Levels of Circulating Biomarkers at Rest and after Exercise in Coronary Artery Disease Patients

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Running title: Biomarkers at Rest and after Exercise

Abstract

Background: As traditional risk factors are unable to fully explain the pathogenesis of coronary artery disease (CAD), novel mechanisms became a target of many investigations. Our aim was to study the response of selected markers to physical exercise.

Methods: High-sensitive C-reactive protein (hs-CRP), matrix metalloproteinases 2 and 9 (MMP-2, MMP-9), advanced oxidation protein products (AOPP), soluble receptor for advanced glycation end-products (sRAGE), pregnancy-associated plasma protein A (PAPP-A), E-selectin, vascular endothelial growth factor (VEGF) and B-type natriuretic peptide (BNP) levels were measured in serum of 21 CAD patients and in 22 healthy controls at rest and after exercise bicycle stress test performed up to the maximal tolerated effort.

Results: At rest, hs-CRP, AOPP, MMP-9 and BNP were significantly elevated in the CAD patients as compared with controls. In contrast, P-selectin was significantly lower in CAD patients and a tendency to lower levels of sRAGE was noted. After exercise MMP-9 and BNP, increased significantly in both groups.

Conclusion: CAD patients have elevated hs-CRP, AOPP, MMP-9 and BNP - novel markers related to cardiovascular risk or left ventricular overload. MMP-9 and BNP increase significantly with exercise both in healthy individuals and CAD patients.

Key words: coronary artery disease, inflammation, metalloproteinases, natriuretic peptides, oxidative stress

Introduction

Although coronary artery disease (CAD) mortality decreases due to primarily preventive measures and success in therapeutical field, it still remains the most frequent cause of death in developed countries. (Cífková and Škodová 2002). In the last decade the research efforts were focused on better understanding of pathogenetic mechanisms and development of novel noninvasive methods to detect early stages of coronary atherosclerosis (Pohost et al, 2000) and identifying subjects at high risk for destabilization of coronary plaques (Aschermann 2004). The presence of "classical" risk factors such as smoking, hypertension, dyslipidemia or diabetes mellitus (Racek and Racková 2002) are unable to explain the whole risk of CAD and its complications. Novel biochemical markers of risk factors were developed to assess subclinical inflammation, oxidative stress, endothelial dysfunction and ventricular overload.

AGEs (Advanced Glycation End-products) result from non-enzymatic glycation and oxidative stress protein modification. RAGEs is a multi- ligand type I transmembrane glycoprotein potentially involved in several pathological processes (Bucciarelli et al. 2002) including atherogenesis. Deleterious effects mediated by AGEs are prevented in part by this receptor which *in vivo* occurs as soluble RAGE (sRAGE). It was repeatedly shown, that the level of sRAGE is decreased in CAD and in hypertensive patients (Geroldi et al. 2005).

AOPP (Advanced Oxidation Protein Products) were described as proteins modified by chlorinated oxidants (Witko-Sarsat et al. 1996) and subsequently identified as a risk factor for coronary atherosclerosis (Kaneda et al. 2002).

PAPP-A (Pregnancy Associated Plasma Protein-A) is a zinc metalloproteinase used for screening of Down syndrome and also a marker of acute coronary syndromes (Bayes-Genis et al. 2001). Its concentration increases in the presence of unstable coronary plaques.

MMPs (Matrix metalloproteinases) constitute a family of endopeptidases that are involved in the breakdown of extracellular matrix and in cancerogenesis and cardiovascular diseases

(Parks 1998). MMP-2 together with MMP-9 are involved in degradation of collagen, elastin and fibronectin, and influence the production of several other molecules (McQuibban et al. 2000). The increase in MMPs activity was shown to be associated with the progression of atherosclerotic plaques to their rupture (Beaudeux et al. 2004).

E- and P- selectins mediate initial interaction of leukocytes and thrombocytes with endothelial cells (Shimizu et al. 1991), a reaction playing a role in pathogenesis of many cardiovascular pathological processes. Their soluble components reflect their turnover and may be readily assessed as markers of endothelial injury and thrombosis.

Vascular Endothelial Growth Factor (VEGF) is an important regulator of angiogenesis and vasculogenesis. While processes of vasculogenesis occur during embryonic period, angiogenesis is also activated during the whole life in processes associated with neovascularization (Neufeld et al. 1999).

BNP (B-type Natriuretic Peptide) is a sensitive marker of both left and right ventricular overload (Maisel et al. 2001). BNP is currently used in diagnosing congestive heart failure and stratifying the risk of patients with pulmonary embolism.

High sensitivity C-reactive protein (hs-CRP) was shown to be associated with increased risk of coronary atherosclerosis. It appears that CRP is not only a marker but an active mediator of atherogenesis (Sung et al. 2003).

Although all above mentioned biochemical parameters are established markers of atherogenesis, unstable plaque presence, ischemia or heart failure, relatively little is known about their immediate response to exercise in healthy individuals and patients with known coronary artery disease. This information is of potential clinical relevance as stress-induced changes, if present, may significantly influence patients´ risk stratification. The aim of our study was to analyze the effect of a standard exercise stress test on selected novel cardiovascular biochemical markers (Stern 2002).

Patients and methods

Study population

In the group of patients with coronary artery disease we included 21 patients (18 men and 3 women) of the average age 65±7 years. All patients had established CAD defined as confirmed history of either myocardial infarction or percutaneous coronary intervention or aortocoronary bypass grafting or angiographically documented coronary disease (presence of at least one ≥ 50% stenosis of proximal major coronary artery) (Hess et al. 2006). Patients who had recently (in last 3 months) suffered from acute coronary syndrome (ACS), patients with stenosis of the main arterial trunk of left coronary artery or its equivalent and patients with CCS class 3 and 4 angina pectoris were not included in the study. All generally recognized contraindications to exercise tests were also respected (Stern 2002). All CAD patients received recommended therapy including ACE inhibitors, statins and low dose (100 mg) acetylsalicylic acid. All patients were invited to withdraw all antianginal treatment (calcium channel blockers, betablockers, long-acting nitrates) at least 24 hours before the test. The control group included 22 otherwise healthy individuals (17men and 5 women) of the mean age 30±5 years. All subjects were enroled from a group of individuals undergoing stress testing to measure their physical performance (e.g. employer-initiated preventive programs). Only subjects with clinically and electrically negative exercise test were evaluated.

The study was approved by local institutional ethical committee and all subjects have given their informed consent prior to entering the study.

Design of the study

Venous blood samples at baseline were taken in patients after 30 min of rest at least. All patients were invited not to perform any vigorous exercise before the baseline blood sampling.

Blood pressure of the patients was taken by the sphygmomanometric method and their resting 12-lead ECG with standard electrode placement in modification by Mason and Linkar was recorded before the exercise test.

The bicycle exercise test was performed using 50W starting load which was afterwards gradually increased by stages lasting 3 minutes and exercise workload increasing by 50W. The ECG curve was continuously monitored and graphically recorded at the end of each minute along with blood pressure measurement. Exercise was terminated when the predicted heart rate maximum was achieved (determined as 220-age) or when limiting symptoms occurred. (In the group of patients in CAD the reason for ending of the test was also a rise in blood pressure above 260/130mmHg and horizontal ST depressions of 4mm and more). Five minutes after the end of exercise blood sample was collected for evaluation of stress induced changes in laboratory parameters.

Laboratory parameters

Blood samples were centrifuged for 10 minutes at 1450 g and serum was frozen at -20ºC. Analyses of samples were performed within six months.

PAPP-A and hs-CRP were measured by TRACE (Time Resolved Amplified Cryptate Emission) on the KRYPTOR analyser (BRAHMS GmbH, Henningsdorf, Germany) using standard kits (BRAHMS GmbH, Henningsdorf, Germany).

sRAGE, MMP-2, MMP-9, (all kits RD Systems, USA), P-selectin, E-selectin and VEGF (Biosource, USA) were assessed with standard ELISA (enzyme linked immunosorbent assay) kits.

AOPP were determined spectrophotometrically according to Witko-Sarsat (Witko-Sarsat et al 1996). Concentration of AOPP is expressed in µmol/l referred to the calibrator.

BNP was measured with chemiluminiscent method (CLIA) using standard kits (Merck, USA) on automated analyser Access, Beckman Counter, USA.

Other routine biochemical parameters were determined by standard clinical-chemistry methods recommended by IFCC (International Federation of Clinical Chemistry).

Statistical analysis

All values are given as mean ± standard deviations. Comparisons of continuous variables between the studied groups were performed using unpaired t-test in normally distributed parameters and Mann-Whitney test in presence of skewed distribution. Paired t-test and Wilcoxon paired t-test were used to examine the influence of exercise on laboratory parameters within the study groups. All analyses were performed using MedCalc 9.3 (MedCalc Software Comp. Mariakerke, Belgium).

Results

Principal demographic data and parameters of the exercise are shown in Table 1. The tolerance of exercise in the group of CAD patients was significantly lower than in the control group in which the average maximum recorded exercise workload 275 W is exceedingly high, proving good physical efficiency of controls.

Routine biochemical characteristics of both studied groups are listed in Table 2. Table 3 depicts studied non-traditional markers related to cardiovascular risk in both groups before and after the exercise. At rest, hs-CRP, AOPPs, MMP 9, and BNP were elevated in the patients group as compared to controls ($p<0.02$, $p<0.05$, $p<0.0001$, respectively). On contrary, P-selectin was decreased ($p<0.05$) and a nonsignificant trend to lower levels of sRAGE in CAD patients was observed.

After exercise MMP-9 and BNP increased significantly in both groups as shown in Figure 1 and 2. After the exercise, the difference in AOPP and BNP levels between the studied groups was still significant, while the MMP-9 concentration did not statistically differ.

Both baseline and post-stress levels of PAPP-A, MMP-2, E-selectin and VEGF were similar in both study groups.

In order to avoid the effect of haemoconcentration on the evaluation, we have corrected all values for the albumin content. However, no change in the statistical evaluation was found.

Discussion

This study shows elevated hs-CRP, AOPP, MMP-9 and BNP and decreased P-selectin and tendency to lower sRAGE in coronary artery disease patients. The principal study finding however is the significant influence of acute exercise on MMP-9 and BNP levels both in CAD patients and healthy subjects.

Exercise is physiologically connected to a certain degree of damage of skeletal muscles and connective tissue, which leads to activation of tissue metalloproteinases (MMP) and to increased expression of cytoadhesive molecules (selectins) and inflammatory substances (TNF α, IL6, CRP) (Carmeli et al. 2005, Signoreli et al. 2003). The elevation of these substances is usually influenced by the exercise intensity. In contrast to long-term physical activity which leads to reduction of parameters of inflammation, the short-term exercise was shown to be associated with their elevation (Brixius et al. 2008).

The difference in AOPP level between the CAD group and healthy controls was an expected result. The change during the exercise was not statistically significant and the difference between both studied groups remained statistically significant also after the exercise. As the elevation of AOPP caused by exercise was described only in those cases when the exercise was linked to hypoxemia (Pialoux et al. 2006), thus minimal changes in AOPP could be explained by the fact that most patients in our CAD group were previously successfully revascularized. We observed a tendency to lower sRAGE values in the group of CAD patients. This observation is in agreement with published data (Nakamura et al. 2007, Falcone et al. 2005). However in contrast to other studies, the difference between CAD and control subjects did not reach the statistical significance. This might be due to small number of studied subjects. Our study is the first showing minimal if any immediate response of sRAGEs induced by the acute exercise.

The increase of MMP-9 level in CAD patients in comparison to controls is in agreement with the results of Wu and co-authors who described the elevation of matrix metalloproteinases on relatively large number of CAD patients compared to patients with microvascular angina (coronary syndrome X) and healthy controls (Wu et al. 2005). Only limited data evaluating the influence of exercise on MMP-9 levels were published. Suhr et al. demonstrated that maximum after-exercise elevation occurred after longer interval from the end of the exercise (Suhr et al. 2007). The demonstration of immediate exercise induced elevation of MMP-9 is difficult to explain by increased expression of genes of MMP-9 (Büttner et al. 2007) However, exercise is associated with prompt leukocyte increase which may produce many substances, including MMP-9.

Important and expected differences were recorded in BNP levels between the study groups. Elevation of BNP within the context of increased wall stress is physiologically present during the exercise. Of note, we have shown that exercise induced a substantial increase also in healthy individuals. Although in CAD patients or patients with left ventricular dysfunction the elevation of BNP is usually more clearly expressed, we observed an important elevation of BNP after exercise even in CAD patients with clinically and electrically negative stress test (Win et al. 2005). Foote as well as Vanzetto and co-workers suggested more than double increase in treadmill test sensitivity without loss of its specificity when BNP was taken into account. In both cases nuclear cardiology methods were used for correlation with the analysis of BNP in blood samples. Authors explain the increase of BNP by the elevation of wall stress during exercise mainly in ischemic myocardium (Foote et al. 2004, Vanzetto et al. 2007). As our study shows the physiological character of BNP increase after exercise even in healthy individuals with excellent physical performance, the BNP as a tool for detection of ischemia should be evaluated with caution taking into account this phenomenon.

In agreement with the observation from the large PREVEND study (Geluk et al. 2008) we found statistically highly significant differences in basal levels of hs-CRP between patients with CAD and control group. This difference existed in spite the fact that we did not include patients who recently suffered from the acute coronary syndrome into the group of CAD patients (Geluk et al. 2007). As previously described chronic forms of CAD are associated with elevated hs-CRP in about 20%. This number increases up to 70% of cases in patients with unstable angina pectoris (Ridker 2003). The difference in hs-CRP observed in our study may be in part explained by the differences of overall physical performance of our study groups. This hypothesis is supported by observations of Kullo et al. These authors demonstrated in asymptomatic men that hs-CRP is inversely correlated with the maximal oxygen consumption and the maximal working capacity (Kullo et al. 2007).

The absence of a pronounced difference in determination of VEGF when comparing the monitored groups and the resting and post-exercise states in both groups is in compliance with the data that exercise positively influences angiogenesis, which, however, is not directly given by the VEGF level elevation, but by influencing of the VEGF rate and antiangiotically functioning endostatin.

Study limitations

The main limitation of our study is the important age difference between the groups. However, the objective of the study was to constitute a control group free from CAD. If older patients were recruited, we would probably need to perform more invasive testing including the coronary angiography to eliminate the presence of serious coronary disease. This approach would be difficult to justify in otherwise healthy asymptomatic individuals. Additionally, our previously published papers we included older controls and observed similar values for PAPP-A as well as for sRAGE (Kalousová et al. 2003, Kalousová et al. 2007).

Conclusion

We conclude that CAD patients have elevated several non-traditional markers related to cardiovascular risk. Moreover, we demonstrated that MMP-9 and BNP increase significantly with exercise both in CAD patients and healthy controls. However after exercise only BNP remains significantly higher in CAD patients than in healthy controls. These observations are of potential clinical relevance and should be taken into account when setting up preanalytical conditions for blood sampling in CAD patients. The physiological elevation of BNP in healthy controls indicates that its use in diagnosing CAD in adjunction to stress testing should be made with caution and only important elevations should be considered as pathological.

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Table 1. Demographic and clinical parameters of study populations

	CAD patients	Healthy controls
	n=21	n=22
Male/female ratio	18 / 3	17 / 5
Age (years)	65 ± 7 ***	30 ± 5
	(51-81)	(22-55)
Diabetes mellitus	5	0
Smokers	4	0
Myocardial infarction	10	0
PCI	7	0
CABG	5	0
Blood pressure at rest (mmHg)	126 ± 19 / 71 ± 16	118 ± 13 / 72 ± 8
Blood pressure after exercise (mmHg)	218 ± 40 / 71 ± 16	238 ± 31 / 65 ± 14
Maximum exercise workload (W)	150 ± 9 ***	275 ± 37
Heart rate at rest (beats/min)	76 ± 12	77 ± 12
Maximum heart rate (beats/min)	137 ± 23 ***	179 ± 12

Data are expressed as mean ± SD, (range).
*** $p<0.0001$ CAD vs. controls
MI = myocardial infarction, PCI = percutaneous coronary intervention, CABG = coronary artery bypass graft

Table 2. Classical biochemical markers related to cardiovascular risk

	CAD patients	Healthy controls
	n=21	n=22
Glucose (mmol/l)	6.64 ± 2.47 ***	4.59 ± 0.63
Uric acid (mmol/l)	381 ± 107**	286 ± 82
Cholesterol (mmol/l)	4.71 ± 0.89	4.62 ± 0.63
HDL cholesterol (mmol/l)	1.25 ± 0.34	1.37 ± 0.34
LDL cholesterol (mmol/l)	2.53 ± 0.93	2.72 ± 0.56
Triacylglycerols (mmol/l)	2.13 ± 1.17**	1.37 ± 0.77
hs – CRP (mg/l)	2.96 ± 3.09**	1.19 ± 0.81

** $p<0.02$, *** $p<0.0001$ CAD vs. controls.

Table 3. Changes in the parameters studied after the exercise, comparision CAD patients with healthy controls

	CAD patients		Healthy controls	
	before the exercise	after the exercise	before the exercise	after the exercise
PAPP-A (mU/l)	8.6 ± 3.0 8.3 (6.4 - 10.0)	8.7 ± 2.1 8.1 (7.3 - 9.4)	8.3 ± 2.6 8.7 (6.4 - 10.4)	8.0 ± 3.2 8.4 (5.5 - 10.2)
AOPP (μmol/l)	111.3 ± 46.6 * 102.6 (73.8 - 141.6)	127.5 ± 60.8 $^{\times\times}$ 107.6 (89.2 - 164.4)	82.8 ± 46.3 72.3 (55.2 - 83.1)	82.8 ± 30.1 78.9 (63.9 - 90.7)
RAGE (ng/l)	1578 ± 864 1452 (1087 - 1806)	1999 ± 1624 1606 (908 - 2164)	1824 ± 744 1711 (1360 - 2252)	2349 ± 1601 1741 (1239 - 3140)
MMP-2 (μg/l)	239.8 ± 60.2 224.0 (189.4 - 300.4)	241.0 ± 60.4 224.0 (202.0 - 296.2)	236.0 ± 48.6 238.3 (191.6 - 283.6)	248.3 ± 52.5 246.3 (206.0 - 299.4)
MMP-9 (μg/l)	742.3 ± 378.8 * 652.2 (536.7 - 920.7)	892.6 ± 459.9 ^{a} 813.8 (537.4 - 1093.9)	565.6 ± 273.8 512.7 (396.3 - 634.3)	768.7 ± 348.9 ^{b} 751.1 (473.2 - 922.9)
BNP (ng/l)	111 ± 88 *** 98 (30 - 172)	150 ± 117 $^{\times\times, b}$ 169 (32 - 217)	32 ± 27 24 (16 - 38)	53 ± 44 ^{a} 40 (30 - 58)
sE-selectin (μg/l)	35.5 ± 20.7 31.8 (16.8 - 51.5)	37.5 ± 22.8 36.5 (17.8 - 54.9)	29.2 ± 13.2 24.5 (18.0 - 41.3)	29.7 ± 13.6 25.7 (20.9 - 39.9)
sP-selectin (μg/l)	148.3 ± 53.2 * 149.3 (99.5 - 189.3)	146.6 ± 48.6 120.8 (113.4 - 175.3)	190.1 ± 63.3 195.5 (146.5 - 248.0)	193.2 ± 90.1 205.3 (121.7 - 241.7)
VEGF (ng/l)	217.4 ± 122.2 187.7 (127.8 - 291.9)	227.9 ± 152.4 167.8 (143.0 - 277.6)	197.6 ± 110.8 168.7 (131.4 - 268.6)	226.1 ± 123.5 176.3 (136.2 - 313.6)

Results are expressed as mean ± SD, median (interquartile range).*p<0.05, *** p<0.0001 CAD vs. controls before the exercise;

$^{\times\times}$p<0.01 CAD vs. controls after the exercise;

[a] p<0.01, [b] p<0.001 effect of the exercise .

Legend to figures

Fig. 1. Influence of exercise on plasma BNP, *****$p<0.01$, ****** $p<0.001$

Fig. 2. Influence of exercise on MMP-9 levels, *****$p<0.01$, ****** $p<0.001$

Figure 1

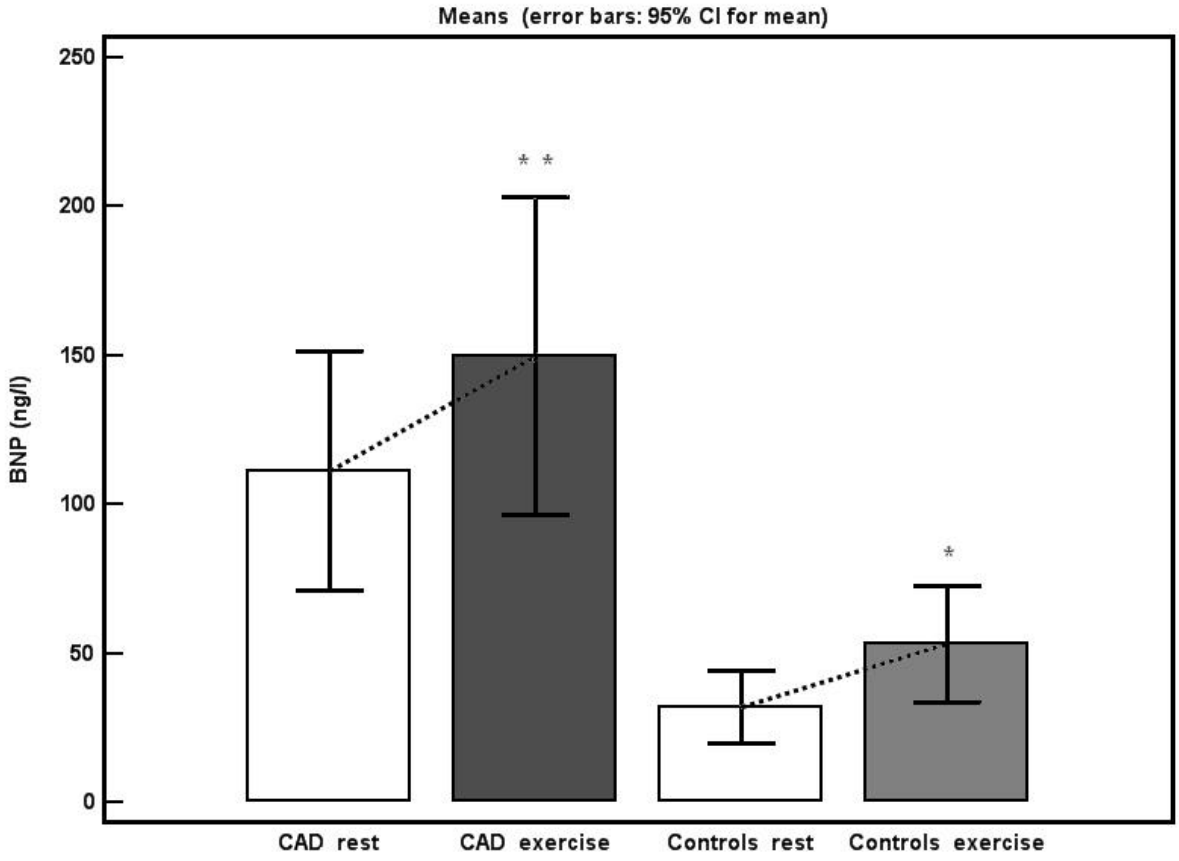
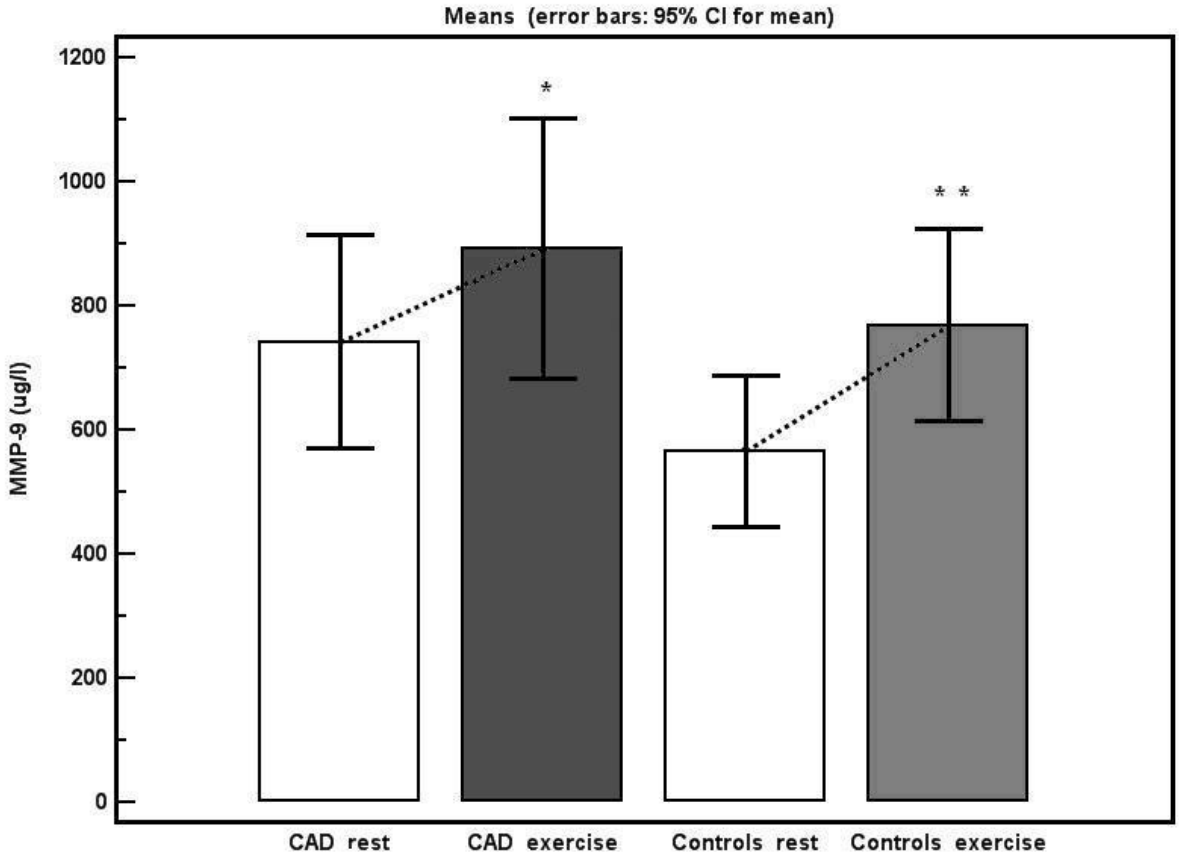

Means (error bars: 95% CI for mean)
BNP (ng/l)
250
200
150
100
50
0
* *
*
CAD rest
CAD exercise
Controls rest
Controls exercise

Figure 2


Means (error bars: 95% CI for mean)
MMP-9 (ug/l)
1200
1000
800
600
400
200
0
*
* *
CAD rest
CAD exercise
Controls rest
Controls exercise