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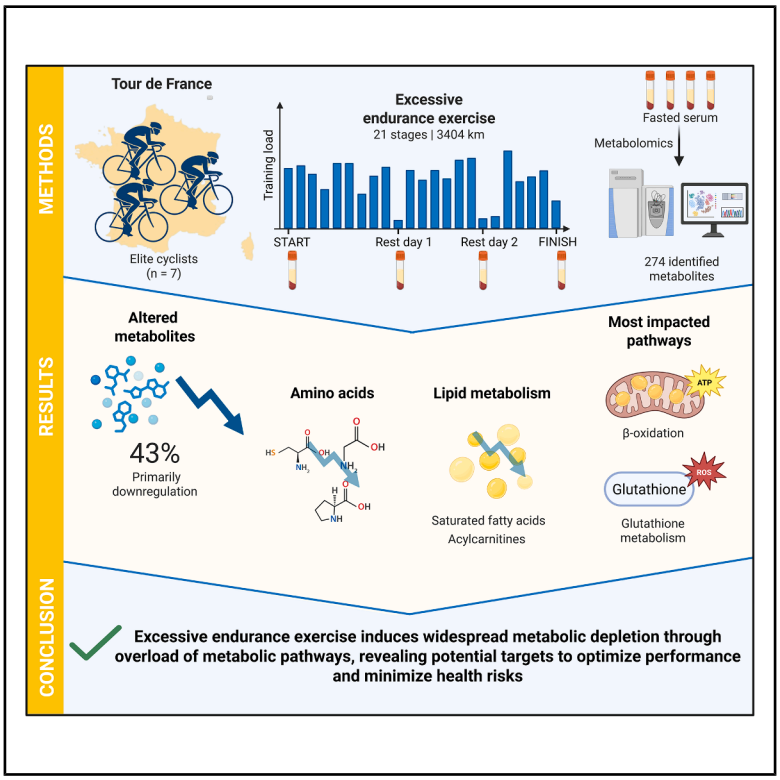
# The metabolic signature of excessive endurance exercise—A prospective study in Tour de France cyclists

## Graphical abstract




## Highlights

- 43% (118/274) of circulating metabolites altered during Tour de France
- Excessive endurance exercise depletes, rather than elevates, circulating metabolites
- Most impacted pathways are β-oxidation and glutathione metabolism
- Major depletion of amino acids, saturated fatty acids, and carnitines

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## In brief

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# The metabolic signature of excessive endurance exercise—A prospective study in Tour de France cyclists

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## SUMMARY

Despite well-established health benefits of regular exercise, molecular mechanisms driving performance adaptations and potential adverse health effects of excessive endurance exercise warrant further investigation. We leveraged the Tour de France (TdF) as an ecological model to characterize the systemic metabolic response to excessive endurance exercise in elite cyclists using targeted and untargeted metabolomics on fasted blood samples. Our analyses revealed significant alterations in 43% of measured metabolites, with pronounced changes during the initial 10 days, followed by further deterioration throughout the race. In contrast to acute exercise responses, the TdF predominantly decreased circulating metabolite availability across multiple pathways, including depletion of amino acids, specific saturated fatty acids, and medium/long-chain acylcarnitines. These findings provide mechanistic insights into the impact of excessive endurance exercise on systemic metabolism and identify potential targets for intervention strategies to maximize performance while minimizing adverse health consequences.

## INTRODUCTION

Endurance exercise training is widely recognized as a cornerstone for preventing and managing chronic metabolic diseases, contributing to the widely endorsed concept of ''exercise is medicine''.[1] These beneficial effects are considered to be driven by the ability of endurance exercise to challenge cellular homeostasis, triggering system-wide responses that ultimately enhance cardiorespiratory fitness and metabolic health. However, emerging evidence suggests a non-linear relationship between exercise dose and health benefits, with a physiological threshold beyond which additional exercise may become detrimental. Recent studies demonstrate that excessive exercise—characterized by sustained periods where training load exceeds recovery capacity—is associated with pathophysiological adaptations that compromise metabolic and overall health.[2–4]

The pathophysiological consequences of excessive exercise manifest across multiple organ systems and are particularly evident in professional athletes. Elite endurance athletes exhibit, for instance, an increased prevalence of maladaptive cardiovascular adaptions (e.g., reduced left ventricular ejection fraction[5] and myocardial fibrosis[6]), as well as systemic disruptions such as impaired glucose homeostasis,[4] and compromised bone mineral density.[7] Furthermore, at least some of these effects (e.g., atrial and left ventricle dilation) persist for at least 30 years following their competitive career.[8] Also in the general population, endurance exercise above a certain volume is associated with higher prevalence of coronary events,[9] mitochondrial functional impairment,[4] reduced insulin sensitivity,[4] and mortality risk.[10]

These maladaptive responses likely originate from prolonged, exercise-induced metabolic strain, resulting in disrupted metabolic homeostasis. In support of such a hypothesis, excessive endurance exercise has been shown to impair mitochondrial respiration in otherwise healthy participants,[4] while excessive exercise-induced overreaching and overtraining are characterized by progressive alterations in metabolic pathways.[11,12] These metabolic disturbances appear to show a sequential pattern, starting with dysregulations in glucose metabolism, followed by lipid and protein metabolism disturbances.[12] However, due to the employed techniques in earlier research (e.g., Fourier transform infrared spectroscopy), the specific metabolites or metabolic pathways impacted by excessive exercise are still unknown.

To identify the impact of excessive endurance exercise, studies either use a cross-sectional approach or simulate excessive exercise by subjecting recreationally active subjects or trained individuals (i.e., tier 1 or 2 subjects[13]) to high-volume, high-intensity endurance training programs for up to 4 weeks.[14] In addition to the evident lack of causality in cross-sectional studies, the relevance of these short-term intervention studies

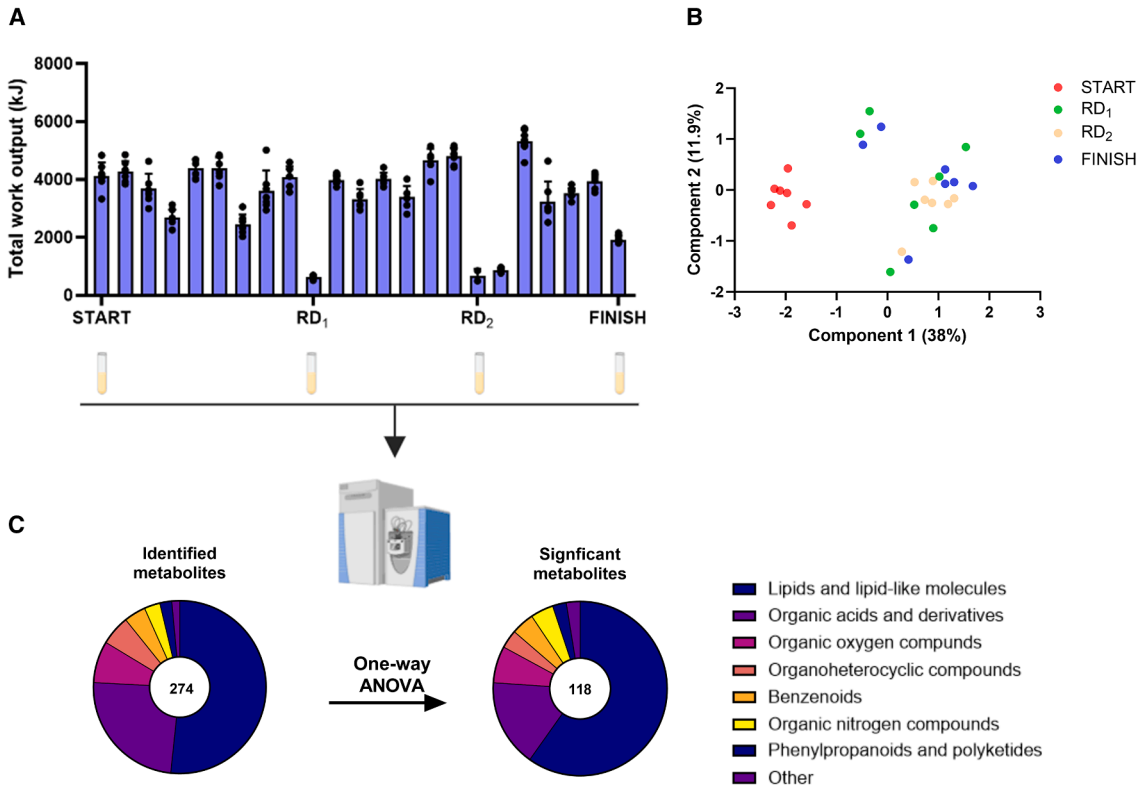


**Figure 1. Metabolomic profiling of fasted serum samples of elite cyclists collected during the Tour de France**

(A) Schematic representation of the study design, including total work output ± SD for every stage of the race for each cyclist ($n$ = 7).

(B) Principal-component analyses of fasted serum samples collected on the morning of the first stage (START), first rest day ($RD_1$), second rest day ($RD_2$), and the final stage (FINISH) ($n$ = 7).

(C) Donut charts displaying the total number of identified metabolites from both targeted and untargeted metabolomics, categorized by compound class, as well as the number of metabolites that showed significant changes throughout the TdF ($n$ = 7). Significant changes ($p < 0.05$) were determined using one-way repeated measures ANOVA with FDR correction, followed by Šidák post hoc test for pairwise comparisons.

in recreational subjects is limited. First, these studies fail to account for the (beneficial) metabolic adaptations acquired through years of intensive endurance training, such as an increased antioxidant defense capacity.[15] Second, the training load in these studies—in both absolute and relative terms—remains far below these in real-world conditions (e.g., due to ethical committee restrictions and the lack of performance pressure and financial incentives). This is particularly problematic as it is precisely elite athletes who experience the adverse health impact of excessive endurance exercise.

Therefore, providing ecologically valid data on the metabolic impact of excessive endurance exercise and its potential link with adverse health outcomes requires the inclusion of top-level athletes (i.e., tier 4 or 5). In this respect, grand tours in professional cycling (i.e., Tour de France, Vuelta a España, and Giro d'Italia) provide an unparalleled opportunity to investigate the metabolic impact of excessive exercise in international to world-class athletes. Evidence for the validity of such grand tours as a model of excessive exercise is provided by earlier studies showing their ability to induce widespread, adverse adaptations including impaired exercise performance,[16] a dysregulated hypothalamic-pituitary-adrenal axis,[17] decreased bone mineral density,[18] and compromised pulmonary function.[19] These findings position grand tours as a valid model for investigating the metabolic impact of excessive exercise.

Therefore, by performing targeted and untargeted metabolomics on fasted blood samples collected from elite cyclists throughout the Tour de France (TdF), we aim to provide a comprehensive temporal profiling of metabolic alterations induced by excessive endurance exercise. This is with the ultimate aim to provide mechanistic insights into the metabolic impact of excessive exercise and the potential causes of its adverse health effects, as well as to identify metabolic alterations indicative of the development of fatigue.

## RESULTS

### The TdF remodels the fasted serum metabolome of elite cyclists

We studied seven elite (including 3 world-class)[13] cyclists during the TdF 2023. Each cyclist completed the entire race (RACE) with a distance of 3,404 km and 56,482 m of elevation gain over 19 race days and 2 rest days (RDs). This resulted in a total work output of ~75,500 ± 7,500 kJ (Figure 1A). Fasted blood samples

were collected immediately upon awaking (7:30 to 9:00 a.m.) on (1) the first racing day (day 1, START), (2) the first rest day (day 10, $RD_1$), (3) the second rest day (day 17, $RD_2$), and (4) the final racing day (day 23, FINISH). Derived serum samples were subsequently analyzed using both targeted and untargeted metabolomics (details are available in the STAR Methods).

A total of 274 serum metabolites were identified (targeted: 29, untargeted: 245 metabolites). The quantified metabolites were primarily lipids and lipid-like molecules (52%) and organic acids (including derivatives, 24%). Principal-component analyses (PCAs) revealed substantial remodeling of the serum metabolome throughout RACE, with the most pronounced alterations occurring from START to $RD_1$ (Figure 1B). A one-way repeated measures ANOVA with false discovery rate (FDR) correction showed that 43% ($n$ = 118) of the measured metabolites were significantly altered throughout the TdF (Figure 1C). This provides robust evidence that the TdF remodels the circulating metabolome.

## Remodeling of the circulating metabolome occurs primarily during the early phase of a grand tour

Volcano plots showed that the TdF affected fasted serum metabolite levels, with most metabolites decreasing ($n$ = 86) and only a small subset increasing ($n$ = 20) when comparing START vs. FINISH samples (Figure 2A). The most dramatic changes occurred during the first week (START vs. $RD_1$; Figure 2B), where 26 metabolites decreased and 13 increased. These changes became less pronounced as the TdF progressed. In the second week ($RD_1$ vs. $RD_2$; Figure 2C), we observed 30 decreased and 2 increased metabolites. By the final week ($RD_2$ vs. FINISH; Figure 2D), changes were minimal with only 7 decreased and 2 increased metabolites. To identify the primary metabolites driving the shift in the metabolome, partial least square discriminant analysis (PLS-DA) and variable importance projection (VIP) analyses were conducted (Figures 2E–2H). These analyses confirmed that the most pronounced alterations occurred between START and $RD_1$, with component 1 explaining 44% of the total covariance. The model for START to $RD_1$ demonstrated excellent predictive ability ($Q_2$: 0.79) and perfect classification accuracy (1.0) using only the first component. The performance of the model decreased substantially during the last 2 weeks of the tour. For $RD_1$ vs. $RD_2$, the first component explained only 20% of the covariance, and the model showed poor predictive ability ($Q_2$: −0.28) with near-random classification accuracy (0.55). Similarly, for $RD_2$ vs. FINISH, the first component explained 19% of covariance, with poor predictive power ($Q_2$: −0.09) despite better classification accuracy (0.83). These results suggest that while metabolic changes were highly consistent across cyclists during the first week, the response became increasingly variable in subsequent weeks.

## Alterations in fatty acid metabolism predominantly drive the change of the circulating metabolome

The main circulating metabolites driving alterations in the metabolome from START to FINISH were largely associated with fatty acid metabolism (Figure 2E). This included decreased levels of long-chain acylcarnitines (pentadecanoylcarnitine and heptadecanoylcarnitine) alongside increased levels of long-chain fatty acids (myristic acid, elaidic acid, and palmitoleic acid), suggesting heightened fatty acid mobilization and oxidation. Additional contributors to the metabolomic shift included fatty acid ethanolamides (linoleoyl ethanolamide [LEA]and oleoylethanolamide [OEA]), as well as metabolites related to drug (O-desmethylnaproxen) and supplement intake (theobromine).

When analyzing the alterations across the different time points (Figures 2F–2H), it was clear that metabolites involved in fat metabolism exerted a significant impact on the serum metabolome at all time points. Of the top 15 contributing metabolites from START to FINISH, 11 were already major contributors from START to $RD_1$. New metabolic signatures occurring following $RD_1$ included a drop in tetrahydrodeoxycortisol, indicating reduced cortisol biosynthesis. Furthermore, O-cresol sulfate showed a dynamic pattern throughout the TdF, with levels decreasing from $RD_1$ to $RD_2$ but subsequently increasing from $RD_2$ to FINISH. From $RD_2$ to FINISH, the tricarboxylic acid (TCA) cycle intermediate succinic acid increased while sphinganine—a key precursor in sphingolipid synthesis—decreased, suggesting enhanced cell membrane turnover (Figure 2H).

## Cluster analysis reveals predominantly temporal patterns of progressive downregulation in circulating metabolites during the TdF

We subsequently performed hierarchical cluster analyses to identify temporal patterns of serum metabolite alterations during the TdF (Figure 3). This revealed eight distinct temporal patterns, one cyclic pattern (i.e., decrease – increase – decrease, cluster 1), five clusters showing an overall downregulation (clusters 2–6), and two clusters showing an overall upregulation of metabolites (clusters 7–8). To enable a comparison between metabolites and clusters, all metabolite levels were standardized to $Z$ scores. Most metabolites showed moderate changes throughout the TdF, with $Z$ scores typically ranging between −1.5 and 1.5.

Cluster 1 (Figure 3B) is characterized by a notable pattern with an initial decrease from START to $RD_1$, followed by a marked increase at $RD_2$ and a subsequent reduction at FINISH. This cluster encompasses two acylcarnitines (3-hydroxyhexanoylcarnitine and octenoyl-L-carnitine) and four amino acids (L-tyrosine, L-valine, L-proline, and D-tryptophan). Cluster 2 (Figure 3C) includes metabolites that exhibit a significant downregulation from START to $RD_1$, followed by sustained low levels throughout the remaining part of the TdF. This pattern is most likely the result of elevated fatty acid oxidation (reduction in L-carnitine) and heightened amino acid catabolism (decrease of L-alanine, glycine, and L-histidine). Cluster 3 (Figure 3D) comprises metabolites that show initial upregulation during the first week, followed by a pronounced downregulation in the subsequent 2 weeks. This pattern involves metabolites associated with oxidative stress and inflammation (12-13-EpOME and 3-methoxybenzenepropanoic acid) and lipid metabolism (xi-10-hydroxyoctadecanoic acid, heptadecanoic acid, undecanedioic acid, and ethyl decanoate). Cluster 4 (Figure 3E) includes metabolites that exhibit a linear drop from START to FINISH. This cluster primarily comprises lipids (OEA and LEA), acylcarnitines (heptadecanoylcarnitine and pentadecanoylcarnitine), and amino acids (L-tryptophan and 1-methylhistidine). The

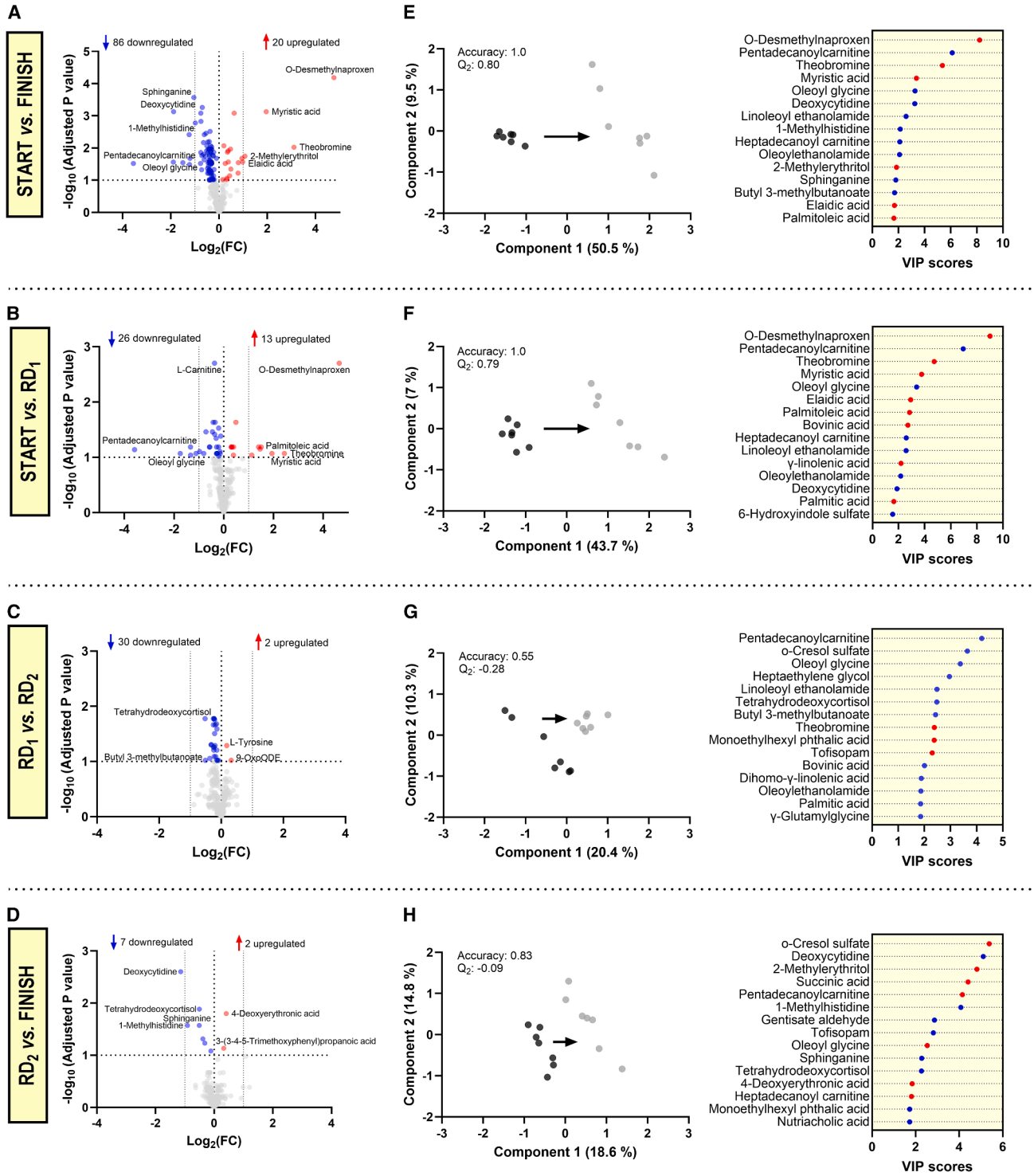


**Figure 2. Volcano plots, principal least square discriminant analyses, and variable importance in projection scores of metabolites impacted by the Tour de France**

Volcano plots, partial least squares discriminant analyses (PLS-DAs), and variable importance in projection (VIP) scores for metabolite level changes from START to FINISH, START to rest day 1 ($RD_1$), $RD_1$ to rest day 2 ($RD_2$), and $RD_2$ to FINISH ($n = 7$).

(A–D) Volcano plots displaying all identified metabolites at the specified time points, with significantly depleted ($p < 0.10$) and upregulated metabolites represented by blue and red dots, respectively ($n = 7$). Significance was determined using paired $t$ test with FDR correction ($p < 0.10$).

(E–H) PLS-DA plots for metabolites at the indicated time points (left), alongside the top 15 compounds ranked by VIP scores (right) ($n = 7$). Blue dots represent depleted metabolites, while red dots indicate upregulated metabolites.

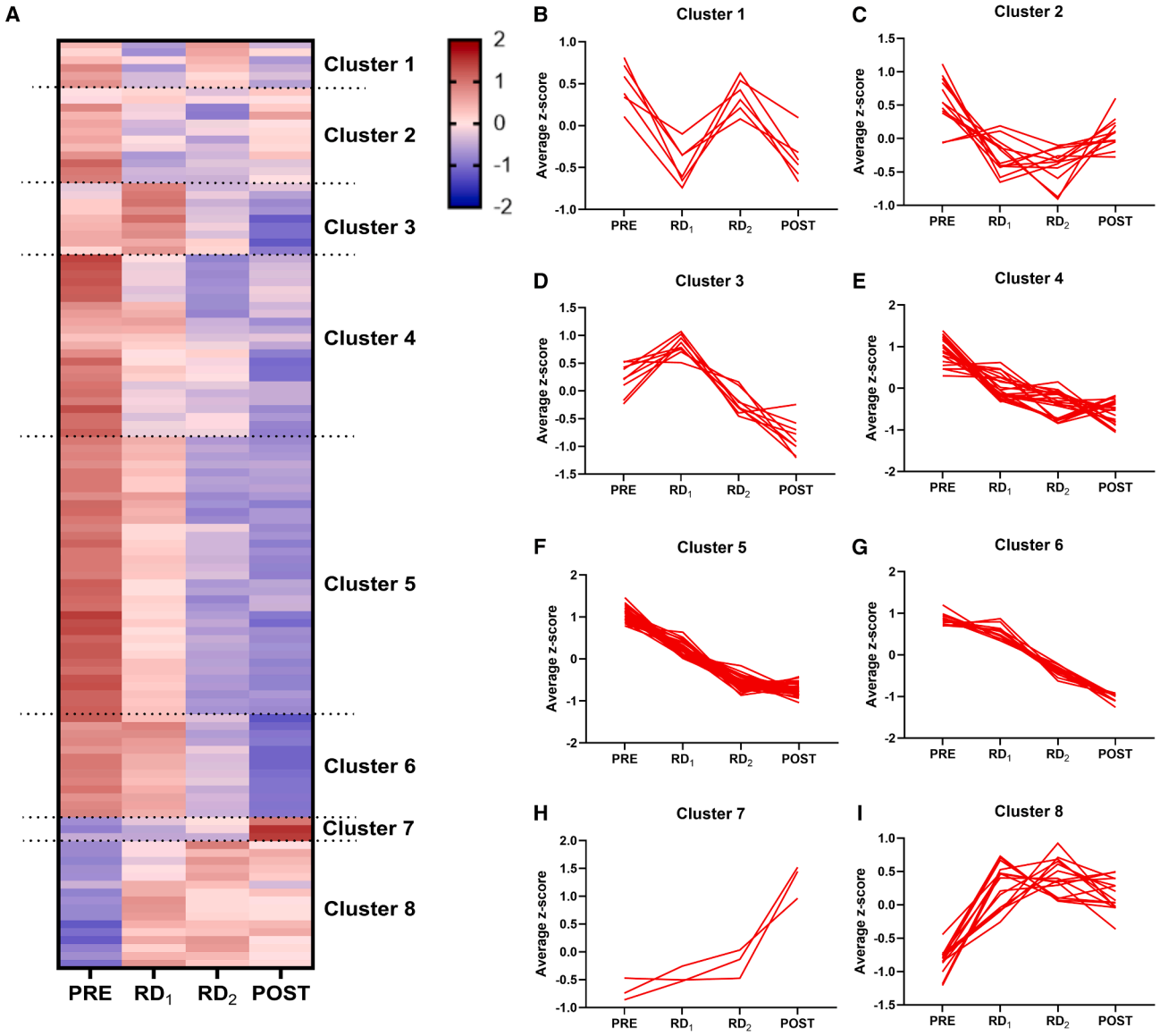


**Figure 3. Hierarchical cluster analyses of serum metabolites impacted by the Tour de France**

(A) Heatmap of *Z* scores with significantly altered serum metabolites during the TdF ($n = 7$). Significance was determined using one-way repeated measures ANOVA with FDR correction ($p < 0.05$). Hierarchical clustering identified eight distinct temporal clusters.

(B–I) Graphs displaying the mean *Z* scores of metabolites over time within each identified cluster ($n = 7$).

$RD_1$, rest day 1; $RD_2$, rest day.

presence of testosterone sulfate and 5α-dihydrotestosterone sulfate in this cluster indicates a progressive suppression of anabolic signaling throughout the TdF. Cluster 5 (Figure 3F), the largest cluster, is composed of metabolites that linearly decrease during the first 2 weeks and remain suppressed in the final week. These metabolites are involved in lipid metabolism (dodecanoic acid and heptanoic acid), energy production (ADP), and cell membrane metabolism (pentacosanoic acid and undecanoic acid). Cluster 6 (Figure 3G) displays a comparable trajectory but with a continued decline during the final week. This cluster encompasses medium- and long-chain fatty acids (ricinoleic acid, 2-hydroxyhexadecanoic acid, and behenic acid). The presence of sphinganine and nervonic acid in this cluster also indicates alterations in sphingolipid metabolism. The remaining two clusters include metabolites that increase over the course of the TdF. Cluster 7 (Figure 3H) consists of three metabolites (2-hexenoylcarnitine, 4-deoxyerythronic acid, and 2-methylerythritol) that notably rise only during the final week. Cluster 8 (Figure 3I) shows a pronounced increase from START to $RD_1$, followed by a sustained elevation. This cluster is enriched with sphingolipids (Cer(d18:1/18:1(11Z))), phospholipids (PE(22:2(13Z-16Z)/15:0)), lipids (myristic acid and bovinic

acid), and TCA cycle intermediates (citric acid and fumaric acid), suggesting upregulation of aerobic metabolism and cell membrane turnover during the TdF. Notably, the largest group of metabolites exhibited a stabilized pattern after either $RD_1$ (clusters 2 and 8) or $RD_2$ (clusters 4 and 5), indicating that various metabolic pathways reach a new steady state within 1 to 2 weeks of excessive exercise.

## Systemic metabolome alterations correlate with perceived fatigue and reveal potential novel biomarkers for training monitoring

We subsequently investigated if we could identify circulating metabolites that were associated with the extent of perceived fatigue. This is with the aim to propose potential blood-based biomarkers that can subsequently be evaluated for their validity in training monitoring (e.g., to detect when training imbalances occur). Cyclists rated their perceived fatigue immediately following each blood sample collection using a 0–10 Likert visual analog scale with the question: "*Your general perception of fatigue in this Grand Tour is?*" (0: no fatigue to 10: completely exhausted). Perceived fatigue increased from 0.5 ± 0.8 at START to 5 ± 1.7 at $RD_1$ (START vs. $RD_1$, $p < 0.001$) and continued to rise throughout the TdF, reaching 8 ± 1.5 at FINISH (START vs. FINISH, $p < 0.001$; Figure 4B). Subsequently, Spearman's correlation coefficients were calculated between perceived fatigue and each metabolite, with FDR correction applied to account for multiple comparisons (Figure 4A). This analysis revealed 84 significant correlations, with 77 being negatively correlated. The negatively correlated metabolites represented diverse biochemical classes, including lipids, sphingolipids, fatty acids, and amino acids. Notably, most of these metabolites have not been previously associated with fatigue or exercise-induced overreaching, underscoring their potential as novel biomarkers for training monitoring. The strongest negative correlations were observed for LysoPC (16:0/0:0) ($r = -0.807$, Figure 4C) and sphinganine ($r = -0.762$, Figure 4D), both key metabolites in cell membrane metabolism. This suggests that cell membrane dynamics may play a role in fatigue development. Interestingly, also dodecanedioic acid showed a strong negative correlation with fatigue ($r = -0.570$, Figure 4E). This aligns with previous research showing its ability to reduce muscle fatigue in diabetic patients.[20] Only seven metabolites showed positive correlations with perceived fatigue, including the TCA cycle intermediates citric acid (Figure 4F) and fumaric acid (Figure 4G), suggesting that enhanced TCA activity increases with increasing perceived fatigue.

## The TdF primarily downregulates the semi-essential amino acid pool

Amino acids serve as modulators in numerous physiological processes essential for optimal exercise performance, including energy metabolism, muscle protein turnover, immune function, and neurotransmitter biosynthesis.[21,22] Consequently, perturbations in amino acid metabolism likely represents a central mechanism underlying the adverse adaptations during periods of excessive exercise. To elucidate the metabolic impact of excessive exercise on the serum amino acid profile, we conducted targeted metabolomics on the collected samples during the TdF. Quantitative analysis revealed significant alterations in 10 of the 22 identified amino acids, all exhibiting an overall downregulation, except for L-tyrosine showing a biphasic pattern ($p < 0.05$, FDR-corrected; Figure 5A). The downregulated amino acids included essential (L-tryptophan, L-histidine, and L-valine), semi-essential (L-cysteine, glycine, and L-proline), non-essential (L-alanine), and D-amino acids (D-tryptophan and D-lysine) indicating broad dysregulation of amino acid homeostasis.

Earlier research primarily focused on the importance of branched-chain amino acids (BCAAs) for endurance athletes, given their preferential oxidation compared to other amino acids during exercise.[23] Furthermore, longitudinal research has demonstrated that excessive exercise is associated with reduced blood levels of BCAAs.[24–26] However, our metabolomic platform revealed that among the BCAAs, only L-valine exhibited a significant depletion during the final week of the TdF (Figure 5I), while the combined isoleucine-leucine signal remained stable. Isoleucine and leucine could not be distinguished due to their molecular weight and chromatographic properties. The depletion of L-valine occurred despite the systematic post-exercise intake of whey protein-enriched carbohydrate-protein recovery beverages. Furthermore, metabolomic profiling revealed significant reductions in three of the six semi-essential amino acids (glycine, L-cysteine, and L-proline) throughout the TdF. In addition, L-arginine ($p = 0.080$) and L-tyrosine ($p = 0.093$) exhibited a downward trend during the first 10 days of the TdF but did not reach statistical significance (Figures 5C, 5E, 5G, and 5J). Furthermore, after one week, a significant reduction in L-alanine concentration was observed, suggesting an elevated gluconeogenic demand (Figure 5D). In contrast, other markers previously associated with overreaching/overtraining, such as an increase in the glutamine/glutamate ratio[27] and an increase in circulating levels of L-tryptophan,[28] were respectively not altered or showed an opposing trend. A notable pattern emerged in L-tyrosine, characterized by reduced levels after the first week followed by an elevation during the second week. This biphasic response may reflect increased catecholamine synthesis during the first week, followed by suppressed production during the final two weeks of the TdF due to decreased β-adrenoreceptor sensitivity.[29]

## The TdF increases β-oxidation and glutathione metabolism

To identify the metabolic pathways most affected throughout the TdF, pathway enrichment analyses (with FDR correction) was applied. This revealed significant alterations in the β-oxidation of very long-chain fatty acids ($p = 0.003$) and glutathione metabolism ($p = 0.047$, Figure 6A). The pronounced decrease in the glutathione precursors, cysteine and glycine, suggests a reduction in glutathione synthesis and, consequently, diminished antioxidant capacity. Given the statistical overrepresentation of metabolites involved in β-oxidation, we conducted an in-depth analysis of the alterations in saturated fatty acids (Figure 6B), unsaturated fatty acids (Figure 6C), acylcarnitines (Figure 6D), and TCA-cycle intermediates (Figures 6E–6J). In general, increased fatty acid oxidation during exercise typically results in elevated blood levels of both fatty acids and acylcarnitines.[30–32] However,

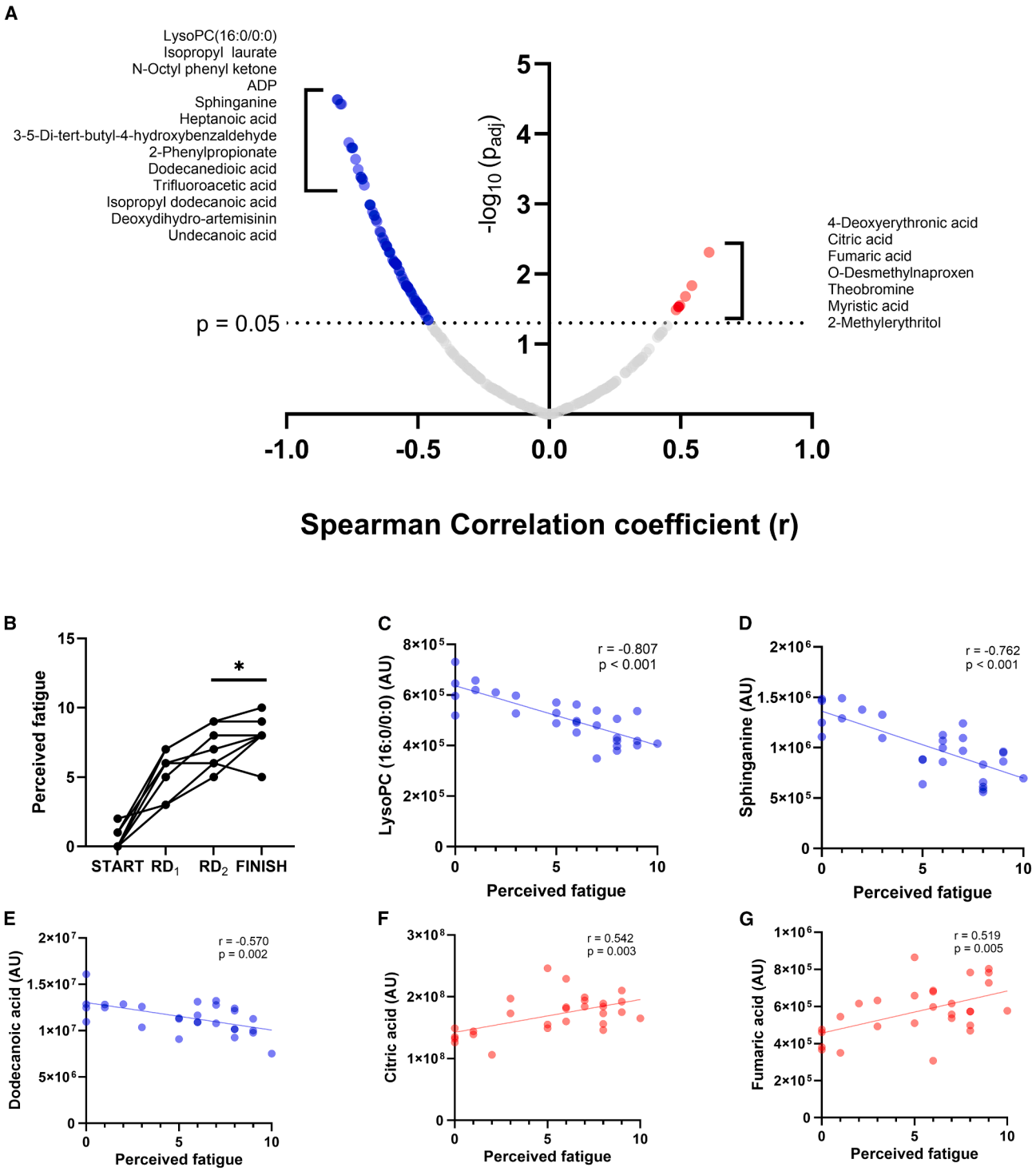


**Figure 4. Correlation analyses between serum metabolite levels and perceived fatigue**

(A) Significant correlations with perceived fatigue are shown, with negative correlations in blue and positive correlations in red ($n = 7$). The $x$ axis represents the Spearman correlation coefficient (r), while the $y$ axis shows $-\log(p)$ adjusted with FDR correction. Statistically significant correlations ($\log(p) > 1.3$, $p < 0.05$) are displayed above the dashed line.

(B) Absolute perceived fatigue values for cyclists at START, rest day 1 ($RD_1$), rest day 2 ($RD_2$), and FINISH ($n = 7$). *$p < 0.05$ vs. PRE.

(C–G) Selection of metabolites showing significant ($p < 0.05$) Spearman correlation coefficients with perceived fatigue ($n = 7$).

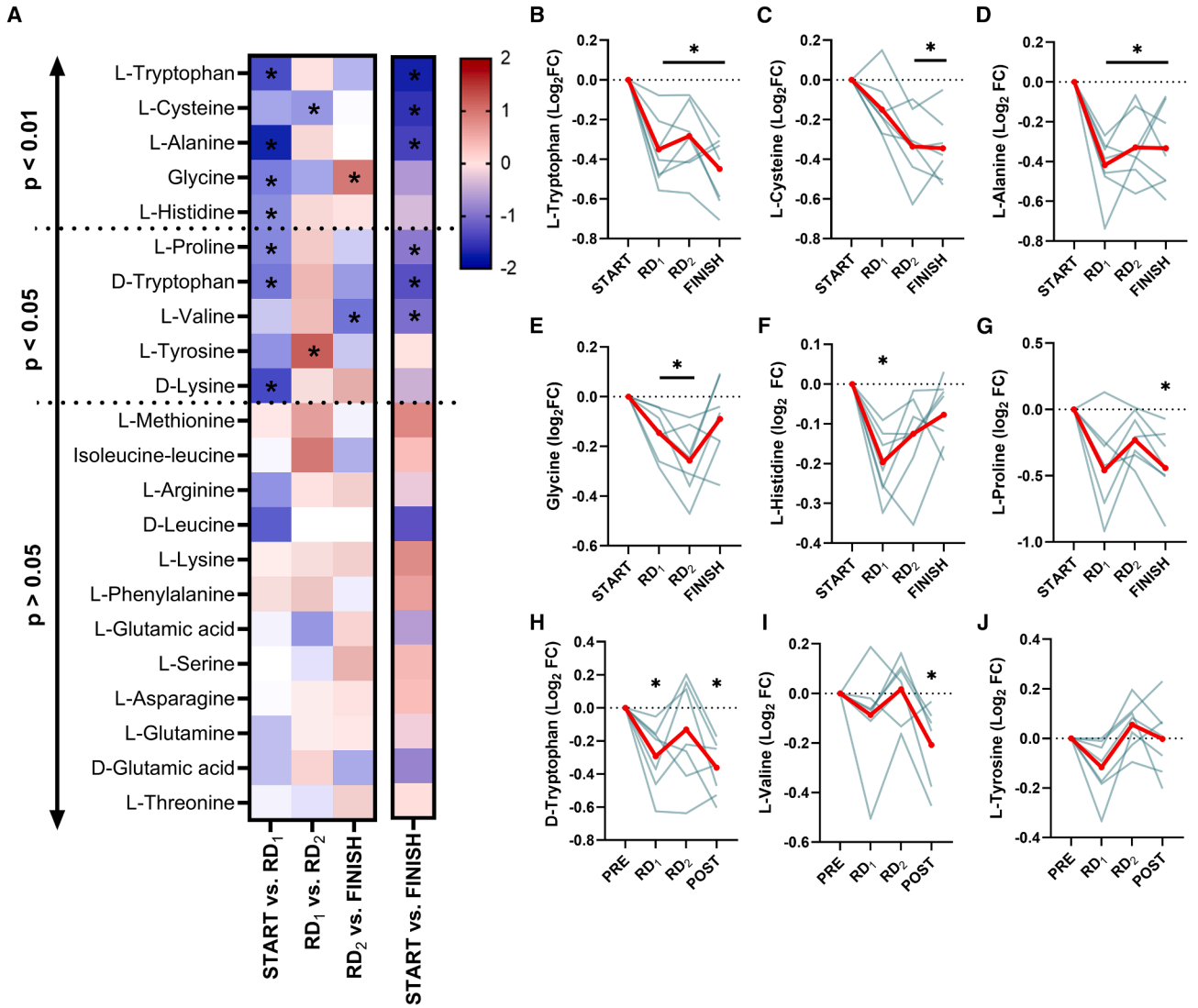


**Figure 5. Impact of the Tour de France on the serum amino acid profile**

(A) Heatmap of change in *Z* scores between the indicated time points of serum amino acids during the TdF ($n = 7$). Data were analyzed using one-way repeated measures ANOVA with FDR correction, followed by Šidák post hoc test for pairwise comparisons ($p < 0.05$ for both). *$p < 0.05$ between indicated time points. (B–J) Individual plots of the $\log_2$ fold change (FC) over time for the 9 most impacted amino acids, with gray lines representing individual values and the red line showing the mean. Data were analyzed using one-way repeated measures ANOVA with FDR correction, followed by Šidák post hoc test for pairwise comparisons ($p < 0.05$ for both) *$p < 0.05$ vs. START.

$RD_1$, rest day 1; $RD_2$, rest day 2.

approximately 16 h after the preceding stage, the fatty acid profile demonstrated a significant reduction in saturated fatty acids, with 64% (7 of 11) showing downregulated levels. This decline was most substantial during the first 10 days, but saturated fatty acids continued to decline toward the end of the TdF. Only one saturated fatty acid, myristic acid, exhibited a minor upregulation during the first 10 days. Conversely, unsaturated fatty acids remained predominantly stable, with only 19% (3/16) exhibiting decreased levels, while elaidic and palmitoleic acid showed an upregulation during the first 10 days. Notably, the downregulated fatty acids are typically already present in low concentrations in the blood of healthy individuals, while the more abundantly present serum fatty acids (palmitic, stearic, and oleic acids) remained stable throughout the TdF.[33,34]

Next, we investigated the acylcarnitine profile to provide insight into mitochondrial fatty acid transport and oxidation. Acylcarnitines, formed by the conjugation of fatty acids to carnitine, are essential intermediates that facilitate the transport of fatty acids across the mitochondrial membrane.[35] Of the 14 identified acylcarnitines, 5 showed a decreasing pattern. This included L-carnitine, medium-chain acylcarnitines (octanoylcarnitine and 3-hydroxyhexanoyl-carnitine), as well as long-chain acylcarnitines (heptadecanoyl-carnitine and pentadecanoyl-carnitine). Only 2-hexenoyl-carnitine showed an increase

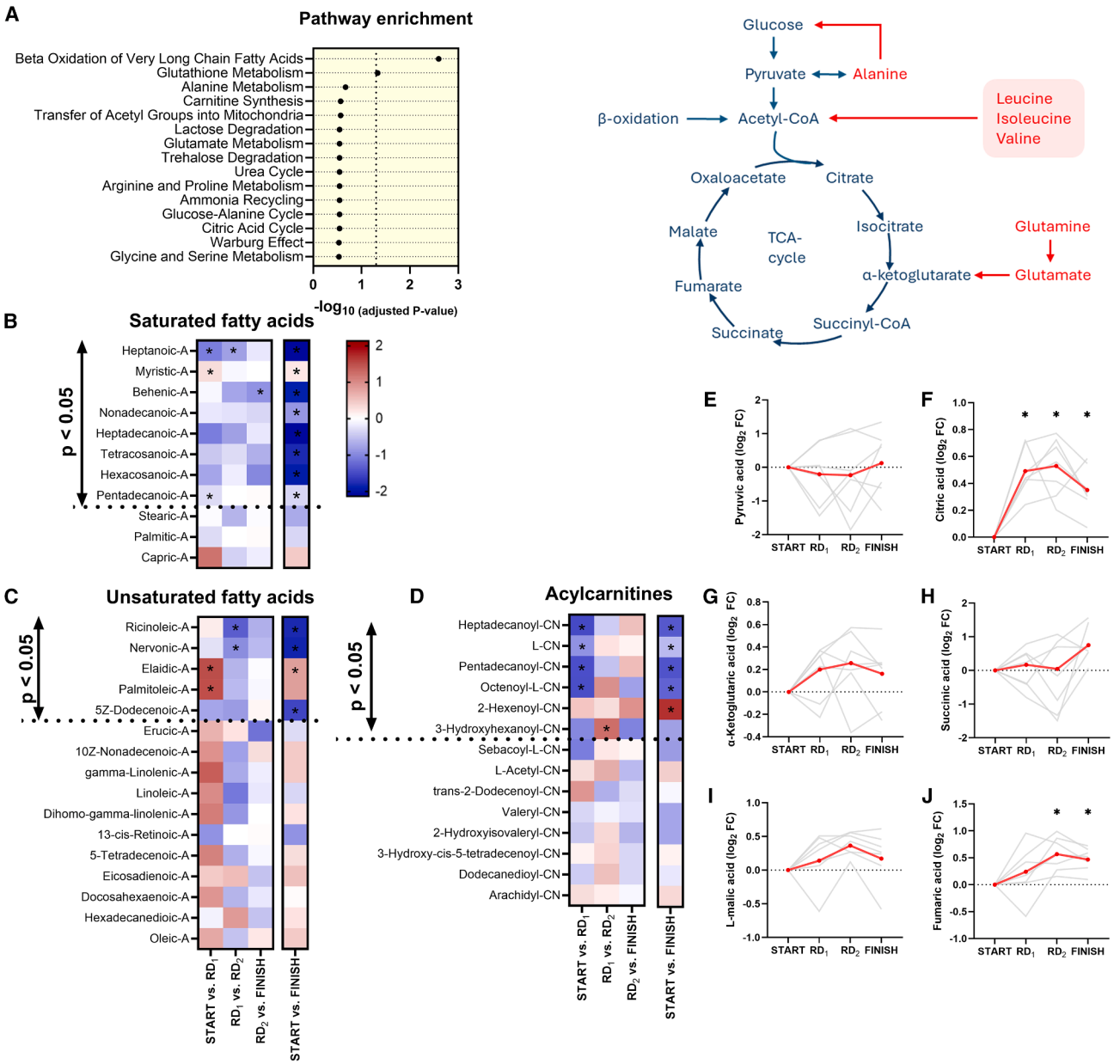


**Figure 6. Impact of the Tour de France on metabolic pathways and lipid metabolism**

(A) Pathway enrichment analyses ($n$ = 7).

(B–D) Heatmaps of change in $Z$ scores between the indicated time points for saturated fatty acids, unsaturated fatty acids, and acylcarnitines ($n$ = 7). Data were analyzed using one-way repeated measures ANOVA with FDR correction, followed by Šidák post hoc test for pairwise comparisons ($p < 0.05$ for both). *$p < 0.05$ between indicated time points.

(E–J) Individual plots of the $\log_2$ fold change over time for pyruvate and tricarboxylic acid cycle metabolites, with gray lines representing individual values and the red line showing the mean ($n$ = 7). Data were analyzed using one-way repeated measures ANOVA with FDR correction, followed by Šidák post hoc test for pairwise comparisons ($p < 0.05$ for both). *$p < 0.05$ vs. START.

A, acid; CN, carnitine; $RD_1$, rest day 1; $RD_2$, rest day 2.

toward the end of the TdF compared to the start. However, short-chain acylcarnitines such as acetylcarnitine and valerylcarnitine, which constitute ~80% of the total plasma acylcarnitine pool,[35] remained stable. In general, downregulation of the serum acylcarnitine pool indicates a decrease in fatty acid oxidation.[32] However, concurrent with the changes in fatty acids and acylcarnitines, we also observed a significant increase in TCA-cycle intermediates citrate and fumaric acid and an upward trend in malic acid, succinic acid, and α-ketoglutaric acid, suggesting enhanced TCA cycle activity toward the end of the

TdF. The stability of the most abundant acetylcarnitines and fatty acids further indicates that fatty acid oxidation was not reduced during the TdF. Instead, the selective reduction in L-carnitine likely reflects increased tissue uptake to support enhanced mitochondrial fatty acid transport. The observation that only fatty acids and acylcarnitines that typically occur in low concentrations in the blood were downregulated indicates selective adaptations in fatty acid transport and oxidation capacity throughout the TdF rather than global changes in lipid metabolism.

## DISCUSSION

Despite that elite endurance athletes live longer compared to the general population,[36] they present with multiple physiological responses that are associated with adverse health outcomes and that may persist beyond athletic retirement.[8] These negative effects are hypothesized to originate from excessive exercise-induced metabolic overload, yet the specific metabolic alterations resulting from excessive endurance exercise remain to be elucidated. By performing comprehensive metabolomic analysis on serum samples obtained during the TdF, we identified that excessive exercise induces profound remodeling of the circulating metabolome in elite athletes. In contrast to acute endurance exercise, which typically increases circulating metabolite availability,[31] the TdF predominantly decreased blood metabolite availability, with particularly pronounced effects on metabolites involved in fat metabolism (e.g., saturated/unsaturated fatty acid, free carnitine, and medium/long-chain acylcarnitines) and semi-essential amino acids (e.g., L-cysteine, glycine, L-proline, and L-tyrosine). Pathway analysis revealed that β-oxidation and glutathione metabolism were the most significant disrupted pathways. Furthermore, we identified 84 circulating metabolites that correlated with perceived fatigue, with cell membrane metabolites showing the strongest associations. These findings suggest novel biomarkers for training monitoring and provide mechanistic insight into fatigue development during excessive endurance exercise.

In total, 43% of the identified serum metabolites were altered during the TdF. The most profound metabolic remodeling occurred during the first 10 days of competition and was sustained throughout the TdF. Additional metabolic changes continued to occur in the second and third week of the TdF. Consequently, the metabolome at FINISH exhibited the greatest divergence from START, indicating a progressive and sustained disruption of metabolic homeostasis rather than transient adaptations. This extensive modulation of the circulating metabolome appears to be a hallmark of excessive exercise. For comparison, a 10-week endurance training intervention in recreational athletes consuming a diet with a high glycemic index induced changes in only 20% of the plasma metabolome.[37] While both excessive and moderate endurance training predominantly induce metabolite depletion rather than accumulation, the proportion of depleted metabolites is substantially greater following excessive (34%) versus moderate exercise (14%).[37] This finding suggests that during excessive endurance exercise, metabolite utilization exceeds athletes' capacity for replenishment. The underlying mechanism for this metabolic depletion likely relates to the extreme energetic demands imposed by grand tour cycling. Cyclists competing in the TdF operate at the upper limits of sustained human metabolic energy expenditure, with daily energy expenditure reaching 4–5 times the basal metabolic rate.[38–40] Such elevated rates of energy expenditure must be supported by primarily the oxidation of carbohydrates and fatty acids, suggesting that the adverse health effects of excessive endurance exercise stem from chronic overload of these metabolic pathways. Disturbed glucose metabolism appears to be an early indicator of non-functional overreaching and overtraining. This is evidenced by Petibois et al.[11] showing that one of the first metabolic signs of overtraining is a reduction in saccharide chains of glycoproteins, while other studies report attenuated blood lactate concentrations[41,42] and impaired glucose tolerance.[4] However, we found no evidence for dysregulations in glucose metabolism throughout the TdF (e.g., unaltered pyruvic acid levels). This observation should be interpreted cautiously, as our mass spectrometry platform did not profile all glycolytic intermediates, potentially obscuring specific metabolic alterations. This limitation stems from the phosphorylated state of many glycolytic metabolites, which causes intracellular retention resulting in circulating concentrations below mass spectrometry detection thresholds.[31]

Athletes diagnosed with overtraining syndrome (vs. well-trained athletes) exhibit significant disturbances in lipid metabolism, including decreased triglyceride, hepatic lipase, and HDL-C levels.[43] Furthermore, research indicated that a 4-week overload training period impaired intrinsic mitochondrial respiration in healthy adults.[4] In this respect, we also observed profound alterations in lipid metabolism, primarily manifested as reductions in specific saturated fatty acids during the first 10 days, accompanied by decreased L-carnitine, as well as medium- and long-chain acylcarnitines. However, given that the acylcarnitines (e.g., acetylcarnitine) and fatty acids (e.g., palmitic acid) that comprise the majority of their respective metabolic pools remained stable, the observed depletions appear to reflect selective metabolic adjustments in specific, less abundant lipids rather than global impairments of lipid metabolism. The observed depletion of L-carnitine and specific acylcarnitines pools can result from several mechanisms, including reduced biosynthesis of L-carnitine and specific fatty acids, diminished activity of carnitine palmitoyl transferase 1 (CPT1), or enhanced activity of L-carnitine/acylcarnitine translocase due to elevated rates of β-oxidation.[44] The latter appears to be the most plausible, given the observed increase in TCA cycle intermediates as well as the decrease in L-carnitine and especially saturated fatty acids. However, reduced availability of fatty acid precursors may also contribute to specific acylcarnitine depletion, as evidenced by the significant positive correlation between pentadecanoylcarnitine and pentadecanoic acid ($r = 0.58$, $p = 0.001$).

Notably, we observed a selective decrease in saturated fatty acids, while the majority of unsaturated fatty acids remained stable or even slightly increased. This pattern diverges from the typical metabolic response to exercise in healthy individuals, where unsaturated fatty acids are preferentially oxidized.[45] This discrepancy may be explained by the metabolic efficiency of saturated fatty acid oxidation, which requires fewer enzymatic steps compared to unsaturated fatty acids with identical carbon

chain length that need two additional energy-consuming reactions during β-oxidation.[46,47] Under conditions of metabolic overload, this preferential utilization of saturated fatty acids may represent an adaptive mechanism to maximize energy yield while minimizing metabolic cost. Our findings on lipid metabolism partially align with those of Nemkov et al.,[48] who reported reduced acylcarnitines levels after a 1-week cycling race in elite cyclists. However, unlike their study where fatty acids remained stable, we observed selective depletion of saturated fatty acids throughout the TdF, with acylcarnitines depleting primarily during the first week. Taken together, this pattern suggests a temporal sequence in metabolic adaptation to excessive endurance exercise, with initial carnitine pool depletion affecting fatty acid transport, followed by subsequent downregulation of saturated fatty acid mobilization or lipolysis.

Research strongly supports a link between serum carnitine status and fatigue development. Reduced plasma L-carnitine and acylcarnitine levels have been reported in patients with chronic fatigue syndrome,[44] post-COVID-19 syndrome,[49] and cancer-related fatigue.[50] Accordingly, L-carnitine supplementation has been shown to attenuate cancer-related fatigue.[51] While the precise mechanism underlying the counteractive effect of L-carnitine on fatigue is not yet completely understood, this likely involves restoration of mitochondrial bioenergetics, enhanced fatty acid oxidation capacity, and attenuation of oxidative stress and proinflammatory pathways.[49,51] Given our observation of decreased L-carnitine and specific acylcarnitines during the TdF, our findings suggest that L-carnitine supplementation may be a potent strategy to attenuate fatigue during periods of excessive endurance exercise.

Amino acids function as energy substrates through gluconeogenesis, facilitate skeletal muscle repair, and modulate central fatigue. All of these processes are particularly challenged during excessive endurance exercise. Despite their critical role in exercise performance and recovery, most studies report limited impact of excessive endurance exercise on the amino acid blood profile, with decreases in glutamine and BCAAs being the most consistently reported changes.[24–26] However, in the present study, 10 of 22 identified amino acids were significantly altered throughout the TdF, with these changes being already evident during the first 10 days of racing. Nemkov et al.[48] reported similar amino acid profile alterations in elite cyclists after a 1-week stage race. These parallel findings indicate that substantial amino acid perturbations occur early in response to excessive endurance exercise rather than developing gradually over prolonged periods. The discrepancies with earlier research likely reflect the extreme physiological demands of professional cycling stage races. In support of this, the daily energy expenditure during grand tours (31,700 kJ $day^{-1}$)[39] substantially exceeds that typically employed in overreaching/overtraining studies performed in recreational athletes (≤15,000 kJ $day^{-1}$),[52,53] potentially explaining the higher amino acid turnover in our study. This substantial energetic demand appears to drive rapid and significant amino acid mobilization and utilization that may not be captured in studies employing less extreme exercise protocols. This underscores the importance of conducting research in elite athletes under real-world competitive conditions to understand the true physiological impact of excessive endurance exercise.

Consistent with this heightened metabolic demand, we observed a significant decline in alanine levels during the initial 10 days, followed by stabilization. Given that alanine is the primary gluconeogenic amino acid, this suggests that there is an initial increase in gluconeogenic demand, followed by a metabolic adaption to preserve alanine pools despite continued metabolic demand.

Notably, three of the six semi-essential amino acids were decreased. While these amino acids are not directly involved as substrates or intermediates in muscle energy metabolism, they become essential whenever their metabolic requirement exceeds their endogenous synthesis capacity.[54] Deficiencies in these amino acids frequently occur during periods of catabolic stress such as surgery, illness, and injury, while supplementation is effective in restoring physiological functions such as tissue repair,[55] nitrogen balance,[54] and immune function.[54,56] These data underscore earlier research indicating that the TdF represents a highly catabolic event[18] and suggest that targeted amino acid supplementation may be beneficial for maintaining metabolic homeostasis during extreme exercise.

Cysteine was the most depleted semi-essential amino acid, exhibiting a continuous decline throughout the TdF across all cyclists. Similar patterns have been observed in blood serum during a 6-day cycling race[48] and in urine during a grand tour in elite cyclists.[57] This cysteine depletion likely results from increased reactive oxygen species (ROS) production, which has been associated with the sustained aerobic energy production during prolonged cycling.[58] Glutathione, the primary intracellular antioxidant, plays a critical role in mitigating ROS-induced damage. As cysteine is the rate-limiting substrate for glutathione synthesis, the progressive depletion of cysteine likely reflects heightened glutathione production to buffer oxidative stress.[59] Furthermore, excessive ROS production during prolonged endurance exercise likely contributes to the adverse cardiovascular effects associated with excessive endurance exercise.[3,60]

Another potential cause for the markedly reduced cysteine levels is increased utilization together with pantothenic acid for coenzyme A (CoA) synthesis.[61] Recent research showed that cysteine depletion in mice reduces liver and muscle CoA levels, resulting in mitochondrial dysfunction and metabolic inefficiency.[62] The depleted cysteine levels during excessive exercise may therefore represent a mechanistic cause of the mitochondrial dysfunction previously reported with excessive exercise,[4] as CoA is essential for mitochondrial fatty acid oxidation and energy production. Interestingly, N-acetyl-cysteine, a widely used supplement to elevate blood cysteine levels, has been shown to enhance both glutathione status and endurance performance, particularly in fatigued states.[63] Consequently, N-acetyl-cysteine supplementation may provide a promising strategy to both enhance endurance performance and attenuate the negative health effects of excessive endurance exercise by upregulating antioxidant capacity and potentially preserving CoA levels.

Both our study and that of Nemkov et al.[48] demonstrated pronounced declines in serum glycine levels among the cyclists. Glycine, together with L-arginine and methionine, constitutes a critical precursor for creatine biosynthesis. We also observed a trend toward L-arginine depletion after the first 10 days of racing.

Research has established that glycine supplementation delays physical fatigue most likely by enhancing muscle creatine content.[64,65] While our study did not quantify creatine concentrations, Nemkov et al.[48] reported modest reductions in serum creatine levels during stage racing.[48] Although creatine supplementation has traditionally been employed to enhance maximal force production and high-intensity exercise performance,[66] our findings suggest potential ergogenic and protective effects of glycine or creatine supplementation. The observed L-arginine reduction may also have significant implications for vascular health during extreme exercise. As the obligate substrate for endothelial nitric oxide synthase, L-arginine availability directly affects nitric oxide production, vascular tone, and endothelial function.[67] As a result, compromised L-arginine status could potentially exacerbate exercise-induced vascular stress, contributing to adverse cardiovascular remodeling associated with chronic excessive endurance training.

A notable finding in our study was the significant depletion of N-acylethanolamines (NAEs) during the first two weeks of the TdF. This decrease was particularly pronounced for LEA and OEA, while stearoyl ethanolamide and palmitoylethanolamide showed only modest reductions. LEA and OEA regulate multiple biological processes, with their primary function being the induction of satiety signals that reduce food intake and body weight in animal models.[68,69] The observed depletion of these NAEs may represent an adaptive response to the extreme metabolic demands of prolonged endurance exercise, potentially maintaining energy balance by attenuating satiety signals and promoting food intake. The mechanism underlying this decline remains to be fully elucidated, but the substantial reductions suggest either decreased biosynthesis or accelerated catabolism during intense exercise. The strong positive correlation between OEA and LEA serum levels ($r = 0.97$) indicates a shared regulatory pathway through which exercise modulates their bioavailability. Importantly, we observed no corresponding decrease in phosphatidylethanolamines, their primary precursors, suggesting that enhanced degradation rather than reduced synthesis drove their depletion.

Beyond characterizing metabolic alterations induced by excessive exercise, we performed correlation analyses to identify potential biomarkers of fatigue. Given that 6 out of 7 cyclists demonstrated a progressive increase in fatigue throughout the TdF, some observed correlations may simply reflect the parallel temporal progression of both fatigue accumulation and metabolic changes rather than mechanistic relationships. Nonetheless, while causality cannot be inferred from these associations, our analyses revealed numerous metabolites strongly correlating with perceived fatigue, which warrant further exploration in controlled settings where inter-individual variability in fatigue responses can be better characterized to distinguish mechanistic relationships from temporal coincidence.

Nevertheless, several identified metabolites have established links to fatigue. Dodecanedioic acid, one of the most strong and negatively correlated metabolites, has demonstrated efficacy in alleviating muscle fatigue in diabetic patients.[20] As an even-chain dicarboxylic acid, dodecanedioic acid differs from conventional fatty acids because its β-oxidation yields both acetyl-CoA and the gluconeogenic intermediate succinyl-CoA.[70] By providing intermediate substrates for both mitochondrial oxidation and ATP production, the metabolic versatility of dodecanedioic acid makes it valuable for individuals with reduced metabolic flexibility, such as type 2 diabetic patients.[70] Given that disturbances in carbohydrate metabolism typically represent the earliest metabolic disruptions during prolonged intense exercise,[11] increased dodecanedioic acid oxidation may serve as a compensatory mechanism, providing anaplerotic substrates for mitochondrial oxidation while preserving muscle glycogen. Another notable finding is the positive correlation between TCA cycle intermediates (citric acid and fumaric acid) and perceived fatigue. This relationship likely reflects a metabolic shift toward enhanced fatty acid oxidation to compensate for diminished glycolytic capacity during overreaching/overtraining states.[41,42] This adaptation may contribute to fatigue development through increased oxygen cost at a given exercise intensity, as fat oxidation requires ~7% more oxygen than carbohydrate metabolism to produce equivalent ATP.

The interpretation of our metabolomic findings must be considered within the context of general nutritional interventions implemented following each stage during the TdF. The most profound impact on the metabolome is expected from the post-exercise recovery shakes containing carbohydrates and whey protein, as well as tart cherry juice. The recovery shakes likely affected the amino acid metabolism patterns observed in the current study as these are rich in essential amino acids. Furthermore, the systematic intake of tart cherry juice, which contains high concentrations of polyphenolic antioxidants,[71] may have influenced pathways involved in antioxidant defense. However, despite this antioxidant supplementation, we observed significant depletion of cysteine, the rate-limiting substrate for glutathione synthesis,[59] suggesting that oxidative stress exceeded the protective capacity of dietary antioxidant intake. This finding indicates that even standardized antioxidant supplementation may be insufficient to counteract the extreme oxidative demands of excessive endurance exercise. Importantly, these nutritional interventions represent standard practices within professional cycling and therefore enhance the ecological validity of our findings.

## Limitations of the study

This study provides unique insights into the metabolic signature of elite male cyclists during one of the most extreme endurance competitions. While the ecological model offers high validity for professional cycling, the findings are specific to this elite population and may differ in recreational or female athletes, or during less extreme training loads. Future studies in larger, more diverse cohorts would help establish which metabolic responses represent universal markers of excessive endurance exercise.

Our analyses did not account for plasma volume expansion associated with intensified endurance training, which could potentially underestimate decreases and overestimate increases in metabolite concentrations. However, due to years of intensive training, plasma volume expansion during a grand tour is minimal (~1%–2%) in elite athletes.[72] Furthermore, plasma volume expansion primarily occurs during the first week of intensified training,[73] while most metabolites in our study continuously suppressed throughout the TdF. This indicates that the observed alterations

in metabolite concentrations predominantly represent true biological alterations rather than hemodilution effects. Furthermore, because we were interested in the chronic effects rather than the acute effects of excessive exercise on the circulating metabolome, blood samples were collected in the morning in the fasted state. However, it cannot be entirely ruled out that the metabolome was still partially affected by subacute changes that resulted from the cycling stage on the previous day. This impact is likely minimal, as the observed alterations in the blood metabolome were largely opposite to those typically observed in the first hours following prolonged endurance exercise.[31] Additionally, our metabolomic coverage identified 274 named compounds, which is comparable to another study with professional cyclists[48] but modest compared to other platforms capable of detecting >600 metabolites. This limitation restricts comprehensive pathway-level insights and makes direct benchmarking against broader metabolomic studies challenging. Future studies would benefit from expanded metabolomic coverage to provide more comprehensive insights into the systemic metabolic impact of excessive endurance exercise. Furthermore, complementary approaches such as plasma proteomics would provide additional mechanistic depth and broaden the range of detectable systemic changes induced by excessive endurance exercise.

## RESOURCE AVAILABILITY

### Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Chiel Poffé (chiel.poffe@uhasselt.be).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

- All serum metabolomics and perceived fatigue data have been deposited at Mendeley Data repository (Mendeley data: https://doi.org/10.17632/5v79d6whz4.1) and are publicly available.
- This article does not report original code.

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## AUTHOR CONTRIBUTIONS

R.R., K.P., P.J., and C.P. conceived and designed the study; R.R., W.L., M.S., P.J., and C.P. performed the experiments; R.R. and C.P. analyzed the data; R.R. and C.P. prepared figures and drafted the manuscript; R.R., W.L., M.S., K.P., P.J., and C.P. revised and approved the final version of the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:



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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Biological samples | | |
| Human serum samples | N/A | N/A |
| Deposited data | | |
| Metabolomics data | Mendeley Data | https://doi.org/10.17632/5v79d6whz4.1 |
| Perceived fatigue data | Mendeley Data | https://doi.org/10.17632/5v79d6whz4.1 |
| Software and algorithms | | |
| MetaboAnalyst | Pang et al.[74] | Version 6.0 |
| GraphPad Prism | https://www.graphpad.com/ | Version 10.6.1 |

## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Eight professional male cyclists from the same Union Cycliste Internationale (UCI) World Tour team were recruited before their participation in the Tour de France 2023 (TdF). One cyclist was not included in the final data analyses due to a crash during the first week of the TdF, resulting in exclusion from the final data analysis. Seven cyclists completed the entire TdF and were included in all analyses. The study was approved by the Ethics Committee Research UZ/KU Leuven (s67595), and conforms to the declaration of Helsinki, and prospectively registered at Clinicaltrials.gov (NCT06386900). Prior to the start of the TdF, cyclists were thoroughly informed about the aims, procedures, and potential risks, after which they provided written informed consent without pressure from team or investigators. All cyclists (mean ± SD: age: 31 ± 6 years, height: 1.83 ± 0.07 m, body mass: 73.0 ± 6.7 kg, ethnicity: Caucasian) completed the entire TdF. Their daily nutritional schedule was designed by qualified sports nutritionists, and was highly similar among the cyclists. In addition to their daily meals, sports nutrition, and snacks, the cyclists received the following supplements: multivitamins, antioxidants, ketone ester, and beetroot juice, aimed at enhancing recovery and performance during the TdF.

## METHOD DETAILS

### Venous blood sampling

Upon voluntary waking up, cyclists rested for 10 minutes in a seated position on their bed. Subsequently, venous blood samples were collected from an antecubital vein (Venoject, Terumo, Tokyo, Japan) into vacuum tubes with silica clot activators (10 mL) by the team physician [Becton Dickinson (BD) Vacutainer]. Tubes were maintained at room temperature for 20 minutes to allow clotting, then centrifuged (1500 rpm, 4°C) for 15 minutes. The serum supernatant was transferred to Eppendorf tubes, transported on dry ice to Belgium, and stored at -80°C until analysis at a later timepoint.

### Targeted and untargeted metabolomics

#### *Untargeted metabolomics*

Samples were extracted using a methanol-chloroform liquid-liquid extraction method. Briefly, 50 μL of serum sample was mixed with 450 μL of extraction buffer (100% methanol with internal standards) and stored overnight at -80°C for protein precipitation. After centrifugation at 17 000 × g for 10 minutes, 300 μL of supernatant was transferred to a fresh tube and mixed with 300 μL chloroform followed by vortexing for 60 seconds. Phase separation was induced by adding 200 μL $H_2O$ and brief vortexing for 15 seconds, then centrifuged for 5 minutes at 5 000 × g. The upper polar phase (200 μL) was transferred to MS vials, while the lower apolar phase was dried using a CentriVap system (Labconco, USA) and reconstituted in 100% ethanol before transfer to MS vials. For both phases, a quality control (QC) sample was made by pooling together 10 μL of each sample. Mass spectrometry measurements were performed using an Infinity II autosampler and pump system (Agilent, USA) coupled to a ScimaX 7T Magnetic Resonance Mass Spectrometer (Bruker, USA) operated in both negative and positive ionization modes. A volume of 10 μL was injected by flow injection analysis using 80% 2-propanol as pushing solvent. Samples were measured in technical triplicates with QC samples measured before, during, and after sample analysis for batch control. The mass spectrometer operated in full scan mode (m/z range: 107-1000) with 35 scans averaged per spectrum at approximately 1 million resolution at 120 m/z. Operating parameters included spray voltages of +4700 V (negative mode) and -4800 V (positive mode), capillary temperature of 200 °C, dry gas at 2 L/min, nebulizer gas at 1 bar, and ion accumulation time of 0.100 s. Data collection was performed using ftmsControl and HyStar software (Bruker, USA), with initial data analysis by MetaboScape 2022b (Bruker, USA) for peak picking, feature detection, and annotation using the Human Metabolome Database (HMDB).

### *Targeted metabolomics*

50 μL of each serum sample was mixed with 450 μL of extraction buffer (100% methanol with internal standards) and stored overnight at -80°C for protein precipitation. After centrifugation at 17 000 × g for 10 minutes, the methanol extract supernatant was used directly for analysis without further liquid-liquid extraction. A volume of 10 μL from each processed sample was analyzed using a Dionex UltiMate 3000 LC System (ThermoFisher Scientific, Bremen, Germany) equipped with a C-18 column (Acquity UPLC – HSS T3, 1.8 μm; 2.1 × 150 mm, Waters) connected to a Q Exactive Orbitrap mass spectrometer (ThermoFisher Scientific) operating in negative ion mode. The separation utilized a step gradient with two solvents: Solvent A (10 mM TBA and 15 mM acetic acid) and Solvent B (pure methanol). The gradient started with 5% of solvent B and 95% solvent A, maintained until 2 minutes post-injection. A linear gradient to 37% B was carried out until 7 minutes and increased to 41% until 14 minutes. Between 14 and 26 minutes, the gradient increased to 95% B and remained at this level for 4 minutes. At 30 minutes, the gradient returned to 5% B, and chromatography concluded at 40 minutes. The flow rate remained constant at 0.25 mL/min with column temperature maintained at 40 °C throughout the analysis. The mass spectrometer operated in full scan mode (m/z range: 70-1050), using a spray voltage of 4.80 kV, capillary temperature of 300 °C, sheath gas at 40.0, and auxiliary gas at 10.0. The automatic gain control target was set at $3.0 \times 10^6$ using a resolution of 140 000 with a maximum ion trap fill time of 512 ms. Data collection was performed using ftmsControl and HyStar software (Bruker, USA), with initial data analysis by MetaboScape 2022b (Bruker, USA) for peak picking, feature detection, and annotation using the Human Metabolome Database (HMDB).

## QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were conducted using MetaboAnalyst software (version 6.0)[74] and the Pingouin Python package (version 0.5.5) on log-transformed data. Spearman correlation coefficients were calculated using GraphPad Prism (version 10.4.1) on raw data. Heatmaps were generated using the Matplotlib Python package (version 3.9.0). All figures were created using Graphpad Prism (version 10.5.0). All analyses included n=7 professional male cyclists who completed the entire TdF and provided blood samples at the four timepoints. Data are presented as mean ± standard deviation (SD) unless otherwise stated. All statistical details, including the statistical test, n values, and significance levels, are provided in the figure legends. Changes in metabolite concentrations over time were analysed using one-way repeated measures ANOVA with false discovery rate (FDR) to control for multiple comparisons. For significant one-way ANOVA effects, post-hoc pairwise comparisons were conducted using Šidák correction. Spearman correlation coefficients were calculated to assess relationships between metabolites and perceived fatigue, with FDR correction applied. Statistical significance was set at $p < 0.05$ for all analyses, except for volcano plot analyses where $p < 0.10$ was used.

## ADDITIONAL RESOURCES

Clinical trial registration: This study was prospectively registered at ClinicalTrials.gov under identifier NCT06386900: https://clinicaltrials.gov/study/NCT06386900.