

1 **The effect of exercise intensity on circulating hepatokine concentrations in healthy men**

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31 **Abstract**

32 Fibroblast growth factor 21 (FGF21), follistatin and leukocyte cell-derived chemotaxin 2  
33 (LECT2) are novel hepatokines which are modulated by metabolic stresses. This study  
34 investigated whether exercise intensity modulates the hepatokine response to acute exercise.

35 Ten young, healthy men undertook three 8-h experimental trials: moderate-intensity exercise  
36 (MOD; 55%  $\dot{V}O_2$  peak), high-intensity exercise (HIGH; 75%  $\dot{V}O_2$  peak) and control (CON;  
37 rest), in a randomised, counterbalanced order. Exercise trials commenced with a treadmill run  
38 of varied duration to match gross exercise energy expenditure between trials (MOD vs HIGH;  
39  $2475 \pm 70$  vs  $2488 \pm 58$  kJ). Circulating FGF21, follistatin, LECT2, glucagon, insulin, glucose  
40 and non-esterified fatty acids (NEFA) were measured before exercise and at 0, 1, 2, 4 and 7 h  
41 post-exercise.

42 Plasma FGF21 concentrations were increased up to 4 h post-exercise compared to CON ( $P \leq$   
43  $0.022$ ) with greater increases observed at 1, 2 and 4 h post-exercise during HIGH vs MOD ( $P$   
44  $\leq 0.025$ ). Irrespective of intensity ( $P \geq 0.606$ ), plasma follistatin concentrations were elevated  
45 at 4 and 7 h post-exercise ( $P \leq 0.053$ ). Plasma LECT2 concentrations were increased  
46 immediately post-exercise ( $P \leq 0.046$ ) but were not significant after correcting for plasma  
47 volume shifts. Plasma glucagon (1 h;  $P = 0.032$ ) and NEFA (4 and 7 h;  $P \leq 0.029$ ) responses  
48 to exercise were accentuated in HIGH vs MOD.

49 These findings demonstrate that acute exercise augments circulating FGF21 and follistatin.  
50 Exercise-induced changes in FGF21 are intensity-dependent and may support the greater  
51 metabolic benefit of high-intensity exercise.

52 **Key words:** exercise, physical activity, hepatokines, liver, FGF21, follistatin, LECT2, insulin  
53 resistance

## 54 **Introduction**

55 The liver plays an integral role in the regulation of metabolic homeostasis through inter-organ  
56 crosstalk with other metabolically active tissues (Iroz et al. 2015, Meex and Watt 2017). In  
57 recent years, several exclusively or predominantly liver-secreted proteins, termed hepatokines,  
58 have been identified as molecules possessing the capacity to exert potent metabolic effects,  
59 both locally and distally (Meex et al. 2015). These include fibroblast growth factor 21 (FGF21),  
60 shown to modulate glucose and lipid metabolism (Kharitononkov et al. 2005, Potthoff et al.  
61 2009, Xu et al. 2009, BonDurant and Potthoff 2018); follistatin, demonstrated to regulate  
62 muscle growth (Amthor et al. 2004, Yaden et al. 2014) and influence pancreatic beta cell  
63 function and survival (Zhao et al. 2015, Hansen et al. 2016a); and leukocyte cell-derived  
64 chemotaxin 2 (LECT2), which can promote both skeletal muscle and adipose tissue insulin  
65 resistance (Lan et al. 2014, Jung et al. 2018).

66 Chronic positive energy balance (Toye et al. 2007, Kirpich et al. 2011), hepatic steatosis (Meex  
67 et al. 2015) and obesity-associated metabolic disease, modulate hepatic protein synthesis and  
68 hepatokine secretion (Fu et al. 2012; Meex et al. 2015). Recent research suggests that  
69 hepatokines may also be sensitive to acute perturbations in energy and substrate metabolism  
70 through coordinating metabolic responses between the liver and extra-hepatic tissues (Xu et al.  
71 2009, Lan et al. 2014, Hansen and Plomgaard 2016). For example, hepatokines are implicated  
72 in the coordination of metabolic responses to both fasting and feeding (Lan et al. 2014, Markan  
73 et al. 2014). Furthermore, hepatokines may participate in metabolic responses to exercise;  
74 potentially contributing to the acute metabolic benefit associated with physical exertion such  
75 as improved whole-body substrate metabolism and insulin sensitivity (Kim et al. 2013, Hansen  
76 et al. 2015, Weigert et al. 2018).

77 To date, a handful of studies have examined the acute effects of exercise on circulating  
78 hepatokines. This research has shown that exercise increases circulating levels of FGF21 and  
79 follistatin (Hansen et al. 2011, Kim et al. 2013, Hansen et al. 2015, Hansen et al. 2016a, Hansen  
80 et al. 2016b, Sargeant et al. 2018), whilst circulating LECT2 is suppressed after exercise in  
81 mice (Lan et al. 2014) but is unaffected in humans (Sargeant et al. 2018). Exercise intensity is  
82 a key variable which mediates the metabolic benefit of acute exercise, with protocols of higher  
83 intensity typically providing the greatest therapeutic effects (Wahren et al. 1978, Wen et al.  
84 2011). Given that FGF21, follistatin and LECT2 responses to exercise are mediated via  
85 metabolic factors sensitive to exercise intensity (for example, glucagon, insulin and non-  
86 esterified fatty acids) (Hansen et al. 2015, Hansen et al. 2016a), it is possible that exercise  
87 intensity may also modulate circulating hepatokines.

88 One previous study explored this hypothesis, demonstrating that circulating FGF21 was higher  
89 in healthy men, one hour after 30 minutes of treadmill running at 80% vs 50% of maximum  
90 oxygen uptake (Kim et al. 2013). This study was, however, limited by the fact that the exercise  
91 protocols were not matched for energy expenditure and no control trial was included.  
92 Consequently, further research is required to explore the impact of exercise intensity on  
93 circulating hepatokines by matching exercise-related energy expenditure between exercise  
94 protocols of different intensities; and to compare responses against a non-exercise control trial.

95 Therefore, the present study investigated the effect of exercise intensity, using two energy-  
96 matched exercise trials, on circulating concentrations of FGF21, follistatin and LECT2 in  
97 healthy men. Based on previous findings, it was hypothesised that both exercise intensities  
98 would lead to transient increases in circulating FGF21 and follistatin, whilst circulating LECT2  
99 levels would be reduced following exercise. Furthermore, these responses would be more  
100 pronounced after the performance of high- vs moderate-intensity exercise.

101 **Materials and methods**

102 *Participants*

103 Following approval from the Institutional Ethical Advisory Committee (ref: R16-P067), 10  
104 healthy men participated in the study. Participant characteristics are presented in Table 1. All  
105 participants were non-smokers, metabolically healthy, weight stable for three months prior to  
106 participation (< 2 kg body mass change) and were not currently taking any medications. Written  
107 informed consent was provided by each participant before data collection commenced.

108

109 *Insert table 1*

110

111 *Participant pre-assessment*

112 Participants attended an initial pre-assessment session in which they completed a health screen  
113 questionnaire, the Physical Activity Readiness Questionnaire (PAR-Q+; Bredin et al. 2013)  
114 and food preference questionnaire, before undergoing anthropometric measurements. Height  
115 and body mass were measured using a wireless measuring station (Seca 285, Seca Ltd,  
116 Germany) and body mass index (BMI) was subsequently calculated. Waist circumference was  
117 measured at the smallest circumference between the xiphoid process and the iliac crest, whilst  
118 body composition was determined using bio-electrical impedance analysis (BC-418, TANITA,  
119 Europe BC, Amsterdam, the Netherlands).

120 Participants then undertook a submaximal incremental exercise test on a motorised treadmill  
121 (Technogym Excite Med, Cesena, Italy), consisting of four, 4-min stages at progressively faster  
122 speeds, to determine the relationship between treadmill speed and oxygen consumption ( $\dot{V}O_2$ )

123 for each participant. The first stage commenced at a speed between 7-8 km·h<sup>-1</sup>, with each  
124 subsequent stage increasing by 1-2 km·h<sup>-1</sup>, according to participant fitness. Expired air was  
125 sampled continuously using breath-by-breath indirect calorimetry (Cortex Metalyzer 3B,  
126 Leipzig, Germany) to quantify  $\dot{V}O_2$  and carbon dioxide production ( $\dot{V}CO_2$ ). Heart rate was  
127 recorded continuously using short-range telemetry (Polar T31; Polar Electro, Kempele,  
128 Finland), whilst rating of perceived exertion (RPE; Borg. 1973) was assessed in the last 30 s  
129 of each stage.

130 After a standardised rest period of 10 min, participants then completed an incremental maximal  
131 exercise test to determine peak oxygen uptake ( $\dot{V}O_2$  peak). The test commenced at a gradient  
132 of 0% and increased by 1% every minute until volitional exhaustion. The maximal exercise  
133 test was designed to bring the participants to exhaustion within approximately 8-12 min and  
134 the treadmill speed was determined by the individual performance of each participant in the  
135 submaximal exercise test. The breath-by-breath data were analysed using a 30 s average  
136 (Robergs et al. 2010) to identify the participants'  $\dot{V}O_2$  peak.

### 137 *Experimental design*

138 Participants completed three main experimental trials: a moderate-intensity (MOD) trial, a  
139 high-intensity (HIGH) trial and a control (CON) trial. The main trials were carried out in a  
140 randomised, counterbalanced order, with at least five days separating each trial. In the 24 h  
141 prior to each main trial, participants were asked to standardise their food intake using a weighed  
142 food record and to abstain from exercise, caffeine and alcohol. Each main trial lasted 8 h  
143 (08:00-16:00) as shown schematically in Figure 1.

144

145

*Insert figure 1*

146

147 *Experimental protocol*

148 On the day of the main trials, participants arrived at the laboratory at 08:00 following an  
149 overnight fast of at least 10 h. During MOD and HIGH trials, participants performed a  
150 continuous treadmill run at moderate-intensity (55%  $\dot{V}O_2$  peak) and high-intensity (75%  $\dot{V}O_2$   
151 peak), respectively, resting thereafter. Participants rested throughout the CON trial. Both  
152 exercise protocols were designed to elicit a gross energy expenditure of 2510 kJ (600 kcal);  
153 meaning the exercise duration differed between MOD and HIGH. The treadmill speed required  
154 to elicit the target intensities was predicted via bivariate linear regression using participants'  
155  $\dot{V}O_2$  peak and  $\dot{V}O_2$ -running speed relationship determined in the pre-assessment session. The  
156 rate of energy expenditure for each trial was then calculated using equations by Frayn (1983)  
157 and the exercise duration required to induce a gross energy expenditure of 2510 kJ was  
158 subsequently estimated. Expired air was sampled continuously during exercise to determine  
159 the actual gross energy expenditure and percentage substrate oxidation of the exercise period.  
160 The speed of the treadmill was adjusted, if necessary, to maintain the target  $\dot{V}O_2$ . Heart rate  
161 was monitored continuously throughout; whilst RPE was recorded every 10 min. Exercise  
162 commenced between 08:00 and 09:00 in a staggered fashion so that exercise finished at 09:00.

163 A standardised breakfast (jam sandwich with butter, strawberry breakfast bar, banana and  
164 chocolate milkshake) was provided to the participants at 2 h post-exercise (11:00), containing  
165 an energy content equal to 35% of the participants' estimated daily energy requirement and a  
166 macronutrient content of 69% carbohydrate, 14% protein and 17% fat. Individuals' daily  
167 energy requirements were calculated from their estimated basal metabolic rate (BMR), which  
168 was multiplied by a physical activity correction factor of 1.4 (Mifflin et al. 1990) to reflect the  
169 sedentary nature of prolonged sitting in the laboratory during the main trials. A standardised



170 lunch (cheese sandwich with butter, salted crisps, mini chocolate roll and apple) was then  
171 provided at 6 h post-exercise (15:00), also containing 35% of the participants' estimated daily  
172 energy requirement (42% carbohydrate, 13% protein and 45% fat).

### 173 ***Blood sampling***

174 Venepuncture blood samples were taken from an antecubital vein upon arrival (baseline; 08:00)  
175 and immediately after exercise (0 h; 09:00). At approximately 0.5 h, a cannula (Venflon,  
176 Becton Dickinson, Helsingborg, Sweden) was inserted into an antecubital vein for further  
177 venous blood sample collection at 1, 2, 4 and 7 h post-exercise. Following each collection, the  
178 cannula was flushed with 10 mL of saline (0.9% w/v sodium chloride, Fannin Ltd, Dublin,  
179 Ireland) to maintain patency. To avoid dilution of subsequent samples, residual saline was  
180 drawn off immediately prior to each sample collection using a 2 mL syringe. All blood samples  
181 were taken with the participant in a semi-supine position to control for any postural changes in  
182 plasma volume.

183 Blood samples were collected into pre-chilled EDTA and lithium heparin monovettes (Sarstedt,  
184 Leicester, UK). All monovettes were centrifuged at 1750 g for 10 min at 4°C (Heraeus  
185 Labofuge 400R, Thermo Electron, Osterode, Germany) and the resultant plasma supernatant  
186 was aliquoted into cryovials. All samples were stored at -80°C until subsequent (batch) analysis.

187 Haemoglobin and haematocrit concentrations were determined from each sample using 20 µL  
188 micropipettes and 20 µL heparinised microhaematocrit tubes. These measures were taken in  
189 duplicate and triplicate, respectively; and were used for subsequent estimation of changes in  
190 plasma volume (Dill and Costill 1974).

### 191 ***Biochemical analyses***

192 Commercially available enzyme-linked immunosorbent assays were used to determine plasma  
193 concentrations of FGF21(DF2100, R&D Systems, Oxford, UK), follistatin (DFN00, R&D  
194 Systems, Oxford UK), LECT2 (5327, MBL International, Massachusetts, USA), insulin (10-  
195 113-01, Merckodia AB, Uppsala, Sweden) and glucagon (10-1271-01, Merckodia AB, Uppsala,  
196 Sweden). Samples from each participant were analysed on the same assay plate to eliminate  
197 inter-assay variation. The mean within-batch coefficients of variation (CVs) for these assays  
198 was  $\leq 6.0\%$ . Plasma glucose (A11A01668, Horiba Medical, Montpellier, France) and non-  
199 esterified fatty acids (NEFA; FA115, Randox Laboratories Ltd., County Antrim, UK) were  
200 determined using enzymatic, colorimetric methods using a bench-top analyser (Pentra 400;  
201 Horiba Medical, Montpellier, France). The mean within-batch CVs for plasma glucose and  
202 NEFA were 0.43% and 1.95%, respectively.

### 203 *Sample size calculation*

204 Based on published data by our research group examining the effects of moderate-intensity  
205 exercise on the total area under the curve (AUC) for FGF21 in normal-weight healthy men  
206 (mean difference  $\pm$  SD:  $286 \pm 340 \text{ pg}\cdot\text{mL}^{-1}\cdot 7 \text{ h}^{-1}$ , giving a standardised difference of 0.84, and  
207 with an intra-person correlation of 0.76) (Sargeant et al. 2018), we required nine participants  
208 to complete our three-trial cross-over study to detect a main effect of trial for FGF21 total AUC,  
209 with 90% power and an alpha error rate of 0.05. To account for participant drop-out or non-  
210 compliance, 10 individuals were recruited. Notably, based on similar data from our previous  
211 study for follistatin total AUC (mean difference  $\pm$  SD:  $630 \pm 942 \text{ pg}\cdot\text{mL}^{-1}\cdot 7 \text{ h}^{-1}$ , giving a  
212 standardised difference of 0.67, and with an intra-person correlation of 0.88), we also had over  
213 90% power to detect a main effect of trial on follistatin total AUC in the current study.

### 214 *Statistical analyses*

215 All statistical analyses were performed using SPSS version 23 (SPSS Inc., Chicago, Illinois).  
216 The preprandial (baseline-2 h), postprandial (2-7 h) and total AUC were calculated using the  
217 trapezoidal method for all outcomes during each trial. Shapiro-Wilk tests were used to assess  
218 the distribution of the data and, when not normally distributed, data were log transformed and  
219 reassessed. Differences between exercise characteristics were assessed using paired *t*-tests.  
220 One-way repeated-measures analysis of variance (ANOVA) was used to examine differences  
221 between trials in baseline concentrations and AUC values for each analyte. Two-way, repeated-  
222 measures ANOVA (within-participant factors: trial and time) was used to assess differences in  
223 hepatokine and metabolite responses over the duration of the different trials. Where a  
224 significant trial and interaction effect was observed, *post-hoc* analyses were performed using  
225 one-way ANOVAs and subsequent pairwise comparisons at each time point to locate any  
226 differences. A Bonferroni adjustment for multiple comparisons was then applied. If Mauchly's  
227 test of sphericity was violated, Greenhouse-Geisser corrected values were used. Correction of  
228 FGF21, follistatin, glucose, NEFA, insulin and glucagon data for plasma volume changes did  
229 not alter the statistical significance of findings, therefore unadjusted values are presented. The  
230 statistical outcomes for LECT2 were altered by plasma volume adjustment, therefore statistical  
231 outcomes are presented for both adjusted and unadjusted data. An alpha value of  $P < 0.05$  was  
232 set to indicate statistical significance. Data are presented as means  $\pm$  SD or SEM, where  
233 indicated.

## 234 **Results**

### 235 *Exercise characteristics*

236 The characteristics for the MOD and HIGH exercise protocols are presented in Table 2.  
237 Treadmill speed, oxygen uptake, and relative exercise intensity were greater during HIGH  
238 compared to MOD (all  $P \leq 0.001$ ). However, the exercise duration was shorter during HIGH  
239 than MOD ( $P < 0.001$ ); resulting in similar gross energy expenditures between the two exercise  
240 protocols ( $P = 0.459$ ). Respiratory exchange ratio (RER) and percentage carbohydrate  
241 oxidation were significantly higher, and percentage fat oxidation significantly lower, in HIGH  
242 compared with MOD (all  $P \leq 0.001$ ). Heart rate and RPE were also greater in HIGH in  
243 comparison to MOD (both  $P \leq 0.001$ ).

244

245 *Insert table 2*

246

### 247 *Hepatokine responses to exercise*

248 Baseline plasma concentrations of FGF21 were similar between trials ( $P = 0.235$ ). Two-way  
249 ANOVA showed a significant main effect of trial and trial by time interaction for plasma  
250 FGF21 concentrations (both  $P < 0.001$ ; Figure 2A). *Post-hoc* analyses revealed significantly  
251 higher plasma FGF21 concentrations during MOD compared to CON at 0, 1 and 2 h; and during  
252 HIGH compared to CON at 1, 2 and 4 h (all  $P \leq 0.017$ ). Additionally, plasma FGF21  
253 concentrations were significantly higher during HIGH vs MOD at 1, 2 and 4 h (all  $P \leq 0.025$ ).  
254 Table 3 details the preprandial (baseline-2 h), postprandial (2-7 h) and total FGF21 AUC.

255 Compared with control, the FGF21 AUC was higher preprandially in MOD and HIGH ( $P \leq$   
256 0.009) yet remained elevated postprandially only in HIGH ( $P = 0.001$ ).

257 Boxplot analysis of total trial AUC values for plasma follistatin identified one participant as an  
258 outlier (Field 2009). This was due to the participant exhibiting mean plasma follistatin  
259 concentrations during HIGH which were 13 times greater than the remaining group SD on the  
260 same trial. Consequently, all data for this participant were removed from follistatin analyses  
261 and thus results are presented for  $n = 9$ . No differences were observed in fasting plasma  
262 follistatin concentrations between trials at baseline ( $P = 0.138$ ). Two-way ANOVA showed a  
263 significant main effect of trial, time and trial by time interaction for plasma follistatin  
264 concentrations (all  $P \leq 0.002$ ; Figure 2B). *Post-hoc* analyses revealed that when compared to  
265 CON, plasma follistatin concentrations tended to be higher at 4 h ( $P = 0.053$ ) and were  
266 significantly higher at 7 h during MOD ( $P = 0.007$ ). Furthermore, plasma follistatin  
267 concentrations were significantly higher at 4 and 7 h during HIGH compared to CON (both  $P$   
268  $\leq 0.003$ ). Examination of AUC data showed that circulating follistatin concentrations were  
269 greater in HIGH vs CON postprandially ( $P = 0.003$ ; Table 3).

270 Baseline plasma concentrations of LECT2 did not differ between trials ( $P = 0.108$ ). Two-way  
271 ANOVA showed a significant main effect of time and trial by time interaction for plasma  
272 LECT2 concentrations (both  $P \leq 0.004$ ; Figure 2C). *Post-hoc* analyses identified elevated  
273 plasma LECT2 concentrations immediately post-exercise during MOD and HIGH trials  
274 compared to CON (both  $P \leq 0.046$ ), with a similar response between exercise trials; however,  
275 following adjustment for plasma volume changes across the trials, statistical significance did  
276 not remain (both  $P \geq 0.243$ ). No differences in AUC values were observed between trials (Table  
277 3).

279

*Insert figure 2*

280

281

*Insert table 3*

282

### 283 *Hormone and metabolite responses to exercise*

284 Plasma hormone and metabolite responses at baseline and after exercise are shown in Figure 3.

285 Baseline plasma concentrations of glucose, NEFA and glucagon were not statistically different

286 between trials (all  $P \geq 0.124$ ). However, plasma insulin was significantly higher and the

287 glucagon to insulin ratio was significantly lower at baseline in MOD compared to CON ( $P \leq$

288 0.01). In response to exercise, two-way ANOVA showed a significant main effect of time and

289 trial by time interaction for glucose and NEFA (all  $P \leq 0.001$ ), whilst a significant main effect

290 of trial was also observed for NEFA ( $P < 0.001$ ). *Post-hoc* analyses revealed significantly

291 higher plasma NEFA concentrations during MOD compared to CON at 0 and 2 h; and during

292 HIGH compared to CON at 0, 2, 4 and 7 h (all  $P \leq 0.044$ ). Additionally, plasma NEFA

293 concentrations were significantly higher during HIGH in comparison to MOD at 4 and 7 h

294 (both  $P \leq 0.029$ ). No significant differences in plasma glucose were found between trials at any

295 time point ( $P \geq 0.084$ ).

296 There was a significant main effect of time and trial by time interaction for glucagon, insulin

297 and glucagon to insulin ratio (all  $P \leq 0.002$ ), whilst a significant main effect of trial was also

298 observed for glucagon ( $P < 0.001$ ). *Post-hoc* analyses revealed that when compared to CON,

299 plasma glucagon concentrations were significantly higher at 0, 1 and 2 h during MOD and

300 HIGH (all  $P \leq 0.045$ ). Furthermore, plasma glucagon concentrations were significantly higher

301 at 1 h ( $P = 0.032$ ) and tended to be higher at 2 h ( $P = 0.061$ ) during HIGH compared to MOD.

302 Subsequently, a significantly greater glucagon to insulin ratio was observed at 1 h during MOD,  
303 and at 1 and 2 h during HIGH, when compared to CON (all  $P \leq 0.006$ ). However, no differences  
304 were observed between the two exercise trials ( $P \geq 0.656$ ). No significant differences in plasma  
305 insulin were found between trials at any time point post-exercise ( $P \geq 0.378$ ).

306

307

*Insert figure 3*

## 308 **Discussion**

309 The present study explored the acute effect of exercise intensity on circulating concentrations  
310 of FGF21, follistatin and LECT2 in a population of healthy men. Our novel observations are  
311 that high-intensity exercise elicited a greater post-exercise increase in circulating FGF21 when  
312 compared to moderate-intensity exercise. Furthermore, circulating follistatin was elevated after  
313 exercise independently of exercise intensity; whilst LECT2 appeared not to respond to acute  
314 bouts of exercise.

315 FGF21 is regarded as a metabolically beneficial hepatokine which is upregulated by obesity  
316 and obesity-related diseases (Chavez et al. 2009, Dushay et al. 2010), potentially as a  
317 compensatory mechanism to preserve metabolic homeostasis (Li et al. 2018). FGF21 has also  
318 been shown to be acutely responsive to metabolic stresses such as fasting, protein restriction  
319 and exercise (Galman et al. 2008, Kim et al. 2013, Laeger et al. 2014). The findings from the  
320 present study demonstrate that the FGF21 response to acute exercise is intensity dependent,  
321 with a greater response evident after an isoenergetic bout of high- vs moderate-intensity  
322 exercise. This finding is supported by previous observations suggesting that greater post-  
323 exercise increases in circulating FGF21 may be present with increasing exercise intensity (Kim  
324 et al. 2013). It must be noted, however, this previous study failed to isolate the effect of exercise  
325 intensity from the confounding influence of exercise-related energy expenditure (Kim et al.  
326 2013). Our findings also demonstrate that the exercise-induced increase in circulating FGF21  
327 persisted for up to four hours post-exercise which is consistent with previous reports from our  
328 laboratory (Sargeant et al. 2018) and elsewhere (Kondo et al. 2011).

329 Although it is known that exercise enhances the expression and secretion of FGF21 from the  
330 liver (Kim et al. 2013, Hansen et al. 2015), the mechanisms underpinning the exercise intensity-  
331 dependent increase in circulating FGF21 cannot be delineated from the present study. Our  
16



332 results cannot distinguish whether the identified difference in systemic FGF21 was due to  
333 altered protein expression, secretion or clearance. Our data may, however, indicate an  
334 influential role of glucagon. Specifically, whilst no intensity-dependent differences were  
335 apparent in circulating NEFA, insulin or the glucagon to insulin ratio (all of which have been  
336 shown to modulate hepatic FGF21 secretion; Inagaki et al. 2007, Arafat et al. 2013, Hansen et  
337 al. 2015, Hansen et al. 2016b), circulating glucagon was two-fold higher in response to high-  
338 vs moderate-intensity exercise. It is therefore possible that the greater FGF21 response to high-  
339 intensity exercise was mediated by a relatively larger glucagon response. This possibility is  
340 supported by preclinical data which has demonstrated an independent role of glucagon as a  
341 stimulus of hepatic FGF21 expression and secretion (Arafat et al. 2013, Habegger et al. 2013,  
342 Cyphert et al. 2014, Alonge et al. 2017, Kim et al. 2018); however, the descriptive nature of  
343 the present study prevents us from unpicking this further.

344 It has been suggested that exercise-related augmentation of FGF21 may contribute to the  
345 beneficial metabolic effects of exercise (Hansen et al. 2016b, Weigert et al. 2018). Notably, in  
346 Wistar rats, Loyd et al (2016) recently demonstrated that FGF21 is essential for the beneficial  
347 effects of exercise training on hepatic steatosis and glycaemic control to be realised during a  
348 period of high-fat feeding. In the present study, the physiological relevance of the greater, yet  
349 transient augmentation of circulating FGF21 in response to high- vs moderate-intensity  
350 exercise, is not clear. It is possible that the enhanced response may contribute to the greater  
351 metabolic benefits associated with high-intensity exercise, including greater improvements in  
352 insulin sensitivity and glycaemic control (Hayashi et al. 2005, Rynders et al. 2014); or it may  
353 reflect a participatory role of the protein in the metabolic response to exercise and recovery.  
354 Carefully designed experiments utilising animal models are required to shed more light on this  
355 question.

356 Previous research has identified follistatin as another hepatokine which is transiently increased  
357 in the circulation in response to a single bout of exercise (Hansen et al. 2011, Hansen et al.  
358 2016a, Hansen et al. 2016b, Sargeant et al. 2018). In the present study, both exercise intensities  
359 led to a similar increase in circulating follistatin concentrations. Previous studies have shown  
360 that the circulating glucagon to insulin ratio is a potent stimulus of hepatic follistatin release in  
361 response to exercise (Hansen et al. 2016a, Hansen et al. 2016b). The comparable follistatin  
362 response between exercise intensities may therefore be due to equivalent changes in the  
363 circulating glucagon to insulin ratio. The importance of these gluco-regulatory hormones may  
364 help explain recent findings showing that circulating follistatin was elevated after both high-  
365 intensity interval exercise and resistance exercise; but not low-to-moderate intensity  
366 continuous exercise (Perakakis et al. 2018). Although not measured in this previous study, it is  
367 likely that only the high-intensity exercise modalities would have triggered an increase in  
368 circulating glucagon and/or a decrease in circulating insulin; as opposed to the low-to-moderate  
369 intensity exercise (Wolfe et al. 1986, Lira et al. 2012).

370 Whilst the physiological relevance of the follistatin response to exercise is not fully understood,  
371 follistatin has been identified to play a beneficial role in several metabolic actions which may  
372 be relevant to exercise. This includes promoting skeletal muscle growth and preventing  
373 degradation through its actions as a natural antagonist of myostatin (Amthor et al. 2004, Yaden  
374 et al. 2014). Moreover, chronic overexpression of follistatin promotes pancreatic  $\beta$ -cell  
375 survival (Zhao et al. 2015, Hansen et al. 2016a), whilst acute administration of follistatin may  
376 reduce glucagon secretion from human islets in a potential negative feedback loop (Hansen et  
377 al. 2016a). Therefore, an exercise-induced increase in circulating follistatin may contribute in  
378 some capacity to the positive metabolic effects of exercise; however, the exact role needs to be  
379 delineated further.

380 When interpreting the follistatin results in the present study, one important methodological  
381 consideration warranting recognition was the decision to feed participants after exercise. In  
382 previous studies, follistatin responses to exercise have been examined with study participants  
383 remaining fasted throughout experimental trials (Hansen et al. 2011; Hansen et al. 2016b).  
384 Notably, the magnitude of increase in circulating follistatin after exercise in these previous  
385 studies was considerably greater than observed in the present study. It is therefore possible that  
386 food consumption may have blunted the follistatin response after exercise in the present study.  
387 In particular, evidence suggests that insulin may inhibit follistatin production (Tao et al. 2018);  
388 including the response to exercise (Hansen et al. 2016b). Raised postprandial insulin may have  
389 blunted our ability to detect an effect of exercise intensity on circulating follistatin within our  
390 post-exercise observation period. An additional experiment is needed to test this hypothesis.

391 LECT2 is a novel hepatokine whose circulating levels are elevated in obesity, type 2 diabetes  
392 and non-alcoholic fatty liver disease (Okumura et al. 2013, Zhang et al. 2018). *In vitro* studies  
393 have shown that LECT2 triggers inflammation in vascular endothelium (Hwang et al. 2015)  
394 and directly inhibits insulin signalling in C2C12 myocytes (Lan et al. 2014) and 3T3-L1  
395 adipocytes (Jung et al. 2018). It has therefore been suggested that LECT2 may provide a  
396 mechanistic link between NAFLD, T2DM and cardiovascular disease (Meex and Watt 2017).

397 In our previous research, 60 min of moderate-intensity treadmill exercise had no impact on  
398 circulating LECT2 concentrations for up to 6 h post-exercise in healthy lean and overweight  
399 men (Sargeant et al. 2018). Based on previous rodent data showing a negative regulatory  
400 influence of hepatic AMPK-phosphorylation on LECT2 expression (Lan et al. 2014), we  
401 hypothesised in the present study that high-intensity exercise would suppress circulating  
402 LECT2. In contrast, we observed a significant increase in circulating LECT2 concentrations  
403 immediately after both moderate- and high-intensity exercise; with no differences apparent in

404 the hours thereafter. It must be noted, however, that statistical significance was lost at the 0 h  
405 time point following adjustment for plasma volume changes, despite similar effect sizes (ES)  
406 remaining (Cohen's *d* before vs after plasma volume adjustment: ES = 0.41 vs ES = 0.49 and  
407 ES = 0.42 vs ES = 0.45 for MOD and HIGH compared to CON, respectively). Therefore,  
408 exercise-induced haemoconcentration may have contributed, in part, to the observed increase  
409 in plasma LECT2 immediately after exercise.

410 Lan et al (2014) previously showed that hepatic LECT2 expression and circulating  
411 concentrations were suppressed immediately after 3 h of treadmill running in C57BL/6J mice.  
412 This contrasting finding with our work may be related to species differences but may also be  
413 due to the nature of the exercise utilised and/or the study participants. Specifically, given the  
414 negative regulatory influence of hepatic AMPK, longer duration exercise with a greater  
415 metabolic challenge may be needed to suppress LECT2 in humans. It is also possible that this  
416 response may only occur in individuals with obesity in which circulating LECT2 is elevated;  
417 however additional studies in populations with obesity are needed to test these hypotheses.

418 A key strength of our study is the precision with which exercise-related energy expenditure  
419 was matched. Conversely, a limitation of the present study is that the observed hepatokine  
420 responses occurred in a population of young, healthy men. Circulating levels of hepatokines  
421 are modulated by adiposity and associated metabolic disease and, therefore, we cannot be  
422 certain that our findings translate to different populations. The therapeutic benefits of exercise  
423 are greater in clinical populations and therefore additional studies should investigate the  
424 influence of exercise of varied intensities and durations in individuals with obesity and  
425 associated metabolic disease.

426 In conclusion, this study demonstrates that circulating concentrations of FGF21 and follistatin  
427 are transiently increased in response to acute bouts of aerobic exercise, whereas circulating  
20

428 LECT2 concentrations may not be sensitive to acute exercise stimuli. Furthermore, exercise  
429 increases circulating FGF21 in an intensity-dependent manner, whilst the exercise-induced  
430 increase in circulating follistatin may be independent of exercise intensity. These findings  
431 support the notion that FGF21 and follistatin may contribute to the favourable metabolic effects  
432 of exercise and a dose-dependent increase in FGF21 secretion may be partly responsible for  
433 the additional benefits of high-intensity exercise. Additionally, these data extend our  
434 understanding of the acute regulation of hepatokines in humans and may help to inform future  
435 therapeutic interventions targeted at manipulating hepatokine concentrations.

436 **Conflict of interest statement**

437 The authors declare no conflicts of interest.

438

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616 **Tables**617 **Table 1.** Participant characteristics ( $n = 10$ ).

Characteristic	Mean $\pm$ SD
Age (years)	26 $\pm$ 2
Height (m)	1.77 $\pm$ 0.07
Body mass (kg)	80.2 $\pm$ 6.2
BMI ( $\text{kg}\cdot\text{m}^{-2}$ )	25.6 $\pm$ 1.7
Waist circumference (cm)	83.7 $\pm$ 4.1
Body fat (%)	15.4 $\pm$ 4.4
$\dot{V}\text{O}_2$ peak ( $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ )	49.8 $\pm$ 5.3

618

**Note:** BMI, body mass index;  $\dot{V}\text{O}_2$  peak, peak oxygen uptake.

619 **Table 2.** Exercise characteristics ( $n = 10$ ).

	MOD	HIGH	<i>P</i> -value
Treadmill speed (km·h <sup>-1</sup> )	8.2 ± 1.4	11.6 ± 1.6	<0.001
Oxygen uptake (L·min <sup>-1</sup> )	2.17 ± 0.26	2.98 ± 0.37	<0.001
Exercise intensity (% $\dot{V}O_2$ peak)	54.6 ± 1.7	74.7 ± 1.8	<0.001
Exercise duration (min)	57 ± 8	42 ± 6	<0.001
Gross energy expenditure (kJ)	2475 ± 70	2488 ± 58	0.459
Respiratory exchange ratio	0.90 ± 0.03	0.99 ± 0.03	<0.001
Carbohydrate oxidation (%)	67 ± 10	94 ± 8	<0.001
Fat oxidation (%)	33 ± 10	6 ± 8	<0.001
Heart rate (beats·min <sup>-1</sup> )	142 ± 10	168 ± 9	<0.001
Rating of perceived exertion	11 ± 2	16 ± 1	<0.001

620 **Note:** Data are presented as means ± SD.  $\dot{V}O_2$  peak, peak oxygen uptake.



621 **Table 3.** Area under the hepatokine concentration vs time curve during CON, MOD and  
 622 HIGH trials for the preprandial (BL-2 h), postprandial (2-7 h) and total trial (8 h) periods.

	CON	MOD	HIGH
<b>FGF21</b> ( <i>n</i> = 10)			
Preprandial AUC (pg·mL <sup>-1</sup> BL-2 h)	144 ± 124	230 ± 156 <sup>a</sup>	334 ± 249 <sup>a§</sup>
Postprandial AUC (pg·mL <sup>-1</sup> 2-7 h)	264 ± 209	323 ± 233	433 ± 271 <sup>a</sup>
Total AUC (pg·mL <sup>-1</sup> 8 h)	407 ± 330	553 ± 377 <sup>a</sup>	768 ± 491 <sup>a§</sup>
<b>Follistatin</b> ( <i>n</i> = 9)			
Preprandial AUC (pg·mL <sup>-1</sup> BL-2 h)	3848 ± 515	4518 ± 1148	4566 ± 962 <sup>#</sup>
Postprandial AUC (pg·mL <sup>-1</sup> 2-7 h)	6615 ± 823	8504 ± 2118 <sup>#</sup>	9275 ± 1406 <sup>a</sup>
Total AUC (pg·mL <sup>-1</sup> 8 h)	10463 ± 1162	13022 ± 3175 <sup>#</sup>	13841 ± 2110 <sup>a</sup>
<b>LECT2</b> ( <i>n</i> = 10)			
Preprandial AUC (ng·mL <sup>-1</sup> BL-2 h)	157 ± 59	165 ± 64	166 ± 62 <sup>#</sup>
Postprandial AUC (ng·mL <sup>-1</sup> 2-7 h)	253 ± 98	267 ± 102	268 ± 99
Total AUC (ng·mL <sup>-1</sup> 8 h)	410 ± 156	432 ± 166	434 ± 160

623 **Note:** Data are presented as means ± SD. FGF21, fibroblast growth factor 21; AUC, area  
 624 under curve; BL, baseline; LECT2, leukocyte cell-derived chemotaxin 2.

625 <sup>a</sup>Significantly different from CON (*P* < 0.05).

626 <sup>#</sup>Tended to differ from CON (*P* < 0.07).

627 <sup>§</sup>Tended to differ from MOD (*P* < 0.07).

628 **Figure captions**

629 **Figure 1.** Schematic illustration of the main trial protocol. Duration of exercise in MOD and  
630 HIGH =  $57 \pm 8$  and  $42 \pm 6$  min, respectively. Participants were provided with 35% of  
631 estimated daily energy requirements at both 2 h and 6 h.

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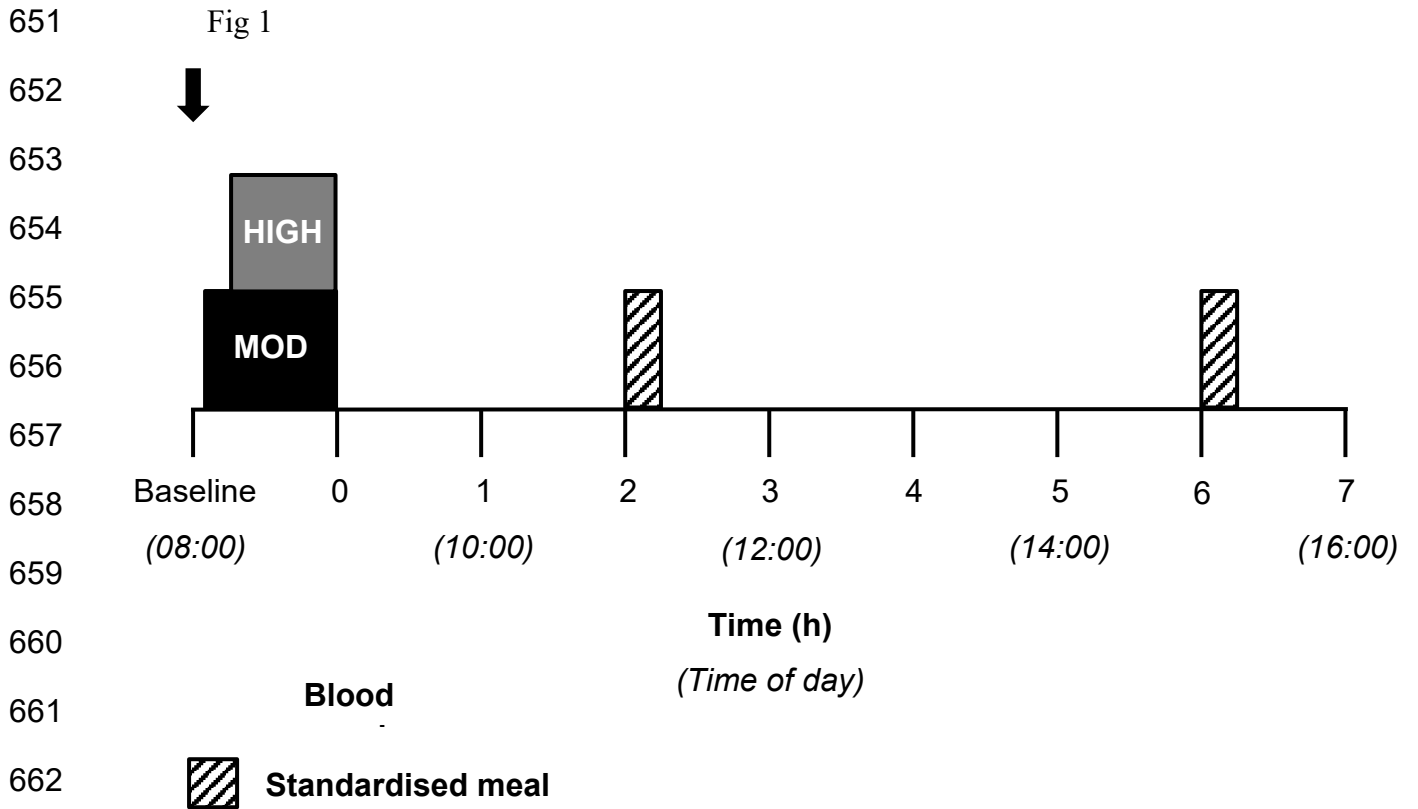
633 **Figure 2.** Plasma concentrations of fibroblast growth factor 21 (FGF21) (A), follistatin (B)  
634 and leukocyte cell-derived chemotaxin 2 (LECT2) (C) during the control (open circles),  
635 moderate-intensity exercise (black circles) and high-intensity exercise (grey circles) trials.  
636 Data are presented as means  $\pm$  SEM,  $n = 10$  for FGF21 and LECT2 data, and  $n = 9$  for  
637 follistatin data. Black rectangle indicates moderate-intensity exercise, grey rectangle indicates  
638 high-intensity exercise and hatched rectangles indicate test meals. BL, baseline. <sup>a</sup>MOD  
639 significantly different from CON ( $P < 0.05$ ); <sup>b</sup>HIGH significantly different from CON ( $P <$   
640  $0.05$ ); <sup>c</sup>HIGH significantly different from MOD ( $P < 0.05$ ).

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642 **Figure 3.** Plasma concentrations of glucose (A), non-esterified fatty acids (NEFA) (B),  
643 glucagon (C), insulin (D) and the glucagon to insulin ratio (E) during the control (open  
644 circles), moderate-intensity exercise (black circles) and high-intensity exercise (grey circles)  
645 trials. Data are presented as means  $\pm$  SEM. Black rectangle indicates moderate-intensity  
646 exercise, grey rectangle indicates high-intensity exercise and hatched rectangles indicate test  
647 meals. BL, baseline. <sup>a</sup>MOD significantly different from CON ( $P < 0.05$ ); <sup>b</sup>HIGH significantly  
648 different from CON ( $P < 0.05$ ); <sup>c</sup>HIGH significantly different from MOD ( $P < 0.05$ ).

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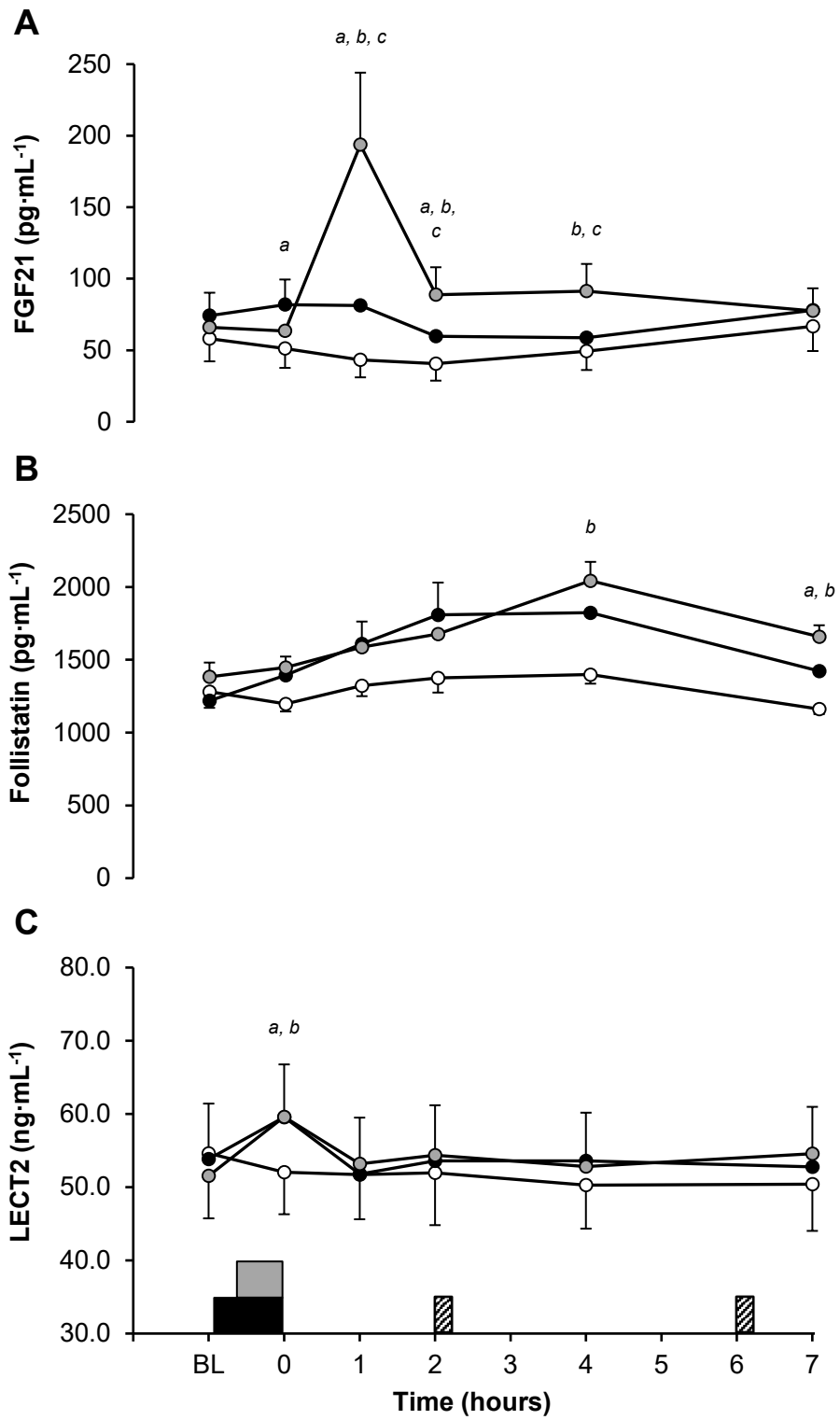
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677 Fig 2

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685 Fig 3

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