

ORIGINAL ARTICLE

Effects of 6-month aerobic interval training on skeletal muscle metabolism in middle-aged metabolic syndrome patients

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Aerobic interval training (AIT) improves the health of metabolic syndrome patients (MetS) more than moderate intensity continuous training. However, AIT has not been shown to reverse all metabolic syndrome risk factors, possibly due to the limited duration of the training programs. To assess the effects of 6 months of AIT on cardio-metabolic health and muscle metabolism in middle-aged MetS. Eleven MetS (54.5±0.7 years old) underwent 6 months of 3 days a week supervised AIT program on a cycle ergometer. Cardio-metabolic health was assessed, and muscle biopsies were collected from the *vastus lateralis* prior and at the end of the program. Body fat mass (−3.8%), waist circumference (−1.8%), systolic (−10.1%), and diastolic (9.3%) blood pressure were reduced, whereas maximal fat oxidation rate and VO_{2peak} were significantly increased (38.9% and 8.0%, respectively) (all $P<.05$). The remaining components of cardio-metabolic health measured (body weight, blood cholesterol, triglycerides, and glucose) were not changed after the intervention, and likewise, insulin sensitivity (CSI) remained unchanged. Total AMPK (23.4%), GLUT4 (20.5%), endothelial lipase (33.3%) protein expression, and citrate synthase activity (26.0%) increased with training ($P<.05$). Six months of AIT in MetS raises capacity for fat oxidation during exercise and increases VO_{2peak} in combination with skeletal muscle improvements in mitochondrial enzyme activity. Muscle proteins involved in glucose, fat metabolism, and energy cell balance improved, although this was not reflected by parallel improvements in insulin sensitivity or blood lipid profile.

KEYWORDS

aerobic interval training, metabolic syndrome, muscle metabolism

1 | INTRODUCTION

The combination of increased life expectancy and sedentary behaviors is alarmingly augmenting the incidence of age-related metabolic pathologies, such as metabolic syndrome (MetS).¹ MetS is a disorder characterized by the co-occurrence of obesity, hyperlipidemia, and insulin resistance. In consequence, patients with MetS have an increased risk for type 2 diabetes, cardiovascular disease, hypertension, and even all-cause mortality compared with age-matched

counterparts.² Low aerobic capacity is also frequently reported in MetS patients, making exercise training a core component in the treatment of the syndrome. Exercise training has proven its efficacy to increase metabolic flexibility and cardiovascular fitness, especially those exercise interventions involving high-intensity interval training.³⁻⁵ Part of those exercise-induced improvements are thought to occur at the skeletal muscle level. In addition to its locomotive function, skeletal muscle is a major determinant of whole-body aerobic capacity,⁶ and plays a crucial role in the regulation of fatty

	
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acid (FA) uptake, mitochondrial FA oxidation,⁷ basal metabolic rate and is the primary tissue responsible for whole-body glucose disposal.⁸ Studies performed by us and others suggest that skeletal muscle energy metabolism may be dysregulated in metabolic disease patients due to decreased mitochondrial function and/or density,^{9,10} or impaired FA oxidation that results in the accumulation of intramuscular lipids.^{11,12} Interestingly, 4 months of aerobic interval training (AIT), that combines periods of light to moderate intensity (~70% HR_{max}) with sets of vigorous intensity (~90% HR_{max}), is a time efficient way to increase aerobic capacity, reverse risk factors, and induce mitochondrial biogenesis in middle-aged MetS individuals.^{9,13} Other AIT studies report mixed results regarding muscle insulin sensitivity, reflected by different effects on the total protein levels of the glucose transporter GLUT4, glycogen, hexokinase II (HKII), and insulin receptor mediated signaling.¹⁴⁻¹⁶ However, the main skeletal muscle signaling cascades activated by exercise have not been sufficiently investigated in response to aerobic interval training in MetS patients. During muscle contraction, the rate of ATP turnover increases and AMPK α phosphorylation on its threonine residue 172 (pThr¹⁷²-AMPK α) can be elicited by both moderate¹⁷ and high-intensity exercise.¹⁸⁻²⁰ When the AMPK signaling cascade is activated, a higher peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1 α) mRNA expression is induced leading to a stimulation of mitochondrial biogenesis and fiber type switching toward type I fibers.^{21,22} Another well-characterized upstream modulator of PGC-1 α is the calcium/calmodulin-dependent protein kinase II (CaMKII) signaling pathway, which is activated by the increased intracellular calcium flux with exercise.^{20,23} This signaling cascade is also thought to mediate the expression of genes controlling FA oxidation, oxidative enzymes, and GLUT4 among others.²⁰

On the other hand, muscle lipases hydrolyze blood triglycerides, triglyceride-rich lipoproteins, and chylomicrons supplying the myocyte with fatty acids to oxidize or store. Upon production by the underlying parenchymal cells, lipoprotein lipase (LPL) is transported and attached to the capillary endothelium by the protein GPIHBP1 forming endothelial lipase (EL). Overexpression of EL decreases high-density lipoprotein (HDL) cholesterol levels, whereas blocking its action increases concentrations of HDL cholesterol.²⁴ EL has been shown to be higher in skeletal muscle of endurance-trained middle-aged men compared to age-matched sedentary men.^{25,26} However, the potential effects of long-term exercise training on LPL, EL, and other lipases important for intramuscular lipid droplets mobilization like the hormone-sensitive lipase (HSL) are not fully elucidated in MetS patients.

In this study, we tested if 6 months of AIT improves metabolic risk factors, insulin sensitivity and upregulates key proteins in skeletal muscle energy metabolism (Thr¹⁷²-AMPK/

Ser²²¹-ACC β phosphorylation, Thr²⁸⁶-CaMKII phosphorylation, glycogen content, hexokinase II (HKII), EL, LPL, and HSL). We also aimed to test whether the intervention is effective to induce mitochondrial biogenesis (citrate synthase activity, CS), and to increase the ability to oxidize fat at the whole-body level (maximal fat oxidation capacity, MFO) and the mitochondrial level (β -hydroxyacyl-CoA-dehydrogenase activity, HAD) in a group of middle-aged MetS with a previous experience in AIT (4 months). Ultimately, results of this study may help to design training based lifestyle intervention programs aimed at improving health among MetS patients.

2 | MATERIAL AND METHODS

2.1 | Participants and study design

Eleven metabolic syndrome patients (eight men and three post-menopausal women) with a previous experience in AIT⁹ volunteered to participate in the study. This study was conducted between December 2012 and June 2013, with a minimum detraining period of 2 months from the previous AIT program. Their mean \pm SD age, height, body weight, BMI, body fat mass, and VO_{2peak} were 54.5 \pm 0.7 years old, 1.65 \pm 0.01 m, 90.1 \pm 0.01 kg, 32.8 \pm 0.3 kg·m⁻², 31.8 \pm 0.3 kg, and 25.6 \pm 0.7 mL·kg⁻¹·min⁻¹. Written consent was given by the participants after they were fully informed about the exercise program and the experimental procedures, and the possible benefits and risks associated with the experiment. All the participants reported no cardiovascular or renal disease, peripheral vascular disease, and any disease associated with exercise intolerance. The study was approved by the local Hospital's Ethics Committee.

Owing to the special characteristics of our participants (middle-aged MetS willing to have a muscle biopsy taken), it was not possible to gather a sufficient number of patients to conduct a randomized controlled trial. In consequence, a quasi-experimental reversal design was used, in which each subject acted as their own control.²⁷

2.2 | Preliminary testing

All volunteers underwent medical screening to exclude individuals with symptoms or signs of cardiorespiratory disease. Prior to the start of the experiment, participants underwent a cycling graded exercise test until volitional exhaustion in an electrically braked cycle ergometer (Ergoselect 200; Ergoline, Germany). Integrated standard 12-lead ECG (Quark T12, Cosmed, Italy) and blood pressure were monitored in every stage to ensure that all subjects had a normal cardiovascular response to exercise. During the maximal test, O₂ consumption was measured by indirect calorimetry (Quark B2; Cosmed), and peak oxygen consumption (VO_{2peak}) and peak heart rate (HR_{peak}) were assessed as explained elsewhere.⁹

2.3 | Exercise training

Participants underwent supervised aerobic interval training (AIT) with a frequency of three times per week during 6 months. Training consisted of pedaling for 10 minutes as warm up at 70% HR_{max} followed by 4×4-minutes intervals at 90% HR_{max} interspersed with 3-minutes active recovery at 70% HR_{max} and a 5-minutes cool-down period for a total of 43 minutes. Exercise intensity was increased as training adaptations developed to maintain the target heart rate (Accurex coded, Polar, Finland). Participants were required to attend at least 85% of all the exercise sessions and instructed to maintain their regular dietary patterns during the duration of the study.

2.4 | Clinical investigation

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Before and after the 6 months training program, all the participants were tested for body composition, anthropometry (weight and waist circumference), resting blood pressure, blood metabolites (fasting glucose, glycated hemoglobin, cholesterol, triglycerides, leptin, and adiponectin), muscle glycogen and protein, exercise MFO, and peak oxygen consumption (VO_{2peak}) using a graded exercise test. Blood was drawn in the morning after a 10-hours overnight fast. Post-training tests were scheduled at least 72 hours after the last exercise training session to avoid measuring the acute effects of the last exercise bout rather than the chronic effects of the exercise training program. Percent body fat, trunk body fat, and fat-free mass were determined by dual energy X-ray absorptiometry (DXA Hologic Serie Discovery Wi QDR). Supine resting blood pressure was recorded using a handheld aneroid sphygmomanometer (Gamma GST; Heine, Germany) as the average of four measurements.

2.5 | Cardio-metabolic health

Peak aerobic capacity (VO_{2peak}) was assessed on an electronically braked cycle ergometer (Ergoselect 200; Ergoline) during a graded exercise testing using indirect calorimetry, (Quark b2; Cosmed) with 12 lead ECG monitoring (Quark T12; Cosmed). The highest heart rate value obtained during the test was considered HR_{peak} . Maximal fat oxidation was assessed in a fasted state using a graded exercise test with 3-minutes stages until respiratory exchange ratio exceeded 1.0. The last minute of each stage was averaged to calculate non protein respiratory quotient and fat oxidation rate.²⁸

2.6 | Blood analysis

Plasma glucose was analyzed using the glucose oxidase-peroxidase method with intra- to interassay coefficient of variation (iCV) of $0.9\pm 1.2\%$. Glycated hemoglobin (HbA1c)

using immune-turbidimetry tests (iCV; $0.7\pm 2.1\%$). HDL-c using accelerator selective detergent method (iCV; $1.7\pm 2.9\%$). Blood triglycerides (TG) with glycerol-3-phosphate oxidize method (iCV; $0.8\pm 1.7\%$). Total serum cholesterol (TC) and high-density lipoprotein cholesterol (HDL-c) by an enzymatic method with a single aqueous reagent (iCV; $1.1\pm 1.4\%$). Plasma leptin and adiponectin concentration were measured using a specific high-sensitivity human ELISA kit (R&D systems, Abingdon, UK).

2.7 | Insulin sensitivity

A 50-minutes-long IVGTT was used to measure insulin sensitivity as proposed by Tura et al.²⁹ (ie, C_{SI} index following the recommendations of the ICARUS group.³⁰ IVGTT was performed using a glucose load of $0.5\text{ g}\cdot\text{kg}^{-1}$ body mass with a maximal dose of 35 g of glucose for participants surpassing 70 kg of body weight. We used a 30% glucose solution (Glucosada 30%, Grifols, Spain) manually infused at an even rate over 3 minutes using two 60-mL syringes (BD Plastipak, Spain). Next, 5-mL blood samples were obtained every 10 minutes (ie, 10, 20, 30, 40, and 50 minutes), and the catheter was flushed with 3 mL 0.9% saline after every sample to ensure patency. Insulin concentration was measured in duplicate using chemiluminescent microparticle immunoassay (iCV; $2.0\pm 2.8\%$) in an automated immunoassay analyzer (Architect ci4100; Abbott Laboratories, USA). The homeostasis model assessment (HOMA) was also calculated following standard procedures.³¹

2.8 | Muscle biopsies analysis

Muscle biopsies were obtained from *m. vastus lateralis* after 30 minutes of rest in the supine position using the Bergström muscle biopsy needle technique modified to include suction. The muscle specimens were immediately inspected to remove any visible blood, fat, or connective tissue. After this, the muscle tissue was frozen in liquid nitrogen within <30 seconds of sampling and stored at -80°C until further analysis. Glycogen concentration was determined from the measurement of glucose after acid hydrolysis, as previously reported.³² The maximal activity of the enzymes β -hydroxyacyl-CoA-dehydrogenase and citrate synthase was determined fluorometrically, using the methodology described previously.³³

2.9 | Total protein extraction, electrophoresis, and Western blot analysis

Muscle protein extracts were prepared as described previously,³⁴ and total protein content was quantified using the bicinchoninic acid assay.³⁵ Briefly, proteins were solubilized in sample buffer containing $0.0625\text{ mol}\cdot\text{L}^{-1}$ Tris-HCl,

pH 6.8, 2.3% (wt/vol) sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, 5% (vol/vol) beta-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue and separated on 10% criterion polyacrylamide precast gels (Bio-Rad, Copenhagen, Denmark). After SDS electrophoresis, the gels were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (0.2 μm pores; Bio-Rad) using the Trans-Blot Turbo Transfer System (25 V in 7-minutes protocol; Bio-Rad) with Trans-Blot Turbo Midi Transfer Packs (Bio-Rad). The membranes were blocked for 1.5 hours at room temperature with either 5% skim milk diluted in Tris-buffered saline (10 $\text{mmol}\cdot\text{L}^{-1}$ Tris Base, 150 $\text{mmol}\cdot\text{L}^{-1}$ NaCl, pH 7.4) with 0.05% Tween-20 (TBS-T), or 5% bovine serum albumin (BSA) TBS-T. The membranes were then incubated with the primary antibody overnight at 4°C. To determine Thr¹⁷²-AMPK α , Ser²²¹-ACC β , and Thr²⁸⁶-CaMKII phosphorylation levels, antibodies directed against the phosphorylated amino acid (MWs 62, 280 and 50 KDa, respectively, Cell Signalling, Danvers, MA, USA) and total form of these kinases (Cell Signalling for AMPK and ACC β and Santa Cruz Biotechnology, Santa Cruz, CA for CaMKII) were diluted in 5% TBS-T; BSA-blocking buffer. GLUT4, HKII, EL, HSL, and LPL total protein expression were assessed in membranes incubated with their specific antibodies (MWs 50, 102, 70, 88, and 56 KDa, respectively, Fischer Scientific, Roskilde, Denmark for GLUT4, Cell Signalling, for HKII, Sigma-Aldrich, Saint Louis, MA for EL and Santa Cruz Biotechnology for HSL and LPL) diluted in 5% BSA-TBS-T blocking buffer. To control for differences in loading and transfer efficiency, the membranes were incubated with a monoclonal mouse anti-alpha-tubulin (MW: 50 KDa; Biosigma, St. Louis, MO, USA) antibody diluted in 5% skim milk TBS-T blocking buffer. No significant changes were observed in alpha-tubulin protein levels during the experiments. Antibody-specific labeling was revealed by incubation with an HRP-conjugated goat anti-rabbit antibody and the HRP-conjugated donkey anti-mouse antibodies from Jackson ImmunoResearch (West Grove, PA, USA) both diluted in 5% skim milk TBS-T blocking buffer and visualized with the ECL Western blotting detection system (Amersham Biosciences, Buckinghamshire, UK) using a CCD system (LAS-3000 Luminescent Image Analyser; Fujifilm, Tokyo, Japan) and quantified by the "Multi Gauge" analysis software (ver. 3.0; Fujifilm). All proteins were measured in duplicate, and the variation coefficient was below 15%.

Pre- and post-intervention samples for each individual were loaded in each gel (26 wells) to reduce intergel variance between groups. In addition, two human muscle samples obtained from a healthy young man were loaded as internal controls on all gels to control for intergel variability. Overall, the variation coefficient for the controls loaded in all gels was 11%.

2.10 | Statistical analysis

Data are presented as mean \pm SD. Normally distributed data were analyzed using Student's two-tailed paired *t* test (pre- to post-training comparison) and differences established by Tukey's post hoc analysis. Statistical significance level was set at $P<.05$. All statistical analyses were performed using SPSS software for windows (v.18; IBM). To determine the magnitude and meaningfulness of findings, effect size statistics were calculated using Cohen's *d* (G*Power Version 3.1.2), being the cutoffs small (from 0.2 to 0.5), medium (from 0.5 to 0.8), or large (over 0.8).

3 | RESULTS

3.1 | Metabolic syndrome factors

Participants responses to exercise training did not differ between sex (46% males and 54% females), and thus, data were analyzed as a group without sex distinctions. Changes in metabolic syndrome factors after 6 months of AIT are detailed in Table 1. Body fat mass ($-3.8\%\pm 0.4\%$; $P<.05$; Cohen's $d=0.45$), waist circumference ($-1.8\%\pm 0.2\%$; $P<.05$; Cohen's $d=0.30$), and blood pressure (-10.1 ± 0.6 and $9.3\%\pm 0.4\%$, respectively, for SBP and DBP; $P<.05$; Cohen's $d=0.91$ and 1.20 , respectively) were the variables that significantly improved after the training program, whereas body weight tended to be lower after the intervention (from 90.1 ± 14.5 to 88.6 ± 14.1 kg, $P=.09$; Cohen's $d=0.11$). In contrast, fasting blood glucose, total cholesterol, HDL, and triglycerides did not change significantly after the 6 months of AIT.

3.2 | Insulin sensitivity, blood parameters, and exercise analysis

Calculated insulin sensitivity index (C_{SI}) from the 50-minutes IVGTT was not significantly altered with 6-month AIT. Accordingly, HOMA values also remained stable with training (Table 2). HbA1c, leptin, and adiponectin were not changed after 6 months of aerobic interval training (Table 2). Regarding exercise variables, $\text{VO}_{2\text{peak}}$ increased with the intervention by 8.0% ($P<.05$; Cohen's $d=0.22$; Table 2). Maximal pedaling workload (W_{max}) and MFO were increased ($9.9\%\pm 0.8\%$ and $38.9\%\pm 4.6\%$, respectively; $P<.05$; Cohen's $d=0.88$ and 0.27 ; Table 2).

3.3 | Mitochondrial biogenesis and fatty acid oxidation capacity

Mitochondrial enzyme citrate synthase activity was increased 26.0% ($P<.05$; Cohen's $d=1.75$) after the training program, whereas mitochondrial β -oxidation measured by

TABLE 1 Metabolic syndrome factors before and after 6 mo of aerobic interval training (AIT)

	Pre-training	After 6 mo of AIT	<i>P</i> value	Cohen's <i>d</i>
Body weight (kg)	90.1±14.5	88.6±14.1	.09	0.11
Body fat mass (kg)	31.8±3.4	30.6±2.6*	.03	0.45
Waist circumference (cm)	107.2±6.8	105.3±6.7*	.02	0.30
Glucose (mmol·L ⁻¹)	6.32±0.81	6.32±0.92	.94	0.00
Triglycerides (mmol·L ⁻¹)	1.15±0.40	1.39±0.80	.24	-0.40
Total Cholesterol (mmol·L ⁻¹)	4.61±0.90	4.55±0.90	.70	0.07
HDL (mmol·L ⁻¹)	1.00±0.12	1.15±0.10	.36	1.42
SBP (mm Hg)	134.0±17.8	120.5±13.1*	.00	0.91
DBP (mm Hg)	81.5±7.9	74.0±4.9*	.00	1.20

HDL, high-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure. Values are mean±SD.

*Significantly different from pre-training (*P*<.05)

TABLE 2 Blood and exercise variables before and after 6 mo of aerobic interval training (AIT)

	Pre-training	After 6 mo of AIT	<i>P</i> value	Cohen's <i>d</i>
HbA1c (%)	5.9±0.5	5.9±0.4	.21	0.00
HOMA	3.6±0.8	4.8±2.1	.21	-0.79
C _{SI} (×10 ⁻⁴ min ⁻¹ [μU·mL] ⁻¹)	4.0±0.7	4.1±0.7	.90	0.00
Leptin (pg·mL ⁻¹)	23 910±19 421	23 889±15 991	1.00	0.00
Adiponectin (pg·mL ⁻¹)	10 789±4420	9949±4424	.40	0.20
VO _{2peak} (L·min ⁻¹)	2.3±0.9	2.5±1.0*	.04	-0.22
VO _{2peak} (mL·kg ⁻¹ ·min ⁻¹)	25.6±7.2	27.9±8.0	.10	-0.32
Maximal workload (W)	194±78	217±97*	.01	-0.27
MFO (g·min ⁻¹)	0.21±0.06	0.29±0.12*	.02	-0.88

HbA1c, glycated hemoglobin; MFO, maximal fat oxidation. Values are mean±SD.

*Significantly different from pre-training (*P*<.05)

enzymatic β-hydroxy-acyl-CoA-dehydrogenase activity was not changed significantly (Figure 1A and B, respectively).

3.4 | Muscle glucose metabolism

Participants muscle glycogen content remained unaltered after 6 months of AIT (223±58 vs 263±75 mmol·mg⁻¹ dry muscle). Similarly, total protein expression of HKII was similar after the intervention (Figure 2E). However, the total protein amount of the glucose transporter GLUT4 was increased by 20.5% (*P*<.05; Cohen's *d*=0.52; Figure 2F).

3.5 | Basal skeletal muscle Thr¹⁷²-AMPKα/Ser²²¹-ACCβ and Thr²⁸⁶-CaMKII levels

Total AMPKα protein expression was a 23.4% higher after the intervention compared to pre-training levels (*P*<.05; Cohen's *d*=0.57; Figure 2A). The Thr¹⁷²AMPKα/Total AMPKα (fractional phosphorylation of AMPKα) was not statistically

significant when comparing pre to post-training (Figure 2B). Neither ACCβ total protein expression nor Ser²²¹-ACCβ phosphorylation changed with the exercise intervention (Figure 2C). Moreover, 24 weeks of stationary cycle aerobic interval training did not alter the basal expression of total and phosphorylated Thr²⁸⁶-CamKII levels (Figure 2D).

3.6 | Muscle lipases

EL expression showed a significant increase (33.3%±0.4%; Figure 3A; *P*<.05; Cohen's *d*=0.85) after training compared to baseline values, with no differences in the HSL and LPL protein levels (Figure 3B-C).

4 | DISCUSSION

The current study reveals that long-term (24 weeks) aerobic interval training induces a number of health benefits related

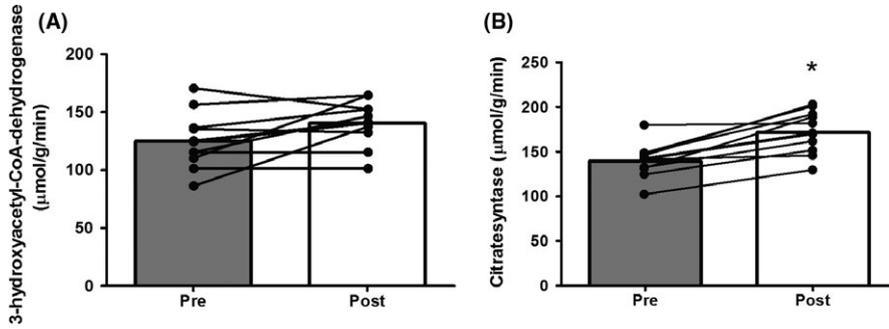


FIGURE 1 Enzyme activity of (A) 3-hydroxy acetyl-coA-dehydrogenase (HAD) and (B) citrate synthase (CS) activity before (PRE) and after (POST) 6 mo of AIT. Solid lines represent individual values for each participant. *Significantly higher from pre-training ($P < .05$)

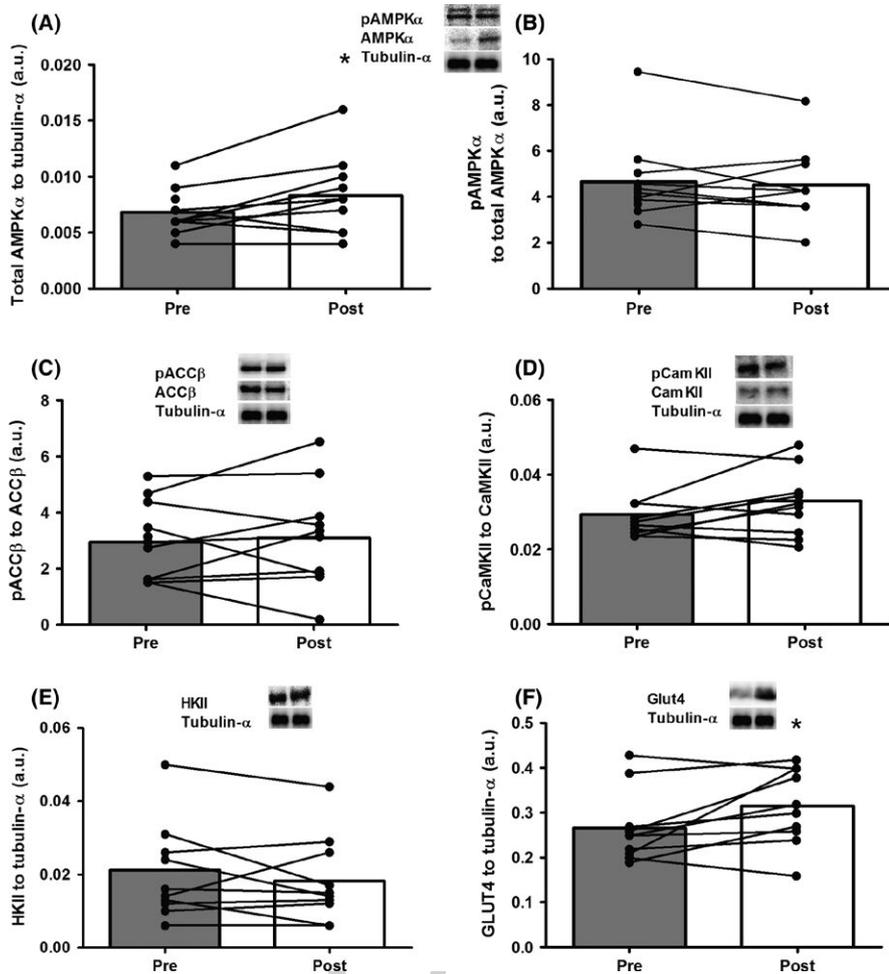


FIGURE 2 Muscle protein expression before (PRE) and after (POST) 6 mo of AIT. (A) Total AMPK α expression; (B) Thr¹⁷²-AMPK α phosphorylation level; (C) Ser²²¹-ACC β phosphorylation level; (D) CamKII phosphorylation level; (E) HKII expression and (F) GLUT4 expression. Solid lines represent individual values for each participant. *Significantly higher from pre-training ($P < .05$)

to metabolic risk factors in middle-aged ~~experienced~~ MetS patients fed “ad libitum.” Namely, subjects reduce their body fat, waist circumference, and blood pressure (Table 1) while slightly increasing their cardiovascular (ie, VO_{2peak}) and more consistently metabolic (MFO) fitness during exercise (Table 2). However, 24 weeks of aerobic interval training (AIT) was not enough to improve their whole-body insulin sensitivity or their blood lipid profile. In addition, using muscle biopsy in the *vastus lateralis*, we analyzed the effects of AIT on key skeletal muscle proteins regulating muscle metabolism to gain insight on how exercise training may attenuate metabolic abnormalities associated with the metabolic syndrome.

4.1 | Changes in mitochondrial biogenesis markers in muscle (CS and upstream modulators AMPK and CaMKII) and whole-body VO_{2peak}

In healthy subjects, it has been established that the main limiting factor determining maximal aerobic capacity is cardiac output rather than skeletal muscle oxygen utilization.³⁶ However, metabolically challenged persons (ie, obese, type 2 diabetics) present impaired skeletal muscle oxidative capacity, often shown by a decrease in mitochondrial density but not function.^{9,37} This misbalance could be counteracted by

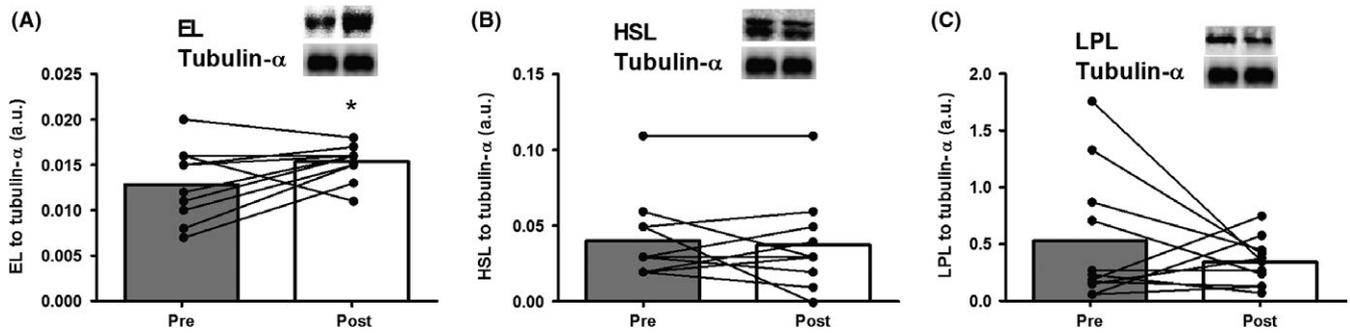


FIGURE 3 Muscle lipase expression before (PRE) and after (POST) 6 mo of AIT. (A) endothelial lipase expression (EL); (B) hormone-sensitive lipase expression (HSL) and (C) lipoprotein lipase expression (LPL). Solid lines represent individual values for each participant. *Significantly higher from pre-training ($P < .05$)

exercise training, because MetS patients experience the habitual cardiovascular adaptations that improve oxygen kinetics in healthy subjects.³⁸ It is somehow surprising the low effect that the training intervention exerted on the aerobic capacity of our subjects (ie, 8.0% increase in VO_{2peak}), in comparison with other studies performed on MetS patients where they observed around a 35% increase in VO_{2peak} .¹⁶ Among the possible causes for this low responsiveness is the fact that the subjects recruited for this study participated in a previous 4 months AIT study performed by our research group,⁹ where they showed a mean increase in VO_{2peak} of 21%. Another explanation could be that the selected training intervention may not be the best therapeutic exercise training regime for longer than 4 months studies, and additional volume and/or intensities are required to increase aerobic capacity. On the other hand, there is a positive relationship between the relative change in VO_{2max} in response to physical training and CS activity (as a surrogate of mitochondrial biogenesis).³⁹ Our participants increased their VO_{2peak} by 8.0%, accompanied by a 26.0% increase in CS activity (Pearson correlation $r = .63$; $P < .05$). Therefore, it seems that AIT-induced improvements in VO_{2peak} are coupled to mitochondrial biogenesis in MetS patients. PGC-1 α mRNA expression is also frequently used as a marker of mitochondrial biogenesis, due its action as coactivator of transcription factors, and nuclear and mitochondrial genes required for the organelle's synthesis.⁴⁰ PGC-1 α expression and activity are regulated by upstream signaling pathways of protein kinases, as AMPK and CaMKII.^{23,41} We have observed that basal pThr¹⁷²-AMPK α phosphorylation degree did not change with the training intervention, maybe because muscle samples were taken in resting conditions where there is lack of an increased energy demand (ie, increased ATP:AMP ratio) that would stimulate AMPK activation. In support for our results, other authors have shown similar basal pThr¹⁷²-AMPK α phosphorylation levels in obese compared with control muscle biopsies obtained from resting vastus lateralis muscle^{42,43} and Kjøbsted et al.⁴⁴ have recently reported an intact regulation of the AMPK signaling network in response to acute exercise in skeletal muscle of male patients with type 2 diabetes.

There is to our knowledge no data about the effects of long-term intermittent perturbations of the cellular energy charge (eg, 24-week interval cycling exercise training) in the skeletal muscle of MetS patients. We observed that this training increases total AMPK α protein level (Figure 2A). Nielsen and colleagues demonstrated an 85% higher level of total AMPK α protein content in endurance trained vs sedentary healthy subjects.⁴⁵ Moreover, 8 weeks of strength training induced a significant increase in total AMPK α in MetS patients.⁴⁶ We observed a 20% higher total AMPK α protein levels after the training intervention, suggesting that an increase in total AMPK protein content is an early event occurring in the adaptation of MetS skeletal muscle toward a more oxidative muscle phenotype. It remains unknown what training program intensity, volume, and duration would exert the best adaptations in MetS individuals, although the metabolic fluctuations induced by intermittent exercise appear to be a potent stimulus for PGC-1 α upstream kinases such as AMPK.

Regarding calcium-mediated muscle signaling, existing literature states that acute high-intensity exercise is able to induce Thr²⁸⁶-CaMKII activation,^{20,47} and short term (3 weeks) of one-legged endurance exercise training provokes a onefold increase in maximal CaMKII activity and CaMKII kinase isoform expression in young healthy men.⁴⁸ Conversely, the resting skeletal muscle levels of either pThr²⁸⁶-CaMKII or total CaMKII remained unchanged in the present investigation. The lack of available data about long-term adaptations on CaMKII signaling pathway in MetS individuals and the different exercise protocols applied make comparisons difficult, and further studies on the effects of AIT on MetS regarding calcium-mediated muscle signaling are therefore warranted.

4.2 | Changes in muscle fat metabolism markers (β -hydroxyacyl-CoA-dehydrogenase, EL, ACC) and maximal fat oxidation induced by exercise

Our data support the classical metabolic adaptations to aerobic exercise training that increase the reliance on fat as energy

substrate during exercise,⁴⁹ confirmed by a significant 36% increase in maximal fat oxidation capacity in our patients. At the muscle cell level, activation of AMPK phosphorylates and inhibits acetyl-coenzyme A carboxylase (ACC), leading to reduced malonyl-coenzyme A and increased FA flux into the mitochondria via carnitine palmitoyl transferase-1.⁵⁰ ACC phosphorylation is therefore considered a marker of increased FA oxidation in the muscle. In concordance with the abovementioned lack of changes in the degree of pThr¹⁷²-AMPK α levels, pSer²²¹-ACC β remained unchanged after 24 weeks of AIT. Moreover, the independent measurement of mitochondrial fatty acid β -oxidation by hydroxy-acyl-CoA-dehydrogenase enzymatic activity confirmed the lack of adaptation at the resting state. Our results imply that in metabolic syndrome patients, the increase in the maximal capacity to oxidize fat during exercise is not explained by an increase in resting mitochondrial FA oxidation capacity or basal activation of key proteins governing skeletal muscle metabolic regulation (AMPK-ACC). It can be alternatively explained by an increased oxygen carrying and delivery capacity of the blood,⁵¹ or increased capacity of FA uptake or release from the lipid droplets for oxidation. We acknowledge that phosphorylation of AMPK-ACC in response to acute exercise after a training intervention could result in substantial changes in the degree of pThr¹⁷²-AMPK α and pSer²²¹-ACC β levels, and such adaptations could also influence the results presented in the study. We made a large effort to obtain muscle biopsies before and after the training intervention, but a limitation is the lack of acute post-exercise muscle biopsies.

Eight weeks endurance training in obese subjects increases the activity of lipases involved in the intramuscular lipolysis regulation, as HSL and ATGL, but not total HSL protein content,⁵² and 2 weeks physical inactivity (induced by leg immobilization) and age negatively affect intramuscular lipolytic capacity in men, leading to intramuscular triglycerides accumulation.²⁵ In agreement, we observed no differences in total HSL and LPL protein content with training, although we observed a novel higher protein amount of EL with 24 weeks AIT in our MetS patients. This lipase encoded by the LIPG gene and synthesized by endothelial cells was initially reported not to be expressed in skeletal muscle⁵³; however, a recent study suggests a possible role for EL in endothelial tissue of human skeletal muscle. Vigelsø et al. showed a 100% higher EL expression in endurance trained compared to healthy middle-aged men and suggested that EL might be an alternative pathway for FA uptake in skeletal muscle.^{25,26} This EL increase can also be explained by a higher microvascular density with endurance training, which has also been observed after sprint interval training in previously sedentary males.⁵⁴ Although we found a higher expression of muscle EL with aerobic interval training, this was not coupled to an increased basal mitochondrial HAD activity and we did not measure angiogenesis in the present

investigation. Consequently, more mechanistically driven studies (ie, combining in vivo protein activity and function and ex vivo mitochondrial FAO respiration) are needed to elucidate the physiological role of EL in human skeletal muscle. Moreover, although we inspected our muscle samples under the microscope to remove any visible blood, fat, or connective tissue, it is not possible to ensure 100% if the origin of the EL expression detected in the blots is from endothelial cells or skeletal muscle fibers.

4.3 | Changes in muscle carbohydrate metabolism muscle markers (HK, GLUT4, glycogen) and insulin sensitivity (CSI, HOMA-IR, blood glucose)

Most of the studies analyzing insulin sensitivity responses to AIT report small or no changes on steady state glucose infusion rate during an euglycemic clamp,¹⁵ oral glucose tolerance tests, and insulin sensitivity indexes.^{9,13,38} Accordingly, the data included in this report (lack of changes in CSI, HbA1c, HOMA-IR, and fasting blood glucose concentration) suggest that 24 weeks of increasing intensity AIT alone is largely futile at decreasing insulin resistance at the whole-body level and that additional interventions (ie, diet or medicine) are necessary to increase insulin sensitivity in metabolic syndrome patients. This lack of adaptation was also reflected at the muscle level, where glycogen content and HKII, a key protein in the regulation of glucose metabolism remained unchanged. Interestingly, we observed a 20.5% increase in the intramuscular levels of the glucose transporter GLUT4, suggesting that discrete adaptations at the cellular level occur before a visible change in whole-body insulin sensitivity can be observed.

4.4 | Metabolic syndrome factors are not further reduced with 24 weeks of AIT

We have previously reported that in sedentary MetS patients 16 weeks of an AIT program could reverse metabolic syndrome factors in roughly one-third of this population despite the absence of a diet intervention. In the present investigation, a 27.3% of the participants reversed metabolic syndrome factors <3. The most important changes were observed in blood pressure (12% decrease), blood HDL-c concentrations (12% increase), and to a lower extent in waist circumference (3.8%). However, plasma glucose and triglycerides concentrations were not significantly reduced.⁹ The question raised from this investigation was whether an AIT program with a longer duration could be more adequate to revert the remaining MetS components to normal clinical values. Surprisingly, we have observed that 24 weeks of AIT significantly reduced blood pressure and waist circumference to a similar extent (10.1 and 1.8%, respectively) than

shorter duration AIT protocols,^{9,16,55,56} and tended to reduce body weight with no significant effects on HDL-c, fasting plasma glucose, and triglyceride concentrations, similarly to shorter studies.^{9,56} In addition, whole-body fat mass was significantly reduced by 3.8%, although systemic concentrations of leptin and adiponectin remained unchanged. This lack of correlation between changes in fat mass stores and leptin concentration has also been observed before in healthy men and women in response to acute and chronic exercise,^{57,58} and indicates that maybe a greater fat mass loss is needed to induce either a reduction in leptin or an increase in adiponectin levels.

This study lacked a control group. However, it is important to say that our study population is very specific and difficult to find (middle-aged MetS willing to donate muscle samples), and that there is enough evidence in the literature about the beneficial effects of exercise training on MetS^{9,13,15,16} to suggest that a randomized trial that condemned the patients willing to participate in the intervention to a passive control group was not ethically justified. Our results suggest that reducing metabolic syndrome factors is not a matter of longer training programs, but maybe training programs with higher training volume or intensity in each bout and/or concomitant diet-induced weight loss.¹³ In consequence, there is definitely a need for longer (≥ 24 weeks) AIT-based studies, combining “ad libitum” and caloric restriction feeding conditions to confirm our results.

5 | PERSPECTIVES

Overall, our findings show that in experienced middle-aged MetS men and women fed “ad libitum,” 6 months of AIT slightly raises their capacity to oxidize fat and consume oxygen during exercise in combination with skeletal muscle improvements in mitochondrial enzyme activity. Moreover, muscle proteins involved in glucose metabolism and energy cell balance also improved although this was not reflected in enhanced insulin sensitivity or blood lipid profile. Therefore, there is certainly a need for additional AIT studies combined with diet and/or medication to better design exercise training interventions able to improve glucose and fat utilization at the muscle and whole-body level. On the other hand, mechanistically driven studies (ie, combining in vivo and ex vivo experiments, or in animal knockout models) are needed to elucidate the physiological role of EL in human skeletal muscle.

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