

## ABSTRACT

Title: CIRCULATING BIOMARKERS OF NITRO-  
OXIDATIVE STRESS IN YOUNG AND  
OLDER ACTIVE AND INACTIVE MEN

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Oxidative stress markers may be novel factors contributing to cardiovascular (CVD) risk. The purpose of this study was to examine the effects of long-term exercise, age, and their interaction on the plasma levels of the oxidative stress markers oxidized LDL (ox-LDL), nitrotyrosine, and myeloperoxidase (MPO), and to investigate whether these levels correlated with plasma NO<sub>x</sub> levels. Older ( $62 \pm 2$  yr) active (n=12) men who had exercised regularly for over 30 years and young ( $25 \pm 4$  yr) active (n=7) men who had exercised regularly for over 3 years were matched to older (n=11) and young (n=8) inactive males. Young subjects showed 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 CVD risk factors, and in older subjects were associated with Framingham risk score ( $r = 0.49$ ,  $P = 0.015$ ). The study found no relationships between plasma markers of oxidative stress and plasma NO<sub>x</sub> levels. The findings suggest that a sedentary lifestyle may be associated with higher ox-LDL levels and that the levels of oxidative stress markers may contribute to CVD risk.

CIRCULATING BIOMARKERS OF NITRO-OXIDATIVE STRESS IN YOUNG  
AND OLDER ACTIVE AND INACTIVE MEN

By

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## List of Abbreviations

- BH<sub>4</sub> – tetrahydrobiopterin
- BMI – body mass index
- CVD – cardiovascular disease
- DBP – diastolic blood pressure
- ELISA – enzyme-linked immunosorbent assay
- eNOS – endothelial nitric oxide synthase
- HDL – high-density lipoproteins
- HOCl – hypochlorous acid
- iNOS – inducible nitric oxide synthase
- LDL – low-density lipoproteins
- MDA – malondialdehyde
- MPO – myeloperoxidase
- NO – nitric oxide
- NO<sub>x</sub> – nitrates/nitrites
- Ox-LDL – oxidized LDL
- RNS – reactive nitrogen species
- ROS – reactive oxygen species
- SBP – systolic blood pressure
- TC – total cholesterol
- TG – triglycerides
- VO<sub>2</sub> max – maximal oxygen uptake

## Introduction

Blood lipid profiles, blood pressure, smoking status, and other conventional risk factors have long been considered in estimating an individual's cardiovascular disease (CVD) risk. However, in the late 1990s, evidence suggested that these conventional CVD risk factors might only be explaining half of CVD cases (16). In addition, only about 60% of the reduction in CVD risk through regular exercise could be attributed to training-induced improvements in conventional CVD risk factors (44). Thus, the search began for novel risk factors that might help provide a more complete picture of a person's CVD risk. Among the most promising novel risk factors recently linked to CVD are oxidative stress markers.

Reactive oxygen species (ROS) are produced as intermediates in a variety of metabolic reactions in the body. In healthy tissues, any increase in ROS is accompanied by an increase in antioxidant capacity, preventing the buildup of excess ROS. When this delicate balance is disrupted and excess ROS results, the body experiences "oxidative stress" as the highly reactive ROS interact with lipids, proteins, and nucleic acids to cause cellular damage. In addition to cell damage, research suggests that oxidative stress can contribute to the development of various pathologies including aging, dementia, and atherosclerosis (47).

The proposed role of oxidative stress in atherosclerosis has been termed the oxidative modification hypothesis (64). According to this hypothesis, oxidized molecules play an important role in atherosclerosis both by embedding in the vascular

wall and promoting plaque formation and by interacting with inflammatory and endothelial dysfunction pathways which negatively affect vascular function. Because of their involvement in the atherosclerotic disease process, various biomarkers of oxidative stress may have predictive value for CVD. Particularly attractive to primary care practitioners are potential systemic markers of oxidative stress that could be measured in a minimally invasive manner, such as plasma levels of oxidized LDL (ox-LDL), nitrotyrosine, and myeloperoxidase (MPO).

Early research has linked plasma levels of ox-LDL, nitrotyrosine, and MPO to CVD (21, 32, 61). Ox-LDL is linked to atherosclerosis because unlike native LDL, ox-LDL migrates into the subendothelial space, is taken up by macrophages, and promotes foam cell formation (51). Ox-LDL also seems to promote endothelial dysfunction, vascular remodeling, plaque rupture, and thrombosis (64). Plasma levels of ox-LDL have been shown to be predictive of future CVD events (39).

Serving as a marker of protein nitration, nitrotyrosine has been associated with inflammation and endothelial dysfunction (7). Elevated nitrotyrosine levels have been found in atherosclerotic lesions, and increased nitrotyrosine levels are associated with various CV disease states (63).

The enzyme MPO plays a role in many of the oxidative modifications associated with atherosclerosis and the resulting endothelial dysfunction by catalyzing the formation of several oxidants (48). Elevated plasma levels of MPO have been associated with CVD (71).

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 long-term physical activity and the interaction of exercise training and age on many of the novel risk factors. Previous studies indicate that short-term exercise training interventions reduce plasma levels of oxidative stress (45), but studies have not investigated the effects of long-term exercise training or age on oxidative stress markers. In addition, while endothelial dysfunction resulting from elevated oxidative stress levels has been implicated in CVD (64), no studies have examined whether the effects of training or age on oxidative stress levels are related to changes in endothelial health or NO bioavailability.

The present study is an important initial step in understanding oxidative stress as an emerging CVD risk factor because it seeks to examine how long-term physical activity and age may affect plasma levels of ox-LDL, nitrotyrosine, and MPO. The findings could justify further research into the changes in oxidative stress levels induced by short-term exercise training interventions, the mechanisms underlying these modifications, and their clinical consequences. In addition, the study attempts to link the effects of training and age on oxidative stress levels to changes in NO bioavailability by measuring plasma levels of the final products of nitric oxide (NO) metabolism, collectively called NOx. The finding of a negative relationship between plasma levels of NOx and plasma levels of ox-LDL, nitrotyrosine, or MPO would lend support to the theory that elevations in oxidative stress levels increase CVD risk by reducing NO bioavailability, which in turn causes endothelial dysfunction.

#### *Specific Aims and Hypotheses*

The first aim of this study is to examine the effects of long-term exercise training, age, and their interaction on the plasma levels of ox-LDL, nitrotyrosine,

MPO, and NOx. It is hypothesized that plasma levels of ox-LDL, nitrotyrosine, and MPO will be lower, and NOx levels higher, in active individuals compared to their inactive peers. In addition, plasma levels of ox-LDL, nitrotyrosine, and MPO will be higher, and NOx levels lower, in older groups compared to their corresponding young group.

The second aim of the study is to determine whether NOx levels are correlated with the plasma levels of ox-LDL, nitrotyrosine, and MPO. It is hypothesized that NOx levels will be inversely related to plasma levels of ox-LDL, nitrotyrosine, and MPO.

## Methods

### Study Design

To study the impact of long-term exercise training and age on oxidative stress, the study used a cross-sectional approach with four groups and 38 total subjects. All subjects were originally recruited, screened, and tested as described in three previous studies (26, 27, 70). All participants were men who provided written informed consent as part of their participation in the Long Term Exercise and Vascular Health Study. In addition, all procedures were approved by the Institutional Review Board at the University of Maryland.

### **Subjects**

Older ( $62 \pm 2$  yr) active (n=12) men who had performed moderate- to high-intensity endurance exercise for over four hours per week for over thirty years were BMI- and age-matched to inactive (n=11) males. The older subjects were from 55 to 77 years of age.

Young ( $25 \pm 4$  yr) active (n=7) men who had performed moderate- to high-intensity endurance exercise for over four hours per week for over three years were matched on the basis of BMI, age, body composition, and conventional CV risk factor profile to inactive (n=8) males. The young subjects were from 18 to 30 years of age.

All subjects were healthy, nonsmoking, and currently free of and with no history of CVD or diabetes. Subjects were excluded if they had Stage 1 or greater

hypertension, or if they were taking cholesterol-lowering, antihypertensive, or antihyperglycemic agents.

The active subjects were located with the help of recruitment flyers posted in local running and cycling stores, on their websites, and in their newsletters. In addition, Department of Kinesiology faculty members who participated in local running clubs talked with fellow members who met the subject qualifications, and other Department of Kinesiology studies with subjects qualified for this study were approached.

The inactive groups consisted of lean, healthy individuals who for at least five years had participated in physical activity less than twice per week for less than 20 minutes per session with a sedentary occupation or retired. The inactive subjects were recruited as matches for the active group on an individual basis. To be considered a match, the inactive male had to be of the same ethnicity, have an age within five years, and a BMI within  $1 \text{ kg/m}^2$  of the active male. Many of the inactive subjects were recruited from subject pools of other studies going on in the Department of Kinesiology.

### **Outcomes**

Venous blood samples were drawn and stored so that the plasma levels of ox-LDL, nitrotyrosine, MPO, and NOx could be compared in the four groups of men: older active, young active, older inactive, and young inactive. In addition to blood sampling, subjects also underwent  $\text{VO}_2$  max and body composition testing.

## Procedures

The study was completed in two visits, with VO<sub>2</sub> max and body composition testing done on the first visit. On the second visit, venous blood samples were drawn in the morning following a 12- to 16-hour fast and stored at -80°C.

Prior to any testing in older subjects, a physician performed a physical examination to screen for CVD and to identify contraindications to exercise testing. All testing occurred the morning after an overnight fast. All subjects had avoided alcohol, vitamins, caffeine, and medications for 24 hours before testing. For active subjects the visits occurred 16 to 24 hours after one of the subject's usual physical activity sessions.

## Instrumentation

### **Maximal Graded Exercise Testing**

Participants performed a maximal treadmill exercise test to screen for CVD and assess VO<sub>2</sub> max. A constant-speed treadmill protocol was used with the grade increasing 2% with each successive 2 minute stage until exhaustion. The treadmill speed was selected by the investigator based on subject experience, typical run/walk speed, and heart rate such that VO<sub>2</sub> max would be achieved in 6-12 minutes. In the older subjects, ECG was used during the test to measure heart rate. Heart rate in the young subjects was measured during testing with heart rate monitors (Polar Electro, Woodbury, NY). Open-circuit spirometry was used to measure maximal oxygen consumption, with the subject's pulmonary ventilation measured and fractions of expired oxygen and carbon dioxide collected. Expired gases were analyzed using an

automated indirect calorimetry system (Oxycon Pro, Viasys). By comparing the amount of oxygen inspired to the amount expired, the amount of oxygen consumed was calculated. For the older subjects, a valid  $\text{VO}_2$  max required that at least three of the following criteria were met: a respiratory exchange ratio  $\geq 1.15$ , a heart rate that was equivalent to the age-predicted max, a plateau in oxygen uptake with increases in workload, or the subject indicated exhaustion. For the young subjects, a valid  $\text{VO}_2$  max required a plateau in oxygen uptake with increasing workload and at least two of the following criteria were met: a respiratory exchange ratio  $\geq 1.10$ , a rating of perceived exertion  $\geq 19$ , or a peak heart rate within ten beats of the age-predicted max.

### **Body Composition Testing**

For all subjects, height, weight, and blood pressure were measured and body mass index (BMI) was calculated. For older subjects, body fatness was assessed with dual x-ray absorptiometry (Hologic). In young subjects, body fatness was estimated using the seven-site skinfold procedure (25).

### **Blood Chemistry Testing**

Blood samples were sent to Quest Diagnostics for analysis. The following levels were measured: glucose, triglycerides, total cholesterol, HDL cholesterol, and LDL cholesterol. In addition, the ratios of total cholesterol to HDL cholesterol and ox-LDL to LDL cholesterol were calculated. Finally, 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 (69).

## **Plasma Ox-LDL Measurement**

Plasma ox-LDL levels in the older subjects were measured previously with a commercially-available competitive Enzyme-Linked Immunosorbent Assay (ELISA) kit (Merckodia) (70). The present study used the same assay to measure ox-LDL in the young subjects. The ELISA was performed in accordance with the manufacturer's directions for use for product number 10-1158-01, which required a dilution of plasma samples with the provided sample buffer. With both the assay previously performed on older subject samples and the assay performed in the present study on young subject samples, two-level control samples (Merckodia) of a known ox-LDL concentration were assayed. These controls confirmed that the procedure was able to detect a positive result.

In the competitive ELISA kit, ox-LDL in the sample competes with a fixed amount of ox-LDL in the well plate for the binding of the mouse monoclonal antibody 4E6. The antibody is able to bind the ox-LDL molecules because during lipoprotein oxidation aldehydes are substituted for lysine residues in the apolipoprotein B-100 region of the molecule, resulting in the generation of a new conformational epitope (20). The 4E6 antibody has been used in several previous studies to measure ox-LDL levels, including a study that found an increase in ox-LDL levels in patients with coronary artery disease (20, 21, 22, 39, 50).

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 interassay variability was minimized. However, when comparing older and young subjects, the interassay variability must be considered.

Previous studies using this ox-LDL assay have cited an interassay variability of 12%, indicating that the measurements are reliable (20). Samples from older and young subjects were run in duplicate to minimize the intra-assay variability and the results were averaged. For the older subjects, the intra-assay coefficient of variation was 6.8% (70). For the young subjects analyzed in the present study, the average intra-assay coefficient of variation for the competitive ox-LDL assay was 9.58%.

### **Plasma Nitrotyrosine Measurement**

Nitrotyrosine was measured using a commercially-available ELISA kit (Cell Sciences). The kit is a solid-phase ELISA based on the sandwich principle that detects nitrotyrosine-containing proteins in plasma. To ensure accurate measurement, the plasma samples were diluted with the provided dilution buffer per manufacturer's instructions for product number HK501. As a part of the constructed standard curve, two wells were filled with dilution buffer only, and they served as negative controls for the assay.

Interassay variability was controlled by running all samples in a single run. In addition, all samples were analyzed in duplicate, minimizing intra-assay variability, and the results were averaged. The average intra-assay coefficient of variation for the nitrotyrosine assay was 9.1%.

### **Plasma MPO Measurement**

MPO was measured with the Human MPO ELISA kit (Cell Sciences). The assay is a solid-phase ELISA based on the sandwich principle that measures the amount of MPO in plasma. To ensure accurate measurement, the plasma samples were diluted with the provided dilution buffer per manufacturer's instructions for

product number HK324. As a part of the constructed standard curve, two wells were filled with dilution buffer only, and they served as negative controls for the assay.

All samples were analyzed in one run to control interassay variation. In addition, all samples were run in duplicate to minimize intra-assay variability and the results were averaged. The average intra-assay coefficient of variation for the MPO assay was 5.0%.

### **Plasma NO<sub>x</sub> Measurement**

The short half-life of NO makes it impossible to accurately measure NO levels directly. However, the final products of NO metabolism, nitrite and nitrate, can be measured in plasma. Collectively called NO<sub>x</sub>, the sum of nitrite and nitrate concentrations serve as an indicator of NO production (53).

In the present study, plasma NO<sub>x</sub> levels were measured using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company). The kit provided an accurate and convenient way to perform a modified version of the Griess assay. First, nitrate was converted to nitrite using nitrate reductase. Then the addition of the Griess Reagents converted nitrite into a purple azo compound. The azo compound is capable of selectively absorbing particular wavelengths of light. Using a plate reader to measure the optical density of each sample at 541 nm, the absorbed light can be quantified. To do this, a standard curve was constructed by plotting the absorbance measured for known quantities of nitrate. As a part of the constructed standard curve, two wells were filled with assay buffer only, and they served as negative controls for the assay. By subtracting the average absorbance of the negative controls from each

sample absorbance and plotting the result on the constructed standard curve, the total concentration of nitrate and nitrite was determined.

As outlined in the manufacturer's instructions for product number 780001, the plasma samples were diluted with the provided assay buffer. All samples were run in triplicate to minimize intra-assay variability and the results were averaged. Although two separate plates were used, inter-assay variation was minimized by creating a standard curve for each plate. The average intra-assay coefficient of variation for the two plates was 7.7%, and the inter-assay coefficient of variation was 13.2%.

### Statistical Analysis

A two-way ANOVA was done 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 the plasma levels of ox-LDL, nitrotyrosine, MPO, and NOx. 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 two-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  $\alpha$  value of 0.05 was used to indicate statistical significance.

## Results

### Subject Characteristics

Active and inactive subjects were successfully matched for age and BMI (Table 1). However, as expected, active subjects had significantly higher VO<sub>2</sub> max values. In addition, older active subjects had significantly lower body fat and significantly better blood chemistry profiles than older inactive subjects.

### Ox-LDL

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 (Figure 1). There were no significant ( $P < 0.05$ ) main effects of age or long-term exercise training on plasma ox-LDL levels, and no significant interaction effect (Figures 2A and 2B). However, there was a trend ( $P = 0.05$ ) suggesting that long-term exercise training is associated with lower ox-LDL levels (Figure 2B). An examination of the relationships between plasma ox-LDL levels and conventional CVD risk factors found significant positive correlations between ox-LDL levels and LDL cholesterol ( $r = 0.325$ ,  $P = 0.046$ ), ox-LDL to LDL ratio ( $r = 0.361$ ,  $P = 0.026$ ), and total cholesterol to HDL ratio ( $r = 0.483$ ,  $P = 0.002$ ) across all study subjects (Figures 3A, 3B, 3C). Also significant was the inverse correlation ( $r = -0.467$ ,  $P = 0.003$ ) between plasma ox-LDL and HDL cholesterol (Figure 3D). The plasma ox-LDL levels reported here are consistent with the values found in other studies in the literature that have used the same or similar ELISA kits (22, 39).

### Nitrotyrosine

Multiple comparisons showed no between-group differences in plasma nitrotyrosine levels (Figure 4). There was a significant main effect of age on plasma nitrotyrosine ( $P = 0.047$ , one-tailed), with older individuals having significantly higher levels (Figure 5A). There was no significant interaction effect between age and long-term exercise training and no significant main effect of long-term exercise training on plasma nitrotyrosine levels (Figure 5B). In addition, across all subjects there was a significant positive correlation ( $r = 0.429$ ,  $P = 0.008$ ) between plasma nitrotyrosine levels and systolic blood pressure (Figure 6). The circulating levels of nitrotyrosine found in other studies vary widely, but the values reported here fall within the range of values reported in the literature (15, 54).

### MPO

Between-group analyses showed that the young active group had significantly higher plasma MPO levels ( $P = 0.012$ ) compared to the older inactive group (Figure 7). However, there were no significant main effects of age or long-term exercise training on plasma MPO levels and no significant interaction effect (Figures 8A and 8B). In addition, there was a significant inverse correlation ( $r = -0.348$ ,  $P = 0.035$ ) across all subjects between plasma MPO levels and triglyceride levels (Figure 9). The numbers reported here for plasma MPO concentrations are consistent with other results in the literature (4, 55).

## NO<sub>x</sub>

Tests of multiple comparisons revealed that the older inactive group had significantly higher plasma NO<sub>x</sub> levels than either the older active ( $P = 0.021$ ) or the young active ( $P = 0.02$ ) groups (Figure 10). However, there were no significant main effects of age or long-term exercise training on plasma NO<sub>x</sub> levels and no significant interaction effect (Figures 11A and 11B). In addition, there were no significant correlations between plasma NO<sub>x</sub> and any of the other outcome variables, ox-LDL, nitrotyrosine, or MPO. Interestingly, a significant inverse correlation ( $r = -0.357$ ,  $P = 0.03$ ) was found between VO<sub>2</sub> max and plasma NO<sub>x</sub> levels across all study subjects (Figure 12A). Also observed across all subjects was a significant positive correlation ( $r = 0.355$ ,  $P = 0.042$ ) between plasma NO<sub>x</sub> levels and the ratio of total cholesterol to HDL cholesterol (Figure 12B). In addition, the NO<sub>x</sub> values found in the present study are comparable to other values reported in the literature (5).

## Within-Age Group Correlation Analysis

In addition to assessing correlations across all subjects, the study examined relationships between outcome variables and conventional CVD risk factors within age groups. These analyses were justified given the lack of any statistically significant age  $\times$  activity interaction effects on the study's main outcomes. If a significant interaction effect had been uncovered, these within-age group analyses would not have been appropriate. In young subjects, plasma ox-LDL levels were negatively correlated ( $P < 0.05$ ) with VO<sub>2</sub> max (Figure 13). In older subjects, levels of ox-LDL were positively associated ( $P < 0.05$ ) with several blood chemistry measures, including total cholesterol and triglycerides (Figures 14A and 14B). Also,

a positive relationship ( $P < 0.05$ ) between plasma ox-LDL levels and the Framingham risk score and risk percentage was observed in older subjects (Figure 15A and 15B). Finally, many of the significant relationships in the combined group that were discussed above were also found within age groups. Because those relationships were already presented, the corresponding within-age group correlations are not shown.

#### *Within-Activity Level Group Correlation Analysis*

When subjects were divided by activity level, plasma MPO levels were positively correlated ( $P < 0.05$ ) with the ox-LDL to LDL cholesterol ratio in inactive subjects (Figure 16). In addition, several of the significant associations already shown for the combined group were also found within-activity level groups, but they are not shown here since the combined group relationships were already presented.

Table 1. Subject Characteristics

Characteristic	Young		Older	
	Active (n=7)	Inactive (n=8)	Active (n=12)	Inactive (n=11)
Age, yr	25 ± 4	25 ± 3	62 ± 6	64 ± 5
Height, m	1.83 ± 0.1	1.81 ± 0.04	1.76 ± 0.07	1.75 ± 0.07
Weight, kg	81.1 ± 12.5	77.9 ± 17.2	70.7 ± 10.6	74.7 ± 8.0
BMI, kg/m <sup>2</sup>	24.4 ± 3.9	23.6 ± 4.4	22.9 ± 2.6	24.3 ± 2.1
Body Fat, %	14.3 ± 5.8	14.8 ± 6.7	18.0 ± 4.5	23.5 ± 5.9 *
Glucose, mg/dL	86 ± 7	81 ± 7	94 ± 7	101 ± 9
TG, mg/dL	69 ± 19	81 ± 31	66 ± 29	103 ± 45 *
TC, mg/dL	146 ± 21	147 ± 25	199 ± 31	194 ± 35
HDL, mg/dL	53 ± 5	49 ± 11	71 ± 12	51 ± 15 *
LDL, mg/dL	79 ± 21	82 ± 22	115 ± 29	123 ± 38
TC/HDL	2.8 ± 0.6	3.1 ± 0.8	2.9 ± 0.7	4.2 ± 1.6 *
SBP, mmHg	118 ± 6	121 ± 5	126 ± 14	133 ± 10
DBP, mmHg	78 ± 5	79 ± 6	81 ± 7	86 ± 6
VO <sub>2</sub> max, mL/kg/min	60.4 ± 5.8	47.4 ± 5.7 †	50.0 ± 6.7	28.1 ± 5.8 *

Values are means ± SD. 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<sub>2</sub> max, maximal oxygen uptake. † P < 0.05 vs. young active. \* P < 0.05 vs. older active.

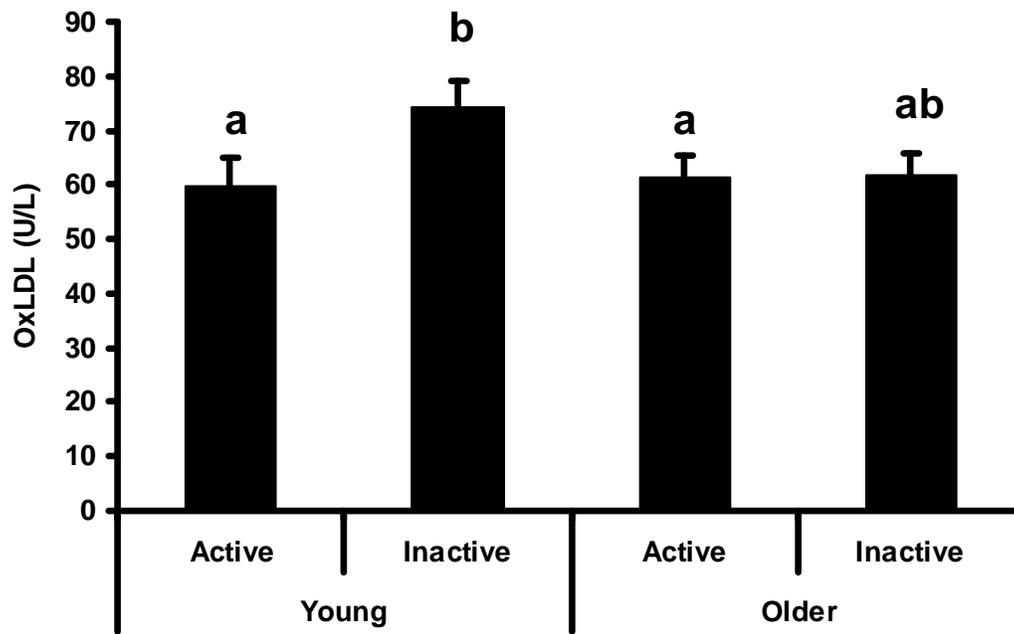
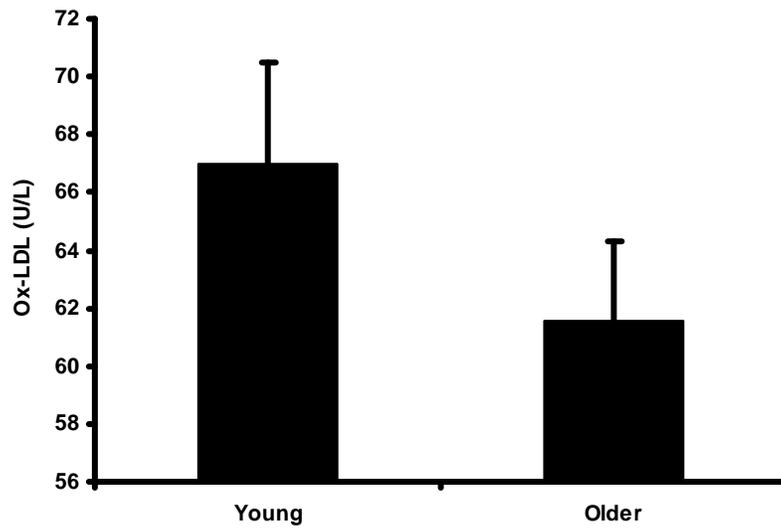


Figure 1. Between-group comparisons showing influence of age and long-term exercise training on plasma levels of ox-LDL. Values are means  $\pm$  SE. Data with like letters are not statistically different from each other ( $P < 0.05$ ).

A



B

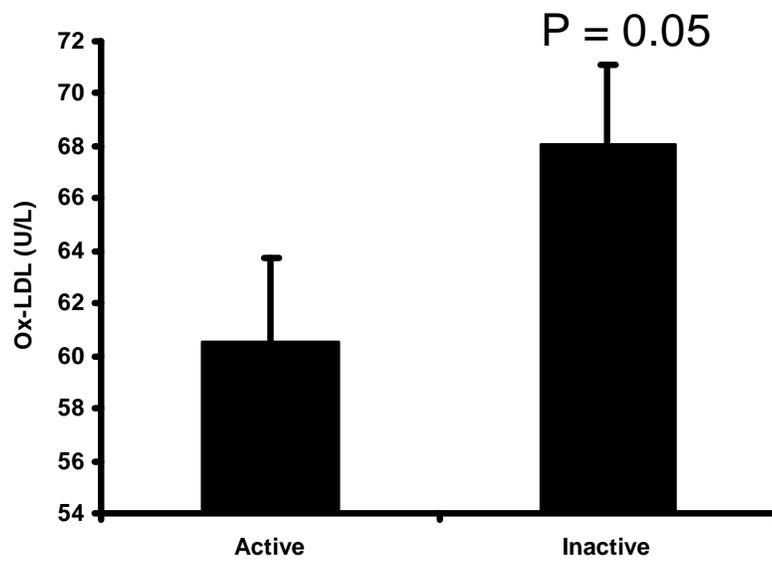
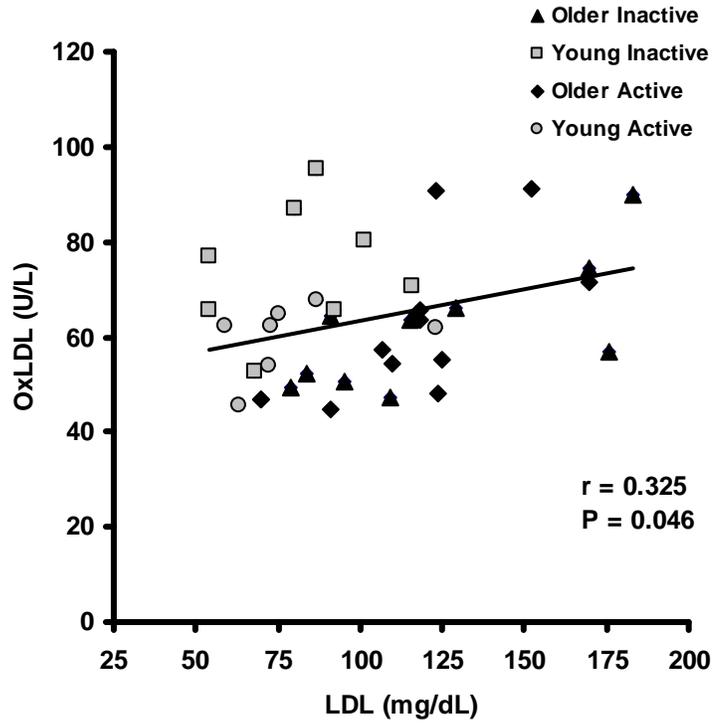


Figure 2. Main effects of age (A) and long-term exercise training (B) on plasma ox-LDL levels. Values are means  $\pm$  SE.

A



B

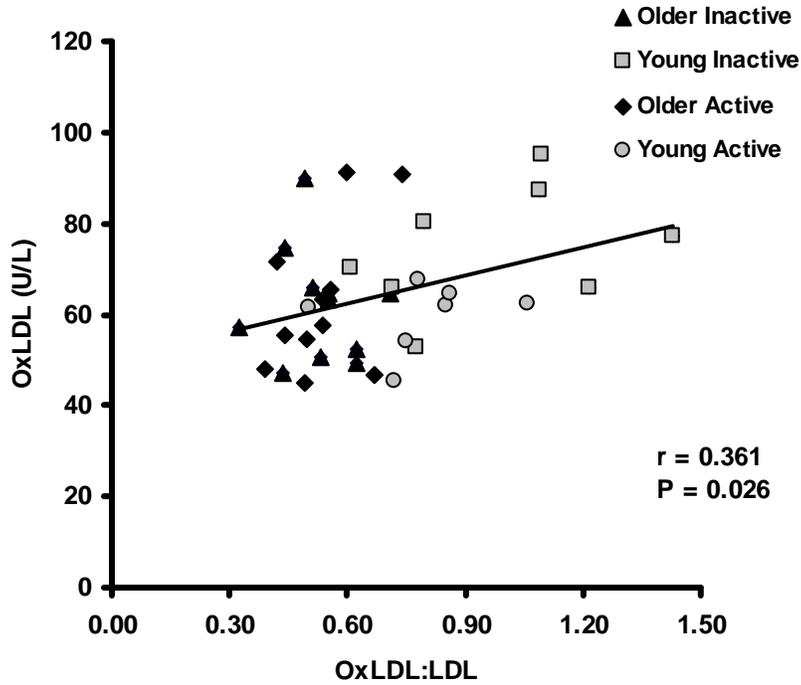
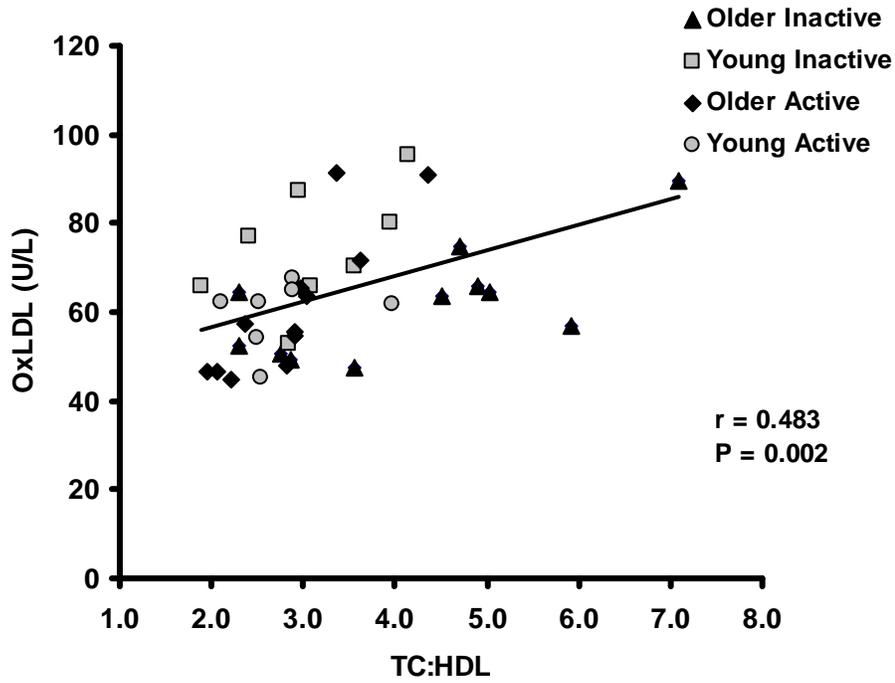


Figure 3A-B. Significant relationships ( $P < 0.05$ ) across all subjects between plasma levels of ox-LDL and the conventional CVD risk factors LDL cholesterol (A) and ox-LDL to LDL cholesterol ratio (B).

C



D

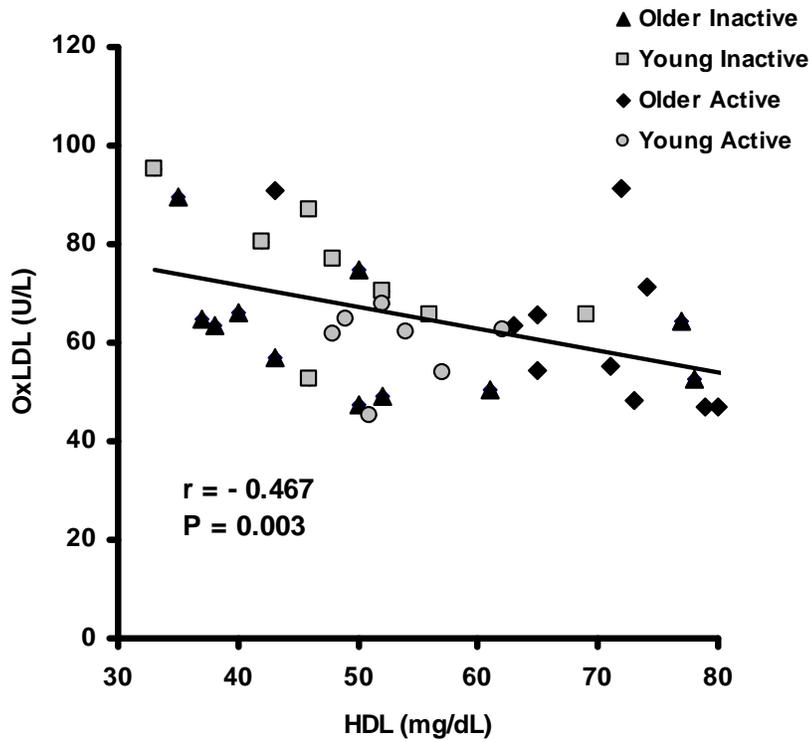


Figure 3C-D. Significant relationships ( $P < 0.05$ ) across all subjects between plasma levels of ox-LDL and the conventional CVD risk factors total cholesterol to HDL cholesterol ratio (C) and HDL cholesterol (D).

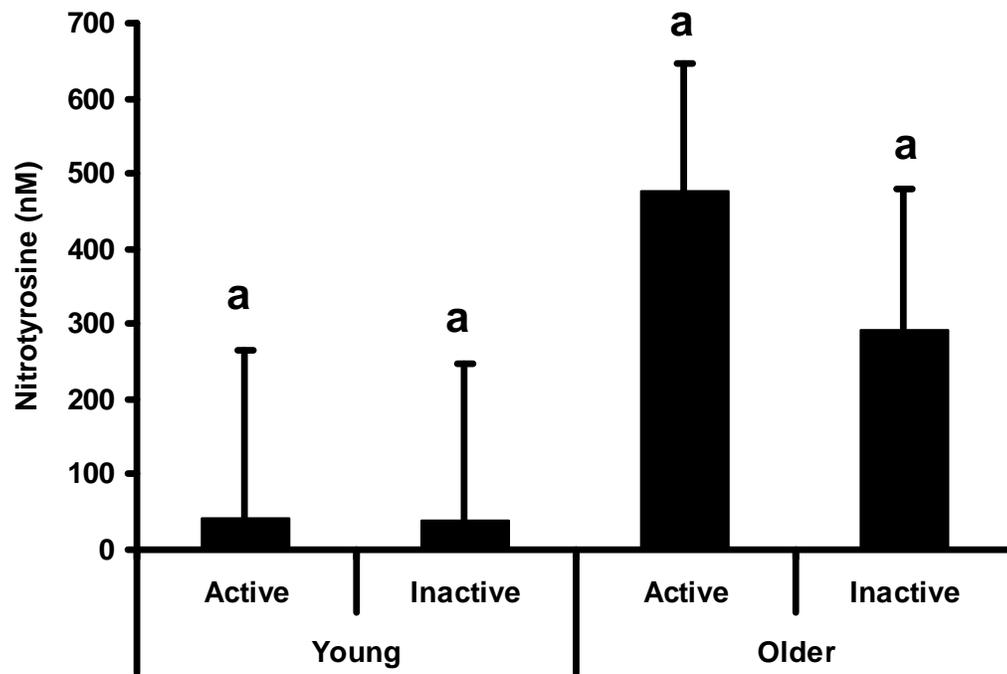
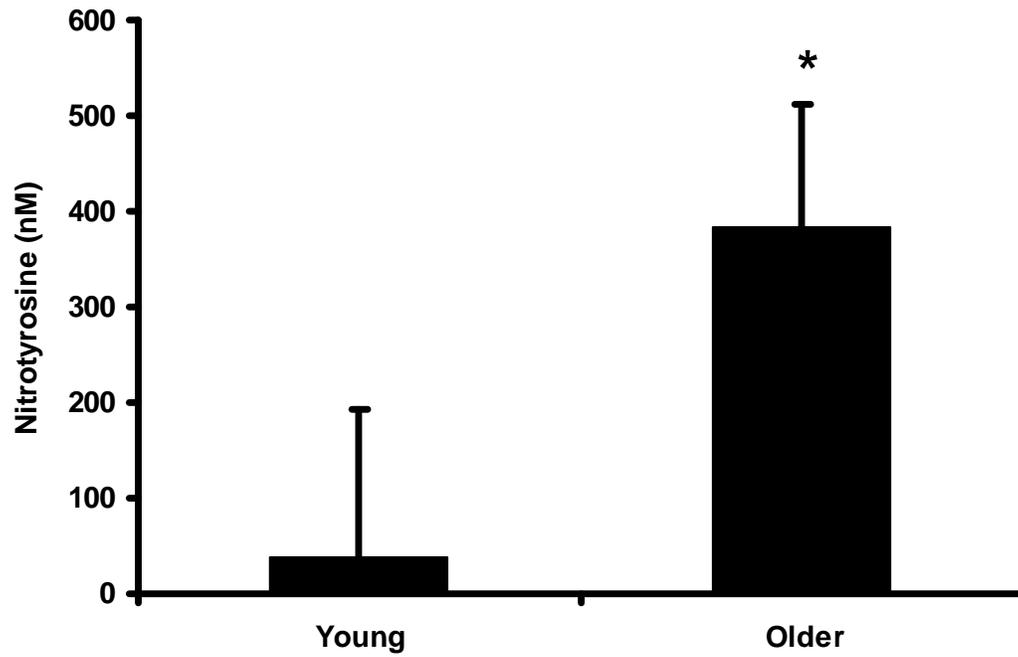


Figure 4. Between-group comparisons showing influence of age and long-term exercise training on plasma levels of nitrotyrosine. Values are means  $\pm$  SE. Data with like letters are not statistically different from each other ( $P < 0.05$ ).

A



B

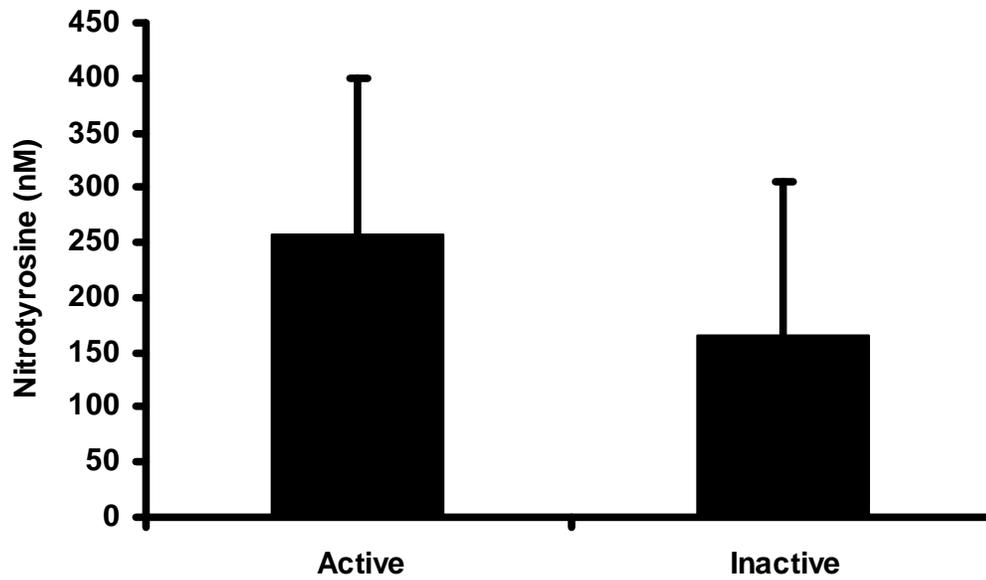


Figure 5. Main effects of age (A) and long-term exercise training (B) on plasma nitrotyrosine levels. Values are means  $\pm$  SE. \*  $P < 0.05$  vs. young.

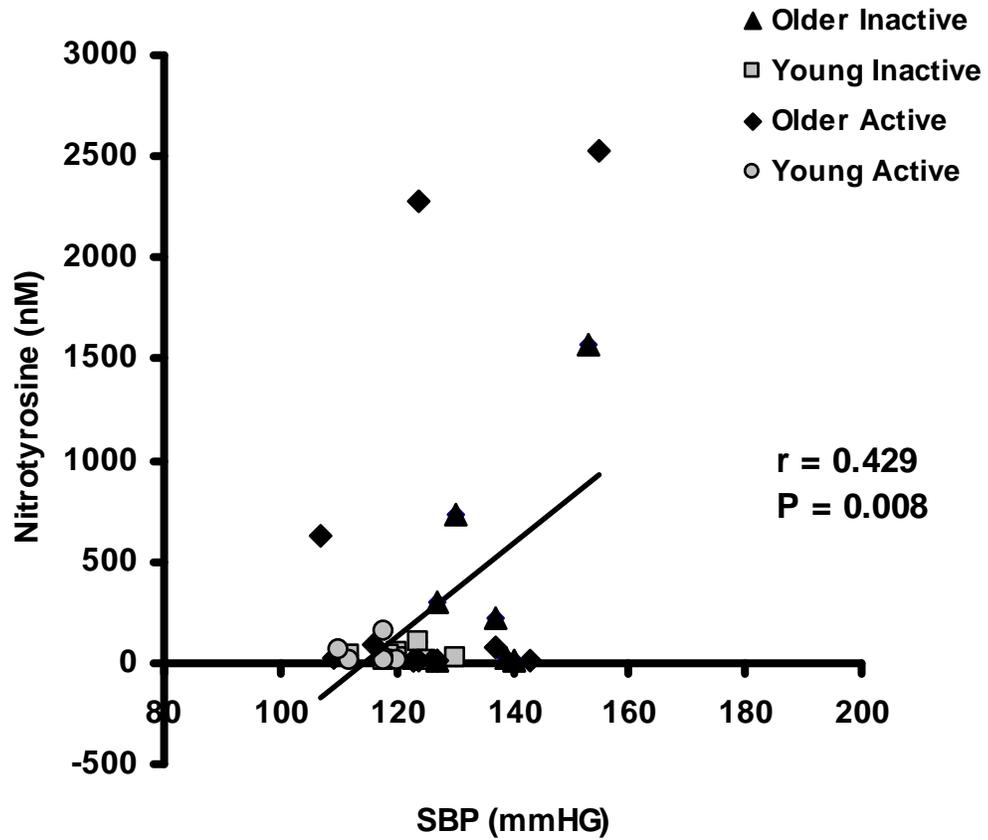


Figure 6. Significant relationship ( $P < 0.05$ ) across all subjects between plasma nitrotyrosine levels and the conventional CVD risk factor systolic blood pressure (SBP).

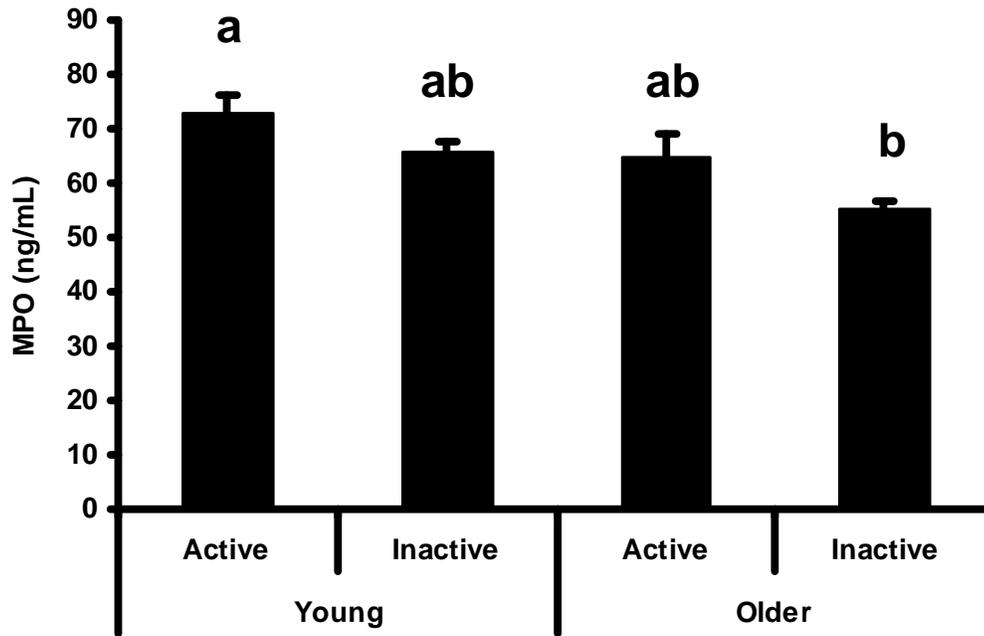
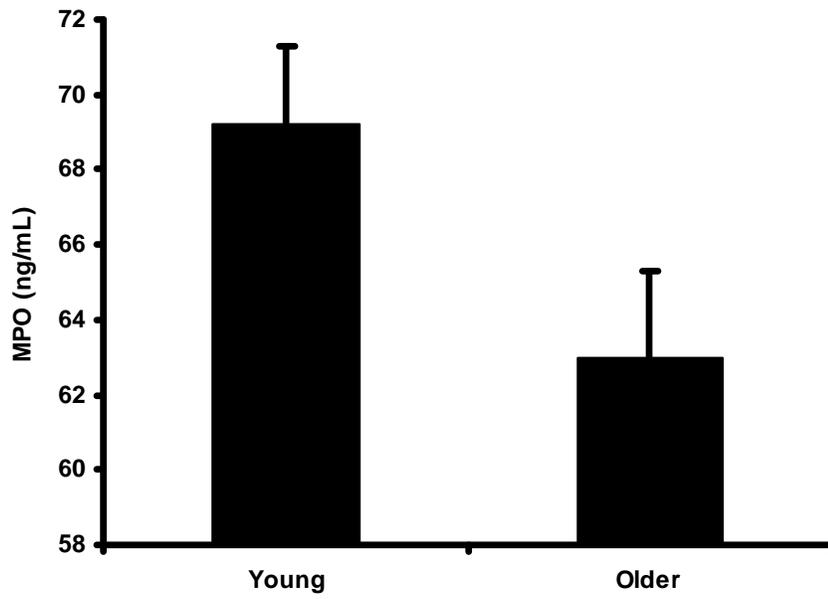


Figure 7. Between-group comparisons showing influence of age and long-term exercise training on plasma levels of MPO. Values are means  $\pm$  SE. Data with like letters are not statistically different from each other ( $P < 0.05$ ).

A



B

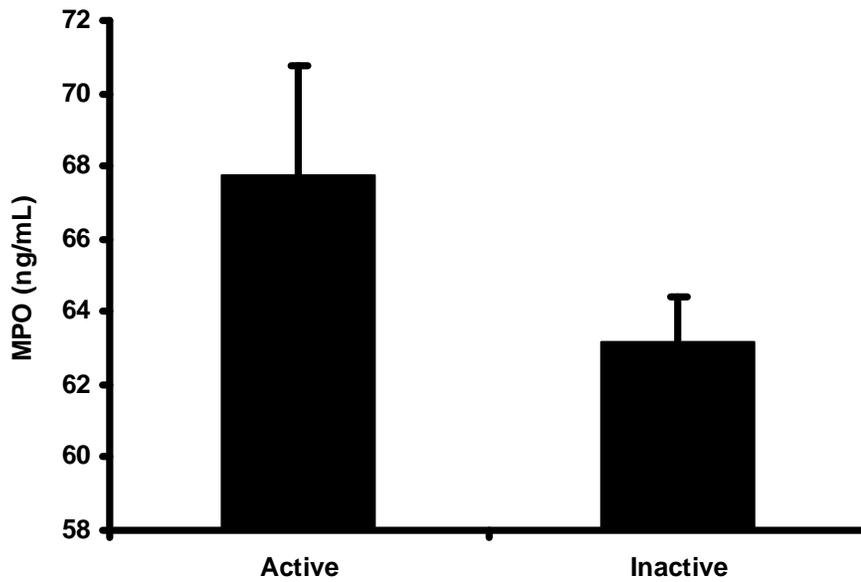


Figure 8. Main effects of age (A) and long-term exercise training (B) on plasma MPO levels. Values are means  $\pm$  SE.

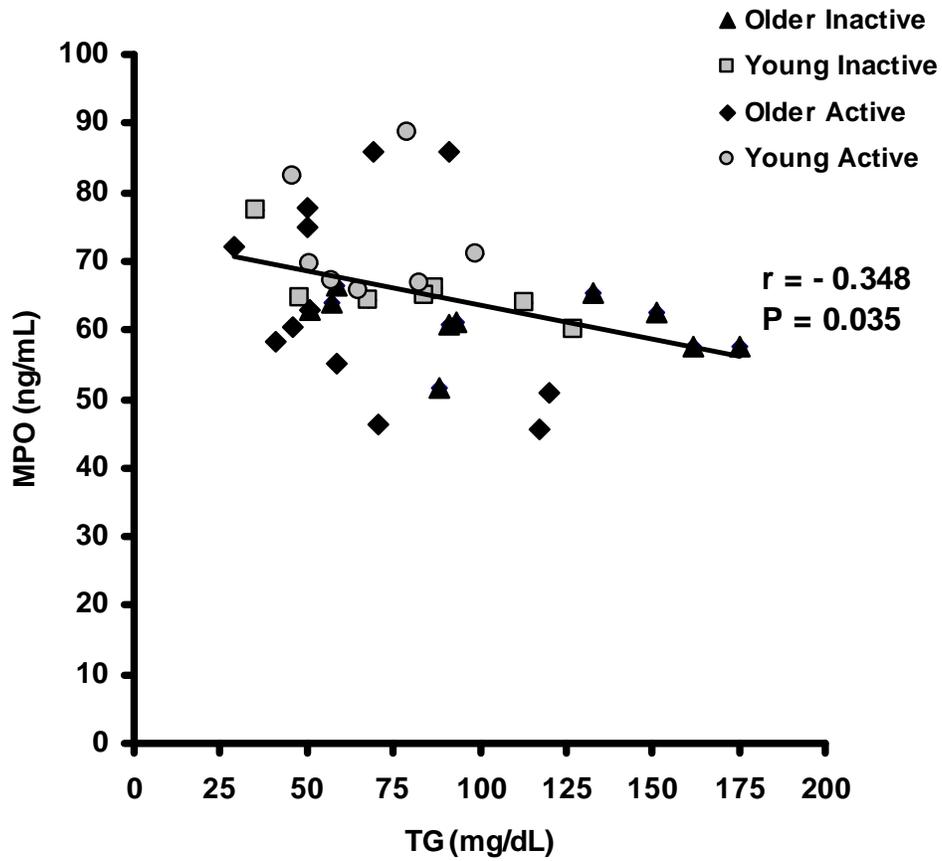


Figure 9. Significant relationship ( $P < 0.05$ ) across all subjects between plasma MPO levels and triglyceride levels.

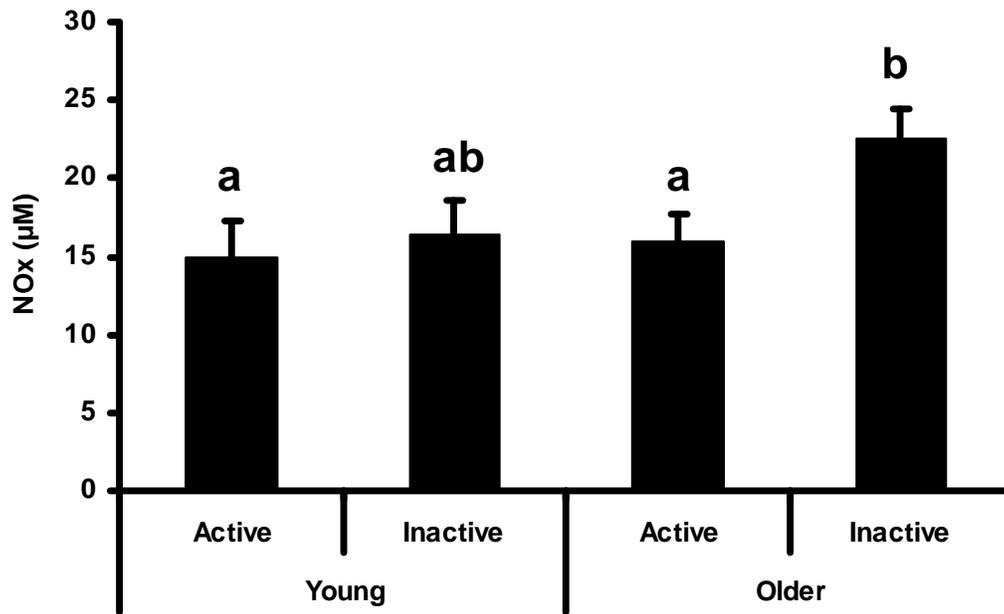
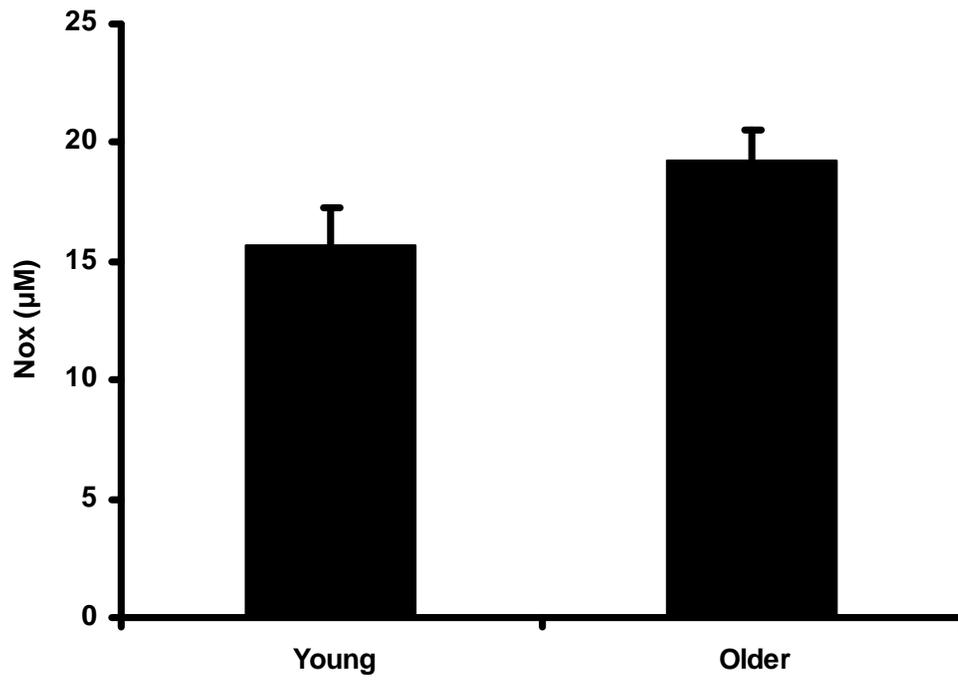


Figure 10. Between-group comparisons showing influence of age and long-term exercise training on plasma levels of NOx. Values are means  $\pm$  SE. Data with like letters are not statistically different from each other ( $P < 0.05$ ).

A



B

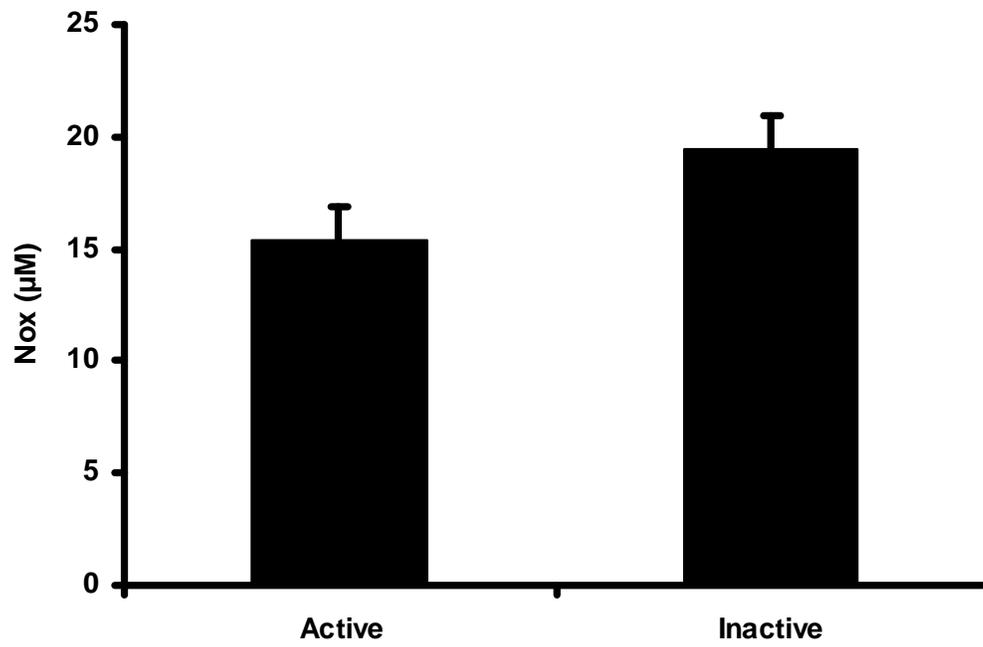
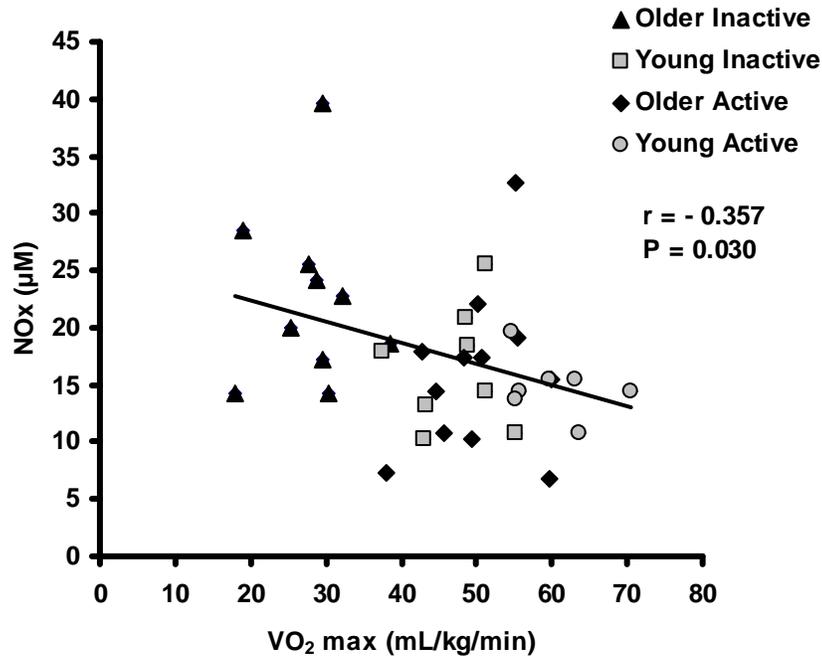


Figure 11. Main effects of age (A) and long-term exercise training (B) on plasma NOx levels. Values are means  $\pm$  SE.

A



B

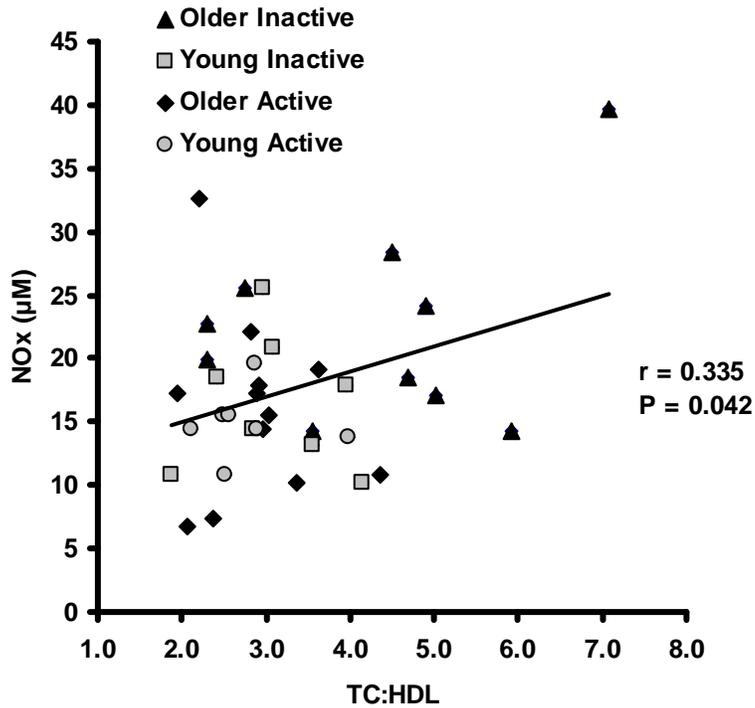


Figure 12. Significant relationships ( $P < 0.05$ ) across all subjects between plasma NOx levels and VO<sub>2</sub> max (A) and the conventional CVD risk factor total cholesterol to HDL cholesterol ratio (B).

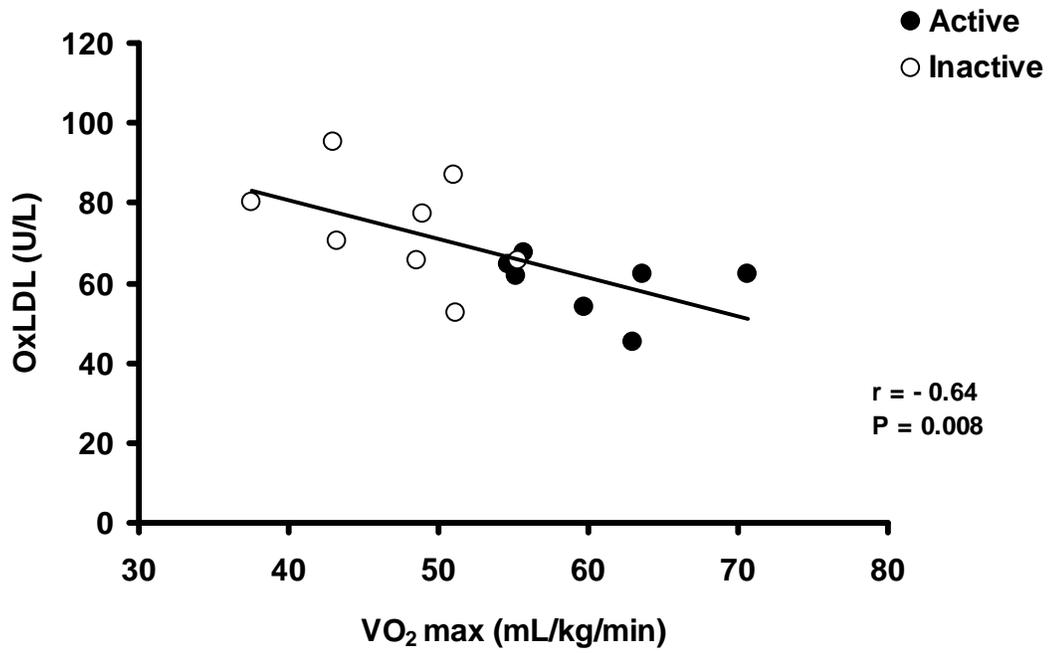
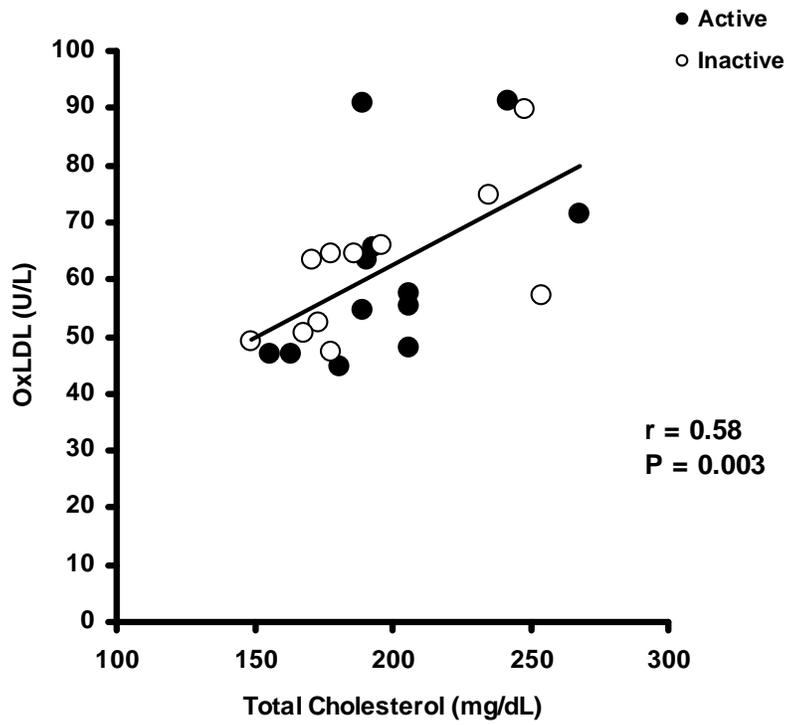


Figure 13. Significant relationship ( $P < 0.05$ ) between plasma ox-LDL levels and VO<sub>2</sub> max in young subjects.

A



B

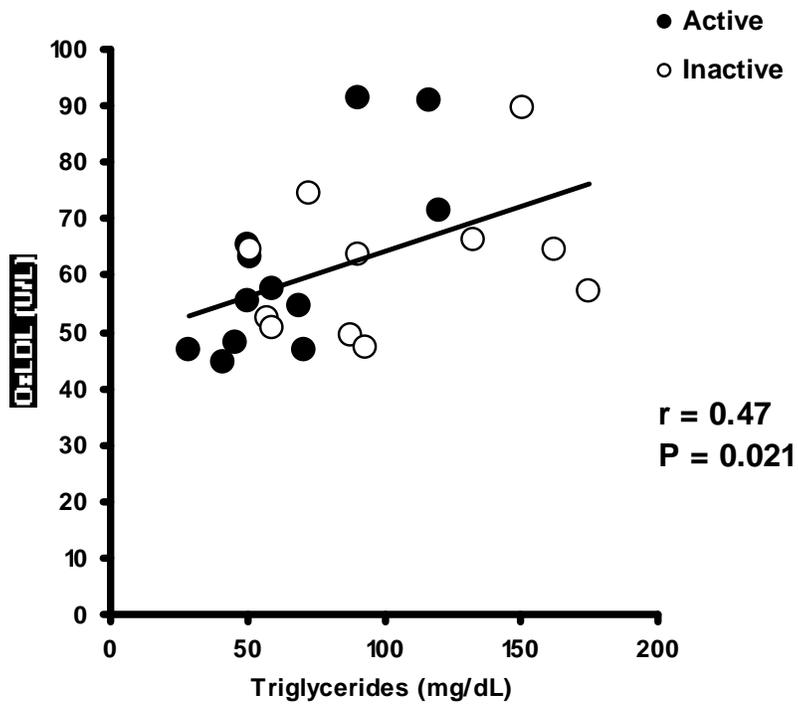
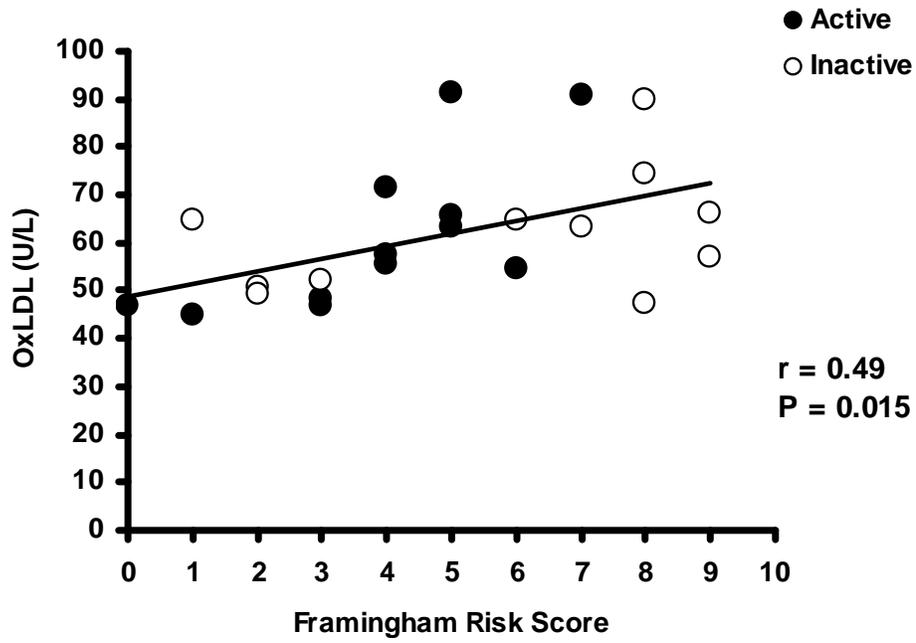


Figure 14. Significant relationship ( $P < 0.05$ ) between plasma ox-LDL levels and total cholesterol (A) and triglycerides (B) in older subjects.

A



B

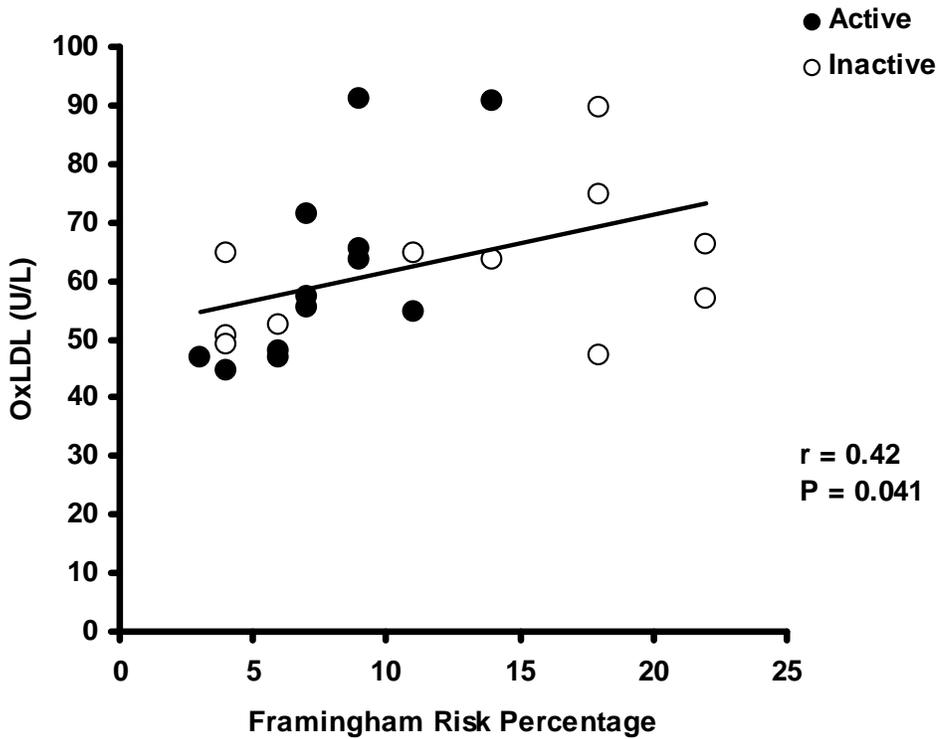


Figure 15. Significant relationships ( $P < 0.05$ ) between plasma ox-LDL levels and Framingham risk score (A) and Framingham risk percentage (B) in older subjects.

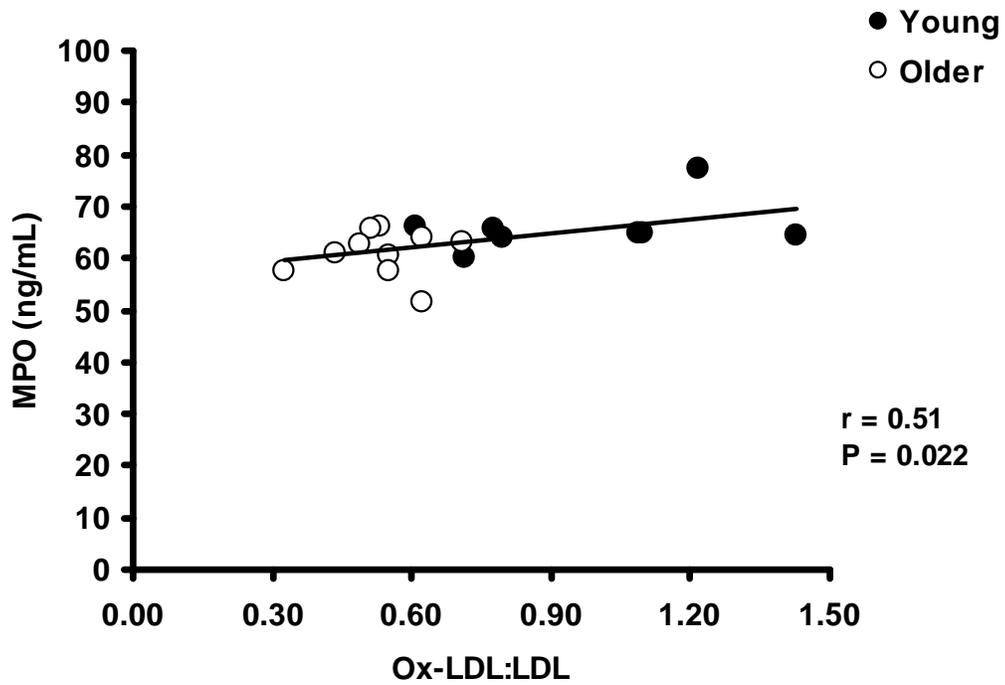


Figure 16. Significant relationship ( $P < 0.05$ ) between plasma MPO levels and ox-LDL to LDL cholesterol ratio in inactive subjects.

## Discussion

The results of this study suggest that plasma oxidative stress levels are affected by long-term exercise training and age, but not consistently in the hypothesized direction. In line with the presented hypotheses, ox-LDL levels did show a trend toward being lower with chronic exercise, with young and older active groups having significantly lower ox-LDL levels than young inactive individuals. In addition, a main effect of age was reported in nitrotyrosine, with older individuals showing significantly elevated plasma nitrotyrosine levels compared to young subjects. However, MPO levels were not reduced and NO<sub>x</sub> levels were not elevated in the chronic exercisers compared to their inactive peers, and older inactive individuals displayed the lowest MPO and the highest NO<sub>x</sub> levels. The study also found that plasma NO<sub>x</sub> levels were not related to plasma oxidative stress levels. However, plasma oxidative stress levels, particularly ox-LDL levels, were related to various conventional CVD risk factors. The relationships between oxidative stress markers and other CVD risk factors suggest that, at the very least, plasma oxidative stress levels may serve as indicators of CVD risk. Plasma ox-LDL levels appear to be particularly attractive as potential markers of CVD risk, as the findings indicate that decreases in ox-LDL levels with chronic exercise could potentially explain some of the CVD risk reduction that occurs with regular exercise. However, the lack of association between plasma oxidative stress and NO<sub>x</sub> levels does not support the hypothesis that reductions in NO bioavailability could explain how elevations in oxidative stress increase CVD risk.

### *Effects of Age and Activity Level on Oxidative Stress Markers*

One of the hypotheses that has been put forth to explain aging has postulated that the loss of functional capacity during senescence can be explained by the irreversible accumulation of molecular oxidative damage (62). 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 subjects compared to the young subjects. The findings suggest that plasma ox-LDL and MPO do not respond to age the same way that nitrotyrosine does. Thus, perhaps not all of the selected oxidative stress markers contribute to the larger increase in oxidative damage that has been hypothesized to occur with age. However, this study appears to be the first to compare the 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.

One alternative explanation for why the present study did not consistently show elevated oxidative stress levels in older groups is the unique nature of the study population. All of the subjects studied were healthy individuals. In contrast, most previous studies have compared ox-LDL, nitrotyrosine, and MPO levels in CVD patients to those in healthy controls, finding elevated oxidative stress levels with the onset and progression of the disease (21, 61, 69, 71). Given the good health of the present study's subjects, perhaps it is not surprising that plasma ox-LDL and MPO did not show age-related changes. A previous study suggested that plasma ox-LDL levels are quite low in healthy people (24). The present findings support this, as the mean plasma ox-LDL levels reported in older subjects (61.6 U/L) and young subjects

(67.0 U/L) are relatively low compared to a previous study that reported an average ox-LDL level of 110 U/L in the plasma of patients who subsequently suffered a CVD event (39). Thus, in the present study's population of healthy subjects, age-related differences in plasma ox-LDL may be difficult to detect. While there is currently no research in the literature on how age affects plasma MPO levels, one can speculate that the use of healthy subjects might also explain the lack of a main effect of age on MPO levels in the present study.

When examining the effects of exercise on systemic levels of oxidative stress, one must consider the apparent contrasting effects of an acute exercise bout versus chronic exercise training. A higher metabolic rate is required during an intense exercise bout, and it results in increased ROS generation during the bout and elevated oxidative stress levels after the bout (9). However, some previous research has indicated that exercise training may reduce systemic levels of oxidative stress (15, 45). Thus, in the present study it was not surprising that a trend was observed linking active individuals to reduced plasma ox-LDL levels. This trend is supported by exercise intervention studies that have found reduced plasma ox-LDL levels in older men and women following a high-intensity exercise intervention (10) and decreased serum ox-LDL concentration after exercise training in sedentary, healthy young men and women (13). The lower plasma ox-LDL levels in active versus inactive subjects in the present study are thus consistent with other results reported in the literature.

However, the present study demonstrated no such reduction in nitrotyrosine or MPO levels in the chronic exercise groups. This finding was somewhat unexpected, as one earlier study found a reduction in plasma nitrotyrosine levels with a 16-week

exercise intervention in older subjects (15). Another study reported a decline in serum MPO levels with a 12-week endurance training intervention in adult subjects with elevated CVD risk (56). Although the present nitrotyrosine and MPO results do not agree with these previously reported findings, they are not without precedent. The effect of training on systemic levels of oxidative stress is still under investigation, and at least one human study concluded that older adults who exercise regularly have higher levels of oxidative stress than their sedentary peers (40). The overall scarcity of information in the literature on changes in plasma ox-LDL, nitrotyrosine, and MPO levels with exercise training prevents definitive conclusions from being drawn on how chronic exercise affects systemic levels of oxidative stress.

In addition, the present findings that nitrotyrosine and MPO levels were not reduced in active individuals compared to their inactive counterparts could be a result of the unique features of this particular study. First of all, the present study examined the effects of long-term exercise training on oxidative stress levels, while all the previous studies have focused on the effects of short-term training. The older subjects in the present study had exercised regularly for over thirty years, and the young subjects for over three years, and these training durations are vastly different from the typical twelve- to sixteen-week training intervention. The beneficial effects of short-term training on plasma oxidative stress levels may disappear with continued training. This would explain how the effects of long-term training on circulating biomarkers of oxidative stress could differ from the effects of short-term training.

The second unique feature of the present study is the nature of the inactive groups. The sedentary subjects selected for the study had no readily apparent health

issues and appeared to suffer no negative consequences from their years of physical inactivity. Despite their sedentary lifestyles, the young inactive subjects had body weights, BMI values, body compositions, blood pressures, and blood chemistry profiles that were no different from their active peers who had been exercising regularly for over three years. The older inactive subjects had body weights, BMI values, and blood pressures that were not statistically different from their active peers who had been exercising regularly for over thirty years. This makes the individuals in both the young and older inactive groups quite exceptional. The inactive subjects may share some genetic advantage that has allowed them to avoid many of the physical consequences typically associated with inactivity. In the same way that the inactive subjects avoided obesity and hypertension, two negative consequences one would expect to occur with many years of inactivity, the inactive subjects in the present study may also have avoided higher oxidative stress levels. This could explain why nitrotyrosine and MPO levels were not reduced in the long-term exercise training groups compared to the inactive groups.

#### *NOx Levels with Age, Activity Level, and Oxidative Stress*

The present study found no main effects of age or activity level on plasma NOx levels. These results contrast with previous research that indicates that NOx levels decline with age, resulting in impaired endothelium-dependent vasodilation that can be reversed with exercise training (12). Other studies have also found that short-term aerobic exercise training increases plasma NOx concentration in healthy young individuals (36). However, the responses to longer-term training ( $\geq 16$  weeks) seem to be more variable. In a group of older men and women, 24 weeks of aerobic

exercise training failed to result in any improvements in plasma NO<sub>x</sub> levels (5). Given the extremely long duration of training in the present study, the lack of association between activity level and plasma NO<sub>x</sub> levels is less surprising.

The present study also found no relationships between plasma NO<sub>x</sub> levels and plasma ox-LDL, nitrotyrosine, or MPO levels. These findings seem to contrast with previous research that has linked increases in oxidative stress to endothelial dysfunction (64). However, the present study's plasma NO<sub>x</sub> measurement is an assessment of NO bioavailability as opposed to a measurement of endothelial function. Some functional test, such as forearm blood flow in response to reactive hyperemia or vasodilation in response to the infusion of an agonist, is needed to assess endothelial health. Therefore, the present study's finding that the older inactive group had significantly higher plasma NO<sub>x</sub> levels than either the older or young active groups does not mean that the older inactive group had the best endothelial function. The only conclusion that can be drawn is that the older inactive group had the highest NO bioavailability. Although a reduction in NO bioavailability is one of the mechanisms thought to cause endothelial dysfunction (5), impairments in endothelial function can occur without decreases in NO<sub>x</sub> 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 (42). Thus, plasma NO<sub>x</sub> 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 levels in the present study is still surprising. Previous research has shown that ox-LDL reduces NO bioavailability by reacting with NO to inactivate it and by stimulating the release of NO scavengers (28). Elevated levels of nitrotyrosine have previously been associated with increased NO consumption and inhibited NO synthesis (7). MPO has previously been shown to decrease NO levels by inhibiting and uncoupling NOS, and consuming NO (48). A possible explanation for why the present study failed to uncover any associations between plasma ox-LDL, nitrotyrosine, and MPO levels and plasma NO<sub>x</sub> levels has already been discussed. In a few words, the particularly long duration of training undertaken by the active subjects in this study may have resulted in different effects on plasma NO<sub>x</sub> levels than a short-term intervention. The previously reported finding that plasma NO<sub>x</sub> levels do not improve with longer-term exercise training (5) suggests that any relationship between oxidative stress levels and NO bioavailability that occurs with short-term training may disappear with continued training.

The present study also found significantly higher plasma NO<sub>x</sub> levels in the older inactive subjects than either the older active or young active groups. There are several possible explanations for this surprising finding. First of all, the elevated NO bioavailability in the older inactive subjects may be the result of the partial uncoupling of endothelial nitric oxide synthase (eNOS) with age and inactivity. When eNOS is fully uncoupled, usually due to the absence of the substrate L-arginine or the reduced availability of the cofactor BH<sub>4</sub>, the ROS superoxide and hydrogen peroxide are produced instead of NO. However, when only partially uncoupled,

eNOS produces superoxide and NO (7). If old age or inactivity triggered the partial uncoupling of eNOS, the result would be runaway NO production. Because NO is a free radical, unchecked NO production could be deleterious. Therefore, the elevated NO bioavailability in the older inactive group could reflect a potentially harmful increase in NO production by the vascular endothelium.

Another possible explanation for the present study's finding that the highest NO<sub>x</sub> levels occurred in the older inactive group is that the excess NO is being produced by some source other than the vascular endothelium. Endothelial cells constitutively produce the NO that plays a role in vasodilation via eNOS (43). However, larger quantities of NO are produced in macrophages by inducible NOS (iNOS) in response to inflammatory stimuli (18). Advanced age and inactivity may activate inflammatory pathways that result in increases in plasma NO via iNOS. Instead of improving endothelial health, the NO produced in response to inflammation has proatherogenic effects, oxidizing lipoproteins and promoting endothelial dysfunction (3). Thus, the synthesis of NO by eNOS may be impaired with age or inactivity, but plasma NO<sub>x</sub> levels may actually increase due to the production of NO by inflammatory cells. This would also explain how oxidative stress, which is known to activate the inflammatory response, might not be linked to reduced plasma NO<sub>x</sub> levels.

#### *Relationships between Oxidative Stress and Conventional CVD Risk Factors*

The present study found several significant relationships between oxidative stress levels, particularly ox-LDL, and conventional CVD risk factors. Across all study subjects, plasma ox-LDL was positively correlated with LDL cholesterol, the

ratio of ox-LDL to LDL cholesterol, and the ratio of total to HDL cholesterol. In addition, plasma ox-LDL was negatively correlated with HDL cholesterol. These relationships between ox-LDL and blood lipid profiles are not unexpected or unprecedented. First, ox-LDL molecules are formed by the oxidative modification of native LDL, so any increase in LDL is likely to be associated with an increase in ox-LDL. A significant positive association between ox-LDL and LDL cholesterol has been reported previously (22). In addition, 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 (41). Indeed, a previous study supports the present finding that plasma ox-LDL levels are inversely correlated with HDL cholesterol levels (21).

Fitting the observed relationship between ox-LDL and blood chemistry, ox-LDL levels were also positively correlated with total cholesterol and triglycerides in the older subjects. These findings are supported by previous research that has shown that hypercholesterolemia and dyslipidemia are strong independent predictors of plasma ox-LDL levels (20). Also 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. 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 (39). Thus, many of the ox-LDL correlations reported in the present study are not surprising.

Within 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 (11). In addition, one of the secondary oxidation products generated by MPO, nitrogen dioxide, has been reported to promote ox-LDL formation (6). 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.

### Limitations

The present study is limited by the cross-sectional design and the relatively small sample size. However, there was sufficient power to detect the differences indicated. In addition, because the plasma ox-LDL levels in older and young subjects were measured separately, the comparisons across age groups for ox-LDL are less convincing. Also, the use of plasma measurements may not be the best way to assess oxidative stress, as the systemic nature of these measurements means that they may not reflect the levels of oxidative stress present locally within every tissue. However, there is substantial evidence to suggest that the plasma levels of the selected oxidative stress biomarkers do have clinical significance (4, 20-23, 39, 50, 61, 63, 66, 71). In addition, the plasma markers of oxidative stress measured in the present study, although chosen because of their prevalence in previous research, may have left out other important plasma measurements such as malondialdehyde (MDA) or thiobarbituric acid reaction substances (TBARS).

Finally, the measurements of plasma ox-LDL, nitrotyrosine, and MPO levels in this study are reliant on the quality of the ELISA kits that were purchased. The

construction of standard curves for each assay act as checks to make sure the values measured are accurate, but one must accept that the assay kits measure what they purport to measure. The validity of any ELISA depends on the specificity of the primary antibody for the molecule of interest. The researcher must accept that the antibodies provided in the kits by the manufacturers are binding the molecules of interest, which in this study were ox-LDL, nitrotyrosine, and MPO.

## Conclusions

The findings of the present study suggest that age and chronic exercise training do modify circulating biomarkers of oxidative stress. While the effects of age and long-term exercise training on plasma markers of oxidative stress were not consistently in the hypothesized direction, there was evidence that a sedentary lifestyle may contribute to elevated ox-LDL levels and that nitrotyrosine levels may increase with age. 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 levels contribute to increases in CVD risk. Additionally, the many associations between plasma oxidative stress levels and conventional CVD risk factors suggest that plasma oxidative stress may serve as a marker of CVD. Future research may even show that oxidative stress plays a role in the pathogenesis of atherosclerosis. The present study also indicates that reductions in plasma oxidative stress, particularly plasma ox-LDL, may explain some of the observed reduction in CVD risk with exercise training. This sets the stage for future research to determine how the training-induced reduction in oxidative stress levels could help prevent CVD. And finally, while more investigation is needed, the present results suggest that adding a measure of plasma oxidative stress to the conventional risk factors used to calculate CVD risk may improve prediction of the disease.

## Review of Literature

### Cardiovascular Disease (CVD)

#### **CVD and Atherosclerosis**

Cardiovascular diseases include high blood pressure, coronary heart disease, stroke, and heart failure. Collectively, these diseases of the heart and vasculature are the number one cause of mortality in the developed world. The process underlying the development of CVD is atherosclerosis. The atherosclerotic process is characterized by the narrowing of arteries due to plaque buildup. In addition to impeding blood flow, the plaques can rupture, compromising the flow of oxygen to critical organs. The response to injury hypothesis of atherosclerosis says that the process begins when the endothelial layer, which is the semi-permeable barrier between the blood and the arterial wall, is injured. While the exact sequence of events is still being studied, the injury to the vessel wall is believed to trigger a change in endothelial permeability that allows LDL cholesterol molecules to enter the intima where they undergo chemical changes. Once modified, LDL molecules first attract monocytes, forming foam cells, and then recruit smooth muscle cells, forming fatty streaks. The buildup of smooth muscle cells and the subsequent development of a connective tissue network results in a fibrous plaque that begins to narrow the artery. When the plaque is disrupted, it may block blood flow or trigger more coagulation and platelet binding to form a more complicated lesion. This general

outline of the atherosclerotic process is commonly used as the foundation for further studies attempting to provide a more complete picture of the process.

### **Conventional Risk Factors**

In the last 50 years, extensive research has identified several factors that independently and significantly increase a person's risk for developing CVD. These intensely-studied risk factors include gender, heredity, smoking status, high cholesterol, hypertension, obesity, and diabetes. Two of the conventional risk factors particularly relevant to this study are age and physical inactivity.

The increase in CVD risk with increasing age is striking. An average American male aged 30-34 has a 3% risk of developing CVD over a ten-year period, while his ten-year risk jumps to 21% when he is 60-64 years old (69).

In addition, studies have repeatedly linked physical inactivity to unfavorable CVD risk factor profiles, including elevated LDL levels and blood pressure, reduced HDL levels, and increased risk of diabetes and obesity (17). Also well-established is the reduction in CVD risk that occurs with exercise training. In previously sedentary individuals, it is generally estimated that exercise training reduces LDL cholesterol by 10 mg/dL, increases HDL cholesterol by 5 mg/dL, decreases systolic blood pressure by 10 mm Hg, and decreases diastolic blood pressure by 8 mm Hg. In addition, only 7 days of exercise training has been found to improve glucose tolerance and decrease insulin resistance in subjects with mild Type II diabetes (58). The importance of physical inactivity as a risk factor for CVD is underscored by a meta-analysis that found a relative risk of death from CVD of 1.9 for inactive individuals compared to their active peers (2).

## **Novel Risk Factors**

The conventional risk factors above have long been considered in estimating an individual's CVD risk. However, in the late 1990s, a growing number of researchers began to question the degree to which conventional risk factors accounted for a person's CVD risk. Several studies concluded that conventional CVD risk factors might explain only 50% of CVD cases (16). In addition, the exercise training-induced reductions in CVD risk could be only partially explained by improvements in conventional risk factors. For example, Mora and colleagues associated higher levels of physical activity with fewer CVD events and found that differences in conventional risk factors could explain only 59% of that inverse relationship (44). The implication that other factors must play a significant role in the development of CVD has not been accepted without debate. Research has emerged to bolster the earlier claims that conventional risk factors account for far more than 50% of an individual's CVD risk (31).

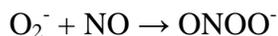
Although the question is not yet settled, the ongoing debate has led to a growing body of research attempting to identify additional CVD risk factors that might help provide a more complete picture of a person's CVD risk. Much of the attention has focused on emerging risk factors in the areas of oxidative stress, inflammation, and endothelial dysfunction. Potential novel CVD risk factors in the area of oxidative stress include plasma levels of oxidized LDL, nitrotyrosine, and myeloperoxidase. Because of the many physiological and biochemical links among oxidative stress, inflammation, and endothelial dysfunction in the pathology of atherosclerosis, the research in all three emerging areas will be discussed.

### Oxidative Stress

Energy is produced in mammalian cells by reducing oxygen to water during aerobic respiration. These reduction-oxidation reactions produce reactive oxygen species (ROS) as intermediates, and these by-products influence a variety of cellular activities in both normal and pathological conditions (47). ROS have been shown to play important roles in cell signaling, vascular growth and differentiation, cell apoptosis, and platelet aggregation (14). To properly execute these roles, ROS production must be carefully modulated and a steady-state balance must be maintained between the levels of oxidants and antioxidants. When this delicate balance is disrupted and excess ROS results, the body experiences “oxidative stress” as the highly reactive ROS interact with lipids, proteins, and DNA to cause cellular damage (47). In addition to cell damage, research suggests that oxidative stress can contribute to the development of various pathologies including aging, dementia, and atherosclerosis (47).

#### **ROS and RNS**

ROS can be divided into two categories: nonradicals and free radicals (64). Important nonradicals in oxidative stress pathways include hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hypochlorous acid (HOCl). Free radicals are highly reactive because they have at least one unpaired electron. When two free radicals meet, they will usually join their unpaired electrons to form a covalent bond, resulting in a nonradical product. An example of this is the reaction between the free radicals superoxide and nitric oxide to form the nonradical product peroxynitrite:



A free radical can also react with nonradical molecules, either by abstracting a hydrogen atom from the nonradical or adding to it (64). Lipids, proteins, and nucleic acids are all examples of nonradicals that are altered in reactions with free radicals (47). Because the products of these reactions are free radicals, ROS production is amplified, potentially leading to oxidative stress.

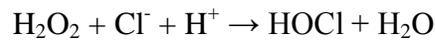
In addition to ROS, reactive nitrogen species (RNS) are often referred to as oxidants because of their tendency to remove electrons. The oxidative stress and resulting cell damage associated with ROS can be equated to the nitrosative stress caused by RNS, and in many studies, including this one, the term “oxidative stress” is used to describe the impact of both excess ROS and RNS. In addition, this paper may at times refer to “nitro-oxidative stress” in an attempt to encompass the damage caused by both of these processes.

### **Sources of ROS**

The major intracellular source of ROS is the mitochondrial electron transport chain, which produces superoxide as a by-product (29). The ROS in blood vessels have several other sources, including NADPH oxidase, xanthine oxidase, eNOS, lipoxygenase, and myeloperoxidase (64).

The NADPH oxidase enzyme complex, present in many cells including vascular smooth muscle cells and endothelial cells, catalyzes the reduction of molecular oxygen to generate superoxide. Xanthine oxidase catalyzes the oxidation of xanthine to uric acid, generating superoxide in the process. Endothelial nitric oxide synthase (eNOS) is responsible for the oxidation of L-arginine to nitric oxide (NO). When uncoupled, eNOS transfers electrons to molecular oxygen rather than L-

arginine, resulting in superoxide formation (64). Lipoxygenases catalyze the oxidation of polyunsaturated fatty acids. Myeloperoxidase (MPO) is a heme-containing enzyme secreted from macrophages and neutrophils. MPO catalyzes the formation of the nonradical oxidant HOCl in the following reaction:



HOCl can oxidize nitrite to the nonradical oxidant nitryl chloride and the radical nitrogen dioxide, both of which promote nitrotyrosine production (64).

By producing various oxidants that can alter lipids and proteins, the above enzymes can contribute to oxidative stress in certain pathological states.

#### **CVD and Nitro-Oxidative Stress**

As discussed previously, atherosclerosis develops in response to an endothelial injury or dysfunction which changes the permeability of the blood vessel wall (59). The resulting movement of LDL molecules into the subendothelial space, where they are subject to uptake by macrophages, results in foam cell formation. These foam cells become fatty streaks and are covered by fibrous plaques and, ultimately, an atherosclerotic lesion, after smooth muscle cells, additional lipids, leukocytes, and platelets are attracted and bound to the site. Recent research indicates that the oxidative stress resulting from ROS and RNS plays a crucial role in CVD. The proposed role of oxidative stress in atherosclerosis has been termed the oxidative modification hypothesis (64). According to this hypothesis, oxidized molecules play an important role in atherosclerosis both by embedding in the vascular wall and promoting plaque formation and by interacting with inflammatory and endothelial dysfunction pathways which negatively affect vascular function.

### Markers of Nitro-Oxidative Stress

One way to evaluate the amount of cell damage that is occurring due to ROS and RNS is to measure products of oxidative processes that can be found in human plasma. Ox-LDL and nitrotyrosine are two such products that can serve as indicators of the body's level of nitro-oxidative stress. In addition, the presence of enzymes that catalyze reactions producing oxidants can provide a measure of excess ROS in the body. MPO, a source of oxidants, is an example of such an enzyme that can be found and measured in human plasma.

#### **Oxidized LDL (Ox-LDL)**

The term ox-LDL does not define a particular molecule, referring instead to a non-homogeneous population of modified forms of LDL. Circulating levels of ox-LDL are the result of oxidation in the arterial wall, most likely by MPO, lipoxygenases, or RNS such as peroxynitrite (41). The oxidation of LDL begins with the loss of LDL's endogenous antioxidants, followed by the conversion of most polyunsaturated fatty acids to hydroperoxides that then react with lysine residues of apolipoprotein B-100 (64). LDL peroxidation leaves the lipoprotein more negatively charged, resulting in a decreased affinity for the LDL receptor and an increased affinity for scavenger receptors in endothelial cells, smooth muscle cells, and macrophages (41). The oxidative modification of LDL in the vascular wall is an important step in the initiation and progression of atherosclerosis because it is oxidized LDL, not native LDL, that is trapped by macrophages in the subendothelial space (64). Macrophage uptake of ox-LDL triggers the migration and proliferation of

smooth muscle cells, resulting in the formation of foam cells which can eventually develop into the fatty streaks characteristic of CVD (34).

An important early step in atherosclerosis is the adhesion of monocytes and lymphocytes to the endothelium, and ox-LDL facilitates this by up-regulating the transcription of adhesion molecules like vascular cell adhesion molecule (30). In addition to its effects on smooth muscle cell migration mentioned above, ox-LDL also supports foam cell formation by promoting monocyte infiltration into the subendothelial space, encouraging monocyte proliferation and differentiation into macrophages, and hindering the ability of resident macrophages to leave the intima (64). Interestingly, one way that HDL cholesterol prevents atherosclerosis is by inhibiting LDL oxidation and by reversing the enhanced monocyte recruitment triggered by ox-LDL (41).

Ox-LDL is also linked to endothelial dysfunction because it can reduce NO bioavailability and lead to vascular remodeling. Ox-LDL reduces NO levels directly, by reacting with NO to inactivate it, and indirectly, by stimulating the release of NO scavengers (28). Ox-LDL may also induce vasoconstriction by inhibiting NO production and stimulating endothelin expression, and a reduction in the arterial lumen occurs with the accumulation of foam cells and the necrosis and apoptosis induced by ox-LDL (41). Collectively, the reduced NO bioavailability and the thickening of the intimal layer impair the function of the endothelium.

Ox-LDL is also associated with increased thrombosis because ox-LDL stimulates platelet adhesion and aggregation, enhances the endothelium's pro-coagulant activity, and decreases fibrinolytic activity (41). In summary, ox-LDL

plays a role in foam cell formation, plaque rupture, endothelial dysfunction, vascular remodeling, and thrombosis.

### **CVD and Plasma Ox-LDL Levels**

As mentioned previously, research has shown that it is ox-LDL, and not native LDL, that makes up aortic lesions (51). Although atherosclerotic lesions are enriched with ox-LDL, the plasma of healthy persons typically has very low levels of ox-LDL (24). However, an increase in plasma ox-LDL levels occurs in certain pathological conditions, including carotid artery atherosclerosis and acute myocardial infarction, when atherosclerotic lesions rupture and expose accumulated ox-LDL to the bloodstream (23).

Further investigation has revealed that elevated levels of circulating ox-LDL are present not only during clinical manifestations of CVD, but also with the symptom-less onset and progression of the disease. For example, plasma ox-LDL levels in clinically healthy men independently predict plaque occurrence in the carotid and femoral arteries and are related to intima-media thickness and levels of the inflammatory marker C-reactive protein (22). In addition, studies have found increased plasma ox-LDL levels in people with coronary artery disease (21).

Even before the onset of CVD, however, circulating ox-LDL levels have been shown to be a useful marker for identifying individuals with a high risk of developing the disease (20). Mounting evidence suggests that plasma ox-LDL levels may contribute as much to the development of CVD and the risk of cardiac events as current conventional risk factors such as total cholesterol. In one study of apparently healthy men, plasma ox-LDL levels were predictive of future CVD events, and they

were stronger predictors than the conventional lipoprotein profile (39). Another study identified an elevated ratio of plasma ox-LDL relative to total cholesterol as a possible indicator of increased acute myocardial infarction risk (50). While more research is needed to definitively establish ox-LDL as a CVD risk factor, the literature points to a strong link between plasma ox-LDL levels and the initiation, development, and clinical outcomes of CVD.

### **Nitrotyrosine**

The formation of 3-nitrotyrosine in proteins results from the nitration of tyrosine, making it an indicator of nitro-oxidative stress. RNS including nitryl chloride, nitrogen dioxide, and peroxynitrite can mediate 3-nitrotyrosine production. As mentioned previously, nitryl chloride and nitrogen dioxide are the ultimate products of the MPO-catalyzed formation of the oxidant hypochlorous acid. Peroxynitrite (ONOO<sup>-</sup>), a powerful oxidant, results from the reaction of the free radicals nitric oxide and superoxide.

Superoxide anions are generated by components of atherosclerotic lesions including endothelial cells, smooth muscle cells, and macrophages, suggesting that peroxynitrite production occurs in the vascular wall (33). While antioxidant defenses appear to protect NO from superoxide scavenging in healthy individuals, diseases like atherosclerosis may partially uncouple eNOS and result in increased generation of both NO and superoxide, leading to peroxynitrite formation (7). Peroxynitrite promotes atherosclerosis by oxidizing lipids, proteins, and DNA, and contributing to cell death and tissue injury (35). While peroxynitrite and other RNS are unstable, the

3-nitrotyrosine production they mediate serves as a stable marker of nitro-oxidative stress.

As indicated previously, peroxynitrite is one of the molecules responsible for LDL oxidation. After in vitro studies showed that 3-nitrotyrosine was a highly specific marker for LDL oxidized by peroxynitrite, an in vivo study found that nitrotyrosine levels were 90-fold higher in LDL isolated from atherosclerotic lesions than in native LDL isolated from the plasma of healthy individuals (33). Thus nitrotyrosine levels reflect the RNS production and subsequent oxidative modifications occurring in the vascular wall.

Nitrotyrosine is associated with endothelial dysfunction because the RNS that lead to its formation reduce NO bioavailability. Peroxynitrite production consumes NO, and once formed, peroxynitrite may uncouple eNOS by oxidizing tetrahydrobiopterin (BH<sub>4</sub>), a cofactor necessary for NO synthesis (7). Any uncoupling of eNOS promotes endothelial dysfunction by decreasing NO production and leads to oxidative stress by generating superoxide. In addition, the depletion of important plasma antioxidants and nitration of proteins that occur because of peroxynitrite lead to endothelium damage that induces thickening of the intima in the arterial wall and impairs vasodilation (65). Nitrotyrosine production clearly has important consequences for endothelial function, and thus has been linked to CVD.

### **CVD and Plasma Nitrotyrosine Levels**

Research continues to examine nitrotyrosine as both a marker of the nitro-oxidative stress present in pathological conditions and a part of the molecular basis of the disease state. While nitrotyrosine levels are low in healthy individuals, they

increase significantly in disease states including inflammatory and cardiovascular disorders (63). For example, patients with celiac disease displayed elevated plasma nitrotyrosine levels compared to controls (66). Patients with established coronary artery disease had plasma nitrotyrosine levels almost twice as high as their healthy peers. These nitrotyrosine levels were significantly reduced with statin therapy, demonstrating the ability of statins to inhibit superoxide formation (61). In a study of clinical CVD patients, atherosclerotic blood vessels had significantly elevated tissue levels of nitrotyrosine when compared to control vessels (65). Thus, there is substantial evidence that elevated nitrotyrosine levels accompany many disease states.

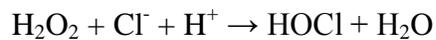
Research also supports the hypothesis that RNS play an important role in CVD by promoting lipid oxidation. In human coronary arteries, the endothelium, foamy macrophages, and inflammatory cells associated with atheroma all display signs of extensive nitration (1). Not surprisingly, an analysis of protein oxidation products in atherosclerotic lesions has revealed enriched nitrotyrosine levels, indicating that nitration induces lipoprotein aggregation (18). So in addition to serving as a strong indicator of nitro-oxidative stress in various disease states, nitrotyrosine formation may be involved in the pathogenesis of atherosclerosis.

### **Myeloperoxidase (MPO)**

By catalyzing the production of ROS and RNS, MPO contributes to nitro-oxidative stress. The enzyme MPO is the single most abundant protein in neutrophils and is also present in monocytes, two types of white blood cells that ingest bacteria and damaged or dead cells by phagocytosis (52). Collectively called phagocytes, these leukocytes typically circulate in the blood. Carr and colleagues described how

activated endothelial cells and macrophages express cytokines that attract phagocytes to sites of acute inflammation in tissues. The neutrophils and monocytes migrate toward the inflammatory site by chemotaxis, and once they reach it they ingest the pathogen or damaged cells and secrete MPO. After being released, active MPO can adhere to endothelial cells, leukocytes, and lipoproteins (8).

As described earlier, MPO catalyzes the production of hypochlorous acid, a cytotoxic oxidant:



In addition to its role in killing engulfed bacteria, hypochlorous acid may also injure normal tissue with its protein-destroying capabilities (11). Once hypochlorous acid is produced, it can lead to the formation of other secondary oxidation products including nitryl chloride and nitrogen dioxide. These MPO-generated oxidants can bind HDL cholesterol in atherosclerotic plaques, rendering the “good” cholesterol molecules dysfunctional (49). So not only does MPO lead to ROS and RNS production and subsequent nitro-oxidative stress, but it also inhibits some atheroprotective mechanisms.

MPO also seems to promote endothelial dysfunction by limiting NO bioavailability through several mechanisms. MPO inhibits and uncouples NOS, reducing NO synthesis, and MPO and its secondary oxidation products consume NO (48). Evidence that MPO impairs endothelium function includes a human study that found an inverse relationship between serum MPO levels and NO-dependent vasodilation (68). These results suggest a link between MPO levels and endothelial dysfunction.

Research also indicates that MPO activity is an important pathway for the conversion of native LDL to its pro-atherogenic form ox-LDL. The enzyme's role in the oxidative modification of LDL was first suggested by the presence of active MPO in human vascular lesions (11). MPO seems to play a role in lipoprotein oxidation because of its production of hypochlorous acid and other secondary oxidation products. LDL molecules that have been modified by hypochlorous acid have been shown to stimulate foam cell formation, increase leukocyte adherence and migration into blood vessels, boost leukocyte ROS production, and encourage the chemotactic activity of neutrophils (64). This MPO-induced migration and aggregation of various leukocytes could contribute to plaque disruption. And similar to hypochlorous acid, other MPO-generated oxidants like nitrogen dioxide have been shown to promote ox-LDL formation (6). Thus, MPO activity is linked to both the oxidative modification of LDL and the activation of the inflammatory response which are essential to the pathogenesis of CVD.

#### **CVD and Plasma MPO Levels**

Plasma MPO levels have been shown to have prognostic value for CVD. Patients with coronary artery disease had elevated blood and leukocyte MPO levels compared to controls, suggesting the enzyme's role as an inflammatory marker of CVD (71). In patients presenting to the hospital with chest pain, plasma levels of MPO independently predicted a person's risk of myocardial infarction and other future major adverse cardiac events in the ensuing six months (4). The evidence shows that plasma MPO levels are a valid marker for CVD.

However, further research suggests that MPO is more than just a marker of pathological conditions. MPO also appears to be a causative factor in the disease states. Both MPO and MPO-generated oxidants have been implicated as participants in tissue injury in inflammatory conditions including kidney disease and asthma (48). In addition, people with partial and total MPO deficiency are less likely to develop CVD (32). While more research is needed, MPO appears to play a role in atherosclerosis through pathways of nitro-oxidative stress, endothelial dysfunction, and inflammation.

### **Effects of Exercise Training on Nitro-Oxidative Stress**

As discussed previously, endurance exercise training has been repeatedly shown to decrease CVD risk by improving both conventional and novel risk factors. Although not studied extensively, evidence shows that exercise training reduces systemic oxidative stress (45). However, an acute bout of vigorous exercise actually increases oxidative stress because the higher respiration rate required during intense exercise leads to the production of more free radicals (9). The contrasting effects of exercise training and an acute exercise bout indicate that progressive adaptations occur with exercise. The plasma oxidative stress induced with each exercise bout may stimulate the arterial antioxidant response, decrease basal oxidant production, and increase eNOS activity, resulting in the ultimate drop in oxidative stress with training (38). However, much of the work supporting these mechanisms has been done in animal models. The effect of training on the antioxidant-oxidant balance in humans is more controversial.

In addition to the research on oxidative stress and exercise training, a few exercise intervention studies have examined changes in the identified nitro-oxidative stress markers with training. Plasma ox-LDL levels were reduced with a high-intensity exercise intervention in sedentary men and women over the age of 55 (10). In sedentary, healthy young men and women, exercise training increased endogenous antioxidant activity, decreased serum ox-LDL concentration, and increased LDL resistance to oxidation (13). Other studies, using baseline levels of conjugated dienes extracted from LDL as an indicator of LDL oxidation, found that levels of these so-called “mildly oxidized LDL” were reduced in young trained girls and older veteran athletes compared to controls, and after exercise training in previously sedentary middle-aged men and women (67).

Even less research has been done on changes in nitrotyrosine and myeloperoxidase with training. In subjects over the age of 65, plasma nitrotyrosine levels were decreased by 20% with a 16-week exercise intervention, and subsequent detraining abolished these adaptations (15). Serum MPO levels declined 28% in adult subjects with elevated CVD risk after a 12-week endurance training intervention (56). While these previous studies provide evidence that relatively short-term exercise training improves plasma levels of oxidative stress markers, little research has examined the effects of long-term exercise training. In addition, while some have speculated about the mechanisms linking oxidative stress to inflammation and endothelial dysfunction, few have linked plasma markers of oxidative stress with NO levels.

## Inflammation

Inflammation is emerging as a risk factor for CVD, with preliminary research finding that levels of inflammatory markers such as C-reactive protein (CRP) are predictive of CVD (57). However, more relevant to the present paper is the intersection of oxidative stress and inflammation in the development of CVD. Research is still needed to determine whether the oxidative stress triggers the inflammatory response or the inflammation initiates atherosclerosis and promotes oxidative modification as a result. But either way, these two emerging CVD risk factors are tightly linked.

First of all, even when properly balanced with antioxidants, ROS and RNS play an important role in host defense and antimicrobial activity. Their role in the immune response is evidenced by the association of impaired ROS/RNS production with susceptibility to bacterial or parasitic infection (64). When excess ROS/RNS accumulates and oxidative modifications result, it is no surprise that inflammatory pathways are involved. The positive relationship between levels of CRP and ox-LDL points to the interaction between inflammation and oxidation, and LDL oxidation has been found to trigger a state of chronic, low-level inflammation that leads to plaque development (20).

During atherosclerosis, the inflammatory response leads to the recruitment and adherence of leukocytes and platelets to the sites of endothelial injury (64). As discussed previously, ox-LDL facilitates this adhesion by up-regulating the transcription of various adhesion molecules (30). Some leukocytes that aggregate at sites of endothelial injury release MPO, the enzyme that can catalyze oxidative

modifications. In addition, leukocytes release proinflammatory cytokines which increase coagulation and cause an unfavorable lipid profile characterized by decreased HDL cholesterol and increased triglycerides (19). Components of the inflammatory response are also responsible for macrophage and smooth muscle cell recruitment into the vascular wall, contributing to foam cell and fatty streak formation. Thus, atherosclerosis can be thought of as a chronic inflammatory process whose pathogenesis is intimately linked to oxidative damage.

### Endothelial Dysfunction

Separating the arterial wall from flowing blood is the endothelium, a thin layer of cells that acts as a selectively-permeable barrier and plays a key role in regulating vascular function by releasing several autocrine and paracrine factors including nitric oxide (NO) (47). NO is a vasodilator that has several antiatherogenic effects, including the regulation of blood pressure and vascular tone, and the inhibition of platelet aggregation, vascular smooth muscle cell proliferation, and leukocyte adhesion (46). Many disease processes, including atherosclerosis, are characterized by a reduction in NO bioavailability. The decline in NO bioactivity results in abnormal vascular homeostatic function, a condition called endothelial dysfunction.

### **Nitric Oxide (NO)**

NO is a diffusible gas that is so rapidly scavenged that it has a half-life of only a few seconds (3). While this short half-life makes measuring NO unfeasible, the final products of NO metabolism, nitrite and nitrate, can be measured in plasma.

Collectively called NO<sub>x</sub>, nitrite and nitrate concentrations serve as an indicator of NO production (53).

NO is synthesized in trace quantities by a reaction of oxygen, arginine, and NADPH that is catalyzed by nitric oxide synthase (NOS). The NO that plays a role in vasodilation is constitutively produced in endothelial cells, platelets, and neutrophils by endothelial NOS (eNOS) in response to homeostatic stimuli (43). However, larger quantities of NO are produced in macrophages by inducible NOS (iNOS) in response to inflammatory stimuli (18). Instead of improving endothelial health, the NO produced in response to inflammation has proatherogenic effects, oxidizing lipoproteins and promoting endothelial dysfunction (3). Thus, in addition to its relationship to oxidative stress, inflammation also plays a role in endothelial dysfunction.

### **CVD and Endothelial Dysfunction**

The link between endothelial dysfunction and CVD is well-established. Measures of endothelial function, such as endothelium-dependent vasodilation, have been shown to predict long-term CVD progression and future CVD events in patients with atherosclerosis (60). The loss of endothelium-dependent vasodilation reflects an impairment in endothelium-derived NO bioactivity (47). Thus, decreased NO bioavailability is indicative of endothelial dysfunction. As outlined previously, ox-LDL, nitrotyrosine, and MPO have all been linked to reduced NO bioavailability by either decreasing NO production, increasing NO scavenging, or uncoupling eNOS. Thus, excess ROS production and oxidative stress are associated with endothelial dysfunction. Whether endothelial dysfunction is an initiating event in atherosclerosis

that triggers oxidative modifications or simply a result of oxidative stress is not known.

### **Effects of Exercise Training on Endothelial Function**

Previous research has shown that exercise training can reverse age-related decrements in endothelium-dependent vasodilation and that active individuals have increased NO bioactivity compared to their sedentary peers (12). In addition, plasma NOx levels have been found to increase with exercise training in various populations. Intense aerobic exercise training increased plasma NOx concentration in healthy young individuals (36). In previously sedentary older women, three months of mild aerobic-endurance exercise significantly increased plasma NOx levels (37). Evidence indicates that physical activity improves endothelial function and can increase NO bioavailability. The apparent links between oxidative stress and endothelial dysfunction suggest that the changes in plasma levels of ox-LDL, nitrotyrosine, and MPO with exercise training are concomitant with changes in plasma NOx. The present study will look simultaneously at the training-induced changes in oxidative stress markers and NO bioavailability to try to better understand the mechanisms underlying the improvements in CVD risk with training. In addition, the study will extend recent findings relative to exercise and age effects on novel CVD risk factors.

### **Summary**

While conventional risk factors explain a significant portion of CVD risk, emerging factors like oxidative stress, inflammation and endothelial dysfunction appear to improve CVD prediction. Ox-LDL, nitrotyrosine, and MPO are oxidative

stress markers that have been linked to atherosclerosis. While the mechanisms by which oxidative stress promotes CVD are not yet fully understood, research suggests that oxidative modifications may impair endothelial function. Endothelial dysfunction, characterized by reduced NO bioactivity, seems to promote the inflammatory response essential to atherosclerosis. Thus, the intersection of oxidative stress, endothelial dysfunction, and inflammation pathways seems to be important in the disease process.

Early evidence also suggests that exercise training may be able to reduce CVD risk in part by decreasing levels of oxidative stress. The training-induced decline in oxidative stress may result in improved endothelial function, which in turn decreases an individual's CVD risk. Great progress has been made in understanding the pathogenesis of atherosclerosis and the effects of exercise training on CVD risk, but more research is needed to elucidate the mechanisms and causes of the disease.

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