

1 **Neuromuscular fatigue and recovery after strenuous exercise**
2 **depends on skeletal muscle size and stem cell characteristics**

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Running title: Neuromuscular Response following muscle damage *in vivo* and *in vitro*

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11 **ABSTRACT**

12 Hamstring muscle injury is highly prevalent in sports involving repeated maximal sprinting.
13 Although neuromuscular fatigue is thought to be a risk factor, the mechanisms underlying the
14 fatigue response to repeated maximal sprints are unclear. Here, we show that repeated
15 maximal sprints induce neuromuscular fatigue accompanied with a prolonged strength loss in
16 hamstring muscles. The immediate hamstring strength loss was linked to both central and
17 peripheral fatigue, while prolonged strength loss was associated with indicators of muscle
18 damage. The kinematic changes immediately after sprinting likely protected fatigued
19 hamstrings from excess elongation stress, while larger hamstring muscle physiological cross-
20 sectional area and lower *myoblast:fibroblast* ratio appeared to protect against fatigue/damage
21 and improve muscle recovery within 48 h after sprinting. Contrastingly, a high
22 *myoblast:fibroblast* ratio appears crucial for the latter stage of muscle regeneration. We have
23 therefore identified novel mechanisms that likely regulate the fatigue/damage response and
24 recovery following repeated maximal sprinting in humans.

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31 INTRODUCTION

32 Hamstring strain is the most frequently occurring injury in sport (Crema *et al.*, 2017),
33 particularly in those sports that involve high-speed running (Ekstrand *et al.*, 2011; Opar *et al.*,
34 2012). Although the aetiology is unclear, numerous risk factors have been proposed, such as
35 short fascicle length (Timmins *et al.*, 2015), poor flexibility (Jonhagen *et al.*, 1994), poor
36 hamstring strength (Opar *et al.*, 2014), and inadequate warm-up (Woods *et al.*, 2007). Further,
37 it is unknown whether hamstring strain is a result of a single event that exceeds the
38 physiological range of hamstring muscle extensibility and contractility, or as a result of an
39 accumulation of eccentric contractions during repeated maximal sprints, causing
40 neuromuscular fatigue (Opar *et al.*, 2012). Neuromuscular fatigue is responsible for acute, as
41 well as prolonged, impairment of muscle function and it can be classified as central fatigue
42 (i.e. originating in the central nervous system), or as peripheral fatigue (i.e. distal to the
43 neuromuscular junction) (Byrne *et al.*, 2004). Although it was recently reported that both central
44 and peripheral fatigue contribute to impaired hamstring muscle function immediately after
45 repeated maximal sprint-related interventions (Marshall *et al.*, 2014; Timmins *et al.*, 2014), the
46 contribution of neuromuscular fatigue to hamstring muscle impairment and recovery over time
47 is insufficiently studied following repeated maximal sprints (Verma *et al.*, 2015). An
48 understanding of hamstring neuromuscular fatigue following repeated maximal sprints may be
49 crucial for understanding hamstring strain aetiology (van der Horst *et al.*, 2015).

50 Peripheral fatigue may be caused by ultrastructural muscle damage, which is indicated by Z-
51 line disturbance (Newham *et al.*, 1983) as well as disruption of the extracellular matrix (Stauber
52 *et al.*, 1990). The extracellular matrix provides structural scaffolding for muscle remodelling
53 and plays an integral role in force transmission (Wang *et al.*, 2009; Gillies & Lieber, 2011). This
54 is referred to as exercise-induced muscle damage and it is exhibited by prolonged strength
55 loss and delayed-onset muscle soreness, as well as the release of muscle-specific proteins
56 [e.g. creatine kinase (CK)] into the circulation over the following days (Howatson & Van
57 Someren, 2008). After substantial muscle damage, myogenic satellite cells (skeletal muscle

58 stem cells), play a key role in skeletal muscle regeneration and remodelling, as activated
59 satellite cells (myoblasts) proliferate and migrate from their niche along the basal lamina to the
60 injury site before terminally differentiating and fusing into myotubes (Tidball, 2011). There is
61 increasing evidence that fibroblasts, the main cell type of muscle connective tissue, play a
62 critical role in supporting muscle regeneration (Murphy *et al.*, 2011; Fry *et al.*, 2017; Mackey
63 *et al.*, 2017). Following damage, infiltrating inflammatory cells activate muscle fibroblasts,
64 which proliferate and migrate to the area of the myotrauma and produce extracellular matrix
65 components in an orchestrated and regulated fashion to support healthy muscle remodelling
66 (Joe *et al.*, 2010; Murphy *et al.*, 2011). However, less is known about the effect of fibroblasts
67 on the initial response and recovery following *physiological* exercise-induced muscle damage,
68 e.g. following repeated sprinting.

69 This acute damage to the muscle-tendon complex may facilitate hamstring strain, which is
70 thought to occur in the late swing phase of sprinting, when the hamstring muscles contract
71 eccentrically, i.e. trying to shorten while being lengthened in an attempt to decelerate the shaft
72 before initial foot-ground contact (Thelen *et al.*, 2005; Chumanov *et al.*, 2012). Therefore, a
73 short biceps femoris long head (BF_{LH}) fascicle length has been suggested to increase
74 hamstring strain risk (Timmins *et al.*, 2016a), as the BF_{LH} is thought to be relatively more
75 eccentrically stretched during the late swing phase of sprinting compared to the other
76 hamstring muscles (Thelen *et al.*, 2005). However, no study has investigated the relationship
77 between BF_{LH} architecture (including muscle fascicle length and cross-sectional area), and the
78 prolonged hamstring muscle response to exercise-induced neuromuscular fatigue. Finally,
79 lower limb neuromuscular fatigue might cause a number of biomechanical alterations in
80 running kinematics (Paschalis *et al.*, 2007). However, it is not known whether repeated
81 maximal sprints influence kinematic patterns, and whether this can lead to prolonged changes
82 in lower-limb kinematics, which may play a role in the development of muscle strain following
83 insufficient recovery (Opar *et al.*, 2012).

84 Here we demonstrated that both central and peripheral fatigue contributed to the immediate
85 loss of muscle function in both the quadriceps and hamstring muscle groups, but that
86 peripheral factors mainly contributed to the sustained loss of hamstring muscle function.
87 Moreover, we established that a lower myoblast:fibroblast ratio in isolated primary human
88 muscle stem cells correlated with improved recovery from both repeated maximal sprints and
89 an *in vitro* artificial wounding assay within the first 48 h. However, at seven days post damage,
90 a higher myoblast:fibroblast ratio appears important for optimal muscle regeneration. We also
91 found that BF_{LH} architecture (i.e. PCSA) was associated with hamstring fatigue, and that
92 neuromuscular fatigue led to reduced hip flexion and knee extension during the late swing
93 phase of steady-state running. Thus, with this interdisciplinary study, we identify novel cellular
94 and neuromuscular mechanisms underpinning central and peripheral fatigue following
95 repeated sprinting, which ultimately led to kinematic changes during the running stride phase
96 associated with hamstring strain injury.

97

98 **RESULTS**

99 **Effect of the Repeated Maximal Sprint Intervention on Neuromuscular Fatigue**

100 To begin examining the effect of repeated maximal sprints on neuromuscular fatigue, we
101 measured different fatigue parameters before (PRE), immediately after (POST), and 48 h after
102 (POST48) the repeated maximal sprint intervention. The average 30 m sprinting speed was
103 $6.48 \pm 0.33 \text{ m s}^{-1}$. There was a main effect of time for heart rate, 30 m sprinting time, rating of
104 perceived exertion and lactate, with all parameters increasing from PRE to POST repeated
105 maximal sprints (all $P < 0.001$). Blood lactate concentration also increased from PRE ($1.63 \pm$
106 0.45 mmol/L) to POST ($9.82 \pm 3.62 \text{ mmol/L}$). The sprinting performance (measured via the
107 performance decrement score (Glaister *et al.*, 2008)) decreased by $3.98 \pm 2.99 \%$, and rating
108 of perceived exertion increased by $96.5 \pm 35.2 \%$ from PRE-to-POST, indicating fatigue had
109 occurred.

110 We then performed *in vivo* functional analysis to assess if repeated maximal sprints resulted
 111 in an increase in central and/or peripheral fatigue. We, therefore, measured BF_{LH} muscle
 112 activation via normalised surface electromyography (sEMG) during hamstring maximum
 113 voluntary contraction (MVC). That assessment showed that a change had occurred in sEMG
 114 ($F_{F2,24}=4.35$, $P=0.022$), with post-hoc pairwise comparisons revealing a decrease from PRE-
 115 to-POST ($P=0.019$). However, this change was no longer evident at POST48 ($P=0.157$, Table
 116 1), suggesting that central fatigue occurred immediately after repeated maximal sprints. No
 117 other changes in muscle (co)activation were observed at any time point ($P>0.05$).

118 **Table 1** Effect of the intermitted sprint intervention on measures of muscle activation. Data are
 119 presented as mean \pm SD. One-way ANOVA, F- and P-values are reported.

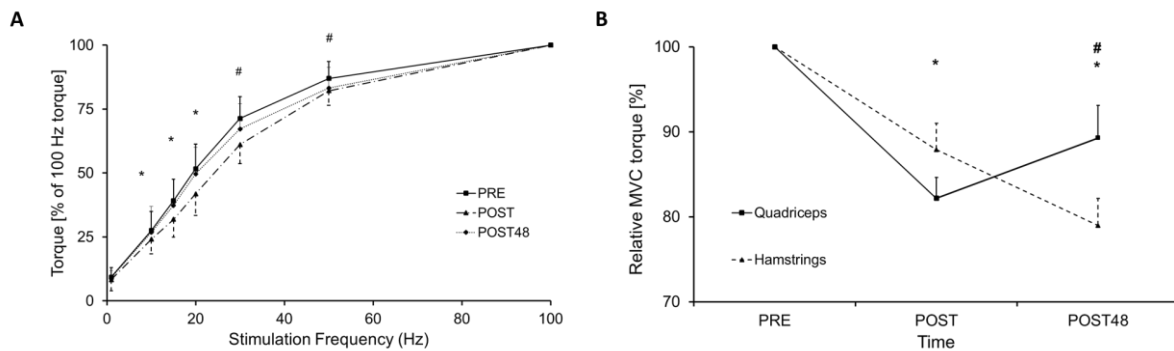
| Assessment [unit] | n | PRE | POST | POST48 | F-Test | P Value |
|--|----|-----------------|------------------|-----------------|------------------|---------|
| Hamstring Muscle Voluntary Activation [ITT, %] | 20 | 98.5 \pm 2.64 | 94.1 \pm 7.83 | 96.9 \pm 5.96 | F(1,4,38) = 2.75 | 0.099 |
| Normalised BF _{LH} knee flexion sEMG _{max} [%] | 14 | 3.32 \pm 1.33 | 2.27 \pm 0.72* | 2.85 \pm 1.16 | F(2,24) = 4.35 | 0.022 |
| Vastus lateralis knee extension sEMG _{max} [mV] | 15 | 0.50 \pm 0.29 | 0.47 \pm 0.34 | 0.51 \pm 0.34 | F(2,24) = 0.17 | 0.772 |
| Quadriceps CoA during 30° hamstring MVC [%] | 12 | 5.74 \pm 7.23 | 4.08 \pm 6.86 | 4.33 \pm 8.76 | F(2,22) = 0.50 | 0.613 |
| Hamstring CoA during 80° quadriceps MVC [%] | 10 | 4.79 \pm 3.37 | 6.62 \pm 3.79 | 7.51 \pm 4.16 | F(2,18) = 1.64 | 0.223 |

ITT – interpolated twitch technique; BF_{LH} – Biceps femoris long head; CoA – Co-Activation; sEMG – surface Electromyography. * Different to PRE ($P<0.05$).

120
 121 We also assessed the torque-frequency relationship *in vivo* via electrical stimulation, as it gives
 122 an indication of peripheral (muscle) fatigue. There was an interaction between time x
 123 stimulation frequency (n=19; $F_{4,9,216}=6.62$, $P<0.001$; Figure 1A). Post-hoc paired t-tests
 124 revealed differences PRE-to-POST for 10-50 Hz, but lower frequencies between 10-20 Hz

125 reverted to baseline values POST48 ($P>0.05$), while the frequencies of 30 and 50 Hz were still
126 decreased POST48 compared to their baseline values ($P<0.05$), providing evidence that
127 peripheral fatigue occurred immediately after the repeated sprints and remained for 48 h

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129

130 **Figure 1 (A)** Torque-frequency relationship, all frequencies were normalised to 100 Hz. * significant
131 differences between before (PRE) and immediately after (POST) the repeated maximal sprint
132 intervention, $P<0.05$; # significant differences between PRE and POST, and between PRE and POST48,
133 $P<0.05$. Data are presented as mean \pm SEM. **(B)** Comparison of relative maximal voluntary contraction
134 (MVC) loss between hamstring and quadriceps muscle group before (PRE), immediately after (POST)
135 and 48h after (POST48) the repeated maximal sprint intervention. * significant differences compared to
136 PRE, $P<0.001$; # significant differences between quadriceps and hamstring MVC, $P<0.05$. Data are
137 expressed as mean \pm SEM.

138

139 Effect of the Repeated Maximal Sprint Intervention on MVC Strength, Muscle 140 Soreness and Serum Markers of Exercise-Induced Muscle Damage

141 To investigate the effect of intermittent sprints on biomarkers of exercise-induced muscle
142 damage, we assessed hamstring (knee flexion) and quadriceps (knee extension) MVC, muscle
143 soreness, serum creatine kinase (CK) activity and interleukin-6 (IL-6) concentration PRE,
144 POST and POST48. Isometric hamstring and quadriceps MVC, muscle soreness (all $P<0.001$)
145 and serum CK activity ($F_{1,3,34}=5.98$, $P=0.017$), as well as IL-6 concentration ($F_{1,3,34}=5.96$,
146 $P=0.018$), showed a main effect of time, which are indicators of muscle damage (Table 2) that
147 was similar to other studies (Howatson & Milak, 2009; Verma *et al.*, 2015; Chen *et al.*, 2017).
148 Post-hoc pairwise comparisons revealed that, compared to PRE, both serum CK activity and
149 serum IL-6 concentration were elevated at POST (both $P=0.027$), and CK activity further

150 increased at POST48 (P=0.012), while serum IL-6 concentration reverted to baseline values
 151 (P>0.05).

152 **Table 2** Effect of the Repeated Maximal Sprint Intervention on Muscle Damage-Biomarkers. Values
 153 are mean \pm SD. One-way ANOVA, F- and P-values are reported.

| Assessment [unit] | PRE | POST | POST48 | F-Test | P Value |
|----------------------------|-------------------|-------------------|-------------------|------------------|---------|
| Quadriceps MVC [N·m] | 270.5 \pm 51.6* | 222.4 \pm 52.5* | 243.0 \pm 71.3* | F(2,38) = 16.55 | <0.001 |
| Hamstring MVC [N·m] | 142.5 \pm 25.0* | 124.8 \pm 29.9* | 112.4 \pm 30.1* | F(2,38) = 25.12 | <0.001 |
| Squat Muscle soreness [cm] | 0.20 \pm 0.41* | 1.95 \pm 1.61* | 2.87 \pm 1.71* | F(2,38) = 28.62 | <0.001 |
| Lunge Muscle soreness [cm] | 0.30 \pm 0.57 | 2.30 \pm 2.08† | 3.48 \pm 2.07† | F(2,38) = 17.02 | <0.001 |
| Range of Motion [°] | 120.3 \pm 6.76 | 115.7 \pm 6.77† | 116.0 \pm 6.27† | F(2,38) = 9.33 | <0.001 |
| CK activity [mU/mL] | 27.9 \pm 23.3 | 53.8 \pm 45.3† | 99.3 \pm 104.5† | F(1,3,34) = 5.98 | 0.017 |
| IL-6 concentration [pg/mL] | 1.89 \pm 3.10 | 7.68 \pm 9.95# | 1.59 \pm 3.46 | F(1,3,34) = 5.96 | 0.018 |

MVC – Maximal voluntary contraction; CK – Creatine Kinase; IL-6 – Interleukin-6; * Significant differences between all time points; † differences PRE-to-POST and PRE-to-POST48 (P<0.05); # differences PRE-to-POST and POST-to-POST48 (P<0.05).

154 Further, there was an interaction between time and muscle groups concerning relative MVC
 155 torque loss (percentage change from PRE MVC) (F_{1,4,38}=7.92, P=0.004). Relative MVC
 156 decreased similarly in both quadriceps and hamstring muscle groups PRE-to-POST (Figure
 157 1B). However, at POST48, hamstring MVC continued to decrease from POST (P=0.010), while
 158 quadriceps MVC began to return to PRE values (P=0.016), and was higher than hamstring
 159 MVC at POST48 (P=0.038).

160 **Effect of the Repeated Maximal Sprint Intervention on Lower-Limb Kinematics**

161 To assess the consequential effect of neuromuscular fatigue on lower-limb kinematics, we
162 captured treadmill running (4.17 m s^{-1}) kinematics with an eight-camera motion capture system
163 over time. Three-dimensional motion analysis showed that there was a non-significant
164 tendency for the duration of the running cycle to take longer POST and POST48, compared to
165 PRE ($P=0.080$; Table 3). Further, treadmill running demonstrated decreases in peak knee
166 extension ($P=0.047$) during the late swing phase at POST compared to PRE, but this reverted
167 to baseline POST48. The percentage change in peak knee extension correlated with the
168 percentage change in relative hamstring MVC torque both measured POST-to-POST48
169 ($r^2=0.258$, $F_{1,2}=5.673$, $P=0.031$).

Table 3 Effect of the Repeated Maximal Sprint Intervention on Kinematics of Treadmill Running at 4.17 m s⁻¹. Values are mean ± SD. One-way ANOVA, F- and P-values are reported.

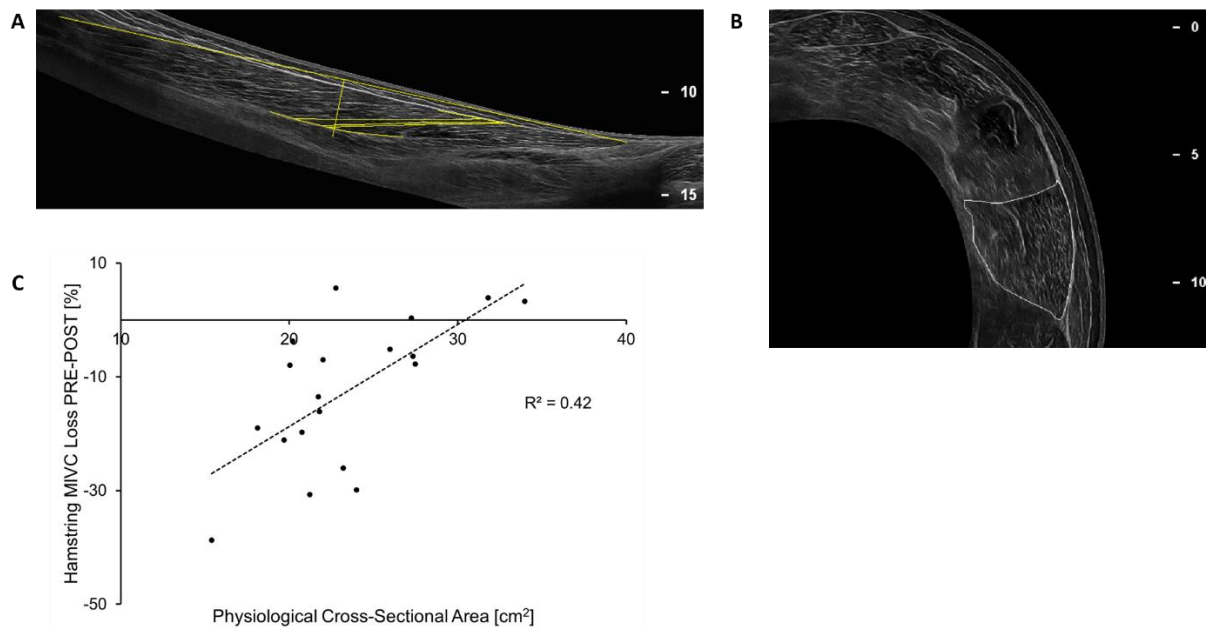
| Kinematics [unit] | PRE | POST | POST48 | F-Test | P Value |
|---------------------------------------|--------------|---------------|--------------|------------------|---------|
| Peak knee flexion (swing phase) [°] | -103 ± 13.9 | -110 ± 12.4 | -108 ± 12.6 | F(2,20) = 2.84 | 0.082 |
| Peak knee extension (swing phase) [°] | -3.66 ± 5.32 | -7.08 ± 5.07* | -4.29 ± 7.06 | F(2,20) = 3.57 | 0.047 |
| Contact hip flexion (toe strike) [°] | 25.2 ± 5.42 | 29.3 ± 7.62 | 17.6 ± 15.6 | F(1,2,20) = 4.05 | 0.062 |
| Contact knee flexion (toe strike) [°] | -15.0 ± 6.25 | -17.6 ± 6.79 | -13.3 ± 9.64 | F(2,22) = 2.79 | 0.083 |
| Duration Running cycle [s] | 0.67 ± 0.03 | 0.68 ± 0.03 | 0.69 ± 0.02 | F(2,20) = 2.88 | 0.080 |
| Stance phase duration [s] | 0.18 ± 0.02 | 0.19 ± 0.02 | 0.19 ± 0.03 | F(2,20) = 1.01 | 0.356 |
| Swing Phase [s] | 0.50 ± 0.04 | 0.50 ± 0.05 | 0.50 ± 0.03 | F(1,2,20) = 0.03 | 0.899 |

Knee fully extended=0°; negative value indicates a flexed knee; * different to PRE (P<0.05).

170

171 **Architecture of the Biceps Femoris Long Head Muscle**

172 To assess whether architectural parameters of the BF_{LH} muscle (Figure 2A and B) were
 173 associated with markers of peripheral fatigue, we performed ultrasound measurements of the
 174 BF_{LH} muscle (Table supplement 5). Muscle fascicle length and pennation angle of the BF_{LH},
 175 which have previously been linked to hamstring muscle strain risk (Timmins *et al.*, 2016b), did
 176 not correlate with any outcome variable of neuromuscular fatigue. However, BF_{LH} PCSA (mean
 177 ±SD: 23.4 ± 4.62 cm²) correlated inversely with relative hamstring MVC loss PRE-to-POST
 178 (R²=0.421, F_{1,17}=12.37, P=0.003, Figure 2C).



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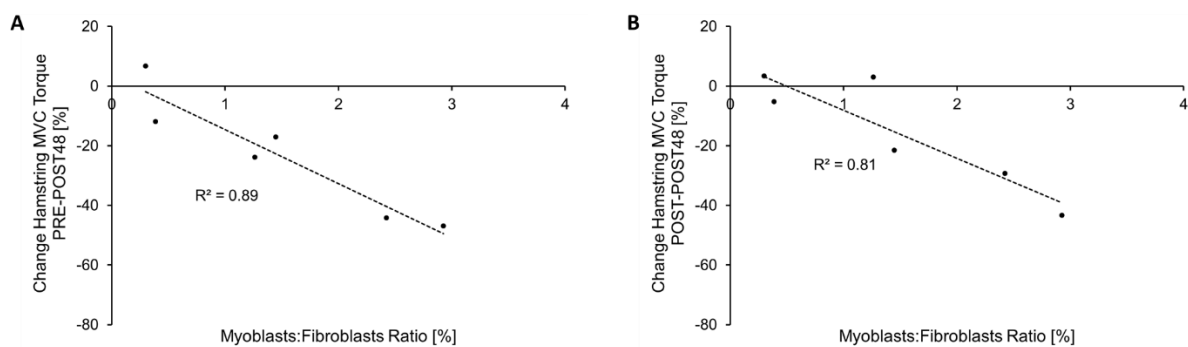
180 **Figure 2 (A)** Longitudinal image of biceps femoris long head, assessment of the biceps femoris long
181 head is highlighted (total muscle length and fascicle length together with pennation angle at 50% of total
182 muscle length). **(B)** Cross-sectional image at 60% muscle length (=100% proximal myotendinous
183 junction), biceps femoris long head is highlighted. **(C)** Correlation between biceps femoris long head
184 muscle physiological cross-sectional area and % hamstring maximum isometric voluntary contraction
185 (MIVC) decrease from before (PRE) to immediately after (POST) the repeated maximal sprint
186 intervention ($P=0.003$).

187

188 **Comparison of the Muscle Response between the Repeated Maximal Sprint** 189 **Protocol and the Muscle Stem Cell Study**

190 We next sought to determine whether skeletal muscle stem cell composition (i.e.
191 myoblast:fibroblast ratio) played a role in muscle strength recovery. Six of the 20 participants,
192 who performed the repeated maximal sprint intervention, also volunteered to provide a muscle
193 biopsy at least three weeks before the repeated maximal sprint intervention. As previous
194 investigations have shown that skeletal muscles of different origin, but with similar
195 physiological functions and fibre type composition, demonstrate similar transcriptome
196 expression patterns of up to 99% (Evangelidis *et al.*, 2016; Terry *et al.*, 2018), we have taken
197 a muscle biopsy from the vastus lateralis as a representative muscle of the quadriceps and
198 hamstring muscle groups. The muscle stem cells were isolated, cultured and then
199 characterized by immunofluorescence staining. The mean \pm SD myoblast:fibroblast ratio of the
200 six participants was 1.46 ± 1.06 (range: 0.299 to 2.93). There was a strong inverse correlation

201 between myoblast:fibroblast ratio and the percentage change in relative hamstring MVC torque
202 measured PRE-to-POST48 *in vivo* ($R=-0.945$, $F_{1,4}=33.73$, $P=0.004$; Figure 3A). Thus, the
203 myoblast:fibroblast ratio explained 89% of the variability in strength recovery PRE-to-POST48,
204 i.e. participants with a high myoblast:fibroblast ratio showed a delayed hamstring strength
205 recovery 48 h after repeated maximal sprints compared to those with a low myoblast:fibroblast
206 ratio. Further, there was an inverse correlation between myoblast:fibroblast ratio and relative
207 hamstring MVC torque measured POST-to-POST48 ($R=-0.943$, $F_{1,4}=17.08$, $P=0.014$; Figure
208 3B). Thus, the myoblast:fibroblast ratio explained 81% of the variability in strength recovery
209 POST-to-POST48. No correlations were found between the myoblast:fibroblast ratio and
210 changes in quadriceps MVC torque or with any other muscle damage and fatigue biomarker
211 following repeated maximal sprints.



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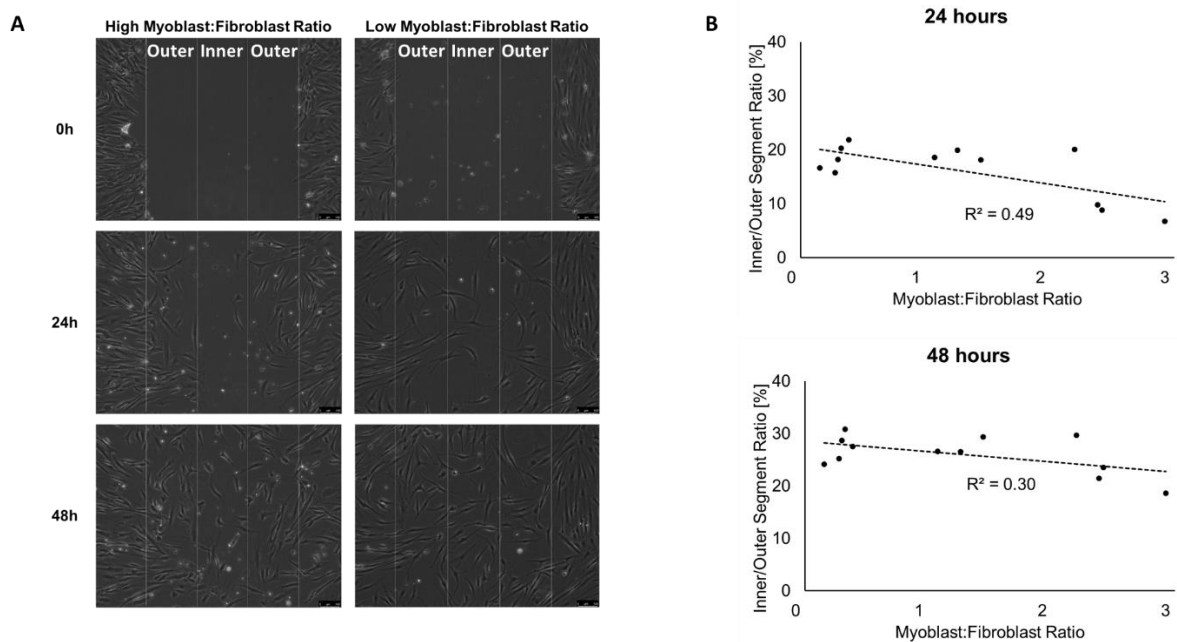
213 **Figure 3** Inverse correlation between the myoblast:fibroblast ratio, assessed in the current *in vitro* study
214 and the change of hamstring MVC torque measured (A) before and 48 h after ($P=0.004$), and (B)
215 measured immediately after and 48 h after ($P=0.014$) (B) the repeated maximal sprint intervention.

216

217 Artificial Wound Healing Assay to Investigate Repair and Regeneration 218 Regarding Myoblast:Fibroblast Ratio

219 To further assess the effect of the myoblast:fibroblast ratio on skeletal muscle recovery, we
220 performed an *in vitro* artificial wounding (scratch) assay. We used primary human skeletal
221 muscle stem cells derived from the six volunteers, who participated in both the repeated
222 maximal sprint intervention and volunteered to provide a muscle biopsy, and another six (two
223 male and four females), who did not participate in the repeated maximal sprint intervention (to

224 increase the power of the *in vitro* study). We did not detect any differences in muscle stem cell
225 characteristics between cells obtained from females and males (data not shown). We,
226 therefore, combined the data from all muscle cells and correlated the muscle characteristics
227 with individual myoblast:fibroblast ratios. We observed no correlations regarding
228 myoblast:fibroblast ratio and the total number of myoblasts and fibroblasts migrating into the
229 artificial wound within all three segments combined at 24 h ($R^2=0.20$, $F_{1,10}=2.56$, $P=0.141$) or
230 48 h ($R^2=0.02$, $F_{1,10}=0.19$, $P=0.671$) after the scratch assay.



231

232 **Figure 4 (A)** Representative images for cell migration of muscle cells with a high myoblast:fibroblast
233 ratio (2.4; left) and with a low percentage of myoblasts (0.3; right) into the artificial wound. The wound
234 area is about 900 μm in width and split into 3 x 300 μm segments (one inner and two outer segments).
235 Magnification is x 10.5, and scale bar is 100 μm . **(B)** Inverse correlations between the myoblast:fibroblast
236 ratio and the migration dynamics of 12 different primary muscle stem cells 24 h ($P=0.011$) and a trend
237 48 h ($P=0.064$) after the artificial wound healing assay.

238 However, there was an inverse correlation between myoblast:fibroblast ratio and migration
239 dynamics for the 12 participants (Figure 4 B). Muscle stem cells with a low myoblast:fibroblast
240 ratio demonstrated more cells in the inner segment than to the outer segment compared to
241 muscle stem cells with high myoblast:fibroblast ratio at 24 h ($R^2=0.49$, $F_{1,10}=9.53$, $P=0.011$)
242 and with a non-significant trend at 48 h ($R^2=0.30$, $F_{1,10}=4.33$, $P=0.064$) after the artificial wound
243 healing assay. Further, there were positive correlations between the myoblast:fibroblast ratio
244 and all parameters at seven and 10 days after the artificial wound healing assay (all $P<0.05$;

245 Table supplement 6). Biopsies with a higher myoblast:fibroblast ratio showed more myotubes
246 per field, which had a higher diameter and area compared to biopsies with a lower
247 myoblast:fibroblast ratio (Figure supplement 5).

248

249 **DISCUSSION**

250 In this study, we have used an interdisciplinary approach to systematically investigate the
251 biomechanical, physiological and cellular factors underpinning neuromuscular fatigue following
252 repeated maximal sprints. We have shown that immediate strength loss was associated with
253 reduced hamstring sEMG activity (indicating impaired hamstring motor unit recruitment) and
254 markers of peripheral fatigue, but the magnitude and sustained changes in MVC torque over
255 time (especially of the hamstrings) was largely associated with indicators of peripheral fatigue.
256 Muscle damage biomarkers indicated that the peripheral fatigue might have been caused
257 predominantly by ultrastructural damage within the hamstring muscle tissue. Further, both
258 central and peripheral fatigue caused by repeated maximal sprints appeared to affect the
259 neuromuscular control of running patterns and BF_{LH} PCSA correlated inversely with the change
260 in hamstring MVC torque immediately after the repeated maximal sprint intervention. A high
261 myoblast:fibroblast ratio showed a delayed wound closure *in vitro* and a delayed MVC torque
262 recovery following repeated maximal sprints *in vivo* within the first 48 h, indicating that stem
263 cells of the non-contractile muscle tissue might positively affect the response to muscle
264 damaging exercises. However, a higher myoblast:fibroblast ratio led to better myotube
265 formation at seven days, and higher CK activity at ten days, after the scratch assay, suggesting
266 that myoblasts are important for the latter stage of muscle regeneration.

267 **Fatigue and Muscle Damage Following Repeated Maximal Sprints**

268 We showed a decreased activity of normalised BF_{LH} sEMG activity immediately after the
269 repeated maximal sprint intervention, but no significant changes in neuromuscular activation
270 using the interpolated twitch technique. The discrepancy between these two methods might

271 be explained by the fact that voluntary activation measured via the interpolated twitch
272 technique investigates all of the hamstring muscles, whilst the normalised EMG analysis was
273 confined solely to the BF_{LH}, which is in line with a previous study (Marshall *et al.*, 2014). We
274 assume that BF_{LH} might fatigue to a greater degree immediately after repeated maximal sprint
275 related interventions compared to the other hamstring muscles. However, we also provide
276 evidence for peripheral fatigue occurring immediately after the repeated maximal sprints, and
277 a delayed recovery at higher frequencies (30-50 Hz) after observing a right shift in the torque-
278 frequency relationship. This may be due to ultrastructural damage predominantly in fast-twitch
279 (which fire at rates from 30-50 Hz) compared to slow-twitch muscle fibres (discharge rates 10-
280 25 Hz) (Friden *et al.*, 1983; Jones *et al.*, 1986), leading to impaired force generation rather
281 than simply fatiguing the muscle fibres.

282 Both the quadriceps and hamstring muscle groups showed similar strength loss immediately
283 after the repeated maximal sprints, but the hamstring muscle group showed further strength
284 loss 48 h later compared to the quadriceps. Other studies did not show this additional strength
285 loss for the hamstring muscle group POST48. Differences in the training status of the
286 participants (Verma *et al.*, 2015; Chen *et al.*, 2017) and in the methodological approaches
287 (Balsom *et al.*, 1992) might partly explain the different outcomes. The peak BF_{LH} EMG activity
288 occurs at a more extended knee angle during hamstring isokinetic muscle contraction
289 compared to the peak EMG occurring at a more flexed knee angle for the other hamstring
290 muscles, such as the semitendinosus (Onishi *et al.*, 2002). Therefore, we suggest that the BF_{LH}
291 is the key hamstring muscle responsible for decelerating the shaft at the end of the late swing
292 phase. After repeated bouts of high-speed running, the semitendinosus might fatigue
293 prematurely (Schuermans *et al.*, 2014) and the BF_{LH} would need to substitute the impaired
294 function of the preceding semitendinosus to decelerate the shaft.

295 **Kinematic Analysis**

296 Our *in vivo* intervention caused changes in the running kinematics with reduced knee extension
297 in the late swing phase immediately after the repeated maximal sprints. Reduced hamstring

298 muscle strength due to neuromuscular fatigue might trigger a protective mechanism directly
299 after repeated maximal sprints. Afferent signals from the fatigued and damaged hamstrings
300 might activate the Golgi tendon organ (Byrne *et al.*, 2004), thus limiting hamstring muscle fibre
301 strain in an attempt to minimise further muscle damage. These kinematic changes were not
302 evident 48 h after the repeated maximal sprints. However, there was a non-significant tendency
303 for prolonged stride duration during running ($P = 0.08$, data not shown) 48 h later and the
304 percentage change of knee extension in the late swing phase of running correlated with
305 changes in hamstring strength both measured from POST to POST48. This indicates that
306 participants with delayed hamstring strength recovery were still not able to fully control running.
307 As hamstring MVC continued to deteriorate 48 h after intermittent sprints but quadriceps MVC
308 started to improve, it could be that lower-limb kinematics in the sagittal plane are controlled by
309 the hamstrings more than the quadriceps. Ultrastructural damage in the hamstring muscles
310 might lead to decelerated movement patterns over time, which could increase the risk for
311 hamstring strain injury during sprinting (Opar *et al.*, 2012).

312 **The Role of the Extracellular Matrix on the Muscle Response Following Repeated** 313 **maximal sprinting**

314 Recent investigations have suggested that hamstring maximum eccentric strength and BF_{LH}
315 fascicle length are predictors of hamstring strain injury (Timmins *et al.*, 2016a). Further, a
316 fatigued muscle is likely to accentuate the risk of muscle strain (Ekstrand *et al.*, 2011). However,
317 we could not find any correlation between BF_{LH} fascicle length and any biomarker of fatigue
318 but BF_{LH} PCSA correlated inversely with hamstring strength loss from PRE to POST. During
319 the late swing phase of sprinting, the hamstring muscles contract eccentrically to
320 decelerate the shaft and to enhance the subsequent concentric shortening contraction for
321 maximal sprinting by using stored elastic energy from the muscle-tendon unit. In comparison
322 to other conventional muscle-damaging interventions (Franchi & Maffiuletti, 2019), this
323 dynamic (stretch-shortening) movements might lead to an additional damage of the hamstring
324 muscle connective tissue structure. Therefore, a larger BF_{LH} PCSA might protect against

325 immediate hamstring MVC loss due to a greater ability to transmit the ground reaction forces
326 laterally (from fibre to fibre) (Turrina *et al.*, 2013), which might disperse the force more
327 efficiently to the tendon, while the muscle fibres themselves undergo less strain. Further, a
328 greater BF_{LH} PCSA reflects more fibres aligned in parallel, which would be accompanied by
329 more muscle connective tissue of the extracellular matrix, thus potentially protecting the
330 muscle fibres from excessive damage during eccentric contractions.

331 The stem cells of the extracellular matrix also demonstrated an important role for muscle
332 strength recovery in the subgroup of participants, as there was a strong inverse correlation
333 between myoblast:fibroblast ratio and hamstring MVC torque recovery POST48. Skeletal
334 muscles with an increased availability of fibroblasts around the area of myotrauma might have
335 a better capacity to reorganise the complex extracellular matrix, thus restoring (lateral) force
336 transmission, which results in a faster recovery of muscle strength after muscle damage. This
337 was in line with the myoblast:fibroblast ratio effect on cellular aspects of muscle regeneration
338 and remodelling assessed in primary muscle stem cells *in vitro*. Muscle stem cells with a low
339 myoblast:fibroblast ratio revealed a faster wound closure (i.e. more cells migrated to the inner
340 part of the artificial injury compared to the outer part), in particular 24 h after performing the
341 scratch assay. However, this effect was less significant 48 h after the scratch protocol, and, at
342 day seven, muscle primary cells with a higher myoblast:fibroblast ratio showed an improved
343 myotube formation, which is in line with the investigation of Fry *et al.* (2014). Together, these
344 results suggest that the abundance and activity of fibroblasts and myoblasts may play different
345 roles, depending on the time points during muscle repair. A larger abundance of fibroblasts
346 has a positive effect at the beginning of muscle repair, but a larger number of myoblasts is
347 more important for the latter stage of muscle regeneration or hypertrophy, when myoblasts
348 differentiate and fuse to become myotubes. We, therefore, assume that repeated maximal
349 sprints with insufficient recovery of previously fatigued and damage muscles (where the fatigue
350 and damage response is modulated by the muscle size and stem cell composition, respectively)
351 might augment the risk of muscle strains, as appropriate damage to the muscle connective

352 tissue is thought to differentiate between exercise-induced muscle damage and muscle strains
353 (Valle *et al.*, 2017; Balius *et al.*, 2018).

354 **Limitations**

355 There was no relationship between the myoblast:fibroblast ratio and any physiological
356 variables regarding the quadriceps femoris, from which the muscle biopsies were obtained. It
357 has previously been shown that skeletal muscles of different origin, but with similar
358 physiological functions and fibre type composition, demonstrate similar transcriptome
359 expression patterns of up to 99% (Evangelidis *et al.*, 2016; Terry *et al.*, 2018). Therefore, the
360 correlation between myoblast:fibroblast ratio and the muscle damage-response of the
361 hamstrings but not the quadriceps muscles is likely explained by more severe ultrastructural
362 damage in the hamstrings than quadriceps. Furthermore, peripheral fatigue can be caused by
363 metabolic perturbations, such as the depletion of intramuscular glycogen (Howatson & Van
364 Someren, 2008). Therefore, because we did not control diet throughout the study, it is possible
365 that inter-individual differences in baseline muscle glycogen may have influenced the ability to
366 maintain maximal intensity throughout the sprints. However, participants were instructed to eat
367 and drink similar foods two hours before each laboratory visit, and to avoid strenuous exercise
368 for at least 48 h prior to the testing. Further, participants were given sufficient recovery between
369 sprint repetitions and there was a low decrement in sprint performance, indicating that
370 glycogen depletion was probably only a minor factor.

371 **CONCLUSION**

372 Repeated maximal sprints induces a greater and more prolonged strength loss in the
373 hamstrings compared to the quadriceps muscles. The immediate loss of hamstring function
374 appears to be due to both central (particularly reduced neuromuscular activation of the biceps
375 femoris long head) and peripheral fatigue, while prolonged hamstring strength loss is
376 predominantly linked to peripheral fatigue. Thigh neuromuscular fatigue following repeated
377 maximal sprints alters hip and knee kinematics during running immediately after the repeated

378 maximal sprints, but this (likely) protective effect is less evident 48 hours after the intervention,
379 which may lead to an increased hamstring muscle injury risk. Furthermore, biceps femoris long
380 head PCSA was inversely related to hamstring strength loss immediately after repeated
381 maximal sprinting, suggesting that the structure of the muscle, including non-contractile tissue
382 (e.g. the extracellular matrix) protects against neuromuscular fatigue. Skeletal muscles with an
383 increased number of fibroblasts might have a better capacity to reorganise the complex
384 extracellular matrix, which results in a faster recovery of muscle function after substantial
385 muscle damage. However, a larger number of myoblasts seems to be more important for the
386 latter stage of muscle regeneration. These novel findings improve our understanding of the
387 physiological, biomechanical and cellular causes and effects of exercise-induced
388 neuromuscular fatigue. The practical implications are that a 48 h recovery period following
389 repeated maximal sprinting is insufficient, and might increase hamstring strain injury risk.
390 Furthermore, increasing hamstring PCSA via resistance training is likely to reduce peripheral
391 fatigue following repeated maximal sprinting, thereby reducing hamstring strain injury risk.

392 MATERIALS AND METHODS

393 Participants

394 *In vivo repeated maximal sprint intervention:* Twenty recreationally active and healthy young
395 men (*mean* \pm *SD*; age 20.3 ± 2.87 years; height 1.79 ± 0.05 m; body mass 75.0 ± 7.89 kg)
396 participated in the repeated maximal sprint intervention.

397 *In vitro muscle stem cell component:* Eight healthy young male (age 21.25 ± 4.27 years; height
398 1.77 ± 0.05 m; body mass 73.78 ± 5.68 kg) and four healthy young female (age 25.5 ± 1.29
399 years; height 1.67 ± 0.08 m; body mass 61.40 ± 2.57 kg) participants provided a biopsy of
400 the vastus lateralis muscle for the *in vitro* muscle stem cell component of this study. Six of the
401 eight males also participated in the repeated maximal sprint intervention at least three weeks
402 after providing a muscle biopsy. Only males were recruited for the repeated maximal sprint
403 intervention, as there is some evidence of sex differences in neuromuscular fatigue (Wüst *et*
404 *al.*, 2008). However, both men and women were recruited for the muscle stem cell component
405 due to there being no reported sex differences in stem cell properties and none within our own
406 pilot studies (data not shown). Prior to starting the study, written informed consent was
407 obtained from each participant and pre-biopsy screening was performed by a physician for
408 those participants who volunteered a muscle biopsy. The study was approved by the Research
409 Ethics Committee of Liverpool John Moores University and complied with the Declaration of
410 Helsinki. Volunteers were physically active but were ineligible to participate if they had
411 performed strength training of the lower limbs within 6 months prior to participation in the study,
412 which was determined during pre-participation screening. Further exclusion criteria were: (i)
413 any lower limb injury in the past 12 months; (ii) age under 18 or above 35 years; and (iii) more
414 than three structured exercise sessions per week.

415 Experimental Design of the Repeated Maximal Sprint Intervention *in vivo*

416 Participants were required to visit the temperature-controlled ($22-24^{\circ}\text{C}$) laboratory on three
417 occasions: (i) familiarisation, (ii) testing day including the PRE and POST assessments; and

418 (iii) POST48 assessments after the repeated maximal sprint intervention. One week prior to
419 the testing day, participants were familiarised with the assessments as well as with repeated
420 maximal sprints (by performing 2-3 submaximal sprints) and BF_{LH} architecture of the hamstring
421 muscle group was assessed via ultrasound. On the test day, participants performed the
422 repeated maximal sprint intervention of 15 x 30 m sprints to induce neuromuscular
423 fatigue/damage in both the quadriceps femoris and hamstring muscle groups. All tests were
424 performed at the same time of the day for each participant. Further, participants were instructed
425 to maintain their normal routine (including eating habits), to refrain from drinking alcohol and
426 to avoid any strenuous exercise 48 h prior to testing and throughout the study, and to refrain
427 from consuming caffeine on testing days. Nothing was consumed throughout the testing
428 sessions except water, which was available ad libitum.

429 The test battery was always performed in the same order with the right leg of each participant
430 and comprised (i) venous blood sampling [for analysing serum interleukin-6 (IL-6)
431 concentration and creatine kinase (CK) activity]; (ii) hamstrings and quadriceps muscle
432 soreness via visual analogue scale; (iii) isometric maximum voluntary contraction (MVC)
433 torque of the quadriceps, as well as both voluntary and involuntary muscle activation and
434 torque-frequency relationship via electrical stimulation] MVC torque of the hamstring together
435 with normalised BF_{LH} sEMG (see below); and (iv) treadmill running (4.17 m s^{-1}) kinematics of
436 the right leg (via an eight-camera motion capture system) PRE and POST48 following the
437 repeated maximal sprint intervention. At POST, kinematic assessments were performed first
438 followed by the aforementioned order of the assessment for practical reasons.

439 **Maximal Repeated Sprint Protocol**

440 Many athletes in team sports are required to perform repeated maximal sprints, which are
441 characterised by short-duration (<10 s) and relatively longer recovery times (>60 s) between
442 maximal sprint bouts, and have a different physiological demand compared to repeated-sprint
443 exercises with shorter recovery times (<60 s) (Bradley *et al.*, 2010; Girard *et al.*, 2011).
444 Therefore, the IS intervention consisted of 15 repetitions of 30 m maximal sprints with a

445 deceleration zone of 12 m. The 30 m distance was chosen as the upper average of both the
446 total sprinting distance (346 ± 115 m) of wide-midfielders and the mean recovery time ($70.2 \pm$
447 25.1 s) between sprint bouts in soccer (Bradley *et al.*, 2009; Bradley *et al.*, 2010), which allows
448 the athlete to maintain the performance of the sprint bouts. Similar protocols have been used
449 elsewhere (Timmins *et al.*, 2014; Verma *et al.*, 2015; Chen *et al.*, 2017). Prior to the repeated
450 maximal sprint intervention, a five-minute warm-up was performed, comprising jogging,
451 dynamic stretching and three self-paced 20 m runs at 60%, 80%, 100% of perceived top speed.
452 During the repeated maximal sprint intervention, the participants were instructed to sprint
453 maximally (verbal encouragement) and to stop within the deceleration zone. Further, they were
454 instructed to move slowly back to the start line and to sit on a chair for the remaining time until
455 the next sprint. The recovery comprised 90 s between repetitions and after every 5th repetition,
456 the participants were allowed to rest for 3 min. Sprinting time during trials was measured and
457 controlled with timing gates (Brower Timing Systems, Draper, UT, USA), which were placed
458 on the start and finish line. Participants started 30 cm before the start line to avoid interfering
459 with timing gates with the arms upon initial acceleration (Howatson & Milak, 2009). Further,
460 heart rate (Polar Oy, Kempele, Finland) and rating of perceived exertion (Borg, 1982) were
461 recorded before and after each repetition. Participants were instructed to wear the same
462 footwear for each testing day. As there was an upsurge in speed of the final sprint, fatigue was
463 assessed with the performance decrement score using the following formula (Glaister *et al.*,
464 2008):

$$465 \quad \text{Fatigue} = (100 \times (\text{total sprint time} \div \text{ideal sprint time})) - 100$$

466 Where total sprint time = sum of time from all 15 sprints; and ideal sprint time = total number
467 of sprints (15) x fastest repetition sprint time. The calculation of this decrement score was also
468 used to quantify changes in heart rate and rating of perceived exertion during the repeated
469 maximal sprint intervention.

470 **Maximal Voluntary Contraction (MVC)**

471 Three test sessions were conducted with an isokinetic dynamometer (Humac Norm, CSMI
472 Solutions, Massachusetts, USA). As *isokinetic* maximum voluntary contractions (MVC) torque
473 tests are only weak predictors of hamstring strain injury (van Dyk *et al.*, 2016), we decided to
474 focus on *isometric* MVC quadriceps and hamstring torque at optimal knee strength angles
475 (optimal torque-joint angle relationship, see below) to avoid further fatiguing the participants.
476 The torque signal was interfaced with an acquisition system (AcqKnowledge, Biopac Systems,
477 Santa Barbara, USA) for analogue-to-digital conversion and sampled at a frequency of 2 kHz.
478 The participant was seated in an upright position and securely fastened with inextensible straps
479 at the chest and waist while the arms were held crossed above the chest. The tibiofemoral
480 epicondyle was aligned with the lever arm rotation axis, and the lever arm shin pad was
481 strapped to the leg, 2 cm above the centre of the lateral malleolus. A Velcro strap secured the
482 distal thigh just above the knee. The hip joint angle was set to 85° (180° = supine position) in
483 order to analyse the knee flexor muscle group at a sprint specific angle associated with the
484 late swing phase of sprinting (Guex *et al.*, 2012). Participants were instructed to maximally
485 extend and flex their leg to measure knee range of motion. Quadriceps MVC was measured
486 at 80° knee flexion (0° = full knee extension), as this is the optimal joint angle for peak
487 quadriceps MVC in healthy young men (Erskine *et al.*, 2009). Hamstring MVC was measured
488 at 30° knee flexion based on this being the optimal joint angle for peak hamstring MVC during
489 our pilot work. Published studies during the time of data collection used a similar angle of
490 hamstring MVC torque (Nedelec *et al.*, 2014; Kirk & Rice, 2016). This was also in line with
491 sprinting kinematics, demonstrating that maximal hamstring muscle lengths during sprinting
492 occur during the late swing phase when the knee is flexed between 30° and 45° (Thelen *et al.*,
493 2005). Prior to isometric MVC assessments, participants underwent a standardised warm up
494 consisting of 10 submaximal isokinetic leg extensions (60°·s⁻¹). Participants then performed
495 three isometric knee extension (quadriceps) and flexion (hamstring) at both joint angles (each
496 MVC lasting 2-3 s), with 60 s rest between MVC of a given muscle group. The highest MVC of

497 the three attempts for each muscle group at each angle was used for subsequent analyses.
498 Throughout the tests, participants received verbal encouragement and biofeedback (MVC
499 outputs) were projected onto a screen in front of the participant.

500 **Hamstring Muscle Voluntary Activation**

501 To measure hamstring muscle voluntary activation capacity via the interpolated twitch
502 technique, stimulation electrodes (12.5 mm x 7.5 mm self-adhesive electrodes (DJO Global,
503 California, USA) were used. The general procedure has been described elsewhere (Erskine
504 *et al.*, 2009; Erskine *et al.*, 2010; Marshall *et al.*, 2014). Briefly, the anode was placed proximal
505 to the popliteal fossa, and the cathode was placed beneath the gluteal fold and slightly medial
506 to avoid activation of the vastus lateralis. Protocols were completed with electrical stimulation
507 pads carefully taped down during the sprinting protocol and were additionally marked on the
508 skin with a permanent marker, to ensure a precise relocation for the POST and POST48 tests.
509 Stimulation was delivered by a high-voltage stimulator (DS7AH; Digitimer Ltd., Welwyn Garden
510 City, United Kingdom), and consisted of a doublet using two 240-V rectangular pulses (200 μ s
511 pulse width) with an inter-pulse duration of 10 ms (100 Hz stimulation). During each
512 experimental session, relaxed hamstring muscles were stimulated while participants were fixed
513 in the isokinetic dynamometer with the same setting for knee flexion MVC (85° hip angle, 30 °
514 knee flexion). The amplitude started with 50 mA to familiarise the participants to the stimulation
515 and was gradually increased in 20 mA increments until a plateau in doublet torque was
516 achieved. We decided to use the individual maximal stimulation (100 %) intensity despite the
517 fact that other publications used supramaximal stimulation (110-130 %) (Marshall *et al.*, 2014)
518 as we experienced lower MVC knee flexion torque output beyond 100 %. That individual
519 amplitude (162.0 \pm 17.4 mA; range: 130–200 mA) was applied during all maximal contractions
520 in the experimental session.

521 The maximal doublet stimulation was used two minutes later to elicit resting maximal doublet
522 torque in the resting state (control doublet), followed 2.5 s later by a second (superimposed)
523 doublet during an isometric knee flexion MVC. The superimposed doublet torque was always

524 calculated manually from careful selection and inspection of the respective time periods
525 compared to a normal increase in voluntary torque. Voluntary activation was calculated
526 according to the following equation:

$$527 \quad VA (\%) = [1 - (\textit{superimposed doublet torque} / \textit{control doublet torque})]$$

528 **Surface Electromyography and Antagonist Muscle Co-activation**

529 Surface electromyographic (sEMG) activity was recorded from the vastus lateralis and BF_{LH} to
530 determine the extent of antagonist muscle co-activation during MVCs of the respective muscle
531 group. Previous reports have shown that the vastus lateralis (Reeves *et al.*, 2004) and BF_{LH}
532 (Kellis & Baltzopoulos, 1999) are representative muscles for the quadriceps femoris and
533 hamstring muscle group, respectively. This procedure has been reported in detail elsewhere
534 (Reeves *et al.*, 2004). Briefly, once the muscles were identified via palpation, and the skin
535 surface was shaved and cleaned with 70% ethanol, two bipolar Ag-AgCl surface electrodes
536 with an inter-electrode distance of 2 cm (Noraxon duel sEMG electrode, Noraxon, Scottsdale,
537 USA) were placed along the sagittal axis over the muscle belly at 33% of the respective muscle
538 length from the distal end [according to SENIAM guidelines (Hermens *et al.*, 2000)] and one
539 reference electrode (Ambu Blue, Ambu, Copenhagen, Denmark) was positioned over the
540 medial tibial condyle. The exact location of the electrodes were marked on the participant's
541 skin with a permanent marker to ensure precise electrode repositioning for the following
542 assessments.

543 Surface EMG signals were sampled at 2000 Hz (Biopac Systems, Santa Barbara, USA) and
544 then band-pass filtered between 10–500 Hz (AcqKnowledge, Biopac Systems, Santa Barbara,
545 USA). Surface EMG activity of both the agonist and antagonist muscles were analysed by
546 calculating the root mean square of the sEMG signal of a 500-ms epoch around peak MVC.
547 To compare BF_{LH} sEMG activity at all three time points, BF_{LH} sEMG of the hamstring MVC at
548 30° was normalised to the evoked maximum compound muscle action potential (M-wave) of
549 the BF_{LH} (see below). Antagonist muscle co-activation (i.e. quadriceps activation during

550 hamstring MVC at 30° knee flexion, or hamstring activation during quadriceps MVC at 80°
551 knee flexion) was calculated with the following formula (where EMG_{max} is the maximum sEMG
552 of the antagonist muscle when acting as an agonist at the same knee joint angle):

$$553 \quad \textit{Antagonist muscle co-activation} = \frac{EMG_{antagonist}}{EMG_{max}} \times 100$$

554 Torque signals, electrical stimuli, and sEMG activity were displayed on a computer screen,
555 interfaced with an acquisition system (AcqKnowledge, Biopac Systems, Santa Barbara, USA)
556 used for analogue-to-digital conversion. Due to technical issues, co-activation data were
557 available for hamstring $n = 12$; and quadriceps $n=10$.

558 **Hamstring Muscle Maximal Compound Muscle Action Potential**

559 The hamstring muscle group was stimulated with single square wave twitch pulses (200 μ s
560 duration) using the same electrical stimulator and stimulating electrodes, as described above.
561 While the participant sat resting on the isokinetic dynamometer with the knee angle set at 30°
562 knee flexion, compound muscle action potentials (M -waves) were evoked with 10 to 20 mA
563 incremental amplitudes until a maximal M -wave (M_{max}) was achieved. The average amplitude
564 necessary to evoke a maximal M -wave was 166.8 ± 19.8 mA; range: 130–210 mA). The
565 maximal M -wave was defined as the mean peak-to-peak sEMG response from the three
566 highest observed M -waves. Due to inter-individual differences in subcutaneous fat and the
567 (re)location of small sEMG electrodes over a relatively large muscle belly, sEMG amplitude is
568 notoriously variable (Araujo *et al.*, 2000). To reduce this inter- and intra-individual variability,
569 we normalised absolute BF_{LH} sEMG to the individual's BF_{LH} maximal M -wave, determined at
570 each testing session (Lanza *et al.*, 2018). Due to technical issues, data of BF_{LH} sEMG
571 normalised to maximal M -wave was only available for $n = 13$.

572 **Torque-frequency Relationship**

573 The torque-frequency relationship was determined by stimulating the hamstring muscle group
574 with single square wave twitch pulses (200 μ s duration) at 1, 10, 15, 20, 30, 50 and 100 Hz for

575 1 s each in a random order and with 15 s rest between each stimulation, using the same
576 electrical stimulator and stimulating electrodes (and location), as described above. The
577 stimulus intensity for 100-Hz stimulation was the amplitude necessary to elicit ~20 % knee
578 flexion MVC torque at PRE, and the same amplitude was used for the same test at POST and
579 48POST. The absolute peak torque at each frequency was normalised to the peak torque at
580 100 Hz for each time point (PRE, POST and POST48).

581 **Delayed Onset Muscle Soreness**

582 Using a visual analogue scale that consisted of a 100 mm line (scale 0-10 cm; 0 cm=no
583 soreness; 10 cm= unbearably painful), in conjunction with both a three-repetition bilateral squat
584 (predominantly to determine quadriceps femoris muscle soreness) (Scott & Huskisson, 1979)
585 and lunges (predominantly to determine hamstring muscle soreness), participants rated their
586 perceived lower limb muscle soreness along the muscle length immediately after each
587 movement. Muscle soreness was also measured by recording the force required to elicit
588 tenderness at nine fixed sites on the skin over the quadriceps (distal, central and proximal
589 locations of the three superficial quadriceps heads, vastus lateralis, vastus medialis and rectus
590 femoris) and six sites on the hamstrings (distal, central and proximal locations of both BF_{LH}
591 and the medial hamstrings), which were previously marked with a permanent marker to ensure
592 the same measuring position PRE, POST and POST48. At each site, a gradually increasing
593 force was applied by the investigator with an algometer (FPK/FPN Mechanical Algometer,
594 Wagner Instruments, Greenwich, USA) with a maximum of 10 kg/cm². Lying in the prone
595 position with the hip and knee fully extended and muscles relaxed, the participant was asked
596 to indicate when the sensation of pressure changed to discomfort, and the force at that point
597 was recorded (Newham *et al.*, 1987).

598 **Ultrasound**

599 Architectural parameters of the BF_{LH} were assessed using B-mode ultrasound imaging.
600 Participants were in the prone position with the hip and knee fully extended and muscles

601 relaxed. The BF_{LH} was investigated, as this muscle is the most commonly injured hamstring
602 muscle in team sports, particularly during sprinting (Ekstrand *et al.*, 2011). Longitudinal and
603 cross-sectional panoramic ultrasound images of the right BF_{LH} were obtained (Philips EPIQ 7
604 Ultrasound System, Bothel, USA). The linear transducer (5-18 MHz; aperture 38.9 mm) was
605 carefully placed on the skin with transmission gel and BF_{LH} was scanned (i) longitudinally from
606 its distal (=0% muscle length) to proximal (=100% muscle length) myotendinous junction along
607 a line drawn with a permanent marker to mark the central pathway between the medial and
608 lateral aspects of the muscle (incorporating the intra-muscular aponeurosis (Evangelidis *et al.*,
609 2014) (Figure 2A); and (ii) cross-sectionally at 20, 40, 60 and 80% along the total muscle length,
610 measured on the skin using a tape measure (Seca, Hamburg, Germany) (Figure 2B).

611 All images were analysed offline (ImageJ, version 1.51s, National Institutes of Health,
612 Bethesda, USA). Two images for each of the four cross-sectional points were recorded and
613 the image of best quality was used to calculate BF_{LH} muscle volume. The volume of the
614 muscular portion between every two consecutive scans was calculated with the following
615 equation:

$$616 \quad \text{Volume} = \frac{1}{3} * d * (a + \sqrt{(ab) + b})$$

617 Where *a* and *b* are the anatomical cross-sectional areas of the muscle of two consecutive
618 cross-sectional scans and *d* is the interval distance between the cross-sectional area
619 measurements. The volume of the entire muscle was calculated by summing up all of the inter-
620 scan muscular volumes (Erskine *et al.*, 2016). Two full-length sagittal images were then
621 recorded to allow for the measurement of resting BF_{LH} muscle fascicle length and pennation
622 angle, which were both assessed in 3 fascicles at 50 % of the total length of BF_{LH}. This point
623 was measured offline (ImageJ). A comparison between offline (sagittal ultrasound) and tape
624 measurements of the total BF_{LH} length revealed a very high correlation ($R^2=0.958$, $P<0.001$).
625 Fascicle length was measured by tracing the fascicular path from the upper aponeurosis to the
626 intra-muscular aponeurosis of the BF_{LH}. Muscle fascicle pennation angle was determined as

627 the angle between the muscle fascicular paths and their insertion into the intra-muscular
628 aponeurosis. The mean of the three measurements for each parameter were used to
629 determine both fascicle length and pennation angle of the BF_{LH} muscle. PCSA was calculated
630 by dividing BF_{LH} volume by its fascicle length. During the time of data collection, a similar
631 methodological approach was published elsewhere (Seymore *et al.*, 2017). One longitudinal
632 image of one participant in the present study was not analysed due to low image quality.
633 Ultrasound scans and image analysis was performed by the same investigator.

634 **Kinematic and Kinetic Data**

635 Three-dimensional kinematic and kinetic data were synchronously collected at 500 Hz using
636 an eight-camera motion analysis system (Qqus 300+; Qualisys, Gothenburg, Sweden),
637 together with a ground-embedded force plate (90 x 60 cm, 9287B; Kistler Holding, Winterthur,
638 Switzerland) at 1,500 Hz. The data were filtered with a digital dual low-pass Butterworth filter
639 at 20 Hz for motion and 60 Hz for force, as previously described (Verheul *et al.*, 2017).
640 Retroreflective markers (12 mm diameter) were placed on anatomical landmarks on the right
641 leg and pelvis, as previously described (McClay & Manal, 1999). One standing static and two
642 functional motion calibration trials were recorded of the participant PRE, POST and POST48.
643 For the static trial, participants stood with their feet approximately shoulder width apart and
644 knees fully extended. This static trial determined local coordinate systems, the location of joint
645 centres, and the foot, shank, thigh, and pelvis segment lengths of each participant. The
646 functional trials defined functional hip joint centres (Schwartz & Rozumalski, 2005) and knee
647 joint axes (Robinson & Vanrenterghem, 2012). Kinematic data were tracked using Qualisys
648 Track Manager Software (Qualisys). Data processing and analysis were undertaken in
649 Visual3D (C-Motion, Germantown, MD). To examine any changes between the time points,
650 joint angles were normalised relative to the static trial of the accompanying time point for
651 minimising the influence of potential slightly different marker positions between the trials.
652 Lower extremity 3D joint angles and angular velocities were calculated using an X-Y-Z Cardan
653 angle rotation sequence. Investigated variables included peak knee and hip angles, as well as

654 range of motion and time during stance and swing phase of the treadmill run, for all three
655 planes, were calculated as described in previous studies (Verheul *et al.*, 2017).

656 **Motorised Treadmill Run**

657 Participants ran on a motorised treadmill (HP Cosmos Pulsar; Nussdorf, Germany) for 30 s at
658 4.17 m s⁻¹ (0° incline), as high-speed running was of interest. The selected speed was based
659 on pilot testing, which demonstrated that 15 km/h was the fastest speed on a motorized
660 treadmill where the participants still felt comfortable. Motion analysis data were recorded for
661 the last 10 s of the run and data were analysed for at least 6 consecutive strides. Peak knee
662 and hip angle data, for all three planes, were calculated (i) between the initial contact and
663 terminal stance of foot; and (ii) between initial and terminal swing phase. The touchdown of
664 the foot during the treadmill run was determined from the kinematic data as occurring at the
665 local minima and the toe-off during running as the local maxima of the vertical velocity of the
666 head of the fifth metatarsal marker on the foot (Maiwald *et al.*, 2009).

667 **Blood Samples**

668 A 10 mL blood sample was drawn from an antecubital vein in the forearm and collected into a
669 serum vacutainer (BD Vacutainer systems, Plymouth, UK). The blood samples were obtained
670 at each time point and left at 22-24°C for 30 min to allow clotting, and then kept on ice when
671 necessary. Serum samples were centrifuged at 1,300 g for 15 min at 4°C. All samples were
672 then aliquoted into 1.5 mL microcentrifuge tubes [Axygen (Corning), New York, USA] and
673 stored at -80°C until subsequent analysis (see below).

674 **Serum Interleukin-6 (IL-6) Concentration**

675 Serum samples were assayed for IL-6 concentration using commercially available human IL-
676 6 enzyme linked immunosorbent assay kits (Quantikine®, R&D systems, Minneapolis, MN,
677 USA) according to the manufacturer's instructions. The intensity of the colour produced after
678 20 min was measured with a Thermo Multiskan Spectrum microplate reader (Thermo Fisher

679 Scientific. Waltham, MA. USA) at 450 nm and values were calculated with Excel 365 (Microsoft,
680 v. 365, USA) by generating a four-parameter logistic curve fit. The minimum detectable dose
681 of human IL-6 was 0.70 pg/mL.

682 **Serum Creatine Kinase Activity**

683 Creatine kinase (CK) activity was assayed using a commercially available CK assay
684 (Catachem Inc., Connecticut, NE, USA), as described in detail elsewhere (Owens *et al.*, 2015).
685 Briefly, 10 μ L blood serum were loaded onto a 96-well UV plate. The CK reaction reagent and
686 diluent (Catachem) were prepared as per the manufacturer's instructions and added to the
687 samples and the change in absorbance monitored continuously over 20 min in a Thermo
688 Multiskan Spectrum plate reader (Thermo Fisher Scientific. Waltham, MA. USA) at a
689 wavelength of 340 nm.

690 **Capillary Blood Lactate Concentration**

691 Capillary blood samples were taken from the finger-tip via a Safety-Lancet Extra 18G needle
692 (Sarstedt; Nümbrecht, Germany) at rest before and immediately after the repeated maximal
693 sprint intervention. Blood samples were analysed within 60 seconds of collection using a
694 portable blood lactate analyser (Arkay Lactate Pro; Kyoto, Japan).

695 **Reagents, Chemicals, and Solvents for Muscle Cell Culture *in vitro***

696 Growth media used for the expansion of human muscle-derived cell populations consisted of
697 Hams F-10 nutrient mix (Lonza, Basel, Switzerland) with added L-glutamine (2.5 mM), 10%
698 heat-inactivated fetal bovine serum (hiFBS; Gibco, Thermo Fisher Scientific, Altincham, UK),
699 1% penicillin-streptomycin (Life Technologies, Warrington, UK), and 1% L-Glutamine (Gibco).
700 Differentiation media consisted of α -MEM (Lonza), 1% hiFBS, 1% penicillin-streptomycin, and
701 1% L-glutamine. Phosphate-buffered saline (PBS; Sigma-Aldrich) was used to wash cell
702 monolayers. Desmin polyclonal rabbit anti-human antibody (Cat# ab15200, RRID: AB_301744)
703 was used (1:200) from Abcam (Abcam, Cambridge, UK), and secondary antibody (TRITC

704 polyclonal goat anti-rabbit; Cat# A16101, RRID: AB_2534775) was used (1:200) from Fisher
705 Scientific.

706 **Muscle Biopsy Procedure**

707 Participants were instructed to avoid any strenuous exercise 48 h prior to the biopsy procedure.
708 Biopsies from the vastus lateralis muscle were obtained under local anaesthesia from each
709 participant, using the Weil-Blakesley conchotome technique as described previously
710 (Baczynska *et al.*, 2016). The conchotome was inserted through the incision into the muscle
711 belly to obtain the 134 ± 82.7 mg muscle biopsy.

712 **Extraction of Human Muscle-Derived Cells**

713 The muscle biopsies analysed in this study were isolated (Blau & Webster, 1981; Crown *et al.*,
714 2000) and cultured (Owens *et al.*, 2015), as reported previously. Briefly, biopsy samples were
715 transferred with precooled growth media from the muscle biopsy suite to the sterile tissue
716 culture hood (Kojair Biowizard Silverline class II hood; Kojair, Vippula, Finland) within 40 min
717 and muscle biopsy samples were washed three times with ice-cold PBS (0.01 M phosphate
718 buffer, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4, in dH₂O). Visible fibrous and fat tissue was
719 removed using sterile scissor and forceps. Samples were cut in small pieces (1 mm³) and
720 digested in 5 ml of trypsin-EDTA for 15 min on a magnetic stirring platform at 37°C to dissociate
721 muscle cells. The trypsinisation process was repeated twice. Supernatant derived following
722 each treatment was collected and pooled with hiFBS at a concentration of 10% of the total
723 volume to inhibit further protease activity. Cell supernatant was centrifuged at 450 g for 5 min.
724 Supernatant was discarded and the cell pellet was resuspended in growth media and plated
725 on a T25 cm² culture flask (Corning, Life Sciences, New York, USA) for cell population
726 expansion. Culture flasks were previously coated with a 2 mg/l porcine gelatin solution (90–
727 110 g, Bloom; Sigma-Aldrich, Dorset, UK) to support cell adhesion.

728 **Expansion of Extracted Cells**

729 The medium was refreshed on the fourth day after the extraction procedure and subsequently
730 every 48 h following two brief washes with PBS. Cells were incubated in a HERAcell 150i CO₂
731 Incubator (Thermo Scientific, Cheshire, UK). T25 cm² culture flasks reached 80% confluence
732 after approximately 10 days and were passaged via trypsinisation. Cells were counted using
733 Trypan Blue exclusion and re-plated on gelatinised T75 cm² culture flasks (Nunc, Roskilde,
734 Denmark). The cells were expanded until passage 3 and then frozen in GM with 10% dimethyl
735 sulfoxide (DMSO) in liquid N₂ as a cryopreservant. All experiments were performed on cells
736 between passages 3 and 6 to avoid potential issues of senescence (Foulstone *et al.*, 2004).

737 **Characterization of Human Muscle-Derived Cells**

738 The mixed population of human skeletal muscle-derived myoblasts and fibroblasts were
739 characterised by immunofluorescent staining for the detection of desmin expressed by
740 myoblasts (desmin positive) and non-myoblasts (desmin negative) to determine the
741 percentage of myoblasts and fibroblasts. Previous investigations have determined that the
742 non-myoblast (desmin negative) fraction is highly enriched in fibroblasts, with up to 99 % of
743 this fraction being fibroblasts (Stewart *et al.*, 2003; Agle *et al.*, 2013). Therefore, non-
744 myoblasts (desmin negative) were referred to here as fibroblasts.

745 Grohmann *et al.* (2005) showed that passaging does not change the percentage of myoblast
746 and fibroblasts and all populations were included for analysis. Monolayers were incubated with
747 25% [vol/vol methanol in Tris-buffered saline (10 mM Tris-HCl, pH 7.8, 150 mM NaCl)], 50%
748 and 100% for 5-min to fix the cells and stored at 4°C wet in Tris-buffered saline until further
749 analysis. Fixed monolayers were permeabilised and blocked for 2 h with 5% goat serum and
750 0.2% Triton X-100 in Tris-buffered saline, prior to staining. Cells were incubated overnight at
751 4°C with anti-Desmin antibody (1:200). After overnight incubation, the primary antibody was
752 removed, and the cells were washed three times with Tris-buffered saline. Secondary TRITC
753 polyclonal goat anti-rabbit antibody (1:200) was then applied and incubated for 2 h at 4°C.

754 Fluorescent images were captured using live imaging microscopy (Leica DMB 6000;
755 Magnification x 10.5) and analysed via ImageJ cell counter plug-in. A total of four randomly
756 selected areas per well were analysed per individual.

757 **Wound-Healing Assay, Migration and Differentiation Analysis**

758 For the wound healing assay, 100,000 cells/ml were seeded in gelatinised six-well plates (Nunc,
759 Roskilde, Denmark). Cells were expanded as described above until cell monolayers reached
760 a confluent state, Growth media was removed, monolayers were washed with PBS and cells
761 were damaged by a vertical scrape with a 1-ml pipette tip (width of the wound area, *mean* ±
762 *S.E.M.*: 896.4 ± 21.24 µm), as previously reported by our group (Owens *et al.*, 2015; Brown *et*
763 *al.*, 2017). PBS was aspirated, damaged cell monolayers were washed twice with PBS to
764 remove cell debris and 2 ml differentiation media was added. Monolayers were imaged with a
765 live imaging microscopy (Leica) for the analysis of cell migration immediately, 24h, 48h and
766 seven days after the wound healing assay. Additional 500 µl differentiation media was added
767 in each well at day 4. TIF images were exported from Leica Application Suite and loaded as
768 TIF image stacks in ImageJ with a cell counter plug-in. Cells in the outer and inner segments
769 were then counted (Figure 4A).

770 Damaged monolayers were imaged at two sites per well in the wound site immediately post-
771 damage (0 h). These image coordinates were tracked and stored to allow subsequent
772 monitoring of the same sites on the wound to reduce this experimental bias. Myotube formation
773 was captured on day 7. Captured images were exported as TIF image files, and analysed in
774 ImageJ. Muscle cell fusion/differentiation was assessed as the number of myotubes per field
775 of view and myotube hypertrophy was determined by measuring myotube length, myotube
776 diameter (the average of three diameters along the length of the myotube), myotube area
777 (determined by manually drawing a line around the sarcolemma of each myotube) and total
778 myotube number via ImageJ cell counter plug-in. By normalising the pixel scale to the micron
779 scale of each image, a value expressed as µm² was obtained for myotube area. A total of two
780 images per well were analysed.

781 **Creatine Kinase (CK) Activity**

782 At 0 and 10 days following the mechanical scrape insult, CK activity was analysed as a marker
783 of muscle cell differentiation/fusion into myotubes. Cell monolayers were first lysed with 300
784 μ l/well of 50 M Tris-mes and 1% Triton-X 100, pH 7.8 (TMT). Ten microliters of TMT cell lysate
785 was loaded in duplicate wells on a 96-well UV plate and used for quantification of CK activity.
786 The CK reaction reagent and diluent (Catachem, Bridgeport, CT, USA) were prepared as per
787 the manufacturer's instructions as previously described (please see subsection Serum
788 Creatine Kinase).

789 **Statistical Analysis**

790 One-way repeated-measures analysis of variances (ANOVA)s were performed to determine
791 whether there was a significant main effect for time (within subject factor) for the following
792 dependent variables: MVC torque, voluntary muscle activation, antagonist muscle co-
793 activation, muscle soreness (for squat lunge via measured via visual analogue scale as well
794 as algometer), rating of perceived exertion, CK activity, IL-6 concentration, and for kinematics
795 data (hip and knee angle parameters). MVC torque data were analysed for interactions and
796 main effects for muscle group and time using two-way mixed design ANOVAs, comparing
797 differences between muscle groups across 3-time points; PRE, POST, and POST48. For
798 within test comparisons, either, independent t-tests, or one-way ANOVAs were used where
799 appropriate. For the torque-frequency relationship, normalised torque at each frequency was
800 analysed using a two-way repeated measures ANOVA, with stimulation frequency (1-100 Hz)
801 and time (PRE, POST and POST48) as the within-groups independent variables. Post-hoc
802 one-way repeated measures ANOVAs were used to determine if the normalised torque at each
803 frequency differed between time points. Bivariate correlations were used to analyse the relation
804 between architectural parameters of the BF_{LH} (volume, fascicle length, fascicle pennation angle
805 and PCSA) and fatigue biomarkers (relative MVC loss normalised to PRE MVC), serum CK
806 activity, serum IL-6 concentration, muscle soreness, knee joint range of motion or changes in
807 range of motion during treadmill running.

808 Bivariate correlations were used to analyse the relation between myoblast:fibroblast ratio and
809 quadriceps and hamstring MVC, migration dynamics (total cell migration, cell proportion of
810 inner to outer segment) and myotube formation (total myotube number, myotube length,
811 average diameter, myotube area and CK activity) of the muscle stem cells. Standard guidelines
812 concerning violation of the sphericity assumption to adjust the degree of freedom of the F-test
813 by the Huynh-Feldt epsilon if epsilon is greater than 0.75 and to use the more stringent
814 Greenhouse-Geisser adjustment if epsilon is less than 0.75 were followed. Results were
815 expressed as mean \pm SD, unless otherwise stated, with statistical significance set at $P < 0.05$.
816 All MVC data were analysed with AcqKnowledge software 4.4 (Biopac-Systems Inc., Goleta,
817 USA) and SPSS 23 Software (IBM Inc., Armonk, NY: IBM Corp) was used for statistical
818 analysis. Occasional missing data are reflected in the reported degrees of freedom.

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822 **DISCLOSURES**

823 No conflicts of interest, financial or otherwise, are declared by the authors.

824 **AUTHOR CONTRIBUTION**

825 R.M.E, P.B., B.D., C.E.S., and M.L. conceived and designed the research; P.B., S.T., M.S.,
826 M.C., J.A.S., and S.O.S performed the experiments; P.B., S.T. and R.M.E analyzed the data;
827 R.M.E., P.B, M.L. and C.E.S. interpreted the results of the experiments; P.B. prepared the
828 figures; P.B. drafted the manuscript; R.M.E., P.B., S.T., M.S., M.L., B.D., C.E.S., M.C., J.A.S.,
829 and S.O.S edited and approved the final version of the manuscript.

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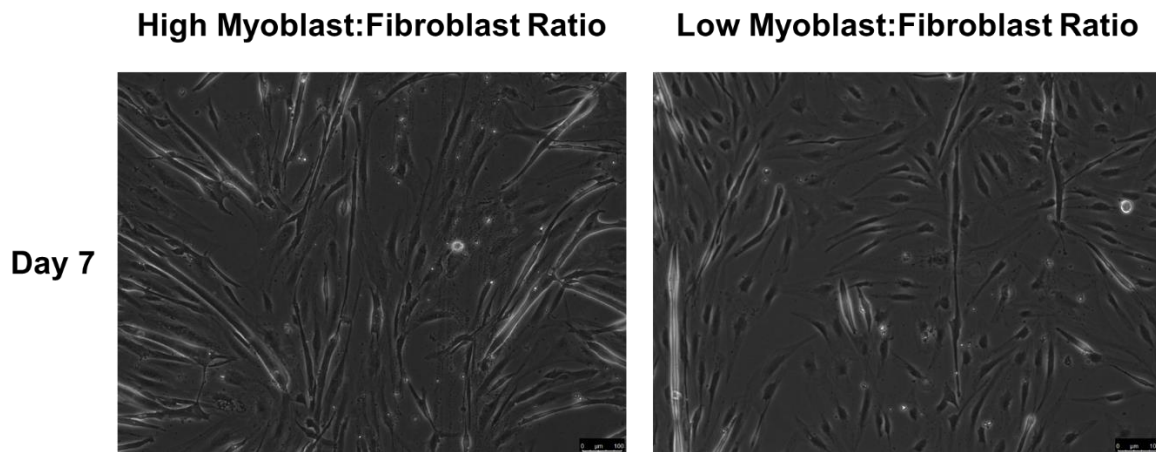
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1098 **SUPPLEMENTARY INFORMATION**



1099
1100 **Figure supplement 5** Representative images for muscle cell differentiation with a high
1101 myoblast:fibroblast ratio (2.4; left) and with a low myoblast:fibroblast ratio (0.3; right) at day seven.
1102 Magnification is $\times 10.5$, and scale bar is $100 \mu\text{m}$.

1103

1104

1105 **Table supplement 4** Architectural parameters of biceps femoris long head (mean \pm SD).

| Muscle length [cm] | Fascicle length [cm] | PCSA [cm ²] | Volume [cm ³] | Fascicle Pennation Angle [°] |
|-----------------------|-------------------------|----------------------------|------------------------------|------------------------------------|
| 27.86 \pm 2.13 | 7.94 \pm 1.38 | 23.4 \pm 4.62 | 182.2 \pm 29.5 | 12.7 \pm 2.77 |

Fascicle length at 50% of BF_{LH} muscle length; PCSA – Physiological cross-sectional area.

1106

1107 **Table supplement 6** *Linear regression analysis between the myoblast:fibroblast ratio and fusion*
1108 *parameters at day 7 and CK activity at day 10 in vitro.*

| | R² | F-Test | P Value |
|------------------------------------|----------------------|----------------|----------------|
| Total myotubes [n] | 0.345 | F(1,10) = 5.27 | 0.045* |
| Myotube Length [μm] | 0.339 | F(1,10) = 5.13 | 0.047* |
| Myotube Diameter [μm] | 0.387 | F(1,10) = 5.56 | 0.031* |
| Myotube Area [μm^2] | 0.404 | F(1,10) = 6.79 | 0.026* |
| CK activity [mU/mL] | 0.409 | F(1,10) = 6.23 | 0.034* |

* significant ($P < 0.05$);

1109