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Nitro-Oxidative Stress Biomarkers in Active and Inactive Men

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Key words
- exercise training
- nitric oxide
- reactive oxygen species
- aging

Abstract

Oxidative stress markers are novel factors shown to be related to cardiovascular (CVD) risk. We examined the effects of long-term exercise, age, and their interaction on plasma oxidized LDL (ox-LDL), nitrotyrosine, and myeloperoxidase (MPO) levels, all biomarkers of oxidative stress, and determined their association with plasma nitric oxide (NOx) levels as an index of NO bioavailability. Older (62±2 yr) active men (n=12) who had exercised for >30 years and young (25±4 yr) active men (n=7) who had exercised for >3 years were age- and BMI-matched to older (n=11) and young (n=8) inactive men. Young subjects had lower plasma nitrotyrosine levels than older subjects (P=0.047). Young inactive subjects had higher ox-LDL levels than either the young active (P=0.042) or the older active (P=0.041) subjects. In addition, plasma oxidative stress levels, particularly ox-LDL, were correlated with various conventional plasma lipoprotein-lipid levels, and in older subjects were associated with Framingham risk score (r=0.49, P=0.015). We found no relationships between plasma oxidative stress markers and NOx levels. The findings suggest that a sedentary lifestyle may be associated with higher ox-LDL levels and that the levels of oxidative stress markers are related to levels of other conventional CVD risk factors and overall CVD risk.

Introduction

Cardiovascular disease (CVD) is the leading cause of death in the developed world, and conventional risk factors such as hypertension and dyslipidemia have long been used to estimate an individual's CVD risk. However, in the late 1990s, growing evidence suggested that these conventional risk factors might only explain half of CVD cases [10]. In addition, only about 60% of the reduction in CVD risk resulting from regular exercise could be attributed to training-induced improvements in conventional CVD risk factors [26]. Among the most promising novel risk factors recently linked to CVD are oxidative stress markers. Oxidative stress results from an imbalance of oxidants and antioxidants that results in excess reactive oxygen species (ROS) production. The excess ROS can damage lipids, proteins, and nucleic acids, and research suggests that oxidative stress may contribute to the development of various pathologies including aging, dementia, and atherosclerosis [28].

Research has linked plasma oxidized LDL (ox-LDL), nitrotyrosine, and myeloperoxidase (MPO) levels to CVD [12,17,32]. Ox-LDL produced by the oxidation of LDL by ROS migrates into the subendothelial space, is taken up by macrophages, and promotes foam cell formation [30]. Ox-LDL also promotes endothelial dysfunction, vascular remodeling, plaque rupture, and thrombosis [35]. Plasma levels of ox-LDL have been shown to predict future CVD events [22]. Nitrotyrosine is a marker of protein nitration that has been associated with inflammation and endothelial dysfunction [3]. Elevated nitrotyrosine levels have been found in atherosclerotic lesions, and increased nitrotyrosine levels are associated with various CV disease states [18,34]. The enzyme MPO catalyzes the formation of several oxidants that are important to the oxidative modifications associated with atherosclerosis [20,29]. Elevated plasma levels of MPO have been associated with CVD [38]. While these biomarkers have been shown to be involved in endothelial dysfunction and the process of atherosclerosis, little is known about their levels in a healthy, pre-clinical population.

The role of physical activity in reducing CVD risk by improving conventional risk factors and the age-related increase in CVD risk is well established. However, less is known about the effect of

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long-term physical activity and the potential interactive effects of exercise training and age on oxidative stress biomarkers. Previous studies indicate that short-term exercise training (10–18 wks) reduces plasma levels of oxidative stress markers [9,19,27], but the effects of long-term exercise training (>26 wks) or age on these markers have not been investigated. Also, while endothelial dysfunction resulting from elevated ROS levels has been implicated in CVD [35], no studies have examined whether the effects of training or age on oxidative stress levels are related to changes in nitric oxide (NO) bioavailability, indexed in the present study as the plasma levels of both nitrites and nitrates (NOx).

We sought to examine the effects of long-term exercise training, age, and their interaction on plasma levels of ox-LDL, nitrotyrosine, MPO, and NOx. We hypothesized that plasma levels of ox-LDL, nitrotyrosine, and MPO would be lower, and NOx levels higher, in active individuals compared to their inactive peers and in the young compared to the older groups.

Methods

All recruiting and screening methods for the subjects in this study were described previously [14,15,37]. Subjects were healthy, nonsmoking men with no history of CVD or diabetes. Older (62±2 yr) active men (n=12) who had performed moderate- to high-intensity endurance exercise for >4 h/wk for >30 years were BMI- and age-matched to inactive men (n=11). Young (25±4 years) active men (n=7) who had performed moderate- to high-intensity endurance exercise for >4 h/wk for>3 years were also matched on the basis of BMI and age to inactive men (n=8). The inactive groups consisted of lean, healthy men who had participated in physical activity <2/wk for <20 min/session for >5 years with a sedentary occupation or retired. All participants provided written informed consent and all procedures were approved by the University of Maryland College Park Institutional Review Board and the study meets the ethical standards of this journal [11]. Maximal oxygen consumption (VO_{2max}) and body composition were measured as described previously [15,37] with all devices calibrated prior to usage and with laboratory environmental conditions standardized and recorded daily. Peripheral venous blood was sampled in the morning before 9 a.m. after an overnight fast. All subjects avoided alcohol, vitamins, caffeine, and medications for 24 h before testing. Subjects were ingesting their habitual diet at the time of testing. Young and older subjects did not take any medications, vitamins, or antioxidant supplementations for 24 and 48 h prior to blood sampling, respectively. For active subjects blood sampling occurred 16–24 h after one of the subject's usual exercise sessions. Plasma lipoprotein-lipid profiles and fasting glucose levels were measured (Quest Diagnostics, Baltimore, MD). To calculate the subject's overall CVD risk, the conventional CVD risk factors that were measured were applied to the equations based on the Framingham study [36].

Plasma ox-LDL levels in the older subjects were published previously and were measured with a commercially-available competitive Enzyme-Linked Immunosorbent Assay (ELISA) kit (Mercodia, Uppsala, Sweden) [37]. The present study used the same assay to measure ox-LDL in the young subjects. In both ox-LDL assay runs, 2-level control samples were used to confirm assay performance. Previously, all samples in the older group were analyzed in a single assay, and in the present study all samples from young subjects were analyzed in a single assay. Thus, within each group the inter-assay variability was eliminated. All samples were measured in duplicate. The intra-assay coefficients of variation were 6.8 [37] and 9.6% for the older and young groups, respectively.

Nitrotyrosine was measured using a commercially-available ELISA kit (Cell Sciences, Canton, MA). The kit is a solid-phase ELISA based on the sandwich principle that detects nitrotyrosine-containing proteins in plasma. For nitrotyrosine, MPO, and NOx, all samples were analyzed in duplicate or triplicate in a single assay to avoid inter-assay variability. The average intra-assay coefficient of variation for nitrotyrosine was 9.1%. MPO was measured with the Human MPO ELISA kit (Cell Sciences, Canton, MA). The intra-assay coefficient of variation for MPO was 5.0%. Plasma NOx levels were measured using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI). The intra- and inter-assay coefficients of variation were 7.7 and 13.2%, respectively.

2-way ANOVAs were run with SPSS software to examine the main effects of long-term exercise training and age, along with the interaction effect of physical activity and age, on plasma levels of ox-LDL, nitrotyrosine, MPO, and NOx. The normality of all variables was verified prior to performing statistical analyses. Multiple comparisons between study groups were analyzed with Fisher's Least Significant Difference method. One-sided p-values are presented for tests of a priori directional hypotheses, unless group means were opposite the direction hypothesized, in which case 2-tailed p-values are presented. Regression analysis was performed to determine whether there were any significant relationships between levels of NOx and levels of the selected oxidative stress markers. In addition, Pearson correlation coefficients were used to assess relationships among study variables. An α value of ≤ 0.05 was used to indicate statistical significance. Data are presented as mean ± SE.

Results

Active and inactive subjects were successfully matched for age and BMI in both the young and the older groups (**Table 1**). However, as expected, active subjects had significantly higher

Table 1 Subject Characteristics.

Characteristic	Young		Older	
	Active (n=7)	Inactive (n=8)	Active (n=12)	Inactive (n=11)
age, yr	25±2	25±1	62±2	64±2
height, m	1.83±0.1	1.81±0.1	1.76±0.1	1.75±0.1
Weight, kg	81.1±4.8	77.9±6.7	70.7±3.0	74.7±2.4
BMI, kg/m^2	24.4±1.5	23.6±1.6	22.9±0.7	24.3±0.6
body Fat, %	14.3±2.2	14.8±2.4	18.0±1.3	23.5±1.7 *
glucose, mg/dL	86±3	81±3	94±2	101±3
TG, mg/dL	69±7	81±11	66±8	103±14 *
TC, mg/dL	146±8	147±9	199±9	194±11
HDL, mg/dL	53±2	49±4	71±4	51±4 *
LDL, mg/dL	79±8	82±8	115±8	123±11
TC/HDL	2.8±0.2	3.1±0.3	2.9±0.2	4.2±0.5 *
SBP, mmHg	118±2	121±2	126±4	133±3
DBP, mmHg	78±2	79±2	81±2	86±2
VO_{2max}, mL/kg/min	60.4±2.2	47.4±2.0 †	50.0±1.9	28.1±1.7*

Values are means ± SE. BMI, body mass index; TG, triglycerides; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure, VO_{2max}, maximal oxygen uptake.
† P<0.05 vs. young active. * P<0.05 vs. older active

VO_{2max} values. In addition, older active subjects had significantly lower body fat and significantly better plasma lipoprotein-lipid profiles, i.e.,– less atherogenic, than the older inactive subjects.

There was a significant main effect of long-term exercise training ($P=0.05$), but not age, on plasma ox-LDL levels (**Fig. 1**). Multiple comparisons revealed that the young inactive group had significantly higher ox-LDL levels than either the young active ($P=0.042$) or older active ($P=0.042$) groups (**Table 2**). Among the significant relationships between plasma ox-LDL levels and conventional CVD risk factors were a significant positive correlation between ox-LDL levels and LDL cholesterol, and a significant inverse correlation between plasma ox-LDL and HDL cholesterol across all study subjects (**Fig. 2**).

There was a significant main effect of age on plasma nitrotyrosine levels ($P=0.047$), with older individuals having significantly higher levels (**Fig. 3**). There was no significant main effect of long-term exercise training on plasma nitrotyrosine levels and no significant interaction effect. Multiple comparisons showed no between-group differences in plasma nitrotyrosine levels (**Table 2**).

There were no significant main effects of age or long-term exercise training on plasma MPO levels and no significant interaction effect. Between-group analyses showed that the young active group had significantly higher plasma MPO levels ($P=0.012$) compared to the older inactive group (**Table 2**).

There were no significant main effects of age or long-term exercise training on plasma NOx levels and no significant interaction effect. Tests of multiple comparisons revealed that the older inactive group had significantly higher plasma NOx levels than either the older active ($P=0.021$) or the young active ($P=0.02$) groups (**Table 2**). There were no significant correlations between plasma NOx and ox-LDL, nitrotyrosine, or MPO levels.

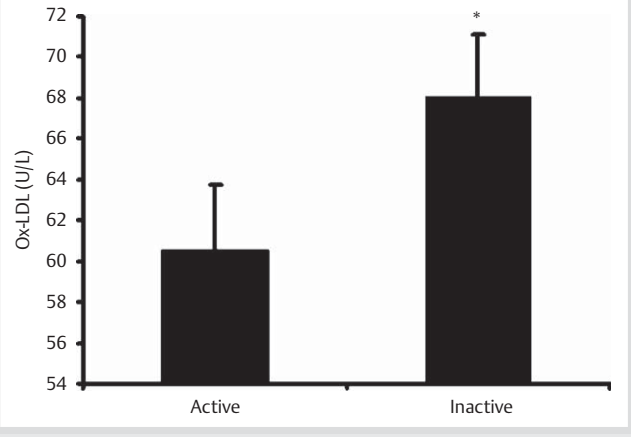

Fig. 1 Main effect of long-term exercise training on plasma ox-LDL level between active (n = 19) and inactive (n = 19) men. Values are means ± SE. *P < 0.05 vs. young.

We also examined relationships between outcome variables and conventional CVD risk factors separately within the young and older age groups. In young subjects, plasma ox-LDL levels were negatively correlated ($r=-0.64$, $P<0.05$) with VO_{2max}. Significant positive relationships between plasma ox-LDL levels and the Framingham risk score and risk percentage were observed in older subjects (**Fig. 4**). When relationships were analyzed separately by activity level groups, plasma MPO levels were positively correlated ($r=0.51$, $P<0.05$) with the ox-LDL to LDL cholesterol ratio in inactive subjects.

Discussion

Our results suggest that plasma oxidative stress marker levels are affected by long-term exercise training and age, but not consistently in the hypothesized direction. The main findings are that (a) plasma ox-LDL levels are lower in long-term exercisers, (b) nitrotyrosine levels are higher in older individuals, (c) MPO levels are lowest and plasma NOx levels highest in older inactive individuals, (d) oxidative stress marker levels are related to various conventional CVD risk factors, and (e) NOx levels are not related to plasma oxidative stress biomarker levels. The relationships between oxidative stress markers and other CVD risk factors suggest that, at the least, plasma oxidative stress marker levels may serve as indicators of CVD risk. However, the lack of association between plasma levels of oxidative stress markers and NOx does not support the hypothesis that elevations in oxidative stress lead to increased CVD risk by reducing NO bioavailability.

It has been proposed that many pathologic changes associated with aging can be explained by the irreversible accumulation of molecular oxidative damage [33]. Therefore, one would expect older individuals to have higher levels of oxidative stress than young individuals. In the present study, however, only nitrotyrosine levels were elevated in the older compared to the young subjects. Our findings suggest that plasma ox-LDL and MPO may not change with age in the same manner as nitrotyrosine. Thus, perhaps not all ROS markers contribute to the increase in oxidative damage that has been hypothesized to occur with age. However, our study is the first to compare plasma levels of the selected oxidative stress markers across age groups, so it is difficult to make definitive conclusions about how the chosen oxidative stress biomarkers are affected by age.

Although acute exercise results in increased ROS generation and elevated oxidative stress [4], some previous studies have found that exercise training may reduce systemic levels of oxidative stress [9, 27]. In the present study we found that active individuals had lower plasma ox-LDL levels than their sedentary peers. This confirms previous results of reduced plasma ox-LDL levels in older men and women [5] and sedentary, healthy young men

	Young		Older	
Outcome	**Active (n = 7)**	**Inactive (n = 8)**	**Active (n = 12)**	**Inactive (n = 11)**
Ox-LDL (U/L)	59.7 ± 5.0 [a]	74.3 ± 4.7 [b]	61.3 ± 3.9 [a]	61.8 ± 4.0 [ab]
nitrotyrosine (nM)	41.1 ± 224.6 [a]	37.3 ± 210.1 [a]	475.2 ± 171.5 [a]	292.2 ± 187.9 [a]
MPO (ng/mL)	73.0 ± 3.4 [a]	65.9 ± 1.8 [ab]	64.7 ± 4.2 [ab]	55.4 ± 1.4 [b]
NOx (μM)	14.9 ± 2.4 [a]	16.4 ± 2.2 [ab]	15.9 ± 1.8 [a]	22.5 ± 2.0 [b]

Values are means ± SE. Ox-LDL, oxidized low-density lipoprotein; MPO, myeloperoxidase; NOx, nitrates/nitrites. Data with like letters are not statistically different from each other ($P<0.05$)

Table 2 Plasma Oxidative Stress Biomarker Levels.

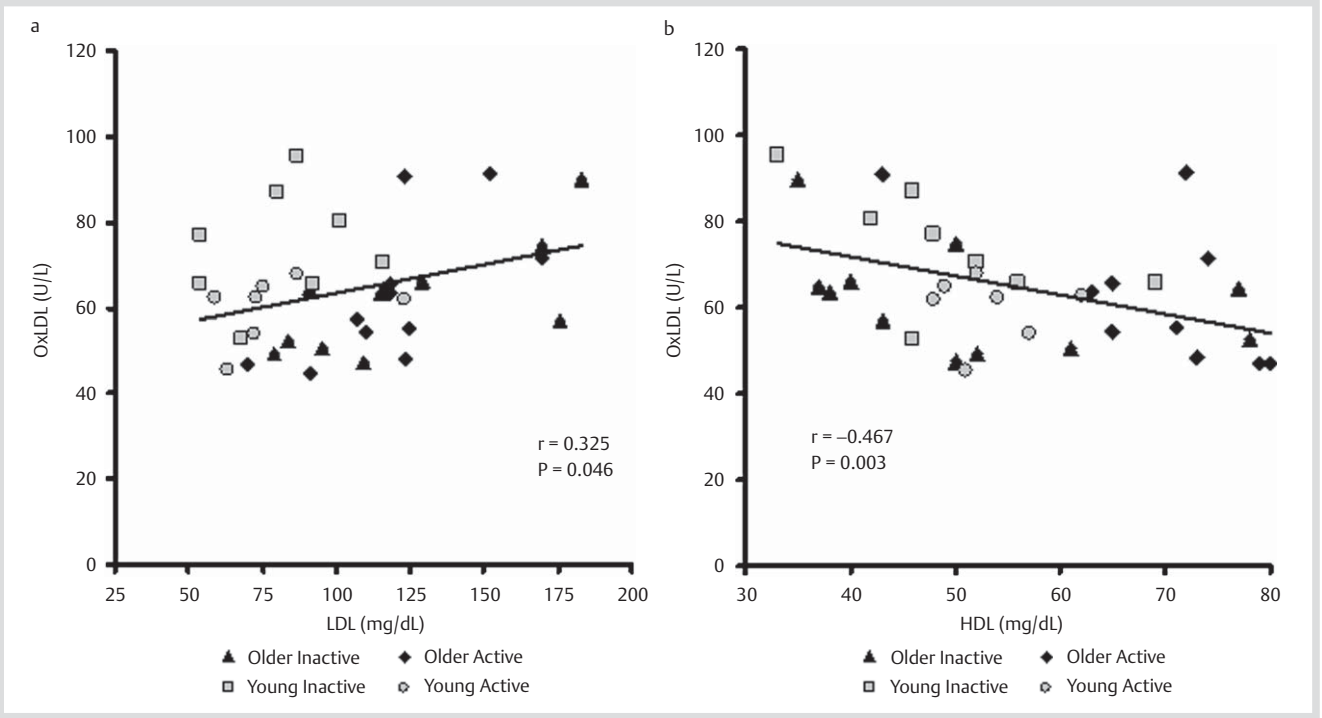

Fig. 2 Significant relationships (P<0.05) across all subjects between plasma levels of ox-LDL and the conventional CVD risk factors LDL cholesterol **a** and HDL cholesterol **b**; sample sizes were as follows: older active men (n=12), older inactive men (n=11), young active men (n=7), and young inactive men (n=8).

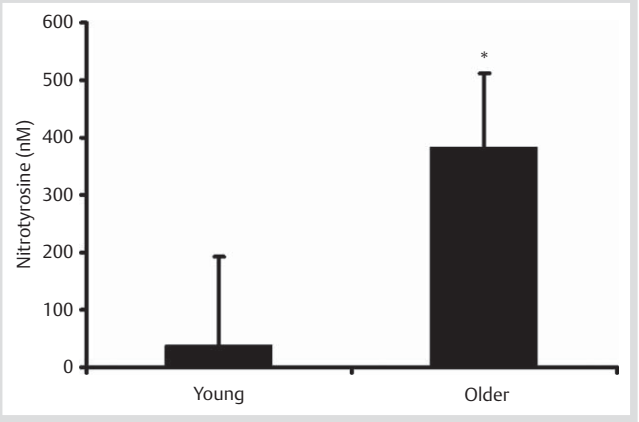

Fig. 3 Main effect of age on plasma nitrotyrosine levels between young (n=15) and older (n=23) men. Values are means ± SE. * P<0.05 vs. young.

and women [8] following an exercise intervention. Thus, our lower ox-LDL levels with long-term training are consistent with these findings. Also, our inverse correlation between ox-LDL levels and VO_{2max} in young subjects further supports the hypothesis that regular physical activity reduces oxidative stress.

We did not find lower nitrotyrosine or MPO levels in the active compared to the inactive groups. This finding was somewhat unexpected, as one earlier study found a reduction in plasma nitrotyrosine levels with a 16-wk exercise intervention in older subjects [9]. Another study reported a decrease in serum MPO levels with 12-wks of endurance training in subjects with elevated CVD risk [31]. Although the present nitrotyrosine and MPO results do not agree with these previously reported findings, they are not without precedent. One study concluded that older adults who exercise regularly have higher levels of systemic oxidative damage than their sedentary peers [23]. The general lack of information in the literature describing plasma ox-LDL, nitrotyrosine, and MPO changes with exercise training prevents definitive conclusions from being drawn relative to how chronic exercise affects systemic levels of oxidative stress.

The present findings that nitrotyrosine and MPO levels were not lower in active compared to the inactive groups could be a result of the unique features of the present study. First, the present study examined the effects of long-term exercise training on oxidative stress levels, while all previous studies have focused on the effects of relatively short-term training. Second, the inactive subjects in the present study are unique because they have no readily apparent health issues and appear to have suffered no negative consequences from their years of physical inactivity. Despite their sedentary lifestyles, the young inactive men had body weight, BMI, body composition, blood pressure, and plasma lipoprotein-lipid values similar to those of their active peers who had been exercising regularly for >3 years. The older inactive subjects had body weight, BMI, and blood pressure similar to those of their active peers who had been exercising for >30 years. This makes the individuals in the young and especially the older inactive groups quite exceptional.

We found no main effects of age or activity level on plasma NOx levels, although there was some evidence that the inactive, and especially the older inactive, groups had higher plasma NOx levels than their trained peers. These results contrast with those from previous studies showing that NOx levels decline with age and that these declines can be reversed with short-term exercise training [7,21]. However, the responses to longer-term training (≥16 wk) seem to be more variable. In a group of older men and women, 24 wk of exercise training did not improve plasma NOx levels [1]. Given the extremely long duration of training in the

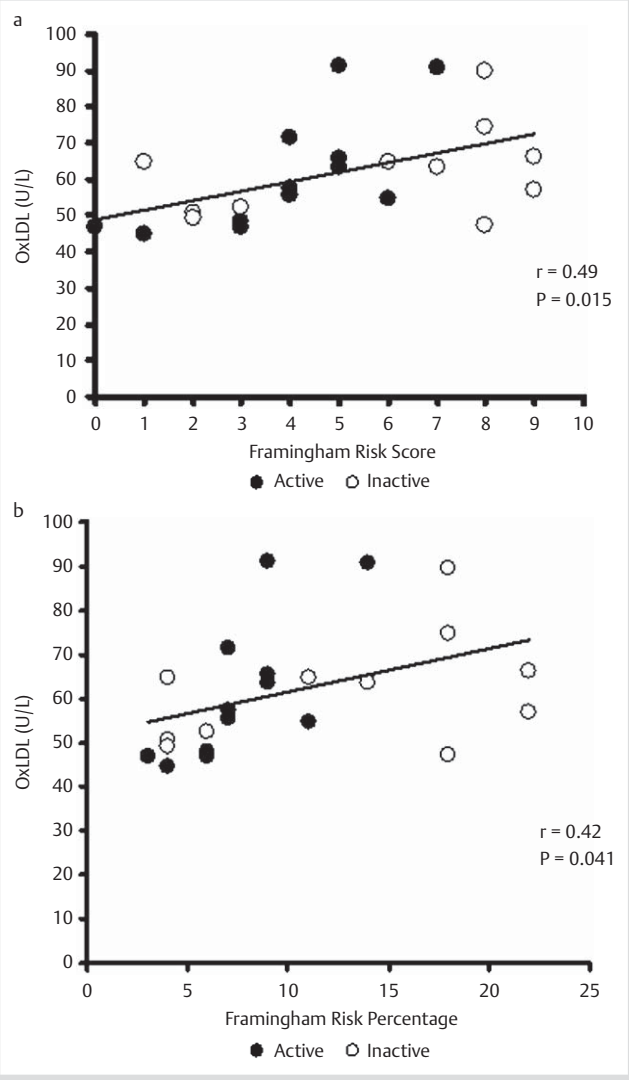

Fig. 4 Significant relationships ($P<0.05$) between plasma ox-LDL levels and Framingham risk score **a** and Framingham risk percentage **b** in the active (n = 12) and inactive (n = 11) older subjects.

present study, the lack of association between activity level and plasma NOx levels may be less surprising.

We found no relationships between plasma NOx levels and plasma ox-LDL, nitrotyrosine, or MPO levels. These findings appear to contrast with previous research that has linked increases in oxidative stress to endothelial dysfunction [35]. However, our plasma NOx measurement is an assessment of NO bioavailability as opposed to a measurement of endothelial function. Although reduced NO bioavailability is one mechanism thought to cause endothelial dysfunction [1], impairments in endothelial function can occur without decreases in NOx levels. For example, an earlier study found that atherosclerotic rabbits had higher plasma NO levels, as determined by quantifying nitrosyl compounds in blood, but impaired endothelium-dependent vasodilation, compared to control rabbits [25]. Thus, plasma NOx levels are not necessarily directly related to endothelial function, and the present study only allows one to make conclusions about how NO bioavailability is affected by age or inactivity.

However, the lack of association between NO bioavailability and plasma oxidative stress marker levels in the present study is still surprising. Previous research has shown that ox-LDL reduces NO bioavailability by inactivating NO and stimulating the release of NO scavengers [16]. Elevated nitrotyrosine levels have been associated with increased NO breakdown and inhibited NO synthesis [3]. MPO has previously been shown to decrease NO levels by inhibiting and uncoupling NOS, and breaking down NO [29]. 2 possible explanations for why the present study failed to find any associations between plasma ox-LDL, nitrotyrosine, and MPO levels and plasma NOx levels have already been discussed. Briefly, the particularly long duration of training undertaken by the active subjects in this study may have resulted in different effects on plasma NOx levels than a short-term intervention. Also, the fact that the inactive subjects in our study were generally very healthy may indicate that these biomarkers are not affected yet in this pre-clinical population.

We found several significant relationships between ox-LDL levels and conventional CVD risk factors. Across all subjects, plasma ox-LDL was positively correlated with LDL cholesterol levels and negatively correlated with HDL cholesterol levels. Such relationships have been previously reported [12,13]. The relationship between ox-LDL and LDL cholesterol makes intuitive sense because ox-LDL molecules are formed by the oxidative modification of native LDL, with an increase in LDL likely to be associated with an increase in ox-LDL. Also, the negative association between ox-LDL and HDL levels fits with evidence suggesting that HDL molecules are atheroprotective, in part, because they inhibit LDL oxidation [24]. In addition, within the older subjects ox-LDL levels were positively correlated with Framingham risk score and percentage, suggesting that ox-LDL could have predictive value for CVD, especially in those at higher risk for CVD. These results are supported by a study that found that plasma ox-LDL levels were predictive of future CVD events, and they were stronger predictors than the conventional lipoprotein profile, in apparently healthy men [22].

In the inactive subjects, a positive relationship was observed between plasma MPO levels and the ratio of ox-LDL to LDL cholesterol. Previous research has indicated that MPO can convert native LDL to ox-LDL [6]. In addition, one of the secondary oxidation products generated by MPO, nitrogen dioxide, has been reported to promote ox-LDL formation [2]. Thus, one would expect an increase in MPO to lead to an increase in ox-LDL formation, resulting in the increased ox-LDL to LDL ratio reported in the present study.

The present study is limited by the cross-sectional design employed to assess potential biomarker differences across age and habitual physical activity level groups. This, combined with the relatively small sample sizes we utilized, has generated results that, while hardly definitive, at the least provide a substantive framework for future longitudinal exercise training intervention studies in larger populations. Also, our findings are limited to plasma biomarker levels, keeping in mind that measuring the localized oxidative stress more directly in tissues or cell compartments may yield different results. In addition, the assessment of plasma levels of oxidative stress markers in the present study may have benefited from the measurement of additional plasma oxidative stress markers, such as malondialdehyde and thiobarbituric acid reaction substances. And finally, we have no mechanistic data to determine if any of these observed relationships or differences across groups can be attributed to specific molecular and cellular mechanisms. Such mechanistic data could include those generated by longitudinal exercise training, dietary, and anti-oxidant studies, labeled plasma biomarker turnover studies, and studies to assess the

release of these biomarkers across specific tissue beds such as adipose tissue and skeletal muscle.

In conclusion, our findings suggest that age and chronic exercise training are associated with different levels of circulating biomarkers of oxidative stress. It appears that a sedentary lifestyle may be associated with elevated ox-LDL levels, and the results indicate that lower plasma ox-LDL levels in trained individuals are related to better CVD risk factor profiles and lower overall CVD risk. In addition, the results did not show a link between plasma oxidative stress and plasma NOx levels, underscoring the need for further research to elucidate how elevations in oxidative stress contribute to increases in CVD risk.

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