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Plasma metabolomic response to acute short-term high-intensity exercise in young soccer players

Resposta metabólica plasmática a exercícios agudos de curta duração e alta intensidade em jovens jogadores de futebol

Respuesta metabólica del plasma al ejercicio agudo de corta duración y alta intensidad en jugadores de fútbol jóvenes

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Abstract
The objective of the present study was to evaluate changes in the metabolomic profile using proton nuclear magnetic resonance in young soccer players after an acute bout of high-intensity exercise. We recruited ten athletes from a first division soccer team. Blood samples were collected at rest and immediately post-test. We observed increased levels with very large effect for lactate (ES = 3.78 [CI95%: 5.04 to 2.20]; p <0.0001) and a very large decrease in N-acetyl glycoproteins (ES = -3.77 [CI95%: -2.19 to -5.03]; p <0.0001). Alanine (ES = 1.19 [CI95%: 2.08 to 0.19]; p = 0.0091) and glutamate (ES = 0.94 [CI95%: 1.82 to –0.02]; p = 0.0044) showed moderate increased levels after exercise test, while pyruvate and acetate showed irrelevant alterations. Decreased levels of lipids were observed in the following chemical shift regions of 0.56 ppm (ES = -1.15 [CI95%: -0.16 to -2.04]; p = 0.0045), 0.72 ppm (ES = -3.3 [CI95%: 0.09 to –1.73]; p = 0.0003) and 2.21 ppm (ES = -1.07 [CI95%: -0.09 to -1.96]; p = 0.0006). These data suggest the participation of different energy transfer pathways, which quite interesting, since the exercise protocol in this study consisted of just 30 seconds of high-intensity effort.
Keywords: Metabolism; High-intensity interval training; Athletes; Magnetic resonance spectroscopy.

Resumo
O objetivo do presente estudo foi avaliar as alterações no perfil metabolômico por meio da ressonância magnética nuclear de prótons em jovens jogadores de futebol após uma sessão aguda de exercícios de alta intensidade. Recrutamos dez atletas de um time de futebol da primeira divisão. Amostras de sangue foram coletadas em repouso e imediatamente após o teste. Observamos níveis aumentados com efeito muito grande para o lactato (ES = 3,78 [IC95%: 5,04 a 2,20]; p <0,0001) e uma diminuição muito grande nas N-acetil glicoproteínas (ES = -3,77 [IC95%: -2,19 a - 5,03]; p <0,0001). Alanina (ES = 1,19 [IC95%: 2,08 a 0,19]; p = 0,0091) e glutamato (ES = 0,94 [IC95%: 1,82 a -0,02]; p = 0,0044) mostraram níveis moderados aumentados após o teste de exercício, enquanto piruvato e acetato mostraram alterações irrelevantes. Níveis diminuídos de lipídios foram observados nas seguintes regiões de mudança química de 0,56 ppm (ES = -1,15 [CI95%: -0,16 a -2,04]; p = 0,0045), 0,72 ppm (ES = -3,3 [CI95%: 0,09 a - 1,73]; p = 0,0003) e 2,21 ppm (ES = -1,07 [CI95%: -0,09 a -1,96]; p = 0,0006). Esses dados sugerem a participação de diferentes vias de transferência de energia, o que é bastante interessante, visto que o protocolo de exercícios neste estudo consistiu em apenas 30 segundos de esforço de alta intensidade.
Palavras-chave: Metabolismo; Treinamento intervalado de alta intensidade; Atletas; Espectroscopia de ressonância magnética.

Resumen
El objetivo del presente estudio fue evaluar cambios en el perfil metabolómico mediante resonancia magnética nuclear de protones en futbolistas jóvenes después de una serie aguda de ejercicio de alta intensidad. Reclutamos a diez atletas de un equipo de fútbol de primera división. Se recolectaron muestras de sangre en reposo e inmediatamente después de la prueba. Observamos un aumento de los niveles con un efecto muy grande para el lactato (ES = 3,78 [IC95%: 5,04 a 2,20]; p <0,0001) y una disminución muy grande en las N-acetilglicoproteínas (ES = -3,77 [IC95%: -2,19 a - 5,03]; p <0,0001). La alanina (ES = 1,19 [IC95%: 2,08 a 0,19]; p = 0,0091) y el glutamato (ES = 0,94 [IC95%: 1,82 a –0,02]; p = 0,0044) mostraron niveles moderados de aumento después de la prueba de esfuerzo, mientras que el piruvato y el acetato mostró alteraciones irrelevantes. Se observaron niveles reducidos de lípidos en las siguientes regiones de desplazamiento químico de 0,56 ppm (ES = -1,15 [IC95%: -0,16 a -2,04]; p = 0,0045), 0,72 ppm (ES = -3,3 [IC95%: 0,09 a - 1,73]; p = 0,0003) y 2,21 ppm (ES = -1,07 [IC95%: -0,09 a -1,96]; p = 0,0006). Estos datos sugieren la participación de diferentes vías de transferencia de energía, lo cual es bastante interesante, ya que el protocolo de ejercicio en este estudio consistió en tan solo 30 segundos de esfuerzo de alta intensidad.
Palabras clave: Metabolismo; Entrenamiento de intervalos de alta intensidad; Deportistas; Espectroscopía de resonancia magnética.

1. Introduction

Team-sports like soccer impose great physiological demands to the body, since it involves a combination of high-intensity sprints with periods of moderate intensity running. Mohr, Krustrup, and Bangsbo (2003) observed that elite soccer players could perform up to 150-250 bouts of high intensity actions during a match. From a biochemical point of view, energy sources during repeated sprint ability (RSA) tests must come basically from muscle phosphocreatine and adenosine triphosphate (ATP) depletion (Bishop, Girard & Mendez-Villanueva, 2011), however anaerobic glycolysis is also involved since high levels of blood lactate are observed in this type of training (Turner & Stewart, 2013).

In fact, high-intensity exercise involves mainly anaerobic energy production that produces high levels of blood lactate as a consequence (Bangsbo, Iaia, & Krustrup, 2007), suggesting that specific adaptations at the skeletal muscle level in humans seem to be determinant for high intensity performance (Faiss et al., 2013). Interestingly, maximal oxygen consumption (VO_{2max}) seems to be inversely associated with RSA suggesting that aerobic capacity could have a major role during recovery to allow adequate energy substrate replenishment (Jones et al., 2013). Previous study by Medbø and Tabata (1989) observed that during high-intensity exercise the contribution of aerobic metabolism shoed a relative importance even during 30s of exercise.

Metabolomics is part of the "omics" trend that starts from a general perspective of a particular fluid or biological sample without a previously established hypothesis (Dunn, Broadhurst, Atherton, Goodacre & Griffin, 2011). The metabolomics research can distinguish the variation of metabolic profile against different training programs and identify markers associated with performance, fatigue, and disorders related to physical exercise (Yan et al., 2009). Horgan and Kenny (2011) point out that the metabolome is the end product of gene transcripts, and therefore reflect the proteomic and transcriptomic changes and is therefore closest to the phenotype of the biological system studied. Thus, metabolites levels are considered as the final response of biological systems, establishing a "fingerprint" of the intrinsic metabolic profile of the individual or influenced by extrinsic factors, respectively (Zhang, Sun, Qiu & Wang, 2013; Dunn, Broadhurst, Atherton, Goodacre & Griffin, 2011). Besides, the metabolites determined with this Nuclear Magnetic Resonance (NMR) metabolomic study are not easily assayed with biochemical methods like N-acetyl moieties or lipids sub-fractions (Le Moyec et al, 2014). Therefore, this study aimed to use the metabolomics method to evaluate possible metabolic changes in young soccer athletes after an acute high-intensity exercise protocol.

2. Materials and Methods

The research was approved by the Ethics Committee of Rio de Janeiro State University (CAAE: 0083.0.228.000-05). The collection of data and biological samples was preceded by the approval of the study participants, by signing the Informed Consent Term. The inclusion criteria were defined by absence of limiting injuries and authorization of participation in the study

by parents or legal responsible adult by completing and signing the consent form. Exclusion criteria for this group were based by the presence of disease or syndrome that prevented cooperation during clinical examinations and blood collections. The number of participants in the study was obtained through convenience sample. Thus, we evaluated ten young soccer players (age, 14.1±2.9 y), with 6.1±2.3 y of previous regular soccer training from a first division soccer team in Rio de Janeiro from the under 15 class.

Anthropometry and Body Composition

The measurement of total body mass (TBM) was held in the balance Filizola® with 0.1 kg accuracy. In the measurement of height, it was used a vertical stadiometer with 200 cm long and 0.1 cm precision. Skinfold measurements were performed by an experienced anthropometrist using Lange™ skinfold caliper with precision of 1 mm. The location of 10 skinfolds (triceps, biceps, chest, axillary, subscapularis, suprailiac, supraspinatus, abdominal, thigh media and medial leg) followed the standardization proposed by the International Society for the Advancement of Kinanthropometry (ISAK) and percentage of fat (%BF) was calculated using the formula proposed by Slaughter et al. (1988). The calculation of fat mass (FM) was obtained by multiplying the% BF by TBM. Lean body mass was calculated by the difference between TBM and FM.

Wingate Test

The standardized 30 s Wingate Anaerobic Test (WAnT) was used as a model of exercise stress according to Inbar, Bar-Or O & Skinner (1996). The session was begun with a brief warm-up, consisting of 5 min at 60 rpm and load established at 50 W. The test started after a countdown of "3, 2, 1, go!", and then, the participant began pedaling on the ergometer at maximum speed and continued for 30 s. After a 10-min rest, the participants were instructed to pedal "all-out'' for 30 s against a resistance of 0.75 kg/kg body mass. Verbal encouragement was provided throughout the test. At the end of test, a 2–3 min cool-down was allowed for all the voluntaries.

Blood collection

All bio-security standards were adopted strictly, as well as the disposal of biological material and the use of individual protection equipment. Blood samples were taken at rest and immediately after the test. Blood was collected from the antecubital vein (10mL) into disposable tubes (Vacuette®). After centrifugation of whole blood (4 ºC for 10 minutes to 1600 g) the obtained plasma was transferred in 700 µL aliquots for plastic tubes (Eppendorffs, Hamburg, Germany), and finally stored in freezer at -80 °C for posterior NMR analysis.

Analysis by Nuclear Magnetic Resonance (NMR)

For the preparation of the final blood sample, a further centrifugation was carried out at 1.200 *g* for 10 minutes at 4 °C, for removal of solid particles from the sample. The final volume was 520 µL, consisting of 130 µL of plasma, 340 µL of sodium chloride (NaCl) and 50 µL of deuterated water (D_2O). The D2 is the reference for aligning magnetic field to the sample (lock). It used an internal reference (hydrogen on the anomeric carbon of glucose), located at $\delta = 5.22$ ppm. The spectra were obtained on a 600 MHz NMR Spectrometer (Bruker Biospin™, Rheinstetten, Germany). It was standardized to 256 scans hydrogen to blood samples. Carr-Purcell-Meiboom-Gill (CPMG) with T2-filter for removing macromolecules signals, with 512 scans pulse sequence, at 300 MHz Some samples were also submitted to 1H-1H-TOCSY technique (TOCSY) with acquisition parameters of 2048 x 256 complex points to solve ambiguity assignments. After acquisition of the spectra 1D and 2D it was performed the correction of phase and baseline of the spectra obtained with TOPSPIN™ (Bruker, Karlsruhe, Germany). The spectra were superimposed in order to verify the presence of possible changes in the chemical shifts of metabolites and variations on the

baseline. For calibration spectra of plasma, an internal reference since DSS interacts with the blood, by adopting the peak of the hydrogen bound to anomeric carbon of glucose was used, located at δ = 5.22 ppm. The assignment of the metabolites was based on TOCSY experiments, Human Metabolome Database (http://www.hmdb.ca/) and Almeida et al. (2017).

Statistical analysis

The intensity data of each peak of biofluids ^{1}H spectra was collected through the 0.03 ppm buckets and were extracted using a specific computer software (AMIX, Bruker Biospin, Rheinstetten, Germany). Regions unaligned were excluded from the analysis (1.46 to 1.48; 2.09 to 2.14; 2.41 to 2.44; 2.66 to 2.83; 3.59 to 6.58; 6.84 to 7.18 and 7.52 to 8.29). Normalizing data was obtained by Pareto technique. To evaluate each metabolite before and after physical test, the matrix generated by AMIX was subjected to multilevel PLS-DA (M-PLS-DA) using Matlab (version 2010a, The Mathworks, Natick, Massachusetts). The M-PLS-DA has been used to assess paired samples, where each individual serves as its own control (Szymańska et al., 2012; van Velzen et al., 2008). For statistical assessment of metabolites, we used GraphPad Software (GraphPad Prism version 7.00 for Windows, La Jolla California USA, www.graphpad.com).

Following data normality confirmation using D`Agostino-Pearson omnibus normality test, we used Student`s t-test. Results were presented as mean ± standard deviation (SD) and statistical significance was set at $p<0.05$. Based on the number of recruited athletes (n=10), considering $\alpha = 0.05$ our study could detect an effect size equal to 0.99 with 80% of power (G*Power software, version 3.1.9.3). This parameter was set as a cut-off point for practical significance over merely statistical significance, also known as equivalence limits (Lakens, 2017). A modified Cohen`s effect size (ES) was used to qualify statements about the magnitude changes as: 0.0-0.2, trivial; 0.2-0.6, small; 0.6-1.2, moderate; 1.2-2.0, large; >2.0, very large; >4.0, extremely large (Hopkins, Marshall & Batterham, 2009). So, based on the effect size categories alterations statistically significant differences could be irrelevant even when were considered “moderate”. Additionally, magnitude-based inferences were calculated to evaluate qualitatively the changes observed in serum metabolites where substantial changes were classified at ≥75% likelihood of the effect being greater than the d_{SESOI}. Effects were classified as unclear (⇔) when 90% confidence interval (CI) crossed both boundaries of smallest worthwhile changes (SWC) and were classified as trivial (⇳) when were not classified as substantial or unclear. This type of data presentation gives the idea of the minimum relevant alterations that should not be classified as trivial. Threshold for assigning qualitative terms to chances of substantial differences were as follows: < 1%, almost certainly not; < 5%, very unlikely; < 25%, unlikely or probably not; > 50%, possibly (+); > 75%, likely or probable (++); > 95%, very likely (+++); > 99%, almost certain (++++).

3. Results

Anthropometric measurements of the study participants are shown in Table 1, where it can be observed that all participants showed adequate levels of body composition parameters.

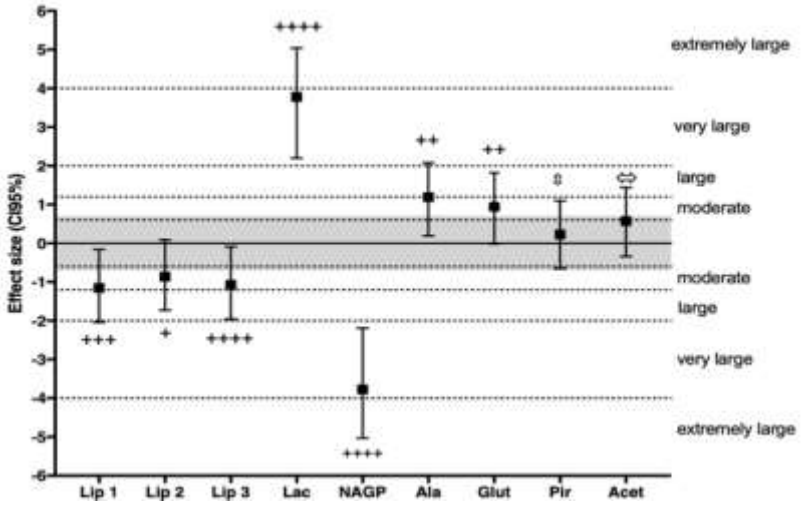
Table 1 – Anthropometric characteristics and training status of study participants.

Age (years)	14.1 ± 2.9
Body mass (Kg)	61.0 ± 5.7
Stature (cm)	167.7 ± 7.4
BMI (Kg/m^2)	21.8 ± 2.6
Percent body fat	18.4 ± 6.0
Fat mass (Kg)	11.2 ± 3.5
Lean body mass (Kg)	49.7 ± 2.3
Training volume (h/week)	5.0 ± 1.8

Source: Authors.

When both conditions were compared we observed increased very large levels of lactate (+3.2 fold, ES = 3.78; p <0.0001) and very large decreases in N-acetyl glycoproteins (-17.5%; ES = 3.77; p <0.0001). Besides, we observed moderate increases in alanine (+21.5%; ES = 1.19; p = 0.0091) and glutamate levels (+32.4%; ES = 0.94; p = 0.0044), while pyruvate levels showed trivial alterations (+32.4%; ES = 0.23; p = 0.5651) after exercise test. Additionally, lipids levels showed moderate decreases in the chemical shift regions of 0.56 ppm (-75.2%; ES = 1.15; p = 0.0045), 0.72 ppm (-20.5%; ES = 3.3; p = 0.0003) and 2.21 ppm (-19.5%; ES = 1.07; p = 0.0006), while increased acetate levels were considered unclear (+20.7%; ES = 0.57; p = 0.024) as showed in Figure 1.

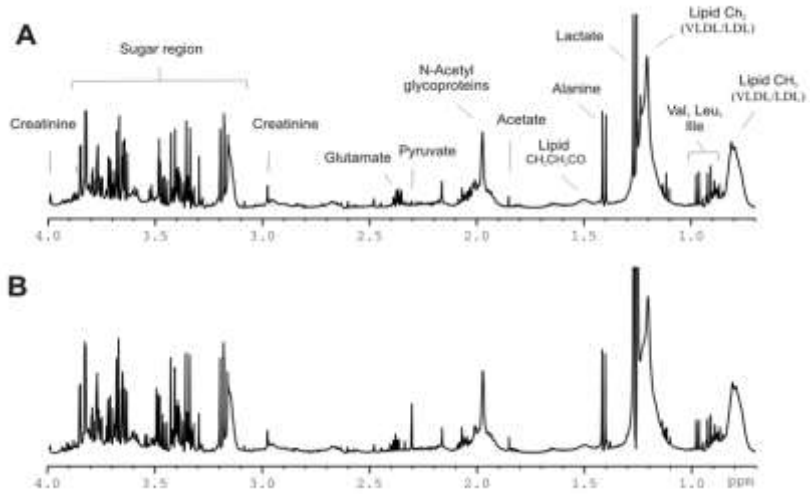
Figure 1 - Effect sizes ^{1}H RMN spectra of serum metabolites in response to Wingate protocol in young soccer athletes.



Lipid 1 (Lip 1), Lipid 2 (Lip 2), Lipid 3 (Lip 3), Lac (lactate), NAGP (N-acetyl glycoprotein), Ala (alanine), Glut (glutamate), Pir (piruvate), Acet (acetate). Gray area denotes trivial zone (small effects were omitted for clarity purposes). Source: Authors.

^{1}NMR spectroscopy revealed distinction between serum metabolite levels when accessed before and after exercise (Figure 2).

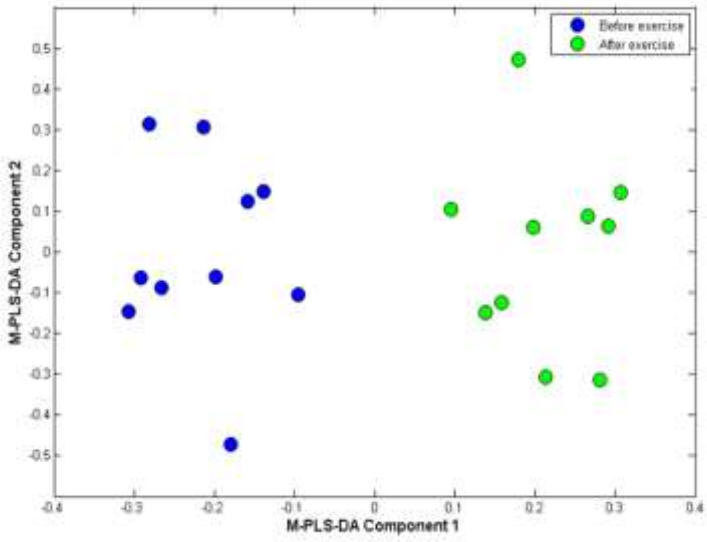
Figure 2 - [1]NMR spectroscopy of serum markers in young soccer players before (A) and immediately after (B) a high-intensity exercise bout.



Source: Authors.

Multilevel-paired data analyses PLS-DA analysis of serum metabolites showed good separation (65%) considering rest and exercise conditions (Figure 3).

Figure 3 - MPLSDA demonstrating the metabolites profile differences before and after exercise.



MPLSDA component 1 and 2 keep 65% of total variation. Loading factor of relevant metabolites responsible for the separation. Source: Authors.

4. Discussion

The need for ATP availability is expressively increased during acute high-intensity exercise. So, metabolic demands must be met basically by glucose and fatty acids (Catoire et al., 2014). Acute WAnT protocol used in this study was able to increase lactate levels, corroborating previous studies (Dror, Oren, Pantanowitz, Eliakim & Nemet, 2017; Öztürk, Özer & Gökçe,

1998). Indeed, high-intensity exercise produces increased amounts of lactate through pyruvate oxidation by lactate dehydrogenase, suggesting the predominance of anaerobic energy production. Besides, we also observed increased levels of alanine and glutamate, while pyruvate levels were unaltered, suggesting the maintenance of the metabolic flux.

Increased levels of alanine after WAnT protocol, suggests increased skeletal muscle protein catabolism and, consequently, hepatic glucose production through alanine-glucose cycle. The conversion of pyruvate to alanine, by shifting the amine grouping mediated by alanine transaminase (ALT) is mediated by glutamate and allows ammonia transport to the bloodstream in a non-toxic manner (Felig & Wahren, 1971). Our results showing significant increases of alanine after 30s of high-intensity exercise corroborates the previous work of Berton et al. (2017) that named alanine a rapid response metabolite.

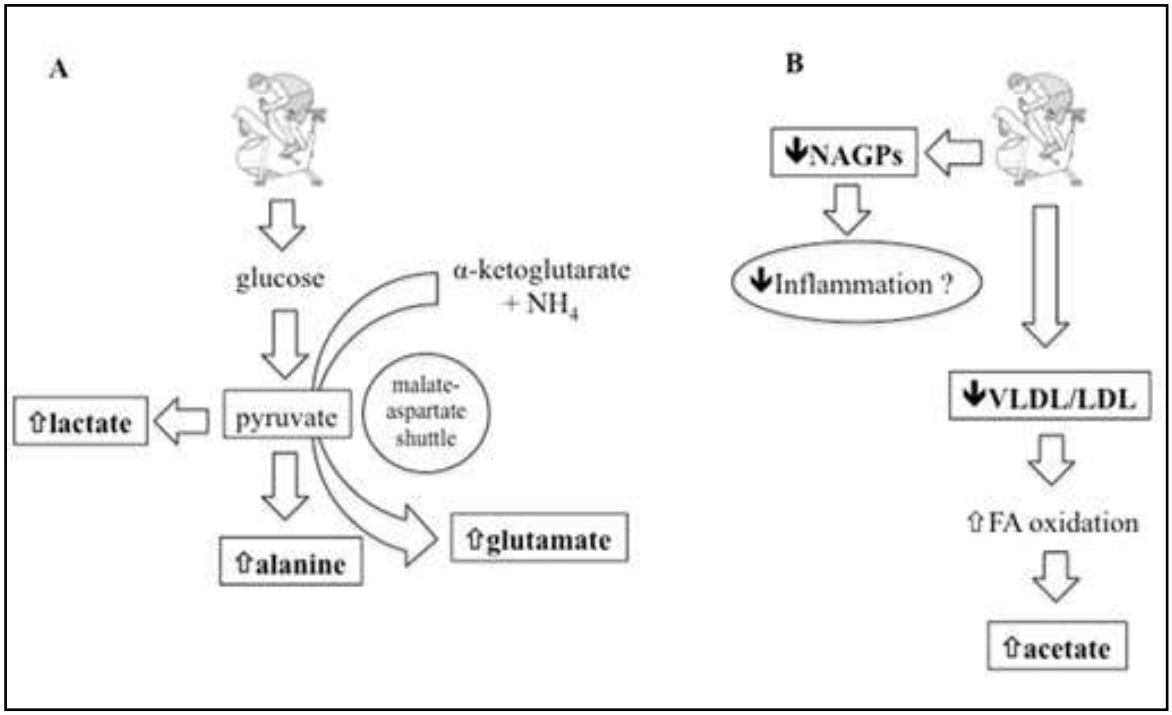
Mechanical damage to active skeletal muscles was previously considered the main mechanism responsible for increased blood glutamate during intense exercise (Leibowitz et al., 2012). However, posterior experiments using metabolome analysis observed that glutamate was also produced by a direct interaction between glucose metabolism and malate-aspartate shuttle (Gheni et al., 2014), suggesting also the participation of aerobic metabolism during anaerobic exercises, as proposed by (Valério et al., 2018). These authors observed increased levels of succinate metabolite after an acute protocol of resistance training. Glutamate also seems to have a relevant role in this process through malate-aspartate shuttle, since it's an active site during physical exercise (Hittel, Kraus, Tanner, Houmard & Hoffman, 2005).

N-acetyl glycoproteins (NAGPs) are acute phase proteins related with innate immunity (Bell, Brown, Nicholson & Sadler, 1987). Increased levels of NAGPs can be observed during conditions of inflammation, infection, stress, and trauma or in disease states that conjugates these features in some manner (Sun et al., 2012; Bell, Brown, Nicholson & Sadler,1987). Pechlivanis et al. (2013) observed decreased levels of NAGPs in healthy males undergoing an acute bout of high-intensity exercise that could be related to a higher demand for glycoprotein anabolism (Le Moyec et al., 2014). Acute exercise protocols normally are related with a posterior increased inflammation response due to many biochemical and molecular pathways. However, the short duration of WAnT protocol could be related with a delayed response for inflammatory signaling together with a complex balance between pro and anti-inflammatory cytokines (Suzuki, Yamada, Sato & Sugawara, 2002).

Acetyl-CoA is partially metabolized to ketone bodies that are circulated into the bloodstream with subsequent uptake by extra hepatic tissues and resynthesized into acetyl-CoA for use as an energy source in tricarboxylic acid (TCA) cycle (Yamashita, Itsuki, Kimoto, Hiemori & Tsuji, 2006). During exercise skeletal muscle can uptake fatty acids derived from VLDL to meet its energetic demands. Recently, a fatty acid induced myokine named ANGPTL4 was described as a LPL antagonist, which is counteracted by AMPK-mediated suppression during exercise conditions to maintain fatty acid uptake in skeletal muscle (Catoire et al., 2014). The decreased level of lipids (VLDL/LDL) observed in this study suggests high rates of fatty acid oxidation reinforcing these mechanisms.

Indeed, aerobic metabolism can assist energy recovery from high-intensity exercise through fatty acid oxidation (Jones et al., 2013). In such conditions, the production of acetyl-CoA is enhanced in liver mitochondria, as a result of beta-oxidation. The increase in acetate concentration could support the hypothesis of an lipolytic effect, since acetate levels seems to rise as a consequence of increasing rates of fatty acid oxidation (Shimazu et al., 2010; Seufert, Graf, Janson, Kuhn & Söling, 1974). However, in our study when effect sizes were considered the acetate levels did not show relevant alterations, probably due to the short duration of WAnT protocol. Our results suggest that short-duration high intensity physical exercise could elicit significant changes in anaerobic metabolism through diverse pathways to attend different metabolic requirements. Interestingly, acetate as an early fat oxidation metabolite could be involved in epigenetics signaling related with additional adaptions from physical exercise (Figure 4).

Figure 4 - Schematic representation of the main events related with metabolites obtained in the study.



Source: Authors.

5. Conclusion

Taken together, these results suggest a simultaneous interaction between anaerobic and aerobic metabolism, during 30s of high-intensity exercise. Pyruvate oxidation seems to be mainly directed to lactate production, but a minor fraction is also directed to alanine and glutamate as well. Future research with athletes are needed to evaluate and compare the demands and adjustments from various sports and training processes, to allow the adequate development of different strategies associated with performance, fatigue and disorders related to physical exercise.

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