

The presence of the NOS3 gene polymorphism for intron 4 mitigates the beneficial effects of exercise training on ambulatory blood pressure monitoring in adults

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Submitted 25 October 2013; accepted in final form 12 April 2014

Sponton CH, Esposti R, Rodovalho CM, Ferreira MJ, Jarrete AP, Anaruma CP, Bacci M Jr, Zanesco A. The presence of the NOS3 gene polymorphism for intron 4 mitigates the beneficial effects of exercise training on ambulatory blood pressure monitoring in adults. *Am J Physiol Heart Circ Physiol* 306: H1679–H1691, 2014. First published April 18, 2014; doi:10.1152/ajpheart.00844.2013.—The number of studies that have evaluated exercise training (ET) and nitric oxide synthase (NOS)3 gene polymorphisms is scarce. The present study was designed to evaluate the relationship between exercise training and NOS3 polymorphisms at $-786\text{T}>\text{C}$, $894\text{G}>\text{T}$, and intron 4b/a on blood pressure (BP) using 24-h ambulatory BP monitoring (ABPM), nitrate/nitrite levels (NO_x), and redox state. Eighty-six volunteers (51 ± 0.6 yr old) were genotyped into nonpolymorphic and polymorphic groups for each of the three positions of NOS3 polymorphisms. Auscultatory BP, ABPM, SOD activity, catalase activity, NO_x levels, and malondialdehyde levels were measured. DNA was extracted from leukocytes, and PCR followed by sequencing was applied for genotype analysis. Aerobic ET consisted of 24 sessions for 3 days/wk for 40 min at moderate intensity. This study was performed in a double-blind and crossover format. ET was effective in lowering office BP (systolic BP: 3.2% and diastolic BP: 3%) as well as ABPM (systolic BP: 2% and diastolic BP: 1.3%). Increased SOD and catalase activity (42.6% and 15.1%, respectively) were also observed. The NOS3 polymorphism for intron 4 mitigated the beneficial effect of ET for systolic BP (nonpolymorphic group: -3.0% and polymorphic group: -0.6%) and diastolic BP (nonpolymorphic group: -3.2% and polymorphic group: -0.5%), but it was not associated with NO_x level and redox state. Paradoxical responses were found for positions T786-C and G894T for the NOS3 gene. Consistently, the presence of the polymorphism for intron 4 blunted the beneficial effects of ET in middle-aged adults. Possibly, this effect might be as consequence of intron 4 acting as a short intronic repeat RNA controlling endothelial NOS activity epigenetically.

blood pressure; nitric oxide synthase 3 polymorphisms; intron 4; exercise training

ENDOTHELIAL NITRIC OXIDE (NO) synthase (eNOS) activity is considered a key step for NO production, which, in turn, plays an important role in a number of physiological systems, such as the regulation of blood pressure (BP) by acting on vascular tone, antiatherosclerotic effects by inhibiting monocyte/macrophage adhesion, prevention of platelet-dependent thrombosis by inhibiting platelet aggregation, and anti-inflammatory effects by inhibiting nuclear transcription factors (NF- κ B) (29). The expression/activity of eNOS can be modulated by cofac-

tors such as tetrahydrobiopterin, molecular oxygen, and the substrate L-arginine, which are essential for eNOS activity (23). It is believed that deficiency of any of these cofactors is associated with the pathogenesis of several cardiovascular diseases, including coronary artery disease, hypertension, and peripheral vascular disease (50). eNOS expression/activity can also be modulated at transcriptional or posttranslational levels, such as by the presence of NOS3 gene polymorphisms or factors interfering with eNOS activation/degradation involving the phosphorylation of several kinases (24).

The most studied NOS3 gene polymorphisms related to cardiovascular disease are at position T786-C (promoter region), exon 7 G894T (substitution of a glutamate for aspartate in eNOS synthesis, also named Glu298Asp), and the sequence of 27-bp variable number of tandem repeats for intron 4. Nevertheless, the functional relevance of these three polymorphisms in the pathogenesis of cardiovascular diseases is still under investigation. A number of studies have found a positive relationship between NOS3 gene polymorphisms and cardiovascular diseases (11, 31, 59, 63); however, other studies found no association (1, 3, 33, 46). The importance of larger-scale efforts to assess the association between genetic and disease prevalence has been repeatedly stated in meta-analysis studies (10, 45); however, other issues should also be addressed, such as the environmental-genetic interaction focusing primarily on the effect of physical exercise on volunteers who present NOS3 gene polymorphisms. Indeed, physical inactivity is considered one of primary cause of cardiometabolic diseases such as atherosclerosis, type 2 diabetes mellitus, obesity, dyslipidemia, and arterial hypertension (34). On the other hand, subjects that are physically active throughout their lifetime have a lower risk of cardiometabolic diseases (22). Additionally, adults who regularly practice physical exercise at a moderate intensity (at least 150 min/wk) have decreased risk factors for cardiovascular and metabolic-endocrine diseases (27). However, some subjects are not responders to the beneficial effects of exercise training or even show adverse metabolic responses on resting BP, fasting insulin, high-density lipoprotein-cholesterol, and triglycerides (9). Therefore, well-controlled studies evaluating the interaction between physical exercise and genetic factors are extremely relevant in attempting to understand how exercise training can regulate the physiological system in association with different genotypes in the human population.

To our knowledge, few studies have been published evaluating exercise training and NOS3 gene polymorphism. Overall, these studies have shown that the presence of a polymorphism for the NOS3 gene attenuates the beneficial effect of exercise

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training measured by flow-mediated dilatation (19, 43), BP (46, 53, 54), and NO production (54). In contrast, some studies have shown that exercise training overcomes the unfavorable effect of NOS3 gene polymorphisms on cardiometabolic responses (64, 65), whereas others have shown that volunteers with NOS3 gene polymorphisms are equally responsive in lowering BP to exercise training compared with subjects without NOS3 gene polymorphisms (20, 49). Of note, only two studies analyzed the three positions of T786-C, G894T, and intron 4 for the NOS3 gene (20, 53), and none has examined ambulatory BP monitoring. Therefore, in the present study, we investigated the influence of NOS3 gene polymorphisms for all three positions (T786-C, G894T, and intron 4) on cardiovascular parameters using ambulatory BP monitoring as well as cardiometabolic biomarkers in middle-aged adults trained for 24 sessions of aerobic exercise training in a double-blind manner and crossover design. We also carried out haplotype analysis to detect interactions among alleles.

MATERIALS AND METHODS

Study Participants

This study was approved by the Institutional Review Board of the Institute of Bioscience at the University of São Paulo State (UNESP). Volunteers were recruited by advertisement in the surrounding area of the UNESP. The inclusion criteria of the study were as follows: to be sedentary, nonobese (body mass index < 30 kg/m²), nonsmoking, nondiabetic (fasting glucose level < 110 mg/dl), and in a physical condition able to perform exercise training. The exclusion criteria were volunteers with cardiovascular disease (angina, valvular disease, or stroke), arthritis, alcohol consumption > 3 drinks/day, and neurological or psychiatric diseases. A general physician examined all volunteers to detect cardiovascular, pulmonary, or other diseases before the exercise training program. Physical activity readiness and Baecke questionnaires were also applied to the participants before inclusion in this study to obtain medical and physical activity data. A total of 198 middle-aged volunteers were eligible to participate in the study. After all procedures (tests, control period, and aerobic exercise training program), only 86 volunteers (24 men and 62 women) finished the study. Volunteers were informed of the procedures and risks of the study and provided written informed consent in accordance with the policies and Ethical Committee of the Institute of Bioscience from UNESP.

Study Design

Eighty-six volunteers participated in this randomized, double-blind, and crossover study. This study lasted 16 wk and was divided into initial, intermediate, and final periods, as shown in Fig. 1. Briefly, each volunteer started the study, and all measurements (blood collection and cardiovascular and anthropometric parameters) were collected (initial period). After that, the participant was instructed not to perform any regular physical exercise as well as to maintain habitual daily life activities (control period, intermediate period). This control period lasted 8 wk. After that, blood samples were taken, and cardiovascular and anthropometric parameters were measured. During this period, the aerobic capacity of each volunteer was determined using a 1-mi. walk test, and maximal lactate steady state (MLSS) was measured for individual prescription of the aerobic exercise training intensity. The aerobic exercise training lasted 8 wk, with 24 sessions, and blood samples and all cardiovascular and anthropometric parameters were collected 24–72 h after the last session of the exercise training program (final period).

Ambulatory BP was monitored only at the beginning and end of the study for each participant. For more details, see Fig. 1.

Anthropometric Measurements

Height and body weight were measured using a stadiometer and scale (Toledo 2096 PP). Body mass index was determined using body weight divided by height (in m²). Waist circumference was measured at the level of the umbilicus. Anthropometric parameters were evaluated at initial, intermediate, and final time points of the study.

Cardiovascular Measurements

Auscultatory BP and heart rate. Volunteers were instructed not to exercise outside of the laboratory before BP measurements. After 15 min of sitting quiet rest, BP was measured by auscultation with an aneroid sphygmomanometer (Tycos, Raleigh, NC) in three sessions. Resting and exercise heart rates were measured after 15 min in a seated position and 30 min at the intensity of MLSS on a treadmill, respectively, using a beat-by-beat heart rate monitor (Polar RS 800CX, Kempele, Finland).

Ambulatory BP monitoring and heart rate. Ambulatory BP readings were performed using a noninvasive automated device (Dyna-MAP⁺) with the cuff fitted on the nondominant arm, as described in previous studies (12, 53a). BP and heart rate were recorded every 15 min during the day (daytime) and every 30 min during the night (nighttime). For the present analyses, we defined daytime and nighttime according to the habitual awake and sleep behaviors reported by each volunteer. To avoid any

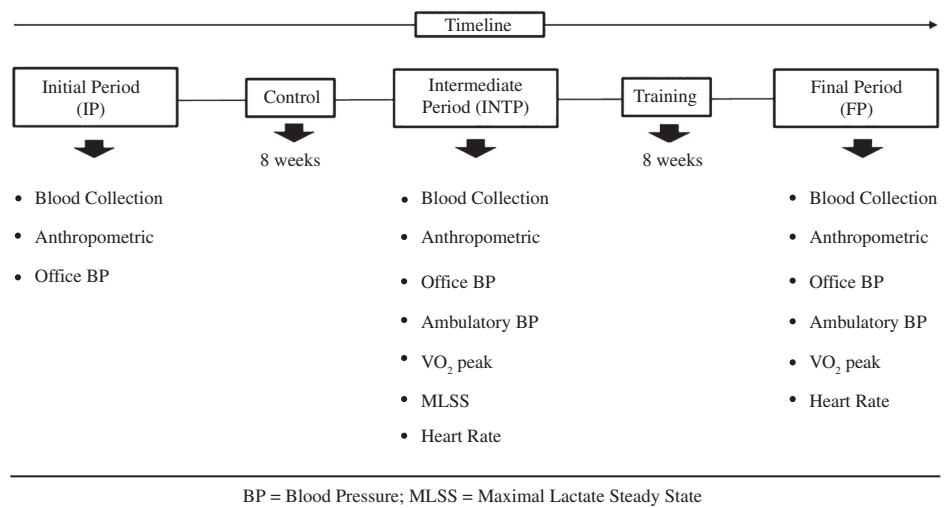


Fig. 1. Protocol design. BP, blood pressure; $\dot{V}O_{2peak}$, peak O₂ consumption; MLSS, maximal lactate steady state.

Table 1. General characteristics of the study participants

Parameters	IP	INTP	FP
Age	50.8 ± 0.6		
Men/women, <i>n</i>	24/62		
Body mass index, kg/m ²	26.7 ± 0.3	26.7 ± 0.2	26.6 ± 0.3
Waist circumference, cm	85.2 ± 0.9	84.8 ± 0.9	83.8 ± 0.9*
SBP, mmHg	116.8 ± 1.6	116.2 ± 1.5	112.5 ± 1.3*
DBP, mmHg	76.7 ± 0.9	76.4 ± 1.0	74.1 ± 0.8*
ABP daytime SBP, mmHg		120.3 ± 1.2	118.2 ± 1.1*
ABP daytime DBP, mmHg		78.2 ± 1.1	76.9 ± 1.0*
ABP nighttime SBP, mmHg		109.7 ± 1.2	107.9 ± 1.2*
ABP nighttime DBP, mmHg		69.1 ± 1.0	68.5 ± 1.0
ABP 24-h SBP, mmHg		118.0 ± 1.1	115.8 ± 1.1*
ABP 24-h DBP, mmHg		76.2 ± 1.0	75.3 ± 1.0
Peak O ₂ consumption, ml·kg ⁻¹ ·min ⁻¹		32.7 ± 0.5	33.8 ± 0.5*
Resting HR, beats/min		76.1 ± 1.1	73.4 ± 1.2*
MLSS exercise HR, beats/min		148.6 ± 1.5	135.7 ± 1.4*
MLSS threshold, μM		3.6 ± 0.1	
NO _x , μM	24.2 ± 1.4	21.7 ± 1.3	24.2 ± 1.6
SOD activity, U/ml	6.9 ± 0.3	6.4 ± 0.4	9.2 ± 0.5*
Catalase activity, nmol·min ⁻¹ ·ml ⁻¹	36.0 ± 1.6	35.1 ± 1.5	40.4 ± 2.0*
Malondialdehyde, μM	8.1 ± 0.2	8.1 ± 0.2	7.8 ± 0.1
Dyslipidemia, <i>n</i> (%)		9 (10.5)	
Statin treatment, <i>n</i> (%)		7 (8.1)	
Fibrate treatment, <i>n</i> (%)		2 (2.3)	
Hypertension, <i>n</i> (%)		31 (36)	

Data are mean ± SE; *n*, number of subjects (*n* = 86 subjects total). IP, initial period; INTP, intermediate period; FP, final period; SBP, systolic blood pressure; DBP, diastolic blood pressure; ABP, ambulatory blood pressure; HR, heart rate; MLSS, maximal lactate steady state; NO_x, nitrate/nitrite. **P* < 0.05, compared to IP and INTP.

interference in the ambulatory BP analysis, a report of the normal daily activity of each participant was evaluated.

Biochemical Analyses

Blood sampling. Venous blood samples (10 ml) were collected after 12 h of overnight fasting. Samples were centrifuged (8,000 *g*, 10 min), and the white buffy layer was removed and discarded. The supernatant was collected and kept in ice for immediately assay or stored at -80°C for future analyses.

Lipid profile, glycemia, and creatinine. Serum samples were used to analyze total cholesterol, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, very-low-density lipoprotein-cholesterol, triglycerides, glycemia, and creatinine using automated standardized methods (Cobas Mira Plus).

Determination of plasma nitrite/nitrate levels. To evaluate NO production, plasma levels of nitrite and nitrate (NO_x) were measured as previously described (54). Briefly, immediately after venous blood collection, the samples were centrifuged (8,000 *g*) for 10 min, and the resulting plasma supernatant was stored at -80°C. Plasma samples were ultrafiltered through microfilter cups (Microcon Centrifugal Filter Units, 10 kDa, Millipore, Bedford, MA). The NO_x concentration of the resulting filtrate solution was determined by ELISA using a commercially available kit (Cayman Chemical, Ann Arbor, MI). This assay determines total NO based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. After the conversion, the spectrophotometric measurement of nitrite is accomplished using the Griess reaction. The resulting deep purple azo compound absorbs light at 540–550 nm.

SOD and catalase enzyme activity and malondialdehyde levels. SOD activity was determined using a nitroblue tetrazolium reduction method (40) for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The absorbance was monitored at 440–460 nm. The catalase activity method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H₂O₂ (30). The formaldehyde produced was measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole with the

purple color absorbance monitored at 540 nm. The thiobarbituric acid-reactive substance assay was determined by the reaction of malondialdehyde (MDA) with thiobarbituric acid under high temperature (90–100°C) and acidic conditions and was measured colorimetrically at 530–540 nm (62). Commercially available kits were used for all analyses according to the manufacturer's instructions (Cayman Chemical). Assays were performed using plasma or serum samples in duplicate or triplicate under different dilutions.

Table 2. Genotype and allele frequencies of the study participants

	Allele Frequency	Percentage
<i>Genotypes</i>		
T-786C		36.1
T,T	0.36	52.3
T,C	0.52	11.6
C,C	0.11	
G894T		
G,G	0.59	59.3
G,T	0.25	25.6
T,T	0.15	15.1
Intron 4b/a		
b,b	0.68	68.6
a,b	0.26	26.7
a,a	0.04	4.7
<i>Alleles</i>		
T-786C		
T	0.62	62.2
C	0.37	37.8
G894T		
G	0.72	72.0
T	0.28	28.0
Intron 4b/a		
b	0.82	82.0
a	0.18	18.0

Aerobic Exercise Tests

Familiarization. Before the exercise tests and training program, volunteers were familiarized to the treadmill by walking during 3–5 days, depending on each participant. All volunteers were asked about fatigue and other symptoms during the exercise. For all exercise tests, the risk of adverse events was controlled according previous recommendations of cardiopulmonary exercise testing guidelines (5).

Peak aerobic capacity measurement. Peak oxygen consumption ($\dot{V}O_{2\text{peak}}$) was determined using the 1-mi. walk test adapted to the treadmill (60). After 5 min of warmup, the volunteer walked as briskly as possible until the distance of 1 mi. was reached. At the end of the treadmill 1-mi. walk, time spent on the test and heart rate were evaluated to calculate $\dot{V}O_{2\text{peak}}$ using a specific equation for walking on the treadmill (47).

MLSS. Volunteers performed two to five test sessions with constant workload on a treadmill (Movement RT 250 PRO) with a fixed, sex-specific speed (6.0 km/h for men and 5.5 km/h for women) according to a previously described study (6). The intensity was controlled by the inclination of the treadmill, with the grade adjusted according to the aerobic capacity of the volunteer in each session. The inclination ranged between 1% (0.5°) and 15% (7.5°) during the test sessions. The blood lactate concentration was measured at three different periods (rest, 20th, and 30th) in all sessions. MLSS was calculated when the maximal difference of blood lactate concentration between the 20th and 30th periods was not higher than 1 mM (7).

Aerobic Exercise Training

The aerobic exercise training program began with appropriate warmup and cooldown activities, and a physical trainer supervised all exercise sessions. Volunteers submitted to a walking exercise on a treadmill in a quiet room with environment-controlled temperature (22–24°C) and humidity (40–60%). Exercise was performed 3 days/

wk, with each session consisting of 30 min (first 4 wk) and 40 min (last 4 wk). The intensity of exercise was prescribed according to the previous individual MLSS test. Heart rate and the rate of perceived exertion (Borg's scale) were recorded every 10 min in each session.

NOS3 Polymorphism Analyses

Genomic DNA was extracted from leukocytes of blood cells through chloroform-phenol, and the PCR-restriction fragment length polymorphism method was used to amplify the NOS3 T-786C, G894T, and intron 4b/a sites of all subjects, as previously described (20). The PCR cycling conditions were 1 cycle at 96°C for 2 min followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min, ending with extension at 72°C for 5 min for all positions.

T-786TC. The NOS3 T-786C region encompassed 180 bp using PCR amplification with the following flanking primers: sense 5'-CACCCAGGCCACCCCAACT-3' and antisense 5'-GCCGAG-GTCGACAGAGAGACT-3'.

Glu298Asp. The following flanking primers were used with PCR to amplify the NOS3 gene at the G894T region: sense 5'-AAGGCAG-GAGACAGTGGATGGA-3' and antisense 5'-CCCAGTCAATC-CCTTTGGTGCTCA-3'.

Intron 4b/a. The following flanking primers were used with PCR to amplify the NOS3 27-bp repeat region in intron 4: sense 5'-AGGC-CCTATGGTAGTGCCTTG-3' and antisense 5'-TCTCTTAGTGCT-TGGTTCACAG-3'.

Sequencing

The sequencing procedure was performed by Macrogen (Seoul, Korea). After that, identification of polymorphisms was determined using sequencing alignment editor (BIOEDIT) software.

Table 3. Characteristics of the study participants grouped by genotypes

Parameter	T-786C					
	TT (<i>n</i> = 31)			TC + CC (<i>n</i> = 55)		
	IP	INTP	FP	IP	INTP	FP
SBP, mmHg	117.5 ± 2.5	116.0 ± 2.6	111.2 ± 2.0*	116.4 ± 2.1	116.4 ± 1.8	113.2 ± 1.8*
DBP, mmHg	76.1 ± 1.5	75.9 ± 1.8	72.8 ± 1.4*	77.1 ± 1.2	76.8 ± 1.2	74.8 ± 1.1*
Resting HR, beats/min	—	74.3 ± 1.6	71.3 ± 1.8	—	76.7 ± 1.4	74.2 ± 1.6*
MLSS exercise HR, beats/min	—	148.1 ± 2.3	134.8 ± 2.3*	—	149.2 ± 2.0	137.5 ± 1.9*
Hypertension, <i>n</i> (%)	—	11 (35.5)	—	—	20 (36.4)	—
Parameter	G894T					
	GG (<i>n</i> = 51)			GT + TT (<i>n</i> = 35)		
	IP	INTP	FP	IP	INTP	FP
SBP, mmHg	118.0 ± 2.2	117.2 ± 2.1	113.1 ± 1.8*	115.0 ± 2.4	114.8 ± 2.1	111.6 ± 1.9*
DBP, mmHg	76.2 ± 1.1	76.6 ± 1.3	74.1 ± 1.1*	77.4 ± 1.6	76.3 ± 1.4	74.0 ± 1.3*
Resting HR, beats/min	—	74.7 ± 1.3	70.7 ± 1.4*	—	77.7 ± 1.7	77.0 ± 1.0†
MLSS exercise HR, beats/min	—	148.4 ± 1.9	135.9 ± 1.8*	—	149.4 ± 2.5	137.5 ± 2.4*
Hypertension, <i>n</i> (%)	—	19 (37.3)	—	—	12 (34.3)	—
Parameter	Intron 4b/a					
	bb (<i>n</i> = 59)			ab + aa (<i>n</i> = 27)		
	IP	INTP	FP	IP	INTP	FP
SBP, mmHg	116.8 ± 1.8	116.6 ± 1.7	112.1 ± 1.5*	116.8 ± 3.3	115.4 ± 2.8	113.3 ± 2.7
DBP, mmHg	76.8 ± 1.1	76.4 ± 1.2	73.8 ± 1.0*	76.6 ± 1.6	76.6 ± 1.7	74.7 ± 1.5
Resting HR, beats/min	—	76.1 ± 1.2	74.3 ± 1.4	—	75.4 ± 2.0	70.9 ± 2.2*
MLSS exercise HR, beats/min	—	147.8 ± 1.8	136.5 ± 1.7*	—	150.8 ± 2.8	136.6 ± 2.7*
Hypertension, <i>n</i> (%)	—	20 (33.9)	—	—	11 (40.7)	—

Data are means ± SE; *n*, number of subjects. **P* < 0.05 compared with IP and INTP; †*P* < 0.05, compared with the respective nonpolymorphic group.

Genotypes and Haplotype Groups

According to the low frequency of $-786CC$ ($f = 0.11$) and intron 4 amino acid genotypes ($f = 0.04$), the groups formed associated the heterozygous and homozygous polymorphic genotypes ($-786TC + CC$, $894GT + TT$, and intron 4 $ab + aa$) apart from nonpolymorphic ($-786TT$, $894GG$, and intron 4 bb) groups. Haplotypes were determined using a Bayesian statistical based program (PHASE, version 2.1, <http://www.stat.washington.edu/stephens/software.html>) (42, 55). The possible haplotypes, including genetic variants of the three NOS3 -786 , 894 , and intron 4 polymorphisms studied, were H1 (TGB), H2 (TGA), H3 (TTb), H4 (TTa), H5 (CGB), H6 (CGa), H7 (CTb), and H8 (CTa).

Statistical Analyses

Data are presented as means \pm SE. Mauchly's sphericity test was used to validate the repeated-measures ANOVA performed to analyze the differences within groups (initial period \times intermediate period \times final period) followed by Tukey's post hoc test. The Kolmogorov-Smirnov test was performed to evaluate normality of the data. A paired Student's t -test was used to analyze differences within the groups at two periods (intermediate period \times final period). Furthermore, an unpaired Student's t -test was used to compare nonpolymor-

phic against polymorphic groups at each period as well as assess changes in values. Finally, a χ^2 -test was used to analyze the association of hypertension or drug therapy and the genotype groups. To increase the statistical power of the haplotype analysis, we excluded a priori uncommon haplotypes (haplotype frequency $< 5\%$) from analysis. The power of the study was 95% for all analyzed groups. $P < 0.05$ was considered statistically significant.

RESULTS

General Data

Twenty-four session of aerobic exercise training promoted a reduction in waist circumference ($\sim 1.2\%$) and decreased both systolic and diastolic BPs ($\sim 3.2\%$ and 3.0% , respectively) in middle-aged adults. Ambulatory BP was also decreased after exercise training (~ 2 and 1.3 mmHg for systolic BP and diastolic BP, respectively) during diurnal time. However, when we analyzed both parameters during 24 h, only systolic BP was significantly reduced in response to exercise training (~ 2.2 mmHg).

Regarding aerobic capacity and the effectiveness of the exercise training, we observed that 24 sessions of aerobic exercise

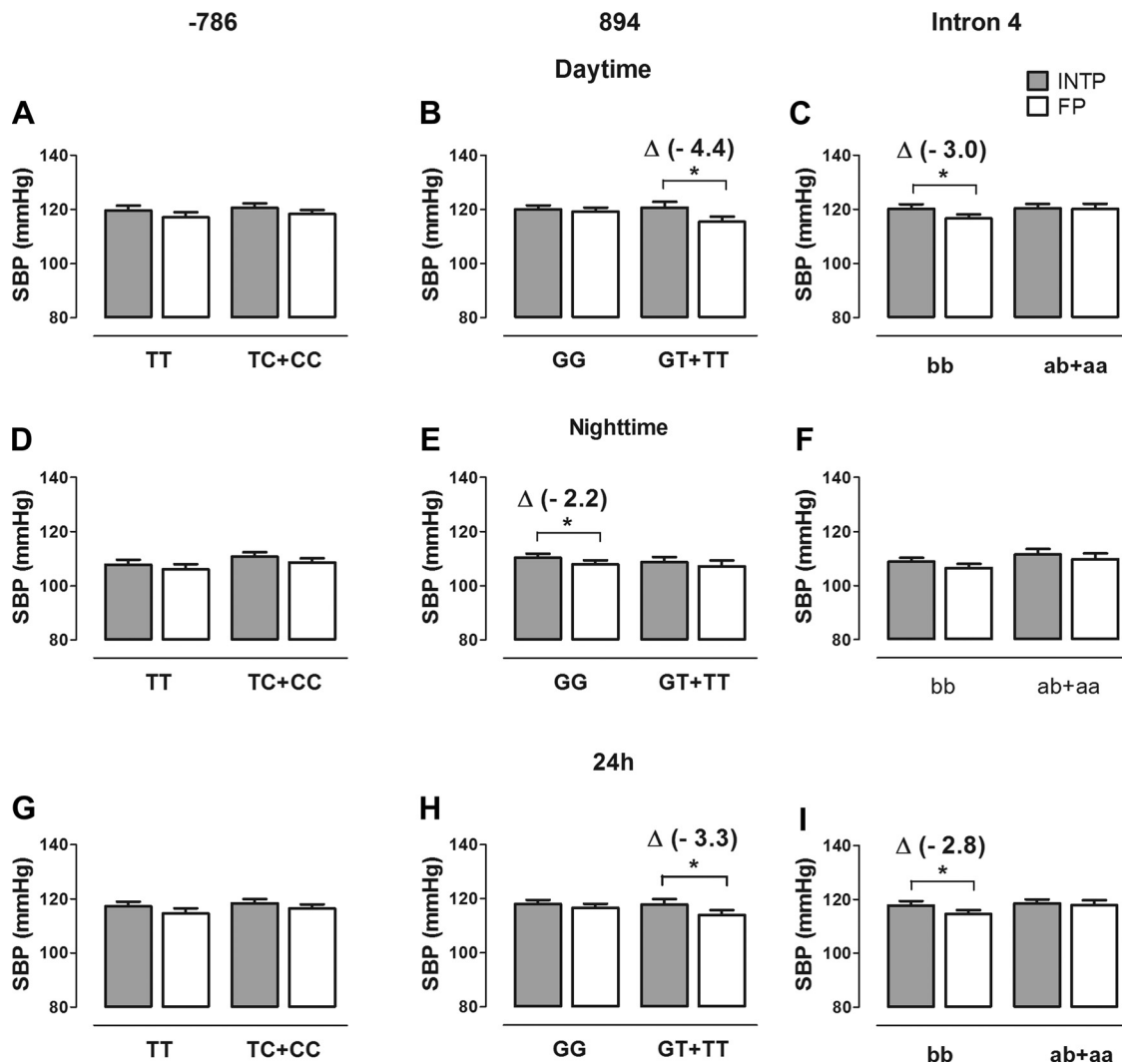


Fig. 2. Effects of aerobic exercise training on ambulatory systolic BP (SBP) monitoring at $-786C>T$, $G894T$, and intron 4b/a nitric oxide synthase (NOS)3 gene polymorphisms according to genotype groups. $-786C>T$ (A), $G894T$ (B), and intron 4b/a (C) illustrate daytime. $-786C>T$ (D), $G894T$ (E), and intron 4 (F) illustrate nighttime. $-786C>T$ (G), $G894T$ (H), and intron 4 (I) illustrate over 24 h. * $P < 0.05$, compared with INTp within the same group.

training significantly increased aerobic capacity, as measured by $\dot{V}O_{2\text{peak}}$, and reduced resting heart rate in the studied population. The activity of antioxidant enzymes (SOD and catalase) was significantly increased in response to exercise training (~ 42.6 and 15.1% , respectively). No changes were found in MDA and NO_x levels. Data are shown in Table 1.

Creatinine levels were significantly increased (8.4%) after exercise training in all studied groups (data not shown). Approximately 10.5% of the volunteers were taking hypolipidemic drugs and 36% were on antihypertensive therapy (Table 1).

Genotype Analyses

Table 2 shows the distribution of all genotypes according to Hardy-Weinberg equilibrium. After that, we evaluate the effects of exercise training on BP by office measurements in all three analyzed genotypes. Volunteers with (TC + CC genotypes) or without (TT genotype) polymorphisms showed a significant decrease in systolic BP after 24 sessions of aerobic exercise training ($\sim 5.1\%$ and 2.7% , respectively). Similarly, volunteers with (GT + TT genotypes) and without (GG genotype) polymorphisms showed a significant reduction in systolic BP in response to aerobic exercise training (3.0% and $\sim 4.2\%$, respectively). Volunteers without polymorphism (bb genotype) showed a reduction

of 4% , and no changes in subjects with polymorphism (ab + aa genotypes) were found. Data are shown in Table 3.

Regarding diastolic BP, similar results to systolic BP were found. Exercise training promoted reduction in diastolic BP values that was genotype independent for the NOS3 gene at position T-786C (TT: 4.3% and TC + CC: 3%) and G894T (GG: 2.8% and GT + TT: 4.4%). In contrast, the presence of the NOS3 gene polymorphism for intron 4 significantly mitigated the health-promoting effects of exercise training. Diastolic BP was reduced 3.9% in volunteers without polymorphism for intron 4 (bb genotype), and no changes were found in subjects with polymorphism (ab + aa genotypes) after 24 sessions of aerobic exercise training (Table 3). Heart rate values were significantly reduced in all three genotypes during physical exercise performance (range of $7\text{--}9\%$). Additionally, the number of hypertensive subjects was similar for all three genotypes (Table 3).

To assure the beneficial health effects as well as sustained lower BP in response to aerobic exercise training, we monitored BP during 24 h in all 86 volunteers. Volunteers with (TC + CC, $n = 55$) or without (TT, $n = 31$) polymorphisms showed no changes in systolic BP in all analyzed time points after exercise training (daytime, nighttime, and 24 h; Fig. 2, A, D, and G). Intriguing, for position G894T of the NOS3 gene,

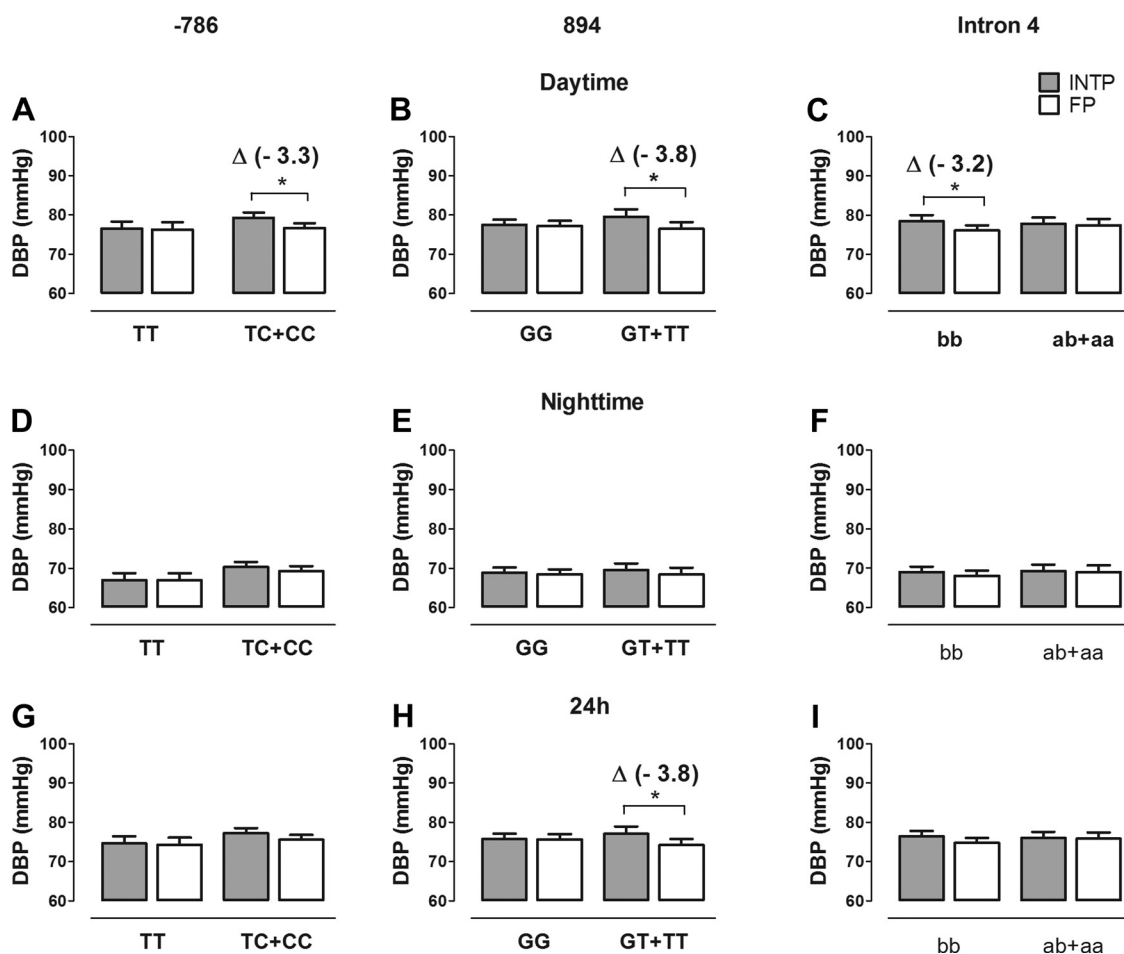


Fig. 3. Effects of aerobic exercise training on ambulatory diastolic BP (DBP) monitoring at $-786>C$, G894T, and intron 4b/a NOS3 gene polymorphisms according to genotype groups. $-786>C$ (A), G894T (B), and intron 4b/a (C) illustrate daytime measurements. $-786>C$ (D), G894T (E), and intron 4 (F) illustrate nighttime measurements. $-786>C$ (G), G894T (H), and intron 4 (I) illustrate over 24 h of measurements. $*P < 0.05$, compared with INT within the same group.

we found that both groups had a reduction in systolic BP, but at different times of the day (Fig. 2, *B*, *E*, and *H*).

Regarding the NOS3 gene for intron 4, ambulatory BP monitoring confirmed the data obtained in office measurements. Only volunteers without polymorphism in the variable number of tandem repeats for intron 4 (bb genotype, $n = 59$) showed a significant reduction in systolic BP during the diurnal period as well as with 24-h monitoring, with an $\sim 3\%$ reduction compared with those with polymorphism (ab + aa genotypes, $n = 27$; Fig. 2, *C* and *I*). No changes were seen during nighttime for both groups for this position (Fig. 2*F*).

Regarding ambulatory diastolic BP monitoring, we found that exercise training promoted a sustained reduction in diastolic BP only for polymorphic volunteers for the NOS3 gene (TC + CC and GT + TT genotypes, $n = 55$ and 35, respectively) during the diurnal period (Fig. 3, *A* and *B*). No changes were found during nighttime for both positions (Fig. 3, *D* and *E*). During 24 h of BP monitoring, diastolic BP values were significantly reduced only for volunteers with the polymorphism for the G894T position of the NOS3 gene (Fig. 3*H*). Confirming office BP measurements, volunteers with the polymorphism for intron 4 of the NOS3 gene showed no changes in diastolic BP in response to exercise training during the diurnal period compared with volunteers without the polymorphism (bb genotype), who showed a significant reduction of this parameter (~ 3.2 ; Fig. 3*C*). No changes were found for both nighttime and 24 h of BP monitoring for volunteers with or without NOS3 gene polymorphisms (Fig. 3, *F* and *I*).

Given that both antioxidant enzymes (SOD and catalase) are the first steps in buffering anion superoxide actions preventing NO inactivation and/or increasing NO bioavailability to vascular smooth muscle cells, we evaluated the activity of both enzymes in all volunteers before and after exercise training.

Our findings show that SOD activity was markedly increased after 24 h of aerobic exercise sessions in all volunteers independent of genotype (Fig. 4, *A–C*). Interestingly, catalase activity was increased only in volunteers with the polymorphism for intron 4 of the NOS3 gene (ab + aa genotypes) after aerobic exercise training (Fig. 4*F*). In contrast, no changes were found in catalase activity in response to exercise training for positions T-786C and G894T of the NOS3 gene independent of genotype (Fig. 4, *D* and *E*). We also analyzed NO_x concentration as well as MDA levels in all volunteers. Unexpectedly, no changes were found in both parameters for all studied genotypes (Fig. 5, *A–F*).

Haplotype Analyses

To verify the interaction of the NOS3 gene polymorphism on ambulatory BP monitoring as well as on cardiometabolic biomarkers in response to exercise training in 86 volunteers, we carried out haplotype analysis for all three NOS3 gene positions. It is believed that this approach is a more powerful tool in detecting the influence of polymorphism on physiological responses. Only five subgroups were formed considering the three studied positions T786-C, G894T, and intron 4 as well as the number of volunteers in each subgroup that allows statistical analysis, named H1 (TGb), H2 (TGa), H3 (TTb), H5 (CGb), and H7 (CTb). Interestingly, haplotype groups H2 and H5 showed no changes in both systolic and diastolic BPs measured by the office method, whereas a significant reduction in both parameters was found in the H1, H3, and H7 groups. Resting heart rate values were not reduced in the H3 and H7 haplotype groups, whereas exercise training was effective in lowering MLSS in all haplotype groups. Data are shown in Table 4.

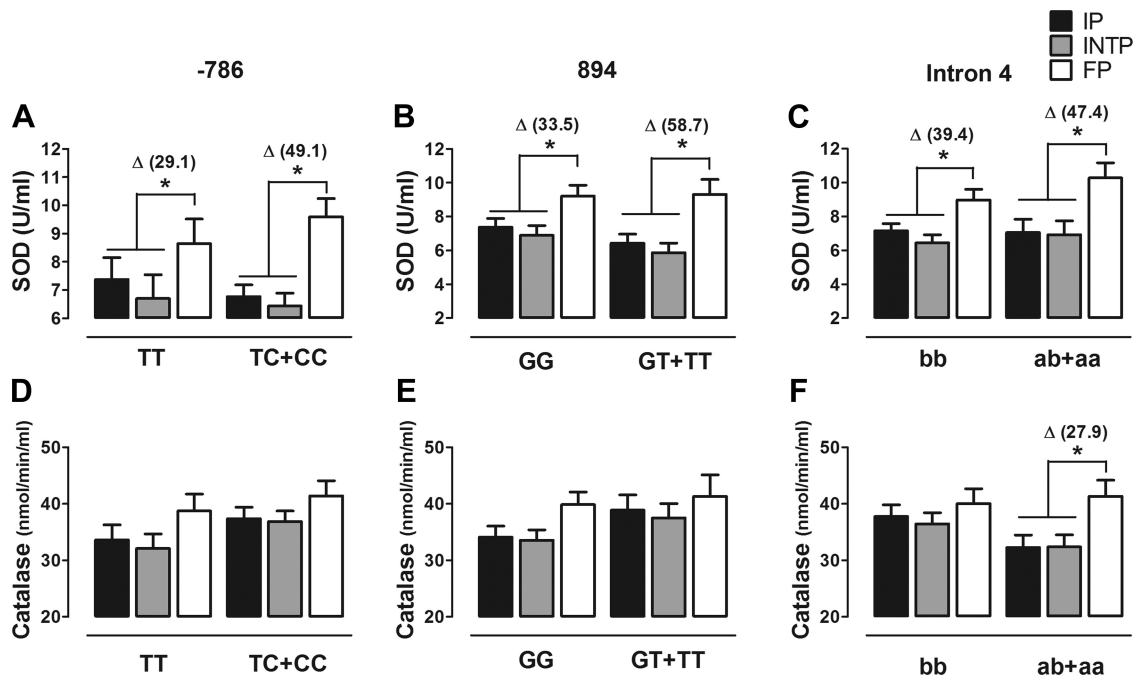


Fig. 4. Effects of aerobic exercise training on antioxidant enzyme activity (superoxide dismutase – SOD and catalase) at $-786>C$, G894T, and intron 4b/a NOS3 gene polymorphisms according to genotype groups. $-786>C$ (*A*), G894T (*B*), and intron 4b/a (*C*) illustrate SOD activity. $-786>C$ (*D*), G894T (*E*), and intron 4 (*F*) illustrate catalase activity. * $P < 0.05$, compared with IP and INTP within the same group.

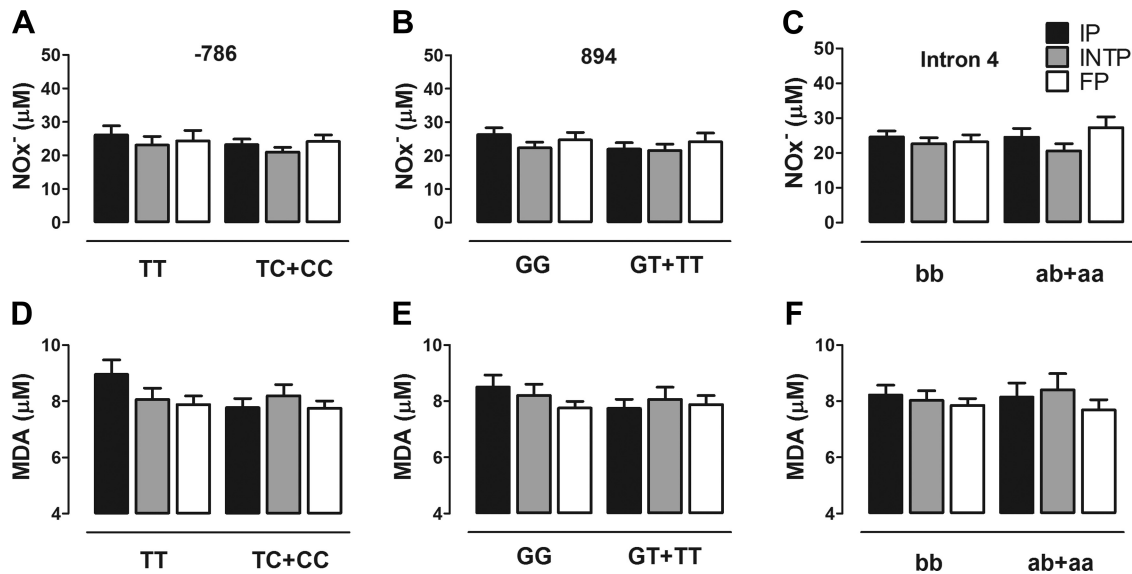


Fig. 5. Effects of aerobic exercise training on nitrate/nitrite (NOx⁻) and malondialdehyde (MDA) concentrations at -786>C, G894T, and intron 4b/a NOS3 gene polymorphisms according genotype groups. -786T>C (A), G894T (B), and intron 4b/a (C) illustrate NOx⁻ level. -786T>C (D), G894T (E), and intron 4 (F) illustrate MDA levels.

Similar to the BP office measurements, exercise training for 24 sessions promoted a significant decrease in systolic BP during daytime and 24 h in H1, H3, and H7 groups (Fig. 6, A and C). Ambulatory diastolic BP values were also significantly reduced in response to exercise training in H3 and H7 groups during daytime and 24 h of measurements (Fig. 7, A and C). On the other hand, cardiometabolic biomarkers failed to show any association with the reduction of BP values in response to exercise training. SOD activity was significantly increased in haplotype groups H1, H2, H5, and H7, except for H3, where no change

was observed (Fig. 8A). Catalase activity exhibited a different pattern in response to exercise training when haplotype groups were evaluated; thus, only H1 and H2 showed an increase in catalase activity (Fig. 8B). Unexpectedly, no changes were found in NO_x concentrations as well as MDA levels in response to exercise training in all formed haplotype groups (Fig. 8, C and D).

DISCUSSION

Major Findings

The rationale of the present study was to investigate whether the presence of NOS3 gene polymorphisms for all three positions T786-C, G894T, and intron 4 influence the health-promoting effect of aerobic exercise training in middle-aged adults, as analyzed by each single-nucleotide polymorphism as well as haplotype analyses. The main findings were as follows:

1. The presence of NOS3 gene polymorphisms for positions T786-C and G894T did not affect the beneficial effect of aerobic exercise training on BP, but no changes were detected in cardiometabolic biomarkers.

2. The presence of the NOS3 gene polymorphism for intron 4 blunted the beneficial effect of aerobic exercise training on BP that could possibly be a consequence of intron 4 acting as a short intronic repeat RNA controlling eNOS activity epigenetically in this population.

General Data

The beneficial effects of shear stress have been largely reported to be induced by exercise training on the eNOS/NO/cGMP signaling pathway in human studies (58, 67) and experimental models (17, 66); however, few studies have examined the interaction between exercise training and NOS3 gene polymorphisms on the cardiovascular system in human populations (19, 20, 48, 49, 53, 54).

Animal studies have shown that mice lacking one allele for the eNOS gene (+/-) are less responsive to exercise training

Table 4. Characteristics of the study participants grouped by haplotypes

	Parameters			
	SBP, mmHg	DBP, mmHg	Resting HR, beats/min	MLSS exercise HR, beats/min
H1 (n = 68)				
IP	117.9 ± 1.6	76.3 ± 0.9		
INTP	116.6 ± 1.7	76.0 ± 1.1	74.7 ± 1.0	147.1 ± 1.6
FP	111.8 ± 1.3*	73.1 ± 0.9*	72.5 ± 1.1*	135.7 ± 1.5*
H2 (n = 27)				
IP	115.5 ± 3.4	75.8 ± 1.7		
INTP	114.1 ± 3.0	75.7 ± 1.9	74.7 ± 1.9	150.4 ± 2.7
FP	112.6 ± 2.8	74.3 ± 1.6	70.2 ± 2.0*	136.7 ± 2.6*
H3 (n = 12)				
IP	116.4 ± 5.2	79.0 ± 3.6		
INTP	117.7 ± 4.2	78.2 ± 3.1	77.4 ± 3.6	148.9 ± 2.0
FP	113.0 ± 4.2*	74.5 ± 2.7*	73.6 ± 3.7	134.4 ± 2.8*
H5 (n = 25)				
IP	117.9 ± 3.5	76.6 ± 1.7		
INTP	119.6 ± 3.0	77.4 ± 1.9	75.5 ± 2.0	148.3 ± 2.8
FP	116.6 ± 3.0	76.0 ± 1.8	70.9 ± 2.1*	136.2 ± 3.0*
H7 (n = 36)				
IP	115.6 ± 2.3	77.9 ± 1.5		
INTP	114.7 ± 2.0	76.7 ± 1.3	78.4 ± 1.6	149.8 ± 2.8
FP	111.6 ± 1.8*	74.6 ± 1.2*	78.6 ± 1.7	139.6 ± 2.7*

Data are means ± SE; n, number of subjects. H1, TGb group; H2, TGa group; H3, TTb group; H5, CGb group; H7, CTb group. *P < 0.05, compared with IP and INTP.

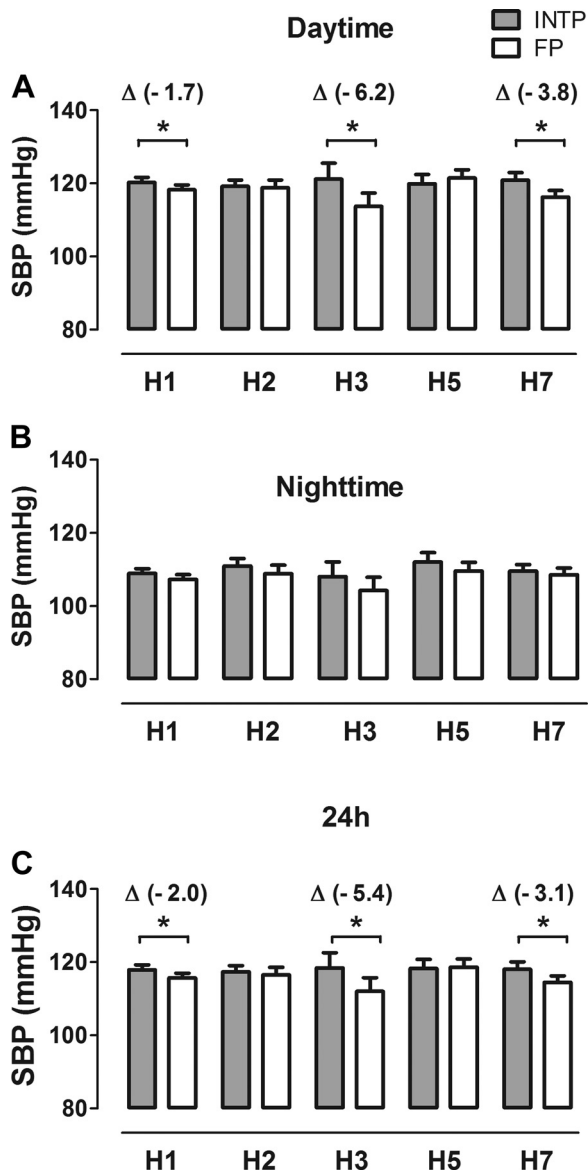


Fig. 6. Effects of aerobic exercise training on ambulatory SBP monitoring at $-786>C$, $G894T$, and intron 4b/a NOS3 gene polymorphisms according to haplotype groups. Measurements during daytime (A), nighttime (B), and over 24 h (C) are shown. * $P < 0.05$, compared with INTTP within the same group.

in activating the eNOS/NO/cGMP signaling pathway (32) or showing improvements in cardiac function (16); however, in human populations, the interaction between NOS3 gene expression and physical exercise is more complex. In our study, when we analyzed all volunteers together, exercise training during 24 sessions was effective in lowering systolic and diastolic BPs in office measurements; however, when we analyzed values over 24 h, these beneficial effects were not found. We believe that the beneficial effects were still there but that they were blunted by the daily activities of each volunteer. Indeed, epidemiological studies have consistently shown that exercise training reduces the prevalence of chronic disease in physically active subjects (4, 22). Exercise training increased aerobic capacity and decreased heart rate, showing that the total volume of the exercise program as well as the intensity used for this population based on MLSS promoted beneficial

effects on the cardiovascular system. These effects were accompanied by increases of SOD and catalase activities even though NO_x levels, which indirectly reflect NO production, were not changed after 24 sessions of aerobic physical exercise. Previous studies from our laboratory have shown that the beneficial effects of exercise training were associated with the upregulation of SOD-1 in obese (15) or diabetic (14) animals without a change in NO_x levels. The lack of any change in NO_x levels in response to exercise training in a human population is intriguing because previous studies from our laboratory have shown increased NO_x levels in obese women (68) and in healthy women after menopause (54). It is well known that nitrate is excreted by kidneys (26, 28); likely the lack of NO_x levels in response to exercise training could be due to increased clearance found in trained volunteers. It should also be pointed

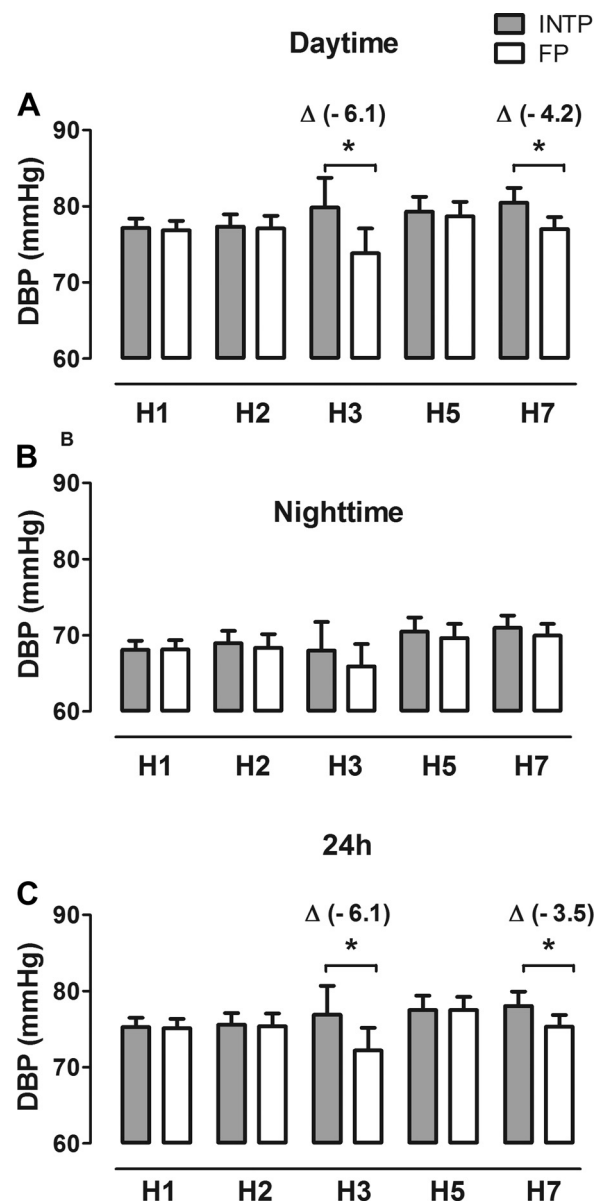


Fig. 7. Effects of aerobic exercise training on ambulatory DBP monitoring at $-786>C$, $G894T$, and intron 4b/a NOS3 gene polymorphisms according to haplotype groups. Measurements during daytime (A), nighttime (B), and over 24 h (C) are shown. * $P < 0.05$, compared with INTTP within the same group.

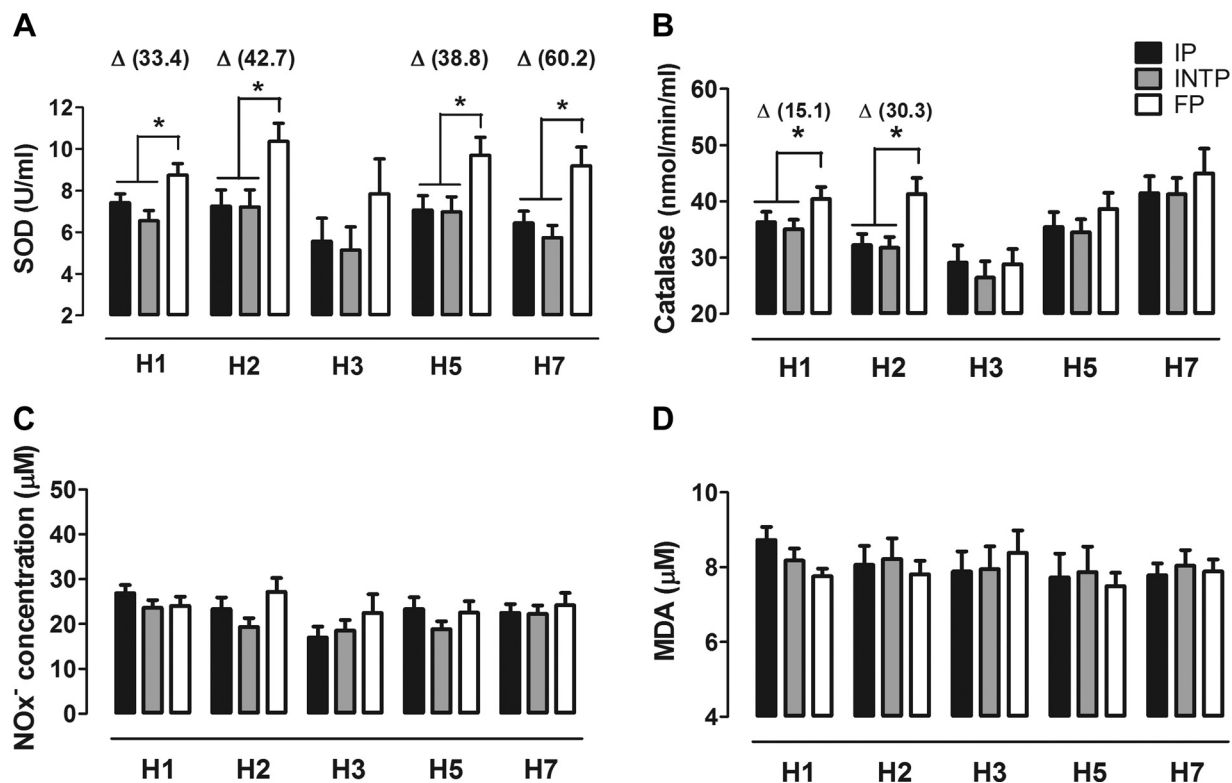


Fig. 8. Effects of aerobic exercise training on antioxidant enzyme activity (superoxide dismutase – SOD and catalase; A and B), NOx⁻ (C), and MDA (D) concentration at -786>C, G894T, and intron 4b/a NOS3 gene polymorphisms according to haplotype groups. **P* < 0.05, compared with IP and INTP within the same group.

out that the exercise training was longer (6 mo) in these previous studies and in a different population compared with the present study.

Oxidative stress plays an important role in the pathogenesis of cardiovascular disease that is linked to endothelial dysfunction, reduced NO bioavailability, and increased oxidant enzyme activity (36). Alternatively, exercise training is considered an important approach in maintaining redox state balance either in a normal state or under pathological conditions (18). In the present study, we did not find any change in MDA levels in response to exercise training. Lack of changes in lipid peroxidation after 24 sessions of exercise training would be expected, since all volunteers presented very low levels of MDA. Indeed, data from our laboratory showed a higher concentration of MDA in diabetic patients on insulin therapy (38 μM) compared with the subjects studied in the present study (8 μM), indicating the nonexistence of oxidative stress in middle-aged adults.

Genotype Analyses

In our crossover study, we found that the presence of the NOS3 gene polymorphism for intron 4 mitigated the beneficial effects of aerobic exercise training on BP in office measurements, which was confirmed by ambulatory BP monitoring in middle-aged adults. However, in the present study, we failed to show any mechanistic insights related to BP regulation in volunteers who carry the b allele for intron 4 of the NOS3 gene since exercise training was effective in increasing SOD activity in a genotype-independent way. Contrary to this, a previous study from our laboratory showed that exercise training for 2

mo was effective in lowering BP in a genotype-independent manner in postmenopausal women (20). This discrepancy could be due to the differences in the protocol design carried out in each study. In the present study, all subjects were followed up for 16 wk (8 wk of control period and a further 8 wk of exercise training). The previous study was carried out without a control period. Another relevant issue is the statistical analysis performed in the present study, where we used repeated-measures ANOVA as a more robust analysis compared with the *t*-test used in the previous study. Considering that ambulatory BP monitoring is a gold standard to make the best diagnosis of cardiovascular function as well as to provide better assessment to detect any change of BP in response to exercise training from a clinical perspective, our findings show that NOS3 gene polymorphisms for intron 4 (ab + aa genotypes) blunted the beneficial effect of exercise training in middle-aged adults as measured by ambulatory BP monitoring. Different from the old concept that the intron is a useless genomic sequence, it is now believed that the intron plays a key role in cell gene transcription controlling translational efficiency (37). Indeed, previous studies have shown that NOS3 gene intron 4 may act as an enhancer/repressor in the regulation of eNOS expression (69, 71). Additionally, it has been reported that 27-nt repeats in NOS intron 4 can be converted to short intronic repeat RNA, increasing histone acetylation and negatively regulating eNOS expression (70). Different from the two other NOS3 gene polymorphisms (T786-C and G894T), the presence of the NOS3 gene polymorphism for intron 4 interferes with the beneficial effects of exercise training, suggesting that the epigenetic influence of

this short intronic repeat RNA might be one possible explanation for subjects who present resistance to the health-promoting effect of physical exercise. Accordingly, a recent study has shown that exercise training for 18 wk did not modify the diminished parasympathetic activity found in healthy young subjects with NOS3 gene polymorphism (53).

Therefore, our hypothesis is that NOS3 gene polymorphisms for intron 4 interfere with mechanisms that regulate BP in dynamic conditions, for instance, exercise training, even though no changes were detectable in plasma cardiometabolic biomarkers. Indeed, few studies have evaluated, in a successful way, the relationship between NO_x measurements and exercise training in human populations (35, 38, 39). Data from our laboratory have shown an increase in NO_x levels after long-term aerobic exercise training (54, 62), whereas other study did not show any changes (20). Even studies involving vasomotor function in response to exercise training as measured by flow-mediated dilatation have shown controversial data (2, 8, 13, 25, 56). We have also looked at antioxidant enzyme activity in our protocol design since we have been studying this response successfully in several experimental models in the exercise science field. Antioxidant SOD activity in response to exercise training showed a genotype-independent pattern, indicating that the NOS3 gene polymorphism for intron 4 does not affect enzyme activity. Unfortunately, human studies evaluating SOD activity have shown great variability, making any comparison among the data difficult, ranging from 1 to 18 U/ml (21, 61). We also did not find a relationship between the genotype analysis and catalase activity, excluding the contribution of the NOS3 gene polymorphism for intron 4 in this antioxidant enzyme.

Paradoxical responses were found for positions T786-C and G894T for the NOS3 gene for the BP monitoring during 24 h; during the diurnal period, we observed a reduction in subjects with polymorphisms, whereas during nighttime, a reduction in BP in subjects without polymorphisms for the NOS3 gene was observed.

Haplotype Analyses

So far, no studies have evaluated the effect of exercise training on ambulatory BP monitoring grouped by haplotypes in middle-aged adults. Despite previous studies that have reported the relevance of NOS3 gene haplotype analysis on the susceptibility to cardiovascular disease (41, 44, 51, 52), our findings show that the interaction of physical exercise and haplotypes is a very complex issue; to establish a clear conclusion, further studies should be performed. Furthermore, most studies have used haplotype analysis as an important tool to detect the association between one particular disease and haplotypes (cross-section studies), but no one has analyzed haplotypes in longitudinal studies (with intervention), as we have. Our data consistently showed that two haplotype groups (TGa and CGb) were unresponsive in lowering systolic and diastolic BPs to exercise training in both office measurements and ambulatory BP monitoring. However, the mechanism by which these haplotype groups are resistant to the beneficial effect of exercise training is unknown since the cardiometabolic biomarkers were not reliable in detecting any changes in the studied population.

Conclusions

Our findings show that the presence of the NOS3 gene polymorphism for intron 4 blunted the beneficial effects of aerobic exercise training, as measured by office and ambulatory BP measurements, in middle-aged adults. Possibly, this effect might be a consequence of intron 4 acting as a short intronic repeat RNA controlling epigenetic eNOS activity in this population.

GRANTS

This work was supported by Sao Paulo Research Foundation Grants 2011/17437-7 and 2010/00866-0 (fellowship for C. H. G. Sponton).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s). The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Author contributions: C.G.S., R.D.E., C.M.R., M.J.F., A.P.J., C.P.A., and A.Z. performed experiments; C.G.S., R.D.E., C.M.R., M.J.F., A.P.J., C.P.A., M.B.J., and A.Z. analyzed data; C.G.S., R.D.E., C.M.R., M.J.F., A.P.J., C.P.A., M.B.J., and A.Z. interpreted results of experiments; C.G.S., A.P.J., and A.Z. prepared figures; R.D.E., C.M.R., M.J.F., A.P.J., C.P.A., M.B.J., and A.Z. approved final version of manuscript; A.Z. conception and design of research; A.Z. drafted manuscript; A.Z. edited and revised manuscript.

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