# Treatment with beta-blockers is associated with lower levels of Lp-PLA2 and suPAR in carotid plaques.

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**Title page**

# Treatment with betablockers is associated with lower levels of Lp-PLA2 and suPAR in carotid plaques.

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Text pages: 29

**Short title**

**Betablockers are associated with lower levels of Lp-PLA2 and suPAR in carotid plaques.**

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**SUMMARY**

This study could confirm the hypothesis that a long–term treatment with betablockers can decrease the expression of two potential markers of inflammation and vulnerability such Lp-PLA2 and suPAR in the carotid plaque, suggesting their possible protective role in plaque inflammation and occurrence of neurological symptoms.

Our findings support an even more selective Lp-PLA2 and suPAR inhibition as a possible strategy in the prevention of cardiovascular disease.

**ABSTRACT**

OBJECTIVES:

To determine whether a long-term treatment with betablockers influences the inflammatory activity in carotid artery disease by reducing the carotid plaque levels of lipoprotein-associated phospholipase A2 (Lp-PLA2), its enzymatic products lysophosphatidylcholine (lysoPCs), and of soluble urokinase plasminogen activator receptor (suPAR).

MATERIALS AND METHODS:

One hundred and thirty-four patients with significant symptomatic or asymptomatic carotid stenosis undergoing surgery were prospectively included and divided into two groups (group A or B) based on the absence or presence of an on-going long-term oral treatment with betablockers. The harvested carotid plaques were analysed for the levels of lysoPCs using mass spectrometry and Lp-PLA2 and suPAR by ELISA.

RESULTS:

Plaques of patients on long-term treatment with beta-blockers revealed lower levels of Lp-PLA2 (group A 0.752 ± 0.393 ug/g *vs* group B 0.644 ± 0.445 ug/g, P=.049) as well as suPAR (group A 0.044 ± 0.024 µg/g *vs* group B 0.036 ± 0.025 µg/g, P=.028).

Levels of Lp-PLA2 and suPAR were positively correlated (r= .637, P < .0001). Lp-PLA2 and suPAR levels were also correlated (P < .0001) with the three lysoPC species tested

(lysoPC 16:0, lysoPC 18:0. lysoPC 18:1). All the above-mentioned findings were confirmed after correction for age, gender, hypertension, coronary artery disease, and statin usage.

CONCLUSIONS:

The reduced levels of Lp-PLA2 and suPAR in human carotid plaques of subjects on long-term treatment with betablockers suggest their possible protective role in plaque inflammation. Our findings support an even more selective Lp-PLA2 and suPAR inhibition as a possible strategy for the prevention of cardiovascular disease.

Key words: treatment/therapies, betablockers, carotid artery stenosis, inflammation, Lp-PLA2, suPAR, lysoPC

## INTRODUCTION

Carotid endarterectomy (CEA) outcomes are influenced by multiple factors such as preoperative medical therapies.[1]

In recent decades, multiple efforts have been carried out in order to reduce the risk of atherogenesis that showed to be triggered by an underlying inflammatory process.[2,3] Among the factors initiating the inflammatory response which lead to the atherogenic process a major role has been attributed to a high sympathetic nerve beta1-activity.[4]

The laminar flow patterns produced by inhibiting the beta-adrenergic system in patients with carotid artery stenosis (CAS)[5,6] have been suggested as one of the mechanisms behind the protective effect of betablockers with respect to the development of carotid artery plaque.

The aim of this study was to investigate the influences of long-term treatment with betablockers beyond the ones attributed to their hemodynamic effect. More specifically, two potential biomarkers which have shown to play a key role in regulating the inflammatory process leading to the atherogenesis such as suPAR (soluble urokinase plasminogen activator receptor) and Lp-PLA2 (lipoprotein-associated phospholipase A2), as well as its hydrolytic products lysoPCs were analysed in human carotid plaques and related to the current use of betablockers and the development of cerebrovascular symptoms. Our hypothesis was that long-term treatment with betablockers would decrease the expression of these potential markers of inflammation and indirectly the vulnerability of the carotid plaques.

## MATERIAL AND METHODS

Patients undergoing CEA at our Vascular Department between November 2005 and June 2008 were enrolled in this prospective study after giving informed consent. The study protocol was approved by the Regional Ethical Committee. Information about comorbidities and past medical history were obtained through preoperative interviews, as well as the available medical records.

Cardiovascular risk factors such as hypertension (systolic blood pressure > 140 mmHg), diabetes, coronary artery disease, smoking, dyslipidemia, immunological disorders and body mass index (BMI) were recorded. Patients with ipsilateral carotid artery occlusion, radiation induced CAS or restenosis after previous CEA or endovascular treatment were excluded from the study cohort.

The regional medication register was analysed to identify and determine the duration of an eventual ongoing treatment with betablockers. This is a health care database where all the prescriptions are registered. Long-term treatment was assumed in patients receiving betablockers of any type and dose for at least six months before surgery, regardless of the primary indication.

All patients underwent a standardized US examination of the carotid arteries the day before surgery and were clinically assessed by an independent accredited neurologist preoperatively. The indications for surgery were CAS associated with ipsilateral symptoms and a stenosis degree > 70 % or, in patients without neurological symptoms, a CAS degree > 80 %. The stenosis degree was assessed with ultrasound based on flow velocities as previously validated.[7] Patients were considered to have asymptomatic disease if they had had no

*amaurosis fugax* (AF), transient ischemic attacks (TIAs) or strokes in the 6 months prior to surgery.

All patients undergoing CEA at our institution were routinely on long-term treatment with statins (simvastatin 40 mg) and acetylsalicylic acid (75 mg) or clopidogrel (75 mg) daily. If no contraindications were present, patients not on long-term treatment with betablockers received a single dose of metoprolol succinate (50 mg) on the day before surgery, and continued on this for the first 30 postoperative days, and were defined as not on long-term treatment for the statistical analysis.

## SAMPLE PREPARATION

The plaques removed by CEA were immediately snap frozen in liquid nitrogen. A one mm-thick fragment from the most stenotic region of the plaque was removed for histological examination. The remaining parts of the plaque were weighed, cut into pieces while still frozen, and homogenized as previously described.[8]

## LP-PLA2 ASSESSMENT

ELISA was used for measuring Lp-PLA2 protein levels. Plaque homogenate (50 µl) were centrifuged at 13000 g for 10 minutes. The supernatant (25 µl) was then removed and used to measure Lp-PLA2. The procedure was performed according to the manufacturer's instructions (The PLAC Test ELISA Kit, diaDexus Inc, San Francisco, California, USA). The Magellan V 6.4 program was used to measure LpPLA2 (absorbance 450 nm) in a Sunrise ELISA reader (Tecan, Austria GmbH, Grödig, Austria).

## LYSO-PC ASSESSMENT

Homogenates were analyzed as described for other tissues.[9] Pre-cooled methanol (160 µL) was added to tubes containing frozen plaque homogenate (40 µL). Samples were homogenized further using a Precellys 24 homogenizer (PEQLAB Biotechnology GmbH, Erlangen, Germany). Homogenization was repeated three times for 20 seconds at 5500 rpm, with 30 seconds cooling intervals between the homogenization steps. Homogenates were then centrifuged (5 minutes at 10000 g, room temperature). Supernatants (20 µL) were analyzed for lysoPC 16:0, lysoPC 18:0 and lysoPC 18:1 using Absolute*IDQ*™ p150 kit (BIOCRATES Life Sciences AG, Innsbruck, Austria). Further details on quantification and assays on API 4000 mass spectrometer (AB Sciex, Darmstadt, Germany) have been previously described.[9,10]

## MEASUREMENT OF suPAR IN HUMAN CAROTID PLAQUES

Measurement of suPAR levels in carotid plaque homogenates were performed using suPARnostic® Standard ELISA Kit (ViroGates, Birkerød, Denmark) with suPAR standards (1.0-20.7 ng/mL), and a blank control (2.6-3.4 ng/ml). Samples were mixed with peroxidase-conjugate monoclonal mouse anti-suPAR antibody and added to a clear microwell plate that were pre-coated with a monoclonal rat anti-human suPAR antibody. Then 3,3´,5,5´-tetramethylbenzidine (TMB) was added to the wells and they were incubated for 20 minutes in the dark. The absorbance at 450 nm, reference filter 650 nm, was measured with TECAN sunrise Absorbance Reader, Magellan, version 6.4 (Tecan, Austria GmbH, Grödig, Austria). The concentrations were determined by interpolation on the standard curve.

## STATISTICAL ANALYSIS

LysoPC´s, Lp-PLA2 and suPAR have been normalized to the wet weight of the plaque. Continuous variables are presented as mean (standard deviation, SD) when not stated otherwise, while categorical variables are presented as percentages. Pearson´s Chi-square is used for categorical variables. Student's t-test is used for continuous variables whenever normally distributed, while Mann-Whitney U test was used for non-normally distributed variables.

Pearson´s correlation is used for normally distributed variables, while Spearman´s rank correlation is used for non-normally distributed variables. Simple and multiple linear regressions are used to explore the relationship between two or more variables.

Correction for usual atherosclerosis risk factors as well as for statin usage was done. Information about comorbidities were incomplete only for the occurrence of inflammatory diseases and immunotherapy.

A P-value of $< .050$ was considered statistically significant. Statistical analysis was performed using SPSS 19.0 (SPSS Inc, Chicago, Ill, USA).

**RESULTS**

One-hundred and thirty-seven plaques from 134 patients (68 ± 9 years, 93 males) undergoing CEA (bilateral in 3 cases) were included. Seventy eight (57%) plaques were associated with ipsilateral hemispheric symptoms while fifty nine(43%) were not.

The study cohort was divided into two subgroups based on the absence (group A, n=78 plaques) or presence (group B, n=59 plaques) of long-term treatment with betablockers.

As shown in table 1, group B patients had more frequently known coronary artery disease (P <.0001), as well as arterial hypertension (P <.0001), higher BMI (P= .034), and simultaneous treatment with statins (P=.003).

Median age (P=.914), the degree of stenosis (P=.909), incidence of preoperative neurological symptoms related to the operated CAS (P=.110), and the time between neurological symptoms and CEA (P=.410) were not different in the two groups.

Patients of group B revealed to have lower plaque levels of Lp-PLA2 (group A 0.752 ± 0.393 ug/g *vs* group B 0.644 ± 0.445 ug/g, P=.049; figure 1A). This was confirmed after further statistical analysis using the time interval between symptoms and CEA as a confounding factor, but could not be confirmed when the presence/absence of preoperative neurological symptoms were used as confounding factors. In particular, patients of group B who had suffered neurological events before CEA had higher levels of Lp-PLA2 than those asymptomatic (0.794 ± 0.505 ug/g vs 0.498 ± 0.323 ug/g, P=.011).

Moreover, patients of group B revealed lower levels of suPAR (group A 0.044 ± 0.024 μg/g *vs* group B 0.036 ± 0.025 μg/g, P=.028; figure 1B) than those of group A.

Levels of Lp-PLA2 and suPAR were positively correlated (r= .637, P < .0001, figure 2). These findings were confirmed after correction for age, gender, hypertension, CAD, and the occurrence of preoperative symptoms as confounding factors (r = .608, P < .0001).

Lp-PLA2 and suPAR levels were also correlated (P < .0001) with the three dominant lysoPC species (16:0, 18:0 and 18:1), together (figures 3 and 4) representing 70 % of the total lysoPC content assessed in the plaque. Also these findings were confirmed after correction for age, gender, hypertension, and coronary artery disease. (Lp-PLA2 to LysoPC 16:0 r2 = .574 P <.0001, Lp-PLA2 to LysoPC 18:0 r2 = .612 P <.0001, Lp-PLA2 to LysoPC 18:1 r2 = .460 P <.0001; suPAR to LysoPC 16:0 r2 = .539 P <.0001, suPAR to LysoPC 18:0 r2 = .506 P <.0001, suPAR to LysoPC 18:1 r2 = .584 P <.0001).

## DISCUSSION

Perioperative betablockade has become standard therapy in the prevention of myocardial infarction in patients undergoing vascular surgery.

Beside their aforementioned protective effect, betablockers have shown inhibiting effects on early stages of carotid atherosclerosis development as it has been reported in humans treated with metoprolol succinate in two long-term placebo controlled studies. [11,12]

In animal models several mechanical explanations of their protective effect have been suggested. One mechanism is that an endothelial injury caused by activation of the sympathetic nervous system also leads to increased permeability in the arterial wall allowing further accumulation of low density lipoproteins (LDL) particles,[12] triggering plaque development.

It has also been suggested that an increase in shear stress and decrease in pressure related cyclic stretching of the artery induced by a decrease in heart rate and pulse pressure, leads to a reduced endothelial injury and thereby to a hampering effect on atherogenesis.[13]

The hypothesis of an intrinsic anti-inflammatory action of betablockers has also been supported by the results of an animal study that showed that treatment with metoprolol attenuated the expression of the inflammatory cytokines tumor necrosis factor-alfa and interleukin 1-beta.[14,15]

Furthermore, betablockers induce an increased stimulation in production of prostacyclin, which prevents both growth of fibrous tissue and cholesterol accumulation in the vessel wall. An increase in prostacyclin has been reported in metoprolol-treated rabbits compared to placebo treated animals, when exposed to high sympathetic activity.[16]

In our study population, plaques of patients on long-term treatment with betablockers revealed a lower level of the content of Lp-PLA2.

Lp-PLA2 is a $Ca^{2+}$ -independent serine lipase, which has been shown to be a prognostic biomarker for cardiovascular and coronary heart disease. Specifically, systemic Lp-PLA2 levels seem to predict the risk of ischemic stroke but do not correlate with carotid artery intima-media thickening or the presence of carotid plaques.[17]

This epidemiological data relates to pathohistological studies, indicating an association between plaque expression of Lp-PLA2 and coronary plaque progression and instability in CAD but are yet to be consolidated for carotid artery disease.[18]

Lp-PLA2 is secreted predominantly by macrophages.[19] Its expression and secretion significantly increase as human monocytes differentiate into macrophages and increase dramatically during activation of macrophages in the atherosclerotic lesion.[20] Recently, Lp-PLA2 has been localized to necrotic cores and inflammatory areas of coronary vulnerable plaques.[18]

LysoPCs are some of the main products of Lp-PLA2 hydrolytic activity. Importantly, lysoPCs have been shown to stimulate both proliferation and apoptosis of endothelial and smooth muscle cells at low and high concentrations, respectively.[21,22] LysoPCs also contribute to the recruitment of monocytes to the arterial wall and to matrix-metalloproteinase production.[23]

The current study shows significant lower levels of Lp-PLA2 and its enzymatic products lysoPC 16:0, 18:0 and 18:1 in plaques of patients on long-term treatment with betablockers. Lower levels of lysoPCs might result in less inflammatory activity knowing that lysoPCs induce inflammation by recruiting inflammatory cells, causing release of inflammatory cytokines/chemokines and contributes to more vulnerability in the plaque by inducing apoptosis in vascular smooth muscle cells and endothelial cells as mentioned above.[24]

It is clear that, if an important fraction of the clinically relevant Lp-PLA2 activity is derived from inflammatory cells within the plaque and not from outside the plaque, simple reduction of circulating LDL levels might not be clinically sufficient. Indeed, statins have been shown to decrease circulating Lp-PLA2 levels in conjunction with a decrease in LDL serum

concentrations, but they do not reduce de novo synthesis and secretion by macrophages.[25] This may explain why the Lp-PLA2-attributable risk of cardiovascular events was not reduced by statins in the WOSCOPS study[26] and why even a high-dose statin therapy is still associated with a 20% cerebrovascular event rate in a population at risk.[25]

Lp-PLA2 inhibitors may therefore fill an important therapeutic gap and a better characterization of the lowering effect of betablockers on Lp-PLA2 levels could bring more light into this unclear field.

In the present study patients on long-term treatment with betablockers also showed to have a lower plaque content of suPAR, which is the soluble form of the membrane bound glycoprotein urokinase-type plasminogen activator receptor (uPAR), known to be present on many of the inflammatory cells in the atherosclerotic process as monocytes, macrophages, neutrophiles and activated T-lymphocytes.[27]

The levels of suPAR have been shown to increase during inflammatory conditions and has recently gained interest as a possible biomarker predicting cardiovascular events.[28]

Higher levels of suPAR have also been found in symptomatic atherosclerotic lesions compared to asymptomatic lesions.[29]

Furthermore, it has been shown that cultured human monocytes release suPAR when stimulated by oxidated low density lipoprotein (oxLDL).[30] Thus, it is possible that the synthesis of suPAR in plaque macrophages is stimulated by oxLDL in atherosclerotic lesions, as part of the inflammatory process which initiates and promotes atheromatosis and constitutes the connecting point between suPAR and Lp-PLA2.

Our findings of a reduced content of suPAR in patients on long-treatment with betablockers could corroborate the hypothesis of their protective effect through an inhibition of the inflammatory process which lays at the basis of atherosclerosis.

The mutual correlations between suPAR, Lp-PLA2 and LysoPCs were checked for mass significance by the Bonferroni test.

A limitation of our non-randomized study, beside its observational character, is the lack of follow-up controls as well as the different therapeutic regimes in terms of drug type, absolute dose and absolute duration.

Moreover, the frequent simultaneous treatment with statins, that have already shown a stabilizing effect on atherosclerotic plaques, can mask the intrinsic effect of betablockers, even if we could confirm our findings after statistical correction using statins usage as a confounding factor.

The benefits of the betablockers treatment are known to be very large as well as their undesired side effects and interaction with simultaneous medical treatments, such as anti-inflammatory drugs.[31] A comparison taking into account this last mentioned treatment did not reveal a significant influence on the plaque levels of both LpPLA2 and suPAR. However, this finding could be related to the low number of patients of our cohort (4% in group A vs 5% in group B) who were under immunotherapy.

The fact that patients on long-term treatment with betablockers revealed to have known coronary disease, arterial hypertension, and a higher BMI could suggest a different phenotype than those without, which could be representative of a metabolic syndrome. This point needs further investigation.

The limited number of patients constituting our cohort does not allow any kind of treatment recommendation but seems to reinforce the protective effect of beta-blocker treatment that seems to go beyond hemodynamics.

The slowing effect of betablockers on development and progression of carotid atherosclerosis could be at the same time the result of a combination of its above-mentioned hemodynamic and pharmacological effects, as well as its complementary interaction with other treatment approaches.

Advances in the understanding of the vascular biology of atherosclerosis raise the possibility of novel therapies that address more directly the various aspects of endothelial dysfunction and the role of endothelial dysfunction in atherogenesis. Potential cellular targets include vascular smooth muscle cells, monocyte/macrophage cell lines, platelets, and endothelial cells. Evidence shows that antiplatelet agents, antioxidant therapies, amino acid supplementation, ACE inhibitors, and angiotensin-receptor blockers may be able to prevent or slow the progression of atherosclerosis.

## CONCLUSIONS

The reduced levels of Lp-PLA2 and suPAR in human carotid plaques of subjects on long-term betablockers therapy suggest their possible protective role in plaque inflammation and vulnerability. The correlation of Lp-PLA2, as well as suPAR levels with the three dominant lysoPC species (16:0, 18:0 and 18:1) corroborate the hypothesis of a generalised and extensive inflammatory response in atherosclerotic tissues, indirectly supporting even more selective Lp-PLA2 inhibition as a possible strategy for the prevention of cardiovascular disease.

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## CONFLICT OF INTEREST STATEMENT

All authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work.

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# LEGENDS

Table 1. Clinical characteristics of the patients using betablockers or not. Categorical variables are presented as percentages. Continuous variables are presented as mean (standard deviation). AF = *amaurosis fugax*; BMI = Body mass index; CAD = coronary artery disease; PAD = peripheral artery disease; TIA = transient ischemic attack; Time to surgery = time between symptoms (if present) and operation. * = Pearson´s Chi-square; ** = Mann-Whitney U test, *** = Student's t-test.

Fig. 1a, b. Box-plots showing the levels of Lp-PLA2 and suPAR according to the absence (group A) or presence of a long-term treatment with betablockers (group B).

Fig. 2. Scatter-plot showing the correlation between plaque levels of Lp-PLA2 and suPAR ($r = 0.63$, $P < .0001$).

Fig.. 3. Scatter-plot showing the correlation analysis between plaque levels of Lp-PLA2 and lysoPCs (16:0, 18:0 and 18:1).

Fig. 4. Scatter-plot showing the correlation between plaque levels of suPAR and lysoPCs (16:0, 18:0 and 18:1).

**Table 1.**

| | **Group A no betablockers (78)** | **Group B beta-blockers (59)** | **P-value** |
|---|---|---|---|
| **Age** | 68.9 (9.3) | 68.7 (8.7) | NS** |
| **Male** | 71 % | 64 % | NS* |
| **BMI** | 26.1 (3.5) | 27.4 (4.1) | .034*** |
| **Diabetes** | 37 % | 51 % | NS* |
| **Hypertension** | 62 % | 90 % | < .0001’ |
| **CAD** | 21 % | 54 % | < .0001* |
| **Smoking** | | | NS* |
| **No** | 23 % | 23 % | |
| **Current** | 33 % | 34 % | |
| **Ex** | 44 % | 43 % | |
| **Statins** | 82 % | 98 % | .003* |
| **Degree of stenosis** | 85.1 (12.4) | 84.8 (11.5) | NS’’ |
| **Symptoms** | 63 % | 49 % | NS* |
| **number of events** | 1.7 (2.7) | 1.2 (1.9) | NS*** |
| **type of symptoms** | | | NS* |
| **AF** | 8 % | 7 % | |
| **TIA** | 31 % | 14 % | |
| **major stroke** | 24 % | 29 % | |
| **Time to surgery - days** | 14.7 (8.2) | 14.3 (8.7) | NS** |

**Asciutto**

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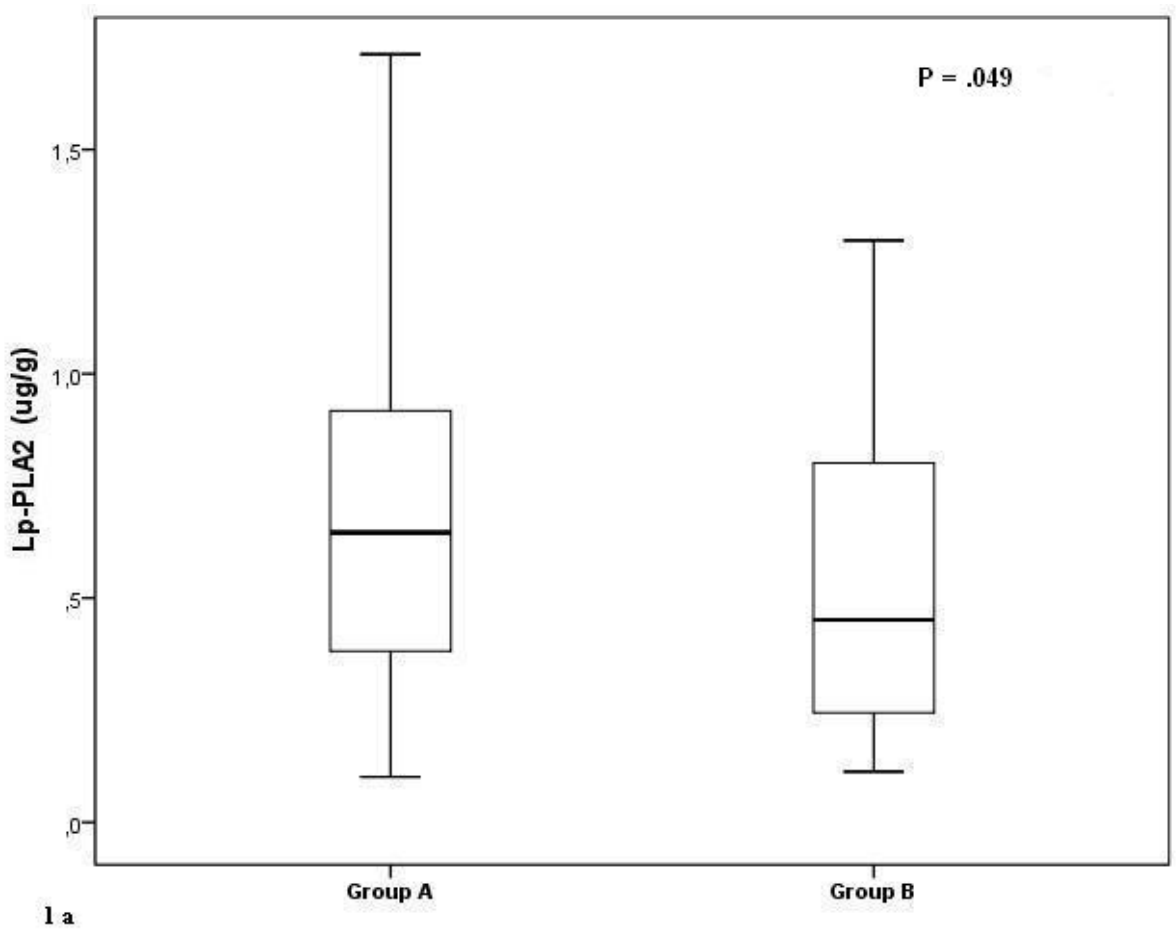

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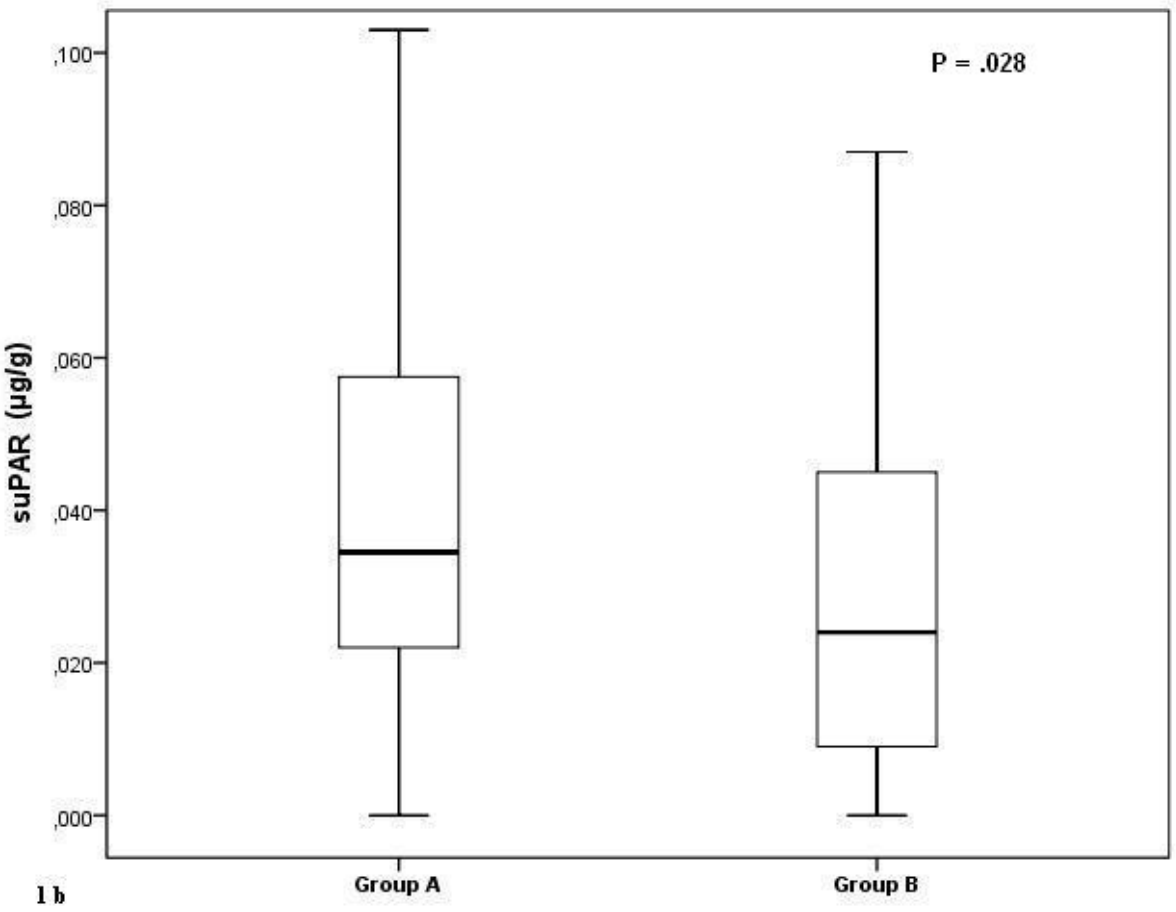

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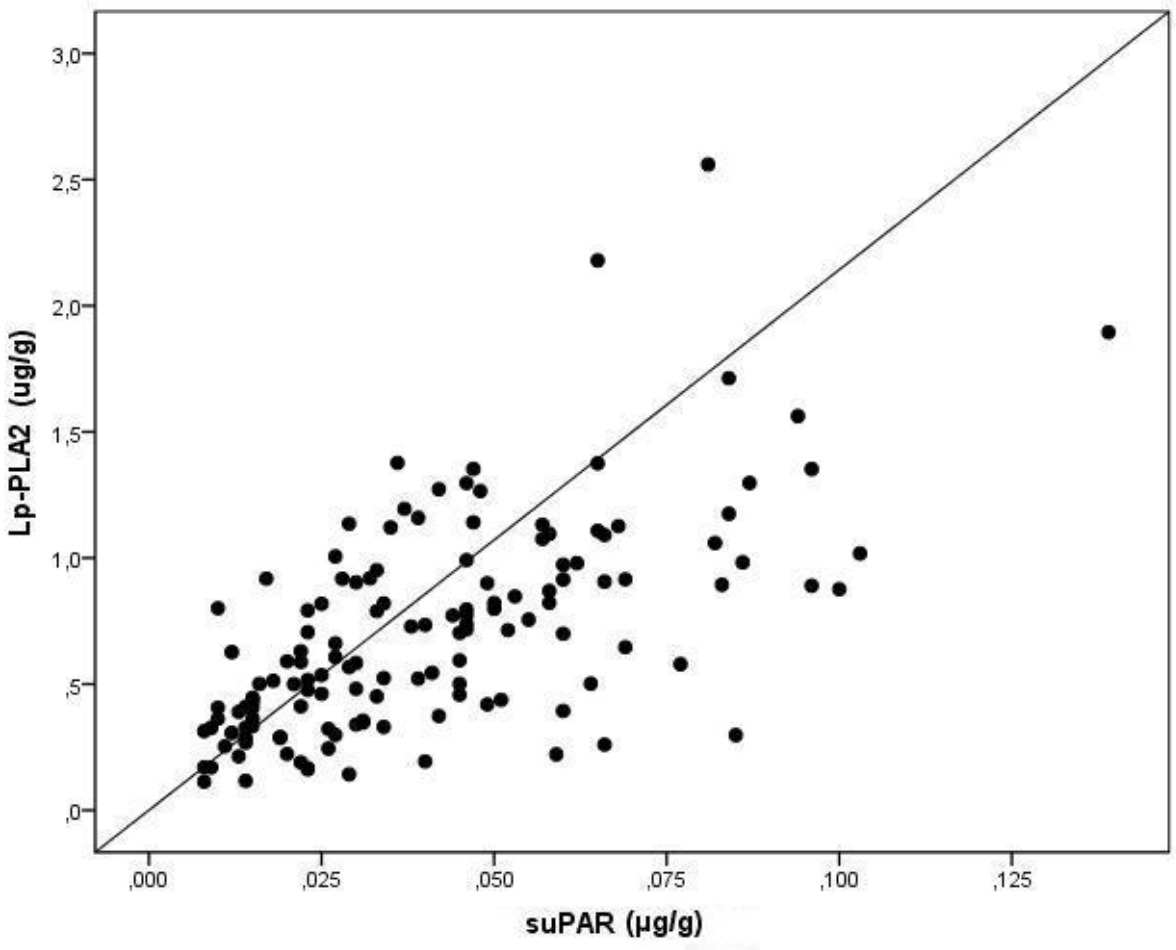

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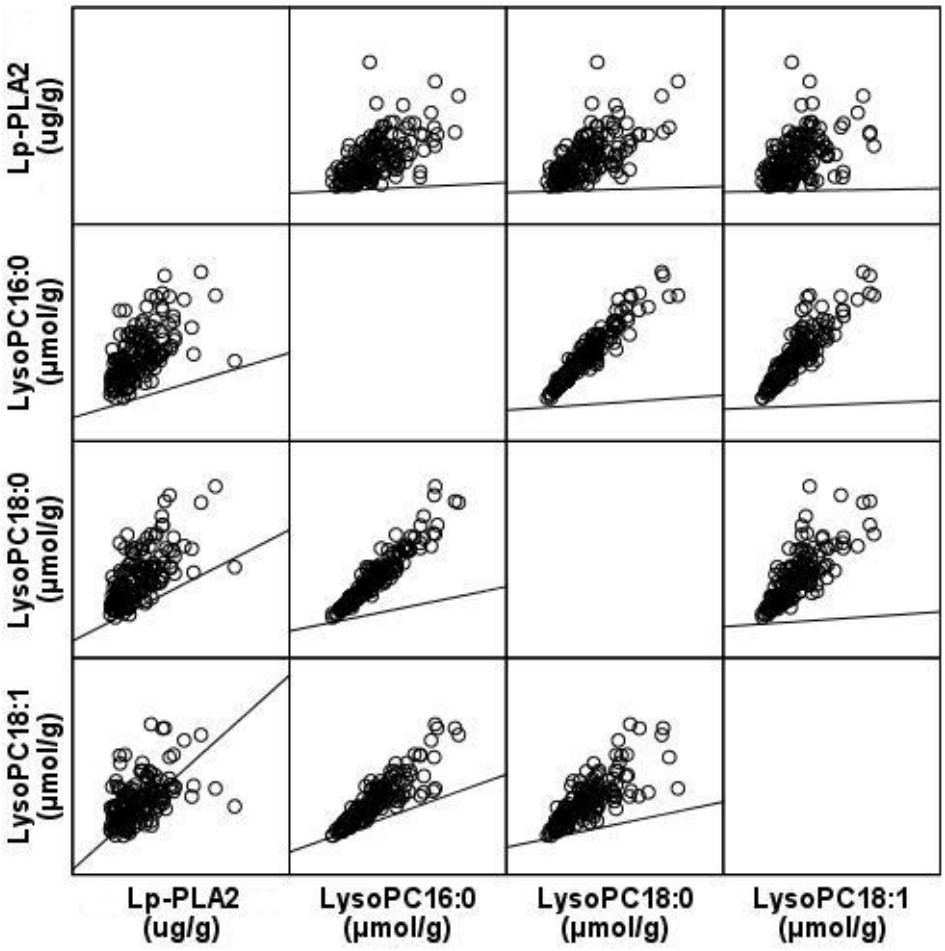

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