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Brief communication (Original)

Comparison of exercise treadmill test, flow mediated dilatation, and inflammation in individuals with low risk of adverse cardiovascular events

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Background: The relationship between endothelial dysfunction, a risk factor for coronary artery disease, and the incidence of cardiovascular disease (CVD) in the general population is not well known.

Objectives: To determine the utility of an exercise treadmill test (ETT) combined with inflammatory markers to show endothelial dysfunction for individuals with a low risk of adverse cardiovascular (CV) events.

Methods: Biomarkers of inflammation (lipoprotein-related phospholipase A_2 (Lp-PLA_2) and high-sensitive C-reactive protein (hs-CRP)) and biomarkers of endothelial dysfunction (nitric oxide, oxidized low-density lipoprotein (Ox-LDL), and sialic acid) were assessed in 60 apparently healthy patients with a positive (+) or negative (–) ETT and across endothelial function assessed by flow mediated dilatation (FMD) and Lp-PLA_2 tertiles.

Results: Lp-PLA_2 levels were increased in ETT (–) compared with ETT (+) patients. Half of ETT (–) patients were found to have levels of Lp-PLA_2 in the highest tertile. There was a significant inverse relationship between ETT and inflammatory biomarkers when adjusted for age, Lp-PLA_2 (r = –0.28, P = 0.04), or hs-CRP (r = –0.35, P = 0.01). No differences were found for biomarkers of endothelial dysfunction. All variables were reassessed across FMD tertiles. Total lipids, Ox-LDL, triglyceride, and Lp-PLA_2 were higher for the lowest FMD tertile.

Conclusion: The elevation of Lp-PLA_2 in ETT (–) patients and the inverse relationship with inflammatory biomarkers, suggest that ETT cannot address endothelial dysfunction for individuals with apparently low risk of adverse CV events, and cannot be used for risk stratification of the general population.

Keywords: Endothelial dysfunction, ETT, exercise treadmill testing, lipoprotein related phospholipase-2, LP-PLA_2, vascular inflammation

The vascular endothelium is a large paracrine organ, which plays a critical role in vascular homeostasis by secreting several mediators regulating vessel tone and diameter, coagulation factors, vascular inflammation, cell proliferation and migration, platelet and leukocyte interaction/activity and thrombus formation [1, 2]. Endothelial dysfunction has been implicated as an important cause of vascular disease and is a primary sign of the early stage of atherosclerotic disease [3, 4].

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The pathophysiological role of endothelial dysfunction in the development of atherosclerosis and cardiovascular disease (CVD) is well established [5-7]. However, little is known of its best and cost-effective assessment and clinical outcome for risk evaluation. Exercise treadmill testing (ETT) is performed routinely to identify high risk individuals. ETT helps clinicians by helping them to decide whether to refer patients for cardiac catheterization. However, ETT cannot predict angiographic findings or a poor prognosis with absolute certainty [8]. Beyond flow mediated dilatation (FMD) and ETT, there is a growing interest in the detection of endothelial dysfunction at the level of microcirculation to predict early stages of atherosclerosis. An important question arises in this

situation as to whether biomarkers of endothelial dysfunction are useful for evaluating a low risk cohort and whether they support ETT results of presumed coronary disease. Inflammation and oxidative stress play a crucial role in the pathogenesis of CVD, contributing both to the early stages and the development of atherosclerosis [9-13]. Generally, C-reactive protein (CRP) is an acute-phase reactant used for evaluation of systemic and vascular inflammation. In a recent meta-analysis of 23 prospective studies of CRP, high CRP level showed a combined risk ratio of 1.60 for coronary heart disease compared with low CRP levels [14]. In addition to CRP, as a marker of vascular inflammation; lipoprotein-associated phospholipase A_2 (Lp-PLA_2) has been shown to predict CVD. Lp-PLA_2 produced by inflammatory cells involved in atherogenesis (macrophages, T-cells, and mast cells), is predominantly bound to atherogenic lipoproteins, and accumulates in human atherosclerotic lesions [15]. Increased expression of Lp-PLA_2 has been observed in atherosclerotic lesions in animal and human models of CVD [16, 17]. Furthermore, the circulating level of Lp-PLA_2 has been proven as an independent predictor for the development of coronary artery disease (CAD) [18]. Recently, assessment of Lp-PLA_2 levels has been recommended as a supplemental risk marker for evaluation of patients with high risk of CVD [19]. However, the association of inflammation with impaired endothelial function, ETT, and FMD in patients with low risk of an adverse cardiovascular (CV) event has not been fully investigated.

In the present study, we evaluated endothelial dysfunction among patients with a positive or negative ETT test to improve our ability to assess the risk of CV events in a noninvasive and cost-effective manner in patients with low risk of an adverse CV event.

Materials and methods

From a total of 289 patients attending the Cerrahpasa Medical School Department of Cardiology at the University of Istanbul, we selected 60 (37 men and 23 women) for further investigation after they had provided their written informed consent to be tested. The study protocol was previously reviewed and approved by the ethics committee of the University of Istanbul, Cerrahpasa Medical School (No. 8237). Thirty patients with a negative exercise effort test and 30 patients with positive exercise effort test were included in our study. Physical examinations of patients were conducted before testing. Patients who were hypertensive took their medication before performing the test. Patients were excluded if they had a systolic blood pressure >170 mmHg and diastolic blood pressure >110 mmHg before the test. Individuals who had a chronic liver disease, chronic renal failure, cancer, serious systemic infections, chronic lung disease, or any endocrine disease were also excluded.

The exercise effort test was performed according to the Bruce's "treadmill" method (exercise treadmill testing (ETT) [20]. Typical chest pain and/or precipitating or raising the ST segment on an ECG by >1 mm were used as positive criteria to evaluate the test. Routine biochemical parameters such as low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, total cholesterol, triglyceride, and fasting plasma glucose were measured at the Fikret Biyal Central Laboratory. Patient blood samples, which were taken into dry tubes, were centrifuged for 15 minutes at 1000 ×*g*. Serum samples were stored as aliquots in suitable tubes with labeling at –20°C obtained until analysis.

The plasma Lp-PLA_2 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (catalog No. E08319h, Cusabio, Wuhan, Hubei Province, P.R. China). Plasma oxidized LDL levels were measured by using an ELISA kit (catalog No. K7810, Immundiagnostik, Bensheim, Germany). Plasma sialic acid levels were determined by Warren's thiobarbituric acid method [21]. Sialic acid, with periodate oxidation generates β-formylpyruvic acid. Absorbance by the pink chromophore complexes resulting from the reaction of β-formylpyruvic acid with thiobarbituric acid was measured spectrophotometrically at 549 nm. Plasma nitric oxide (NO) levels were measured using a colorimetric assay kit (catalog No. 22110, Oxis international Inc, Foster City, CA, USA). Plasma hs-CRP levels were measured using an ELISA kit (catalog No. EIA-4584, DRG Diagnostics, Marburg, Germany).

We evaluated endothelial function based on the measurement of flow-mediated dilatation (FMD) using brachial artery ultrasonography. Brachial artery ultrasonography was performed in the Echocardiography Laboratory, Department of Cardiology, Cerrahpasa Medical School. A transducer connected to a Vingmed System V ultrasound instrument (GE Healthcare, Little Chalfont, Buckinghamshire, UK) at the appropriate frequency was used to achieve this aim. After 12 hours of fasting,

examination was performed by two experienced practitioners in a quiet room at 20–24°C. Longitudinal images of the brachial artery were taken from the antecubital fossa. Baseline brachial artery diameter measurements of patients were made after being rested for at least 10 minutes. A sphygmomanometer cuff was inflated to 300 mmHg and this pressure was maintained for 4–5 minutes. The second measurement was made 45–60 seconds after removing the inflated cuff (reactive hyperemia). Patients had 15 minutes rest. Then, 0.5 mg diluted glyceryl trinitrate (GTN or nitroglycerine) was administered and the final measurement was made after 3–4 minutes. Vessel diameters and flow rates measured after reactive hyperemia and administration of GTN were compared with resting values. FMD results were calculated according to the method descried by Celermajer et al. [22-24].

Statistical analyses were performed with SPSS for Windows software (version 17.0; SPSS Inc, Chicago, IL, USA). Differences in biomarkers between patients who had ETT (+) and ETT (–) were analyzed using a Student *t* test. $P < 0.05$ was considered significant. Comparison of FMD and Lp-PLA_2 tertiles among groups were made using an ANOVA. Pearson correlation coefficients were used to examine the relationship of Lp-PLA_2 levels and markers of oxidative stress.

Results

The clinical characteristics of the study population are listed in **Table 1**. As seen in the Table, age, total cholesterol levels, and number of blocked vessels differed significantly across exercise effort test ($P = 0.000$, $P = 0.017$, $P = 0.005$, respectively). Subjects in the ETT (+) group were older than ETT (–) group. Total cholesterol levels were significantly lower for ETT (+) subjects whereas the percentage of the number of blocked vessels were shown an adverse pattern. Notably, other parameters did not differ significantly across the ETT results.

Next, we assessed the relationship of Lp-PLA_2, Ox-LDL, hs-CRP, NO, sialic acid levels, and FMD between ETT results (**Table 2**). Compared with patient participants with ETT (+), participants who were ETT (–) had higher median levels of Lp-PLA_2, hs-CRP, and NO levels ($P = 0.009$, $P = 0.07$, $P = 0.013$, respectively); however, there were no changes for any other parameters tested.

We next analyzed patient characteristics and biochemical markers across FMD tertiles as shown in **Table 3**. Subjects in the each tertile were at the same age and their BMIs were similar. For each FMD tertile, the percentage of smoking and family history of CV event were very low. Triglyceride, total, and LDL-cholesterol levels tended to be higher for the lowest tertile when compared with the highest tertile. However, the difference was not significant. Notably among endothelial biomarkers, Lp-PLA_2 and Ox-LDL levels tended to be higher for the lowest tertile of FMD as compared with the highest tertile, again, none of these differences were significant. We did not observe any differences in any of the other parameters tested.

Table 1. Clinical characteristics of patients between exercise treadmill test (ETT) results

Clinical Characteristics	ETT (–) N = 30	ETT (+) N = 30	*P*
Age (years)	48 ± 7	56 ± 7	<0.05
Men/women (number)	17/13	20/10	0.057
BMI (kg/m^2)	27.8 ± 3.6	26.2 ± 2.8	NS
Systolic blood pressure (mmHg)	130 ±20	140 ± 10	0.08
Diastolic blood pressure (mmHg)	80 ± 10	80 ± 10	NS
Diabetic (%)	10	16.7	NS
Triglyceride (mg/dL)	154 ± 77	141 ± 64	NS
Total cholesterol (mg/dL)	215 ± 46	191 ± 28	0.017
LDL cholesterol (mg/dL)	140 ± 37	136 ± 30	NS
HDL cholesterol (mg/dL)	53 ± 17	46 ± 8	0.07
Glucose (mg/dL)	102 ± 32	102 ± 14	NS
Blocked vessel (%)	0%	23, 3%	0.005

BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Data are expressed as means ± SEM. NS, not significant. Group means were compared using a Student *t* test.

Table 2. Endothelial biomarkers and flow mediated dilatation (FMD) between exercise treadmill test (ETT) results

Endothelial biomarkers	ETT (-) N = 30	ETT (+) N = 30	*P*
Lp-PLA$_2$ (IU/ml)	296.64 (223.59–365.43)	173,86 (124.92–305.07)	0.009
Oxidized LDL (ng/ml)	66.22 (62.55–93.48)	63.88 (57.90–102.31)	NS
hs-CRP (mg/L)	1.54 (0.86–3.06)	1.11 (0.56–2.26)	0.07
Nitric oxide (µM)	55.67 ± 13.43	46.76 ± 13.53	0.06
Sialic acid (mg/ml)	43.15 ± 10.92	45.04 ± 10.35	NS
FMD1 (%)	48.48 (32.21–65.78)	35.27 (16. 99–62.54)	NS
FMD2 (%)	56.37 ± 39.54	51.41 ± 33.18	NS

Lp-PLA$_2$, lipoprotein-associated phospholipase A$_2$. Data are expressed as means ± SEM or median (interquartile range) for normally distributed variables. NS, nonsignificant. Values for Lp-PLA$_2$, high-sensitive C-reactive protein (hs-CRP), oxidized low-density lipoprotein (LDL), and flow mediated dilatation (FMD) were logarithmically transformed before analyses. Nontransformed values are shown. Group means were compared using a Student *t* test.

Table 3. Patient characteristics and endothelial biomarkers across FMD tertiles

	1st tertile (<16%)	2nd tertile (16%–54%)	3rd tertile (>54%)	*P*
Age	51 ± 8	52 ± 9	53 ± 8	NS
BMI	28.4 ± 3.9	26.3 ± 2.8	26.5 ± 3.3	NS
Smoking (%)	20% (2)	40% (4)	40% (4)	NS
Family history (%)	44% (4)	33% (3)	22% (2)	NS
Systolic BP (mmHg)	134 ± 17	130 ± 14	131 ± 12	NS
Diastolic BP (mmHg)	80 ± 6	80 ± 5	80 ± 8	NS
Triglyceride (mg/dl)	149 (101–237)	123 (82–165)	127 (89–199)	NS
Total cholesterol (mg/dl)	214 ± 45	191 ± 32	203 ± 39	NS
LDL cholesterol (mg/dl)	144 ± 36	130 ± 28	141 ± 36	NS
HDL cholesterol (mg/dl)	51 ± 18	47 ± 10	50 ± 12	NS
Glucose (mg/dl)	96 (87–104)	103 (91–109)	94 (87–117)	NS
Lp-PLA2 (IU/ml)	274.9 (193.9–365.8)	269.6 (147.3–369.6)	254.8 (141.1–310.7)	NS
Oxidized LDL (ng/ml)	66.9 (63.2–82.4)	63.9 (60.5–69.1)	64.9 (59.9–168.7)	NS
hs-CRP (mg/L)	1.4 (0.8–4.7)	1.3 (0.7–2.2)	1.5 (0.4–2.4)	NS
Nitric oxide (µM)	53 ± 12	47 ± 14	54 ± 15	NS
Sialic acid (mg/ml)	43.5 ± 10.7	43.2 ± 10.9	45.6 ± 10.5	NS

Data are expressed as means ± SEM or median (interquartile range) for normally distributed variables. NS, nonsignificant. BMI, body mass index. BP, blood pressure. HDL, high-density lipoprotein. hs-CRP, high-sensitive C-reactive protein. Values for triglyceride, glucose, lipoprotein-associated phospholipase A$_2$ (Lp-PLA$_2$), oxidized low-density lipoprotein (LDL), and CRP were logarithmically transformed before analyses. Nontransformed values are shown. Group means were compared using an ANOVA.

We then separated subjects into tertiles according to the Lp-PLA$_2$ levels and analyzed the relative frequency of FMD. There was a stepwise decrease in Lp-PLA$_2$ levels between the FMD tertiles. The highest Lp-PLA$_2$ levels were seen for the highest FMD tertile (**Figure 1**). We also observed higher Lp-PLA$_2$ levels for the ETT (–) subjects (**Figure 2**). Finally, we assessed associations between ETT, FMD tertiles, and clinical and biochemical parameters after adjusting for age. There was significantly negative relationship between ETT and BMI (r = –0.36, P = 0.006), total cholesterol (r = –0.32, P = 0.02), Lp-PLA$_2$ (r = –0.283, P = 0.04) and hs-CRP (r = –0.35, P = 0.01). There were also stronger association between Lp-PLA$_2$ and CRP levels for the first tertile of FMD than in the other tertiles (r = 0.66, P = 0.004).

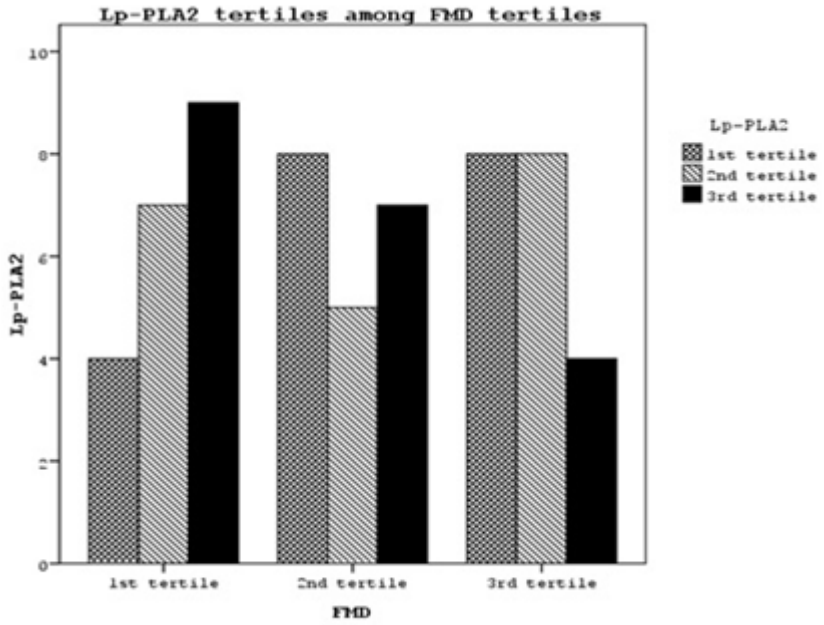

Figure 1. Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) tertiles between flow mediated dilatation (FMD) tertiles

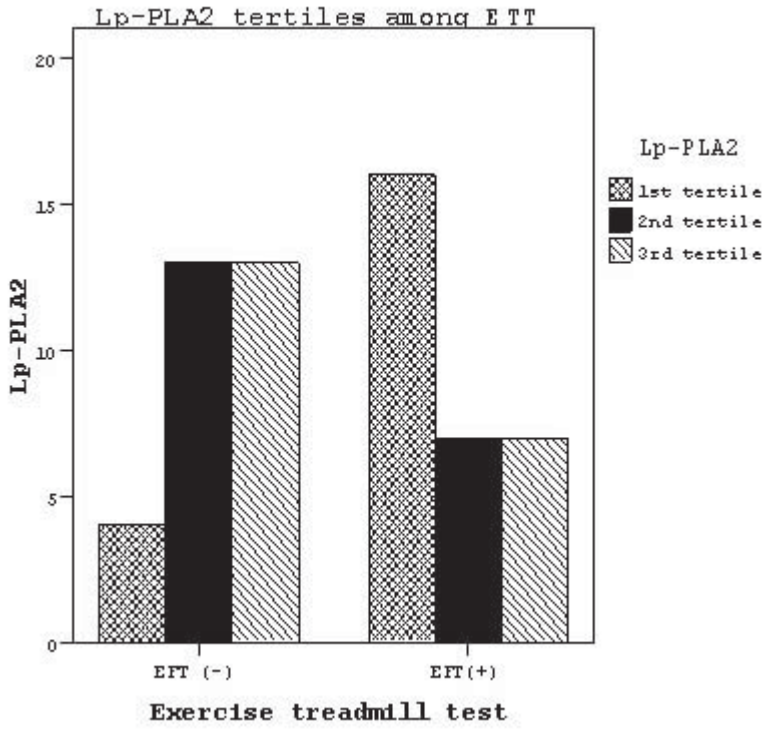

Figure 2. Lipoprotein-associated phospholipase A2 (LP-PLA_2) tertiles among exercise treadmill test (ETT)

Discussion

The main new finding of our study was the role of Lp-PLA_2 as a marker of vascular inflammation and its relationship between ETT and FMD in apparently healthy individuals. When Lp-PLA_2 and ETT result alone were considered, the ETT result was negatively associated with a higher degree of inflammation. Further, the Lp-PLA_2 level was highly associated with CRP levels in the lowest tertile of the FMD irrespective of the ETT result. We could not find any significant difference in any of the endothelial biomarkers between patients in the ETT (+) and ETT (–) groups. The ETT is conventionally used to evaluate myocardial ischemia according to the presence or absence of changes in the ST segment, producing only a positive or negative result. It is very difficult to define the magnitude of ischemia [25-28]. Our patient group consisted of apparently healthy subjects without any cardiac complaints so the sensitivity of the conventional ETT may be too low for them. Evaluating endothelial dysfunction by invasive and noninvasive methods has been used to address the risk of future CV events [29-31]. In particular, ultrasound measurement of brachial artery flow mediated dilation (FMD) during reactive hyperemia provides prognostic information about CV events in patients with chest pain and in those undergoing surgery for vascular disease [31, 32].

There are a limited number of studies that have revealed the relationship between endothelial dysfunction and adverse CV events in patients without CVD. Shecter et al. showed that branchial artery median FMD independently predicts long-term adverse CV events in healthy subjects in addition to conventional risk factor assessment [33]. Witte et al. stated that FMD and intercellular adhesion molecule 1 are related in healthy subjects, and pointed out the risk of coronary heart disease [34]. Yeboah et al. showed that brachial FMD is a predictor of incident CV events in population-based adults, and that it improved the classification of individuals as low, intermediate, and high CVD risk compared with the Framingham Risk Score [35]. The contributions of biochemical endothelial markers to endothelium derived dilatation are difficult to define because their importance may vary lots of confounders. Determining endothelial function by noninvasive and easy methods is challenging for scanning the apparently healthy population for their risk of CV events. To our knowledge, there are limited studies that have examined the relationship between ETT and inflammation to address endothelial dysfunction in patients without CVD.

We assessed the relationship between endothelial function and CV risk factors in Turkish adults who were in a "high CV risk population" according to the European Society of Cardiology. Individuals in the "low CV risk" categorized by the European Society of Cardiology score Risk Charts. Patients taking any medication that could affect FMD and biomarker measurements including statins and antihypertensive agents were excluded from the study. Limitations of our study include the small population. Samples were collected from apparently healthy healthcare workers from Cerrahpasa Medical School who were not in the same age range. Further the sex difference, age range, and menopause status of the small population might affect the ETT or biochemical test results.

Conclusions

Our findings have several clinically important implications. First, ETT did not add prognostic information beyond established CV risk factors in apparently healthy subjects. Second, ETT could not be used to screen the healthy population, and did not indicate endothelial dysfunction in addressing future risk of an adverse CV event, in subjects with or without signs of inflammation. Although our findings need to be confirmed in a larger population, they suggest that screening the healthy population using cost-effective, noninvasive, and accurate methods according to risk profile might be of value in establishing the risk of future CV events.

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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