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Title Page

Global metabolic stress of isoeffort continuous and high intensity interval aerobic exercise: a comparative ¹H NMR metabonomic study

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ABSTRACT

The overall metabolic/energetic stress occurring during an exercise-bout is proposed as the main driving force for long-term training adaptations. Continuous and high-intensity interval exercises (HIIE) are currently prescribed to acquire the muscular and metabolic benefits of aerobic training. We applied ^1H NMR-based *metabonomics* to compare the overall metabolic perturbation and the activation of individual bio-energetic pathways of three popular aerobic exercises matched for effort/strain. Nine men performed continuous, long-interval (3min-bouts), and short-interval (30s-bouts) exercise-bouts under isoeffort conditions. Blood was collected before and after exercise. The multivariate PCA and OPLS-DA models showed a distinct separation of pre- and post-exercise samples in three protocols. The two models did not discriminate the post-exercise overall metabolic profiles of three exercise types. Analysis focused on muscle bio-energetic pathways revealed an extensive up-regulation of carbohydrate-lipid metabolism and TCA-cycle in all three protocols; there were only few differentiations among protocols in post-exercise abundance of molecules when long-interval bouts were performed. In conclusion, continuous and HIIE exercise protocols, when performed with similar effort/strain, induce comparable global metabolic response/stress despite their marked differences in work-bout intensities. This study highlights the importance of NMR-metabonomics in comprehensive monitoring of metabolic consequences of exercise training in blood of athletes and exercising individuals.

Key words: metabolomics, exercise, aerobic, interval exercise, metabolites, metabolism, carbohydrates, lipids, amino acids, NMR

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1. INTRODUCTION

Aerobic (endurance) exercise improves muscle metabolism, increases cardiorespiratory fitness, and reduces the metabolic risk factors associated with cardiovascular diseases in healthy and diseased adults¹⁻³. Continuous and high-intensity interval protocols (HIIE) are two aerobic exercise regimens that are currently prescribed to acquire the benefits of endurance training. The beneficial metabolic long-term training adaptations are, at least in part, specific to the extent of metabolic perturbation and to the activation of biochemical signaling pathways that occur during an acute exercise bout³⁻⁵. Thus, understanding the metabolic fingerprint of continuous and HIIE, by monitoring changes in overall metabolic/energetic profile and relevant metabolites, may provide insights into the muscular adaptive responses and the preventive/therapeutic effects of each exercise regimen on markers of health. It may also allow clinicians and sports scientists to prescribe exercise with greater clarity to the expected adaptations.

In this study we applied the metabonomic approach to investigate the *overall* metabolic variations of three popular aerobic exercise regimens, the continuous exercise and two HIIE, the short (30s-bouts) and the long (3min-bouts). In contrast to conventional approaches focusing on few preselected metabolites, metabonomics encompasses the integrative metabolic response of several biochemical pathways providing a global unbiased (non-targeted) “snapshot” of the organism’s overall cellular metabolic status and physiological function⁶. This approach enables also the discrimination of the *overall* metabolic state between various physiological or pathological stimuli. ¹H-NMR, in particular, allows the assessment of small molecules involved in different bio-energetic metabolic pathways (e.g. TCA cycle intermediates, amino acids). Therefore, metabonomics is being increasingly used to examine the metabolic stress to a single exercise bout⁷⁻¹⁴.

Continuous protocols use exercise bouts of long duration at steady sub-maximal intensities, while interval protocols employ shorter exercise bouts at higher intensities (near or above VO₂max) interspersed with recovery phases. The integration of exercise duration and intensity determines the overall metabolic/energetic stress, the main driving force for cellular and physiological processes and the long-term training adaptive responses³⁻⁵. The activation of individual molecular and metabolic

1 pathways during exercise may be influenced by the independent and interactive effects of (i) the
2 repeated rest to work transitions^{15,16}, (ii) the configurations in intensity and duration of work and rest
3 intervals^{17,18}, and (iii) the hormonal responses¹⁹.

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9 Comparison of the cellular metabolic/energetic stress of continuous exercise and HIIE was the subject
10 of recent communication that used targeted metabolomics on specific biochemical pathways
11 (glycolysis, lipolysis) and molecules¹². Targeted approaches, however, do not provide information on
12 changes in *overall* metabolic stress, an important governor of molecular signaling for muscular
13 adaptations^{3,4}, and do not integrate metabolic fluxes and stimuli of several bio-energetic pathways.
14 Importantly, the continuous and HIIE, although equalized for external mechanical work-load in that
15 previous study, differed in overall effort/strain (internal physiological load). A more demanding
16 exercise may result to greater sympathoadrenal drive and different hormonal responses affecting the
17 metabolic flux. Thus, it is not clear whether the different metabolic responses in two protocols were
18 attributed to the type of aerobic regimen *per se* (continuous and HIIE) or to the higher *overall*
19 effort/strain in HIIE. Finally, the overall metabolic stress of the short-interval protocols (~30s) has yet
20 to be examined; despite that such exercise regimes have been widely used and studied in sport and
21 clinical settings.

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27 The current investigation applied a multivariate untargeted NMR profiling of blood from athletes
28 before and after intense continuous and HIIE exercise protocols. The study includes several novel
29 elements: (i) the study of the *overall* metabolic/energetic stress of a high-intensity short-interval (30s)
30 exercise protocol, (ii) a comparison of global metabolic/energetic stress and metabolic fingerprint of
31 continuous, long-interval (LI) and short-interval (SI) aerobic exercise protocols, and (iii) the use of
32 isoeffort approach when comparing the metabolic stress of three popular aerobic exercise regimens.
33 The latter is of particular importance since the rise in rate of perceived exertion (effort) is highly
34 associated with changes in peripheral metabolic compounds during moderate to high intensity
35 exercise²⁰. We also focused on molecules related to major bio-energetic pathways (glycolysis,
36 lipolysis, amino acid metabolism, TCA cycle).

2. EXPERIMENTAL SECTION

2.1 Subjects

Nine young healthy men volunteered to participate in this study. All participants were healthy and participated in soccer training 4-5 times per week. Their mean ($\pm$ SE) age, body mass, height, maximal heart rate (HRmax), VO₂max, and maximal aerobic velocity were 20.5 $\pm$ 0.7 years, 74.7 $\pm$ 1.3 kg, 179.1 $\pm$ 1.5 cm, 196 $\pm$ 2 beats $\cdot$ min⁻¹, 59.7 $\pm$ 1.2 mL kg⁻¹ $\cdot$ min⁻¹, and 15.3 $\pm$ 0.3 km h⁻¹, respectively. Before the start of the study, the institutional review board committee approved the experimental protocol, and the participants provided the written informed consent. All participants completed a medical questionnaire to ensure that they were not using any medications, and were free of cardiac, respiratory, renal, or metabolic diseases.

2.2 Maximal graded exercise test

On the first visit, following the body mass and height measurement (Seca, Hamburg, Germany) the participants performed a maximal incremental test on a motorized treadmill (Runrace, Technogym, Gambettola, Italy) for the assessment of maximal oxygen consumption (VO₂max), maximal aerobic velocity (MAV), and maximal heart rate (HRmax). The initial speed was set at 8 km h⁻¹ (0% slope) and was increased by 1 km h⁻¹ every 2 min until volitional exhaustion. The test was terminated when the participant was no longer able to maintain the speed. Respiratory gas exchange was measured breath-by-breath (Oxycon Pro, Jaeger, Germany) and heart rate was recorded (Polar Electro, Kempele, Finland) continuously during the incremental test. The criteria for VO₂max (maximum effort) were: (i) heart within 10 beats $\cdot$ min⁻¹ of the predicted HRmax (210 – (0.65 $\times$ age)), (ii) a respiratory exchange ratio (RER) >1.15; (iii) visible exhaustion and inability to sustain exercise despite continuous verbal encouragement; and (iv) a plateau in oxygen consumption (VO₂) (increment of less than 2.1 mL kg⁻¹ min⁻¹ despite the increase in workload). All participants attained at least three of the above criteria ensuring that maximum effort and VO₂max were achieved. VO₂max was defined as the highest mean of two successive 15-s periods VO₂ values. MAV was defined as the speed at which VO₂max was first achieved and maintained for at least 1 min²¹.

2.3 Continuous and Interval exercise protocols

On three separate visits, the participants performed, in a randomized order, the intense continuous, the LI, and the SI exercise protocols. Upon arrival to the laboratory and after a 15-min seated rest, a pre-exercise blood sample was obtained from the antecubital vein. Following a standardized warm-up, the participants completed either the intense continuous (at 80% of MAV), the LI protocol (3-min runs at 95% of MAV with 3-min recovery at 35% of MAV) or the SI (30-s runs at 110% of MAV with 30-s recovery at 50% of MAV) exercise protocols. The average VO_2 of work-bouts for each exercise protocol was calculated by dividing the sum of all the 15-sec segment VO_2 values by the total time of work-bouts. A post-exercise blood sample was collected within 5 min of completion of exercise. The three exercise protocols were performed within two weeks at the same time of the day (± 1 h) and in similar atmospheric conditions (temperature, 22–23°C; relative humidity, 40%–45%). To ensure that all participants would provide a similar overall effort (i.e. the same physiological strain would be exerted) in three exercise protocols, the end-point for the continuous and interval exercise protocols was defined as a HR of 5 beats below their HRmax (i.e. about 97.5–98% of HRmax); this HR was maintained for 4 consecutive, 5-s readings before terminating the exercise. At the completion of exercise, the rate of perceived exertion (RPE) was also obtained. HR and RPE scores are being consistently used as measures of physiological strain and training load²²⁻²⁴. As evident in the results' section, the RPE scores were similar across protocols, indicating that equal overall effort was achieved in three protocols.

The participants followed a diet consisting of 55% carbohydrates, 30% of fat and 15% of proteins for 2 days before the tests. Two and the half hours prior to each testing, they consumed a standardized meal of similar nutrient composition with the energy content of 9 kcal per kg of body weight. Participants were also instructed to abstain from consuming alcoholic drinks the day prior the sessions and caffeinated drinks the day of the testing, to refrain from any exercise activity for 48 h before each test, and to have sufficient rest the night prior to the sessions.

2.4 Blood samples collection & preparation

Blood samples (10 ml) were collected into EDTA tubes before and within 5 minutes after the completion of each aerobic exercise protocol. The samples were immediately centrifuged at $1800 \times g$ at 4°C for 15 min (Centrifuge 5702R, Eppendorf AG, Hamburg, Germany). The plasma was removed, separated into aliquots, and stored at -80°C until ^1H NMR analysis. On the day of analysis, the samples were thawed at room temperature and were prepared for analysis in a random sequence. 150 μL of buffer $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ in D_2O (0.5 mol/L, pH 7.2) were diluted with 400 μL of EDTA-plasma. In that mixture, 50 μL of sodium maleate dibasic (6 mmol/L) were added as internal standard. After vortexing and centrifuging at $12000 g$ (4°C) for 10 min, 550 μL of the supernatant were placed in 5 mm wide NMR tubes. Plasma volume changes were calculated using the hematocrit (Hct) and hemoglobin (Hb) concentrations before and after each protocol with the following equation: $\text{PV}_A = (100 \times \text{Hb}_B/\text{Hb}_A) - [(100 \times \text{Hb}_B/\text{Hb}_A) \times \text{Hct}_A]^{25}$; where PV_A is plasma volume after exercise, Hct_A is the ml of red cells per ml of blood after exercise, Hb is hemoglobin concentration before and Hb_A is hemoglobin after exercise. The percentage change in plasma volume ($\% \Delta\text{PV}$) after exercise was calculated as $\% \Delta\text{PV} = 100 \times (\text{PV}_A - \text{PV}_B)/\text{PV}_B$; where PV_B (plasma volume before exercise) is equal to 100 minus the Hct (%)²⁵.

2.5 ^1H NMR Spectroscopy and Data pre-processing

NMR spectra were recorded on a Varian 600 MHz NMR spectrometer operating at 599.828 MHz, with the temperature held at 293.15 K. Each spectrum was acquired using the Carr-Purcell-Meiboom-Gill (CPMG) pulse, with 128 scans over a spectral width of 6613.8 Hz, acquisition time of 4.832 s and relaxation delay of 6.00 s. The CPMG pulse suppresses the broad lipoprotein signals. The suppression of water signal was achieved by applying the Presat pulse sequence with presaturation power set to 0 dB. All spectra were referenced to sodium maleate dibasic at 5.994 ppm, phased and baseline-corrected using the MestReNova 9.0. The residual water signal was excluded (4.40-5.24 ppm) and the full spectra were binned in increments of 0.0005 ppm. The resulted data were normalized to sodium maleate to compensate for possible differences in solutions concentrations.

DEPT135-HSQC 2D NMR spectra were recorded for assisting the assignment of metabolites, in combination with the reference metabolic ^1H NMR database (Chenomx) and literature data.

2.6 Statistical Analysis

Spectral data were analyzed using multivariate statistics including unsupervised principal component analysis (PCA) and supervised orthogonally filtered PLS-DA (OPLS-DA) pareto scaled on Simca 13 statistical software (Version 13.0.2.0, Umetrics, Umea, Sweeden). PCA-X and OPLS-DA analyses were applied to examine the differentiation in overall metabonomic profile (i) between the pre and the post exercise blood samples within each exercise protocol (exercise effect) and (ii) among the three aerobic exercise protocols at prior to and after exercise time points (effect of protocol). For each valid OPLS-DA model S-line plots were generated to identify the main discriminators and their contribution to the separation. S-line plot is a type of plot targeting mainly in NMR and other spectral data, displaying the original spectra based on the predictive loading. S-line plot is a pseudo-NMR spectrum projection of the OPLS-DA coefficient's plots. In that plot the relevance to the model is indicated by the abundance of the signal while the significance by the color code. Variables having significant effect usually combine high absolute numerical loading value and red/orange color. Each cross-validation parameter (R^2X , R^2Y and Q^2Y) was checked in order to assess the robustness and the predictability of the models and only models with high R^2Y and Q^2Y values corresponding to good discrimination and predictability were further investigated. Another validation tool for PLS-DA models was the permutation test ($n=999$). Two-way ANOVAs with repeated measures were used to determine the effect of endurance protocol (continuous, LI, and SI) and of exercise (pre to post) on 15 metabolites related to muscle bioenergetics and on 2 indices showing the transition from aerobic to anaerobic metabolism (Statistica 7.0, Statsoft, France). Significant main effects were followed by Newman post-hoc tests. Percent change from pre to post exercise was also calculated for each metabolite. Data for univariate statistics are presented as mean $\pm$ SE. The level of statistical significance for all analyses was set at $\alpha = 0.05$.

3. RESULTS

3.1 Exercise protocols characteristics

The mean values for exercise protocol characteristics are presented in Table 1. Total exercise time and completed distance (including the active recovery time in interval protocols) were significantly higher in LI vs. the respective values in intense continuous and SI ($p < 0.01-0.05$). There were no differences, however, among protocols in duration and distance covered at or above the lower intensity (40% VO_2max) that is prescribed for enhancing aerobic fitness in healthy individuals. The three protocols were matched for overall effort (RPE: 18.67 ± 0.21 , 18.50 ± 0.19 , and 18.44 ± 0.18 and RPE medians (IQ range_{1 to 3}): 18.5 (0.5), 18.5 (1.0), and 18.5 (0.5) for intense continuous, LI and SI, respectively) and physiological strain (HR_{peak} : 191.8 ± 2.0 , 192.0 ± 2.0 , 192.6 ± 1.5). The kcals consumed during the entire protocols (including the recovery time in SI and LI) were similar in the continuous and SI but higher in LI protocol ($p < 0.05$). However, the average VO_2 as well as the kcal consumed during work-bouts performed above the lower functional intensity for enhancing aerobic capacity were not different among protocols. There were no differences in plasma volume changes among protocols ($-7.1 \pm 1.4\%$, $-8.6 \pm 0.6\%$, and $-7.6 \pm 1.2\%$, in continuous, LI, and SI, respectively).

3.2 Metabonomic investigation after endurance exercise (effect of exercise)

Typical ^1H NMR plasma spectrum is presented in Figure 1. Selected regions corresponding to metabolites with the most distinctive variations after exercise are shown in the insert figure. To determine the effect of intense endurance exercise on shifts of overall metabonomic spectra, an unsupervised principal component analysis (PCA) was developed including the pre- and the post-exercise samples of all three protocols. When testing all plasma samples in one set, PCA scores plot provides a distinct separation between pre- and post-exercise samples with a clear left to right shift for all subjects (Figure 2), indicating that exercise was responsible for the majority of the variation in the original spectra. In this PCA score plot no discrimination can be seen in metabonomic spectra between the three exercise protocols for either pre-exercise or for post-exercise samples. Next, supervised OPLS-DA models were built to identify the main discriminators of the metabolic shift. The profound increase in lactate was found to be the key differentiator (Figure S1). New OPLS-DA model excluding

the chemical shifts corresponding to lactate still showed separation between pre and post exercise samples and revealed glucose, pyruvate, succinate and alanine as major separating factors (Figure 3).

3.3 Comparison of metabonomic spectra of three exercise protocols

To study metabolic changes within each exercise protocol, OPLS-DA models were generated for each exercise protocol separately. Figure 4 shows OPLS-DA scores plots before and after the exclusion of lactate from the dataset and the corresponding S-line plots illustrating the contribution of NMR shifts (peaks) in group discrimination. Clear separation of pre- from post-exercise samples was observed for each protocol. For continuous and for SI exercise protocols the separation was attributed to increases in pyruvate, glucose, alanine, and succinate (Figure 4A and 4C); for the LI protocol, the separation, in addition to the above compounds, was also ascribed to increase in citrate and proline (Figure 4B).

Despite the substantial shift of metabolome after exercise in all three protocols, no valid pairwise OPLS-DA model could be developed to compare the metabolome of exercise protocols for pre- or for post-exercise samples. This finding indicates the absence of statistically significant differences in plasma overall metabolic composition at pre and at post-exercise time points among intense continuous, LI, and SI protocols.

3.4 Metabonomic analysis targeted on individual bio-energetic pathways

Analysis of variance (ANOVA) targeted on 15 metabolites related to muscle bioenergetics (e.g. glycolysis, lipolysis, amino acid metabolism, and TCA cycle intermediates) and 2 indices of transition from aerobic to anaerobic metabolism was subsequently performed. Pre- to post-exercise changes within each protocol and pairwise comparisons between protocols at pre and post-exercise time points are presented in Table 2.

ANOVAs indicated a significant main effect of exercise (pre to post) on 15 of 17 metabolites (except for tyrosine and histidine) and significant endurance "protocol" $\times$ "exercise" interactions on 3 of 18 molecules (citrate, alanine, glutamine). Pairwise analyses *within* each exercise protocol revealed: (i) ten molecules significantly increased ($p < 0.001$ - 0.05) after the completion of all three exercise protocols (glucose, lactate, pyruvate, glycerol, alanine, citrate, succinate, lactate/pyruvate and lactate/citrate), (ii) two amino acids (glutamine and proline) decreased only after continuous and LI

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3 protocols ($p < 0.01$ - 0.05), and (iii) six amino acids (leucine, isoleucine, valine, tyrosine, and histidine)
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5 did not demonstrate significant pre to post-exercise changes. Pairwise comparisons *between* protocols
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7 did not show significant differences for pre-exercise values of all metabolites, as also shown by
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9 multivariate analysis. At the completion of exercise, however, the concentrations of alanine and citrate
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11 were significantly higher in LI vs. the continuous and SI protocols ($p < 0.01 - 0.05$).
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16 This study was to the first to compare the global cellular metabolic/energetic stress of a continuous
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18 and two types of HIIE matched for overall effort (as determined by RPE scale) and strain. We applied
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20 ^1H NMR-based *metabolomics* to compare the overall metabolic shifts (multivariate approach) as well
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22 as the activation of individual biochemical branches involved in energy production (targeted strategy).
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24 The novel findings of our study are: (i) a clear substantial shift of plasma metabolome (an increase in
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26 overall metabolite composition) after all three types of exercise, (ii) no discrimination of the overall
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28 metabolic snapshot among the three isoeffort high-intensity endurance exercise protocols, (iii) an
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30 extensive up-regulation of carbohydrate/lipid metabolism, and activation of the TCA cycle (as
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32 suggested by increase in lactate, pyruvate, glycerol, citrate, and succinate); the plasma amino acids
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34 levels were affected to a lesser degree, and (iv) no differences among protocols, with a few exceptions,
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36 in post-exercise metabolic profiles related to specific biochemical pathways.
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39 The isoeffort approach employed in this study integrates the contribution of intensity and duration of
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41 exercise to overall effort/strain. This is critical, since the overall effort/stress may affect hormonal and
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43 molecular signaling and hence, the metabolic response. For example, an exercise protocol with greater
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45 *overall* effort and physiological strain, results to greater sympathoadrenal drive and hence metabolic
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47 responses confounding the effects of the protocol *per se* on metabolic stress. Thus, matching for the
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49 *overall* exercise stimulus (effort), when comparing different training modalities, is an important aspect
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51 when examining the effects of the type of endurance exercise *per se* (continuous vs. interval exercise)
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53 on metabolic response. The idea of matching for overall effort, when comparing the
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55 responses/adaptations of aerobic training modalities, has been recently advocated by several studies as
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57 the most appropriate matching method²⁶⁻²⁹.
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4.1 Global (overall) metabolic response

Changes in the overall cellular metabolic/energetic stress during each type of endurance training may provide insights into the training load and cellular long-term adaptations⁴. PCA and OPLS-DA clearly differentiated pre from post-exercise samples for each endurance protocol, suggesting an increased substrate flux; the main discriminators were metabolites related to glycolysis (lactate, pyruvate, and glucose) and TCA cycle (succinate). When OPLS-DA models were developed separately for each exercise type to reveal protocol-specific changes in metabolome, citrate was also identified, but only in LI.

The most important finding was that the PCA and the OPLS-DA models did not distinguish the post-exercise overall metabolic spectra of the three protocols. This may be rooted in the similar overall effort/strain exerted during the three endurance protocols; a more demanding protocol would conceivably elicit a greater shift of plasma metabolome. Our findings are in accordance with the centrally regulated effort model suggesting a high association between the rise in rate of perceived exertion (RPE) and changes in peripheral metabolic compounds during exercise at moderate to high intensities²⁰. In fact, RPE has been proposed as an indicator of metabolic reserves as sensed by the brain. Our results imply that when matched for overall effort and strain (RPE and HR_{peak}) the three endurance protocols may produce similar overall metabolic challenge and possibly similar muscular long-term adaptations^{3-5, 29}. This is in line with a recent study showing that two different sprint training programs consisting of maximal runs showed similar acute and long-term impact on serum metabolic fingerprint¹⁴. Thus, the overall metabolic response is mostly tied up to the overall effort and strain rather than to the characteristics (intensity, duration, work-rest transitions) of the endurance protocol *per se*.

Notably, no direct comparisons with other studies is possible, since the only study that compared continuous and LI endurance protocols using metabolomics did not carry a global multivariate strategy¹². Our results are in context with previous studies that used conventional analytical techniques and isoeffort/isostrain exercise conditions (as suggested by RPE and/or HR) and reported similar responses between continuous and interval endurance exercises for metabolites (glucose, glycogen, free fatty acids), for circulating microRNAs, and for molecular signaling pathways (AMPK and p38MAPK

phosphorylation) and genes (PGC-1 α , PRC, PPAR β/δ , and PDK4 mRNA) associated with adaptations to endurance exercise³⁰⁻³³.

4.2 Anaerobic Glycolysis

All three protocols caused a significant rise in plasma metabolites associated with carbohydrate catabolism. The rise in plasma pyruvate and lactate clearly suggest an enhanced rate of “anaerobic” glycolytic pathway; the observed increase in plasma glucose is most likely related to its greater rate of appearance, due to the enhanced hepatic glycogen breakdown during exercise³⁴. Although these responses were expected for HIIE, due to their high-intensity of work-bouts, they were also observed in continuous exercise. The reason is that the continuous protocol was performed above the anaerobic threshold and near to exhaustion. Hence, an increase in glucose, pyruvate, and lactate seems realistic and in context with other studies using ¹H-NMR^{10, 35-37} or conventional analytical techniques^{34, 38}.

To the best of our knowledge this is the first study comparing indicators of carbohydrate metabolism after isoeffort continuous, SI, and LI endurance exercise. An interesting observation was that glucose, pyruvate, and lactate increased in the continuous protocol to values comparable to those in HIIE. If the continuous protocol was performed at lower intensity (below the anaerobic threshold) the results might have been different. The most likely explanation for this response is that all three aerobic protocols were performed with similar overall effort and near to exhaustion. In previous studies, RPE and HR have been highly associated with sympathoadrenal activation and noradrenaline levels during moderate to high intensity exercise^{20, 39, 40}. In fact, changes in blood noradrenaline, lactate, and glucose have been proposed as peripheral signals influencing RPE⁴¹. Increased catecholamines and sympathoadrenal drive during moderate to high-intensity exercise may stimulate muscle glycogen utilization⁴² contributing to elevated pyruvate and lactate and increase plasma glucose through enhanced hepatic glucose production⁴³ and/or inhibition of insulin secretion and reduction in peripheral glucose uptake^{38, 42}. Meyer et al. showed that when exercising at similar HR and RPE, continuous and interval exercise protocols result to similar *average* glucose, norepinephrine, epinephrine, and dopamine levels³⁰. Another possible explanation for the similar increase in indicators of glycolysis may be related to changes in energy fuels during work to recovery transitions in HIIE⁴⁴.

All three protocols resulted to similar rise in lactate to pyruvate ratio. The change in lactate to pyruvate ratio has been used as an index of tissue hypoxia, a transition from aerobic to anaerobic metabolism carbohydrates during exercise⁸, and as a marker of oxidative stress and cellular oxidative damage⁴⁵. Tissue hypoxia is among the main mechanisms promoting mitochondrial biogenesis and capillary proliferation through the stimulatory action of HIF-1 and VEGF. This finding is in context with previous study reporting similar O₂ saturation in vastus medialis muscle when the three exercise protocols were executed with similar effort⁴⁶.

4.3 Oxidative metabolism

One fundamental adaptation to endurance exercise training is the increase in skeletal muscle oxidative capacity. In support, we found a significant rise in plasma glycerol in all three protocols suggesting increase in lipolysis^{37,47}. We also observed a significant rise in circulating span 1 and 2 TCA intermediates (TCAi, citrate and succinate), indicative of increased TCA cycle flux, after all three endurance protocols. The increase in plasma citrate and succinate most likely resulted from the expansion of muscle TCAi pool, due to activation of oxidative pathways and excessive pyruvate accumulation, and their spill over into the circulation. Our findings corroborate previous metabonomic studies reporting the accumulation of TCAi in plasma after intense continuous^{10, 35-37} and LI¹² protocols. The functional significance, though, of changes in blood TCAi is still unclear⁴⁸.

We add new information to prior findings showing that the intense continuous and HIIE protocols elicit comparable rise in glycerol. This may be attributed to similar effort and strain in three protocols and their association with sympathadrenal activation and catecholamine release, known modulators of lipid mobilization. However, in lack of more comprehensive lipid metabolism analysis in our study, the suggestion of analogous lipolytic rates in three protocols should be interpreted with caution. The greater plasma citrate observed after LI vs. the other two protocols is possibly related to the very low intensity and the long duration of the rest phase in LI. Support to this stems from previous studies reporting an abrupt accumulation in plasma FFA during the first minutes of recovery after exercise⁴⁹,⁵⁰ contributing to high citrate through acetylCoA formation. The mechanisms, of course, underlying

the differences in circulating citrate in LI vs. the other two protocols should be examined experimentally.

The present study provides evidence that under isoeffort conditions the three endurance exercise regimes have a similar potential to increase oxidative metabolism. An interesting observation was that the oxidative metabolic phenotype was similarly up-regulated in continuous and SI protocols, despite their marked differences in exercise intensity. Thus, provided an adequate intensity and duration, the continuous aerobic exercise may induce the oxidative pathways to an extent observed in high-intensity SI protocols. In support, Essen et al. also reported comparable glycerol response in SI (15s) and continuous (60min) protocols⁴⁴, and a recent metabonomic study reported lower plasma citrate and succinate after moderate-intensity continuous exercise compared to a more demanding LI protocol¹².

4.4 Amino Acid metabolism

So far, no study has compared the changes in circulating amino acids among three popular endurance exercise protocols. In general, we did not observe substantial changes in plasma amino acids immediately after the three exercise protocols. Alanine increased in all three types of exercise which is in accordance with most previous studies that used either short or prolonged endurance exercise protocols^{12, 51-53}. Based on previous studies, the increase in plasma alanine seems likely the result of an excessive pyruvate accumulation in the muscle as part of the TCAi pool anaplerotic reactions and the activation of alanine-glucose cycle to replenish the carbohydrate/glycogen supplies^{48, 53}. We have also observed a marked reduction in glutamine, also an important gluconeogenic precursor; however, this was evident only after intense continuous and LI exercise protocols. Based on previous reports, three reasons may collectively explain this finding: (i) the increase in hepatic, gut, and renal glutamine uptake from the circulation to support the gluconeogenic pathway⁵⁴⁻⁵⁶, (ii) the increased renal uptake of glutamine during metabolic acidosis^{54, 55}, (iii) the decreased glutamine release from the muscle due to the reduced intramuscular glutamate availability during exercise^{48, 53}. The lack of changes in plasma glutamine after the SI protocol in our study is in accordance with previous investigations that employed repeated exercise bouts of short duration (30-60s)^{57, 58}. The changes in plasma glutamine and proline levels after endurance exercise are not clear. Most studies reported a

reduction^{10, 51, 52, 55, 56, 59, 60} or an increase^{10, 12, 52, 56, 61} after a prolonged submaximal or an exhaustive endurance exercise, while fewer works showed no change^{12, 51, 56}.

BCAA (leucine, isoleucine, and valine) originate from muscle protein breakdown and are used during exercise as an energy substrate to support gluconeogenesis and the production of TCAi. We did not reveal changes in plasma BCAA in all three exercise protocols. A recent metabonomic study also failed to observe significant changes in these molecules immediately after a moderate intensity continuous or a high-intensity LI exercise¹². Other studies have reported increase¹¹, reduction^{35, 52} or no change^{9, 60} in circulating or urine BCAA levels. Definite conclusions on BCAA metabolism during the three endurance protocols could not be drawn, since plasma levels of BCAA reflect the balance between their production and utilization.

5. CONCLUSIONS

All three endurance protocols caused marked perturbations of the overall metabolic profile. The holistic view emerging from our study is that, when performed with similar effort and strain, the intense continuous and high-intensity interval exercise protocols (short and long) induce comparable global cellular metabolic/energetic stress. This is despite their marked differences in the intensity of work-bouts. Thus, the overall metabolic response during endurance exercise appears to depend mostly on the overall effort and strain rather than on the specific characteristics of the endurance protocol *per se*. These findings are in accordance with the centrally regulated effort model suggesting a high association between the rise in effort and changes in peripheral metabolites during exercise²⁰. The metabonomic analyses focusing on individual metabolic bio-energetic pathways (glycolysis etc.), generally support the multiivariate global metabolic approach. Under isoeffort conditions, the continuous and the SI protocols resulted not only to comparable global metabolic stress but also to analogous changes in metabolites related to muscle bioenergetics. Although our findings pertain to comparable cellular/metabolic challenge and long-term training adaptations when the three different exercises are performed with similar effort, this view should be evaluated experimentally.

Supporting Information. The following files are available free of charge.

Figure S1- OPLS-DA model including the chemical shifts corresponding to lactate.

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Table 1. The mean $\pm$ SE of exercise characteristics for isoeffort intense-c (ontinuous, long-interval, and short-interval exercise

Variable	Continuous	Long-Interval (3min-3min)	Short-Interval (30s-30s)
Entire exercise protocol			
Duration (sec)	1081 $\pm$ 48	1720 $\pm$ 188*	1097 $\pm$ 101
Distance (m)	3831 $\pm$ 213	4986 $\pm$ 539*	3773 $\pm$ 342
kcal	341 $\pm$ 19	447 $\pm$ 50*	352 $\pm$ 34
kcal/min	18.9 $\pm$ 0.5	15.6 $\pm$ 0.4*	19.2 $\pm$ 0.6
Exercise above the lower functional intensity ($> 40\% \text{VO}_{2\text{max}}$) ^a			
Duration	1081 $\pm$ 48	950 $\pm$ 94	1097 $\pm$ 101
Distance	3831 $\pm$ 213	3840 $\pm$ 395	3773 $\pm$ 342
kcal	341 $\pm$ 19	300 $\pm$ 93	352 $\pm$ 34
kcal/min	18.9 $\pm$ 0.5	19.0 $\pm$ 0.5	19.2 $\pm$ 0.6
$\text{VO}_{2\text{average}}$	50.7 $\pm$ 1.2	50.8 $\pm$ 1.1	51.3 $\pm$ 1.5
RPE			
mean	18.67 $\pm$ 0.21	18.50 $\pm$ 0.19	18.44 $\pm$ 0.18
median (IQ range _{1 to 3})	18.50 (0.5)	18.50 (1.0)	18.50 (0.5)
HR_{peak}	191.8 $\pm$ 2.0	192.0 $\pm$ 2.0	192.6 $\pm$ 1.5

^a Of note, the entire duration and distance in continuous and the short-interval exercise protocols were performed at or above the lower intensity (40-50% $\text{VO}_{2\text{max}}$) that is prescribed for enhancing aerobic fitness in healthy individuals; in LI protocol only the work-bouts were performed above this intensity.

* $p < 0.05$ vs. respective value in intense-continuous and short-interval

Table 2. The mean ±SE signals of detected metabolites before and after isoeffort intense-continuous long-interval, and short-interval exercise protocols

	Shift (ppm)	Variable	Pre Exercise	Post Exercise	% Change	ANOVA significant main effects
Carbohydrate Metabolism	4.60	Glucose				
		Continuous	3.41 ± 0.21	4.30 ± 0.16*	30 ± 10	Exercise: p < 0.001
		Long-Interval	3.43 ± 0.16	4.09 ± 0.22*	20 ± 6	
		Short-Interval	3.05 ± 0.20	4.65 ± 0.50*	52 ± 11	
	4.09	Lactate				Exercise: p < 0.001
		Continuous	0.16 ± 0.01	0.73 ± 0.08*	372 ± 45	
		Long-Interval	0.17 ± 0.02	0.90 ± 0.09*	508 ± 109	
		Short-Interval	0.19 ± 0.02	0.78 ± 0.08*	346 ± 63	
	2.34	Pyruvate				Exercise: p < 0.001
		Continuous	0.15 ± 0.01	0.26 ± 0.02*	85 ± 13	
		Long-Interval	0.15 ± 0.01	0.27 ± 0.01*	86 ± 23	
		Short-Interval	0.14 ± 0.01	0.25 ± 0.01*	78 ± 13	
Lipid Metabolism	3.63	Glycerol				Exercise: p < 0.001
		Continuous	1.19 ± 0.21	1.82 ± 0.21*	76 ± 23	
		Long-Interval	0.98 ± 0.12	1.82 ± 0.23*	90 ± 21	
		Short-Interval	1.26 ± 0.20	1.81 ± 0.28*	58 ± 22	
TCA cycle	2.62	Citrate				Exercise: p < 0.001 Interaction: p < 0.01
		Continuous	0.112 ± 0.006	0.139 ± 0.009*	25 ± 5	
		Long-Interval	0.101 ± 0.005	0.159 ± 0.011*#	57 ± 8	
		Short-Interval	0.104 ± 0.007	0.143 ± 0.010*	39 ± 7	
	2.37	Succinate				Exercise: p < 0.001
		Continuous	0.107 ± 0.012	0.256 ± 0.016*	155 ± 20	
		Long-Interval	0.129 ± 0.011	0.211 ± 0.016*	84 ± 33	
		Short-Interval	0.118 ± 0.014	0.275 ± 0.036*	139 ± 24	
		Citrate/Succinate				Exercise: p < 0.001
		Continuous	0.219 ± 0.014	0.395 ± 0.022*	82 ± 22	
		Long-Interval	0.230 ± 0.013	0.370 ± 0.023*	68 ± 53	
		Short-Interval	0.222 ± 0.019	0.417 ± 0.042*	89 ± 37	
Anaerobic/Aerobic		Lactate/Pyruvate				Exercise: p < 0.001
		Continuous	1.13 ± 0.13	2.85 ± 0.34*	156 ± 18	
		Long-Interval	1.13 ± 0.11	3.44 ± 0.40*	252 ± 72	
		Lactate/ Citrate				Exercise: p < 0.001
		Continuous	1.46 ± 0.16	5.69 ± 0.96*	284 ± 42	
		Long-Interval	1.76 ± 0.27	6.02 ± 0.88*	301 ± 79	
		Short-Interval	1.86 ± 0.17	5.71 ± 0.71*	219 ± 39	
Amino acids metabolism	1.46	Alanine				Exercise: p < 0.001 Interaction: p < 0.05
		Continuous	0.39 ± 0.03	0.58 ± 0.03*	52 ± 7	
		Long-Interval	0.37 ± 0.03	0.63 ± 0.04*#	77 ± 10	
		Short-Interval	0.36 ± 0.02	0.54 ± 0.02*	53 ± 7	
	0.93	Leucine				Exercise: p < 0.05
		Continuous	0.28 ± 0.01	0.32 ± 0.02	13 ± 7	
		Long-Interval	0.29 ± 0.01	0.31 ± 0.01	10 ± 5	
		Short-Interval	0.28 ± 0.01	0.30 ± 0.02	7 ± 6	
	0.99	Isoleucine				Exercise: p < 0.001
		Continuous	0.070 ± 0.006	0.089 ± 0.005	33 ± 13	
		Long-Interval	0.067 ± 0.008	0.093 ± 0.007	47 ± 12	
		Short-Interval	0.074 ± 0.009	0.080 ± 0.004	21 ± 16	

	1.02	Valine Continuous Long-Interval Short-Interval	0.24 ± 0.01 0.21 ± 0.02 0.23 ± 0.02	0.26 ± 0.01 0.25 ± 0.01 0.22 ± 0.01	12 ± 8 23 ± 9 -2 ± 5	Exercise: p < 0.05
Amino acids metabolism	2.40	Glutamine Continuous Long-Interval Short-Interval	0.169 ± 0.007 0.194 ± 0.010 0.176 ± 0.013	0.140 ± 0.009* 0.147 ± 0.014* 0.172 ± 0.013	-17 ± 5 -22 ± 9 -2 ± 3	Exercise: p < 0.01 Interaction: p = 0.05
	1.99	Proline Continuous Long-Interval Short-Interval	0.23 ± 0.02 0.25 ± 0.02 0.21 ± 0.02	0.17 ± 0.02* 0.17 ± 0.02* 0.19 ± 0.01	-23 ± 11.8 -33 ± 7.04 -0.4 ± 11.7	Exercise: p < 0.05
	6.86	Tyrosine Continuous Long-Interval Short-Interval	0.038 ± 0.003 0.038 ± 0.002 0.038 ± 0.002	0.041 ± 0.003 0.046 ± 0.005 0.038 ± 0.001	11 ± 9 22 ± 14 3 ± 3	
	7.08	Histidine Continuous Long-Interval Short-Interval	0.089 ± 0.003 0.085 ± 0.005 0.087 ± 0.002	0.087 ± 0.002 0.088 ± 0.005 0.086 ± 0.004	-1 ± 3 7 ± 10 -1 ± 3	

* p < 0.01-0.05 vs. pre-exercise value in the same protocol

# p < 0.01-0.05 vs. the post-exercise value in continuous and short-interval protocols

Figure Legends

Figure 1. Typical 600 MHz CPMG ^1H NMR blood plasma spectra with peak assignment. All metabolites were identified and assigned in the spectra, using NMR online databases for 1D and 2D spectra and ChenomxNMR Suite software. Two-dimensional (HSQC) spectra were also acquired in order to unravel and elucidate the assignment of peaks present in 1D spectra. Insert represents the most fluctuating molecules of pre (grey) and post exercise (red) conditions for a subject.

Figure 2. PCA scores plot of all plasma samples for pre (open symbols) and post (closed symbols) exercise. Colors represent different type of exercises (described in the legend). A distinct separation between pre and post exercise samples is observed.

Figure 3. OPLS-DA plot (a) ($R^2Y = 82.2\%$, $Q^2Y = 66.5\%$, $p < 0.001$) and the corresponding S-line plot (c) generated after the exclusion of lactate. Clear separation of pre and post exercise samples is observed according to the valid permutation plot (b) ($n=999$ permutations). The metabolites labeled are pyruvate (M2), glucose (M3), alanine (M4) and succinate (M5). The colour bar on the right side corresponds to the weight of a variable in the discrimination between pre and post exercise samples, ranging from a weak correlation (blue) to a strong correlation (red) to the discrimination.

Figure 4. OPLS-DA plots (upper: including signals of lactate, lower: excluding signals of lactate) and the corresponding S-line plots (excluding signals of lactate to highlight other metabolites) for pre- (open symbols) and post-exercise (closed symbols) samples for (CE) intense continuous, (LI) long-interval, and (SI) short-interval exercise protocols. The OPLS-DA model clearly separated the pre and the post exercise samples within each exercise protocol. The metabolites labeled are pyruvate (M2), glucose (M3), alanine (M4), succinate (M5), citrate (M6), and proline (M7). The colour bar on the bottom corresponds to the

weight of a variable in the discrimination between sets of samples, beginning from a weak correlation (blue) to a strong correlation (red) to the discrimination.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

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