Enzyme Immunoassay for the Quantitative Determination of Lp-PLA₂ in Human Plasma and Serum

FOR IN VITRO DIAGNOSTIC USE [IVD]
STORE ALL TEST COMPONENTS AT 2 TO 8°C

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INTENDED USE

The diaDexus PLAC® test is an enzyme immunoassay for the quantitative determination of Lp-PLA₂ (lipoprotein-associated phospholipase A₂) in human plasma and serum, to be used in conjunction with clinical evaluation and patient risk assessment as an aid in predicting risk for coronary heart disease, and ischemic stroke associated with atherosclerosis.

SUMMARY AND EXPLANATION

Lp-PLA₂ is a calcium-independent serine lipase that is associated with low-density lipoprotein (LDL) in human plasma and serum [1] and is distinct from other phospholipases such as cPLA₂ and sPLA₂ [2]. Lp-PLA₂ is produced by macrophages and is expressed in greater concentrations in atherosclerotic lesions [3]. Several lines of evidence suggest that oxidation of LDL plays a critical step in the development and progression of atherosclerosis [4,5]. Lp-PLA₂ participates in the oxidative modification of LDL by hydrolyzing oxidized phosphatidylcholines, generating lysophosphatidylcholine and oxidized free fatty acids, both of which are potent proinflammatory products that contribute to the formation of atherosclerotic plaques [6,7,8].

The diaDexus PLAC test is a sandwich enzyme immunoassay that uses two highly specific monoclonal antibodies for the direct measurement of Lp-PLA₂ concentration in human plasma and serum [9]. Elevated levels of Lp-PLA₂, as measured by immunoassay, were found in patients with angiographically proven coronary heart disease (CHD), when compared to age matched controls [1]. Gender-specific clinical studies regarding the association of Lp-PLA₂ and the risk of cardiovascular events have been limited [10, 11], warranting additional research in this area. In a retrospective case-control study, using samples from hypercholesterolemic men (n=6595) in the West of Scotland Coronary Prevention Study (WOSCOPS), a 2-fold greater risk of coronary heart disease was observed for subjects in the highest quintile of Lp-PLA₂ levels, compared to the lowest quintile. Furthermore, the CHD risk association of Lp-PLA₂ was shown to be independent of LDL and other markers of inflammation: C-reactive protein, white cell count and fibrinogen. The authors of the study stated in their conclusions, “Elevated levels of lipoprotein-associated phospholipase A₂ appear to be a strong risk factor for coronary heart disease, a finding that has implications for atherogenesis and the assessment of risk” [10]. In another report, using samples from the Atherosclerosis Risk in Communities (ARIC) study, which followed 12,819 apparently healthy middle-aged (45-64 years) men and women for six to eight years, Lp-PLA₂ was found to be an important predictor of CHD risk. For individuals with LDL less than 130 mg/dL, Lp-PLA₂ was significantly and independently associated with a 2-fold higher risk for CHD events including myocardial infarction, revascularization, and death from cardiac disease [12].
The ARIC study was re-analyzed to determine the risk of stroke associated with increased levels of Lp-PLA₂. A total of 223 stroke events were identified from the study group; of this, 194 (87%) were ischemic stroke associated with atherosclerosis, as classified by the ARIC investigators. This proportion of ischemic stroke to the total is consistent with the percentage found in the general population [13]. The results of this study indicated that Lp-PLA₂ was a strong predictor of these strokes, with an increased risk of nearly 2-fold, even after adjustment for blood pressure, lipids, diabetes, body mass index, and other inflammatory markers [14].

**PRINCIPLE OF THE TEST**

The diaDexus PLAC test is based on the principle of a sandwich enzyme immunoassay using two specific monoclonal antibodies described by Caslake et al. [1]. The assay system utilizes monoclonal anti-Lp-PLA₂ antibody (2C10) directed against Lp-PLA₂ for solid phase immobilization on the microwell strips. Assay Buffer is added to wet the wells of the plate. Sample is added to the plate and incubated for 120 minutes at 20-26 °C. The wells are washed with distilled water to remove any unbound antigen. A second monoclonal anti-Lp-PLA₂ antibody (4B4) labeled with the enzyme horseradish peroxidase (HRP) is then added and reacted with the immobilized antigen at 20-26 °C for 60 minutes, resulting in the Lp-PLA₂ molecules being captured between the solid phase and the enzyme-labeled antibodies. The wells are washed with distilled water to remove unbound labeled antibodies. The substrate, tetramethylbenzidine (TMB), is then added and incubated at 20-26 °C for 20 minutes, resulting in the development of a blue color. Color development is stopped with the addition of Stop Solution, changing the color to yellow. The absorbance of the enzymatic turnover of the substrate is determined spectrophotometrically at 450 nm and is directly proportional to the concentration of Lp-PLA₂ present. A set of Lp-PLA₂ Calibrators is used to plot a standard curve of absorbance versus Lp-PLA₂ concentration from which the Lp-PLA₂ concentration in the test sample can be determined.

**REAGENTS AND MATERIALS**

Materials supplied with the kit:
(Sufficient for 96 wells)

- **Antibody Coated Strips**  
  Mouse monoclonal anti-Lp-PLA₂ (2C10) antibody coated microwell strips

- **Calibrators 1-6**  
  (0, 50, 100, 250, 500, and 1000 ng/mL)  
  diaDexus recombinant Lp-PLA₂ antigen (DDX-RA) in a protein stabilizing diluent

- **Assay Buffer** 23 mL, Blue color  
  Buffered reagent with bovine serum albumin and mouse serum

- **Conjugate** 23 mL, Red color  
  Mouse monoclonal anti-Lp-PLA₂ (4B4) antibody conjugated to horseradish peroxidase in a buffered reagent with carrier proteins (fetal calf serum and mouse IgG)

- **TMB Reagent** 11 mL  
  3,3',5,5'-tetramethylbenzidine in a mildly acidic buffer

- **Stop Solution** 11 mL  
  1N HCl
Materials required but not provided:

• Precision single and multi-channel pipettors: 0.02, 0.10, 0.20 mL
• Disposable pipette tips (A new pipette tip must be used for each addition of different samples or reagents during the assay procedure.)
• Distilled water
• Vortex mixer or equivalent
• Plate shaker capable of running at 600 rpm
• A microwell plate reader with a bandwidth of 10 nm or less and an optical density (O.D.) range of 3 or greater at 450 nm
• Computer software capable of point-to-point curve fit for calculating concentration of analyte from optical density (optional)
• Controls (available separately, contact Technical Support)

WARNINGS AND PRECAUTIONS

• For In Vitro Diagnostic Use.
• Treat all blood samples as potentially biohazardous material.
• TMB reagent contains 3,3',5,5'-tetramethylbenzidine (TMB). If exposed to skin, immediately flush area with soap and water. If eyes are exposed, flush with copious amounts of water and seek immediate medical attention.
• Stop Solution contains 1N HCl. If exposed to skin, immediately flush area with soap and water. If eyes are exposed, flush with copious amounts of water and seek immediate medical attention.
• Dispose of test kits and reagents in a manner consistent with relevant regulations.
• Do not use reagents past their expiration dates.
• Do not mix components from other lots of diaDexus PLAC test kits.
• Controls are not provided with the kit but can be purchased separately. It is recommended that the “high” and “low” controls be included in each patient run. If control values are not within acceptance limits, repeat the assay. Additional quality control testing may be necessary according to state and local requirements.

REAGENT PREPARATION AND STORAGE

Store unopened test kits at 2-8 °C upon receipt. In addition, keep the microwell strips sealed in the foil pouch with desiccant to minimize exposure to moisture. Opened test kits will remain stable until the expiration date shown, provided they are stored as described above.

SPECIMEN COLLECTION AND STORAGE

• Collect blood samples in EDTA or heparin plasma collection tubes or any serum collection tubes, including gel separation tubes.
• Prepare all samples by removing or separating serum or plasma from red blood cells using standard separation procedures.
• Store all samples refrigerated (2-8 °C) immediately upon collection; however, Lp-PLA₂ in samples is stable for up to twelve hours total at room temperature (20-26 °C).
• Centrifuge and separate samples within four hours of venipuncture; however, the analyte remains stable when separation is delayed up to seven days following collection.
• Test plasma or serum within seven days of collection when stored at 2-8 °C.
• For longer storage of up to three months prior to analysis, freeze processed samples stored in an aliquot tube at or below –20 °C.
• Processed plasma or serum samples may be frozen and thawed up to six times without affecting the Lp-PLA₂ quantitation.
• When transporting samples, ship samples at 2-8 °C on ice packs.
ASSAY PROCEDURE

Preparatory Steps

1. Allow patient samples and controls to thaw, and place on ice or at 2-8 °C as soon as thawed.
2. Bring Assay Buffer, Conjugate and TMB to room temperature (20-26 °C) before use.
3. Vortex the samples and controls to mix thoroughly. Avoid foaming.
4. Remove the stripwell frame and required number of coated microwell strips from the foil pouch after bringing the plate to room temperature. It is recommended that each Calibrator and Control be run in duplicate. Ensure that the foil pouch containing any unused strips is completely resealed with the desiccant that came in the pouch and is stored at 2-8 °C.

Sample Incubation

1. Pipette 200 µL of room temperature Assay Buffer into the appropriate wells of the coated microwell strip.
2. Using a pipettor and tip with appropriate low volume precision, dispense 10 µL of Calibrators, samples, and controls into the appropriate wells after vortexing. It is recommended that Calibrators and Controls be run in duplicate. Use a calibrated pipette and new pipette tip for each calibrator, control, or sample.
3. Incubate on a plate shaker set at 600 rpm for 120 minutes at room temperature.
4. At the end of the incubation period, wash the microwell wells four (4) times with at least 300 µL of distilled or deionized water. (DO NOT USE TAP WATER.)
5. Blot the plate on absorbent paper after the final wash. Immediately (in less than 2 minutes) proceed to the next step. Do not allow the microwell strip to dry.

Conjugate Incubation

1. Pipette 200 µL of room temperature Conjugate into each well.
2. Incubate the plate at room temperature for 60 minutes. Do not use the plate shaker.
3. Remove the TMB Reagent from the refrigerator and bring to room temperature before use.
4. At the end of the incubation period, wash the microwells four (4) times with at least 300 µL of distilled or deionized water. (DO NOT USE TAP WATER.)
5. Blot the plate on absorbent paper after the final wash. Immediately (in less than 2 minutes) proceed to the next step. Do not allow the microwell strip to dry.

Substrate Incubation

1. Pipette 100 µL of room temperature TMB Reagent into each well.
2. Gently swirl the plate on a flat surface for 10-15 seconds to ensure mixing.
3. Incubate the plate at room temperature for 20 minutes in the dark. Do not use the plate shaker.
4. Stop the reaction by adding 100 µL of room temperature Stop Solution to each well.
5. Gently swirl the plate on a flat surface for 20-30 seconds to ensure mixing. It is important to make sure that the blue color completely changes to yellow color.
6. Wipe moisture from the bottom of the plate using a paper towel.
7. Within 15 minutes of adding the Stop Solution, read the optical density (O.D.) at 450 nm using a microwell plate reader.

PROCEDURAL NOTES

• Store all test reagents at 2-8 °C. Except for the calibrators, allow the reagents to equilibrate to room temperature prior to use. Equilibration of a 23 mL bottle of reagent may require up to an hour to reach room temperature.
• Bring the microwell plate to room temperature before opening the bag. Store the strips in the foil pouch with desiccant to minimize exposure to moisture. Always keep the unused microwell strips in the foil pouch with desiccant.
• Always have the next step reagent ready 2–3 minutes before each washing step.
• For accurate measurement of samples, the addition of samples, calibrators, and controls must be precise. Pipette carefully using only calibrated equipment.
• This assay may be performed using any validated washing method.
• Do not use plate sealers during incubations.

CALCULATION OF RESULTS

1. Construct a standard calibration curve by plotting the mean absorbance obtained for each Calibrator on the y-axis versus the Lp-PLA₂ concentration in ng/mL on the x-axis. Use a point-to-point curve fit with appropriate computer software to construct the standard calibration curve.
2. Using the mean absorbance value for each sample and control, determine the corresponding concentration of Lp-PLA₂ in ng/mL from the calibration curve.

EXAMPLE OF CALIBRATOR CURVE

Results of a typical standard calibration curve with O.D. readings at 450 nm are shown on the y-axis against Lp-PLA₂ concentrations (ng/mL) shown on the x-axis. This calibration curve is for the purpose of illustration only. A standard calibration curve should be generated by the user for each assay performed.

<table>
<thead>
<tr>
<th>Lp-PLA₂ (ng/mL)</th>
<th>Mean Absorbance (O.D. at 450 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.048</td>
</tr>
<tr>
<td>50</td>
<td>0.287</td>
</tr>
<tr>
<td>100</td>
<td>0.507</td>
</tr>
<tr>
<td>250</td>
<td>0.944</td>
</tr>
<tr>
<td>500</td>
<td>1.409</td>
</tr>
<tr>
<td>1000</td>
<td>2.014</td>
</tr>
</tbody>
</table>
LIMITATIONS

Procedure
- Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the package insert instructions and with adherence to good laboratory practice.
- The wash procedures are critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
- As with any assay system employing mouse monoclonal antibodies, the possibility exists for interference by human anti-mouse antibodies (HAMA) present in the sample that could cause falsely elevated or depressed values.

Clinical Interpretation
- Lp-PLA₂ levels should be interpreted in conjunction with clinical findings and other diagnostic tests.
- This test does not replace blood cholesterol tests or other traditional risk factors identified for coronary heart disease or ischemic stroke.

EXPECTED VALUES

Samples from apparently healthy males (n=251) and apparently healthy females (n=174), in the clinically relevant age range of 40-70 years, were evaluated with the diaDexus PLAC test. The reference population was represented by the following ethnic backgrounds: African-American n=26, Caucasian n=390, Hispanic n=8, and not specified n=1. The distributions of Lp-PLA₂ values across the entire population and divided by gender appear in the following table:

<table>
<thead>
<tr>
<th>Percentile</th>
<th>Lp-PLA₂ ng/mL</th>
<th>All (n=425)</th>
<th>Females (n=174)</th>
<th>Males (n=251)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>126</td>
<td>120</td>
<td>131</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>174</td>
<td>169</td>
<td>179</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>201</td>
<td>188</td>
<td>205</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>235</td>
<td>228</td>
<td>244</td>
<td></td>
</tr>
<tr>
<td>67</td>
<td>262</td>
<td>252</td>
<td>268</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>289</td>
<td>285</td>
<td>293</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>369</td>
<td>342</td>
<td>376</td>
<td></td>
</tr>
</tbody>
</table>

The reference interval calculated from the samples (central 90%) was found to be 120-342 ng/mL for females and 131-376 ng/mL for males. These ranges are provided as guidelines only and are not intended to address “critical values” or medical decision limits. Each laboratory should establish its own reference intervals. Guidance for establishing reference intervals can be found in NCCLS Standard C28-A2 (How to Define and Determine Reference Intervals in the Clinical Laboratory; Approved Guideline - Second Edition). Recent published studies [10,12,15,16,17] have demonstrated an increased risk of cardiovascular disease (CVD) associated with Lp-PLA₂ values in the second and third tertiles (upper 67% of the studied populations) vs. the first tertile (lower 33% of the studied populations). Therefore, a more conservative approach for identifying individuals with a significant increased risk for CVD attributable to Lp-PLA₂ may be the 50th percentile value of the population. In the current PLAC test, this threshold value corresponds to 235 ng/mL.
PERFORMANCE CHARACTERISTICS

Sensitivity

The minimum detection limit is 1.3 ng/mL, as calculated by interpolation of the mean plus two standard deviations of 24 replicates of the 0 ng/mL Lp-PLA₂ calibrator.

Assay Precision

Intra-assay and inter-assay variability were determined by testing three human plasma pools with Lp-PLA₂ concentrations distributed throughout the calibration range of the assay. The three plasma pools were assayed, using a single lot of reagents, in duplicate, on two separate stripwells per day, for twenty days. The data are summarized below:

<table>
<thead>
<tr>
<th>Plasma Pool</th>
<th>Mean Concentration Lp-PLA₂ (ng/mL)</th>
<th>Intra-assay % CV n=80</th>
<th>Inter-assay % CV n=20</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>208</td>
<td>4.3</td>
<td>6.3</td>
</tr>
<tr>
<td>2</td>
<td>494</td>
<td>5.8</td>
<td>8.7</td>
</tr>
<tr>
<td>3</td>
<td>770</td>
<td>5.0</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Linearity

Six EDTA-plasma samples with known high Lp-PLA₂ levels were intermixed with six plasma samples with known low Lp-PLA₂ levels. Percent recovery was determined as the measured value divided by the expected value, multiplied by 100. The average recovery was 100%, demonstrating linearity of the diluted samples over a range of 90–897 ng/mL Lp-PLA₂.

Interfering Substances

Five endogenous substances found in blood were evaluated for interference in the assay. Five individual plasma samples with Lp-PLA₂ values ranging from 118-154 ng/mL were spiked with potential interferents endogenous to blood. No appreciable interference was observed at spiked levels of 500 mg/dL hemoglobin, 3000 mg/dL triglycerides, 500 mg/dL cholesterol, 20 mg/dL bilirubin, and a total of ~6 g/dL albumin.

BIOVARIABILITY

Plasma samples were collected from 43 non-fasting, apparently healthy adults. At least seven time points were taken over a 4-week period, for a total of 364 samples. Intra- and inter-individual biological variations and critical differences were determined using the entire sample set. Lp-PLA₂ demonstrated minimal biovariability, commensurate with other cardiovascular lipid markers.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lp-PLA₂ Biovariability Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-individual (%CV average)</td>
<td>14.7</td>
</tr>
<tr>
<td>Inter-individual (%CV)</td>
<td>32.8</td>
</tr>
<tr>
<td>Critical Difference (%)</td>
<td>47.9</td>
</tr>
</tbody>
</table>
CLINICAL STUDIES

Coronary Heart Disease

To determine the efficacy of the diaDexus PLAC test as a predictor of risk for coronary heart disease (CHD), Lp-PLA$_2$ levels were measured in 1348 banked EDTA-plasma samples from a large, multi-center epidemiology study, the Atherosclerosis Risk In Communities (ARIC) study, sponsored by the National Institutes of Health’s National Heart, Lung, and Blood Institute. Participants were followed for the development of CHD for six to eight years. Samples used for the PLAC test were from participants, age 47-69, who were free of CHD at the time of blood drawn. This is a case-cohort study where samples from all the CHD cases (607) were tested together with 741 appropriately matched participants without CHD at the time of censor (controls).*

Cox regression models were used to evaluate the association of Lp-PLA$_2$ and CHD in a univariate analysis (Model 1), a univariate analysis adjusted for demographics (Model 2), and a multivariate model adjusted for demographics and other prognostics factors (Model 3). Using high and low tertile cutpoints of Lp-PLA$_2$, generated from the ARIC data set (420 and 310 ng/mL, the 67$^{th}$ and 33$^{rd}$ percentiles, respectively), the hazard ratios of the Cox regression analyses demonstrated that Lp-PLA$_2$ is a significant predictor of risk for CHD, for the highest and intermediate levels when compared to the lowest level of Lp-PLA$_2$, for all participants (see Table 1). It should be noted that different cutpoints may be appropriate for different clinical populations.

* NOTE: 86 results (5.5%) were outside the assay acceptance criteria and were excluded from data analyses.

Table 1. Risk Ratios of CHD for Subjects Across All LDL Levels

<table>
<thead>
<tr>
<th>Lp-PLA$_2$ Risk Ratio (95% CI, p value)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lp-PLA$_2$ Tertile</strong></td>
</tr>
<tr>
<td><strong>#CHD cases/total subjects in category</strong></td>
</tr>
<tr>
<td><strong>Model 1</strong></td>
</tr>
<tr>
<td><strong>Model 2</strong></td>
</tr>
<tr>
<td><strong>Model 3</strong></td>
</tr>
</tbody>
</table>

*The lowest tertile with Lp-PLA$_2$ values <310 ng/mL is used as the reference group.

Model 1: univariate analysis  
Model 2: adjusted for age, race, and gender  
Model 3: Model 2, plus adjustment for current smoking status, blood pressure, diabetes, HDL, LDL, CRP, and Lp-PLA$_2$ - LDL interaction

A statistical interaction was found between Lp-PLA$_2$ and LDL. Therefore, it was appropriate to evaluate Lp-PLA$_2$ risk ratios in the high and low LDL subgroups. The median value of LDL for the cohort population was 130 mg/dL. This defined the high and low LDL subgroups. Tables 2a and 2b represent the univariate analysis of the risk ratios in the high and low LDL subgroups. The risk ratios are calculated from Cox regression employing the weighted case-cohort method with Barlow adjustment, n=1348.
Table 2a. Risk Ratios of CHD for Subjects with LDL <130 mg/dL

<table>
<thead>
<tr>
<th>Lp-PLA2†</th>
<th>Tertile 1</th>
<th>Tertile 2</th>
<th>Tertile 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk Ratio</td>
<td>1.0</td>
<td>2.17 (1.41-3.36)</td>
<td>3.52 (2.25-5.49)</td>
</tr>
<tr>
<td>#CHD cases/total subjects in category</td>
<td>51/215 (23.7%)</td>
<td>75/195 (38.5%)</td>
<td>77/163 (47.2%)</td>
</tr>
</tbody>
</table>

*The lowest tertile with Lp-PLA2 values <310 ng/mL is used as the reference group.
†Lp-PLA2 cutpoints based on the ARIC study population across all LDL levels.

Table 2b. Risk Ratios of CHD for Subjects with LDL >130 mg/dL

<table>
<thead>
<tr>
<th>Lp-PLA2†</th>
<th>Tertile 1</th>
<th>Tertile 2</th>
<th>Tertile 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk Ratio</td>
<td>3.15 (2.08-4.77)</td>
<td>3.66 (2.43-5.51)</td>
<td>5.10 (3.43-7.57)</td>
</tr>
<tr>
<td>#CHD cases/total subjects in category</td>
<td>110/234 (47.0%)</td>
<td>126/247 (51.0%)</td>
<td>169/294 (57.5%)</td>
</tr>
</tbody>
</table>

*The lowest tertile for LDL <130 subgroup, with Lp-PLA2 values <310 ng/mL, is used as the reference group.
†Lp-PLA2 cutpoints based on the ARIC study population with LDL >130 mg/dL.

In the high LDL subgroup, the subgroup specific tertile groups yielded cutpoints of 350 and 460 ng/mL; the risk ratio increased with higher Lp-PLA2 values. Therefore, for individuals with high LDL, a higher Lp-PLA2 cutpoint should be considered. Further research is warranted to evaluate the Lp-PLA2 - LDL interaction in the subgroup with high LDL. For the total population, Lp-PLA2 was a significant predictor of risk for CHD for the high and intermediate groups versus the low Lp-PLA2 (reference) group.

Ischemic Stroke

The levels of Lp-PLA2 were evaluated in the ARIC study to determine its efficacy as a predictor of risk for stroke. A total of 223 stroke events were identified from the study group; of this, 194 (87%) were ischemic stroke associated with atherosclerosis, as classified by the ARIC investigators. A similar case-cohort study was designed, where samples from all the available ischemic stroke cases (194) were tested together with 762 appropriately matched participants without CHD or stroke at the time of censor (controls).

As with the study for CHD risk, Cox regression models were used to evaluate the association of Lp-PLA2 and stroke in a univariate analysis (Model 1), a univariate analysis adjusted for demographics (Model 2), a multivariate model adjusted for demographics and other prognostic factors (Model 3), and all factors including CHD status (Model 4). The same tertile cutpoints (420 and 310 ng/mL, the 67th and 33rd percentiles, respectively) were applied to this study as for the earlier analyses. CHD status itself was found to be a predictor of risk, with a hazard ratio of 2.26 in a fully adjusted model. The hazard ratios of the Cox regression analyses demonstrated that Lp-PLA2 is a significant and independent predictor of risk for ischemic stroke for the highest tertile, when compared to the lowest tertile of Lp-PLA2, for all participants, with an increase of up to nearly 2-fold, even after adjustment for diabetes, lipids, blood pressure, smoking status, body mass index (BMI), other inflammatory markers, and CHD status (see Table 3).
Table 3. Risk Ratios of Ischemic Stroke for All Subjects

<table>
<thead>
<tr>
<th>Lp-PLA2</th>
<th>Tertile 1</th>
<th>Tertile 2</th>
<th>Tertile 3</th>
</tr>
</thead>
<tbody>
<tr>
<td># ischemic stroke cases/total subjects</td>
<td>47/283 (16.6%)</td>
<td>44/305 (14.4%)</td>
<td>103/368 (28.0%)</td>
</tr>
<tr>
<td>Model 1</td>
<td>1.0</td>
<td>0.85 (0.57-1.29, p=0.45)</td>
<td>1.79 (1.27-2.52, p=0.0010)</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.0</td>
<td>0.89 (0.59-1.35, p=0.58)</td>
<td>2.09 (1.46-3.01, p=0.0001)</td>
</tr>
<tr>
<td>Model 3</td>
<td>1.0</td>
<td>0.89 (0.58-1.36, p=0.59)</td>
<td>1.81 (1.22-2.69, p=0.0034)</td>
</tr>
<tr>
<td>Model 4</td>
<td>1.0</td>
<td>0.86 (0.56-1.31, p=0.48)</td>
<td>1.75 (1.18-2.60, p=0.0057)</td>
</tr>
</tbody>
</table>

* The lowest tertile with Lp-PLA2 values <310 ng/mL is used as the reference group.

Model 1: univariate analysis
Model 2: adjusted for age, race, and gender
Model 3: Model 2, plus adjustment for diabetes, LDL, HDL, blood pressure, smoking, BMI, and CRP
Model 4: Model 3, plus adjustment for CHD

Further analyses were performed to determine if Lp-PLA2 was predictive of ischemic stroke across the complete range of systolic blood pressure (SBP) in the population, and to determine whether blood pressure and Lp-PLA2 were additive in assessing risk for ischemic stroke. Systolic blood pressure tertile cutpoints were assigned by the 33rd and 67th percentiles of the population (113 and 130 mm Hg, respectively). The study population was divided into the low, mid, and high range (1st, 2nd, and 3rd tertile) of SBP and the low and high range of Lp-PLA2 (below and above the median, 377 ng/mL in the ARIC study). The relative risk of each group was compared to the risk of events associated with the group in the 1st tertile of SBP and the group below the median of Lp-PLA2 (Table 4).

Table 4. Risk Ratios of Ischemic Stroke: Additive Effects of Lp-PLA2 and Systolic Blood Pressure

<table>
<thead>
<tr>
<th>SBP (mm Hg)</th>
<th># ischemic stroke cases/total subjects in category</th>
<th>Below Median</th>
<th>Above Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;113</td>
<td>29/270 (10.7%)</td>
<td>1.00 (p=0.03)</td>
<td>2.29 (p=0.03)</td>
</tr>
<tr>
<td>113-130</td>
<td>60/337 (17.8%)</td>
<td>2.05 (p=0.06)</td>
<td>3.53 (p=0.0004)</td>
</tr>
<tr>
<td>&gt;130</td>
<td>105/349 (30.1%)</td>
<td>3.52 (p=0.0005)</td>
<td>6.75 (p&lt;0.0001)</td>
</tr>
</tbody>
</table>

The individuals above the median of Lp-PLA2 concentration in the ARIC study and in the top tertile of systolic blood pressure (>130 mm Hg) had a risk ratio of 6.75 (p<0.0001), compared to those individuals below the median of Lp-PLA2 and in the lowest tertile of blood pressure. These results indicate that Lp-PLA2 and blood pressure are additive in their ability to predict risk, and that individuals in the highest groups of both variables are at the greatest risk of suffering an ischemic stroke associated with atherosclerosis.
**PRODUCT SAFETY INFORMATION**

<table>
<thead>
<tr>
<th>Calibrator Set (1-6)</th>
<th>Assay Buffer/Stop Solution</th>
<th>Antibody Coated Strips/ TMB Reagent/Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xn R22 R36/38 S24/25-26-36</td>
<td>Xi R36/38 S24/25</td>
<td>S24/25-26-36</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>R22</td>
<td>Harmful if swallowed</td>
</tr>
<tr>
<td>R36</td>
<td>Irritating to eyes</td>
</tr>
<tr>
<td>R38</td>
<td>Irritating to skin</td>
</tr>
<tr>
<td>S24</td>
<td>Avoid contact with skin</td>
</tr>
<tr>
<td>S25</td>
<td>Avoid contact with eyes</td>
</tr>
<tr>
<td>S26</td>
<td>In case of contact with eyes, rinse immediately with plenty of water and seek medical advice</td>
</tr>
<tr>
<td>S36</td>
<td>Wear suitable protective clothing</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Authorized Representative</th>
<th>In vitro diagnostic medical device</th>
<th>Batch</th>
<th>Expiry Date</th>
<th>Storage conditions</th>
<th>Harmful</th>
<th>Irritant</th>
<th>Catalog Number</th>
<th>European Conformity</th>
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</thead>
<tbody>
<tr>
<td>Bevolt-nächtigung</td>
<td>In-vitro-Diagnostikum</td>
<td>Charge</td>
<td>Verfallsdatum</td>
<td>Lagerbedingungen</td>
<td>Gesundheitsschädlich</td>
<td>Reizend</td>
<td>Katalognummer</td>
<td>CE-Konformitätszeichen</td>
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<tr>
<td>Représentant agréé</td>
<td>Dispositif de diagnostic in vitro</td>
<td>Lot</td>
<td>Date de péremption</td>
<td>Conditions de stockage</td>
<td>Nocif</td>
<td>Irritant</td>
<td>Numero di catalogo</td>
<td>Conformità europea</td>
</tr>
<tr>
<td>Representante autorizado</td>
<td>Dispositivo médico para diagnóstico in vitro</td>
<td>Lote</td>
<td>Fecha de caducidad</td>
<td>Condiciones de almacenamiento</td>
<td>Perjudicial</td>
<td>Irritante</td>
<td>Número de catalogo</td>
<td>Conformidad europea</td>
</tr>
<tr>
<td>Rappresentante autorizzato</td>
<td>Dispositivo medico-diagnostico in vitro</td>
<td>Lotto</td>
<td>Data di scadenza</td>
<td>Condizioni di conservazione</td>
<td>Nocivo</td>
<td>Irritante</td>
<td>Numero de catalogo</td>
<td>Conformidad europea</td>
</tr>
</tbody>
</table>
REFERENCES


This product is covered by U.S. Patent Nos. 5532152, 5641669, 5698403, 5847088, 5968818, 5981252, and 6177257. Additional patents pending.

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