

## **Association between serum secretory phospholipase A2 and risk of ischemic stroke**

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## **Abstract**

**Background and Purpose:** Previous literature has demonstrated an association between high serum levels of type-II secretory phospholipase A2 (sPLA2) concentration and an increased risk of coronary artery disease. However, such association has not been established in terms of ischemic stroke risk. We aimed to evaluate the association between both sPLA2 concentration and activity as continuous variables with risk of future ischemic stroke.

**Methods:** We conducted a nested case-control study using data from the European Prospective Investigation into Cancer - Norfolk study. Cases (n=145) in the current study were participants who developed ischemic stroke during follow-up, with controls (n=290) matched in a 2:1 ratio based on age and sex. Statistical analyses were performed using SPSS (version 25.0, Chicago, Ill) software. Logistic regression was used to determine odds ratios (OR) and corresponding 95% confidence intervals (95% CIs) for ischemic stroke.

**Results:** After adjusting for a wide array of cardiovascular confounders, sPLA2 activity was found to be associated with an increased risk of ischemic stroke using both multiple imputations with chained equations and complete case analysis: OR 1.20 (95% CI: 1.01-1.43) and OR 1.23 (95% CI: 1.01 -1.49), respectively. However, sPLA2 concentration was not found to be associated with increased risk of ischemic stroke.

**Conclusions:** The activity of sPLA2, but not sPLA2 concentration, is associated with an increased risk of future ischemic stroke. This finding may be significant in risk group stratification, allowing targeted prophylactic treatment, or the development of novel therapeutic agents.

## Introduction

Stroke is a major cause of morbidity and mortality and is currently the 5<sup>th</sup> leading cause of death in the United States.<sup>1</sup> Better understanding of the pathophysiological processes that increase the risk of stroke may improve prediction, allowing targeted prophylactic treatment, or the development of novel therapeutic agents.

The phospholipase A<sub>2</sub> (PLA<sub>2</sub>) family are a group of intracellular and secreted enzymes that hydrolyse the acyl bond at the sn-2 position of phospholipids composing cell membranes and lipoproteins.<sup>2,3</sup> This hydrolysis reaction produces non-esterified fatty acids (NEFAs) and lysophospholipids, which can act as precursors for proinflammatory mediators such as leukotrienes, eicosanoids, and prostaglandins.<sup>3,4</sup> The secreted PLA<sub>2</sub> (sPLA<sub>2</sub>) enzymes represent more than a third of this family, and currently only the sPLA<sub>2</sub>-IIA isoform is detectable in the circulation with a commercially available ELISA.<sup>3,5</sup>

It is postulated that sPLA<sub>2</sub> may promote atherosclerosis through several factors. By decreasing the number of phospholipids in the bilayer of low-density lipoprotein (LDL), it thereby exposes more proteoglycan binding sites. This leads to accumulation of LDL within the intima of arteries, allowing uptake by macrophages to produce foam cells.<sup>4,6-8</sup> The conformational changes in lipoproteins are also thought to make them more prone to oxidation in the intima and subsequently increases their uptake by macrophages.<sup>4</sup> Another factor is the production of pro-inflammatory mediators from sPLA<sub>2</sub> mediated hydrolysis, as it is known that atherosclerosis is an inflammatory disease that begins with the accumulation of LDL within the intima of arteries.<sup>4,9,10</sup>

Previous prospective studies have found an association between high sPLA<sub>2</sub> concentration and activity and an increased risk of coronary artery disease (CAD).<sup>11,12</sup> Given that atherosclerosis is also an important contributor to the pathophysiology of ischemic

stroke, we explored whether there is an association between sPLA2 concentration and/or activity and incident ischemic stroke.

## **Methods**

We conducted a nested case-control study using data from the European Prospective Investigation into Cancer-Norfolk (EPIC-Norfolk) study, which has previously been discussed in detail.<sup>13</sup> In brief, it was a prospective study consisting of 25,663 members of the general population from Norfolk, UK, aged 45 – 79. The participants in this study were drawn from a previous EPIC-Norfolk CAD nested case-control study, in which both cases and controls were selected from participants of the EPIC-Norfolk study.<sup>11</sup>

Cases (n=1105) in the EPIC-Norfolk CAD study were identified as either having had a hospital admission, or died, due to CAD during follow-up until November 2003. Controls (n=2209) were matched by sex, age (within five years), and time of enrolment (within three months) in an attempted 2:1 ratio. Participants with a history of heart attack or stroke at baseline clinic visit were excluded.

From the participants of the EPIC-Norfolk CAD study, we identified 145 ischemic stroke cases from either death certificates or hospital discharge records during an extended follow-up until March 2016. Controls (n=290) were then matched from the remaining participants in a 2:1 ratio based on age and sex, excluding participants who developed CAD or haemorrhagic stroke during follow-up.

Blood samples were obtained at baseline clinic visit between 1993 and 1997 using venepuncture and expressed into plain and citrate bottles. Blood samples were processed for assay at the Department of Clinical Biochemistry, University of Cambridge, or stored at -80°C. Serum concentrations of sPLA2 were measured with a sandwich-type enzyme-linked

immunosorbent assay and serum sPLA2 activity was measured by a selective fluorometric assay, as previously described.<sup>14,15</sup> Data-linkage to the East Norfolk Health Authority Database enabled the identification of patients who developed ischemic stroke during follow-up. Ischemic stroke was identified using codes I63, I65, and I66 according to the 10<sup>th</sup> edition of the International Classification of Disease.

The odds of ischemic stroke was calculated using logistic regression to determine odds ratios (ORs) and corresponding 95% confidence intervals (95% CIs) based on both sPLA2 concentration and activity as continuous variables. Odds ratios were adjusted for: age, sex, body mass index (BMI), smoking status, diabetes, systolic blood pressure, diastolic blood pressure, high density lipoprotein (HDL), LDL, and either sPLA2 activity or concentration. Analysis was performed using both multiple imputation using chained equations (MICE) and complete case analysis to increase the statistical power.

Statistical analyses were performed using SPSS (version 25.0, Chicago, Ill) software.<sup>13</sup> Baseline characteristics of ischemic stroke cases and matched controls were compared. The Chi-squared test was used for categorical variables and either the 2-sample t-test or the Mann Whitney U test for parametric or non-parametric continuous variables, respectively. Both sPLA2 concentration and activity, as well as HDL, LDL, and age were found to have skewed distributions and were recorded in the tables as median values with corresponding interquartile ranges (IQR). The normally distributed variables were recorded in the tables as mean values with associated standard deviation (SD). The categorical variables were recorded as total number with associated percentage.

## Results

The sample population consisted of 435 patients with median age 71.2 (IQR 66.6 – 73.7) years; 42.5% female. This included 145 patients who developed ischemic stroke over the follow-up period (1993 – 2016), with the remainder being matched controls. The range of time in days from participant inclusion in the study to incident ischemic stroke was 576 – 8140; mean 4141 (SD  $\pm$  1496). No patients were lost to follow-up during the study.

Table 1 presents the baseline characteristics of ischemic stroke cases and matched controls. It was found that individuals who smoke and have diabetes were more likely to have an ischemic stroke in the future. Systolic blood pressure, diastolic blood pressure, and LDL were also significantly higher in cases compared to controls. Furthermore, sPLA2 activity levels were found to be significantly higher in cases than controls (4.6 nmol/min/mL; IQR 4.1 - 5.4 versus 4.3 nmol/min/mL; IQR 3.7 - 4.9;  $p = 0.001$ ). However, differences in sPLA2 concentration were not found to be statistically significant.

Table 2 presents the odds of ischemic stroke by sPLA2 concentration and sPLA2 activity analysed by logistic regression as continuous variables with associated 95% CIs using MICE. After adjusting for all confounding factors, sPLA2 concentration was found not to be associated with an increased risk of ischemic stroke: OR 1.02 (95% CI 0.99 - 1.04). However, sPLA2 activity was found to be associated with an increased risk: OR 1.20 (95% CI 1.01 - 1.43).

Table 3 presents the odds of ischemic stroke by sPLA2 concentration and sPLA2 activity analysed by logistic regression as continuous variables with associated 95% CIs using complete case analysis. After adjusting for all confounding factors, sPLA2 concentration was again found not to be associated with an increased risk of ischemic stroke:

OR 1.02 (95% CI 0.99 - 1.04). However, sPLA2 activity was found to be associated with an increased risk: OR 1.23 (95% CI 1.01 - 1.49).

## **Discussion**

This nested case-control study has shown that sPLA2 activity, but not concentration, is associated with an increased risk of ischemic stroke. To the best of our knowledge, no previous studies have assessed the association between sPLA2 and risk of ischemic stroke. However, previous studies have assessed the association between ischemic stroke and a similar marker: lipoprotein-associated phospholipase A2 (Lp-PLA2), which also belongs to the A2 phospholipases family of enzymes.

In a collaborative analysis of 32 prospective studies, Lp-PLA2 concentration was shown to be associated with increased risk of developing both ischemic stroke and CAD.<sup>16,17</sup> Furthermore, patients in the highest quartile of Lp-PLA2 activity following a first episode of ischemic stroke have been found to be at an increased risk of recurrence.<sup>18-20</sup>

Other inflammatory biomarkers apart from the A2 family of phospholipases have been studied and shown to predict ischemic stroke. A prospective study has found that higher leukocyte levels are associated with an increased risk of having an ischemic stroke.<sup>21</sup> Elevated CRP levels have also been shown to be associated with both an increased risk of developing cardiovascular disease, including ischemic stroke, and an increased risk of ischemic stroke recurrence after a first incident.<sup>22-26</sup>

The secretion of sPLA2 is stimulated by pro-inflammatory mediators, such as tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6.<sup>2,27</sup> Findings have shown that the majority of sPLA2 is secreted from hepatocytes and arterial smooth muscle, with expression modulated by systemic inflammation.<sup>2</sup>

The mechanism by which sPLA2 promotes atherosclerosis has three key components. First, the hydrolysis of LDL by sPLA2 in the plasma or tunica intima changes the configuration of apolipoprotein B molecules, resulting in the modified-LDL having an increased affinity for proteoglycans of the tunica intima extracellular matrix. This results in LDL retention – a key component in atherogenesis.<sup>2,7</sup> Secondly, sPLA2 hydrolysis releases NEFAs and lysophospholipids. NEFAs induce the expression of proteoglycans from the tunica media and therefore a vicious circle ensues, with high NEFA levels from modified-LDL retention resulting in more proteoglycan production and, therefore, retention capacity.<sup>2,28</sup> Thirdly, modified-LDL becomes more susceptible to oxidation.<sup>29</sup> Oxidised LDL stimulates macrophage growth, resulting in increased macrophage take-up of oxidised LDL, creating the foam cells which are characteristic of early atherosclerotic lesions.<sup>30</sup> The action of sPLA2 also results in an increase in bioactive phospholipids which increase monocyte-endothelial binding affinity and thereby facilitates the accumulation of macrophages in the lesion.<sup>31</sup>

Based on the association between sPLA2 and CAD, the VISTA-16 trial was carried out to investigate the effect of the non-specific sPLA2 inhibitor varespladib in patients with acute coronary syndrome (ACS). Surprisingly, it was found that varespladib did not reduce the risk of further cardiovascular events in patients with ACS and was in fact shown to increase the risk of myocardial infarction, resulting in early termination of the trial. Of interest, this trial also showed that varespladib did not have a statistically significant effect on the risk of developing stroke.<sup>32</sup>

The measurement of sPLA2 activity encompasses three different isoforms: sPLA2-IIA, sPLA2-V, and sPLA2-X, whereas sPLA2 concentration only measures the level of the sPLA2-IIA isoform in the serum.<sup>4</sup> Atherosclerotic plaques have been found to contain all three of these isoforms, which are each thought to play distinct roles in the process of

atherosclerosis.<sup>4</sup> This may explain our finding of sPLA2 activity, but not concentration, being associated with increased risk of ischemic stroke. This hypothesis is supported by a study which shows that sPLA2 activity is a better predictor than concentration of risk of developing CAD in healthy individuals.<sup>12</sup>

Despite the decline in stroke mortality from 2008 - 2013, it remains the fifth leading cause of death in the United States as of 2017,<sup>1,33</sup> with substantial economic cost associated with stroke survival.<sup>34</sup> A greater understanding of enzymatic pathways that are associated with increases in the risk of future stroke may contribute to a better understanding of pathophysiological processes that can be targeted by novel therapeutic agents.

The current study had some limitations. The participants were drawn from an EPIC-Norfolk CAD case-control study and hence the prevalence of cardiovascular risk factors in both cases and controls may be higher than in the general population, thus attenuating the effect size observed. As an observational study, the possibility of residual confounding cannot be excluded and causal inferences cannot be made based on the findings from the current study. Furthermore, the confounding effect of medications that can influence sPLA2 were not adjusted for. Finally, the current study was unable to stratify analysis by sub-type of ischemic stroke. Due to the differences in pathophysiology underlying these, it is possible that sPLA2 activity is predictive of large artery atherosclerosis, but not other sub-types, such as cardioembolic.

The current study also had several strengths. Firstly, we included data collected from a prospective study. Secondly, we adjusted for many major cardiovascular confounders to determine as accurately as possible if sPLA2 is an independent biomarker. Thirdly, only a small proportion of data was missing (< 5%), and both MICE and complete case analysis

were performed with corroborating results, thereby maximising the validity of our statistical methodology.

## **Conclusions**

The activity of sPLA2 as a continuous variable was found to be associated with an increased risk of future ischemic stroke, independent of traditional cardiovascular risk factors and sPLA2 concentration. However, the large heterogeneity between different sub-types of ischemic stroke may attenuate the observed relationship in this study. Future studies evaluating the association between sPLA2 and ischemic stroke risk may benefit by stratifying analysis by pathophysiology of ischemic stroke sub-type.

Further research with a larger sample size is required to establish a normal range for the activity of sPLA2. This would allow subsequent risk group stratification of patients and the potential for targeted prophylactic treatment.

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### **Disclosures**

None.

### **Consent Statement**

All participants gave written informed consent.

### **Approval Statement**

Ethical approval was obtained from the Norwich District Health Authority Ethics Committee.

### **Data Availability Statement**

Data requests can be submitted to the EPIC-Norfolk Steering Committee.

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**Table 1.** Baseline Characteristics of Ischemic Stroke Cases and Matched Controls in EPIC-Norfolk

Variables	Controls	Cases	P-Value	Total
Age (years)	71.2 IQR 67.0 – 73.4	71.1 IQR 66.5 – 73.3	0.750	71.2 IQR 66.6 – 73.7
Sex			0.373	
Male	171 (68.4)	79 (31.6)		250 (57.5)
Female	119 (64.3)	66 (35.7)		185 (42.5)
Body Mass Index (kg/m <sup>2</sup> )	26.5 (± 3.5)	26.4 (± 3.4)	0.499	26.5 (± 3.5)
Smoking Status			<0.001	
Never	115 (74.2)	40 (25.8)		155 (35.6)
Previous	163 (65.5)	86 (34.5)		249 (57.2)
Current	12 (38.7)	19 (61.3)		31 (7.1)
Diabetes			0.005	
Yes	3 (27.3)	8 (72.7)		11 (2.5)
No	287 (67.7)	137 (32.3)		424 (97.5)
Systolic Blood Pressure (mmHg)	140.6 ± 16.3	147.6 ± 17.0	<0.001	142.9 ± 16.9
Diastolic Blood Pressure (mmHg)	83.1 ± 10.7	85.8 ± 10.2	0.008	84.1 ± 10.6
HDL (mmol/L)	1.3 IQR 1.0 – 1.6	1.3 IQR 1.0 – 1.6	0.353	1.3 IQR 1.0 – 1.6

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LDL (mmol/L)	4.0 IQR 3.4 – 4.7	4.3 IQR 3.6 – 5.0	0.005	4.1 IQR 3.5 – 4.7
sPLA2 (ng/mL)	8.9 IQR 6.1 – 14.7	9.5 IQR 6.7 – 15.6	0.109	9.0 IQR 6.3 – 14.8
sPLA2 activity (nmol/min/mL)	4.3 IQR 3.7 – 4.9	4.6 IQR 4.1 – 5.4	0.001	4.4 IQR 3.8 – 5.0

Data are presented as mean ( $\pm$  SD), for normally distributed continuous variables; n (%), for categorical variables; median (IQR), for continuous variables with a skewed distribution.

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; sPLA2, type II secretory phospholipase A2.

**Table 2.** Odds of ischemic stroke by sPLA2 concentration and sPLA2 activity as continuous variables with 95% confidence intervals (analysis using multiple imputation with chained equations)

	sPLA2 Concentration	sPLA2 Activity
Unadjusted	1.02 (0.99 – 1.04)	1.31 (1.11 – 1.55)
Model 1	1.01 (0.99 – 1.04)	1.31 (1.10 – 1.54)
Model 2	1.02 (0.99 – 1.04)	1.24 (1.05 – 1.47)
Model 3	1.02 (0.99 – 1.04)	1.20 (1.01 – 1.43)

Model 1 adjusted for: Age, Sex. Model 2 adjusted for: Model 1 + BMI, Smoking Status, Diabetes, SBP, DBP. Model 3 adjusted for: Model 2 + HDL, LDL.

**Table 3.** Odds of ischemic stroke by sPLA2 concentration and sPLA2 activity as continuous variables with 95% confidence intervals (complete-case analysis)

	sPLA2 Concentration	sPLA2 Activity
Unadjusted	1.02 (0.99 – 1.04)	1.32 (1.12 – 1.56)
Model 1	1.01 (0.99 – 1.04)	1.31 (1.11 – 1.55)
Model 2	1.02 (0.99 – 1.04)	1.21 (1.01 – 1.44)
Model 3	1.02 (0.99 – 1.04)	1.23 (1.01 – 1.49)

Model 1 adjusted for: Age, Sex. Model 2 adjusted for: Model 1 + BMI, Smoking Status, Diabetes, SBP, DBP. Model 3 adjusted for: Model 2 + HDL, LDL, sPLA2 Concentration or sPLA2 Activity. Model 3 contained 393 cases; 42 excluded due to missing data.