Supplementary Appendix

This appendix has been provided by the authors to provide readers with additional information about the authors' work.

Supplement to: França-Pinto A, Mendes FAR, Carvalho-Pinto R, Agondi RC, Cukier A, Stelmach R, Saraiva-Romanholo BM, Kalil J, Martins MA, Giavina-Bianchi P, Carvalho CRF. **Aerobic training decreases bronchial hyperresponsiveness and systemic inflammation in patients with moderate or severe asthma: a randomized controlled trial**

Supplementary Appendix

This supplementary material section has been provided by the authors to provide readers with additional information about the methods and results.

The components in this Supplementary Material document are as follows:

Online publication Supplementary Material for **Aerobic training decreases bronchial hyperresponsiveness and systemic inflammation in patients with moderate or severe asthma: a randomized controlled trial**

METHODS

Experimental design

All patients underwent the baseline assessment and performed all the tests in the following sequence: during week 1, the asthma control questionnaire (ACQ) and asthma quality of life questionnaire (AQLQ) were administered, and the patients received a daily diary for symptoms and underwent pulmonary function testing; during week 2, induced sputum and fractional exhaled nitric oxide (FeNO) were collected; during week 3, a maximal aerobic exercise test was performed, and a blood sample was collected; and during week 4, bronchial hyperresponsiveness was assessed. Persons involved in data collection were blinded to the group allocation. After completing the 24 treatment sessions, the patients were re-evaluated following the same sequence as the baseline assessment. All patients were clinically stable (i.e., at least 30 days without exacerbation) at baseline and re-evaluation. In addition, the patients were asked to refrain from vigorous exercise 24 h before all assessments.

Interventions

Education program

Both groups completed an educational program consisting of 2 classes, lasting 2 hours each, during week 4. The topics discussed included asthma pathophysiology, diagnosis and treatment, environmental control, disease control using the daily diary, proper medication use and daily peak expiratory flow measurements.[1]

Breathing exercise program

Both groups completed a breathing exercise program twice a week for 12 weeks. Each session consisted of 30 minutes of yoga breathing exercises, including Kapalabhati (fast expiratory breathing exercise followed by passive inhalation), Uddhiyana (full exhalation followed by

forced inspiration performed without air inhalation apnea), and Agnisara (full exhalation followed by a sequence of retractions and protrusions of the abdominal wall in apnea).[2, 3] Each exercise was executed in sets of 3, lasting 2 minutes each, followed by 1 minute of rest. For the TG patients, breathing exercise sessions were performed before the aerobic training sessions.

Aerobic training program

The aerobic training was initially performed at the heart rate (HR) corresponding to one-third of the difference between the anaerobic threshold (AnT) and the respiratory compensation point (RCP) (HR AnT + 33% x [HR RCP – HR AnT]) obtained from cardiopulmonary exercise testing (see below). After two weeks of the adaptation, the exercise intensity was increased to two-thirds of the difference between the AnT and the RCP (HR AnT + 66% x [HR RCP – HR AnT]).[4] If a patient maintained this intensity for 2 consecutive exercise sessions without symptoms, the exercise intensity was increased by 5% of HR (until 85% of patient's maximal heart rate was achieved) by increasing either the treadmill speed or the inclination. The use of salbutamol (200 μg) before an exercise session was recommended only if the peak expiratory flow was <70% of the patient's best value. Peak expiratory flow and asthma symptoms were monitored at the end of every exercise session.

Assessments

Bronchial hyperresponsiveness

The bronchial provocation test was conducted according to American Thoracic Society (ATS) guidelines.[5] Patients inhaled increasing concentrations of histamine in the following sequence: 0.0625 mg/mL, 0.25 mg/mL, 1.0 mg/mL, 4.0 mg/mL and 16.0 mg/mL using the dosimeter method (DeVilbiss 646 nebulizer, DeVilbiss Health Care, Somerset, PA, USA).

This method consists of 5 deep inhalations from each concentration, starting at functional residual capacity and holding the breath (near total lung capacity) for 5 seconds. Forced expiratory volume in the first second (FEV_1) was measured 30 and 90 seconds after the inhalations. The test is considered positive when the histamine concentration promotes a decrease ≥20% in FEV_1 (PC_{20}) in relation to the post-saline value or when the maximum concentration is reached (16 mg/mL). We followed the criteria of absolute and relative contraindications recommended by the ATS guidelines.[5] One double dose of concentration is considered an important clinical improvement.[6, 7]

Serum cytokines and total IgE

Plasma levels of inflammatory mediators were evaluated before and after 3 months of intervention. The patients were instructed to fast for 8 hours and not to drink alcohol or caffeine for 12 hours preceding the blood collection. Venous blood samples were centrifuged, and the supernatants were frozen in aliquots at -80 °C and analyzed at the end of the study. The cytometric bead array method (BD Biosciences, San Jose, CA, USA) was used to analyze the levels of IL-4, IL-5, IL-6, IL-8, IL-10, tumor necrosis factor (TNF)-α, IL-12p70, IL-8/CXCL8, monocyte chemotactic protein-1 (MCP-1/CCL2) and RANTES/CCL5. Samples were analyzed in a flow cytometer (LSR, model Fortessa) using FACSDiva™ software (both from BD Biosciences). The assay was performed according to the manufacturer's protocol; the detection limits for each interleukin or chemokine were as follows: IL-4 (144.4 fg/mL), IL-5 (67.8 fg/mL), IL-6 (68.4 fg/mL), IL-8/CXCL8 (0.2 pg/mL), IL-10 (13.7 fg/mL), TNF-α (67.3 fg/mL), IL-12p70 (12.6 fg/mL), MCP-1/CCL2 (2.7 pg/mL) and RANTES/CCL5, IL-8 (69.9 fg/mL). Total serum IgE was measured by nephelometry and commercially available kits (Dade Behring/Siemens, Deerfield, USA). The cut-off value for elevated IgE was set at 100 IU/mL.

Fractional exhaled nitric oxide (FeNO)

FeNO was collected offline, and the evaluation was made before spirometry. The patients were advised to blow into a Mylar bag, with a breath pressure of 12 cmH_2O, monitored by the pressure gauge, reaching a flow rate of 200 mL/s. All measurements were determined by chemiluminescence (Sievers 280) in accordance with the ATS recommendations.[8] This procedure and analysis were performed by a blinded investigator.

Induced sputum

After each patient was pre-medicated with 400 μg salbutamol, 3% hypertonic saline inhalation was administered using an ultrasonic nebulizer for 15 minutes.[9] The patients were asked to blow their nose, rinse their mouth with water and swallow the water to reduce contamination of the sputum specimen with post-nasal drip or saliva. Sputum samples were visually separated from saliva, the aliquot was treated with 0.1% dithiothreitol (Sigma-Aldrich, SP, Brazil), and the mixture was briefly stirred with a vortex mixer. Total cell counting was performed with a hemocytometer;[10, 11] the cell suspensions were adjusted to 1.0×10^6/mL.[12] The sputum was processed using the cytospin method, and the cells were classified as eosinophils, lymphocytes, neutrophils, macrophages, squamous cells, goblet, and ciliated cells on the basis of their morphology by a single-blinded investigator.

Asthma symptoms and exacerbation

Asthma symptoms and exacerbation were evaluated using a daily diary of symptoms according to previous methods.[3, 13] A daily diary was used to record asthma symptoms, such as cough, diurnal and nocturnal dyspnea, wheezing and use of relief medications. All

patients received the diary on the first day of assessment and filled it out 30 days before intervention and every month during intervention.

Asthma Control Questionnaire

ACQ-7, a standardized tool for assessing clinical control in asthmatic patients, consists of 7 questions: 5 questions related to asthma symptoms (daytime and nighttime symptoms, activity limitations, dyspnea, wheezing); one question related to the use of short-acting β_2 agonists, such as rescue medication; and one question related to FEV_1 before bronchodilator in the percent of predicted values. The ACQ score is the average of the 7 items obtained over a 7-day period and ranges from 0 to 7.[14] The patients were classified based on ACQ-7 score as having controlled asthma (<0.75), partially controlled asthma (0.75-1.5) or uncontrolled asthma (>1.5).[15] A minimum clinically important difference is 0.5 on a 7-point scale.[16]

Asthma quality of life

The total score was obtained using the average score of the 32 questions.[17] A higher AQLQ score indicates a better quality of life. A minimum clinically important difference is 0.5 on a 7-point scale.[18]

Cardiopulmonary exercise test

This test was performed on a treadmill (h/p/cosmos®, Pulsar, Nussdorf-Traunstein, Germany) using a ramp protocol with fixed speed and increments of 2% inclination every minute. Pulmonary gas exchange was measured using a breath-by-breath automated gas analysis system (CPX/D) with a disposable pneumotach flowmeter (CPX/D, Medgraphics®, USA). Heart rate, blood pressure and subject perception of effort were assessed throughout the test; 200 μg of salbutamol was used 15 min before the test to allow patients to reach maximum

oxygen consumption. All asthmatic subjects achieved physical exhaustion as determined by the following criteria: reaching the plateau or peak VO_2 independent of the increased workload; reaching the maximum predicted heart rate (±5%); or a respiratory coefficient ≥1.10 with the subject unable to maintain the speed test. The anaerobic threshold (AnT) was determined using the following combination of factors: (i) loss of linearity between VCO_2 and VO_2 and (ii) the point at which the ventilatory equivalent for oxygen (VE/VO_2) and the final expiratory pressure oxygen ($P_{ET}O_2$) reached their lowest value before their increase during the test that was associated with an increased respiratory exchange ratio (RER) and an abrupt increase in the pulmonary ventilation.[19, 20] The respiratory compensation point (RCP) was defined as the point where the VE began to change out of proportion to VCO_2; that is, a systematic increase in VE/VCO_2 with a consequent decline in final expiratory carbon dioxide ($P_{ET}CO_2$). A blinded exercise physiologist expert performed the analysis. The mechanical power load was calculated for each subject from the following equation: power (watts) = weight (kg) × 9.81 × sine of the angle of inclination × speed (meters/second).[21]

Pulmonary function

The following variables were recorded: forced vital capacity (FVC, in liters); forced expiratory volume in the 1st second (FEV_1, in liters); and FEV_1/FVC ratio. The percentage off the predicted normal values were calculated for the Brazilian population.[22]

Atopy

Patients were considered atopic if they presented a clinical history suggestive of respiratory allergy and specific IgE antibodies on the following tests: *in vivo* (skin prick test) and *in vitro* (Phadiatop test). The skin prick test was performed using 5 classes of 9 common aeroallergens: house dust mites (*Dermatophagoides pteronyssinus* and *Blomia tropicalis*),

animal dander (*Felis domesticus* and *Canis familiaris*), pollens (*Lolium perenne*), molds (*Aspergillus fumigatus* and *Penicillium notatum*) and cockroach (*Blattella germanica* and *Periplaneta americana*). The allergens were supplied by Asac pharma (Sao Paulo, Brazil), and the test was considered positive in the presence of a mean wheal diameter 3 mm greater than the negative control. The Phadiatop test was used to determine atopic status using the fluoroenzymeimmunoassay (FEIA) method. It was performed on the ImmunoCAP system according to the manufacturer's instructions (Phadia AB – Sweden). The test was considered positive (atopic) when the IgE concentrations ≥0.35 KU/L.

RESULTS

A total of 464 subjects were assessed for eligibility: 406 were excluded, 103 refused to participate and 58 patients were randomized into 2 groups. Fifteen patients (7 CG/8 TG) withdrew from the study: 3 patients withdrew because of health problems not related to asthma (cataract surgery, renal or cardiovascular disease), 10 because of scheduling difficulties related to work and 2 for personal reasons. Therefore, 43 patients completed the study and were analyzed (21 GC / 22 GT). All patients used moderate- to high-dose corticosteroids, and both groups used β_2 agonists as rescue medication. Before treatment, 29 patients (67.4%) did not present airway obstruction, 8 patients (18.6%) had mild obstruction and 6 patients (14.0%) had moderate obstruction.

During the first two weeks, the patients trained at 65% of maximal HR. By the 3th to 8th weeks, the patients trained at approximately 75% of HRmax. During the last 4 weeks, 95% of the patients trained at 85% of HRmax.

TABLE S1 – Within-group comparison and between-group comparison for cardiopulmonary exercise testing in asthmatic patients before and after the intervention

Outcomes	Control Group (n=21)			Training Group (n=22)			Treatment effect	
	Before	Mean (95% CI) within-group difference	p-value time	Before	Mean (95% CI) within-group difference	p-value time	Mean (95% CI) between-group difference	p-value treatment
Maximal Aerobic Capacity								
VO_2 ($mLO_2.kg^{-1}.min^{-1}$)	25.5 (5.9)	2.2 (-0.2-4.5)	0.053	27.0±4.2	-0.97 (-2.43-0.49)	0.182	-4.84 (-8.86- -0.82)	0.019
Power (W)	202.8 (67.3)	-3.3 (-25.4-18.9)	0.762	190.3±32.3	-57.07 (-73.08- -41.07)	<0.001	-44.08 (-83.37- -4.80)	0.029
RCP								
VO_2 ($mLO_2.kg^{-1}.min^{-1}$)	21.0 (3.8)	1.3 (0.3-2.3)	0.045	22.6±3.6	-0.56 (-.2.29-1.16)	0.502	-3.51 (-6.99- -0.45)	0.017
Power (W)	124.8 (50.1)	0.5 (-16.2-40.5)	0.965	115.5±32.1	-32.14 (-54.08- -10.20)	0.006	52.68 (13.44-91.90)	0.010
Anaerobic Threshold								
VO_2 ($mLO_2.kg^{-1}.min^{-1}$)	17.8 (3.9)	1.9 (0.5-3.4)	0.011	18.7±3.1	-0.01 (-2.00-1.98)	0.993	-3.11 (-5.40- -0.82)	0.009
Power (W)	76.2 (33.6)	6.0 (-15.3-27.3)	0.560	65.5±32.3	-57.80 (-69.08- -46.52)	<0.001	4.82 (-22.95-32.58)	0.728

Values are presented as the means and (standard deviations). VO_2, oxygen consumption; RCP, respiratory compensation point.

TABLE S2 – Within-group comparison and between-group comparison for pulmonary function in asthmatic patients before and after the intervention

Outcomes	Control Group (n=21)			Training Group (n=22)			Treatment effect	
	Before	Mean (95% CI) within-group difference	p-value	Before	Mean (95% CI) within-group difference	p-value	Mean (95% CI) between-group difference	p-value
$FEV_{1,}$ L	2.00 (0.7)	-0.1 (-0.2-0.1)	0.471	2.1 (0.8)	0.0 (-0.1-0.1)	0.952	-0.0 (-0.5-0.4)	0.930
% predicted	66.3 (19.0)	-2.3 (-8.6-3.9)	0.447	69.0 (21.0)	-1.1 (-4.8-2.6)	0.546	2.5 (-11.5-16.5)	0.721
FVC, L	2.7 (0.9)	-0.0 (-0.1-0.1)	0.640	2.8 (0.9)	-0.01 (-0.1-0.1)	0.800	-0.1 (-0.6-0.4)	0.762
% predicted	77.0 (18.0)	-1.0 (-4.4-2.3)	0.525	78.2 (17.6)	-1.9 (-4.5-0.7)	0.148	1.2 (-11.8-12.4)	0.961
FEV_1/FVC	72.2 (10.0)	-0.2 (-0.5-0.0)	0.432	73.0 (10.5)	0.0 (-0.0-0.0)	0.769	-0.0 (-0.1- 0.1)	0.659
$FEF_{25-75\%}$, $L.s^{-1}$	1.6 (0.7)	-0.1 (-0.4-0.2)	0.550	1.7 (0.9)	0.0 (-0.2-0.2)	0.942	-0.0 (-0.6-0.6)	0.963
% predicted	51.0 (23.2)	-2.6 (-13.0-7.6)	0.595	54.5 (33.0)	0.1 (-5.1-5.4)	0.958	3.6 (-11.4-18.6)	0.633

Values are presented as the means (standard deviations). FEV_1, forced expired volume in the first second; FVC, forced vital capacity; $FEF_{25-75\%}$, mean forced expiratory flow (FEF) between 25% and 75% of FVC; $L.s^{-1}$, liters per second**.**

REFERENCES FOR SUPPLEMENT

1. Rees J, Kanabar D. ABC of Asthma. 5th ed. Oxford, England: Blackwell Publishing 2006.
2. Svatmarama S. The Hatha Yoga Pradipika. Translation by Akers BD. 1st ed. New York, NY: YogaVidya.com 2002.
3. Mendes FAR, Mendes FA, Almeida FM, et al. Effects of aerobic training on airway inflammation in asthmatic patients. *Med Sci Sports Exerc* 2011;43:197-203.
4. Fanelli A, Cabral ALB, Neder JA, et al. Exercise training on disease control and quality of life in asthmatic children. *Med Sci Sports Exerc* 2007;39:1474-80.
5. Crapo RO, Casaburi R, Coates AL, et al. Guidelines for methacholine and exercise challenge testing-1999. *Am J Respir Crit Care Med* 2000;161:309-29.
6. Inman MD, Hamilton Al, Kerstjens HAM, et al. The utility of methacholine airway responsiveness measurements in evaluating anti-asthma drugs. *J Allergy Clin Immunol* 1998;101:342-8.
7. Reddel HK, Taylor DR, Bateman ED, et al. An official American Thoracic Society/European Respiratory Society statement: asthma control and exacerbations: standardizing endpoints for clinical asthma trials and clinical practice. *Am J Respir Crit Care Med* 2009;180:59-99.
8. Dweik RA, Boggs PB, Erzurum SC, et al. An Official ATS Clinical Practice Guideline: Interpretation of exhaled nitric oxide levels (FE_{NO}) for clinical applications. *Am J Respir Crit Care Med* 2011;184:602-15.
9. Pizzichini E, Pizzichini MM, Leigh R, et al. Safety of sputum induction. *Eur Respir J* 2002;20:9-18.

10. Pizzichini E, Pizzichini MM, Efthimiadis A, et al. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. *Eur Respir J* 1996;9:1174-80.
11. Saraiva Romanholo BM, Barnabé V, Carvalho AL, et al. Comparison of three methods for differential cell count in induced sputum. *Chest* 2003;124:1060-6.
12. Gibson PG, Norzila MZ, Fakes K, et al. Pattern of airway inflammation and its determinants in children with acute severe asthma. *Pediatr Pulmonol* 1999;28:261-70.
13. Mendes FA, Gonçalves RC, Nunes MP, et al. Effects of aerobic training on psychosocial morbidity and symptoms in patients with asthma: a randomized clinical trial. *Chest* 2010;138:331-7.
14. Juniper EF, O'Byrne PM, Guyatt GH, et al. Development and validation of a questionnaire to measure asthma control. *Eur Respir J* 1999;14:902-7.
15. Juniper EF, Bousquet J, Abetz L, et al. Identifying 'well-controlled' and 'not well-controlled' asthma using the Asthma Control Questionnaire. *Respir Med* 2006;100:616-21.
16. Juniper EF, Svensson K, Mork AC, et al. Measurement properties and interpretation of three shortened versions of the asthma control questionnaire. *Respir Med* 2005;99:553-8.
17. Juniper EF, Guyatt GH, Epstein RS, et al. Evaluation of impairment of health related quality of life in asthma: development of a questionnaire for use in clinical trials. *Thorax* 1992;47:76-83.
18. Juniper EF, Guyatt GH, Willan A, et al. Determining a minimal important change in a disease-specific Quality of Life Questionnaire. *J Clin Epidemiol* 1994;47:81–7.
19. Wasserman K, Hansen JE, Sue DY, et al. Normal values. In: Wasserman K, Hansen JE, Sue DY, et al., eds. Principles of Exercise Testing and Interpretation. Philadelphia, PA: Lippincott Williams & Wilkins 2005:160–82.

20. Gaskill SE, Ruby BC, Walker AJ. Validity and reliability of combining three methods to determine ventilatory threshold. *Med Sci Sports Exerc* 2001;33:1841-8.

21. Bush A, Busst CM, Johnson S, et al. Rebreathing method for the simultaneous measurement of oxygen consumption and effective pulmonary blood flow during exercise. *Thorax* 1988;43:268-75.

22. Pereira CAC, Neder JA. Sociedade Brasileira de Pneumologia e Tisiologia: diretrizes para testes de função pulmonar. *J Pneumol* 2002;28:s1-238.