in this study. The median time between incident stroke and interview was 8 days (interquartile range [IQR], 5–28 days) in Hai and 35 days (IQR, 10–84 days) in Dar-es-Salaam. Table 1 shows baseline characteristics and established stroke risk factors, including their OR. As previously reported,\(^4\) hypertension, diabetes mellitus, smoking, and
a high ratio of total to high-density lipoprotein cholesterol were associated with stroke in univariate analysis. In addition, a positive result of anti-*Treponema pallidum* IgG antibodies (TP-IgG), indicating current or past syphilis, was more common in cases (24.7%) than in controls (12.7%), and a positive TP-IgG was significantly associated with stroke risk (OR, 2.8; 95% CI, 1.6–4.6). Cases from Hai also had significantly higher high-sensitive C-reactive protein (hs-CRP) concentrations compared with controls from the same area. In contrast, hs-CRP concentrations in cases and controls from Dar-es-Salaam did not differ significantly.

Table 2 shows the results of the prevalence and OR of aPL corrected for age, sex, and residence area. Given the lack of previous normative data from SSA on which to establish cut-offs for positive findings, samples were considered positive for anticardiolipin or anti-β₂-GPI antibodies when the concentration exceeded the 95th percentile of the values obtained in the Tanzanian controls. A positive LA was found in 30 (19%) of the cases and in 4 controls (1%) (OR, 20.8; 95% CI, 7.2–60.5). The OR of LA was higher in stroke patients aged ≤65 years (OR, 49.0; 95% CI, 6.4–374.6) compared with those aged >65 years (OR, 11.7; 95% CI, 3.2–43.3; Table 3). LA

<table>
<thead>
<tr>
<th>Table 1. Baseline Characteristics</th>
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<tr>
<th></th>
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<td>Controls</td>
<td>Cases</td>
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<td>56</td>
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<td>Hs-CRP, µg/mL, median (IQR)</td>
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<td>203/223 (91)</td>
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Data are number with percentage, mean with SD or median with IQR. CI indicates confidence interval; HDL, high-density lipoprotein; Hs-CRP, high-sensitive C-reactive protein; IQR, interquartile range; OR, odds ratio; and TP-IgG, *Treponema pallidum* IgG assay.

*P < 0.001 vs cases.
had a trend for a higher prevalence in cases from Hai than in cases from Dar-es-Salaam (22.6% versus 12.5%; P=0.12), but the ORs of a positive LA for stroke were similar in both areas (Table 3). Further adjustment for the presence of traditional risk factors, such as diabetes mellitus, hypertension, dyslipidemia, or smoking, did not affect the risk of stroke associated with LA (data not shown). A computed tomography scan, performed within 15 days of stroke, was available in 15 LA-positive stroke cases; 12 of them had an ischemic stroke and 3 a hemorrhagic stroke.

Of the other 2 aPL subpopulations, anticardiolipin and anti-β2-GPI antibodies, only the presence of anticardiolipin IgG was associated with an increased risk for stroke (OR, 2.1; 95% CI, 1.0–4.3). None of the patients had anticardiolipin IgG levels exceeding 40 GPL units. Anticardiolipin antibodies are frequently found in patients with treponemal antibodies, and these infection-related anticardiolipin are not considered true aPL. In our study population, 12 of 33 individuals (36%) with a positive anticardiolipin IgG also had a positive TP-IgG, and the significant association of anticardiolipin IgG with stroke risk disappeared when the results were adjusted for results of TP-IgG (OR, 1.7; 95% CI, 0.8–3.7). Five of 34 LA-positive individuals (14.7%) had a positive TP-IgG, and adjustment for TP-IgG result did not markedly change the OR for stroke of a positive LA. IgM anticardiolipin antibodies were not associated with an increased risk of stroke. Although the presence of IgG anti-β2-GPI antibodies had no effect on the risk of stroke, the presence of anti-β2-GPI IgM was associated with a strongly reduced risk for stroke (OR, 0.3; 95% CI, 0.1–1.1). This inverse relationship was even stronger when the 90th percentile cutoff value was used (OR, 0.2; 95% CI, 0.0–0.6). Interestingly, when the Dutch diagnostic cutoff value for anti-β2-GPI antibodies (determined as the 95th percentile in 40 healthy Dutch adult controls) was applied, 67% of the Tanzanian study population had an increased anti-β2-GPI IgG titer, a prevalence that was similar for cases and controls from both areas.

The simultaneous presence of LA and anti-β2-GPI or anticardiolipin antibodies was uncommon. Only one out of 30 patients with LA also had anti-β2-GPI antibodies, and 3 out of 30 patients with LA had anticardiolipin antibodies. None of the controls had both LA and antibodies against β2-GPI or anticardiolipin. LA has been reported to arise because of antibodies against β2-GPI or prothrombin.11,12 Given the rarity of anti-β2-GPI in LA-positive individuals in our cohort, we measured the prevalence of antibodies against the complex of prothrombin and the phospholipid phosphatidylserine (aPS/PT antibodies). Only 3 out of 30 patients with LA had aPS/PT antibodies. There was also no significant association between presence of these antibodies and stroke, with 8% of cases and 5% of controls having a positive aPS/PT IgG (OR, 1.9; 95% CI, 0.9–4.0) and 6% of cases and 5% of controls being positive for aPS/PT IgM (OR, 1.3; 95% CI, 0.6–1.8). None of cases or controls were positive for all 3 aPL subtypes. The presence of anti-β2-GPI antibodies, aPS/PT, or anticardiolipin antibodies next to LA did not increase the risk of stroke compared with the risk of only LA (data not shown).

LA positivity has been associated with increased concentrations of inflammatory markers.13,14 In the Hai population, the 23 LA-positive cases had a significantly higher median hs-CRP concentration (9.3 µg/mL; IQR, 3.8–25.2 µg/mL) than the 79 LA-negative cases (5.1 µg/mL; 1.4–12.6 µg/mL; P=0.03). In contrast, median hs-CRP between the 7 LA-positive and 49 LA-negative cases in Dar-es-Salaam area were similar (3.0 µg/mL; IQR, 2.2–45.6 µg/mL versus 2.8 µg/mL; IQR 0.7–9.7 µg/mL; P=0.4).

**Discussion**

The current findings from a large community-based, case–control study with prospective case ascertainment identify LA as an important and independent stroke risk factor in this setting. The incident cases of stroke in this study should be broadly representative of people with stroke in Tanzania who survive long enough to give a blood sample.2,4 Overall, 19% of the stroke cases had a positive LA compared with 1% in controls. The association of LA with stroke was highest in individuals aged ≤65 years. Most of the cases had an isolated LA positivity, that is, they did not have concurrent anticardiolipin, anti-β2-GPI IgG, or aPS/PT antibodies, and the latter 3 antibodies were not associated with an increased stroke risk. In contrast, presence of anti-β2-GPI IgM was associated with protection...
against stroke. High anti-β2-GPI IgG titers were common in both cases and controls in this study population. When cutoff values of a Dutch control population were applied instead of the Tanzanian controls, 67% of the entire study population would be classified as being positive for anti-β2-GPI IgG.

Our current finding of a strong association of LA positivity and stroke risk is consistent with previous studies showing a high prevalence of aPL in patients with stroke and especially in young adults. In a systematic review conducted by Antiphospholipid Syndrome Alliance For Clinical Trials and International Networking, the estimated frequency of aPL in stroke patients of all ages was 13.5%. Another recent systematic review calculated a 17.4% prevalence of aPL in patients with cerebrovascular events aged <50 years. Most LA-positive individuals in our current study had an isolated LA, and this proportion was higher than that reported in other studies. For example, Fabris et al found that 32% of 41 LA-positive individuals had concurrent anticardiolipin or anti-β2-GPI antibodies and 56% had concurrent aPS/PT. LA comprises a heterogeneous group of antibodies reacting with phospholipids, and the exact nature of the inhibitors responsible for the isolated LA in the participants in our study remains unknown. About the pathogenic nature of an isolated LA activity, previous findings by our group and our current findings lend further support that LA assays are by far superior in detecting pathological subpopulations of aPL antibodies. In women presenting with a first stroke under the age of 50 years, we previously showed that a positive LA was much more strongly associated with stroke (OR, 43.1; 95% CI, 12.2–152.0) compared with anti-β2-GPI (OR, 2.3; 95% CI, 1.4–3.7), whereas no association was found for anti-2-GPI (OR, 2.3; 95% CI, 1.4–3.7), whereas no association was found for anti-cardiolipin and antiprothrombin antibodies. Most of these women had an isolated LA. A recent prospective observational study in 151 LA-positive individuals also found LA positivity to be associated with increased mortality, independent of concomitant positivity for anticardiolipin or anti-β2-GPI. In contrast, Pengo et al recently reported that the risk of thromboembolic events was low in a prospective cohort of individuals with an isolated LA, suggesting that the risk associated with an isolated LA is dependent on the population studied.

Nearly all studies on aPL antibodies and stroke risk have been performed in high-income countries. To the best of our knowledge, there are no studies from SSA. Our current study suggests that LA may be one of the leading risk factors for stroke in SSA next to traditional stroke risk factors such as diabetes mellitus, smoking, hypertension, and dyslipidemia. We also found a positive TP-IgG to be common among stroke cases (24.7%) and to be significantly associated with stroke risk. TP-IgG was determined to correct for the fact that positive anticardiolipin antibodies are frequently found in syphilis, and these antibodies do not differentiate between past or current syphilis. Syphilis and especially meningovascular neurosyphilis is a well-recognized cause of stroke in young patients. Our results should be interpreted with caution, because no additional diagnostic tests for (neuro)syphilis were performed, including nonreponenmal tests or cerebrospinal fluid examination. Still, our data suggest that syphilis testing should be considered part of the work-up of stroke in SSA.

Apart from syphilis, LA and other aPL subpopulations may arise in the course of other infections, including those common in SSA such as HIV, mycobacterial infections, syphilis, malaria, and viral hepatitis. The observation that the prevalence of LA was higher in cases from rural Hai region may be explained by a higher infectious burden in this area, whereas traditional stroke risk factors may be more important in urban Dar-es-Salaam. A systematic screening for infections, including tuberculosus and viral hepatitis, was beyond the scope of this study. Screening for HIV was performed in this cohort previously and HIV infection turned out to be a major stroke risk factor with a prevalence of 24.5% in cases and 6.5% in controls. All participants were naive for antiretroviral therapy. Because HIV testing was performed anonymously, results could not be linked to results of aPL antibodies.

Infection-associated aPL usually interact solely with phospholipids such as cardiolipin and have no anti-β2-GPI activity. They are usually not associated with thrombosis or other pathology. An ever-increasing number of infectious diseases has also been related with the presence of anti-β2-GPI antibodies. As recently reviewed, many healthy individuals have low circulating titers of low-affinity anti-β2-GPI antibodies. These antibodies may belong to the natural antibody repertoire and play a role in the removal of apoptotic bodies and host defense against infections. Infections are able to induce low-titer and low-affinity natural antibodies to full-blown autoantibodies, and even though the precise mechanisms responsible for the transition from natural occurring to pathological autoantibodies are currently unknown, it is conceivable that this may occur at a higher rate in patients with chronic or recurrent infections. In this respect, our finding that 67% of the study population had increased anti-β2-GPI IgG titers when Dutch cutoff values were applied is of particular interest. Genetic factors may play a role in this high prevalence, as ethnic differences in reference values of different thrombophilia markers, including aPL, have been described earlier. It is, however, tempting to speculate that the high burden of acute and chronic infections, including malaria, may also account for the high anti-β2-GPI IgG titers in this Tanzanian population.

We cannot exclude with certainty that stroke itself may induce LA and other aPL. Stroke is associated with inflammation, and earlier studies that evaluated the time course of CRP have found an increase in CRP after acute stroke. This might explain why the Hai cases, in whom blood was collected earlier after stroke, had a higher hs-CRP level than the Dar-es-Salaam cases. Nonetheless, LA-positive cases also had a higher hs-CRP level compared with the LA-negative cases. Elevated levels of CRP may interfere with LA testing, but this does not occur with use of the dilute Russell Viper Venom Time system, as was used in our study. LA itself may also lead to inflammation through activation of the endothelium and upregulation of the expression of tissue factor and proinflammatory cytokines. This is supported by previous studies reporting an association of isolated LA with increased levels of inflammatory markers. Our study also reported for the first time a protective effect of anti-β2-GPI IgM in stroke, and this fits the same concept of naturally occurring autoantibodies having a protective effect.
In line with this concept is the observation that presence of anti-β2-GPI IgM protected against lupus nephritis in a large cohort of patients with systemic lupus erythematosus.31

Particular strengths of our study are its prospective design, the fact that both functional (LA) and serological assays (anti-β2-GPI, anticardiolipin, and aPS/PT) were performed, and the availability of adequate control groups, which is essential for calculation of risk estimations. However, different limitations should also be acknowledged. First, blood samples for the current analyses were only available in 158 out of 200 stroke cases and 369 out of 398 controls from the originally described cohort.4 Nonetheless, we have no reason to think that this resulted in systematic bias. Second, the low number of LA-positive controls hampered adjustment for multiple traditional stroke risk factors in one model. Adjustment of single risk factors had no effect on the risk ratio, which is not surprising because diabetes mellitus, smoking, dyslipidemia, and hypertension are not expected to influence the risk for aPL.

Third, we were not able to differentiate between past and active syphilis because only a TP-IgG was available. Whether participants had been treated for syphilis was not specifically asked, but none self-reported that they had. Fourth, only a single sample was available, and LA positivity could therefore not be confirmed after 12 weeks in a second sample as suggested in the International Society on Thrombosis and Haemostasis guidelines.9 This also impedes classifying the proportion of participants having true antiphospholipid syndrome, as this diagnosis requires the persistent presence of antiphospholipid antibodies together with clinical criteria of vascular occlusion or pregnancy morbidity.34 Finally, our study does not allow us to draw definite conclusions on the contribution of aPL in stroke pathogenesis. This would require large, prospective cohort studies, which will be difficult to execute in these areas. A wealth of evidence from basic, animal, and clinical studies has highlighted the prothrombotic effects of aPL, and in our opinion, this makes it unlikely that aPL would merely be a bystander in our stroke cohort.

Conclusions

This study identified LA as a major stroke risk factor in SSA. The pathogenic role of aPL in stroke in this area and contributing factors to the high aPL prevalence in SSA deserve further study.

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Disclosures

None.

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