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The altered human serum metabolome induced by a marathon

Abbreviated title: Altered metabolome induced by a marathon

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ABSTRACT

Introduction: Endurance races have been associated with a substantial amount of adverse effects which could lead to chronic disease and long-term performance impairment. However, little is known about the holistic metabolic changes occurring within the serum metabolome of athletes after the completion of a marathon.

Objectives: Considering this, the aim of this study was to better characterize the acute metabolic changes induced by a marathon.

Methods: Using an untargeted two dimensional gas chromatography time-of-flight mass spectrometry metabolomics approach, pre- and post-marathon serum samples of 31 athletes were analyzed and compared to identify those metabolites varying the most after the marathon perturbation.

Results: Principle component analysis of the comparative groups indicated natural differentiation due to variation in the total metabolite profiles. Elevated concentrations of carbohydrates, fatty acids, tricarboxylic acid cycle intermediates, ketones and reduced concentrations of amino acids indicated a metabolic shift between various fuel substrate systems. Additionally, elevated odd-chain fatty acids and α-hydroxy acids indicated the utilization of α-oxidation and autophagy as alternative energy-producing mechanisms. Adaptations in gut microbe-associated markers were also observed and correlated with the metabolic flexibility of the athlete.

Conclusion: From these results it is evident that a marathon places immense strain on the energy-producing pathways of the athlete, leading to extensive protein degradation, oxidative stress, mammalian target of rapamycin complex 1 inhibition and autophagy. A better understanding of this metabolic shift could provide new insights for optimizing athletic performance, developing more efficient nutrition regimens and identify strategies to improve recovery.

Keywords: marathon; serum; metabolomics; metabolite markers; fuel substrates

1. INTRODUCTION

Although physical activity has been shown to be substantially beneficial to human health (Ojiambo, 2013), various negative effects including cardiovascular dysfunction (Webner *et al.*, 2012), muscle damage (Howatson *et al.*, 2010), increased propensity to upper respiratory tract infection (Robson-Ansley *et al.*, 2012) and severe inflammation (Bonasia *et al.*, 2015) have been associated with running extensive distances, such as that of endurance races. Even though these races have become increasingly popular, limited research is based on the elucidation of the effects of these races using a metabolomics approach. Metabolomics is defined as the identification and quantification of the small metabolite compounds (<1500 Da) present in a biological system in order to determine the physiological effects induced by a specific perturbation (Heaney *et al.*, 2017). Since metabolites are typically the end-products of the genome, transcriptome and proteome, alterations in these are indicative of the overall physiological state of the investigated biological system (Heaney *et al.*, 2017).

Previous metabolomics studies have indicated elevated concentrations of various carbohydrate/glycolysis metabolite intermediates, indicative of free glucose utilization as the preferred energy source during strenuous physical activity (Lewis *et al.*, 2010; Salway, 2012; Waśkiewicz *et al.*, 2012). Furthermore, significant alterations to the tricarboxylic acid cycle intermediates were induced by a marathon (Turer *et al.*, 2014) and could be attributed to additional strain placed on the electron transport chain (ETC), causing an imbalanced NADH:NAD^+ ratio (Esterhuizen *et al.*, 2017). According to previous work (Stellingwerff, 2012), free glucose and other carbohydrate stores can become depleted within approximately 90 min after the start of the race, which most likely lead to the utilization of alternative fuel substrates (lipids and amino acids) for energy production (Waśkiewicz *et al.*, 2012). Increased lipolysis activity results in elevated serum glycerol and free fatty acids (Lewis *et al.*, 2010; Waśkiewicz *et al.*, 2012), the latter of which produce acetyl-CoA via β-oxidation and subsequent energy via the TCA cycle and ETC (Salway, 2012). Furthermore, the increased synthesis of acetyl-CoA could also ascribe the elevated ketone concentrations previously reported (Pechlivanis *et al.*, 2010), as it is a key component of ketogenesis. In the event that the strenuous physical activity continues beyond the capacity of the athlete's lipid stores, or if the traditional lipid oxidation pathways become saturated

51 (Staron *et al.*, 1989), the athlete's metabolism shifts towards protein catabolism (resulting in reduction
52 blood amino acid levels) in an attempt to synthesize the energy required to complete the marathon
53 (Lewis *et al.*, 2010). These amino acids are primarily oxidized to pyruvic acid and acetyl-CoA, both of
54 which can serve as TCA cycle influx substrates for energy production (Salway, 2012). Additionally,
55 protein degradation has been shown to alter purine catabolism, resulting in elevated adenosine-
56 monophosphate, inosine-monophosphate, hypoxanthine, xanthine, uric acid and allantoin (Turer *et al.*,
57 2014), the latter of which is a uric acid derivative and a surrogate index of oxidative stress (Lewis *et*
58 *al.*, 2010).

59 Although these metabolomics studies provide some clues to the metabolic alterations that occur during
60 strenuous physical activity such as long-distance endurance races, very few of these employed an
61 untargeted metabolomics approach. Considering this, an untargeted two-dimensional gas
62 chromatography time-of-flight mass spectrometry (GCxGC-TOFMS) metabolomics approach was used
63 to holistically compare the serum metabolite profiles of 31 recreational marathon athletes before and
64 after the completion of a marathon (42 km), in order to better characterize the acute metabolic changes
65 induced by exercise stress.

66 2. MATERIALS AND METHODS

67 2.1 Participants

68 All participants completed a health and dietary questionnaire (including a menstrual cycle questionnaire
69 for female participants) prior to the marathon to assess their eligibility. Individuals with food allergies,
70 cardiovascular complications, musculoskeletal disorders/injuries, or those receiving anti-inflammatory
71 treatment were excluded from the study. Athlete participation in this investigation was completely
72 voluntary, and all the participants gave written and informed consent. A summary of the participant
73 characteristics is presented in Table 1.

74 2.2 Clinical samples

75 Blood samples were collected by antecubital fossa venesection of 31 marathon athletes (19 males and
76 12 females) 24 hours before and immediately after completing the Druridge Bay Marathon
77 (Northumberland, UK). Pre-marathon samples were collected the day preceding the race (between
78 10am and 6pm) as a means of reducing additional metabolic changes induced by the venesection stress
79 as well as to limit interference to the athletes' pre-marathon regimens. The individuals were required
80 to be in a hydrated, yet fasted state (for a minimum of two hours) at time of baseline sample venesection.
81 The samples were collected in standard 10 mL vacutainer vials, placed on ice and transported to the
82 laboratory (Faculty of Health and Life Sciences, Department of Sport, Exercise and Rehabilitation at
83 the Northumbria University in Newcastle upon Tyne, UK) for immediate processing. Briefly, the blood
84 was allowed to clot for 30 min and centrifuged at 3 000 *g* for 10 min. The supernatant (serum) was
85 then extracted and immediately frozen (-80°C) before being transported (on dry ice) to the North-West
86 University, Human Metabolomics: Laboratory of Infectious and Acquired Diseases. These serum
87 samples were stored at -80°C until metabolomics analyses commenced.

88 **2.3 Chemicals and reagents**

89 Methoxamine hydrochloride, 3-phenylbutyric acid and bis(trimethylsilyl)-trifluoroacetamide (BSTFA)
90 with 1% trimethylchlorosilane (TMCS) were purchased from Sigma Aldrich (St. Louis, Missouri, USA)
91 and the pyridine was from Merck (Darmstadt, Germany). The acetonitrile used, was an ultra-pure
92 Burdick and Jackson brand (Honeywell International Inc., Muskegon, Michigan, USA) and hence did
93 not require any further purification.

94 **2.4 Sample extraction and derivatization**

95 A total metabolome extraction procedure was performed on all the collected serum samples, along with
96 appropriate quality control (QC) samples. During this process 50 µL of internal standard,
97 3-phenylbutyric acid (0.45 µg/mL) dissolved in a chloroform:methanol:water (1:3:1) solution, was
98 added to 50 µL of each serum sample. Hereafter, the samples were placed on ice before 300 µL of
99 acetonitrile was added as a protein precipitation agent. Samples were then subjected to the REAX D-
100 91126 vortex (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) for 2 min and centrifuged

101 at 3 500 *g* for 10 min at 4°C. The supernatant of the biphasic solution was then transferred to a clean
102 GC-MS sample vile and dried at 40ºC under a light stream of nitrogen gas for approximately 45 min.
103 Hereafter, 25 µL of methoxamine hydrochloride dissolved in pyridine (15 mg/mL) was added to each
104 sample and incubated at 50°C for 90 min. Thereafter, 40 µL BSTFA with 1% TMCS was added,
105 followed by derivatization for 60 min at 60°C. Each derivatized sample was transferred to a new GC-
106 MS vile containing a vile insert and capped.

107 **2.5 GCxGC-TOFMS analysis and processing**

108 The derivatized samples were placed on a multi-purpose auto-sampler tray (Gerstel GmbH and co. KG,
109 Mülheim van der Ruhr, Germany) in a randomized order, and analyzed using a Pegasus 4D GCxGC-
110 TOFMS system (LECO Africa (Pty) Ltd, Johannesburg, South Africa), fitted with an Agilent 7890A
111 GC and TOFMS (LECO Africa). During analyses, 1 µL of each sample was injected using a 1:3 split
112 ratio. Purified helium was used as a carrier gas and set at a constant flow rate of 1 mL/min, while the
113 injector temperature was set to operate at a constant 270°C throughout the entire sample analysis. The
114 primary oven was fitted with a Restek Rxi-5MS capillary column (30 m; 0.25 µm diameter and 0.25 µm
115 film thickness) and programmed to start the run at an initial temperature of 70°C for 2 min, followed
116 by an increase of 4°C/min until a final temperature of 300°C was reached and maintained for 2 min.
117 The secondary oven, equipped with a Restek Rxi-17 capillary column (1 m; 0.25 µm diameter and
118 0.25 µm film thickness), was programmed with an initial 85°C, which was increased by 4.5°C/min until
119 a final temperature of 300°C was reached and maintained for 2 min. The thermal modulator was set to
120 pulse streams of cold and hot nitrogen gas every 3 s, for 0.5 s. The detector was set to disregard all
121 mass spectra (ms) information for the first 400 s of each run to exclude solvent detection; however, this
122 was still included on the time axis of the primary column to reflect accurate retention times.
123 Additionally, the transfer line and ion source were respectively held at a constant of 270°C and 220°C
124 for the entire run, with a detector voltage of 1600 V and filament bias of -70 eV. Ms were acquired at
125 a rate of 200 ms per second, over a range of 50–800 m/z. The total run time per sample was 111.28
126 min. Following GCxGC-TOFMS analysis, 838 peaks were identified, which was processed using
127 LECO Corporation's ChromaTOF software (version 4.32), as described by (Luies and Loots, 2016).

128 **2.6 Statistical analyses**

129 Prior to statistical analysis, the data were normalized in relation to the internal standard and subjected
130 to several "clean-up steps", including a 50% zero filter, QC drift correction, QC coefficient of variation
131 filter and zero value replacement (Fernandez *et al.*, 2000; Luies and Loots, 2016). Hereafter, a natural
132 shifted log transformation was performed to correct for skewed variable distribution, as well as auto
133 scaling to align all variables (Van den Berg *et al.*, 2006) (exclusively during multivariate analysis).

134 Both multivariate and univariate statistical analyses were performed using MATLAB software (2012),
135 adjusted with the PLS toolbox (2016), to identify the metabolite markers best describing the variation
136 between the comparative groups. Multivariate approaches included principle component analysis
137 (PCA) to determine whether a natural differentiation occurred between the comparative groups, and
138 partial least square–discriminant analysis (PLS-DA) to characterize the group separation (Luies and
139 Loots, 2016). The univariate data analyses included a Wilcoxon signed rank test, corrected for multiple
140 testing by the Benjamini–Hochberg procedure, to assess the statistical significance of each compound
141 (Benjamini and Hochberg, 1995; Rosner *et al.*, 2006), while an independent effect size calculation
142 (Wilcoxon signed effect size) was used to assess the practical significance of each detected metabolite
143 (Tomczak and Tomczak, 2014).

144 **3. RESULTS**

145 The PCA differentiation between serum metabolite profiles (Fig. 1) of the marathon athletes before and
146 after the completion marathon was clearly defined. The total amount of variance explained by the first
147 three principle components (PCs) (R_2X cum) was 53%, of which PC1 accounted for 24%, PC2 for 20%
148 and PC3 for 9%. Additionally, the PLS-DA model (results not shown) showed a modelling parameter
149 R_2Y (cum) of 48.81%, indicating the total variance of the response Y, and a Q_2 (cum) of 83.37%,
150 indicating the cross-validation variation due to the response Y.

151 Since the aim of this study was to attain a holistic view of the altered human serum metabolome induced
152 by a marathon, compounds with a PCA power value ≥ 0.5 or Wilcoxon *p*-value ≤ 0.017 (BH-critical

153 value) or an effect size ω-value ≥ 0.3 were considered significant and interpreted based on their
154 associated metabolic/biochemical pathways. This multi-statistical approach yielded an initial list of 78
155 metabolite markers, of which 70 metabolite markers could be annotated by comparison of their mass
156 spectra and retention times to that of commercially available and in-house libraries developed from
157 previously injected standards (see Table 2).

158 **4. DISCUSSION**

159 The altered metabolite markers listed in Table 2 are indicative of the major metabolic pathways affected
160 by the marathon and are mainly associated with the macro-fuel substrate utilization pathways
161 (carbohydrates, lipids and amino acids) and the regulation thereof (TCA, oxidative phosphorylation
162 [OXPHOS] and gut microbiome). These metabolite pathways, along with the intermittent dietary-
163 associated metabolite markers, are comprehensively discussed below and schematically presented in
164 Fig. 2.

165 Various carbohydrate metabolites were significantly elevated following the marathon and can be
166 ascribed to gluconeogenic influx (MacLaren and Morton, 2012) and a reduced insulin secretion (Richter
167 *et al.*, 1992), which is typically induced by an initial depletion of glucose and glycogen stores occurring
168 approximately 90 min after the start of a marathon (exercise intensity dependent) at a VO_{2max} >75%
169 (Stellingwerff, 2012). The reduced insulin concentrations temporarily inactivate insulin-dependent
170 glucose uptake systems (i.e. GLUT 4 transporters and glucokinase) (Salway, 2012), preventing glucose
171 absorption into cells. This could ascribe the post-marathon elevations in serum glucose concentrations
172 and various other associated metabolites, including glucaric acid (Żółtaszek *et al.*, 2008) and mannose
173 (Hu *et al.*, 2016) as well as the elevated concentrations of gluconeogenesis-associated metabolites, i.e.
174 myo-inositol (Eisenberg and Parthasarathy, 1987), erythritol (synthesized via erythrose-4-phosphate in
175 pentose pathway) (Hootman *et al.*, 2017), glycerol and glyceric acid (Salway, 2012; Wadman *et al.*,
176 1976). Furthermore, the elevated concentrations of pyruvic acid observed in the post-marathon samples
177 were anticipated since it is an end-product of the glycolysis pathway which feeds into the TCA cycle
178 for further ATP production (Salway, 2012). This is confirmed by the accumulation of various TCA

179 cycle intermediates such as α-ketoglutaric acid, succinic acid, citric acid, fumaric acid and malic acid
180 (Qiang, 2015), which also indicate the accumulation of circulating NADH/$FADH_2$ molecules
181 (Esterhuizen *et al.*, 2017) as a result of a saturated ETC activity. It is also important to mention that
182 many of the aforementioned carbohydrate metabolites, along with elevated concentrations of mannose
183 (Hu *et al.*, 2016), sorbose (Guzik and Stachowicz, 2016), mannitol (McNutt, 2000), tagatofuranose
184 (Kroger *et al.*, 2006), and threonic acid (an ascorbic acid derivative) (Simpson and Ortwerth, 2000), are
185 well-known constituents of fruits/fruit juices, vegetables/vegetable juices, peanuts, energy bars, energy
186 drinks and various other ergogenic aids consumed by the athletes during the course of the marathon
187 (Jeukendrup, 2011; Pfeiffer *et al.*, 2012).

188 The reduction in intracellular glucose due to the aforementioned cellular uptake inhibition is known to
189 activate lipolysis of free and adipose tissue-bound triacylglycerol (TAG) (MacLaren and Morton, 2012)
190 as alternative fuel substrates. This is substantiated by the elevated concentrations of serum glycerol,
191 monopalmitin and various free fatty acids (lauric acid, palmitic acid, palmitoleic acid, 11-eicosenoic
192 acid, 11,14-eicosadienoic acid, myristoleic acid, α-linolenic acid, 5-dodecenoic acid, linoleic acid and
193 oleic acid) (Kujala *et al.*, 2013; Lewis *et al.*, 2010; Peake *et al.*, 2014; Waśkiewicz *et al.*, 2012).
194 Additionally, accumulated 3-hydroxy acids (β-hydroxyhexanoic acid) and 3-keto acids
195 (β-hydroxy-α,β-didehydrosebacic acid) are indications of a saturated β-oxidation pathway, ascribed to
196 the inhibition of the rate-limiting enzyme, β-hydroxyacyl dehydrogenase, which is pursued by
197 3-ketoacyl-CoA thiolase. This saturated β-oxidation pathway results in the catabolism of the
198 accumulated fatty acids via α-oxidation (Roe and Ding, s.a.), thus substantiating the elevated
199 concentrations of α-hydroxyoctanoic acid (C_8; an α-oxidation intermediate) and the odd-chain fatty
200 acids (OCFA; tridecanoic acid [C_{13}], pentadecanoic acid [C_{15}], heptadecanoic acid [C_{17}] and
201 10-heptadecenoic acid [$C_{17:1}$]) detected in the post-marathon serum. It should however be mentioned
202 that these OCFA may also arise from elevated dietary intake (Jenkins *et al.*, 2017) and/or autophagy of
203 various cellular constituents during extensive energy-requiring states (Singh and Cuervo, 2011).
204 Nevertheless, irrespective of their origins, these OCFAs are ultimately catabolized to propionyl-CoA
205 (Pfeuffer and Jaudszus, 2016), hence the elevated β-hydroxypropionic acid observed in the post-

206 marathon serum. The elevated concentrations of β-hydroxybutyric acid and acetoacetic acid are
207 anticipated, as these are alternative fuel substrates for the brain (Cahill and Vech, 2003) and skeletal
208 muscles (Holloszy and Coyle, 1984) in hypoglycemic states, and could also be an indication of an
209 imbalanced redox state (Esterhuizen *et al.*, 2017; Salway, 2012). Furthermore, the post-marathon
210 elevations of malonic acid typically indicate the accumulation of malonyl-CoA, which is a long-chain
211 fatty acid (LCFA) transport inhibitor (Salway, 2012) and could therefore be an additional reason for the
212 increased cytosolic LCFAs (palmitic acid, palmitoleic acid, 11-eicosenoic acid, 11,14-eicosadienoic
213 acid, myristoleic acid, α-linolenic acid, linoleic acid and oleic acid).

214 Most amino acids are catabolized into TCA cycle substrates via propionyl-CoA, succinyl-CoA, pyruvic
215 acid or acetyl-CoA, depending on the specific amino acid (Salway, 2012). Reduced concentrations of
216 amino acids (serine, glycine, alanine, aspartic acid, phenylalanine, tyrosine, threonine and methionine)
217 and altered amino acid-associated metabolite concentrations (dimethylglycine (Holm *et al.*, 2003),
218 pyroglutamic acid (Kumar and Bachhawat, 2012), indole-3-acetic acid (Salway, 2012) and glutaric acid
219 (Peake, 2016)) were detected in the post-marathon serum, which indicates amino acid catabolism during
220 the marathon. Furthermore, elevated concentrations of α-hydroxybutyric acid (an intermediate in the
221 threonine/methionine pathway), further supports the aforementioned $NADH:NAD^+$ imbalance,
222 ketoacidosis, reduced insulin secretion and impaired glucose absorption (Gall *et al.*, 2010).
223 Additionally, elevated *p*-hydroxyphenylacetic acid and
224 *p*-hydroxyphenyllactic acid could be indicative of mild liver injury/damage (Ghoraba *et al.*, 2014;
225 Liebich and Pickert, 1985), which concur with previous findings (Jastrzebski *et al.*, 2015; Lippi *et al.*,
226 2011).

227 In accordance with previous findings (Pechlivanis *et al.*, 2010), reduced serum concentrations of the
228 branched-chain amino acids (BCAAs) isoleucine (0.05 vs 0.02 μg/mL, p=0.018), valine and leucine
229 were detected in the post-marathon samples, while their catabolism intermediates i.e.
230 α-ketoisovaleric acid, β-hydroxyisobutyric acid (valine metabolites) and α-ethylhyrdracrylic acid (allo-
231 isoleucine metabolism) (Wendel *et al.*, 1989) were significantly elevated. Allo-isoleucine catabolism
232 intermediates may serve as alternative substrates in the valine catabolism pathway (Ryan, 2015),

233 however, since the valine catabolism intermediates remained elevated in the post-marathon serum it can
234 be deducted that allo-isoleucine is instead catabolized to α-ethylhydracrylic acid (Korman *et al.*, 2005)
235 via α-keto-β-methylvaleric acid (explaining its reduced concentrations post-marathon) with the
236 subsequent production of $FADH_2$ (Korman *et al.*, 2005). The elevated concentrations of β-
237 hydroxyisovaleric acid detected post-marathon is typically associated with ketone rich environments
238 (as observed in the current investigation), resulting from increased leucine catabolism and the
239 subsequent isovaleryl-CoA production (Mock *et al.*, 2011). This metabolite is also a well-known
240 constituent of athlete supplementation (Brioche *et al.*, 2016) and could be elevated due to dietary
241 ingestion during the marathon. Furthermore, reduced concentrations of these BCAAs, in particular
242 leucine, results in mammalian target of rapamycin complex 1 inhibition (Laplante and Sabatini, 2009),
243 which in turn activates various catabolic processes such as autophagy (of organelle and plasma
244 membrane constituents) to release additional embedded fuel substrates (Singh and Cuervo, 2011).
245 Mammalian target of rapamycin complex 1 (mTOR1) inhibition can also be induced by other factors
246 including elevated 5'-AMP-activated protein kinase during energy deprivation, reduced oxygen levels,
247 reduced essential amino acids and inflammation (Laplante and Sabatini, 2009), all of which are
248 associated with endurance races and evidently occur in the marathon athletes investigated in this study.
249 Considering that autophagy contributes to elevated amounts of cellular debris, the aforementioned
250 accumulation of fatty acids and especially the OCFAs, in the post-marathon serum may also be
251 explained by the autophagosomal degradation of phospholipids, sphingolipids (Kishimoto *et al.*, 1973;
252 Maes *et al.*, 1996) and phytosphingosines (Kitamura *et al.*, 2017; Kondo *et al.*, 2014) found in cell and
253 organelle membranes. Additionally, the elevated concentrations of squalene and 5-pregnene-3β,20α-
254 diol in the post-marathon serum suggest cholesterol degradation (Charlton-Menys and Durrington,
255 2007; Salway, 2012), further supporting the activation of autophagy as the latter is a common
256 constituent in cell membranes (Salway, 2012). Elevated 5-pregnene-3β,20α-diol (also known as 20α-
257 dihydropregnenolone) post-marathon also indicates steroid metabolism activation via cholesterol
258 catabolism as it is produced by the reduction of pregnenolone via 20-hydroxysteroid dehydrogenase
259 (Ebner *et al.*, 2006). Pregnenolone is a precursor for the production of aldosterone and cortisol, the
260 latter of which stimulates lipolysis, gluconeogenesis and protein catabolism (Salway, 2012).

261 Furthermore, a significant decrease in α-aminomalonic acid concentrations was observed in the post-
262 marathon serum samples. Although the precise origin of this metabolite is unknown, it has been
263 associated with abnormal protein oxidation, macrophage accumulation, non-essential amino acid
264 oxidation via reactive oxygen species and pulmonary arterial hypertension indication (Bujak *et al.*,
265 2016).

266 The microbiome plays a crucial role in many essential metabolic processes required during strenuous
267 exercise, including the regulation of energy metabolism, oxidative stress and inflammatory response
268 pathways (Mach and Fuster-Botella, 2017). To this end, various microbial-associated metabolites (i.e.
269 tagatofuranose, talofuranose, ethyl-α-D-glucopyranoside, arabitol, indole-3-propionic acid and
270 D-rhamnose) were significantly altered in the post-marathon samples. Most of these metabolites are
271 synthesized from host carbohydrate intermediates, either via catabolism or fermentation processes
272 (arabitol: a sugar alcohol produced form arabinose/glucose (Kumdam *et al.*, 2014); and ethyl-α-D-
273 glucopyranoside: an ethnolysis product from glucose (Hu *et al.*, 2013)). Indole-3-propionic acid is the
274 deamination product of tryptophan, produced by gut microbes (e.g. *Clostridium sporogenes*) (Wikoff
275 *et al.*, 2009) and further supports the notion of reduced amino acids. The reduced concentrations of D-
276 rhamnose, a component of most Gram-positive bacterial cell walls (Mistou *et al.*, 2016), contradict the
277 above-mentioned microbial product elevations. Since the particular nature of this reduction following
278 completion of the marathon is unclear, it warrants future investigation.

279 In addition to these endogenously and microbially produced metabolites, elevated concentrations of
280 ibuprofen were also observed post-marathon. Ibuprofen is a well-known non-steroidal, anti-
281 inflammatory drug (Nieman *et al.*, 2006) commonly used by athletes for preventing muscle damage,
282 muscle soreness and inflammation and was most likely consumed by some athletes prior to/during the
283 marathon (Mcanulty *et al.*, 2007).

284 **5. CONCLUSION**

285 The results of this metabolomics investigation suggest that the body utilizes various fuel substrate
286 pathways to comply with the high energy demands required during the marathon, including catabolism

287 of carbohydrates, lipids (β-oxidation and α-oxidation) and amino acids, as well as activation of
288 ketogenesis and autophagy via mTOR1 inhibition. Considering the results of the current investigation
289 as well as previous literature, the possible cascade of events contributing to this metabolic "snapshot"
290 could be summarized as follows: (a) A proposed initial reduction in carbohydrate catabolism and
291 glucose uptake via the insulin-dependent transporters lead to glycolysis dysregulation, ketogenesis
292 activation and increased serum glucose. (b) A metabolic shift towards fatty acid utilization (from either
293 endogenous or dietary TAGs) is induced, which (c) overwhelms/saturates the β-oxidation pathway,
294 resulting in the α-oxidation of fatty acids. (d) Amino acids (from either endogenous or dietary protein
295 catabolism) are also used as alternative fuel substrates, resulting in (e) mTOR1 inhibition and autophagy
296 as the body desperately tries to generate the necessary fuel substrates to comply with the energy demand.
297 (f) Lastly, various metabolic processes are activated to reduce oxidative stress and regulate/correct the
298 redox imbalance.

299 Possible limitations of this study include human genotype/phenotype variation (an inevitable
300 confounder) and the uncontrolled dietary intake of the athletes during the marathon. However,
301 convincing athletes to deviate from their individualized supplementation protocols would be extremely
302 difficult, if not impossible. Validation using a larger sample cohort could further substantiate the current
303 findings. Nonetheless, these findings indicate the extensive metabolic changes induced by the marathon
304 perturbation. Possible future prospects could be to investigate the effects of supplementing with amino
305 acids, pre- and probiotics, and β-hydroxyisovaleric acid as a means of improving aerobic exercise
306 performance, reduce skeletal muscle and liver damage, and enhance recovery.

307 **6. FOOTNOTES**

308 **6.1 Author contribution**

309 The concept and study were designed by DTL, ZS, GH, TC, KMK and EJS; samples were acquired
310 from the Northumbria University in collaboration with GH, TC, KMK and EMS. ZS was responsible
311 for manuscript drafting, data analysis and interpretation, the latter of which was assisted by DTL, LL
312 and LJM. LL, LJM and DTL were involved in repeated manuscript reviewing, of which LL was greatly

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320 **6.4 Data availability statement**

321 The current analysis is part of a larger study consisting of multiple aims which are being drafted into
322 various manuscripts. Considering this, the datasets generated from this investigation are not publically
323 available, but can be acquired from the corresponding author on reasonable request. The authors declare
324 that all the results included within this study has been presented clearly, honestly and without
325 fabrication, falsification, or inappropriate data manipulation.

326 **7. COMPLIANCE WITH ETHICAL STANDARDS**

327 **7.1 Conflict of interest**

328 The authors declare that there are no conflicts of interest, and that this manuscript, and the work
329 described therein, is unpublished and has not been submitted for publication elsewhere.

330 **7.2 Ethical approval**

331 Ethical approval for this investigation, conducted according to the Declaration of Helsinki and
332 International Conference on Harmonization Guidelines, was obtained from the Research Ethics
333 Committee of the Faculty of Health and Life Sciences at the Northumbria University in Newcastle upon

334 Tyne, UK (reference number: HLSTC120716). Informed consent was obtained from all individuals
335 included in the study.

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509

510 **9. TABLES**

511 **Table 1** Summary of the participant demographical information

Participant demographical information	Average ± Standard deviation
Age (years)	41 ± 12
Pre-marathon athlete weight (kg)	71.3 ± 10.1
Post-marathon athlete weight (kg)	69.2 ± 9.7
Marathon experience (years)	9 ± 8
Marathon experience (races)	16 ± 29
Finishing time (hh:mm:ss)	04:19:09 ± 00:49:01

512

513 **Table 2** The significant serum metabolite markers best describing the variation between the pre- and

514 post-marathon groups, listed alphabetically

Metabolite name (PubChem ID)	Pre-marathon athletes:	Post-marathon athletes:	Wilcoxon Signed Rank test		PCA
	Concentration (µg/ml) (Standard deviation)		(*p* value)	(ω value)	(Power)
α-Aminomalonic acid (100714)	0.046 (0.022)	0.025 (0.009)	3.1×10^{-4}	0.458	0.031
α-Ethylhydracrylic acid (188979)	0.002 (0.001)	0.005 (0.002)	1.3×10^{-6}	0.615	0.025
α-Hydroxybutyric acid (11266)	0.019 (0.011)	0.045 (0.023)	2.8×10^{-6}	0.595	0.018
α-Hydroxyoctanoic acid (94180)	8.7×10^{-5} (5.3×10^{-5})	1.2×10^{-4} (6.3×10^{-5})	0.001	0.413	0.003
α-Ketoglutaric acid (51)	2.9×10^{-4} (2.1×10^{-4})	4.5×10^{-4} (1.8×10^{-4})	1.1×10^{-4}	0.490	0.009
α-Ketoisovaleric acid (5204641)	0.002 (0.001)	0.003 (0.001)	1.7×10^{-4}	0.478	0.017
α-Keto-β-methylvaleric acid (47)	0.009 (0.003)	0.007 (0.002)	0.005	0.358	0.025
α-Linolenic acid (5280934)	1.6×10^{-4} (8.1×10^{-5})	4.5×10^{-4} (2.6×10^{-4})	2.1×10^{-6}	0.602	0.021
β-Hydroxybutyric acid (441)	0.021 (0.019)	0.215 (0.161)	1.2×10^{-6}	0.617	0.030
β-Hydroxyhexanoic acid (151492)	3.2×10^{-4} (1.6×10^{-5})	8.9×10^{-4} (3.1×10^{-4})	2.1×10^{-6}	0.602	0.033
β-Hydroxyisobutyric acid (87)	2.9×10^{-5} (4.9×10^{-5})	2.9×10^{-4} (1.4×10^{-4})	1.2×10^{-6}	0.617	0.034
β-Hydroxyisovaleric acid (69362)	0.002 (0.001)	0.002 (0.001)	0.011	0.321	0.009

β-Hydroxypropionic acid (68152)	0.003 (0.001)	0.004 (0.001)	0.007	0.343	0.013
β-Hydroxy-α,β-didehydrosebacic acid (5366445)	0.005 (0.002)	0.006 (0.002)	0.009	0.333	0.013
5-Dodecenoic acid (5312377)	$1.3x10^{-4}$ ($2.8x10^{-5}$)	0.002 (0.001)	$1.2x10^{-6}$	0.617	0.037
5-Pregnene-3β,20α-diol (312224064)	$3.0x10^{-4}$ ($2.7x10^{-4}$)	$6.1x10^{-4}$ ($4.3x10^{-4}$)	$3.4x10^{-6}$	0.590	0.007
10-Heptadecenoic acid (86289714)	0.001 (0.001)	0.003 (0.001)	$1.2x10^{-6}$	0.617	0.030
11-Eicosenoic acid (142770)	0.001 (0.001)	0.004 (0.001)	$1.3x10^{-6}$	0.615	0.033
11,14-Eicosadienoic acid (3208)	0.002 (0.001)	0.003 (0.001)	$9.3x10^{-4}$	0.421	0.008
Acetoacetic acid (96)	$7.5x10^{-5}$ ($5.4x10^{-5}$)	$2.1x10^{-4}$ ($1.5x10^{-4}$)	0.002	0.393	0.014
Alanine (5950)	$4.2x10^{-4}$ ($2.9x10^{-4}$)	$2.6x10^{-4}$ ($1.5x10^{-4}$)	0.004	0.368	0.003
Arabitol (439255)	0.004 (0.001)	0.005 (0.002)	$9.9x10^{-4}$	0.418	0.006
Aspartic acid (5960)	0.020 (0.012)	0.013 (0.007)	0.014	0.311	0.019
Citric acid (311)	$9.6x10^{-4}$ ($4.5x10^{-4}$)	0.002 (0.001)	$8.9x10^{-5}$	0.498	0.012
Dimethylglycine (673)	0.023 (0.013)	0.017 (0.008)	0.011	0.324	0.008
D-Rhamnose (5460029)	0.001 (0.001)	0.001 (0.001)	$1.1x10^{-2}$	0.324	0.006
Erythritol (222285)	0.007 (0.002)	0.012 (0.004)	$1.6x10^{-6}$	0.610	0.016
Ethyl-α-D-glucopyranoside (91733361)	0.169 (0.085)	0.213 (0.094)	0.008	0.336	0.003
Fumaric acid (444972)	0.005 (0.002)	0.007 (0.002)	$7.5x10^{-4}$	0.428	0.018
Glucaric acid (33037)	$4.1x10^{-4}$ ($1.8x10^{-4}$)	0.001 (0.001)	0.002	0.391	0.010
Glucose (5793)	0.001 (0.001)	0.003 (0.005)	$8.7x10^{-6}$	0.565	0.025
Glutaric acid (743)	$3.1x10^{-4}$ ($1.9x10^{-4}$)	$4.7x10^{-4}$ ($2.8x10^{-4}$)	0.009	0.331	0.009
Glyceric acid (752)	0.005 (0.002)	0.008 (0.003)	$6.9x10^{-5}$	0.505	0.015
Glycerol (753)	0.088 (0.042)	0.447 (0.143)	$1.2x10^{-6}$	0.617	0.041
Glycine (750)	0.042 (0.019)	0.026 (0.013)	$7.5x10^{-4}$	0.428	0.024
Heptadecanoic acid (10465)	0.004 (0.001)	0.006 (0.001)	$1.2x10^{-6}$	0.617	0.020
Ibuprofen (3672)	$7.5x10^{-6}$ ($1.8x10^{-5}$)	0.004 (0.013)	$6.4x10^{-5}$	0.508	0.011
Indole-3-propionic acid (3744)	0.002 (0.001)	0.001 (0.001)	$1.8x10^{-4}$	0.475	0.004
Indole-3-acetic acid (802)	0.003 (0.002)	0.002 (0.001)	$8.1x10^{-4}$	0.426	0.021
Lauric acid (3893)	0.011 (0.003)	0.020 (0.005)	$1.2x10^{-6}$	0.617	0.026

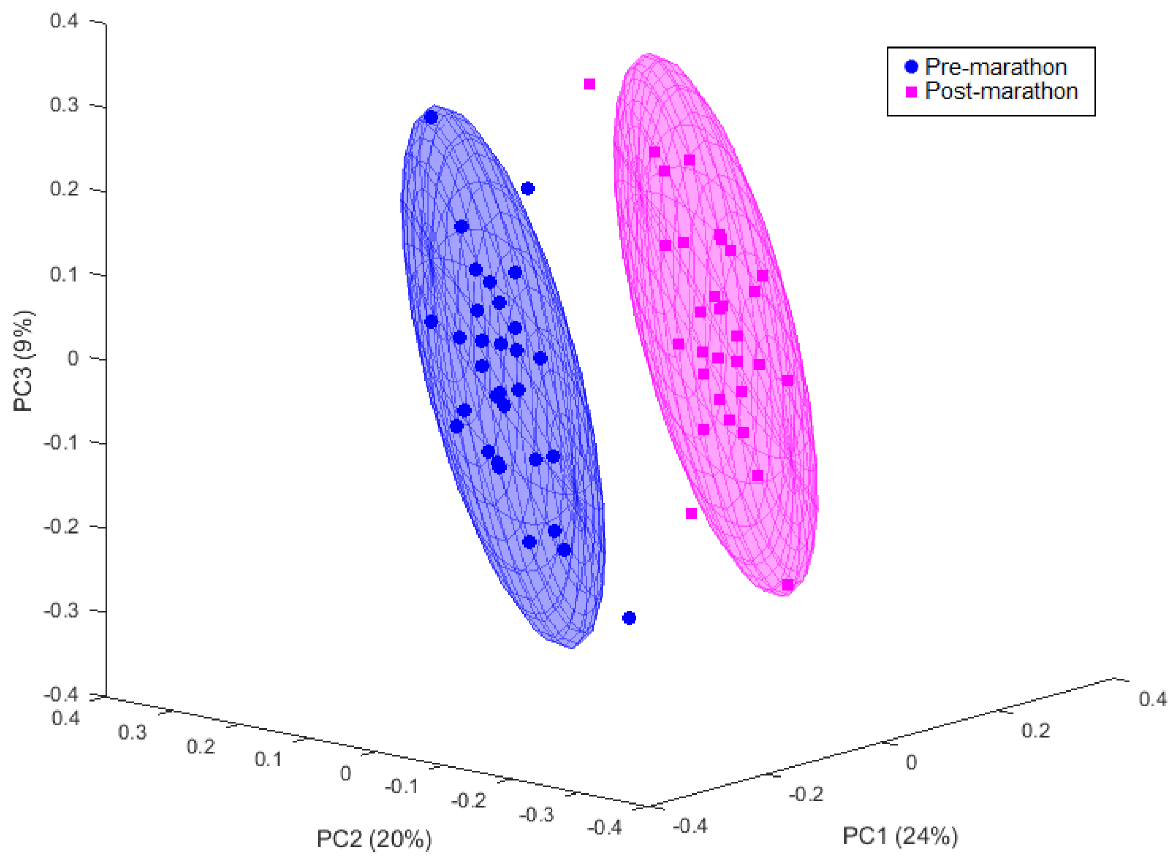
Leucine (6106)	0.130 (0.087)	0.064 (0.044)	0.002	0.398	0.022
Linoleic acid (5280450)	0.124 (0.050)	0.168 (0.055)	4.9×10^{-4}	0.443	0.006
Malic acid (525)	0.004 (0.002)	0.007 (0.003)	3.1×10^{-6}	0.592	0.018
Malonic acid (867)	3.1×10^{-4} (1.2×10^{-4})	4.1×10^{-4} (1.7×10^{-4})	0.005	0.353	0.006
Mannitol (6251)	0.004 (0.004)	0.007 (0.010)	0.006	0.348	0.004
Mannose (18950)	3.2×10^{-4} (4.3×10^{-4})	0.003 (0.005)	5.5×10^{-6}	0.577	0.024
Methionine (6137)	0.006 (0.004)	0.003 (0.002)	6.9×10^{-4}	0.431	0.017
Myo-inositol (892)	0.031 (0.030)	0.045 (0.035)	8.1×10^{-4}	0.426	0.011
Monopalmitin (14900)	3.9×10^{-4} (2.1×10^{-4})	6.7×10^{-4} (3.6×10^{-4})	0.002	0.401	0.011
Myristoleic acid (5281119)	0.001 (0.001)	0.006 (0.002)	1.2×10^{-6}	0.617	0.037
Oleic acid (445639)	0.127 (0.072)	0.511 (0.196)	1.3×10^{-6}	0.615	0.022
Palmitic acid (985)	0.423 (0.124)	0.633 (0.128)	1.6×10^{-5}	0.548	0.016
Palmitoleic acid (445638)	0.009 (0.008)	0.042 (0.022)	7.2×10^{-6}	0.570	0.026
Pentadecanoic acid (13849)	0.004 (0.002)	0.008 (0.002)	1.2×10^{-6}	0.617	0.022
Phenylalanine (6140)	0.030 (0.015)	0.020 (0.009)	0.002	0.393	0.021
p-Hydroxyphenylacetic acid (127)	0.002 (0.001)	0.003 (0.002)	0.001	0.408	0.008
p-Hydroxyphenyllactic acid (9378)	0.003 (0.002)	0.005 (0.002)	2.9×10^{-4}	0.460	0.014
Pyroglutamic acid (7405)	0.060 (0.014)	0.050 (0.023)	2.3×10^{-4}	0.468	0.009
Pyruvic acid (1060)	0.013 (0.009)	0.024 (0.012)	7.5×10^{-4}	0.428	0.016
Serine (5951)	0.033 (0.024)	0.018 (0.011)	0.014	0.314	0.021
Sorbose (439192)	0.015 (0.006)	0.022(0.01)	6.1×10^{-4}	0.436	0.008
Squalene (638072)	0.002 (0.001)	0.003 (0.004)	0.006	0.351	0.011
Succinic acid (1110)	0.011 (0.008)	0.016 (0.010)	5.5×10^{-6}	0.577	0.006
Tagatofuranose (12306016)	0.101 (0.058)	0.190 (0.122)	3.9×10^{-4}	0.450	0.012
Talofuranose (15560229)	0.147 (0.050)	0.253 (0.090)	2.3×10^{-5}	0.538	0.011
Threonic acid (5460407)	0.011 (0.004)	0.016 (0.005)	1.1×10^{-5}	0.557	0.025
Threonine (6288)	0.021 (0.014)	0.011 (0.007)	0.003	0.381	0.017
Tridecanoic acid (12530)	2.4×10^{-4} (1.0×10^{-4})	3.8×10^{-4} (1.7×10^{-4})	9.5×10^{-6}	0.562	0.010
Tyrosine (6057)	0.019 (0.008)	0.014 (0.006)	0.007	0.343	0.018
Valine (6287)	0.188 (0.115)	0.094 (0.061)	9.3×10^{-4}	0.421	0.027

515 Abbreviations: PCA: Principle component analysis

516

517 ## 10. FIGURE LEDGENDS

518 **Fig. 1** Principle component analysis scores plot showing clear differentiation of the serum samples of
519 marathon athletes before (denoted by circles) and after (denoted by squares) the completion of a
520 marathon. The variance accounted for are indicated in parenthesis. Abbreviations: PC: principle
521 component



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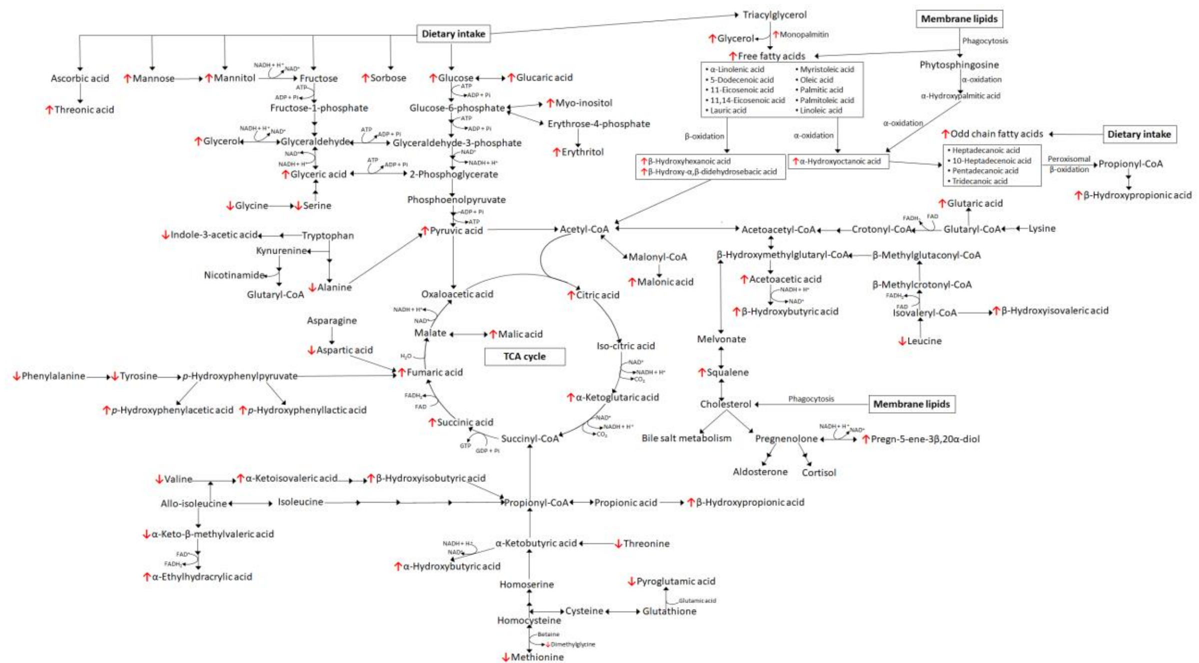
524 **Fig. 2** A schematic representation of the altered serum metabolome induced by a marathon. The altered
525 metabolites are either donated as increased (↑) or decreased (↓) relative to the pre-marathon group.
526 Abbreviations: FAD: flavin adenine dinucleotide, FADH: flavin adenine dinucleotide + hydrogen,
527 NAD: nicotinamide adenine dinucleotide, NADH: nicotinamide adenine dinucleotide + hydrogen,
528 ATP: adenosine triphosphate, ADP: adenosine diphosphate



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