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1 **Lifelong exercise, but not short-term high intensity**
2 **interval training (HIIT), increases GDF11, a marker**
3 **of successful ageing: A preliminary investigation**

4

5

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24 ABSTRACT

25 Lifelong exercise is associated with regulation of skeletal mass and function, reductions in
26 frailty, and successful ageing. Yet, the influence of exercise on myostatin and myostatin-
27 interacting factors is relatively under examined in older males. Therefore, we investigated
28 whether serum total myostatin, free myostatin, follistatin, and growth and differentiation
29 factor 11 (GDF11) were altered following high intensity interval training (HIIT) in a group of
30 13 lifelong sedentary (SED; 64 [6] years) and 11 lifelong exercising (LEX; 62 [6] years)
31 older males. SED follistatin was moderately greater than LEX pre-HIIT (Cohen's $d = 0.66$),
32 and was largely greater post-HIIT (Cohen's $d = 1.22$). The HIIT-induced increase in
33 follistatin was large in SED (Cohen's $d = 0.82$) and absent in LEX (Cohen's $d = 0.03$).
34 GDF11 was higher in LEX pre- (Cohen's $d = 0.49$), and post- (Cohen's $d = 0.63$) HIIT
35 compared to SED. HIIT resulted in no change to GDF11 in LEX or SED (Cohen's $d = 0.00$ -
36 0.03). Peak power output and GDF11 correlated ($r = 0.603$), independent of grouping.
37 Differences in GDF11 with lifelong exercise training, paired with the correlation between
38 GDF11 and peak power output, suggest GDF11 may be a relevant myostatin-interacting
39 peptide to successful ageing in humans, and strategies to maintain this need to be further
40 explored.

41

42

43 KEYWORDS

44 Ageing · Exercise · Follistatin · GDF11 · HIIT · Myostatin

45

46 RUNNING TITLE

47 GDF11 is increased in successful ageing

48

49 INTRODUCTION

50 Myostatin (originally growth and differentiation factor 8 [GDF8]) is a pro-catabolic, anti-
51 anabolic peptide hormone that is a central regulator of skeletal muscle mass (Elliott et al.,
52 2012). Secreted by skeletal muscle, myostatin is found in an active unbound (free) form, or
53 bound to its own pro-peptide, or separate peptides such as follistatin, or follistatin-related
54 gene (FLRG; Amthor et al., 2004, Gilson et al., 2009, Hill et al., 2002), each inhibiting its
55 biological function. Myostatin has both paracrine and endocrine effects (Zimmers et al.,
56 2002), although it is the endocrine function which appears key for regulation of muscle mass,
57 due to an observed inverse correlation with muscle mass in humans (Gonzalez-Cadavid et al.,
58 1998). Moreover, inhibition of this endocrine function results in muscle hypertrophy in mice
59 (Whittemore et al., 2003).

60

61 Ageing is associated with a progressive loss of muscle mass and associated function (Metter
62 et al., 2002) The rate of loss of muscle mass and function with ageing is noted to differ
63 between individuals, which gave rise to ‘usual’ and ‘successful’ ageing hypothesis (Rowe
64 and Kahn, 1987). A more recent definition of successful ageing being “optimisation of life
65 expectancy while minimising physical and mental deterioration and disability” (Bowling and
66 Dieppe, 2005), a trait that is often seen in life-long masters athletes (Pollock et al., 2015).
67 Whilst the role of myostatin in regulation of muscle mass is well described, there are few
68 data, and no prospective studies to contextualise the influence of myostatin within the ‘cycle
69 of frailty’ that precedes sarcopenia. From the few cross-sectional studies, one observed ~50%
70 higher plasma myostatin in older sedentary (~63-75 years of age) compared with younger
71 healthy (~20-35 years of age) men (Yarasheski et al., 2002). However, this was not replicated
72 in a study of men aged ~22, ~69, and ~76 years of age, regardless of sarcopenic severity
73 (Ratkevicius et al., 2011). Recently, we have observed an inverse association between age

74 and plasma myostatin in a large group (n = 88) of healthy individuals aged 18-72 years of age
75 (Elliott et al., 2016). Considering the current incomplete understanding concerning the role of
76 myostatin and myostatin-interacting peptides in the ageing process, the pool of evidence
77 needs to be extended.

78

79 Growth and differentiation factor 11 (GDF11) is a peptide with similar sequence homology
80 as myostatin, and it is possible that both peptides share similar signalling pathways and
81 biological influence within skeletal muscle. Unlike myostatin however, the expression of
82 GDF11 is not limited to skeletal muscle tissue (Lee and McPherron, 1999, Walker et al.,
83 2016). There also appears to be an indicated role for GDF11 in the ageing process; higher
84 circulating GDF11 in middle-aged mice has been positively associated with longevity and
85 exposure of aged mice to a youthful systemic environment led to restoration of skeletal
86 muscle and hepatic cellular function (Zhou et al., 2016). Similarly, the ageing muscle
87 phenotype is partially offset by provision of recombinant GDF11, as demonstrated by
88 increased grip strength and running endurance in mice (Sinha et al., 2014).

89

90 Whilst it remains to be seen whether these findings can be consistently replicated, or indeed
91 translated to the human model of ageing, only a small number of studies that have examined
92 the effects of exercise training on serum myostatin and associated mRNA expression, whilst
93 GDF11 remains unexamined in the human exercise model. Indeed, 2–3 months' resistance
94 training in healthy young individuals resulted in increased muscle mass and decreased muscle
95 mRNA and serum myostatin (Roth et al., 2003, Walker et al., 2004). To the best of these
96 authors' knowledge, no reports on the effect of exercise (in any form) on GDF11 expression
97 currently exists.

98

99 Recently, high intensity interval training (HIIT) has received much attention due to its
100 physiological and sociological benefits. Indeed, HIIT is noted to be more enjoyable than
101 traditional, continuous training (Thum et al., 2017), has higher compliance in patient
102 populations than continuous training (Shirayev and Barclay, 2012), and is noted to have equal
103 or improved clinical outcomes in a number of ageing-related cardiovascular or metabolic
104 disorders (Cassidy et al., 2016, Ramos et al., 2015). Whilst not optimized for muscle
105 hypertrophy, HIIT improves myofibrillar protein synthesis (Bell et al., 2015), muscle power
106 (Sculthorpe et al., 2017), and fat free mass (FFM) (Herbert et al., 2017) in older males.

107

108 Therefore, in order to progress our understanding of the biological relationship between
109 myostatin and myostatin-interacting peptides with ageing and exercise, the aim of this
110 preliminary study was twofold: 1) To compare resting levels of plasma myostatin and
111 myostatin-interacting peptides between lifelong sedentary (SED) and a positive control group
112 of lifelong exercising (LEX) ageing men, and 2) to examine the influence of 6 weeks' HIIT
113 on plasma myostatin and myostatin-interacting peptides in SED and LEX. We hypothesised
114 that, on enrolment to the study, SED would exhibit higher myostatin, follistatin, free
115 myostatin, and lower GDF11. We further hypothesized that 6 weeks' HIIT would decrease
116 plasma myostatin, follistatin, and free myostatin in SED, and increase GDF11.

117

118

119 METHODS

120 *Participants*

121 Participants provided written informed consent prior to enrolment to a larger study (Hayes et
122 al., 2017, Herbert et al., 2017, Knowles et al., 2015) which was approved by the University of
123 the West of Scotland Ethics Committee (Reference: UEC16_042012/Herbert). Participants
124 were familiarised with experimental procedures and approval to exercise was given by their
125 general practitioner. Subsequently, a subgroup of 24 males were analysed for this pilot
126 investigation. Thirteen males participated in the SED group, whilst 11 males participated in
127 the LEX group (Table 1). Participants in the SED group did not participate in any formal
128 exercise training and had not done so for >30 years. The LEX group were active exercisers
129 and had been so for the previous >30 years. They consisted primarily of current masters
130 competitors in sports including water-polo, triathlon, sprint cycling, road cycling and distance
131 running. For six weeks prior to commencing HIIT training, LEX recorded their normal
132 weekly exercise, which included type, frequency, intensity (recorded by heart rate telemetry),
133 and duration of training. Time spent in low to medium intensity (<65% heart rate reserve
134 [HRR]), and high-intensity (>65% HRR) training totalled $214 \pm 131 \text{ min}\cdot\text{wk}^{-1}$ and 67 ± 52
135 $\text{min}\cdot\text{wk}^{-1}$ respectively. Group selection was affirmed by differences in aerobic conditioning
136 (peak oxygen uptake; $\text{VO}_{2\text{peak}}$) between groups (table 1). Participants were tested pre- and
137 post-HIIT at the same time of day, seven weeks apart. Order of measurements was blood
138 sampling, body composition, peak power assessment, and determination of $\text{VO}_{2\text{peak}}$.

139

140

Table 1 about here

141

142

143 *Blood draws and analysis*

144 Participants arrived at the exercise physiology laboratory between 07.00–09.00 h, following
145 an overnight fast and having abstained from strenuous exercise for a minimum of 48 h.
146 Participants were reminded to maintain standardized conditions prior to each assessment
147 point which included arriving in a hydrated state having abstained from caffeine and alcohol
148 consumption for 36 h. Following 20 min supine rest blood was sampled from the
149 nondominant arm using the standard venepuncture method into sterile serum separator
150 vacutainer tubes (Becton Dickinson, Rutherford, NJ) that were kept at room temperature in
151 the dark, for 30 min, to allow for clotting, after which samples were centrifuged at 1100 g at
152 4°C for 15 min. Serum was then extracted, aliquoted, and stored at –80°C until subsequent
153 analysis. Blood samples were collected at the same time of day for each participant to control
154 for biological variation and minimise inter-participant analytical variation.

155

156 Concentrations of serum myostatin protein (both total and free fractions) were quantified by
157 ELISA (DGDF80, R&D Systems, UK). Briefly, aliquots of serum were brought to room
158 temperature, before 100 µL of plasma was diluted with 1:4 diluent buffer (free myostatin) or
159 activated with 50 µL HCl (6 mol, 10 minutes at room temperature) for removal of myostatin
160 binding proteins, before neutralization (50 µL of NaOH 6 mol + 1.2 mol HEPES) and
161 dilution with provided diluent buffer (200 µL) to produce a final 1:4 dilution. Recombinant
162 myostatin was used as a standard (33.3-2,000 pg·mL⁻¹). Concentrations of serum follistatin
163 (DFN00, R&D Systems, UK) and serum GDF11 (DY1958, R&D Systems, UK) were
164 quantified by ELISA, per manufacturer's instructions. Recombinant follistatin (250-16,000
165 pg·mL⁻¹) and GDF11 (15.6 – 1000 pg·mL⁻¹) was used as a standard. Plates were read
166 spectrophotometrically at 450 nm and blanked to 570 nm (VersaMax, Molecular Devices,

167 USA). Coefficient of variability of standards and samples were 7% and 6%, 6% and 4%, and
168 4% and 8%, for myostatin follistatin, and GDF11, respectively.

169

170 *Body composition and performance measures*

171 Height was measured to the nearest 0.1 cm using a stadiometer (Seca, Birmingham, UK), and
172 a multi frequency bioelectrical impedance analyzer (BIA; Tanita MC-180MA Body
173 Composition Analyzer, Tanita UK Ltd.) was used to determine body mass and body
174 composition as described elsewhere (Hayes et al., 2013b). Participant peak power output was
175 assessed using the Herbert 6 s cycle test (Herbert et al., 2015b) and participants' individual
176 values were used to calculate the resistance (40% peak power output) during HIIT. VO_{2peak}
177 was determined by indirect calorimetry as previously described (Knowles et al., 2015).

178

179 *Exercise training*

180 HIIT sessions were performed once every five days, for six weeks (nine sessions in total) as
181 previously described (Hayes et al., 2017, Herbert et al., 2017, Knowles et al., 2015).
182 Rationale for this programme is provided by our previous work which identified that five
183 days of recovery was required for recovery of peak power output in ageing men (Herbert et
184 al., 2015a). Each session consisted of 6 x 30 s sprints at 40% predefined peak power output
185 interspersed with 3 min active recovery on a cycle ergometer (Wattbike Ltd., Nottingham,
186 UK). Sessions were conducted in groups of between four and six participants and were the
187 sole exercise performed by both groups during this time.

188

189 *Statistical Analysis*

190 Following confirmation of parametricity by a Shapiro-Wilk test of normality and Levene's
191 test for homogeneity of variance, a mixed (between group [SED, LEX] x within individual

192 time [pre-HIIT, post-HIIT]) repeated measures analysis of variance (ANOVA) was used for
193 differences in groups and time points with Bonferroni *post-hoc*. Non-parametric data were
194 examined by Fishers exact test, with correction for multiple comparisons by Bonferroni's
195 method. Alpha level was set *a priori* at $p < 0.05$, and effect size for paired comparisons is
196 reported as Cohen's d throughout, interpreted as trivial (<0.2), small (≥ 0.2), moderate (≥ 0.5),
197 and large (≥ 0.8). Parametric data sets are summarized in text as mean [SD], whilst non-
198 parametric are given as median (upper - lower quartile). Figures are presented as grouped dot
199 plots, as recommended by Drummond and Vowler (2012).

200

201 RESULTS

202 Pre-HIIT, SED individuals were heavier ($p = 0.131$, Cohen's d = 0.66) with a greater body fat
203 percentage ($p = 0.120$, Cohen's d = 0.66) than LEX. SED had a lower VO_{2peak} ($p < 0.001$,
204 Cohen's d = 2.00), absolute peak power output ($p = 0.036$, Cohen's d = 0.90) and relative
205 peak power output (a surrogate for muscle quality; $p = 0.020$, Cohen's d = 1.08) than LEX
206 (table 1).

207

208 There was no group x time interaction for total myostatin protein ($p = 0.750$), nor was there
209 an effect of group ($p = 0.081$) or time ($p = 0.701$). However, large effect sizes were noted
210 between SED and LEX total myostatin both pre-HIIT (4217 [317] $pg \cdot mL^{-1}$ and 3394 [391]
211 $pg \cdot mL^{-1}$ in SED and LEX respectively; Cohen's d = 2.06; Figure 1A) and post-HIIT (4163
212 [337] $pg \cdot mL^{-1}$ and 3678 [438] $pg \cdot mL^{-1}$ in SED and LEX respectively; Cohen's d = 1.24).
213 Following HIIT, SED experienced only trivial increases in total myostatin (Cohen's d = 0.17)
214 whilst LEX moderately increased total myostatin (Cohen's d = 0.68).

215

216

217 In a similar manner to total myostatin, there was no group x time interaction for free
218 myostatin protein ($p = 0.790$), nor and effect of group (0.996) or time ($p = 0.601$). No notable
219 effect size changes were observed for free myostatin pre-HIIT (1182.0 [372.2] $\text{pg}\cdot\text{mL}^{-1}$ and
220 1159.3 [418.1] $\text{pg}\cdot\text{mL}^{-1}$ in SED and LEX respectively; Cohen's $d = 0.06$; Figure 1B) or post-
221 HIIT (1203.3 [533.3] $\text{pg}\cdot\text{mL}^{-1}$ and 1224.5 [404.1] $\text{pg}\cdot\text{mL}^{-1}$ in SED and LEX respectively;
222 Cohen's $d = 0.05$). Moreover, neither SED (Cohen's $d = 0.05$) nor LEX (Cohen's $d = 0.16$)
223 had any more than a trivial effect on free myostatin from pre- to post-HIIT.

224

225

226 There was a significant main effect of group ($p = 0.002$), but not time ($p = 0.171$), or a group
227 x time interaction ($p = 0.561$) for serum follistatin. SED follistatin was greater than LEX
228 follistatin pre-HIIT (2508 [628] $\text{pg}\cdot\text{mL}^{-1}$ and 2102 [598] $\text{pg}\cdot\text{mL}^{-1}$ in SED and LEX
229 respectively; $p = 0.132$, Cohen's $d = 0.66$). SED follistatin was also greater than LEX
230 follistatin post-HIIT (3043 [676] $\text{pg}\cdot\text{mL}^{-1}$ and 2126 [809] $\text{pg}\cdot\text{mL}^{-1}$ in SED and LEX
231 respectively; $p < 0.001$, Cohen's $d = 1.22$). The HIIT-induced increase in follistatin was large
232 in SED ($p = 0.011$, Cohen's $d = 0.82$), whilst LEX experienced no change ($p = 0.443$,
233 Cohen's $d = 0.03$).

234

235 GDF11 data were examined by Fishers exact test, and presented as median (upper - lower
236 quartile). GDF11 was higher in LEX pre- ($p = 0.012$, Cohen's $d = 0.49$), and post- ($p = 0.009$,
237 Cohen's $d = 0.63$) HIIT compared to SED. HIIT resulted in no change to GDF11 in SED
238 (70.7 [52.6 – 193.1], 77.1 [73.1 – 104.3] $\text{pg}\cdot\text{mL}^{-1}$ pre- and post-HIIT respectively; $p = 0.74$,
239 Cohen's $d = 0.03$) or LEX (272.7 [219.2 – 387.2], 305.0 [243.8 – 399.4] $\text{pg}\cdot\text{mL}^{-1}$ pre- and
240 post-HIIT respectively; $p = 0.72$, Cohen's $d = 0.00$).

241

242

243

Figure 1 about here

244

245

246 As we have previously reported in a larger cohort (Hayes et al., 2013a), peak power output
247 was higher in LEX individuals relative to SED ($p = 0.036$, Figure 2A). There was no
248 correlation between peak power output and total myostatin ($p = 0.196$, $r = -0.273$), free
249 myostatin ($p = 0.812$, $r = 0.051$), or follistatin ($p = 0.569$, $r = -0.113$). However, strong
250 positive correlations were observed between GDF11 and both absolute peak power output (p
251 $= 0.002$, $r = 0.603$; Figure 2B) and relative peak power output ($p < 0.001$, $r = 0.636$; figure
252 2C).

253

254

255 *Figure 2 about here*

256

257 DISCUSSION

258 The main finding of this preliminary study was that SED presented greater concentrations of
259 serum total myostatin and follistatin, and lower concentrations of GDF11, compared with
260 LEX pre-HIIT. Serum follistatin alone responded significantly to HIIT but was confined to
261 the SED group. A notable and novel finding from this study is the observed association
262 between peak power output and GDF11, which has not been previously demonstrated in the
263 human. These data provide preliminary evidence that the role of GDF11 in healthy ageing
264 observed in mice is maintained in humans.

265

266 With regards to healthy ageing, our finding that LEX displayed significantly higher GDF11
267 than SED at baseline is novel and noteworthy. It has been noted that older mice treated with
268 plasma from younger mice show a younger phenotype (Horrington et al., 1960, Lunsford et
269 al., 1963), which has since been partially attributed to GDF11 differences in older and
270 younger mice. In mice, mid-life GDF11 is predictive of longevity (Zhou et al., 2016). Aged
271 mice show a typical 'older muscle' phenotype which results in lower muscle volume,
272 endurance, and grip strength relative to young mice. Moreover, treatment with recombinant
273 GDF11 returned grip strength to near young levels, and improved running endurance
274 performance (Sinha et al., 2014). However, it has also been noted that GDF11 may inhibit
275 myoblast differentiation into mature myotubes in a myostatin-like manner (Egerman et al.,
276 2015), perhaps unsurprising, as the myostatin and GDF11 peptide share ~90% homogeneity.
277 It should be further noted that Egerman et al. (2015) used *in vitro* doses of 10-100 ng·mL⁻¹,
278 whilst both their data, and our data reported here, suggests circulating GDF11 in older males
279 is 100-1000 pg·mL⁻¹, an order of magnitude lower in concentration, possibly explaining the
280 disparity of these findings.

281

282 This argument that GDF11 concentration play a role in successful ageing is supported by two
283 separate findings we report here. Firstly, we note GDF11 is significantly higher in LEX than
284 SED, with some overlap between these groups. Further, we note a significant moderate
285 positive correlation between peak power output (both absolute power and relative to FFM)
286 and GDF11, independent of grouping. Whilst our data does not allow us to suggest causality,
287 it is exciting to note this correlative relationship. To the best of our knowledge, this is the first
288 dataset linking successful ageing and improved muscle function in the human with GDF11,
289 and directly links our findings with those of Sinha et al. (2014), that exogenous GDF11
290 protects older mice against ageing- and sedentarism-associated frailty. It is thus tempting to
291 suggest GDF11 plays a similar role in ageing humans, and this hypothesis needs to be further
292 explored with experimental approaches to increase GDF11 expression in humans.

293

294 Circulating myostatin is noted to correlate with lean muscle mass across both healthy and
295 cachexic individuals (Gonzalez-Cadavid et al., 1998). As SED and LEX presented with
296 different body composition at baseline, the moderately lower concentrations of total
297 myostatin in LEX at baseline is understandable. Whilst others have reported decreases in
298 plasma myostatin and gains in muscle mass following resistance exercise (Walker et al.,
299 2004, Saremi et al., 2010), limited research regarding interaction between HIIT and myostatin
300 exists. Pugh et al. (2015) reported reduced muscular myostatin mRNA in healthy individuals
301 2 and 6 hours following a single bout of HIIT (although a different protocol to that employed
302 herein), yet we are the first group to report chronic changes to resting serum myostatin
303 following HIIT. The aim of HIIT is not primarily to build muscle mass, so whilst our HIIT
304 protocol did not significantly alter serum total or free myostatin, expectations of an alteration
305 in this peptide may have been ambitious in the absence of muscle mass alteration.

306

307 Whilst our findings concerning GDF11 are noteworthy, we acknowledge certain limitations
308 of the present investigation. Whilst we attribute differences in GDF11 to life-long activity
309 differences, we acknowledge that we cannot separate how much exercise was required to
310 produce these observed differences. The addition of a moderately active group (meeting
311 physical activity guidelines), would allow for comparison of multiple exercise habits, rather
312 than the two extremes presented here. Moreover, our lack of inactive control group (no HIIT)
313 and relatively small sample size may limit interpretations. The present investigation formed
314 part of a larger research study with other primary outcome variables (Grace et al., 2015,
315 Herbert et al., 2017, Knowles et al., 2015), and therefore only a subset of participants were
316 analysed. As such, our results remain preliminary until the influence of exercise habits on
317 serum GDF11 is investigated with either a large-scale randomized control trial (RCT) or
318 prospective observational trial.

319

320 To date, much attention has been placed on myostatin itself, with alterations in myostatin
321 expression resulting in significant and striking alterations in muscle mass in animal models
322 (Kambadur et al., 1997, Mosher et al., 2007). However, here we show that total myostatin
323 only moderately differs in a model of successful ageing, suggesting the role of myostatin may
324 not be as important in successful ageing as other factors reported here. Instead, greater focus
325 may need to be placed on these myostatin-interacting factors, as we showed follistatin was
326 lower, and GDF11 was higher in our LEX model of successful ageing. Further, the
327 correlation between GDF11 and muscle quality is exciting, and may suggest a protective role
328 of GDF11 against ageing-associated muscular frailty in the human.

329

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487 MANUSCRIPT TABLES

488 **Table 1:** Participant anthropometric and performance parameters on enrolment to the
 489 investigation in lifelong sedentary (SED), and lifelong exercising (LEX), older males. Data
 490 presented as mean [SD].

	SED (n=13)	LEX (n=11)
Age (years)	64 [6]	62 [6]
Stature (cm)	174 [6]	174 [6]
Body mass (kg)	91 [19]	80 [12]
Body fat (%)	24 [16]	16 [6]
FFM (kg)	66 [6]	66 [7]
Peak oxygen uptake ($\text{ml}\cdot\text{kg}\cdot\text{min}^{-1}$)	28 [6]	40 [7]*
Peak power output (W)	663 [147]	831 [221]*
Peak power output ($\text{W}\cdot\text{kg FFM}^{-1}$)	10 [2]	12 [2]*

491 *Denotes significantly different than SED ($p < 0.05$). FFM = fat free mass

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