

The double product breakpoint as predictor of stress and the correlation between IL-12 and biomarkers in athletes

SÉRGIO LUÍS PRAMPERO¹, LUIS CARLOS OLIVEIRA GONÇALVES², NÁDIA RAQUEL DUTRA MORAIS MOURÃO³, MÁRCIO VINÍCIUS DE ABREU VERLI⁴, EDUARDO LUZÍA FRANÇA⁵, ADENILDA HONORIO FRANÇA⁶, ANÍBAL MONTEIRO DE MAGALHÃES NETO⁷
^{1,2,3,5,6,7}Graduate Program in Basic and Applied Immunology and Parasitology, Federal University of Mato Grosso (UFMT), BRAZIL;
^{2,4,7}Graduate Program in Physical Education, Federal University of Mato Grosso (UFMT), BRAZIL;
^{2,4}Graduate Program in Health Sciences, Federal University of Mato Grosso (UFMT), BRAZIL

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Abstract:

Immunometabolism emerges as the target for future research in health and exercise sciences. The impact caused by a treadmill running test on different biomarkers, changes in the cardiovascular system, and the correlation between IL-12 and performance. Ten male high-performance athletes performed using a gas analyzer, and the treadmill exercise protocol was progressively increased until exhaustion. The athletes ran an average distance of 3 km, characterized by a gradual load increase in stages until voluntary exhaustion. Thus, the initial speed was 10 km/h with an increment of 1 km every 400 meters. Correlation of 1.0 ($P=0.00278$) between IL-12 and $\dot{V}O_2$ in the respiratory compensation time and of 0.943 ($P=0.0167$) between pre-exercise IL-12 and the time to reach maximal effort, with the same correlation and P-value between pre-exercise IL-12 and the distance covered, and pre-exercise IL-12 and the test time. Post-exercise IL-12 presented a correlation of 0.9 ($P=0.0167$) with the heart rate of the respiratory compensation point. A breakpoint for both the double product and heart rate was observed between minutes 2 and 3 of the test, both measurements being more acute than the anaerobic threshold and the respiratory compensation point, and even that breakpoint for systolic blood pressure. The average percentage change in heart rate and the double product obtained the same value (213%). The heart rate and the double product breakpoint are sensitive and acute markers of stress level, and the production of IL-12 depends on the intensity and duration of the exercise, which may correlate with performance.

Key Words: - Immunometabolism, Cytokines, Sportomics, Data mining. **Introduction**

Introduction

Physical exercise, traditionally used by science to evaluate the performance of athletes or to test the effectiveness of different supplements in this performance, gains a new perspective with the emergence of sportomics sciences (Gonçalves et al., 2012; Magno-França et al., 2013; Coelho et al., 2016).

Sportomics aims to reproduce the natural conditions of different sports and exercise methods, not to improve the conditioning of athletes and gain titles, but to study the immunometabolism in different lines, from the influence of drugs, nutrients, types of stress, pathologies, and limitations, in different populations (Gonçalves et al., 2012; Bachini et al., 2021; Gonçalves et al., 2022; Lopes et al., 2022), observing modulations in the immune system, metabolism and the integration between organs and systems in these conditions (Gonçalves et al., 2012; Lopes et al., 2019; Verli et al., 2021).

Metabolic alterations and cardiovascular diseases are a growing trend in the 21st century and represent a concern for health professionals and researchers worldwide (Antunes et al., 2016).

Physical exercise is essential in preventing different diseases, mainly through myokines, a group of peptides produced, expressed, and released by muscle fibers under contraction. Myokines such as interleukin 6 (IL-6), interleukin 10 (IL-10), and interleukin 1 (IL-1) help configure the metabolic-immune factor interface and the health-promoting effects of physical exercise by releasing humoral factors capable of interacting with other tissues, organs, and systems (Leal et al., 2018).

However, not only the cytokines mentioned above influence this immunometabolism. For example, interleukin 12 (IL-12) is a pro-inflammatory cytokine produced and secreted by B cells (Germann & Rude, 1995) and antigen-presenting cells such as monocytes, macrophages, and dendritic cells (Trinchieri & Gerosa, 1996), with a primary role in immunity (Stern et al., 1996) as a regulator of innate and acquired immunity, indicating a promising future for research in the field of immunometabolism (Bashyam, 2007).

Physical exercise may be paramount in regulating plasma IL-12 concentration, positively or negatively modulating the immune system, metabolism, and integration and functioning of different organs (Silveira et al., 2016).

The present study sought the acute impact caused by a running test on a treadmill, on different biomarkers, changes in the cardiovascular system, and a possible correlation between IL-12 with kidney and liver function biomarkers and its association with performance in racing athletes.

Material & methods

Subjects

The study included ten male individuals who are high-performance athletics athletes without recent musculoskeletal injuries or any other health conditions that represent medical contraindications for exercise and who participate in municipal, state, and national competitions. The number of participants that make up the sample is justified because they are very high-level athletes, among the first ranked in the country, who live in the same training center, eat, sleep, and train simultaneously, leaving the sample as homogeneous as possible. Constantly and have medals on their resume. Anthropometric characteristics are presented in table 1.

Table 1. Subject's characteristics (n = 10).

	Average	Median	SD	SE
Age (years)	21.2	19.5	5.5	1.7
Weight (kg)	60.6	59.0	6.4	2.0
Height (cm)	173.5	171.0	7.5	2.4
BMI (kg/m ²)	20.1	20.1	1.4	0.4

BMI – Body Mass Index; **SD** – Standard Deviation; **SE** – Standard Error.

Ethical approval

The participants received all the information about the study's objectives, procedures, and risks. Only after agreeing they were invited to sign an informed consent form (TCLE), which assured their rights of privacy and freedom to withdraw from the study when they agreed. The study was previously submitted and approved by the Ethics Committee in Research involving Human Beings of the Federal University of Mato Grosso (UFMT), Araguaia campus, having been approved under opinion number: 5,716,414

Experimental design

This is a cross-sectional study. Data collection was performed at the Cardiology Institute; the cytology analyzes were carried out in the Chronoimmunomodulation laboratory at the Federal University of Mato Grosso (UFMT), Araguaia campus; and the other blood analyzes were carried out in a private laboratory, all located in the city of Barra do Garças – MT. Collections were carried out between February and April 2021, always taking place at a standardized time, between 2:00 pm and 3:30 pm, to avoid possible influences of the circadian cycle. Also, the environmental conditions were standardized at a temperature maintained at 21° degrees.

Procedures

Exercise. The athletes performed dynamic and static stretching in all body segments, lasting 5 minutes as an essential warm-up.

Using a gas analyzer, the treadmill exercise protocol was progressively increased until exhaustion. The specific warm-up that preceded the test consisted of light running, which lasted 15 minutes. Afterward, the athletes ran an average distance of 3 km, characterized by a gradual load increase in stages until voluntary exhaustion. Thus, the initial speed was 10 km/h with an increment of 1 km every 400 meters.

In turn, the anaerobic threshold was determined by ventilatory parameters. The parameters from the applied exercise protocol sought to expose the participants to a distance similar to those faced in natural field environments and trigger physiological changes similar to what would occur in a real running scenario.

Ergospirometric assessment. Cardiovascular assessment during the stress test was performed using an exercise electrocardiogram (ErgoPC Elite) with monitoring at lead points (D1, D2, D3, AVR, AVL, AFV, MC5, V1, V2, V3, V4, V5, and V6). In addition, blood pressure (BP) was measured indirectly using a Missouri sphygmomanometer and a Rappaport stethoscope.

The metabolic evaluation was performed using a computerized analyzer (MetaLyzor 3B), a face mask (V2 mask Small), and software for capturing and demonstrating data, as well as for storing and processing all cardiorespiratory and metabolic variables evaluated.

Flow cytometry. The concentration of cytokine IL-12 present in serum samples was evaluated using the "Cytometric Bead Array" kit (CBA, BD Bioscience, USA). These cytokines were analyzed using flow cytometry, and the data were analyzed using the FCAP Array software.

Blood sampling. Two extractions of 5 mL each of venous blood were collected from the antecubital vein of each participant by a qualified professional with experience in field collections. Blood collection occurred with a stainless-steel needle syringe, the first sample being extracted before the stress test and the second after the test.

After collection, the samples were stored in a metal-free polypropylene tube and centrifuged at 2500 rpm for 10 minutes at room temperature.

Statistical analysis

Initially, descriptive statistics were performed on the data, with position measurements (mean, median, mode, and percentiles) and dispersion (amplitude, variance, standard deviation, and standard error).

Afterward, the univariate analysis of these data was performed using the Shapiro-Wilk normality test (because the sample was smaller than 30 individuals). The equal variance test would be applied if the Shapiro-Wilk test presented a result indicating normal distribution ($P > 0.05$). For results with $P > 0.05$, the paired T-Student test would follow; if $P \leq 0.05$, the paired T-Student test would follow the non-parametric Mann-Witney test. If the Shapiro-Wilk test presented a result indicating non-normal distribution ($P \leq 0.05$), the non-parametric Mann-Witney test would be applied directly. Still, in the phase of the univariate analysis, the analysis of repeated measures ANOVA One Way dependent was performed because they were the same individuals in different conditions and moments.

So, for a better interpretation of the data, the individuals were divided into two groups according to their sex. Then, the calculation of percentage variation was applied:

$$\Delta\% = \frac{(\square\square\square\square\square\square\square\square - \square\square\square\square\square\square\square\square)}{\square\square\square\square\square\square\square\square} \times 100$$

Next, multivariate data analysis was performed using data mining and machine learning techniques.

In this phase, in order to seek a bivariate measure between the data, because the observations contain quantitative values, the Pearson and Spearman correlation tests were applied, with the Spearman correlation being used for a visual analysis using the heat map strategy and the Pearson test as an initial measure for the following machine learning analyses. As exploratory models of machine learning: CLUSTER - Classical Clustering (Agglomerative Hierarchical Method) and Nearest neighbor (single linkage); ORDINATION - Principal Component Analysis (PCA) and Correspondence Analysis (CA).

The Z score was previously applied to adjust observations measurement units, and the Fruchterman-Reingold algorithm was applied with Euclidian Similarity Index (Fruchterman & Reingold, 1991; Karmen et al., 2019). SigmaPlot 14.5 (Academic Perpetual License - Single User - ESD Systat® USA), R and R Studio (Free version for Windows) and, Past 4.03 (Free version for Windows) was used to carry out the different statistical tests and produce the graphs. Finally, the correlation coefficients were presented using heat maps (Gonçalves et al., 2022).

Results

Supplementary table 1 presents the mean and standard error values for the pre- and post-exercise times and the percentage variation between the means for the mentioned times. Because the hematocrit had a slight percentage variation (4%) and did not present a significant difference between the means, there was no need to apply a correction factor to the values of all blood biomarkers because when there is an acute change in the hematocrit, this indicates an alteration in the state of hydration, with hemoconcentration or hemodilution, which would alter the measured values, not due to a difference in production, but due to a change in the amount of plasma. Among the data presented in supplementary table 1, there was no difference in markers of renal function (urea and creatinine) and liver function (Gamma GT, TGP, and TGO), leaving the entire discussion to changes in IL-12 and their correlations.

Figure 1 shows the behavior of individualized systolic blood pressure, with a steady state in the initial and middle thirds and changes in the final third of the test (Figure 1A). Figure 1B, also in individual analysis, reveals no change in diastolic blood pressure throughout the test.

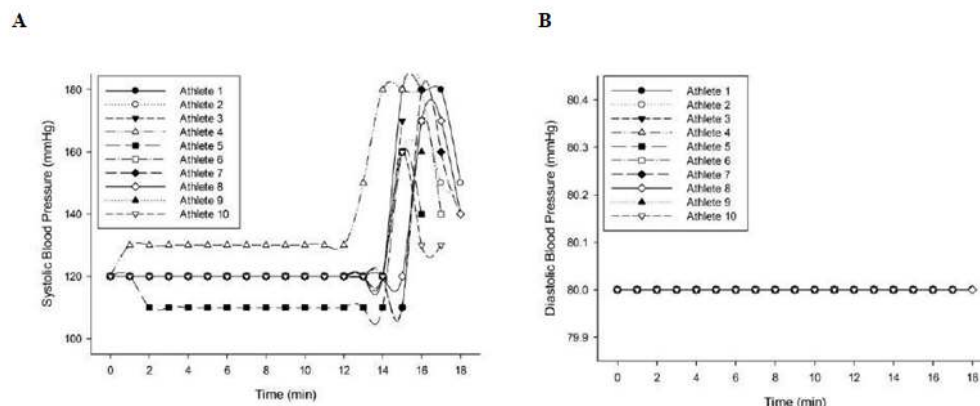


Figure 1. Individualized behavior of systolic blood pressure (A) and diastolic blood pressure (B).

Figure 2 shows that the heart rate has already changed in the initial third of the test, revealing itself as the most acutely affected cardiovascular component in the present protocol (Figure 2A). The graphic design was similar to that of Figure 2B, indicating that heart rate is the main factor that alters the double product during the test.

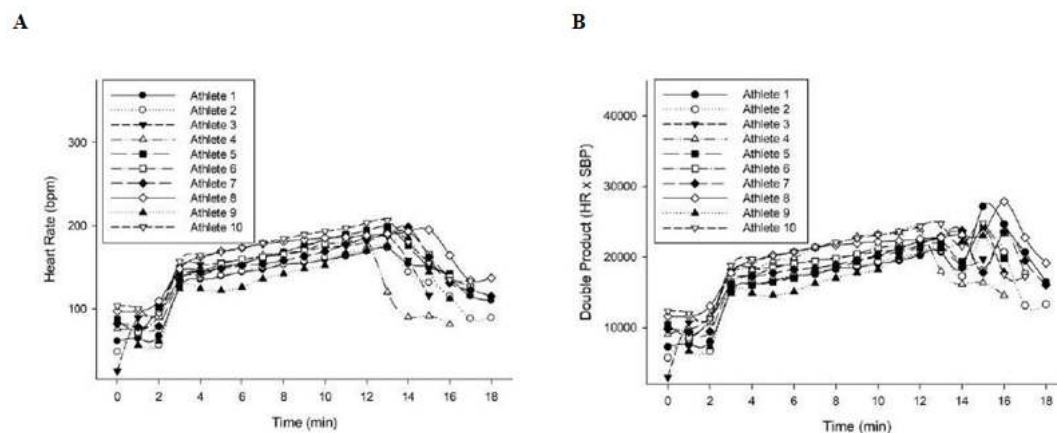


Figure 2. Individualized heart rate (A) and double product (B) behavior.

The values of the correlation coefficients obtained from the Spearman test were presented as a heat map, with a dark blue scale for the coefficients from -1.0, passing through lighter shades of blue until reaching the color green for the zero value, passing through shades of yellow and light red until reaching dark red for the correlation of 1.0.

The heat map contained in Figure 3 shows a positive correlation of 1.0 ($P=0.00278$) between the variation in IL-12 concentration and VO_2 in the respiratory compensation time.

The graph, as mentioned above, also revealed a positive correlation of 0.943 ($P=0.0167$) between pre-exercise IL-12 and time to reach maximum effort, with the same correlation coefficient and P value between pre- and IL-12 the distance covered, and between the pre-IL-12 and the test time (Figure 3). The post-exercise IL-12 presented a correlation coefficient of 0.9 ($P=0.0167$) with the heart rate at the respiratory compensation point.

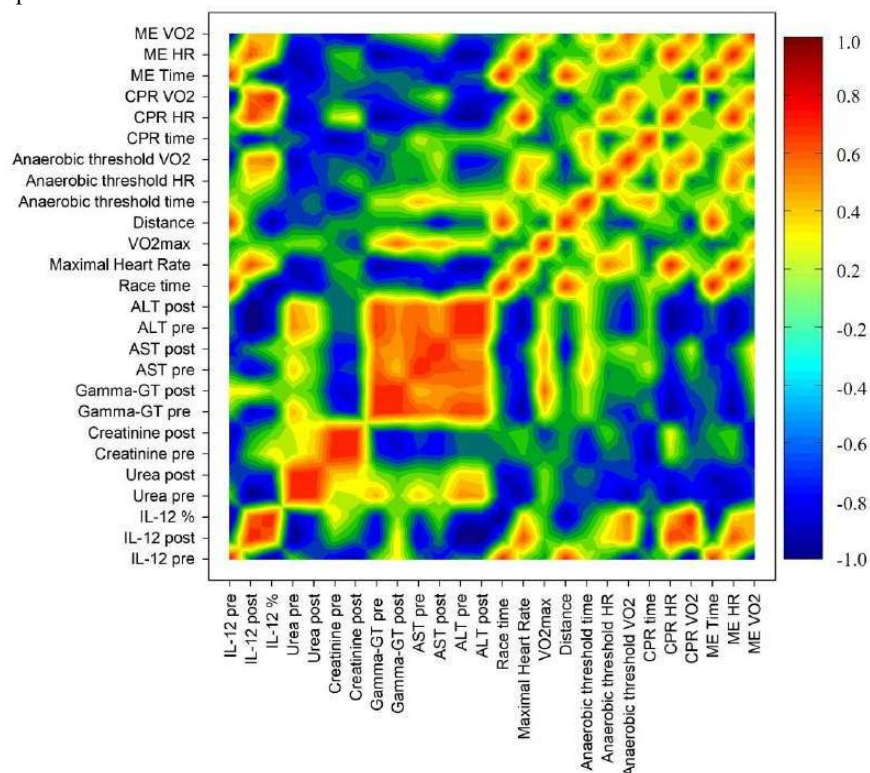


Figure 3. A heat map with the correlations obtained in the Spearman test

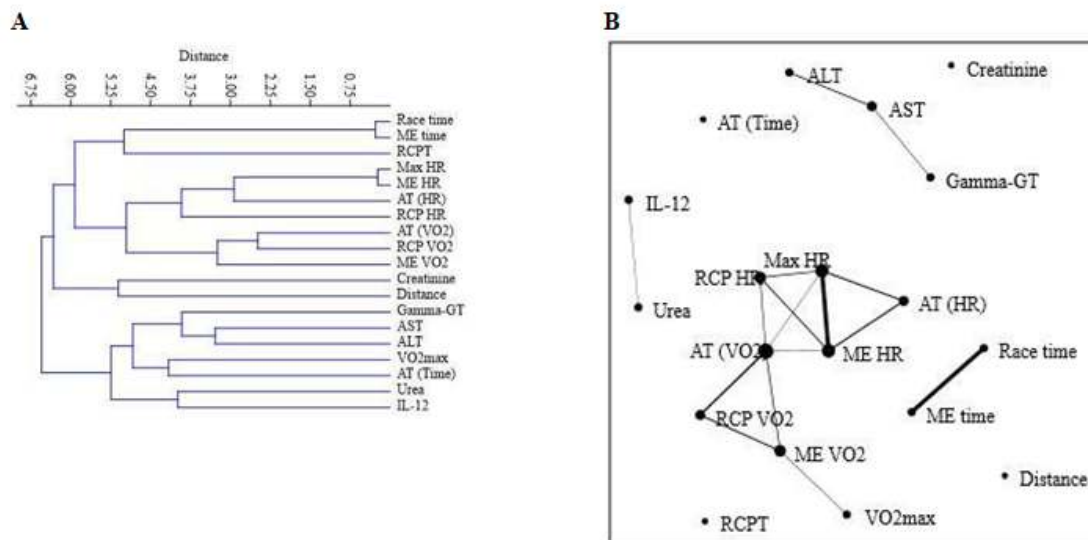


Figure 4. (A) Dendrogram and (B) network plot with Fruchterman-Reingold algorithm after calculating the Euclidean Similarity Index.

Figure 4 revealed the main similarities between the study variables when evaluated by the Euclidean Similarity Index. In this new form of analysis, a group with a more significant similarity between IL-12 and the time

to reach the anaerobic threshold was indicated, in addition to Gamma GT, AST, ALT, VO2 max, and urea. Finally, the supervised machine learning technique by linear regression presented a model adjustment coefficient (R^2) of 0.80, indicating that 80% of the variation in VO2 at the respiratory compensation point can be explained or determined by the variation in IL-12, passing through on the Shapiro-Wilk normality test with a P- value of 0.593, and passing the Constant Variance Test by Spearman Rank Correlation ($P = 0.060$) with a final value of $P=0.016$ (Figure 5).

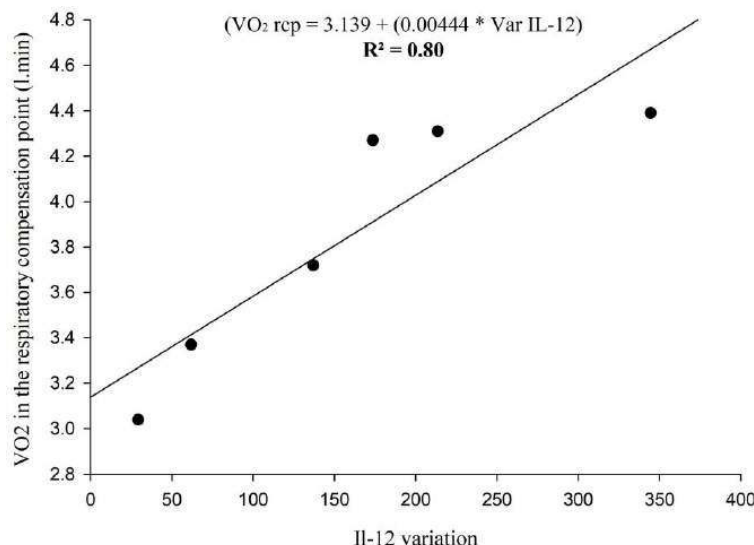


Figure 5. Linear regression as Supervised Machine Learning technique

Dicussion

Systolic and diastolic blood pressure measurements are often used to assess the risk of stroke. In contrast, the calculation of the double product (heart rate x systolic blood pressure) is applied as a cardiac workload marker and as an index of myocardial oxygen consumption (Schutte et al., 2013).

It has already been identified that the double product breakpoint coincides with the lactate and ventilatory threshold during physical exercise. Therefore, this is called the "breakpoint," when there is an abrupt increase, visually identified, in the value of the double product (Omiya et al., 2004).

Because the present study uses an incremental test on a treadmill as a protocol, a breakpoint was observed, both for the double product and for the heart rate, between minutes 2 and 3 of the test (Figure 2), both measurements being more acute than the anaerobic threshold (mean of 7:52 minutes) and the respiratory compensation point (mean of 11:08 minutes) and even the breakpoint for systolic blood pressure (between minutes 12 and 15 – Figure 1A), indicating DP as the most sensitive non-invasive marker for the type of protocol. Considering the pre and immediately post-exercise times, the mean percentage change in heart rate and the double product obtained the same value (213%). No significant variation was observed in diastolic blood pressure measurement (Figure 1B).

Interleukin 12 presented an average percentage variation of 160% between the pre- and post-exercise times (Supplementary Table 1), different from that observed in low-intensity running on a treadmill, which may not present alteration in its concentration, or even a slight reduction (Cox et al. 2007), the same occurs with the practice on a cycle ergometer (Hallberg et al., 2010), which seems to be dose-dependent, that is, to be related to the intensity or duration of the exercise (Patino et al., 2018).

It has already been observed that physical exercise modulates the maturation and development of dendritic cells (Chiang et al., 2007), which may be one of the mechanisms involved in changes in the plasmatic concentration of IL-12. Aiming to deepen the observation of the increase in the concentration of IL-12, the correlation of this alteration with other study data was sought from the Spearman test. Its findings were plotted in figure 3 through a heat map, where it was detected a correlation coefficient of 1.0 ($P=0.00278$) between IL-12 variation and VO_2 at the respiratory compensation point, in addition to a correlation of 0.943 ($P=0.0167$) between pre-exercise and maximum effort time, distance and test time. The post-exercise IL-12 correlated 0.9 ($P=0.0167$) with the heart rate at the respiratory compensation point. All these findings suggest that the production of IL-12 during physical exercise is dose-dependent. It depends on the intensity and duration; moreover, this cytokine may be linked to performance modulation in athletes. Aiming to estimate models that, although simplifications of reality present adequate adherence between absolute and estimated values, different researchers can choose different statistical models from the same database (Silberzahn & Uhlmann, 2015).

Machine learning techniques and the new concept in metabolic studies suggested by sportomics may dictate the future pace of research in immunometabolism (Bassini et al., 2014; Nahon et al., 2023; Gomes et al., 2023; Galvão et al., 2023; Gonçalves et al., 2023; Xie et al., 2023; Asadi et al., 2023; Bongiovanni et al., 2019; Pintus et al., 2021; Bragazzi et al., 2020; Bassini et al., 2022; França et al., 2023; Inbar et al., 2022).

Conclusions

Sportomics studies show changes in immunometabolism obtained in real situations of different sports. The present sports study, using an exploratory machine learning technique, showed that in running tests on a treadmill with incremental load, the heart rate and the double product breakpoint are sensitive and acute markers of stress level. Although the production of IL-12 during exercise depends on the intensity and duration of the exercise, it may correlate with biomarkers of renal and hepatic function, in addition to the performance of athletes, bringing to light possible immunomodulation of the performance-metabolism triad -Immune system.

Conflicts of interest - The authors declare that there is no potential conflict of interest

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Appendix

Supplementary table 1. Exercise-induced changes in key biomarkers – mean (SE).

	PRE	POST	Δ %
Hematocrit (%)	42 (1.0)	44 (1.1)	4%
Urea (mg/dL)	32.8 (2.4)	34.0 (2.3)	2%
Creatinine (mg/dL)	1.0 (0.1)	1.2 (0.2)	16%
Gamma GT (U/L)	23.4 (2.7)	25.5 (2.7)	16%
TGO (U/L)	37.8 (3.3)	44.1 (4.6)	16%
TGP (U/L)	36.9 (4.6)	39.1 (4.8)	6%
IL-12 (pg/ml) *	3.4 (0.5)	7.8 (0.5)	160%

*Difference.