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Original research

Metabolomics reveals that fittest trail runners show a better adaptation of bioenergetic pathways

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ABSTRACT

Objectives: To analyze the effect in the blood metabolome of trail running, a demanding sport that takes place in the natural environment, places considerable strain on both muscles and joints. While metabolic responses to aerobic exercise have been analyzed in-depth, few studies have focused on trail running.
Design: Observational study to analyze changes in 35 different metabolites - representative of aerobic exercise-induced by a simulated 21-km trail race with an uphill gradient of 1400 m.
Methods: We performed a semiquantitative metabolomics study consisting of capillary blood microsampling and targeted screening with liquid chromatography and mass spectrometry to analyze, in 33 licensed athletes, changes concerning 35 metabolites.
Results: We observed significant changes in many metabolites, including increased acetyl-carnitine and taurine concentrations (false discovery rate–corrected paired *t*-test P value 1.63×10^{-13}, and P value 5.021×10^{-12}, respectively) and decreased carnitine and proline concentrations (P value 6.33×10^{-10}, and P value 1.21×10^{-9}, respectively). Metabolic responses to trail running were largely independent of sex but were influenced by the level of training, with runners with a higher level showing resistance to exercise-induced changes in taurine, 1-methyl histidine, acetyl-carnitine, and hypoxanthine concentrations. Performance (measured as race time) was inversely correlated with changes in specific metabolites (including taurine, serotonin, and hypoxanthine) and directly correlated with increases in glutathione.
Conclusions: Our findings demonstrate the usefulness of metabolomics studies for analyzing exercise-induced physiological changes and show individual differences associated with the level of training and performance.

Practical implications

- Running in the natural media, trail running, induces profound changes in metabolism.
- These changes are measurable by microsampling of blood before and after the run.
- Despite training *and* performance significantly influencing these changes, they are individually determined, and their knowledge may be helpful for individualized training.

1. Introduction

Trail running is a sport that takes place on unpaved surfaces such as dirt tracks and forest paths, often in mountainous terrain. In race situations, paved sections must not exceed 15%.[1] Trail running is a high-intensity, long-duration activity that places considerable physiological, muscular, and biomechanical strain on the body. This practice is characterized by high aerobic and anaerobic demands over a prolonged time in which performance is influenced by the availability of sufficient metabolic fuel substrates to meet high energy expenditure requirements. Numerous studies have analyzed the physiological and energy requirements to determine which factors affect performance, including the mean aerobic capacity (and oxygen uptake [VO_2]), running economy, heart morphology, blood lactate concentrations, adipose mass, and women's iron levels.[2]

System biology and derived tools, such as metabolomics, transcriptomics, and lipidomics, provide an objective means of investigating the molecular effects of exercise. This quantitative dimension includes

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evaluating the changes of specific biomolecules (mRNA, metabolites, lipids) in a transversal and individualized manner. This knowledge could help to link, comprehensively, the effects of physical activity in specific modules of the cell, tissue, and organ physiology. Metabolomics, the comprehensive study of low-molecular-weight intermediates (metabolites) involved in bodily processes, is an exciting approach, as the physiological state of cells influences metabolic reactions, tissues, organs, and organisms.[3] Specific families of metabolites, such as the lipid eicosanoids, enhancing or reducing inflammation, pain perception, cell growth regulation, blood pressure control, and tissue blood flow modulation,[4] are all involved in exercise and its physiological response.

In this sense, the metabolome study in sport sciences has disclosed tricarboxylic acid cycle (TCA), glycolysis, aminoacyl-tRNA biosynthesis, urea cycle, arginine biosynthesis, branch-chain amino acids, and estrogen metabolism are all linked to higher physical activity and fitness and less sedentary time.[5] Since the magnitude of changes depends on the individual, it has been postulated that the use of molecular markers (obtained by genomics, transcriptomics, and metabolomics), which are linked to the development and manifestation of physical qualities (speed, strength, endurance, agility, and flexibility), in sport sciences is expected to be appropriate.[6] This use includes selecting a sport specialization, optimizing the training process, and determining an athlete's current functional state (overtraining).[7]

The application of metabolomics to assess the biological effects of given interventions, for example, could contribute to a greater understanding of metabolic responses and lead to evidence-based recommendations and strategies for high-performing athletes, complementing other approaches, such as observational studies.[8] Nonetheless, trail running has not been characterized by metabolomics yet. This study aimed to perform a metabolomics analysis to study trail running effects on circulating aerobic exercise metabolites and investigate noticeable changes in individual runners.

2. Methods

2.1. Subjects and trail run design

We recruited 33 runners (28 men and five women with a mean ± SD age of 35.02 ± 8.59 years, Supplemental Table 1) with varying performance levels, training, and competition goals. The study was conducted under the principles of the Declaration of Helsinki and was approved by the ethics committee at Hospital Arnau de Vilanova in Lleida, Spain (registration number 1665).

The study trial consisted of a simulated trail running race, held on March 21, 2017. The route was duly signposted as it had been used two days earlier for the Trail Montroig Extreme race held in Sant Llorenç de Montgai (https://www.wikiloc.com/trail-running-trails/trail-montroig-extrem-2017-no-name-15423919). The route covered 21 km and had a cumulative uphill gradient of 1400 m (Supplemental Fig. 1). A note was made of training type and volume for each participant by interview. This information was used to split the groups into a low-level training group (<6 h a week), a moderate-level training group (6–10 h a week), and a high-level training group (>10 h a week). Each participant's performance (race time) was recorded (Supplemental Table 1).

2.2. Sample preparation

Capillary blood samples were obtained by digital punctures on participants' hands just before and after the end of the race (with a maximum time lapse of 10 min between the end of the race and the sampling) in volumetric absorptive microsampling devices[9] (10 microliter size, Mitra® Clamshell, Neoteryx), according to manufacturer instructions. The blood was left to dry in the open air until the next day and then stored frozen at −80 °C. The metabolites were extracted from the devices employing a mixture of water:acetonitrile (1:3, v:v), containing ^{13}C-labeled phenylalanine as internal standard, for 1 h, keeping the samples at 4 °C. The extract was then centrifuged (13,000 rpm, 15 min at room temperature), and the supernatant was stored at −20 °C to allow any proteins to precipitate. The next day it was thawed and centrifuged again, and the supernatant was used for analysis using liquid chromatography coupled to mass spectrometry (LC-MS). These conditions were chosen after assaying the recovery of known amounts of the standards (Supplemental dataset 1) of microsampling devices after several storage conditions (comprising the combination of 3 different times: 24 h, 48 h, and 72 h; with 5 different temperatures: −80 °C, −20 °C, 4 °C, 25 °C, and 37 °C), as well as solvents for extraction (water, methanol, acetonitrile, and mixtures).

2.3. LC-MS conditions

For analysis, we have developed a method, detailed in Supplemental materials, using a targeted approach based on liquid chromatography coupled to mass spectrometry to detect and quantify a metabolomic panel of 35 metabolites belonging to the amino acid, purine, and nitrogen metabolism found in human blood (see Supplemental Table 2). Samples were decoded and randomized before injection.

2.4. Statistics

All analytical and statistical procedures were performed in a blinded fashion (i.e., not knowing to which training level or moment of sampling). After unblinding, the peak areas were log-transformed and auto-scaled in the Metaboanalyst Platform[10,11] which was employed also to perform principal component analyses (PCA), hierarchical clustering, receiver operating characteristic (ROC) curve calculation, partial least squares discriminant analyses (PLS-DA), and paired *t*-tests, according to published guidelines.[10,11] We also evaluated the effect of sex and timing of sample extraction (before vs. after running) in the levels of each metabolite by two-way repeated-measures analysis of variance, performed in IBM SPSS v 25 (Armonk, NY, USA).

As a web resource, all metabolite concentrations and their correlations with sample timing, performance and training are presented in the Supplementary data (https://metatrail.herokuapp.com/).

3. Results

According to the use of authentic standards, the conditions employed for maximizing most metabolite recoveries involved drying after field sampling at ambient temperature for 24 h. After that, the devices were left at −80 °C for long-term storage. A mixture of water:acetonitrile (1:3, vol:vol) was chosen to extract metabolites of intermediate polarity without extracting most apolar (lipid) molecules. A hydrophilic interaction liquid chromatography system was chosen for this reason, allowing to resolve the separation of metabolites of high polarity in the middle of the chromatographic run (see retention times in Supplemental Table 2). The use of this chromatographic mode prevents ion suppression effects that may be present employing reverse phase systems, where most polar compounds elute in the chromatographic front. Since we did not apply labeled internal standards for each metabolite, we offer the peak area as representative for semiquantitative analyses of blood concentrations of the metabolites.

The trail race significantly affected the blood concentrations of the metabolites analyzed (Supplemental Table 3, Supplemental Fig. 2). Paired analyses showed significant changes in blood concentrations for 21 of the 35 metabolites analyzed, even after stringent false discovery rate correction. Acetyl-carnitine, taurine, and kynurenic acid all had ROC values of higher than 0.9 (Fig. 1A), indicating their potential value as biomarkers for use in trail runners.

Multivariate analyses clearly distinguished between pre- and post-race samples (Fig. 1B). A two-component model explained more than 42% of the variance in the PCA. Using these data in a prediction model based on discriminant analyses (PLS-DA) (Fig. 1C), we could achieve

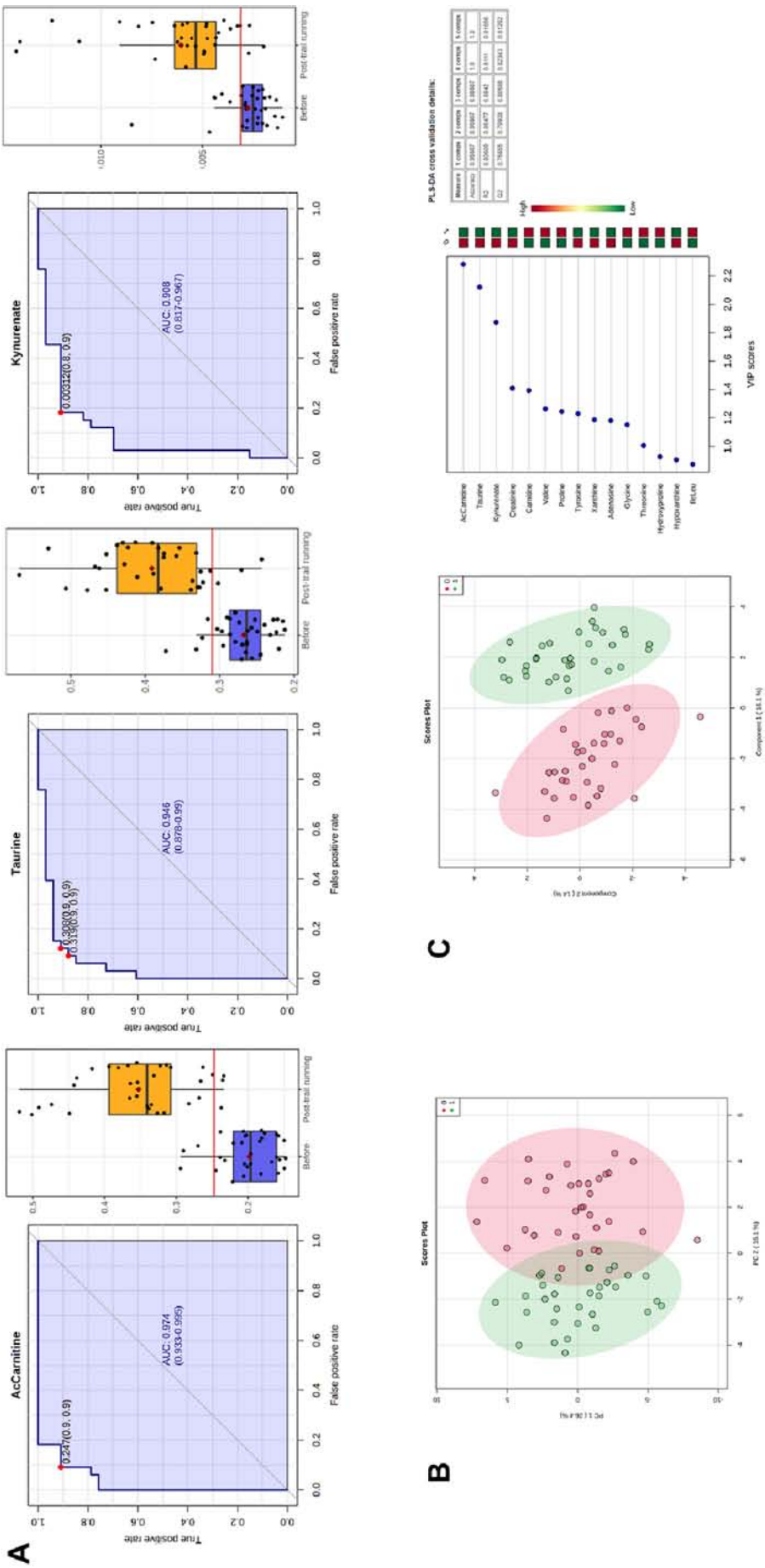

Fig. 1. Trail running-induced metabolic changes. The receiver operating characteristic curves show (A) increased whole blood concentrations of acetyl-carnitine, taurine, and kynurenic acid, indicating their potential as biomarkers of trail running performance. Both principal component analysis (B) and partial least squares discriminant analysis (PLS-DA) (C) showed apparent differences in pre- and post-race concentrations in the multivariate analyses. The importance of these metabolites is supported by the variable importance in projection (VIP) scores (C, middle panel). The results of the PLS-DA model are shown in the table in figure C, right panel. Figures B and C, red (or 0) and green (or 1), show results for pre-and post-race concentrations, respectively.

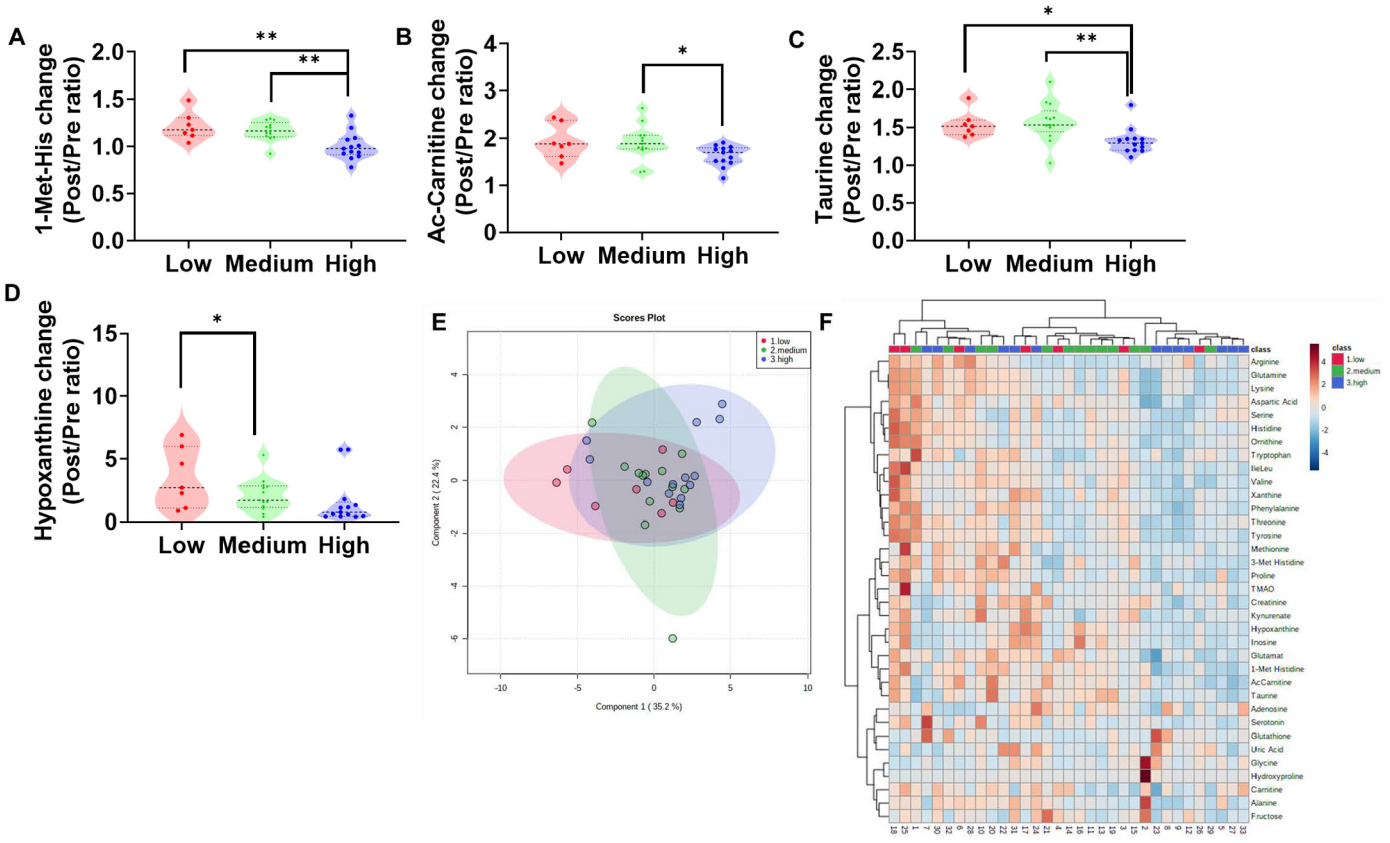

Fig. 2. Associations between training level and metabolic responses to trail running. Changes in blood concentrations of selected metabolites were influenced by training level. Individuals who trained more showed smaller increases in blood 1-methylhistidine (A), acetylcarnitine (B), taurine (C), and hypoxanthine (D) concentrations, as shown by the violin plots. Neither multivariate analyses by partial least squares discriminant analysis (E) nor hierarchical clustering (F) revealed a metabolomics profile for the metabolites analyzed. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively (uncorrected Fisher's least significant differences posthoc analyses after analysis of variance).

very high accuracy (96%) in revealing whether a sample belonged to pre- or to post-race. Cross-validation of this two-component model offered an R2 value of 0.79 (substantial predictive value) and Q2 value of 0.86 (almost optimal fitting of data with the model).[12] Overfitting of the model was ruled out by permutation testing. The test evaluates whether the specific classification of the samples in the two designed groups is significantly better than any other random classification in two arbitrary groups (separation distance statistic $p < 0.01$). The metabolites contributing to the model's discriminative ability were acetyl-carnitine, taurine, kynurenic acid, creatinine, and carnitine, all with VIP (variable importance in prediction) scores of higher than 1.2 (Fig. 1C). The race induced significant decreases in the concentrations of the branched-chain amino acids (BCAAs) leucine and isoleucine, and several other amino acids, including glutamine and alanine. Hierarchical clustering did not distinguish between pre- and post-race samples (Supplementary Fig. 3), highlighting the importance of studying individual responses.

Sex did not significantly influence metabolic responses induced by trail running, as significant differences were observed for just nine metabolites in the sex-stratified analyses (Supplemental Table 4). Notably, none of the metabolite concentrations was influenced by the interaction between sex and metabolic response. We recognize as a limitation the low number of women evaluated here.

On comparing responses according to training level (low, moderate, high), quantified as the number of hours spent training a week, four metabolites—taurine, 1-methylhistidine, acetyl-carnitine, and hypoxanthine—had a p-value below or near 0.05 (Fig. 2). In most cases, minor post-race increases were observed in the top tertile (high-level training). Despite some clustering of samples from the low- versus high-level training group in the multivariate analyses, the distinction was not perfect (Fig. 2).

In analyzing correlations between metabolite concentrations and performance, measured as race time, none of the pre-race concentrations were correlated with performance (data not shown). Significant correlations were, however, observed between performance and post-race concentrations (Fig. 3, Supplemental Table 5) for seven metabolites: kynurenic acid, serotonin, tyrosine, hypoxanthine, adenosine, and taurine (positive correlation), and glutathione (negative correlation) ($p < 0.06$, Spearman rank correlation). Significant correlations were observed between pre- and post-race ratios for taurine, serotonin, and hypoxanthine (Supplemental Table 6, Fig. 3), with increased concentrations correlating with performance.

4. Discussion

The method employed here, volumetric absorptive microsampling, has been extensively evaluated for the obtention of dried blood specimens at a fixed volume for application to many bioanalytical purposes.[13] We propose its usefulness in the on-field collection of blood samples in situations such as the trail-running where access to specialized personnel or storage equipment might be limited. This technique has been recently proposed for quick and easy blood collection without the need for dedicated personnel or dedicated spaces.[14] The present metabolomics analysis examined the effects of trail running on critical metabolic pathways involved in physical activity (energy, amino acid, and redox metabolism), mainly comprising metabolites of aerobic exercise.

4.1. Energy metabolism

Analysis of metabolites involved in energy obtention showed increased acetyl-carnitine and decreased carnitine concentrations after

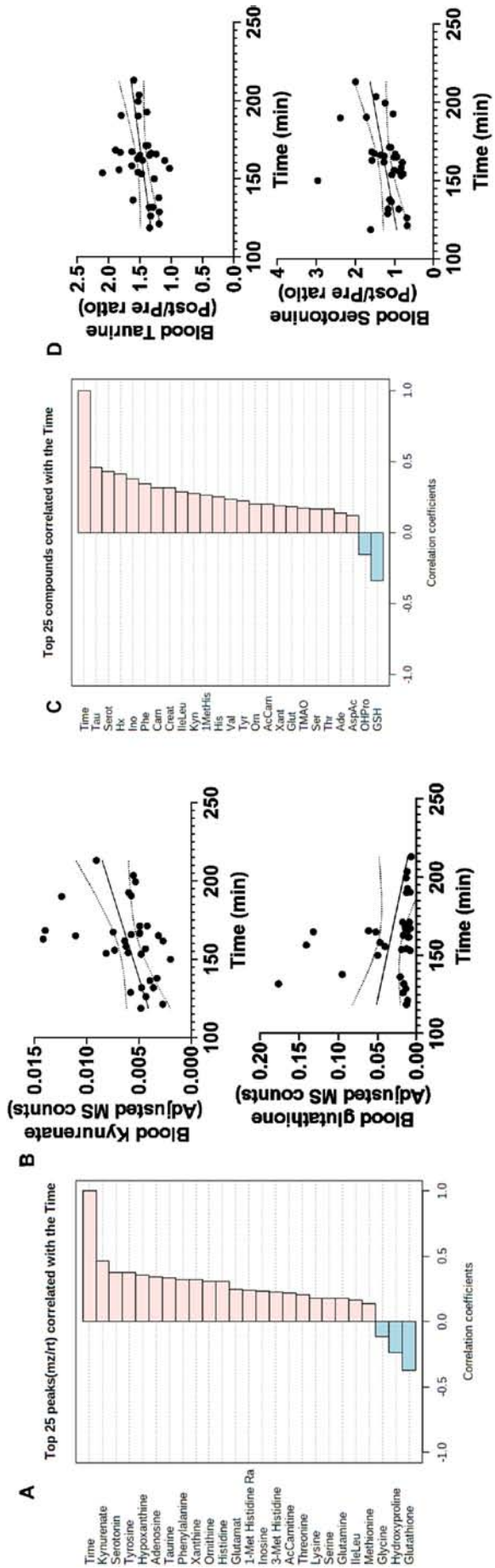

Fig. 3. Associations between trail race performance and metabolic responses. Spearman's Rho correlation analyses show correlations between race performance (race time) and post-race metabolite concentrations (A) and pre- to post-race metabolite ratios (B). As examples, B and D show individual correlations for post-race concentrations of kynurenic acid (B, upper panel) and glutathione (lower panel). Similarly, changes in taurine (D, upper panel) and serotonin (D, lower panel) were correlated with race time. The lines in B and D indicate linear regression, with discontinuous lines showing 95% confidence intervals.

the race, suggesting the involvement of accelerated lipid metabolism to produce carnitine as a source of energy. Numerous studies have reported reductions in carnitine concentrations after endurance exercise.[15]

4.2. Amino acid metabolism

Extreme physical activity results in diminished blood amino acid concentrations.[16] Also, decreases in blood BCAA levels may contribute to fatigue during prolonged exercise.[17] Elevated post-race hypoxanthine, xanthine, and uric acid concentrations were also observed in our series, supporting reports that increases in these metabolites might be due to protein degradation.[18]

At the end of an exercise session, plasma taurine concentrations reach a peak, interpreted as a response to osmolar regulation and lipid oxidation in skeletal muscle fibers.[19] The most likely explanation for increased plasma taurine concentration after exercise is increased taurine efflux from skeletal muscle via Na^{++}-mediated membrane depolarization and incorporation of taurine carriers to the plasma membrane, as the taurine concentration is two orders of magnitude lower in the plasma (20–100 μM) than in the tissue.[20] In comparing plasma taurine concentrations in trained athletes after different types of endurance exercise, increases were associated with both exercise duration and intensity, suggesting that blood taurine may be derived from skeletal muscle, positioning it as a possible biomarker of muscle damage and lipid oxidation.[21] In the present study, post-race changes in taurine and acetyl-carnitine concentrations were closely correlated in our study (data not shown), suggesting a close relationship between lipid oxidation, oxidative stress, and muscle damage. Our data showing that fittest individuals had lower increases in taurine, and the fact that taurine increases correlated inversely with performance (based on the run time) agree with its role as a biomarker of skeletal muscle stress (metabolic, osmotic, redox, Ca^{++}).

Tryptophan is another amino acid that deserves special mention, via its metabolism through the kynurenine pathway. Inflammatory cytokines and cortisol increase the conversion of tryptophan to kynurenine via indoleamine 2,3-dioxygenase and tryptophan dioxygenase, respectively. Kynurenine is subsequently metabolized via kynurenine aminotransferase (KAT) to produce kynurenic acid, a neuroprotective agent.[22] Our results, showing increased kynurenate values after trail running, align with recently published data showing increased kynurenine, kynurenic acid, cortisol, and cytokine levels after acute endurance exercise.[23] Athletes who engage in regular endurance training have been found to have increased levels of KAT mRNA in skeletal muscle, meaning that, like the athletes in our series, they can produce higher levels of kynurenic acid, which could enhance adipose tissue energy expenditure, aspartate biosynthesis, and mitochondrial respiration.[24] These changes would be consistent with the increase in mitochondrial fatty acid oxidation discussed above.

Like alanine and glutamine, proline is involved in the anaplerotic pathway of the TCA cycle. Our observation of decreased proline levels after exercise is consistent with this usage. Sufficient carbohydrate intake could prevent the metabolization of proline and alanine via the anaplerotic pathway, leading to higher blood concentrations.[25]

4.3. Redox metabolism

We found that post-race blood glutathione (GSH) levels were directly correlated with race performance, with higher concentrations observed in faster runners in our series. Depleted levels, however, were observed in other runners up to 1 month after participating in an ultra-endurance race.[26] Our observation of increased post-race GSH levels in faster runners indicates the potential value of GSH replenishment in runners with lower levels and highlights the usefulness of individual metabolomics-guided strategies. We also found decreased post-race serotonin blood concentrations in our series. Serotonin is produced mainly in the digestive system and is stored in circulating platelets (reviewed in 27). Its metabolism by skeletal muscle, which leads to kynurenic acid via increased KAT expression, may contribute to reductions in depressive mood states linked to endurance exercise.[28] Our observation of increased post-race serotonin levels supports the idea that serotonin could be used as a circulating biomarker of hypoxia. In particular, we found smaller increases in individuals who performed better. Reflecting the complexity of the causes and consequences of changes in just a single metabolite, increases in serotonin levels appear detrimental (hypoxia), whereas decreases, coupled with kynurenic acid production, appear beneficial. Changes in kynurenic acid levels, however, were not correlated with changes in serotonin levels in our series, highlighting the complex relationships between metabolites (data not shown).

Our study has some limitations, including our small sample size, which may have prevented us from detecting significant results for certain metabolites and determining the specific influence of sex on metabolite concentrations. Further, the use of semiquantitative analyses might have obscured quantitative relationships between metabolites. However, the study's goal was to obtain individualized, paired analyses of the effect of trail-running on the circulatory levels of selected metabolites. The changes in these metabolites are not specific for trail running, as they represent mostly aerobic exercise. In addition, by collecting whole blood samples, we could not analyze metabolite concentrations detected in cellular versus noncellular components (e.g., platelets or red blood cells versus plasma). Among the metabolites assessed here, some (glutathione, glutamate, aspartate, and acetyl-carnitine) are enriched in red blood cells.[29] Indeed, whole blood samples collected employing a similar device have been employed recently in the study of running.[30] As a result, metabolomics of human blood can provide detailed information on metabolic mechanisms underlying physiological responses, health, aging, and disease, as well as the biological effects of drugs, nutrients, and environmental cues, including sport practice.

5. Conclusions

In this paper, we describe a new methodology for analyzing exercise-associated metabolic physiology, using new tools such as microsampling and metabolomics. Used together, both allow us to have a global picture of the metabolic changes in athletes in a high-performance test such as trail running, but which could be extrapolated to other races and physical activities. Metabolic responses to trail running were strongly influenced by the level of training, with runners with a higher level of training showing resistance to exercise-induced changes in taurine, 1-methyl histidine, acetyl-carnitine, and hypoxanthine concentrations. Performance (measured as race time) was also inversely correlated with changes in specific metabolites (including taurine, serotonin, and hypoxanthine) and directly correlated with increases in glutathione. In all athletes, trail run increased blood acetyl-carnitine and taurine concentrations and decreased carnitine and proline concentrations. Globally, metabolomic blood analyses in trail running could improve our understanding of physiological responses to this increasingly popular endurance sport.

Extended information on methods and discussion, as well as Supplementary figures, tables, and datasets are presented in electronic form. Full details of metabolite concentrations and correlations are freely available at https://metatrail.herokuapp.com/. Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsams.2021.12.006.

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Declaration of interest statement

The authors declare that they have no conflict of interest.

Confirmation of ethical compliance

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the ethics committee at Hospital Arnau de Vilanova in Lleida, Spain (registration number 1665).

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