scientific reports

Check for updates

OPEN

Muscle transcriptome profiles in elite male ultra-endurance athletes acclimated to a high-carbohydrate versus low-carbohydrate diet

Catherine Saenz[1,4,5]✉, Kaleen M Lavin[2,5], Elaine C Lee[3], Carl M Maresh[1], William J Kraemer[1], Marcas M Bamman[2], Timothy J Broderick[2] & Jeff S Volek[1]

Low-carbohydrate, high-fat diets enhance lipid metabolism and decrease reliance on glucose oxidation in athletes, but the associated gene expression patterns remain unclear. The purpose of this study was to determine whether coordinated molecular pathways in skeletal muscle may be revealed by differential expression of genes driven by dietary profile, exercise, and/or their interaction. We investigated the skeletal muscle transcriptome in elite ultra-endurance athletes habitually (~ 20 months) consuming a high-carbohydrate, low-fat (HC, *n* = 10, 33 ± 6y, VO2max = 63.4 ± 6.2 mL O2•kg-1•min-1) or low-carbohydrate, high-fat (LC, *n* = 10, 34 ± 7y, VO2max = 64.7 ± 3.7 mL O2•kg-1•min-1) diet. Skeletal muscle gene expression was measured at baseline (BL), immediately-post (H0), and 2 h (H2) after 3 h submaximal treadmill running. Diet induced a coordinated but divergent expression pattern at BL where LC had higher expression of genes associated with lipid metabolism. Exercise resulted in a dynamic but uniform gene response, with no major differences between groups (H0). At H2, gene expression patterns were associated with differential pathway activity, including inflammation/immunity, suggesting a diet-specific influence on early muscle recovery. These results indicate that low-carbohydrate, high-fat diets lead to differences in resting and exercise-induced skeletal muscle gene expression patterns, underlying our previous findings of differential fuel utilization in elite ultra-endurance athletes.

Previous studies have consistently reported that several weeks of very low-carbohydrate intake leads to marked increases in fat oxidation at rest and during exercise beyond that achieved with endurance training alone[1]indicating that maximal rates of fat oxidation during exercise are achieved only when the habitual diet is very low in carbohydrates. Peak fat oxidation rates have been shown to more than double from less than 1 g/min in athletes consuming moderate-to-high-carbohydrate diets to mean values above 1.5 g/min and approaching 2 g/min in some athletes consuming lower-carbohydrate diets[2,3]. There is a dearth of research focused on elucidating the molecular details surrounding the cellular machinery required to support the shift away from glucose oxidation to a high flux of fatty acid and ketone metabolism in the face of low exogenous carbohydrate intake.

[1]Department of Human Sciences, The Ohio State University, Columbus, OH, USA. [2]Florida Institute for Human and Machine Cognition, Pensacola, FL, USA. [3]Department of Kinesiology, University of Connecticut, Storrs, CT, USA. [4]College of Education and Human Ecology, Department of Human Science, The Ohio State University, Exercise Science Program A048 PAES Building 305 Annie & John Glenn Avenue, Columbus, OH 43210, USA. [5]Catherine Saenz, Kaleen M Lavin have contributed equally to this work. ✉email: saenz.11@osu.edu

Keto-adaptation refers to the global process of adapting to a very low-carbohydrate diet that achieves a sustained state of nutritional ketosis[4]. While enhanced fat oxidation is a hallmark of keto-adaptation, the time course and full description of genetic, metabolic and physiological alterations that take place remain relatively unexplored, particularly in humans. Aspects of nutritional ketosis, such as elevated ketogenesis and circulating beta-hydroxybutyrate (BHB) levels between 0.5 and 5.0 mmol/L[4], appear within the first week of starting a ketogenic diet[5]. In contrast, several weeks to months appear to be required for endurance exercise performance and other features such as preservation of glycogen to manifest[2,6]. Until recently, there was minimal research conducted on keto-adapted athletes who maintained a ketogenic diet beyond four weeks. In the Fat-Adapted Substrate Use in Trained Elite Runners (FASTER) study, we reported metabolic differences in high-level competitive ultra-endurance athletes who habitually consumed either a high-carbohydrate, low fat (HC) or a very, high-fat (LC) eating pattern[2]. Despite similar competitive achievements, training regimens, and maximal aerobic capacities between groups, the keto-adapted athletes demonstrated peak fat oxidation rates 2.3-fold higher (HC: 0.67 ± 0.14 g/min vs. LC: 1.54 ± 0.18 g/min)[2], which occurred at a higher percentage of VO_2max. Previous research consistently describes similar metabolic shifts in keto-adapted athletes[7]. These works often report decreased skeletal muscle glycogen stores combined with decreased carbohydrate and increased fat oxidation rates[2]. One unexpected finding in FASTER was that there were no differences in skeletal muscle glycogen concentrations at any time point between groups including when compared at rest, immediately following a 180-minute run (H0), and 120 min into recovery(H2)[2]. In contrast from previous research, this study is one of the few to investigate metabolic shifts in ultra-endurance athletes habitually consuming a ketogenic diet while competing as opposed to more short-term dietary shifts, suggesting that long-term keto-adaptation results in a remarkable conservation of muscle glycogen, most likely by repurposing carbons derived from non-carbohydrate sources such as lactate and glycerol. Additionally, evidence suggests gene expression shifts associated with carbohydrate restriction, independent of fat content, are linked to muscle glycogen levels (i.e., the lower the muscle glycogen, the more robust the gene expression patterns associated with fat oxidation)[8]. Given the similar muscle glycogen patterns demonstrated between low- and high-carbohydrate athletes in our previous report on FASTER[2], it might be assumed that differences in gene expression would also be attenuated.

Beyond promoting high-fat oxidation rates and decreasing reliance on glucose oxidation, keto-adaptation is associated with cellular, molecular, and tissue-specific effects extending beyond substrate utilization[9–11]with unique impacts on skeletal muscle tissue and metabolism[12–14]. Much of this body of work has been investigated in murine models. For example, Ma et al. found gene expression profiles associated with fat oxidation, ketolysis, recovery, and endurance capacity were all upregulated in mice after an 8-week ketogenic diet and consistent endurance exercise regime[10]. Interestingly, these changes were explicitly observed in slow-twitch muscle fibers, the fibers with the highest oxidative capacity, which are fully recruited during endurance exercise. It is important to note that energy metabolism, and by extension ketoadatpation, in murine models does not mirror nutritional ketosis in humans, particularly as it relates to oxidative capacity[15], limiting this body of work's translatability. A limited number of investigations in humans have used targeted approaches to assess underlying molecular adaptations to short-term carbohydrate restriction (2–5 days) combined with acute, endurance exercise bout[16–18]but this information does not account for the distinct and uniform phenotypes observed with longer-term, chronic carbohydrate restriction (> 6 mo.). There is one study that compared circulating and skeletal muscle glucose metabolism in trained cyclists following either a low-carbohydrate, high fat diet or a more traditional, mixed diet for a minimum of 6 months. The authors reported differences in glucose tolerance molecular metabolism between the two dietary groups[19] but the underlying global gene expression signatures related to the observed differences were not explored.

To shed light on gene expression patterns in response to high and low carbohydrate habitual diets and the acute response to exercise, we performed transcriptome analysis on skeletal muscle tissue obtained from participants in FASTER to probe potential differences in underlying mechanisms observed in keto-adapted ultra-endurance athletes. The purpose of this study was to determine whether coordinated molecular pathways in skeletal muscle may be revealed by differential expression of genes[20] driven by dietary pattern, exercise, and/or their interaction. In contrast to a hypothesis-driven approach, this transcriptomics exploration was a discovery, hypothesis-generating analysis to help propel the field forward.

Results

Twenty highly trained and accomplished male ultra-endurance athletes participated in this study. Participant characteristics and primary metabolic results have been previously reported[2]. Relevant physical and metabolic characteristics are presented again here in Table 1. Athletes were habitually following their respective dietary approach for an average of 20 months (range 9–36 months) and were matched for training styles and competition performance. The primary difference between the two groups was their habitual diet patterns, which were assessed using detailed 3-day diet records. There were no differences in total calories consumed (HC (kcal): 2756 ± 638; LC (kcal): 2721 ± 700). There were significant differences in macronutrient composition between HC and LC groups, specifically higher fat and lower carbohydrate content in the LC group (Fig. 1). As previously reported, the LC group exhibited significantly higher fat oxidation rates at rest and during submaximal exercise[2] (Table 1)

	High-Carbohydrate Diet Athletes *(n=10)*	Low-Carbohydrate Diet Athletes *(n=10)*
Age (y)	33 ± 6	34 ± 7
Height (cm)	174 ± 5	176 ± 8
Weight (kg)	66.5 ± 6.8	68.8 ± 8.2
Body Fat (%)	9.6 ± 4.3	7.8 ± 2.4
VO_2max (L/min)	4.25 ± 0.46	4.46 ± 0.39
Competitive Running Experience (y) *Distance Ran during 180 min Treadmill Run (km)*	9 ± 6 37 ± 3.9	11 ± 8 35.6 ± 2.1
Baseline Metabolic Characteristics		
Muscle Glycogen (mmol/kg wet weight)	143.1 ± 50.9	140.5 ± 49.2
Fat Oxidation (mean g/min)*	0.083 ± 0.031	0.117 ± 0.030
Carbohydrate Oxidation (mean g/min)*	0.100 ± 0.060	0.027 ± 0.071
H0 Metabolic Characteristics		
Muscle Glycogen (mmol/kg wet weight)	54.3 ± 14.7	47.2 ± 17.1
Fat Oxidation (mean g/min)*	0.866 ± 0.118	1.213 ± 0.163
Carbohydrate Oxidation (mean g/min)*	1.159 ± 0.253	0.436 ± 0.246
H2 Metabolic Characteristics		
Muscle Glycogen (mmol/kg wet weight)	89 ± 27.3	92 ± 19.9
Fat Oxidation (mean g/min)*	0.131 ± 0.037	0.171 ± 0.032
Carbohydrate Oxidation (mean g/min)*	0.089 ± 0.058	−0.028 ± 0.066

Table 1. Participant physical and metabolic characteristics. Values are means ± standard deviations. Some values have been previously reported in publications by our research team. *Denotes $P<0.05$ between diet groups at a given time point. BL: baseline, H0: immediately postexercise, H2: 120 min postexercise.

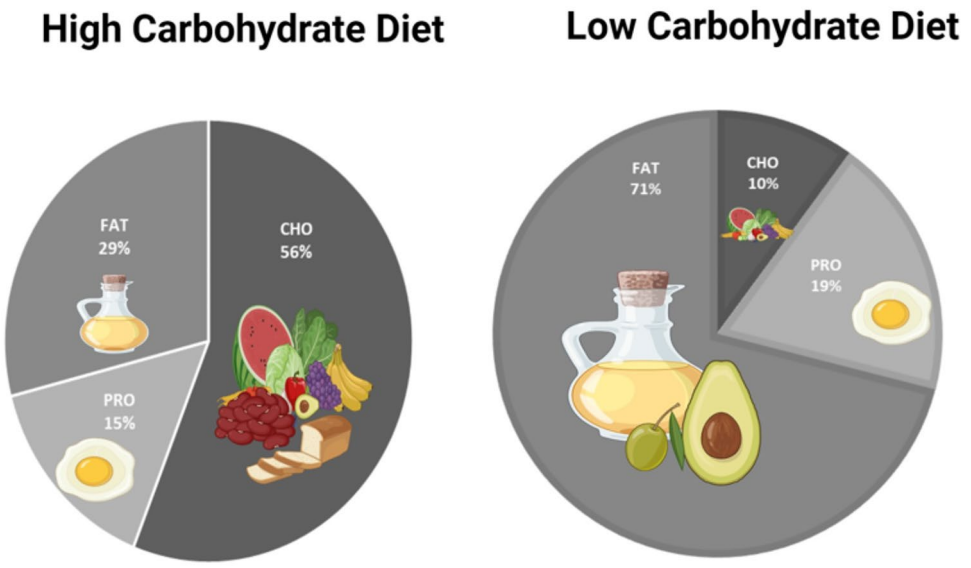

Fig. 1. Habitual macronutrient dietary profiles for LC and HC athletes. LC athletes had significantly higher dietary fat while consuming lower dietary carbohydrate but no difference in dietary protein intake when compared to HC (p <0.05). This figure was created using BioRender.com.

Skeletal muscle gene expression patterns

A total of 25,282 genes were reliably sequenced across all muscle samples. A group-by-time analysis of variance revealed main effects for diet in 653 (2.6%) transcripts ($p<0.01$, FDR < 0.05) and for exercise in 8,413 (33.3%) transcripts ($p<0.01$, FDR < 0.05). Diet-by-exercise interaction was seen for the expression levels of 312 transcripts ($p<0.01$, FDR < 0.05). Specific comparisons of interest were investigated in follow-up post-hoc analysis using Benjamini-Hochberg adjustment for multiple comparisons and are described below.

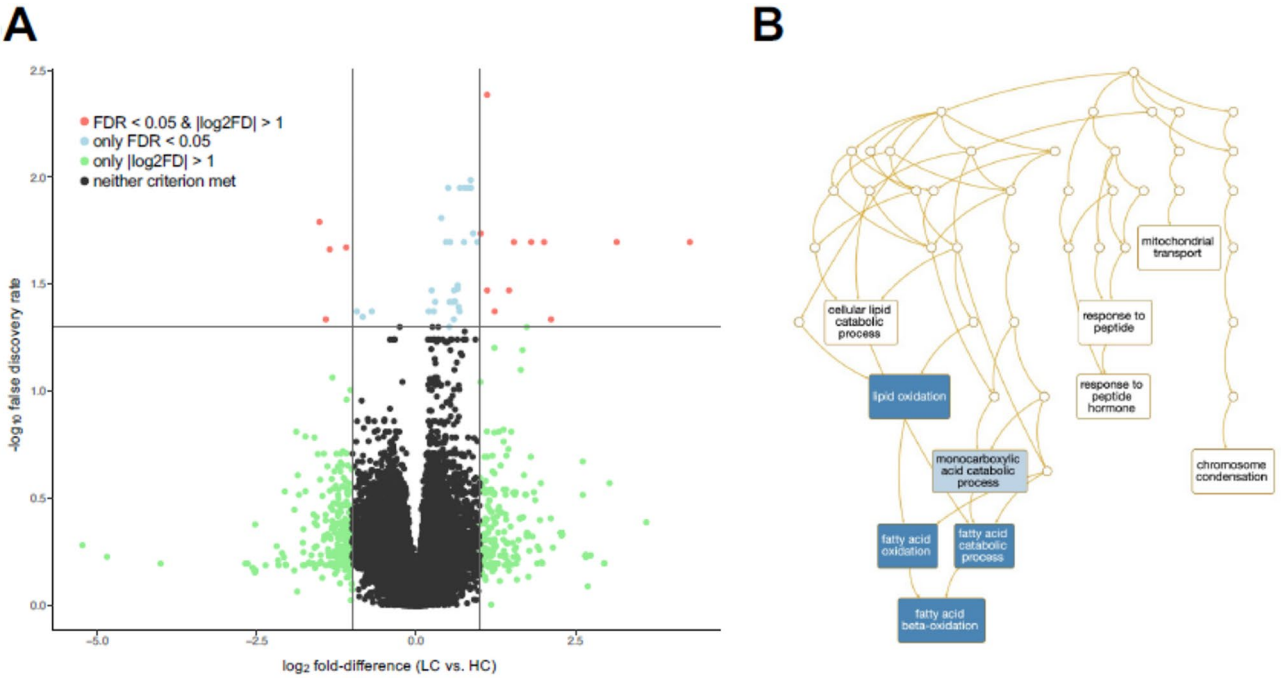

Fig. 2. Volcano plot showing differential gene expression in skeletal muscle of elite ultra-endurance athletes adapted to low-carbohydrate (LC) or high-carbohydrate (HC) diet. Color denotes whether individual gene reached false-discovery rate (FDR)-level significance, $\log_2$fold-difference between groups, both, or neither. **3B**: gene ontology for processes associated with genes more highly expressed in muscle of individuals adapted to LC diet.

HC and LC athletes demonstrated divergent gene expression patterns at Rest

To determine differences in skeletal muscle gene expression between athletes adapted to the two diets at rest (i.e., prior to exercise), differentially expressed genes (DEG) were identified using ANOVA, with post-hoc tests focused on differential expression between LC and HC at baseline. Baseline skeletal muscle gene expression patterns differed between HC and LC (Fig. 2A). Seven genes showed lower expression, and 37 genes had higher expression in LC vs. HC. Of the seven genes with lower expression, 4 had a $\log_2$FD of < -1, and of the 37 genes with higher expression, 11 had $\log_2$FD $> +1$. No DEGs more lowly expressed in LC annotated to known protein-coding genes. However, several DEGs higher in LC vs. HC were associated with fatty acid beta-oxidation via overrepresentation analysis (Fig. 2B, nominal $P < 0.0001$). These included acetyl-CoA acyltransferase 2 (*ACAA2*, +1.6-fold higher in LC-adapted muscle), enoyl-CoA hydratase 1 (*ECH1*, +1.8-fold in LC), and hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha (*HADHA*, +1.7-fold in LC). Additionally, 3-hydroxymethylglutaryl-CoA synthase 2 (HMGCS2), involved in ketogenesis, demonstrated the highest fold difference between LC and HC athletes (+4.3-fold). This difference held up at the immediate post-exercise (H0) time point (+4.9fold higher in LC-adapted muscle) but was diminished 2 h post-exercise (H2) (Table 2).

Exercise resulted in a dynamic response in Gene expression

Exercise-responsive DEGs were identified based on significant change from baseline within a diet group at each postexercise timepoint. Overlapping patterns in the time course of diet-dependent gene expression are shown in Fig. 3A. Of the 44 DEGs at baseline, 17 remained more highly expressed in LC than HC at the H0 time point, whereas 19 additional DEGs were identified (16 upregulated, 3 downregulated). Only 1 gene [*PPP1R1A* (protein phosphatase 1 regulatory inhibitor subunit 1 A] was higher in LC muscle 2 h post-exercise; this gene was different across all time points between diets (Fig. 3B**).** Table 2 lists all DEGs between diets at each time point based on FDR < 0.05 and $|\log_2 FC| > 1$.

Figure 4A and Fig. 4B represent numbers of unique and shared DEGs within each time point relative to the baseline for each diet group. For example, 330 genes were differentially expressed vs. baseline in HC only. These are primarily annotated to biological pathways including osteoblast differentiation, angiogenesis, and bone morphogenic protein (BMP) signaling. Relevant DEGs included SRY-box transcription factor 8 (*SOX8*),

	ENSEMBL Gene ID	Gene Symbol	Gene Name (if available)	log_2fold-difference
BL	ENSG00000134240	HMGCS2	3-Hydroxy-3-Methylglutaryl-CoA Synthase 2	4.30
	ENSG00000237560	AC004562.1	*[unavailable]*	3.15
	ENSG00000153303	FRMD1	FERM Domain Containing 1	2.12
	ENSG00000130707	ASS1	Argininosuccinate Synthase 1	2.01
	ENSG00000233947	RP11-336N8.2	*[unavailable]*	1.81
	ENSG00000185507	IRF7	Interferon Regulatory Factor 7	1.54
	ENSG00000266049	RP11-703M24.5	*[unavailable]*	−1.52
	ENSG00000253844	RP11-546K22.1	*[unavailable]*	1.46
	ENSG00000261480	RP11-578F21.6	*[unavailable]*	−1.42
	ENSG00000232344	AC087163.2	*[unavailable]*	−1.35
	ENSG00000183780	SLC35F3	Solute Carrier Family 35 Member F3	1.23
	ENSG00000197576	HOXA4	Homeobox A4	1.12
	ENSG00000135447	PPP1R1A	Protein Phosphatase 1 Regulatory Inhibitor Subunit 1 A	1.12
	ENSG00000250137	RP11-380P13.1	*[unavailable]*	−1.10
	ENSG00000175567	UCP2	Uncoupling Protein 2	1.02
H0	ENSG00000134240	HMGCS2	3-Hydroxy-3-Methylglutaryl-CoA Synthase 2	4.92
	ENSG00000237560	AC004562.1	*[unavailable]*	4.14
	ENSG00000233947	RP11-336N8.2	*[unavailable]*	2.17
	ENSG00000130707	ASS1	Argininosuccinate Synthase 1	2.09
	ENSG00000153303	FRMD1	FERM Domain Containing 1	1.95
	ENSG00000237437	ASS1P12	Argininosuccinate Synthetase 1 Pseudogene 12	1.86
	ENSG00000154102	C16orf74	Chromosome 16 Open Reading Frame 74	1.66
	ENSG00000185507	IRF7	Interferon Regulatory Factor 7	1.24
	ENSG00000100100	PIK3IP1	Phosphoinositide-3-Kinase Interacting Protein 1	1.17
H2				
	ENSG00000135447	PPP1R1A	Protein Phosphatase 1 Regulatory Inhibitor Subunit 1 A	1.19

Table 2. Differentially expressed genes in skeletal muscle of low-carb diet adapted athletes vs. high-carb adapted athletes at each study time point. All genes are low-carb diet (LC) vs. high-carb diet (HC), such that a positive log2fold-difference indicates higher expression in LC. BL: baseline, H0: immediately postexercise, H2: recovery (2 h postexercise).

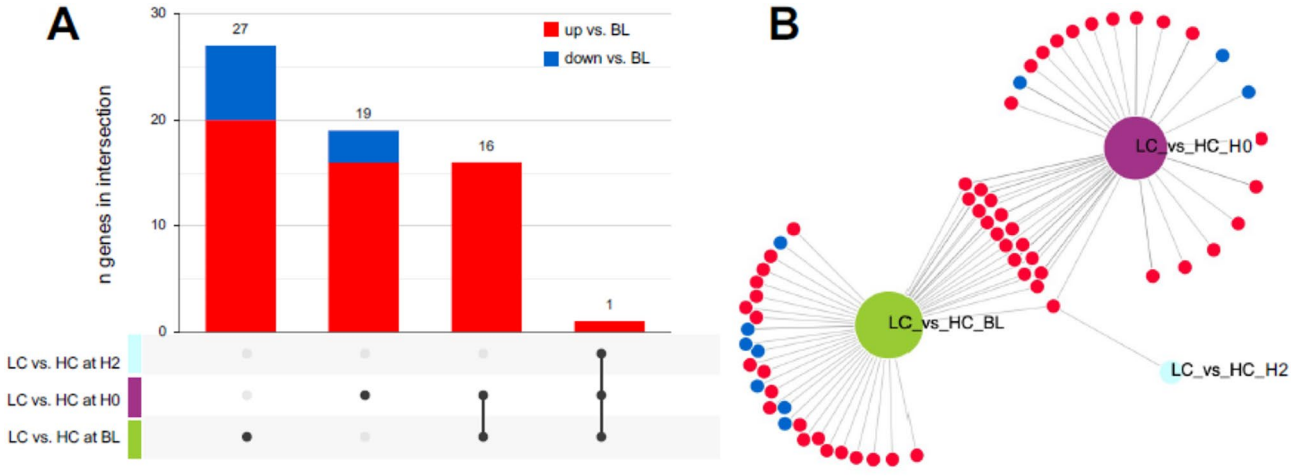

Fig. 3. A UpsetR plot showing numbers of genes differentially expressed between diet groups at each time point, with intersections (overlaps) denoted by lines connecting rows of the bottom panel. Downregulated genes are shown in blue and upregulated in red. B: DiVenn diagram showing differentially expressed genes between diets at each time point. Each cluster corresponds to a bar on the chart in panel A. HC: high-carbohydrate, LC: low-carbohydrate; BL: baseline; H0: immediately postexercise, H2: 120-min postexercise (2 h recovery).

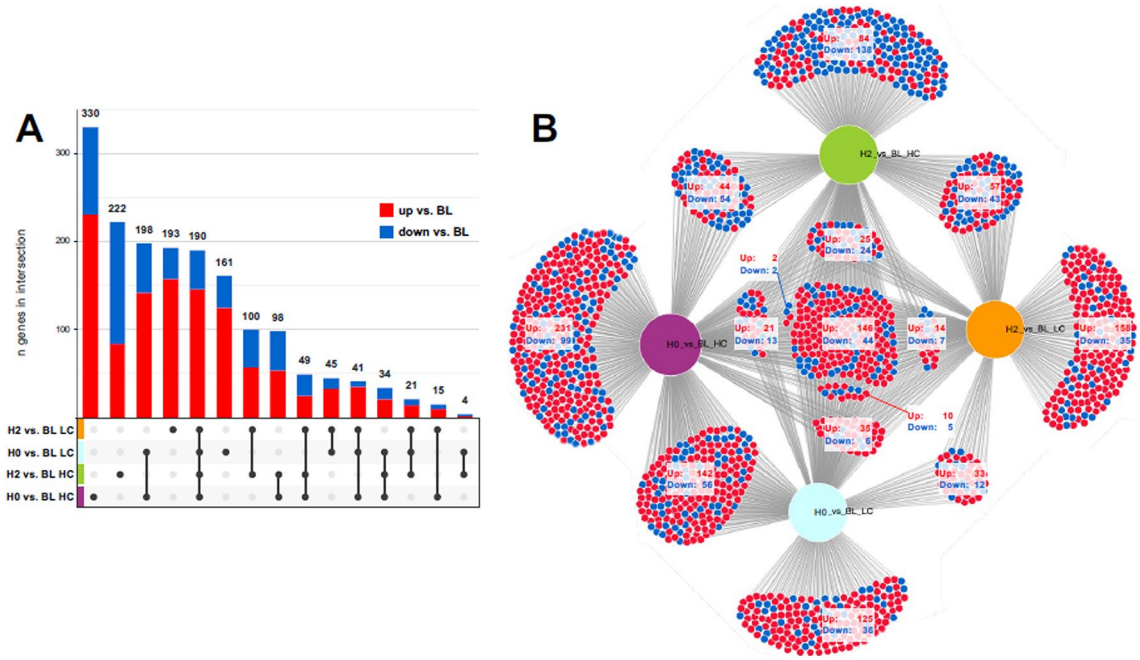

Fig. 4. UpsetR plot showing numbers of genes differentially expressed relative to baseline for each of the two diet groups, with intersections (overlaps) denoted by lines connecting rows of the bottom panel. Downregulated genes are shown in blue and upregulated in red. DiVenn diagram showing differentially expressed genes between diets at each time point. Each cluster corresponds to a bar on the chart in panel A. HC: high-carbohydrate, LC: low-carbohydrate; BL: baseline; H0: immediately postexercise, H2: 120-min postexercise (2h recovery).

homeobox A2 (*HOXA2*), bone morphogenic protein 2 (*BMP2*), and melanoma cell adhesion molecule (*MCAM*). A total of 161 genes were different at H0 vs. baseline in LC.

Irrespective of diet (both HC and LC combined), 198 genes were differentially expressed at the immediate postexercise timepoint (H0) and were primarily affiliated with angiogenesis and related processes, e.g., NFE2-Like BZIP transcription factor 2 (*NFE2L2*), phosphoinositide-3-kinase regulatory subunit 3 (*PIK3R3*), and hypoxia-inducible factor 3 subunit alpha (*HIF3A*). The 198 shared genes were also associated with a range of processes including but not limited to negative regulation of muscle adaptation [e.g., ERBB receptor feedback inhibitor 1 (*ERRFI1*), forkhead box O1 (*FOXO1*), and nitric oxide synthase (*NOS3*)], vasculature development [e.g., histone deacetylase 9 (*HDAC9*), nuclear factor of activated T-cells 2 (*NFATC2*) and Nodal growth differentiation factor (*NODAL*)], and the cellular response to starvation [e.g., cytoplasmic polyadenylation element binding protein 4 (*CPEB4*) and cyclin-dependent kinase inhibitor 1 A (*CDKN1*)]. Genes that overlapped between diet and/or timepoint were assessed for ontological relationships; when available, these are shown in **Supplementary Table 1** for each of the 15 intersections (overlaps) plotted in Fig. 4A.

Visual representation of overrepresented biological processes via ClusterProfileR (Fig. 5) shows the overlapping and distinct biological pathways associated with DEG sets. Immediately after the cessation of running exercise relative to baseline, both groups demonstrated differential expression of genes related to transcription coregulator activity, nuclear hormone receptor binding, and RNA-polymerase II-specific DNA-binding transcription factor binding. At the same time, the HC group demonstrated differential expression of unique gene clusters including actin binding and ubiquitin protein ligase binding, whereas LC demonstrated unique gene clusters annotating to processes such as catalytic activity on RNA and histone acetyltransferase activity.

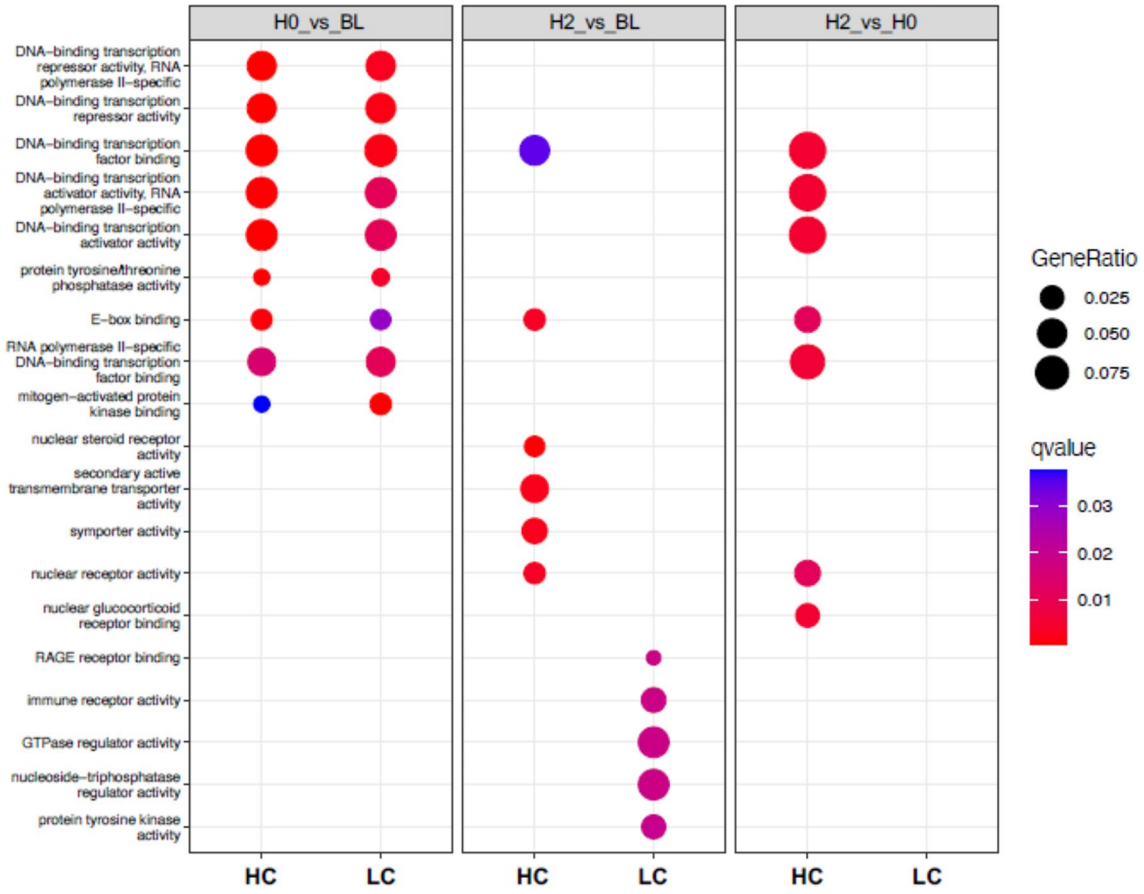

Fig. 5. ClusterProfiler representation of top pathways associated with differentially expressed genes in each diet group at each time point. Color indicates Q-value (FDR-adjusted P-value) and size indicates the proportion of genes in the input gene set that are enriched in a given pathway. HC: high-carbohydrate, LC: low-carbohydrate; BL: baseline; H0: immediately postexercise, H2: 120-min postexercise (2 h recovery).

The Early Recovery Period led to a Dynamic Response in Gene Expression, but Revealed Divergences in Diet.

Recovery-responsive DEGs were identified based on significant change from BL within a diet group at the post two hour-exercise timepoint (H2). At H2 vs. BL, the HC group showed differential expression of 222 different genes, (Fig. 3A) for which no significant prominent biological ontological association was available. A separate group of 193 genes were differently expressed based in the LC group two hours into recovery. Overrepresentation analysis shows associations with immune response and neutrophil activation, among others. As shown in Fig. 3, there were 100 DEGs shared between diets and associated with pathways related to metal ion handling and homeostasis (e.g., metallothioneins B, F, X, and H).

Figure 5 shows the top biological processes associated with DEGs at each postexercise time point by diet group. Noticeably, HC and LC were different when considering the H2 time point, indicating that recovery from exercise may be distinct as a function of diet. In HC, pathways such as symporter and nuclear receptor activity were differentially affected, but LC demonstrated differential expression of genes associated with inflammation and immunity, e.g., RAGE receptor signaling and general immune receptor signaling, among others (Fig. 5). Compared to the H0 time point, no significant biological pathways were identified from DEG in LC, whereas HC had differential expression of genes that annotated to DNA binding and nuclear receptor activity. Thus, while exercise immediately minimizes the gene expression differences between groups, recovery with divergent nutrient provisions may partially reestablish them.

Gene Expression Signatures of Metabolic Characteristics of Interest

A linear model testing the association between changes in substrate use and baseline gene expression was performed for both major fuel sources. Baseline carbohydrate oxidation rate was related to the baseline expression of 24,531 genes at FDR < 0.05. The change in carbohydrate oxidation from BL to H0 was related to baseline expression of 4,021 genes. However, no genes were related to the change in carbohydrate oxidation from baseline to H2 postexercise. The top genes are shown in Table 3 and elaborated in **Supplementary Table 2**. Baseline fat oxidation rate was related to the baseline expression of 1,957 genes. Change in fat oxidation from BL to H0 exercise was related to baseline expression of 3,704 genes, whereas 7,545 were related to the change from BL to H2. The top ten genes in each gene set are shown in Table 4 and elaborated on in **Supplementary Table 2**.

Gene Symbol	Gene Name	log_2FC	FDR	Fiber Type Marker
ACTA1	actin alpha 1	390,735	2.10E-03	*N/A*
CKM	creatine kinase, M-type	284,738	2.19E-03	*N/A*
MYL2	myosin light chain 2	141,858	6.95E-03	type I
TNNC1	troponin C1, slow skeletal and cardiac type	121,746	3.89E-03	type I
TNNT1	troponin T1, slow skeletal type	89,629	7.63E-03	type I
TNNC2	troponin C2, slow skeletal and cardiac type	85,879	2.07E-03	type IIa
CTD-2201G16.1	unknown	84,990	1.25E-02	*N/A*
MYL1	myosin light chain 1	84,093	2.07E-03	type IIa
MYH7	myosin heavy chain 7	77,538	8.56E-03	type I
MB	myoglobin	64,708	2.18E-03	N/A

Table 3. Top ten genes related to change in carbohydrate oxidation following exercise (H0 vs. baseline) across all participants (n=20). Genes are ranked by log_2fold-change per unit (mmol/kg wet weight). N/A indicates gene was not previously found to be more highly expressed in one pure fiber type vs. another.

Gene Symbol	Gene Name	log_2FC	FDR	Fiber Type Marker
hsa-mir-6723	microRNA 6723	3398	3.67E-04	*N/A*
CA3	carbonic anhydrase 3	1107	1.22E-04	type I
MYH1	myosin heavy chain 1	639	4.07E-04	type IIa
RNA28S5	RNA, 28 S Ribosomal 5	621	1.29E-03	*N/A*
FABP3	fatty acid binding protein 3	606	9.77E-04	type I
RP11-451G4.2	*not available*	583	1.49E-04	*N/A*
AC002398.12	*not available*	524	2.87E-04	*N/A*
MYL12A	myosin light chain 12 A	510	2.77E-04	type I
RP5-857K21.11	*not available*	389	7.29E-03	*N/A*
HSPB6	Heat shock protein B6	361	3.30E-04	*N/A*

Table 4. Top ten genes related to change in total fat oxidation during exercise and recovery across all participants (n=20). Genes are ranked by log_2fold-change per unit (mean g/min). Please note that the extremely small scale for fat oxidation measurement results in a very large number for this value in this analysis.

Gene Symbol	Gene Name	log_2FC	FDR	Fiber Type Marker
MYL1	myosin light chain 1	−129.4	1.42E-04	type IIa
MYH2	myosin heavy chain 2	−57.9	1.82E-04	type IIa
TNNI2	troponin I2, fast	−44.8	1.90E-04	type IIa
TNNT3	troponin T3, fast	−43.0	1.55E-04	type IIa
CA3	carbonic anhydrase	−38.3	1.12E-04	type I
RNA28S5	RNA, 28 S ribosomal 5	−21.1	1.52E-03	*N/A*
RP11-451G4.2	*not available*	−20.4	1.08E-04	*N/A*
ATP2A1	ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 1	−20.4	1.39E-04	type IIa
MYH1	myosin heavy chain 1	−16.9	1.10E-02	type IIa
MYBPC2	myosin binding protein C2	−12.2	1.15E-04	type IIa

Table 5. Top ten genes related to change in skeletal muscle glycogen during exercise and recovery (H2 vs. baseline) across all participants (n=20). Genes are ranked by log_2fold-change per unit (mmol/kg wet weight). N/A indicates gene was not previously found to be more highly expressed in one pure fiber type vs. another.

Similarly, genes associated with baseline glycogen availability, as well as genes that might predict the tendency to deplete glycogen during exercise and/or recovery, we used a continuous linear model to assess all genes for relatedness to glycogen levels in skeletal muscle samples collected at the same biopsy time points. Baseline skeletal muscle glycogen content was correlated to the expression of 1,680 genes. Furthermore, 2,372 genes had baseline expression patterns related to the change in glycogen from BL to H0, and 9,121 genes were associated with the change from baseline to H2. Table 5shows the top 10 based on unit-wise correlation and denotes those that have been previously associated with isolated skeletal muscle fiber types, where applicable[21]. **Supplementary Table 3**

shows the results for each time point (absolute value at baseline and change from baseline for each postexercise time point).

Discussion

For the first time, this investigation targeted skeletal muscle gene expression in elite ultra-endurance athletes adapted to vastly different dietary approaches surrounding an acute bout of exercise. Habitual dietary carbohydrate restriction combined with higher fat intake is associated with a series of consistently observed phenotypes, such as higher fat oxidation levels and reduced carbohydrate oxidation rates. Despite these well-documented effects, the extent to which skeletal muscle gene expression is altered in human muscle responses to keto-adaptation has been examined but there is a lack of information and the full extent to muscle adaptations have not been fully examined. This knowledge gap has implications for multiple relevant phenomena in exercise, including early recovery, repeated exposures, interference, and response heterogeneity. To date, the gene expression patterns in skeletal muscle amongst LC athletes and how this may reflect the keto-adapted phenotype in humans remain unknown. The current study aimed to address these research gaps.

A primary finding of this investigation is that distinct dietary patterns varying primarily in carbohydrate and fat in male elite ultra-endurance athletes were associated with diet-specific divergent skeletal muscle gene expression patterns at rest, that became more uniform between diet groups during the immediate recovery from an acute bout of submaximal exercise yet began to emerge again two hours post exercise. A secondary finding was that the gene expression patterns observed in LC athletes reflected the metabolic characteristics associated with keto-adapted athletes such as increased fat oxidation, fatty acid catabolic processes, ketone metabolism, and beta-oxidation[2]. An unexpected finding was that *PPP1R1A*, a gene sensitive to carbohydrate levels and involved in glycogen synthesis, was a prominent gene differentially expressed between groups at every time point and remained higher in LC athletes at all time points in this study.

Baseline (i.e., at rest, before exercise) provided the greatest number of between-group differences in LC vs. HC. Several gene expression patterns in the LC group supported the well-established findings that endurance exercise training combined with low carbohydrate and high fat intake optimizes lipid metabolism pathways within skeletal muscle[22]. While several genes involved in these processes were identified, some with the highest fold differences between groups included *ACAA2*, *ECH1*, and *HADHA*. All of these genes are closely involved with beta-oxidation in various tissues throughout the body, and we identified them specifically in skeletal muscle. For example, *ACAA2*is specifically involved in the final steps of mitochondrial beta-oxidation and has also been linked to adipocyte differentiation in in vitro models[23]. *ECH1*, encoding for a peroxisomal beta-oxidation enzyme, is part of a bifunctional enzyme with *HADHA*[24]. *ECH1*has been associated with improved metabolic health and normalized dyslipidemia in in vivo rodent models[25]. To date, human studies have associated *ECH1*with fatty acid oxidation and as a primary player in lipid metabolism[25,26]. *HADHA*encodes the alpha subunit of the mitochondrial trifunctional protein (MTP)[27]. In addition to fat oxidation, *HADHA* levels coincided with circulating lipolysis biomarkers, such as glycerol and BHB. Increases in *HADHA* imply more MTP production that supports the augmented free fatty acid oxidation observed in LC athletes.

HMGCS2, a rate-limiting step in ketogenesis[28], had the highest fold difference between groups at BL and H0, with higher levels observed in LC when compared to HC athletes. This gene encodes the mitochondrial enzyme that catalyzes the first reaction in ketogenesis. Most research available for HMGCS2 focuses on hepatic tissue, the primary location of ketogenesis[29]. While it has been detected in skeletal muscle, there is limited information on its exact role and how it relates to ketogenesis. Interestingly, *HMGCS2*upregulation mirrored the significant increase in ketone BHB serum levels we observed at BL and immediately post exercise in this group of LC athletes[2]. In vitro studies have shown *HMGCS2*increases in the fasted state and decreases in the fed state in Hep-G2 cell lines. When increased, it induces fatty acid beta-oxidation and ketogenesis and regulates the metabolic adaptations to fasting[30]. In the fasted state, *HMGCS2* is regulated through Sirtuin 3 (*Sirt3*) activity. Sirt3, found in the mitochondria, is part of a highly conserved protein family and most recently has been studied in several comparative genomic models for its role in longevity[31]. Calorie restriction and fasting induce *Sirt3*, both of which also result in increases in beta-oxidation and ketogenesis. It has also been linked with increased mitochondrial respiration while simultaneously decreasing reactive oxygen species production through its induction of *UCP-1*[32], a mitochondrial gene responsive to fasting, as well as a carbohydrate restricted, ketogenic diet[33]. Shimazu et al. observed *HMGCS2* enzyme activity, along with BHB production, as being dependent on *Sirt3*levels[34]. This research has further expanded and now directly links increased *HMGCS2*activity to ketone body production and longevity in mice[35]. The stark difference in *HMGCS2* responses in skeletal muscle between groups observed in this study warrants a more targeted investigation, primarily focused on the relationship between carbohydrate restriction, *HMGCS2*, oxidative stress, exercise training adaptations, and recovery processes.

Despite the incomplete overlap in DE genes between the diets, we did not detect major differences between the two groups' biological processes associated with muscle gene expression following 3 h treadmill running. This highlights the robust effect of acute exercise on gene expression. While some differences were observed, the two groups had more uniform gene responses with the primary biological processes. For example, both had an increase in gene expression pathways associated with metabolic reactions such as gluconeogenesis and lipid metabolism (*FOXO1*), nitric oxide and reactive oxygen species (*NOX3*), and immune function (*NFATC2*). *FOXO1*has been shown to increase after a bout of endurance exercise as well as higher fat (but not necessarily lower carbohydrate) meal[36]. Previous works have suggested that *FOXO*1 is sensitive and increases in response to changes in free fatty acid availability and may be a regulator of skeletal muscle carbohydrate oxidation[36]by potentially inhibiting pyruvate dehydrogenase complex. Others have shown that a short-term (5 d) low-carbohydrate, high-fat diet led to decreased pyruvate dehydrogenase activity in skeletal muscle resulting in increased fat oxidation and decreased carbohydrate oxidation rates during cycling bouts[37]. While short-term

and long-term carbohydrate restriction have been found to have distinct metabolic molecular signatures[19], this phenomenon may be observed in both settings[37]. That is to say, *FOXO*1 may be the upstream regulator of this observed molecular shift during reduced dietary carbohydrate availability, regardless of whether it is a short-term diet change or habitual dietary approach. In our study, both groups saw an increased expression of *FOXO1*at H0, and both HC and LC groups decreased glycogen to the same level H0 despite significantly different carbohydrate and lipid oxidation rates during exercise. These gene expression patterns match the physiological data previously published[2] and continue to emphasize the potentially unique carbohydrate and glycogen metabolism signatures that keto-adapted athletes may present with and also emphasize how powerful a bout of exercise is on skeletal muscle gene expression.

While not as distinct as at rest, gene expression at H2 demonstrated differences between diets in several critical biological processes beyond those observed at H0. While H2 vs. H0 would also have shed light on interesting physiological responses, the rationale for comparing BL to H2 was that the diet differences were most stark at baseline and acutely minimized by exercise; therefore, the clearest indication of recovery would be a return to resting status within each diet group. The biological pathways uniquely associated with DE genes in LC athletes included pathways related to immunity and inflammation. Ketones influence inflammation and immune health through a variety of cellular pathways[38]. Shaw et al. (2020) explored how a short-term ketogenic diet (i.e., 31 days) compared to a habitual, moderate-carbohydrate diet impacted immune markers before and after a run to exhaustion at 70% VO_2max in trained male endurance athletes[39]. The authors found that the ketogenic diet induced differing immune profiles compared to the athletes' habitual diet that, included increases in IL-10 in combination with increases in other cytokines such as IL-2, IL-1β, IL-8, and IFN-γ. Kim et al. (2022) found IL-1β, and TNF-α decreased after healthy adults consumed a very short-term, isocaloric ketogenic diet (i.e. 3 days). The authors concluded that the impact of a ketogenic diet on the inflammasome could be detected within as little as 3-days, which has been observed in other dietary approaches such as extended fasting or very low-calorie diets[40]. It is unknown what the immune and inflammation impacts in skeletal muscle post-exercise translate to in this study. Thus, it is of enormous value to follow up on the present work to establish whether transient changes in genes in the present study led to chronic differences in fatigability and suitability for repeated performance.

PPP1R1A was the only gene significantly upregulated in the LC group at H2 and was differentially expressed in the LC group at all three time points compared to the HC group. *PPP1R1A* encodes for a potent inhibitor of protein-phosphatase 1 (*PP1*). *PP1*, which increases with insulin, can dephosphorylate glycogen synthase to promote glycogen synthesis when glucose levels are high34. Similarly, *PP1*is deactivated when glucose levels are low to allow for activation of glycogen degradation pathways. Very little is known about the mechanisms that regulate PP1 and its inhibitors, such as PPP1R1A70, but it is highly responsive to dietary carbohydrate levels. A consistent phenotype during carbohydrate-restriction is a reduction in carbohydrate oxidation at rest and during submaximal exercise[2]. In contrast, intramuscular glycogen levels did not differ between groups at any point during this study, despite the LC group consuming significantly lower habitual and day-of dietary carbohydrates, indicating glycogen was degraded and resynthesized uniformly across all athletes, irrespective of diet. It is unclear why glycogen was degraded to the level it was in LC athletes, given their efficiency and evidence of fat oxidation for fuel and glycogen sparing[6]. We previously speculated glycogen might be utilized as an alternative fuel source within the pentose phosphate pathway. There is still much that is unknown, but it is clear that habitual carbohydrate restriction results in unique adaptations in glycogen metabolism. This phenotype may depend on the length of time the athlete restricts carbohydrate intake (i.e., weeks vs. months). Furthermore, while substrate levels in this study did not differ between groups, signaling factors related to glycogen metabolism did, warranting further investigation to elucidate this mechanism fully.

Future directions

This novel study lays crucial groundwork for many future directions exploring the role of a carbohydrate restricted, high fat diet on skeletal muscle health and performance. Ketones have been found to impact gene expression and skeletal muscle physiology in several ways, such as reduced oxidative stress and enhanced mitochondrial function[41]. Similarly, a rise in ketones has been associated with reduced muscle damage and improved recovery after a bout of exercise in animal models[42]. However, its primary role in skeletal muscle is to act as a fuel source during exercise. This has been especially prominent in exercise metabolism in highly-trained athletes, regardless of habitual diet[41], but may be even more so in keto-adapted athletes. Furthermore, ketones seem to impact different muscle fiber types uniquely[41], and these changes depend on whether the increase in ketones occurred due to a ketogenic diet al.one or a ketogenic diet combined with exercise training[43]. To date, much of this work has been conducted in murine models. Our study is the most extensive analysis to date to focus on habitual nutritional ketosis and human muscle tissue. Still, it seems to have similar findings of increased gene expression profiles related to ketogenesis and lipid metabolism in keto-adapted athletes. This study also lays the foundation for investigating the impact of ketogenic diets on exercise metabolism in the broader context of the athletes participating in these sports. Our study was limited to male participants; findings should not be extrapolated to female athletes, untrained men, or individuals taking a ketogenic diet to manage an existing metabolic disorder. Given the sex balance in participation in ultra-endurance sports[44]and purported higher reliance on fat resources during intensity-matched exercise in females, it is important to establish whether similar biological patterns underlie women's responses to exercise and recovery[44,45]. Future opportunities could explore how gene expression profiles differ by fiber type in athletic, keto-adapted environments and how this may translate to fuel metabolism, muscle physiology, and more translational aspects related to exercise modality and training, performance, and recovery.

An area for future investigation and discovery is how these results translate to other metabolically active tissues in humans. Skeletal muscle represents a key communicative and locomotive organ system, and these

roles are often dependent on physical location. For instance, the *m. vastus lateralis* (VL)sampled in the present study is a highly-profiled mixed-fiber type muscle with key roles in movement but may be less relied upon for treadmill running than other candidate muscles, such as the soleus or gastrocnemius[46]. Future opportunities to investigate the transcriptomic responses of other such muscles are warranted. Presently, selecting to profile the VL enables our findings to be compared against the multitude of existing data sets that sample this muscle. While the VL may not be the predominant locomotor muscle for treadmill running, it is substantially activated and contributes to overall systemic metabolism. This may partially explain the present similarity in glycogen utilization between the two diet groups. Additionally, the VL's mixed fiber type presents an opportunity to investigate glycogen-dependent metabolism, as the type IIa fiber population preferentially uses glycogen[47]. Our findings support the idea that glycogen utilization is related to the baseline expression of type IIa-fiber marker genes identified in healthy, highly trained male VL muscle[21]. The impact of a ketogenic diet on gene expression in adipose tissue has been studied to some extent in in vivo models. However, this body of literature is still in its infancy. Ma et al. (2018) studied how an 8-week ketogenic diet impacted circulating markers related to energy metabolism and skeletal muscle and lipid gene expression profiles in aerobically exercising mice[10]. After 8 weeks, the authors found exercise capacity increased due to increased lipid and ketone metabolism. Lipid gene expression profiles matched observed patterns in circulating markers, and authors identified an increase in key lipolytic and fat oxidation genes that may be associated with enhanced exercise capacity[10]. Given our finding of increased fat and ketone metabolism in skeletal muscle, a future direction might explore how adipose tissue is affected by nutritional ketosis and how this relates to other phenotypes observed in keto-adapted athletes, such as increased fat oxidation, lipolysis, and ketogenesis.

While it may be argued that the exercise stimulus investigated was not exhaustively challenging for these elite athletes, it is useful to consider this bout characteristic of a typical training regimen. To mimic this further, individuals slightly modulated training to accommodate study testing but did not completely abstain from exercise altogether in the days preceding the testing bout. The employed 3 h submaximal intensity run presents a metabolic and mechanical stress that may be compounded by subsequent training sessions in the same or other modalities (e.g., "brick" training in triathlons, outdoor/trail running). Examining the effect of diet on muscle metabolism following repeated exposures would provide valuable insight into optimal training practices, race preparedness, safe recovery methods, and potentially even injury avoidance. Future studies should examine the muscle gene response to longer or repeated bouts to gain insight into the role of a ketogenic diet in protection against accumulated fatigue. Furthermore, a more detailed time course with later biospecimen collections could be very useful, as others have shown muscle damage following endurance running to last for several days in highly-trained men[48]. Similarly, it is important to note that, while it was a small amount of calories compared to the energy expenditure observed during the protocol, the athletes were not fasted and consumed a protein shake immediately before and after the run. This small amount of dietary intake reflects real-world settings where individuals often consume some level of caloric intake before training and for this study, may have impacted substrate oxidation and glycogen utilization to some capacity. Future investigations exploring how fasting versus specific dietary consumption surrounding exercise have a high opportunity for translation to practical application with a focus on better understanding how immediate dietary decisions impact exercise metabolism.

Conclusion

Low-carbohydrate, high fat, ketogenic diets consistently and uniformly lead to increased fat oxidation and fatty acid metabolism rates even beyond those achieved through endurance training adaptations. Here we establish for the first time the effects of a habitual low-carbohydrate, high fat diet on human skeletal muscle transcriptomic response to accustomed acute exercise. These results indicate that a habitual ketogenic diet leads to differences in constitutive skeletal muscle gene expression at rest and surrounding a bout of accustomed exercise. These gene expression patterns support the keto-adapted phenotype of increased capacity for fat oxidation and accelerated ketogenesis. Presently, the exercise stimulus had a profound influence on gene expression, but differences due to habitual diet could still be detected at every time point. Supporting observed phenotypes associated with keto-adaptation, a LC diet substantially increased genes involved in fat oxidation, lipid metabolism, and ketogenesis. One distinctive discovery was the consistently elevated expression of the glycogen inhibitor *PPP1R1A* in LC athletes. This unexpected differential gene expression did not match substrate outcomes, which found that glycogen breakdown and resynthesis patterns were the same between the two groups at all time points. Future research can further elucidate the connection between diet and gene expression patterns and questions such as the metabolic outcomes, how training status and exercise modality impact gene expression, and how gene expression patterns reflect signatures observed in diet-specific phenotypes. Together, this knowledge can build an understanding of the broad reach of diet manipulation, particularly ketogenic diets, and their impact on skeletal muscle health and athletic performance, with clear ramifications for metabolism in the general population and metabolic disease.

Methods

Experimental approach

This study aims to provide mechanistic context into the molecular shifts that occur at the skeletal muscle level using biospecimens collected as part of the previously published FASTER study[2]. FASTER recruited twenty, highly competitive, elite ultra-endurance male athletes for a cross-sectional comparison of athletes who habitually consumed a high-carbohydrate, low-fat diet (HC, $n=10$) versus those who habitually consumed a low-carbohydrate, high-fat diet (LC, $n=10$). Athletes were actively competing at the time of the study and competed in primarily either ≥50 km running competitions and/or a minimum of half-length Ironman

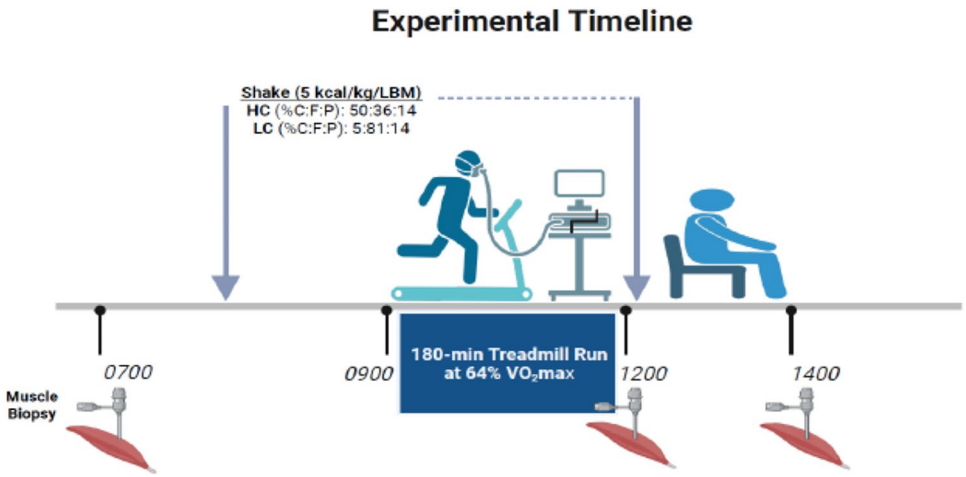

Fig. 6. Experimental Timeline. Athletes ran for 180-minute on a treadmill at 64% of maximal oxygen consumption. About 90 min before and immediately after the run, athletes consumed a shake (5 kcal/kg LBM) with similar ingredients but with a macronutrient composition representative of their respective diets (HC (%C: F:P):50:36:14; LC (%C: F:P): 5:81:14). Skeletal muscle biopsies were obtained from the m. vastus lateralis before (BL), immediately post (H0), and 120 min after the exercise protocol (H2).

triathlons (113.15 km). Collectively, athletes held several athletic accomplishments that included sponsorships, course, state, and/or national-level record-holdings, and national and international appearances for Team USA.

Athletes were matched for age, training status, competition distance, and ultra-endurance performance (Table 1). Primary inclusion criteria centered on athletes who consumed either a very high-carbohydrate, low-fat diet (CHO: >55%, PRO: 20%, FAT: <25%) or a very low-carbohydrate, high-fat diet (CHO: <20%, PRO: 20%, FAT: >60%) for a minimum of 6 months. Interested athletes were asked to submit a detailed, 3-day (2 weekdays, 1 weekend day) diet record to assess inclusion criteria which was reviewed and entered into a commercial nutrient analysis software (Nutritionist Pro, Axxya Systems, Stafford, TX) by a registered dietician. Eligible athletes attended a screening/informational session where they were fully informed of the protocol design and possible risks of the investigation prior to signing an informed consent. Athletes also provided written, informed consent for their biospecimens to be used in future analysis. Athletes were asked to maintain their training level and dietary profile leading up to testing. The experimental protocol was approved by the University of Connecticut Institutional Review Board and all methods were performed in accordance with the Declaration of Helsinki.

A full description of the experimental approach was published previously[2]. Briefly, athletes completed a 180-minute run on a treadmill at 64% of maximal oxygen consumption. About 90 min before and immediately after the run, athletes consumed a shake (5 kcal/kg LBM) with similar ingredients but with a macronutrient composition representative of their respective diets (HC (%C: F:P):50:36:14; LC (%C: F:P): 5:81:14). Shakes were prepared fresh each testing day using whole food sources (i.e. whey protein, strawberries, heavy cream, olive oil, walnut oil, and the HC athletes had additional bananas and agave syrup). Skeletal muscle biopsies were obtained from the *m. vastus lateralis* before (BL), immediately post (H0), and 120 min after the exercise protocol (H2) (Fig. 6). Samples were then processed for mRNA-sequencing analysis.

180-minute treadmill exercise bout

Whole body substrate use, muscle glycogen, and circulating metabolites from this cohort were recently published[2]. Briefly, each subject spent 3 days visiting the laboratory, which was composed of the arrival day, day of testing, and the departure day. Participants were instructed to maintain their habitual diet leading up to testing. To ensure habitual diet matched the athlete's respective group, each athlete recorded a food log for 48 h prior to the testing day. Participants were flown to the laboratory and arrived between 1400 and 1600 h the day before testing. Participants were instructed to minimize running and/or major races for 7 days prior to testing.

The morning of testing, participants arrived at the laboratory at 0600 h following a 10-hr overnight fast. Participants were instructed to drink 0.5 L of water the previous night and another 0.5 L of water upon waking up to ensure a euhydrated state for testing. Immediately upon arrival to the laboratory, urine specific gravity (USG) (Model A300CL, Spartan, Japan) was tested to check hydration status. Athletes were required to have a USG ≤ 1.025 to continue testing procedures. Next, anthropometrical measurements were collected. Height was measured to the nearest 0.1 cm, and body weight was recorded to the nearest 0.1 kg (OHAUS Corp, Fordham Park, NJ). Body composition was assessed via Dual-energy X-ray absorptiometry (Prodigy, Lunar Corporation, Madison, WI). Baseline resting energy expenditure and blood draw samples were collected. Immediately after, baseline muscle biopsy samples were collected and processed as described below.

Athletes were then provided a nutritional shake (5 kcal/ kg) designed to be representative of each dietary group. On average, the HC was 332 kcal shake with a breakdown of CHO: 50%, PRO: 14%, FAT: 36%. Similarly, the LC was an average of 343 kcal shake with a breakdown of CHO: 5%, PRO: 14%, FAT: 81%. All shakes were made with heavy cream, olive oil, whey protein, walnut oil, and strawberries. HC shakes had additional

ingredients to increase carbohydrate content by including bananas and agave syrup. Athletes consumed the shake within 10 min after which each rested quietly in a seated position for 90 min.

Indirect calorimetry measurements were collected immediately before athletes began the exercise protocol. Athletes then ran on a treadmill at a uniform grade and a consistent pace equivalent to 64% VO_2max for 180 min. Breath-by-breath gas exchange measurements of VO_2 and VCO_2 were collected and recovered every 30 s for a total of 10 min at the start of, 60 min into, and 150 min into the run. Oxygen uptake, minute ventilation, respiratory exchange ratio, and carbohydrate and fat oxidation rates were measured at each time point. Participants were able to drink water ad libitum throughout the run, but no other calories were ingested. Fifteen minutes after completing the run, a second muscle biopsy was taken after which athletes then consumed a shake identical to the first one. Indirect calorimetry measurements were collected immediately post, 30 (30+), 60 (60+), and 120 (H2) minutes into recovery. At H2 the final skeletal muscle biopsy was obtained and processed.

Skeletal muscle biopsy procedure and processing

All three muscle biopsies from the vastus lateralis were obtained using the modified Bergstrom technique[49] with added suction described by Evans et al.[50]. Samples were obtained from alternate legs with the final muscle biopsy occurring 3 cm from the baseline sample site. A trained physician sterilized the biopsy location first with alcohol and then with betadine. The aseptic site was numbed using 8–10 cc of local anesthetic (2% lidocaine hydrochloride) injected at a 450 proximal and a 450 distal angle to the injection site in a cone-like formation. A #11 surgical scalpel was used to create a small incision (~1 cm) through the skin and muscle fascia. The muscle sample was obtained using a sterile biopsy needle, 5 mm in diameter (Surgical Instruments Engineering, Midlothian, United Kingdom). It was inserted ~2 cm into the incision site where a double-chop method with suction was used to collect sample. The needle was immediately removed and replaced with gauze to minimize bleeding from the incision site. A single suture was used to close the incision site.

Muscle samples were removed from the needle, cleaned to remove any excess blood or connective tissue, divided into multiple pieces, and processed accordingly. To ensure high quality RNA, immediately following the biopsy, one piece was placed in 1 ml TRIzol Reagent (Ambion, Life Technologies, Grand Island, NY), positioned on ice and immediately homogenized in 4 × 30 s set homogenizing bursts using a rotor-stator handheld homogenizer (D1000, Benchmark Scientific, Edison, NJ). Samples were then immediately frozen in liquid nitrogen and stored in −800 C for subsequent RNA extraction.

RNA extraction and sequencing

Total RNA was extracted using the traditional TRIzol method. Homogenized samples were thawed on ice followed by the addition of 200µL of chloroform and thorough mixing. Samples were left to incubate at room temperature for 5 min and then centrifuged for 15 min at 4°C at a speed of 12,000 g. The aqueous layer containing nucleic acid was removed and nucleic acid was precipitated with 100% cold ethanol (EtOH) to reach total 35% EtOH. Samples were incubated for 30 min at −20°C and centrifuged for 15 min at 4°C, 16,000 g. RNA pelleted together at the bottom of the tube and supernatant was discarded. 75% ethanol was used to wash the RNA pellet twice, with supernatant being removed each time. RNA pellet was left to air-dry after which it was resuspended in 20µL of nuclease-free, ultra-pure diethlpyrocarbonate-treated water.

Samples were further treated with DNAse I (Ambion Life Technologies, Grand Island, NY). First, 1µL of RNAse I and 2 uL of 10X DNAse I buffer were added. Samples were then incubated for 30 min at 37°C. Immediately following, 2.3µL DNase Inactivation Reagent was added. Samples were incubated at room temperature for 2 min and then centrifuged for 2 min at 4°C at a speed of 16,000 g. Total RNA supernatant was removed and transferred to an RNAse and DNAse-free Eppendorf tube.

Extracted total RNA samples were checked for quality and concentration with a Nanodrop (NanoDrop 2000, Thermo Scientific, Wilmington, DE). One µL of sample was used to measure 260/280 ratios and 260/230 ratios. An additional cleanup was performed using an ethanol precipitation with sodium acetate to further purify each sample. One µL of 3 M sodium acetate (pH between 5 and 5.5) was added to the RNA sample and mixed well. 25µL of 100% cold ethanol was added and mixed thoroughly. Samples were incubated for 30 min at −20°C and centrifuged for 15 min at 4°C at a speed of 16,000 g to allow RNA to pellet. Supernatant was discarded and 100µL of 75% cold ethanol was added to the pelleted RNA and inverted several times to mix. Samples were centrifuged for 10 min, and the previous step was then repeated. After the second ethanol wash, all supernatant was removed, and RNA pellet was dried at room temperature for 5 min. 10µL of RNase-free water was added to suspend the RNA pellet. A small amount was aliquoted out to be reserved for the quality control check and the majority of the sample was stored in −80°C for subsequent analysis.

RNA quality and concentration was determined on a Nanodrop (NanoDrop 2000, Thermo Scientific, Wilmington, DE) using 1µL of sample. Afterwards, samples were further assessed for total RNA quality and integrity on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) with the use of a microfluidic chip (Agilent RNA 6000 Nano Kit, Agilent Technologies, Santa Clara, CA). Chip-technology allows for a more sensitive and accurate portrayal of quality and integrity of RNA samples. The technology assesses quality through a patented RNA Integrity Number (RIN) with a custom minimum RIN threshold needed for specific downstream analyses. RIN score for sequencing was set at seven for all samples.

mRNA-sequencing

Library preparation and sequencing for all 60 samples of extracted total RNA was completed by Ocean Ridge Biosciences (Palm Beach Gardens, FL). Libraries were created using a non-strand specific library of polyA RNA (Illumina TruSeq RNA Sample Prep Kit v2, Illumina, San Diego, CA). Further quality control analysis of each DNA library included a repeat chip analysis using a BioAnalyzer and sample quantification using real time PCR.

Samples were sequenced on the Illumina HiSeq 2500 (Illumina, San Diego, CA); all samples met a minimum of 48 million paired-end 100 nucleotide reads (24 million per direction) passing sequencing filters. Mean Phred quality score was at least 30 for 94% of passed-filter reads and averaged 36.5 ± 0.2 across all reads. Across all samples, reads aligned with 69.13 ± 0.01% efficiency to the reference genome Ensembl *H. sapiens* (Hg19), the most up-to-date genome build at the time of the study, as described below.

Statistical approach and bioinformatics

Independent t-tests were used to compare physical characteristics, dietary profiles, and exercise data between the HC and the LC groups. Metabolic characteristics were analyzed with a two-way repeated measures analysis of variance for between (HC versus LC) and within (time-dependent) factors. Significantly different main or interaction effects were further analyzed using Fisher's least significant difference post-hoc test. These data are presented as means ± SD. Statistical significance was defined as $p \leq 0.05$.

Ocean Ridge Biosciences performed the sequencing and preliminary bioinformatic analyses. Alignment was done using both sequenced strands. FASTQ files from the sequencing run were aligned to Ensembl *H. sapiens* (Hg19), the Ensembl human reference genome, using TopHat 1.4.150. Files were used to generate BAM alignment files and Samtools 0.1.1851 was used to generate BAM index files. Normalization counts of reads were done by mapping reads per kilobase of transcript per million (RPKM) to annotated Ensembl genes using the open-source software Bioconductor easyRNASeq v1.6.0 package on R version 3.0 (Lucent Technologies, Inc., Murray Hill, NJ). RPKM values were filtered where the Reliable Quantification Threshold was set at 50 mapped reads. Anything under 10 reads per gene was replaced with the average RPKM value equivalent to 10 reads per gene across all samples. Filtered and adjusted gene RPKM values were used for statistical analysis.

A mixed-effect model was performed that focused on the effects of diet and exercise on gene expression. Type I Analysis of variance via the Companion to Applied Regression (car) package in R (v 3.0) was used to compare differential gene expression due to diet, exercise, and the interaction thereof. When appropriate, Tukey tests were used for post-hoc analyses. False discovery rates (FDR) were calculated from p-values using the Benjamini Hochberg method. Post-hoc tests of interest included comparing expression between both diet groups at each exercise time point (BL, H0, and H2), as well as changes in expression across time within a diet group. Gene expression was declared significantly different where FDR < 0.05 and absolute $\log_2$fold-change ($\log_2$FC) > 1, corresponding to overall expression being halved or doubled relative to the reference condition. Within each diet, DEGs that met FDR and $\log_2$FC criteria vs. baseline and compared qualitatively between diets using UpsetR analysis, as described by our group[51], and graphically represented for directionality using DiVenn[52]. When possible, each intersection (overlapping list) of genes was assessed for biological pathways in the gene ontology database using over-representation analysis via WebGestaltR. ClusterProfileR was used to further investigate the biological similarities and differences for gene sets passing FDR and $\log_2$FC vs. baseline filters.

Finally, as an exploratory direction, baseline gene expression was assessed for correlation to baseline metabolic characteristics including skeletal muscle glycogen concentration as well as carbohydrate and fat oxidation rates. Genes were tested for their predictive capacity via relatedness to the change in glycogen (absolute difference) and carbohydrate and fat oxidation rates (fold-change) from baseline to either H0 or H2. This was performed using linear models in the limma package in R v. 4.2.2. All genes with FDR < 0.05 were pursued for interpretation.

Data availability

The datasets generated and/or analyzed during the current study are available in the NIH Gene Expression Omnibus repository under accession number GSE273041 (reviewers please use access token during review: urgdmskgzhwzvol).

Received: 15 October 2024; Accepted: 3 February 2025
Published online: 11 March 2025

References

1. Yeo, W. K., Carey, A. L., Burke, L., Spriet, L. L. & Hawley, J. A. Fat adaptation in well-trained athletes: effects on cell metabolism. *Appl. Physiol. Nutr. Metab.* **36**, 12–22 (2011).
2. Volek, J. S. et al. Metabolic characteristics of keto-adapted ultra-endurance runners. *Metabolism* https://doi.org/10.1016/j.metabol.2015.10.028 (2016).
3. Webster, C. C. et al. Gluconeogenesis during endurance exercise in cyclists habituated to a long-term low carbohydrate high-fat diet. *J. Physiol.* https://doi.org/10.1113/JP271934 (2016).
4. Saenz, C. & Effect Of A 21-day well-formulated ketogenic Diet On women's Metabolic Health: Glow Pilot Trial. *Med. Sci. Sports Exerc.* https://doi.org/10.1249/01.mss.0000687480.31149.20 (2020).
5. Volek, J. S., Noakes, T. & Phinney, S. D. Rethinking fat as a fuel for endurance exercise. *Eur. J. Sport Sci.* https://doi.org/10.1080/17461391.2014.959564 (2015).
6. Phinney, S. D., Bistrian, B. R., Evans, W. J., Gervino, E. & Blackburn, G. L. The human metabolic response to chronic ketosis without caloric restriction: preservation of submaximal exercise capability with reduced carbohydrate oxidation. *Metabolism* https://doi.org/10.1016/0026-0495(83)90106-3 (1983).
7. Noakes, T., Volek, J. S. & Phinney, S. D. Low-carbohydrate diets for athletes: what evidence? *Br. J. Sports Med.* https://doi.org/10.1136/bjsports-2014-093824 (2014).
8. Diaz-Lara, F. J., Botella, J. & Reisman, E. Are enhanced muscle adaptations associated with carbohydrate restriction regulated by absolute muscle glycogen concentration? *J. Physiol.* **598**, 221–223 (2020).
9. Boison, D. New insights into the mechanisms of the ketogenic diet. *Curr. Opin. Neurol.* **30**, 187–192 (2017).
10. Ma, S., Huang, Q., Tominaga, T., Liu, C. & Suzuki, K. An 8-Week ketogenic Diet Alternated Interleukin-6, Ketolytic and Lipolytic Gene expression, and enhanced Exercise Capacity in mice. *Nutrients* **10**, 1696 (2018).
11. Schnyder, S., Svensson, K., Cardel, B. & Handschin, C. Muscle PGC-1α is required for long-term systemic and local adaptations to a ketogenic diet in mice. *Am. J. Physiol. -Endocrinol Metab.* **312**, E437–E446 (2017).

12. Ogura, Y. et al. Ketogenic diet feeding improves aerobic metabolism property in extensor digitorum longus muscle of sedentary male rats. *PloS One*. **15**, e0241382 (2020).
13. Huang, T. Y. et al. Combined effects of a ketogenic diet and exercise training alter mitochondrial and peroxisomal substrate oxidative capacity in skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **320**, E1053–E1067 (2021).
14. Webster, C. C. et al. Reduced glucose tolerance and skeletal muscle GLUT4 and IRS1 content in cyclists habituated to a long-term low-carbohydrate, high-fat diet. *Int. J. Sport Nutr. Exerc. Metab.* https://doi.org/10.1123/ijsnem.2019-0359 (2020).
15. Miller, V. J., Villamena, F. A. & Volek, J. S. Nutritional ketosis and mitohormesis: potential implications for mitochondrial function and Human Health. *J. Nutr. Metab.* https://doi.org/10.1155/2018/5157645 (2018).
16. Margolis, L. M. et al. Exercising with low muscle glycogen content increases fat oxidation and decreases endogenous, but not exogenous carbohydrate oxidation. *Metabolism* **97**, 1–8 (2019).
17. Arkinstall, M. J., Tunstall, R. J., Cameron-Smith, D. & Hawley, J. A. Regulation of metabolic genes in human skeletal muscle by short-term exercise and diet manipulation. *Am. J. Physiol. Endocrinol. Metab.* **287**, E25–31 (2004).
18. Cameron-Smith, D. et al. A short-term, high-fat diet up-regulates lipid metabolism and gene expression in human skeletal muscle. *Am. J. Clin. Nutr.* https://doi.org/10.1093/ajcn/77.2.313 (2003).
19. Webster, C. C. et al. Reduced glucose tolerance and skeletal muscle GLUT4 and IRS1 content in cyclists Habituated to a long-term Low-Carbohydrate, High-Fat Diet. *Int. J. Sport Nutr. Exerc. Metab.* **30**, 210–217 (2020).
20. Lavin, K. M. et al. Wiley,. State of Knowledge on Molecular Adaptations to Exercise in Humans: Historical Perspectives and Future Directions. in *Comprehensive Physiology* (ed. Prakash, Y. S.) 3193–3279 (2022). https://doi.org/10.1002/cphy.c200033
21. Rubenstein, A. B. et al. Single-cell transcriptional profiles in human skeletal muscle. *Sci. Rep.* **10**, 229 (2020).
22. Chi, M. M. et al. Effects of detraining on enzymes of energy metabolism in individual human muscle fibers. *Am. J. Physiol.* **244**, C276–287 (1983).
23. Zhang, Y. et al. Acetyl-Coenzyme A acyltransferase 2 promote the differentiation of sheep precursor adipocytes into adipocytes. *J. Cell. Biochem.* **120**, 8021–8031 (2019).
24. De Boer, V. C. J. et al. Chronic quercetin exposure affects fatty acid catabolism in rat lung. *Cell. Mol. Life Sci. CMLS.* **63**, 2847–2858 (2006).
25. Huang, D., Liu, B., Huang, K. & Huang, K. Enoyl coenzyme a hydratase 1 protects against high-fat-diet-induced hepatic steatosis and insulin resistance. *Biochem. Biophys. Res. Commun.* **499**, 403–409 (2018).
26. Fiorentino, T. V. et al. Pioglitazone corrects dysregulation of skeletal muscle mitochondrial proteins involved in ATP synthesis in type 2 diabetes. *Metabolism* **114**, 154416 (2021).
27. Rector, R. S., Payne, R. M. & Ibdah, J. A. Mitochondrial trifunctional protein defects: clinical implications and therapeutic approaches. *Adv. Drug Deliv Rev.* **60**, 1488–1496 (2008).
28. Asif, S. et al. Hmgcs2-mediated ketogenesis modulates high-fat diet-induced hepatosteatosis. *Mol. Metab.* **61**, 101494 (2022).
29. Shukla, S. K. et al. HMGCS2 is a key ketogenic enzyme potentially involved in type 1 diabetes with high cardiovascular risk. *Sci. Rep.* **7**, 4590 (2017).
30. Vilà-Brau, A., De Sousa-Coelho, A. L., Mayordomo, C., Haro, D. & Marrero, P. F. Human HMGCS2 regulates mitochondrial fatty acid oxidation and FGF21 expression in HepG2 cell line. *J. Biol. Chem.* **286**, 20423–20430 (2011).
31. Albani, D. et al. Modulation of human longevity by SIRT3 single nucleotide polymorphisms in the prospective study Treviso Longeva (TRELONG). *AGE* **36**, 469–478 (2014).
32. Haigis, M. C. & Guarente, L. P. Mammalian sirtuins—emerging roles in physiology, aging, and calorie restriction. *Genes Dev.* **20**, 2913–2921 (2006).
33. Hasan-Olive, M. M. et al. A ketogenic Diet improves mitochondrial Biogenesis and Bioenergetics via the PGC1α-SIRT3-UCP2 Axis. *Neurochem Res.* **44**, 22–37 (2019).
34. Shimazu, T. et al. SIRT3 deacetylates mitochondrial 3-Hydroxy-3-Methylglutaryl CoA synthase 2 and regulates ketone body production. *Cell. Metab.* **12**, 654–661 (2010).
35. Tomita, I. et al. Ketone bodies: a double-edged sword for mammalian life span. *Aging Cell.* **22**, e13833 (2023).
36. Constantin-Teodosiu, D., Constantin, D., Stephens, F., Laithwaite, D. & Greenhaff, P. L. The role of FOXO and PPAR Transcription Factors in Diet-mediated inhibition of PDC activation and Carbohydrate Oxidation during Exercise in humans and the role of pharmacological activation of PDC in Overriding these changes. *Diabetes* **61**, 1017–1024 (2012).
37. Stellingwerff, T. et al. Decreased PDH activation and glycogenolysis during exercise following fat adaptation with carbohydrate restoration. *Am. J. Physiol. Endocrinol. Metab.* **290**, E380–388 (2006).
38. Stubbs, B. J. et al. Investigating ketone bodies as immunometabolic countermeasures against respiratory viral infections. *Med* **1**, 43–65 (2020).
39. Shaw, D. M., Merien, F., Braakhuis, A., Keaney, L. & Dulson, D. K. Adaptation to a ketogenic diet modulates adaptive and mucosal immune markers in trained male endurance athletes. *Scand. J. Med. Sci. Sports*. **31**, 140–152 (2021).
40. Kim, E. R. et al. Short term Isocaloric Ketogenic Diet modulates NLRP3 Inflammasome Via B-hydroxybutyrate and fibroblast growth factor 21. *Front. Immunol.* **13**, 843520 (2022).
41. Yakupova, E. I., Bocharnikov, A. D. & Plotnikov, E. Y. Effects of Ketogenic Diet on muscle metabolism in Health and Disease. *Nutrients* **14**, 3842 (2022).
42. Mansor, L. S. & Woo, G. H. Ketones for post-exercise recovery: potential applications and mechanisms. *Front. Physiol.* **11**, 613648 (2021).
43. Shimizu, K. et al. Short-term and long-term ketogenic diet therapy and the addition of exercise have differential impacts on metabolic gene expression in the mouse energy-consuming organs heart and skeletal muscle. *Nutr. Res.* **60**, 77–86 (2018).
44. Tiller, N. B. et al. Do sex differences in Physiology Confer a female advantage in Ultra-endurance Sport? *Sports Med.* **51**, 895–915 (2021).
45. Saenz, C. et al. Low energy intake leads to body composition and performance decrements in a Highly-Trained, Female Athlete: the WANDER (Woman's activity and Nutrition during an extensive Hiking Route) Case Study. *J. Am. Nutr. Assoc.* **43**, 296–303 (2024).
46. Dorn, T. W., Schache, A. G. & Pandy, M. G. Muscular strategy shift in human running: dependence of running speed on hip and ankle muscle performance. *J. Exp. Biol.* **215**, 1944–1956 (2012).
47. Pascoe, D. D. et al. Effects of exercise mode on muscle glycogen restorage during repeated days of exercise. *Med. Sci. Sports Exerc.* **22**, 593–598 (1990).
48. Hikida, R. S., Staron, R. S., Hagerman, F. C., Sherman, W. M. & Costill, D. L. Muscle fiber necrosis associated with human marathon runners. *J. Neurol. Sci.* **59**, 185–203 (1983).
49. Bergstrom, J., MUSCLE ELECTROLYTES IN & MAN DETERMINED BY NEUTRON ACTIVATION ANALYSIS ON NEEDLE BIOPSY SPECIMENS. *Scand. J. Clin. Lab. Investig Engl.* **14**, 511-513 (1962).
50. Evans, W. J., Phinney, S. D. & Young, V. R. Suction applied to a muscle biopsy maximizes sample size. *Med. Sci. Sports Exerc.* **14**, 101–102 (1982).
51. Lavin, K. M. et al. Dynamic transcriptomic responses to divergent acute exercise stimuli in young adults. *Physiol. Genomics*. **55**, 194–212 (2023).
52. Sun, L. et al. DiVenn: an interactive and Integrated web-based visualization Tool for comparing gene lists. *Front. Genet.* **10**, 421 (2019).

Acknowledgements
The authors would like to thank the FASTER research team, Peter Defty, for assistance in recruiting participants and the extraordinary research participants for their enthusiasm in participating in this project.

Author contributions
CS, JV, CM, WK contributed to conception, research design, and data collection. CS and EL performed experiments. CS, JV, EL, and KL analyzed data and interpreted results. CS and KL prepared figures and KL prepared all tables. CS, KL, EL, and JV drafted manuscript and CS and KL led manuscript preparation and contributed equally to all aspects needed for submission. All authors contributed to editing and revising the manuscript. All authors approved final version of manuscript.

Funding
This work was supported by contributions from Quest Nutrition and The Robert C. and Veronica Atkins Foundation.

Declarations

Competing interests
The authors declare no competing interests.

Conflict of interest
Dr. Volek is co-founder and has equity in Virta Health, serves on scientific advisory boards for Simply Good Foods and Abbott Biowearables Sensors in Ketogenic Diets, and receives royalties from books on low-carbohydrate diets.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-88963-9.

Correspondence and requests for materials should be addressed to C.S.