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Longitudinal NMR-based Metabolomics Analysis of Male Mountain Ultramarathon Runners: New Perspectives for Athletes Monitoring and Injury Prevention

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Abstract

Background The aim of this study was to explore how a metabolomic approach could provide valuable information on changes in the athletes' metabolome during a mountain ultramarathon race. To achieve this goal, we established a longitudinal cohort of athletes enrolled in the TOR des Géants, a 330 km mountain ultramarathon with 24,000 m of elevation gain. Sixteen healthy male athletes (43.9 ± 10.1 years) were recruited, and blood samples were collected at four time points: pre-race, mid-race, post-race and after 72 h recovery. Using a 1H-NMR-based metabolomic approach, we evaluated metabolic changes that occur during both race effort and recovery, and correlated them with functional muscle, cardiac, inflammatory, and renal biomarkers already used in the clinic. The processed data were analyzed using multivariate analysis tools specific to longitudinal study design, and innovative pathway analysis was used for data interpretation.

Results Mountain ultramarathon running significantly affected the metabolism and physiology of athletes. Multivariate analysis highlighted specific metabolites and functional biomarkers associated with prolonged exercise. Neither metabolite levels nor biomarker concentrations returned to baseline after 3 days of recovery. Finally, innovative pathway analysis shed light on specific metabolic changes resulting from mountain ultramarathon exercise.

Conclusion In this study, we propose an NMR-based metabolomics strategy to assess exercise-associated metabolic changes during and after events such as the Tor des Géants. Using state-of-the-art data representation methods specific to metabolomics analysis, we demonstrated that such a methodology can provide a unique view of the biology associated with such extreme conditions. As this approach provides unique insights into the biology of extreme exercise, it holds promise for the development of new tools for athlete management.

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Key Points

- Mountain ultramarathon running induces significant metabolic changes, with altered levels of key metabolites and clinical biomarkers that do not fully return to baseline after 72 h of recovery.
- NMR-based metabolomics revealed shifts toward ketosis and amino acid degradation during the race, highlighting the importance of lipid metabolism for energy production under extreme conditions
- This approach provides valuable insights into athlete management and injury prevention by offering personalized monitoring of metabolic responses during and after prolonged endurance events.

Keywords Exercise metabolism, Injury prevention, NMR metabolomics, Personalized monitoring, Ultramarathon

Background

Mountain ultramarathons are defined as single or multi-day running events over distances greater than a marathon (42.195 km), held in natural settings (i.e., ≤ 25% paved or asphalted roads), and held in extremely challenging mountainous environments with large elevation gains (e.g., an average of 10–15%) [1]. Over the past few decades, these events have become increasingly popular, especially among women and master athletes (athletes > 35 years of age) [2]. As ultra-trail races can exceed 200 km with more than 20,000 m of positive elevation gain, they can lead to significant physical challenges for participants. In fact, these events require specific and intensive training, averaging 66–83 km/week [3], and may induce excessive stress in athletes, affecting their health status. Several studies in the literature have demonstrated the impact of such a level of physical activity on bone damage [4], musculoskeletal [5], cardiac [6] and acute renal injuries, as well as ionic imbalance problems [7, 8]. Although these changes usually normalize after the race depending on the intensity, duration, and management of the effort [9], their accurate monitoring remains important. Considering the growing popularity of these events in recent years, the significant physiological stress on participants and the self-management required during the race, there is a need to develop innovative follow-up strategies to better assess the impact of mountain ultramarathon running on runners' physiological, biological and health responses.

Metabolomics can characterize and quantify a large number of small molecules present in a biological sample at a precise time point [10] and therefore represents a potentially comprehensive approach to detect metabolic changes related to dietary, pathological, environmental, and lifestyle factors [11]. Due to its non-destructive, highly reproducible and quantitative aspect, proton nuclear magnetic resonance (1H-NMR) spectroscopy analysis of biological samples represents an interesting solution to establish the metabolite profile of patients and follow its variation over time [12, 13]. By detecting the electromagnetic signals emitted by hydrogen atoms on molecules placed in a strong electromagnetic field, 1H-NMR allows the detection and quantification of hundreds of compounds in a biological matrix. Each sample is then described by a large number of metabolite concentration values, representing its metabolic status, rather than by a few carefully selected features. As metabolomics aims to study changes between and/or among cohorts of individuals, it deals with a significant amount of complex data. The complexity of the datasets generated arises from their correlated nature rather than from their size per se. Indeed, as metabolites are modulated by complex biological pathways, the identification of significant changes under specific conditions is a challenging task that requires special data representation methods. Thus, this methodology, in combination with state-of-the-art "big data" analytic tools [14] built to decipher the complex architecture of the generated datasets, leads to innovative representations of an individual's health status [15].

The application of metabolomics in sport has recently been termed "sportomics", and this field has gained interest during the last decade [16]. Indeed, exercise-induced changes in metabolism have already been studied in runners [17], snowboarders, skiers, cyclists, soccer [18], basketball or rugby players [19]. In short, most of these studies aimed to investigate changes in the metabolic profile of athletes during exercise and to assess their training content [18], training intensity [20], performance level [21] or to compare their profiles with those of healthy but sedentary counterparts [22]. Considering the demanding and challenging nature of ultramarathons and the new population involved in these events, the use of metabolomics may improve our understanding of the systemic biological responses to mountain ultramarathon running and help in the development of new tools for athlete monitoring.

In the present study, 1H-NMR-based metabolomics is used to assess metabolic changes that occur during the mountain ultramarathon race and the subsequent

recovery period. We aim to correlate these changes with functional muscle, cardiac, inflammatory, and renal biomarkers already used in routine clinical practice. By using our methodology, we may better understand the metabolic changes experienced by athletes during the mountain ultramarathon race and provide new tools for athlete monitoring and health management.

Methods

Participants and Study Design

As described in our previous work [23], the subjects were registered runners (n = 27) participating in the "Tor des Géants", a 330 km race with significant elevation changes (+24,000 m) held in the Aosta Valley (Italy). All subjects were recruited by the race organizers through e-mail and public announcements with no prior selection or inclusion criteria. Informed written consent was obtained from each participant. The experimental design of the study was approved by the local ethics committee of the Azienda Regionale Sanitaria USL della Valle d'Aosta (n◦900-18/08/2014) and was performed in accordance with the standards of ethics outlined in the Declaration of Helsinki. Food consumption was not monitored during the race or recovery period. However, participants only had access to food provided by the organizer. These included pasta, rice, and cereal bars for carbohydrate intake; meat, dried meat, and cheese for protein; as well as energy gels, energy bars, dried fruits, fruit bars, applesauce, and candies as supplements and snacks. Common exclusion criteria were used, including smoking, substance abuse, regular medication use, medical or psychiatric diseases, or abnormalities detected during laboratory screening. The best finishing time of 71 h 49 min was achieved in the 2014 race (7–14 September), which featured 740 starters and 446 (60%) finishers [24]. The weather conditions were mostly sunny but windy over the 7 days of the race. In the Aosta valley, temperatures were between 12 and 26 °C at low altitudes and between 0 and 12 °C during nights at the highest altitudes. Wind velocity ranged between 5 and 20 km/h.

The final samples set of the present study includes 16 healthy male athletes (43.9 ± 10.1 y/o), with matching time-points from the initial 27 subjects. Our longitudinal study design includes four time points for blood sampling (Fig. 1): 4 days prior the race (T1); at mid-race (T2, 148.7 km, + 9,270 m elevation gain); at the end of the race (T3, 330 km, + 24,000 m); after 72 h of recovery (T4).

Samples Collection and Post-analytic Treatments

Plasma samples were collected at each time point from an antecubital vein into an EDTA (ethylenediaminetetraacetic acid) tube and immediately centrifuged for 10 min at 4 °C at 1507 RCF. Plasma samples were snap frozen and stored at −80 °C prior to 1H-NMR analysis. Blood biomarkers related to cardiac, inflammatory and renal function were collected and analysed as previously described [23]. These parameters include the measurement of N-terminal pro-hormone of natriuretic peptide (NT-proBNP) linked to myocyte-stress; Galectin-3 (Gal-3) and Suppression of Tumorigenicity 2 protein (ST2) linked to cardiac remodeling and fibrosis; heart fatty acid binding protein (hFABP), copeptide, and high sensitive troponin T (hsTnT) representing cardiac ischemia-necrosis; creatine kinase (CK), Myoglobin (MYO), creatine kinase MB (CKMB), for muscle damages; C-reactive protein (CRP) and total protein related to inflammation; Myeloperoxydase linked to oxidative stress; and finally

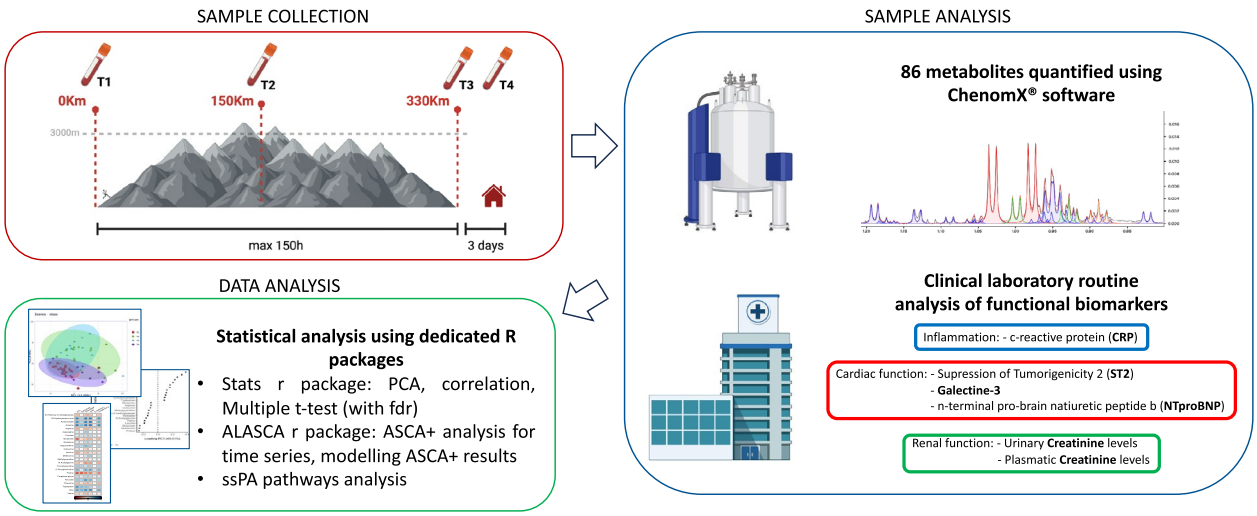

Fig. 1 Longitudinal study design for sample collection, analysis, and data analysis of mountain ultramarathon runners' samples

urinary and plasmatic creatine levels and urea associated with renal function.

Sample Preparation

Samples were prepared according to Chenomx® (Chenomx Inc., Edmonton, AB, Canada) standard operating protocols[25]. Briefly, protein content of plasma was removed upon ultra-filtration step (AMICON ultra 0.5 mL-3KDa filter tube, Merck-Millipore, Burlington, Massachusetts, USA) and 30 µL of Internal Standard solution (trimethylsylilyl-3-propionic acid-d4 5 mM and maleic acid 5 mM diluted in deuterated phosphate buffer at pH 7.4) was added to the 270 µL of filtrated sample. Samples are stored at 4 °C into the Bruker® Sample Jet (Bruker, Billerica, USA) prior ^{1}H-NMR analysis. Internal standards and deuterated buffers were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

^{1}H-NMR Analysis

Proton NMR analysis were conducted on a Bruker Avance HD spectrometer (Bruker, Billerica, USA) operating at 700.17 MHz for signal acquisition. The ^{1}H-NMR sequence used to generate raw data and the basic processing steps were conducted as previously described [26].

Statistics

The Processed spectra were imported into Chenomx profiler 10.0 (Chenomx Inc., Edmonton, AB, Canada) for metabolite quantification. Hence, using this software over 86 unique metabolites were identified and quantified, and the standard deviation of measured concentrations was assessed based on pooled quality control (QC) samples (See supplementary table S1). Prior to multivariate analysis, the data set was 10log transformed and normalized using the autoscaling algorithm available in MetaboAnalystR package (R version 4.1.2). Multivariate and univariate statistical analyses such as Principal Component Analysis (PCA), multiple t-test, fold change analysis, correlation analysis and one-way repeated ANOVA conducted either on normalized or raw data were performed using dedicated R packages (MetaboAnalystR package, Biostatflow web platform, Cor package). For univariate analysis, all p-values were adjusted using False Discovery Rate (FDR) correction, as implemented in Biostatflow wich relies on the Benjamini–Hochberg method, with a significance threshold set at 0.05. Repeated measure ANOVA Simultaneous Component Analysis (RM-ASCA+) analysis dedicated to longitudinal experimental design was performed and modelled using ALSCA R packages describe by Jarmund et al. [27]. Jackknife resampling validation method was used through 500 iterations to validate RM-ASCA+models and estimate the performance, i.e. the model associated with the smallest Confidence Intervals (CIs). Single sample pathway (ssPA) and kernel PCA (kPCA) analysis used to facilitate pathway-based interpretation were performed using ssPA 1.0.2 python package recently described by Wieder et al. [28]. Finally, descriptive statistics were performed using GraphPad Prism (version 8, GraphPad Software, San Diego, USA) and arsenal R packages.

Results

The characteristics of the participants are shown in Table 1. Therefore, apart from the possible metabolic adaptation that could result from more intensive training, no confounding factors can be attributed to our sample set.

During our experiment at the Tor des Géants, the weather conditions in the Aosta Valley were challenging, adding to the complexity of this already extreme endurance race. Throughout the week, runners faced a mix of weather conditions, including rain, fog, and cold temperatures, especially at night and at higher altitudes. Some participants reported being soaked and cold during certain parts of the race, particularly at night, when fog made visibility difficult. In the higher mountain passes, temperatures dropped significantly, and many runners had to seek shelter to warm up and dry off. During the day, conditions were more manageable, with cooler temperatures typical of early autumn in the Alps. However, rain continued to fall in some areas and runners had to deal with wet and muddy terrain, making the technical sections of the course even more challenging. The combination of steep climbs and variable weather, including

Table 1 Descriptive characteristics of the study participants

Overall (N = 16)	
Age (years)	
Mean (SD)	43.6 (9.8)
Range	26–56
BMI (kg/m2)	
Mean (SD)	22.8 (2.3)
Range	19.2–28.8
HR (at rest) (bpm)	
Mean (SD)	54.2 (10.7)
Range	40–80
Training (km per week)	
Mean (SD)	66.2 (28.2)
Range	15–112
Race time (min)	
Mean (SD)	7600 (870)
Range	5747–8714

Training load was evaluated on the last year prior event

occasional sunny spells, tested the resilience of the participants throughout the event. These varied conditions are common in mountain races such as the Tor des Géants, where altitude and exposure to the elements can cause rapid changes in the weather.

In this longitudinal study we quantified 86 circulating metabolites using NMR metabolomics to analyse changes in the metabolite profile throughout the race and recovery. Indeed, as shown in the PCA score plot (Fig. 2a), the information gathered with our metabolomics approach provides a clear description of metabolic changes during the race as shown by T2 (mid-race) and T3 (post-race) samples, which are clearly different from T1 (pre-race) and T4 (recovery) samples. The impact of such prolonged exercise is well illustrated by Principal Component 2 (PC2, y-axis of both score and loading plot Fig. 2a–b) which accounts for approximately 10% of the total variability of the dataset. This shows how such an extreme event affects the metabolism of runners and their subsequent recovery after 3 days of rest as T1 and T4 samples seem cluster together (small PC2 score). PCA loading plot (Fig. 2b) highlights the main metabolites used to represent the samples, mainly branched chain amino acids (BCAAs), other amino acids, metabolites from tricarboxylic acid cycle (TCA) metabolites, ketone bodies, creatinine, and urea.

Multiple t-test with FDR correction applied on *p*-values highlights several metabolites with significant variation between the four different time points (significance threshold set at 0.05). These metabolites (listed in the Table S3) include those previously highlighted in the non-discriminant PCA analysis and responsible for the group separation. Other metabolites such as acetone, glucose or pyruvate were found to be significatively impacted during the race, even though their effect were not well described by the PCA analysis (Fig. 3).

To facilitate the interpretation of longitudinal data, to provide a more accurate description of temporal changes and to better describe the effect of the race and recovery (time effect), respectively, we used the repeated measures ANOVA simultaneous component analysis + (RM-ASCA +)[29] methodology using the ALASCA r package. Using RM-ASCA + the effect of age, training load (km per week) and performance (time to complete the race in minutes) can be isolated by including these parameters as covariates. Nevertheless, no effect of these covariates was found have a significative effect on our dataset (data not shown). The main effect of time is represented by the component PC1 explaining 49.01% of the variability of the metabolomics dataset (Fig. 4a). Metabolites exhibiting positive loadings, as shown in Fig. 4b**,** demonstrated higher concentrations at time-points with elevated PC1 scores, such as T2 and T3. These metabolites include those previously reported by PCA and multiple t-test analysis. This analysis confirms their association with the prolonged exercise and gives a comprehensive picture

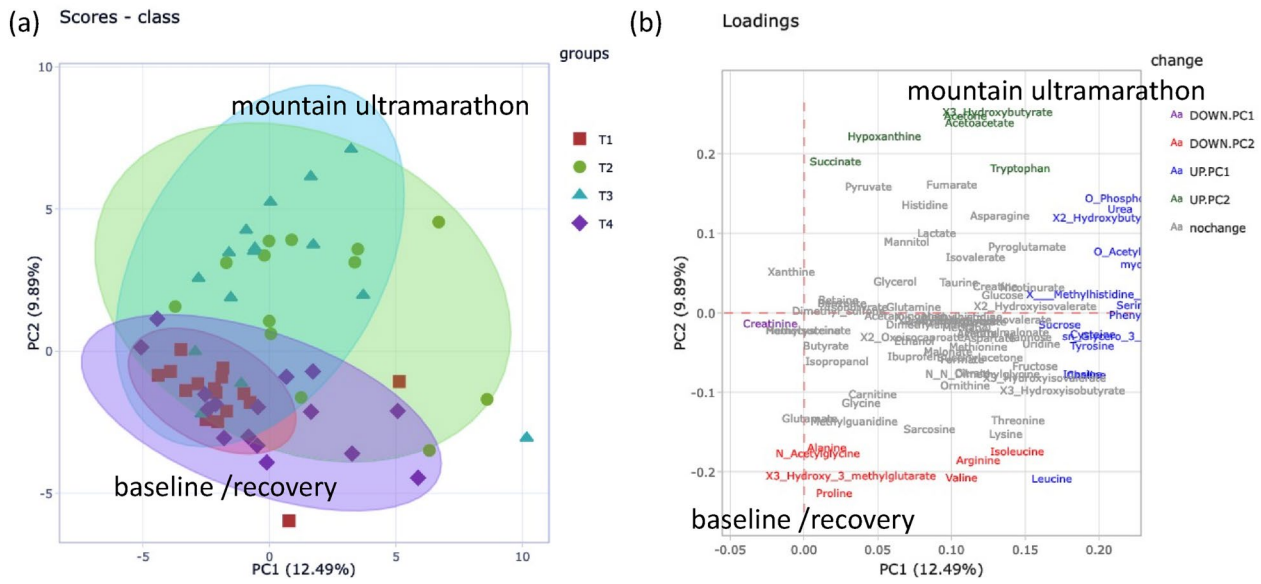

Fig. 2 PCA score, with 95% confidence ellipse drawn for each group (**a**) and loading (**b**) plot of NMR metabolomics data (T1 = pre-race; T2 = mid-race, 148.7 km; T3 = post-race, 330 km; and T4 = recovery, 72 h post-race). The effect of the ultramarathon is described by PC2 that accounts for 9.89% of the whole variation. Downregulated metabolites along PC1 and PC2 are colored in purple and red respectively, upregulated metabolites along PC1 and PC2 are colored in blue and green respectively, metabolites that didn't contribute to the variation are colored in grey (the complete list of association between PCs and metabolites is described in supplementary table S2)

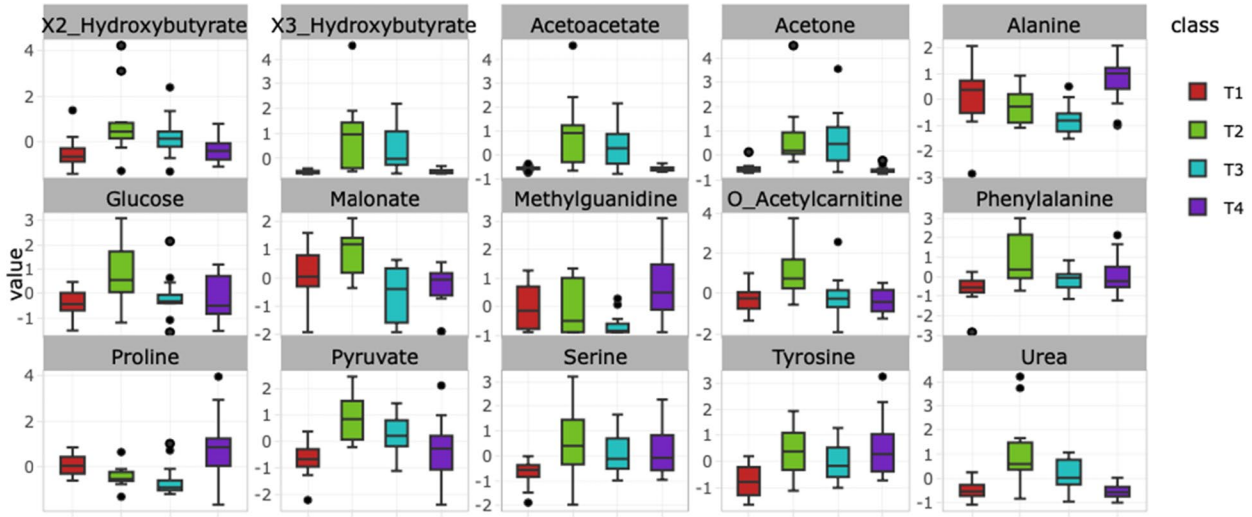

Fig. 3 Multiple t-test on NMR metabolomics data (signif: FDR adj. p-value < 0.05, all *p*-values are available in supplementary table S3; T1 = pre-race; T2 = mid-race, 148.7 km; T3 = post-race, 330 km; and T4 = recovery, 72 h post-race)

of its impact on the metabolism of ultramarathon runners. Furthermore, the lower PC1 score observed in T4 samples, when compared to T1, indicates that the ultramarathon continues to affect the runners' metabolism even after 3 days of recovery. The same methodology was applied to clinical biomarkers related to cardiac, renal function and inflammation revealing that time effect contributes to 99.52% of the total variability according to PC1. Cardiac, renal, and inflammatory biomarkers were found in higher concentration for time-points having positive PC1 score namely T2 and T3 (Fig. 4c and d). NT-proBNP is the biomarker that shows the strongest association with the exercise effect, as it has the highest PC1 score (Fig. 4d). MYO, CK, hsTnT, CRP, Urea, and other functional biomarkers were also found to be affected during the ultramarathon. Noteworthily, as T4 samples have higher PC1 score than T1 samples (Fig. 3d), indicating that the effects of the ultramarathon race are still present after 3 days of recovery. All the features highlighted during this analysis were found to be significantly altered during the race and recovery.

Correlation analyses were performed to analyze possible relationships between the metabolites associated with the time effect identified through RM-ASCA + analysis and functional cardiac, renal, and inflammatory biomarkers. Important correlations are highlighted as bright blue/red circles on the correlogram (Fig. 5). Linear correlation values range from 1 to −1 and interesting correlations are associated with values greater than 0.5 absolute values (see table S4). Matching results with multivariate longitudinal analysis can be extracted from this correlation plot, e.g., metabolites such as acetone positively associated with the effect of exercise are also positively correlated with functional cardiac, inflammatory, and renal biomarkers. Similarly, proline, which is negatively associated with the effect of exercise, is one of the metabolites negatively correlated with the clinical values affected by this extreme exercise (MYO, hsTnT, CK, ST2, Gal-3).

The Single Sample Pathway associated with kPCA analysis was used to highlight metabolic pathways impacted during both the race and the recovery. On the score plot (Fig. 6a), samples from T1 form a distinct cluster, while samples from T2 and T3 are located along negative PC2 values, and samples from T4 are spread along positive PC2 values. The clustering of T2 and T3 samples illustrates the metabolic changes induced by the mountain ultramarathon, while the recovery phase is characterized by the divergence of T4 samples from the T1 cluster. The generated biplot (Fig. 6b) shows the metabolic pathways associated with PC1 and PC2 and facilitate its interpretation. This plot shows that the mountain ultramarathon has a major impact on the synthesis and degradation of ketone bodies, lysine degradation, taurine and hypotaurine metabolism, Vitamin-B5 and CoA signaling pathways. Furthermore, even after 72 h of recovery, the metabolic profile continued to be influenced by the exercise as samples from the T4 group did not cluster with those from the T1 group, indicating a sustained effect on metabolism. In particular, there was a notable increase in the turnover of cysteine, methionine, and histidine metabolism compared to pre-race samples. The results

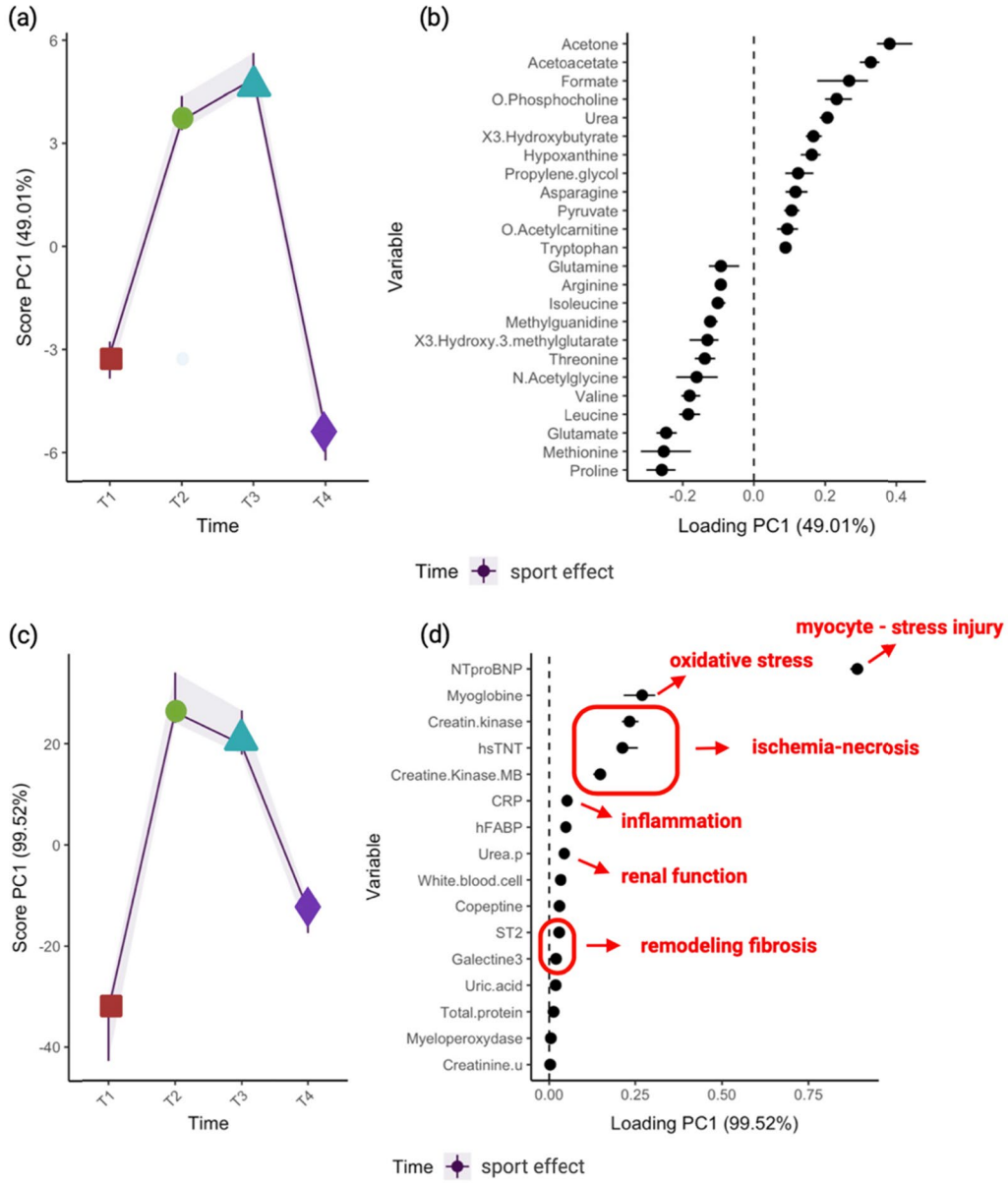

Fig. 4 Time development (T1 = pre-race; T2 = mid-race, 148.7 km; T3 = post-race, 330 km; and T4 = recovery, 72 h post-race) of the plasma metabolome through the race as score (**a**) and loading plot (**b**) from NMR metabolomic data; Time development (T1 = pre-race; T2 = mid-race, 148.7 km; T3 = post-race, 330 km; and T4 = recovery, 72 h post-race) of the clinical biomarkers through the race as score (**c**) and loading plot (**d**). Time-points or feature exhibiting an important and positive PC score have positive association with time effect. This effect is responsible of 49.01% and 99.52% of the variation of the metabolomics and clinical biomarkers datasets, respectively (n.b.: All plots and ASCA + analysis were performed through the use of ALASCA r packages, 95% CI represented in grey shadow were evaluated through 500 iteration validation step, see Methods section)

suggest that ultra-trail runners did not fully return to their baseline metabolic state after the 3-day recovery period. In addition, pathways associated with central carbon metabolism, glycine, serine, and threonine metabolism, and tyrosine and tryptophan metabolism, remained more activated in T1 samples.

Discussion

"TOR des Géants" is considered as one of the most challenging mountain marathons races in the world with a distance of 330 km and a considerable elevation change (+24,000 m). Over the past years, such races have become increasingly popular, attracting a new population

Fig. 5 Correlation plot of metabolites features highlighted by ALASCA analysis and clinical biomarkers. Interesting correlation appears as bright blue or red spot for positive or negative correlation respectively

of athletes beyond the usual ultra-distance runners. In previous studies [23] we analyzed clinical data from a longitudinal cohort of athletes who participated to this challenging event. Our findings highlighted the kinetics of cardiac remodeling and fibrosis biomarkers that arise from this extreme event [23] and its impact on muscles tissues [24] and brain water diffusivity [30].

In this study we demonstrated that mountain ultra-marathon exerts an important influence on the metabolism and physiology of the participants using ^{1}H-NMR metabolomics. Multivariate analysis dedicated to longitudinal dataset highlighted specific metabolites associated with the effects of such prolonged exercise. Moreover, biomarkers linked to myocytes-stress injury, cardiac remodeling-fibrosis, cardiac ischemia-necrosis, inflammation, and renal function are affected by exercise. Neither the metabolite levels nor the clinical biomarker concentrations returned to baseline levels after 3 days of recovery. Finally, innovative pathway analyses shed light on the specific metabolic changes induced by mountain ultramarathon running.

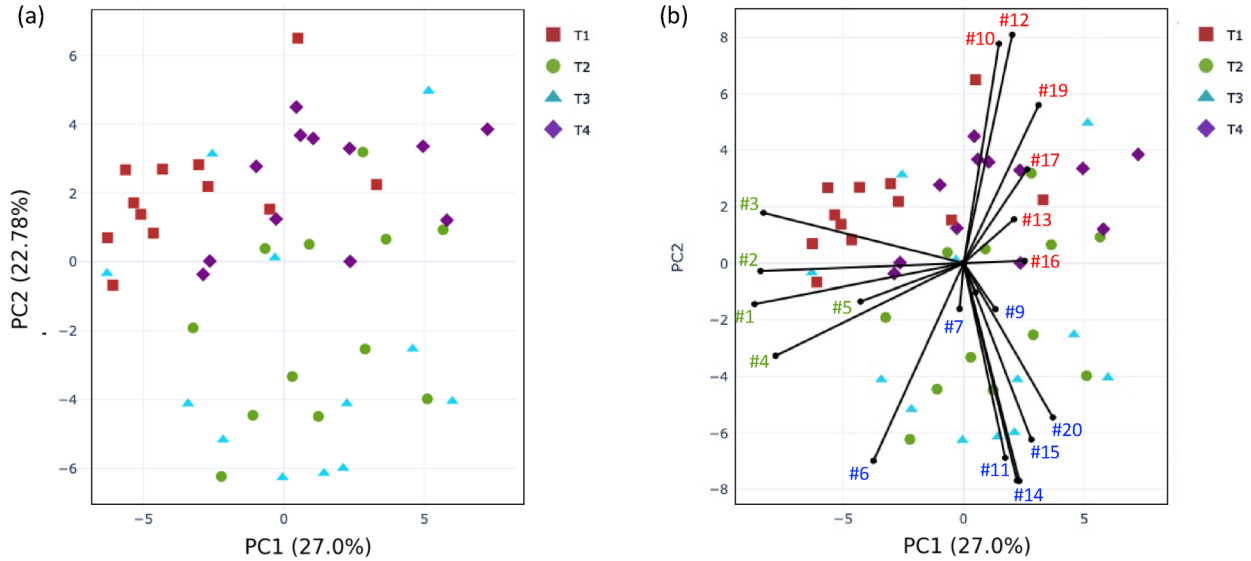

Fig. 6 ssPA score plot (**a**) and biplot (**b**) on NMR metabolomics data (T1 = pre-race; T2 = mid-race, 148.7 km; T3 = post-race, 330 km; and T4 = recovery, 72 h post-race). The sport effect is well described along PC2 axis and account for 22.78% of the total variation of the dataset while PC1 is responsible of the clustering of baseline samples and account for 27.0% of total variance. On the biplot are represented important variables contributing the most to PC1 and PC2 and association between PCs and biochemical pathways are listed in Table S5

Effects of Ultramarathon on Metabolomics Data and Clinical Biomarker

At mid-race (T2) and post-race (T3) acetone and acetoacetate are the metabolites that exhibit the largest increase. These compounds are involved in ketone bodies' metabolism and are produced when the body uses fat as its primary energy resource in a process called ketosis. In addition, increased levels of acetyl-carnitine also suggest an important turnover of lipid metabolism for energy production. Elevated levels of acetyl-carnitine have previously been reported in the serum of trained trail-runners [31]. This is known to facilitate the transport of fatty acids into the mitochondria to further contribute to the formation of acetyl-CoA, a central molecule in energy metabolism. In line with these observations, energy metabolism is affected during the race as metabolites related TCA-cycle, including formate and pyruvate, were increased at mid- and post-race in the plasma of athletes. This metabolic pathway is a key source of energy involving glucose from glycolysis and amino acids through their catabolism. BCAAs and several other amino acids such as proline, methionine, glutamate, glutamine, threonine, and arginine decreased during the race, demonstrating their involvement in energy and muscle metabolism. In fact, in addition to their use as an energy source, BCAAs such as leucine are important stimulators of muscle protein synthesis [32] and are known to decrease in skeletal muscles during exhaustive aerobic exercise [33]. The depletion of amino acids during the race is also evidenced by the elevated levels of 3-hydroxybutyric acid mid- and post-race. This carboxylic acid is part of the ketone bodies and is representative of partial degradation products of BCAAs (mainly valine) released from the muscles for hepatic and renal gluconeogenesis. Simultaneously, as glucose resources decrease, lipolysis is upregulated to provide fatty acids as an alternative energy source, particularly for muscle and heart tissue. During this process, fatty acids are broken down to form acetyl-CoA molecules, which subsequently lead to the formation of ketone bodies, including 3-hydroxybutyric acid. Ketone bodies, as previously highlighted in the discussion as an alternative energy source, are used for up to 60% of the brain's energy requirements, and they continue to serve as an important energy reserve for the heart and skeletal muscles [34, 35]. Interestingly, tryptophan and asparagine are the only amino acids that increased during this extreme sport event. In the context of intense exercise, increased levels of tryptophan are associated with the development of fatigue [36]. When present in large amounts, tryptophan is transported across the blood–brain barrier and converted to serotonin via two enzymatic reactions. Elevated levels of serotonin in the brain may contribute to central fatigue and overreaching/overtraining [37], influence the perception of effort and lead to reduced performance [38]. Our results show that NMR-metabolic profiling of blood derived samples provides access to interesting information about the status of athletes. These results clearly demonstrate how

mountain ultramarathon running affect the metabolism of participant. From the metabolic shift towards ketosis to the development of fatigue, this methodology provides a comprehensive view of the metabolic event that occurs during the race. Gathering such information could help athletes to manage their race by improving their exogenous energy intake and optimizing their race plan. Indeed, given the prolonged effort that athletes are face during such event, the management of food intake and rest periods is imperative. In fact, planning and optimizing the dietary strategy to improve fat oxidation capacity has already been described as a training recommendation for ultramarathon athletes [39]. The use of metabolomics in this context could help to better design and adapt runners' supplementation at a personalized level.

Our study confirms the biphasic responses previously observed for biomarkers of cardiac remodeling and fibrosis (ST2 and Gal-3) [23]. Interestingly, we observed here that the mountain ultramarathon had a strong association with NT-proBNP levels, confirming a large impact on cardiac function. Oxidative stress, ischemia and necrosis markers also contribute to this ultramarathon effect, whereas the influences of inflammation and renal function were moderate, compared to the cardiac ones. Confirming previous observations on cardiac fatigue [40], postural control alteration [41] or cardiac fibrosis [23], all these alterations followed a biphasic response i.e. they peaked in the middle of the race and decreased after the race, remaining even higher than before the race. This may be due to an accumulation of fatigue in the athletes, which would reduce the pace after mid-race (148.7 km, +9270 m elevation gain). Interestingly this biphasic response is not observed at metabolites level. This may indicate that even when runners slow down, thereby reducing the strain on their muscles, heart or kidneys, their metabolism appears to be affected throughout the event.

Correlation analysis shows a stronger association between ketone bodies and muscle, inflammatory, cardiac, and renal biomarkers than with other metabolites. This could be explained by the metabolic shift that occurs during such extreme prolonged efforts where glucose availability becomes limited. Ketone bodies are used as an alternative fuel source and will be used for muscle metabolism, which explains why their concentration remains elevated until post-race time point and therefore correlates with functional biomarkers. Indeed, studies have shown that ultramarathon runners often fail to meet the recommended carbohydrate intake during endurance exercise [42]. As glucose resources are depleted, ketosis is used to fuel the runner's body. When ketone bodies are used as an alternative energy source, nitrogen catabolism is reduced allowing the retention of lean muscle mass [43]. Although ketosis may be beneficial to athletes, it is usually associated with undesirable effects as the initial adaptation period is characterized by lethargy, fatigue, and thus impaired performance [44]. In ultra-endurance events where athletes are faced with extremely long distances and limited energy availability, our data suggests that ketosis becomes an inevitable metabolic response. However, the early challenges associated with this shift—such as fatigue, lethargy, and reduced performance—can significantly hinder athletic output [34]. Adequate metabolic adaptation to ketosis is therefore crucial in helping athletes mitigate these negative effects.

Exercise-induced fatigue is typically characterized by glycogen depletion, lactate accumulation, and oxidative stress. The utilization of fats and ketone bodies offers a more efficient and sustained energy supply compared to glucose oxidation, which requires 11 enzymatic steps to produce ATP [45]. Ketosis not only provides a quicker energy source but also avoids the rapid depletion of glycogen stores commonly seen during endurance exercise. Furthermore, ketogenic diets have shown potential benefits in promoting muscle health due to their anti-inflammatory and antioxidant properties, which can help prevent exercise-induced fatigue, muscle damage, and oxidative stress [46–49]. In contrast, glucose metabolism generates higher levels of reactive oxygen species (ROS), leading to excessive free radicals, chronic inflammation, and mitochondrial damage—factors that negatively impact both muscular and overall health.

In this context, keto-adaptation strategies have emerged to enhance endurance capacity, improve fatigue resistance, and expedite recovery. Our metabolomic approach represents a promising method to evaluate how athletes participating in ultra-endurance events experience these metabolic shifts and to assess the degree of keto-adaptation in athletes following a ketogenic regimen versus those relying on conventional diets. This comparative analysis could provide valuable insights into the impacts of ketosis on both performance and health in extreme endurance conditions.

Post-ultramarathon Recovery and Metabolomics Data and Clinical Biomarkers

A major finding of the present study is that ultramarathon runners did not fully recover after 72 h of recovery. Although this effect is not well described by the PCA analysis as recovery samples (T4) seem to cluster with the pre-race samples (T1) when analyzing the score plot for PC1 and PC2, the ALASCA longitudinal multivariate analysis clearly show that these samples differ for the levels of metabolites and several functional biomarkers.

From our NMR-based metabolomics investigations, we observe that most of the metabolites with significant variations during the race do not return to baseline levels and remain elevated. Indeed, ketone bodies related metabolites such as acetone, acetoacetate, and 3-hydroxybutyrate are present at lower levels compared to pre-, mid-, and post-race concentrations. This may be due to the increased carbohydrate intake during the recovery phase which would optimize muscle glycogen synthesis from glucose [50, 51]. This carbohydrate intake will therefore prioritize the use of glucose as the main source of energy supply and thus lipid based energetic pathways using ketone bodies is reduced. This observation is in line with the increased levels of amino acids during the recovery phase. In this context essential amino-acid intake might be highly effective in increasing muscle protein synthesis [52]. The observed effect on the levels of metabolites associated with recovery samples could therefore be attributed to the specific diet that ultramarathon runners would consume during this crucial period. Gathering such information could therefore be useful to monitor the status of athletes post competition and ensure proper recovery.

The physiological characteristics of the participants were still impacted after 72 h of recovery, as shown by the remaining elevated values of muscle, cardiac, inflammatory, and renal function biomarkers. As previously reported by Le Goff et al. [23], none of these values meet the risk criteria for cardiac or renal injury. Moreover, this study demonstrated that myocardial, and musculoskeletal stress appeared to be most affected by the prolonged exercise, as the levels of associated markers were largely increased. The association with prolonged ultra-endurance exercise is not new [40, 53] it is also known that, such increases are not associated with adverse clinical outcomes [54], even if long-term cardiac and renal damage cannot be excluded [7, 8]. Globally, the practice of ultramarathon running will drastically impact the cardiac, muscular, and renal functions of athletes, but these effects will not reflect acute pathological status as biomarker levels decrease after the race.

Metabolic Pathways During Mountain Ultramarathon and Recovery

Single sample pathways analysis associated with kPCA of metabolomics data gives access to an innovative representation of the studied effects. Indeed, by facilitating pathways analysis interpretation, effect on the metabolism during the race can be deciphered. A notable aspect of this section is the clustering of post-race samples, demonstrating once more that the metabolism of the participants did not fully return to baseline after 72 h of recovery.

One interesting observation arises from the analysis of the amino acid-related (AAs) pathways. While most of AAs pathways seem contributing to the pre-race cluster, three major pathways (i.e., Vitamin-B5 and CoA biosynthesis; cysteine and methionine metabolism; and histidine metabolism) are responsible for the spread of recovery samples along PC1 component. This may indicate that significant changes are still perceivable for these pathways after 3 days of recovery, while athletes may have recovered from other AAs-related signaling routes. As discussed above, mountain ultramarathon is a challenging sport event that depletes glycogen energy store and use ketogenesis as fuel supply. CoA, derived from Vitamin-B5, is essential for the conversion of acetyl-CoA in TCA cycle. Thus, elevated turnover of this pathway is clearly associated with restoration of energy levels during the recovery phase. Moreover, CoA will play a role in amino-acid synthesis essential for muscle repairs and protein synthesis and thus can impact cysteine and methionine metabolic routes through protein synthesis. As histidine is the principal precursor of histamine, a molecule involved in immune response and inflammation, its released could be associated with inflammatory events [55]. One hypothesis could be that inflammatory response is associated as a response to myocardial stress that occurs at mid- and post-race. This could be viewed as a healthy response of the system to cardiac stress accumulated during intensive effort.

In conclusion, the utilization of cutting-edge data representation to analyze metabolic changes occurring during a race enhances our understanding of the biochemical processes experienced by athletes. By discerning key pathways involved in the race, this approach holds promise for revolutionizing athlete management. By optimizing the energy use throughout the race, significant improvements in performance can be achieved, while minimizing the risk of injury. Furthermore, by targeting specific pathways during the recovery phase and implementing appropriate supplementation strategies, athletes can promote healthier practices within their sport and facilitate more effective recuperation.

Limitations, Unique Contribution, and Perspective

While this study demonstrates the usefulness of NMR-based metabolomics to provide valuable information for the monitoring of athletes, this study is not free from limitations.

The first major limitation of this study is that the composition of the cohort prevents us from generalizing the findings to all types of athletes, as neither sex nor gender was taken into account when constructing the dataset. Specifically, this study was conducted solely on male samples, as the few female samples collected were excluded

to avoid introducing additional variability and creating a highly unbalanced sample group. Other important limitations include the relatively small sample size which could hampers the robustness of statistical models generated and prevent us from detecting significant results for some metabolites. Furthermore, such on-field investigation on the participants of a mountain ultramarathon lasting several days has obvious limitations: it is impossible to monitor accurately food and fluid intake during and immediately after the race. In addition, the characterization of the training level of the participants is limited to weekly training loads. It is also impossible to perform a VO_{2max} test shortly prior to the race for time and logistical reasons. Moreover, this would not be accepted by the runners. However, due to the extreme stress induced by the race itself, it is likely that these potential confounding factors are likely to be negligible. Although age, physical fitness (represented here by weekly training load), and performances (time to complete the race) are known to influence athletes' metabolomes post-exercise [56, 57], our study design did not account for these factors when constituting the cohort. As a result, we were unable to detect meaningful effects of these covariates on our metabolome data. Future studies should address this limitation by including a more balanced cohort that better represents the heterogeneity of mountain ultramarathon participants.

Another limitation lies in the analytical technique used for sample analysis. While NMR presents an interesting and valuable option for carrying out metabolomics studies, its lower sensitivity compared to mass spectrometry limits the detection of less concentrated metabolites. The final limitation is the difficulty of comparing our results with those obtained in other ultramarathons. Indeed, due to the extreme distance of the 'Tor des Géants', athletes exert a slower pace than on other races of shorter distance and/or elevation [58].

While previous studies in the field have primarily focused on metabolic adaptation in athletes using "static" study designs— such as investigating the effects of ultramarathons on metabolism under simulated conditions [59], predicting results using pre-race samples [60], characterizing marathon-induced metabolic changes using pre- and post-race samples [61, 62], and investigating runners' recovery using multiple post-race time-point samples [63, 64]—our study stands out by capturing the dynamic metabolic changes that occur in real-time during a mountain ultramarathon. To our knowledge, this is one of the first study conducted under actual mountain ultramarathon race conditions, without controlling for the athletes' race schedules, diets, or supplementation, providing a more authentic representation of the metabolic processes at play. By taking samples at multiple time points throughout the event, we offer a more comprehensive view of the biochemical events that unfold during such an extreme and prolonged effort. This approach is essential to gaining a clearer understanding of how the body responds during the race, as opposed to merely before or after, which can miss critical insights into the physiological demands faced by ultramarathon runners. The methodology we used sets a new precedent for future research, offering a unique framework for studying endurance sports in a real-world context and highlights key aspects of this sport practice that must investigated.

Further development of the application of metabolomics in this context would require additional blood samples from healthy individuals with different fitness status or different physiological profiles. This step is essential to identify key adaptations that emerge from ultramarathon running. Such knowledge could prove invaluable in the assessment of high-risk injury situations and aiding in injury prevention. Another area of interest could be the investigation of specific nutrient supplements (e.g., BCAAs or ketones) and their effects on the metabolism of the ultramarathon runners. Gathering information on the precise need in supplementation requirement for participation, performance, and recovery from such events is imperative for high-performance athletes. In this field, this study identifies metabolomics as an innovative approach that could pave the way for a novel follow-up strategy by using biochemical information at a personalized level.

Conclusion

In this paper, we underscore the potential of NMR-based metabolomics as a powerful tool for evaluating exercise-related metabolic changes occurring during and after mountain ultramarathon races. Leveraging these innovative technologies allows for a more comprehensive and personalized approach for athletes' management. From performance enhancement to risk assessment and injury prevention, metabolomics offers practitioners and coaches the opportunity to build innovative strategies to promote healthy ultramarathon running. By providing a holistic perspective on the biochemical events associated with such races, this study paves the way for a paradigm shift in athlete management. While further research is nedded to address essential questions, metabolomics is emerging as a complementary approach to current methods for studying and monitoring the physiological states of athletes. Our approach shows great potential for application in the context of ketosis, offering valuable insights into athletes' metabolic adaptation to this state. It could be particularly useful for evaluating their preparation, managing race strategies, and tailoring supplementation based on individual metabolic profiles. Furthermore, this

method could be employed to monitor and assess cardiac functionality, shedding light on how endurance races impact cardiovascular health. Ultimately, metabolomics, and more specifically NMR-based metabolomics, holds the promise of becoming a key tool for the individualized monitoring of athletes participating in ultra-endurance events.

Abbreviations

^{1}H-NMR	Nuclear magnetic resonance
EDTA	Ethylenediaminetetraacetic acid
NT-proBNP	N-terminal pro-hormone of natriuretic peptides
Gal-3	Galectin-3
ST2	Suppression of tumorigenicity 2 protein
hFABP	Heart fatty acid binding protein
hsTnT	High sensitive Troponin T
CK	Creatine kinase
MYO	Myoglobin
CKMB	Creatine kinase MB
CRP	C-reactive protein
QC	Quality control
FDR	False discovery rate
PCA	Principal component analysis
PLS-DA	Partial least square – discriminant analysis
ssPA	Single sample pathway
kPCA	Kernel PCA
RM-ASCA+	Repeated measure ANOVA simultaneous component analysis
PC	Principal component
BCAAs	Branched chain amino acids
TCA	Tricarboxylic acid cycle

Supplementary Information

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Supplementary Material

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Author contributions
CLG, MS, EC, GPM, J-FK, and PC contributed to conception and design of the study. CLG and MS organized the database and wrote the first draft of the manuscript. MS, AC, and MC performed samples analysis and statistical analysis. JN, EB, and SP performed sample preparation. EG, JD, LH, JL, PM, TD and PDT contributed to manuscript revision, read, and all authors approved the submitted version.

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Availability of Data and Material
The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Declarations

Ethics Approval and Consent to Participate
The studies involving human participants were reviewed and approved by the local ethical committee of the Azienda Regionale Sanitaria USL della Valle d'Aosta (n°900–18/08/2014) and was performed in accordance with the standards of ethics outlined in the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

Consent for Publication
Not applicable.

Conflicts of interest
The authors have no potential conflicts of interest.

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