

1 **Association of ACTN3 R577X but not ACE I/D gene variants with elite rugby**
2 **union player status and playing position**
3

4 **Authors;** S. M. Heffernan¹, L. P. Kilduff², R. M. Erskine^{3,4}, S. H. Day¹, J. S. McPhee⁵, G. E.
5 McMahon^{1,6}, G. K. Stebbings¹, J. P. H. Neale¹, S. J. Lockey¹, W. J. Ribbans⁷, C. J. Cook⁸, B.
6 Vance⁹, S. M. Raleigh⁷, C. Roberts^{10,11}, M. A. Bennett², G. Wang¹², M. Collins¹³, Y. P.
7 Pitsiladis¹² & A. G. Williams^{1,4}

8 **Affiliations;** ¹MMU Sports Genomics Laboratory, Manchester Metropolitan University, Crewe, UK, ²A-STEM,
9 College of Engineering, Swansea University, Swansea, UK, ³Research Institute for Sport & Exercise Sciences,
10 Liverpool John Moores University, Liverpool, UK, ⁴Institute of Sport, Exercise and Health, University College
11 London, London, UK, ⁵School of Healthcare Science, Manchester Metropolitan University, Manchester, UK,
12 ⁶Northern Ireland Sports Institute, Newtownabbey, Belfast, UK, ⁷Division of Sport, Exercise and Life Science,
13 University of Northampton, Northampton, UK, ⁸School of Sport, Health and Exercise Sciences, Bangor University,
14 Bangor, UK, ⁹Institute of Cardiovascular & Medical Sciences University of Glasgow, Glasgow, UK, ¹⁰Medical and
15 Scientific Department, South African Rugby Union, Cape Town, South Africa, ¹¹Discipline of Sports Science,
16 Faculty of Health Sciences, University of Kwazulu-Natal, Durban, South Africa, ¹² Centre for Sport and Exercise
17 Science and Medicine (SESAME), University of Brighton, Brighton, UK, ¹³MRC/UCT Research Unit for Exercise
18 Science and Sports Medicine, University of Cape Town (UCT), Cape Town, South Africa.

19 **Authors contributions;** Listed alphabetically: MC, SD, RE, SH, LK, YP, AW and GW conceived and designed the
20 study. CC, MC, SD, RE, SH, LK, SL, GMc, JMc, JN, GR, SR, WR, GS, BV, AW and GW contributed to data
21 collection. SH and AW analysed data and drafted the manuscript. All authors contributed to interpretation of data,
22 revised the article critically for important intellectual content and approved the final version of the manuscript.

23 **Running head:** ACE and ACTN3 gene variant frequency in elite rugby athletes

24 **Corresponding author;** S. M. Heffernan¹ MMU Sports Genomics Laboratory, Manchester Metropolitan
25 University, Crewe Green Road, Crewe, UK, CW1 5DU, email: shane.heffernan@stu.mmu.ac.uk, telephone:
26 01612475456

27 **Abstract**

28 We aimed to quantify the *ACE* I/D and *ACTN3* R577X (rs1815739) genetic variants in elite rugby
29 athletes (rugby union and league), compare genotype frequencies to controls and between
30 playing positions. The rugby athlete cohort consisted of 507 Caucasian men, including 431 rugby
31 union athletes that for some analyses were divided into backs and forwards and into specific
32 positional groups: front five, back row, half backs, centers and back three. Controls were 710 Caucasian
33 men and women. Real-time PCR of genomic DNA was used to determine genotypes using
34 TaqMan probes and groups were compared using Chi-square and odds ratio (OR) statistics.
35 Correction of p-values for multiple comparisons was according to Benjamini-Hochberg. There
36 was no difference in *ACE* I/D genotype between groups. *ACTN3* XX genotype tended to be
37 underrepresented in rugby union backs (15.7%) compared to forwards (24.8%; P=0.06).
38 Interestingly, the 69 back three players (wings and full backs) in rugby union included only six XX genotype
39 individuals (8.7%), with the R allele more common in the back three (68.8%) than controls (58.0%; $\chi^2=6.672$,
40 P=0.04; OR=1.60) and forwards (47.5%; $\chi^2=11.768$, P=0.01; OR=2.00). Association of *ACTN3* R577X with
41 playing position in elite rugby union athletes suggests inherited fatigue resistance is more prevalent in
42 forwards while inherited sprint ability is more prevalent in backs, especially wings and full backs. These
43 results also demonstrate the advantage of focusing genetic studies on a large cohort within a single sport,
44 especially when intra-sport positional differences exist, instead of combining several sports with varied
45 demands and athlete characteristics.

46 **Key words:** α -actinin-3, angiotensin converting enzyme, athlete genetics, RugbyGene project

47

48 **Introduction**

49 Rugby is an intermittent team sport comprised of two similar but differing codes, rugby league (RL) and rugby
50 union (RU). Both codes consist of diverse playing positions, each with different physiological, anthropometric
51 and technical attributes (8, 10, 20, 27) including two distinct sub-groups in each code: forwards and backs.
52 Recently, global positioning system (GPS) tracking and time-motion analysis have been used to estimate the
53 physical demands of rugby athletes and compare forwards and backs during high-level match play (8, 20, 27).
54 In RU, backs travelled 12% greater total distance (6545 m versus 5850 m), achieved maximum speeds 16%
55 faster ($30.4 \text{ km}\cdot\text{h}^{-1}$ versus $26.3 \text{ km}\cdot\text{h}^{-1}$) and engaged in over four times (58% versus 13%) high-
56 intensity running activities ($>5.0 \text{ m}\cdot\text{s}^{-1}$), as a proportion of total activity (8, 27) compared to
57 forwards. These data suggest a more sprint-oriented metabolic demand in backs compared to
58 forwards. Furthermore, due to the complexities of forward play, forwards performed sixfold
59 more (9.9%) high-intensity static exertion activities (rucks, mauls, scrums and line-outs) than
60 backs (1.6%) and spent 19.8% more time running above 80% of their maximal speed (8, 27,
61 respectively). This implies that forwards, although often of higher body mass, (14) are more
62 likely to benefit from fatigue-resistant physiological qualities than backs. Accordingly, Deutsch
63 *et al* (10) showed that forwards had a notably higher work-to-rest ratio than backs (1:7 and 1:22,
64 respectively). Given that the roles of backs and forwards differ significantly in terms of
65 physiological demands, these differences may be reflected in distinct genetic characteristics (18).
66 Elite RL athletes cover similar total distances ($\sim 7000 \text{ m}$ versus $\sim 5000 \text{ m}$; backs versus forwards,
67 respectively) and have similar anthropometric characteristics to RU athletes (20). Players regularly transfer
68 between RL and RU codes so investigating both codes (combined and separately) for their genetic
69 characteristics is justified.

70

71 The two most studied gene variants in exercise genomics (*ACE* I/D and *ACTN3* R577X
72 polymorphisms) have recently been considered in meta-analyses. Ma *et al* (23) reported that

73 *ACE* II genotype was associated with physical performance (odds ratio (OR) 1.23), especially
74 endurance performance (OR 1.35). Furthermore, *ACTN3* RR genotype was associated with speed
75 and power performance (OR 1.21; 23), supported elsewhere (2). More extensive information
76 regarding *ACE* I/D and *ACTN3* R577X polymorphisms is available (13, 26). Due to differences
77 in physical characteristics between rugby athletes and the general population and the diverse
78 physiological demands within rugby, these genetic markers could predispose athletes to success
79 or specific roles at the elite level.

80

81 One recent paper examined *ACE* I/D genotype frequency distribution in young, non-elite RU
82 athletes. *ACE* I/D genotype frequencies did not differ between forwards and backs, with no
83 control group included (5). The same group (4) also investigated *ACTN3* R577X in 102 young
84 male RU athletes and reported no association, despite some tendencies for the R allele to be
85 more frequent in backs or subgroups of backs. Studying elite athletes would be better able to
86 answer the question whether these genetic variants are associated with elite status and playing
87 position in rugby.

88

89 Therefore, the purpose of the present study was to investigate whether elite rugby athletes in the
90 RugbyGene project (18) and a control group differed in terms of *ACE* I/D and *ACTN3* R577X
91 genotype distribution, and whether athletes in specialized playing positions similarly differed. It
92 was hypothesized that the *ACTN3* R allele and the *ACE* I allele would be more frequent in rugby
93 athletes than controls. It was further hypothesized that *ACTN3* XX and *ACE* II genotypes would
94 be underrepresented in RU backs compared to forwards, due to differences in overall work-to-
95 rest ratio and differing requirements for high maximum speed.

96 **Methods**

97 **Participants**

98 Ethical approval was granted by Manchester Metropolitan University (MMU), University of
99 Glasgow, University of Cape Town and Northampton University ethics committees and complies
100 with the Declaration of Helsinki. As part of the RugbyGene project, elite Caucasian male rugby
101 athletes (n=507; mean (standard deviation) height 1.85 (0.07) m, mass 101 (14) kg, age 29 (7)
102 years) including 71.2% British, 17.2% South African, 7.1% Irish and 4.5% of other nationalities
103 were recruited, having given written informed consent. Caucasian controls (61% male; n=710;
104 height 1.73 (0.10) m, mass 74 (13) kg, age 29 (16) years) included 89.6% British, 8.9% South
105 African, 0.7% Irish and 0.8% of other nationalities. Athletes were considered elite if they had
106 competed regularly (>5 matches) since 1995 in the highest professional league in the UK, Ireland
107 or South Africa for RU and the highest professional league in the UK for RL. Of the RU athletes,
108 53.4% had competed at international level for a “High Performance Union” (Regulation 16,
109 worldrugby.org) and 38.5% of RL had competed at international level. International status was
110 confirmed as of 1 January 2015. Athletes were taller and heavier ($p<0.0005$) but not older
111 ($p=0.871$) than controls.

112 **Procedures**

113 *Sample collection*

114 Blood (~70% of all samples), saliva (~25%) or buccal swab samples (~5%) were obtained via
115 the following protocols. Blood was drawn from a superficial forearm vein into an EDTA tube
116 and stored in sterile tubes at -20°C until processing. Saliva samples were collected into Oragene
117 DNA OG-500 collection tubes (DNA Genotek Inc., Ontario, Canada) according to the
118 manufacturer’s protocol and stored at room temperature until processing. Sterile buccal swabs
119 (Omni swab, Whatman, Springfield Mill, UK) were rubbed against the buccal mucosa of the

120 cheek for approximately 30 s. Tips were ejected into sterile tubes and stored at -20°C until
121 processing.

122 *DNA isolation and genotyping*

123 DNA isolation and genotyping were performed in the MMU, University of Glasgow, University
124 of Cape Town (DNA isolation only) and University of Northampton laboratories. There are some
125 differences between protocols summarized below; however, there was 100% agreement among
126 reference samples genotyped in the three genotyping centers, i.e. Glasgow, Northampton and
127 MMU laboratories. The majority of samples were processed and genotyped in the MMU
128 laboratory. Genotype calling was successful for both variants in all samples.

129

130 At MMU and Glasgow, DNA isolation was performed using the QIAamp DNA Blood Mini kit
131 and standard spin column protocol, following the manufacturer's instructions (Qiagen, West
132 Sussex, UK). Briefly, 200 µL of whole blood/saliva, or one buccal swab, was lysed, incubated,
133 the DNA washed and the eluate containing isolated DNA stored at 4°C. In Cape Town, DNA was
134 isolated from whole blood using a different protocol (22). Briefly, samples were lysed,
135 centrifuged, the DNA washed and samples stored at -20°C. Genotyping of DNA isolated in Cape
136 Town was performed in Glasgow. At Northampton, DNA was isolated from whole blood using
137 Flexigene kits (Qiagen). Briefly, samples were lysed, DNA precipitated and washed, with
138 samples stored at -20°C.

139 *Genotyping*

140 Genotyping in the Glasgow laboratory was performed on *ACTN3* (rs1815739) and an *ACE* tag
141 SNP (rs4341) in perfect linkage disequilibrium with *ACE* I/D in Caucasians (15). Briefly, 10 µL
142 Genotyping Master Mix (Applied Biosystems, Paisley, UK), 1 µL SNP-specific TaqMan assay
143 (Applied Biosystems), 6 µL nuclease-free H₂O and 3 µL DNA solution (~9 ng DNA) were

144 added per well. In the Northampton laboratory, genotyping was performed for *ACTN3* R577X
145 (rs1815739) by combining 10 μ L of Genotyping Master Mix, 8 μ L H₂O, 1 μ L assay mix with 1
146 μ L of purified DNA (~10 ng). In both laboratories, PCR was performed using a StepOnePlus
147 real-time detector (Applied Biosystems). Briefly, denaturation began at 95°C for 10 min, with 40
148 cycles of incubation at 92°C for 15 s then annealing and extension at 60°C for 1 min. Initial
149 analysis was performed using StepOnePlus software version 2.3 (Applied Biosystems). There
150 was 100% agreement within duplicates of all samples.

151
152 At MMU, samples were genotyped for *ACTN3* R577X (rs1815739) by combining 5 μ L
153 Genotyping Master Mix, 4.3 μ L H₂O, 0.5 μ L assay mix and 0.2 μ L of purified DNA (~9 ng), for
154 samples derived from blood and saliva. For DNA derived from buccal swabs, 5 μ L Genotyping
155 Master Mix was combined with 3.5 μ L H₂O, 0.5 μ L assay mix and 1 μ L DNA solution (~9 ng
156 DNA). Either a Chromo4 real-time system (Bio-Rad, Hertfordshire, UK) or a StepOnePlus was
157 used. Briefly, denaturation began at 95°C for 10 min, with 40 cycles of incubation at 92°C for 15
158 s then annealing and extension at 60°C for 1 min. Initial genotyping analysis was performed
159 using Opticon Monitor software version 3.1 (Bio-Rad) or StepOnePlus software version 2.3.
160 Duplicates of all samples were in 100% agreement. For *ACE* I/D at MMU, 5 μ L of Genotyping
161 Master Mix, 1.55 μ L H₂O, 0.9 μ L of I and D allele-specific probes and 0.38 μ L of *ACE* primer
162 111, 112, 113 (sequences below) were combined with 0.5 μ L DNA solution (~23 ng DNA) per
163 well for blood and saliva. For DNA derived from buccal cells, primer and probe volumes were
164 identical but 0.05 μ L H₂O and 2 μ L DNA solution (~18 ng DNA) were used. Similarly, in the
165 Northampton laboratory, *ACE* I/D was genotyped by combining 11 μ L of Genotyping Master
166 Mix, 2 μ L of I and D probes, 2 μ L of *ACE* primer 111, 112, 113 and 4 μ L DNA solution (~40 ng

167 DNA). Either a Chromo4 real-time system or a StepOnePlus was used. Briefly, there were 50
168 cycles of denaturation at 92°C for 15 s then annealing and extension at 57°C for 1 min. Initial
169 analysis was performed using Opticon Monitor 3.1 software or StepOnePlus software version
170 2.3. Again, there was 100% agreement within duplicates of all samples.

171 *Primers and probes*

172 For rs1815739 and rs4341, the appropriate TaqMan assay was used (Applied Biosystems). For
173 the direct ACE I/D assay, three primers (150 nM each) and probes (VIC, 150 nM and FAM, 75
174 nM; 21) were used;

175 Primer ACE111: 5'-CCCATCCTTTCTCCCATTTCTC-3'

176 Primer ACE112: 5' -AGCTGGAATAAAATTGGCGAAAC-3'

177 Primer ACE113: 5' -CCTCCCAAAGTGCTGGGATTA-3'

178 I Allele specific probe (VIC-ACE100): VIC-5' AGGCGTGATACAGTCA-3'-MGB

179 D Allele specific probe (FAM-ACE100): FAM-5'TGCTGCCTATACAGTCA-3'-

180 MGB

181 **Positional groups**

182 To assess genotype and allele frequencies within the RU group, athletes were allocated to sub-
183 groups; forwards (props, hookers, locks, flankers, number eights) and backs (scrum halves, fly
184 halves, centers, wings, full backs). Also, due to diverse physiological demands within RU (8,
185 27), athletes were further divided into positional groups according to their similar movement
186 patterns (8) front five (props, hookers, locks), back row (flankers, number eights), half backs
187 (scrum halves, fly halves), centers and back three (wings and full backs). Comparisons between
188 positions were not performed for the RL cohort due to low statistical power when it was
189 subdivided.

190 **Data analysis**

191 SPSS for Windows version 19 (SPSS Inc., Chicago, IL) software was used to conduct Pearson's
192 Chi-square (χ^2) tests to compare genotype and allelic frequencies between athletes and controls,
193 and between positional subgroups. For *ACTN3* and *ACE*, 26 and 16 tests, respectively, were
194 subjected to Benjamini-Hochberg (BH; 6) corrections to control false discovery rate and
195 corrected probability values are reported. Where appropriate, OR was calculated to estimate
196 effect size. Alpha was set at 0.05.

197 **Results**

198 All genotype data for athletes and controls were in Hardy-Weinberg equilibrium. There were no
199 differences in genotype frequencies within the athlete or control groups according to nationality.
200 For *ACE* I/D, there were no differences between all athletes (RU and RL combined) and controls in genotype
201 ($\chi^2=1.117$, $P=0.83$), between RU or RL and controls, nor between playing sub-groups for RU (Table 1).
202 Furthermore, for *ACTN3* R577X there were no genotype differences between controls and all athletes
203 ($\chi^2=1.645$, $P=0.44$), RL ($\chi^2=1.829$, $P=0.44$) or RU ($\chi^2=0.216$, $P=0.33$). However, when considering RU playing
204 position, the X allele was overrepresented in forwards (52.5%) compared to backs (37.8%, $\chi^2=8.128$, $P=0.02$;
205 OR=1.49, 95%CI=1.13-1.96, $P=0.004$) and controls (42%, $\chi^2=6.217$, $P=0.02$; OR=1.25, 95%CI=1.02-1.54,
206 $P=0.033$; Table 1 & Figure 1A). Similarly, there was a tendency ($P=0.023$ before BH correction) of the XX
207 genotype to be overrepresented in forwards (24.8%) compared to backs (15.7%, $\chi^2=5.193$, $P=0.08$; OR=1.77,
208 95%CI=1.09-2.89, $P=0.022$) and controls (18.3%, $\chi^2=7.582$, $P=0.08$), with no difference between backs and
209 controls ($\chi^2=3.043$, $p=0.37$).

210

211 Interestingly, the 69 back three athletes (wings and fullbacks) included only six individuals (8.7%) of XX
212 genotype which differed from the forwards (24.8%; $\chi^2=11.082$, $P=0.05$; OR=3.46, 95%CI=1.43-8.34, $P=0.006$)
213 and tended to differ from the combined half backs and centers group (19.8%; $\chi^2=4.151$, $P=0.08$; OR=2.59,
214 95%CI=1.00-6.74, $P=0.049$). Likewise, the R allele distribution was greater in the back three (68.8%) than the

215 controls (58.0%; $\chi^2=6.672$, $P=0.02$; OR=1.60, 95%CI=1.09-2.33, $P=0.014$), forwards (47.5%; $\chi^2=11.768$,
216 $P=0.01$; OR=2.00, 95%CI=1.34-2.99, $P=0.0007$) and the other backs (58.2%; $\chi^2=4.173$, $P=0.05$; OR=1.59,
217 95%CI=1.02-2.48, $P=0.042$; (Figure 1 **B**).

218 **Discussion**

219 The present study is the first to show a genetic association with elite athlete status in rugby
220 union. We found associations for the *ACTN3* R577X polymorphism but not for *ACE* I/D, thus
221 rejecting our hypotheses regarding *ACE* I/D. Furthermore, no difference was observed for the
222 *ACTN3* R577X genotype or allele distribution between all athletes and controls, thus rejecting
223 the hypothesis that differences would exist between non-athletes and all players as a single
224 cohort. Similarly, there were no differences between the RU, RL and control groups when
225 playing position was not considered. However, as hypothesized, in RU backs compared to
226 forwards there was a lower proportion of XX genotype and X allele, which probably reflects the
227 greater need for speed generation in backs and more sustained activity in forwards. The small
228 cohort of RL athletes means that comparisons between playing positions are not feasible until the
229 cohort increases substantially.

230

231 *ACTN3* R577X

232 The most remarkable finding of the present study was the low frequency of the XX genotype
233 among the back three RU athletes (8.7%), approaching although not as low as the frequency
234 observed in elite sprinters (25, 31). The XX genotype is present in ~18% of Caucasians (Table 1)
235 and indicates absence of the α -actinin-3 protein (3, 24). Absence of α -actinin-3, a protein almost
236 exclusively expressed in fast twitch skeletal muscle fibers, could hinder back three (wing and full
237 back) sprint ability. R allele carriers have a greater proportion of type II and IIx fibers and larger

238 relative surface area per IIX fiber than XX carriers (1, 7, 30). Furthermore, Seto *et al* (29)
239 recently showed the likely mechanism for this genotype-phenotype association is via the
240 calcineurin muscle fiber remodeling pathway. They found greater calcineurin activity (which
241 induces slow myogenic programming and a shift towards oxidative phenotype) in α -actinin-3
242 knockout mice (KO) and humans (*ACTN3* 577XX genotype) due to preferential binding of α -
243 actinin-2 (upregulated in the absence of α -actinin-3) to the fast fiber-specific calsarcin-2 (an
244 inhibitor of calcineurin). This could explain the advantage of R allele carriers over α -actinin-3
245 deficient XX individuals for high velocity contractions – particularly important for back three
246 RU players. While backs and forwards previously showed similar fiber type proportions (19),
247 these older data are arguably not relevant to modern rugby athletes, given their changed physical
248 characteristics in recent years (14). Skeletal muscle fiber type proportions are unknown in
249 contemporary elite RU athletes who now compete in a more popular, fully professional sport and
250 complete much higher training loads than previously. Recent *in vivo* data also show that R allele
251 carriers exhibit greater muscle volume and maximal power output (11, 17). While RU forwards
252 show greater maximal power, backs are able to generate greater power relative to body mass
253 ($\text{W}\cdot\text{kg}^{-1}$; 9), which corresponds with the greater R allele frequency in the backs and especially
254 the back three players. These data, plus evidence that type II fibers are larger and more powerful
255 per unit volume than type I (15), suggest the R allele would benefit back three rugby athletes for
256 muscle power and fast fiber characteristics - which supports our findings (Table 1 and Figure 1).

257

258 Arguably, the higher propensity for aerobic enzyme activity (porin, COX IV, hexokinase, citrate
259 synthase, succinate dehydrogenase and β -hydroxyacyl CoA dehydrogenase; 28, 29) and greater
260 force recovery after fatigue observed in α -actinin-3 deficient mice (28), could indicate that XX

261 genotype humans might have a greater capacity for recovery from fatiguing exercise - a trait
262 which would benefit forwards with their more sustained match play intensity and necessity for
263 quick recovery. The shorter rest periods for forwards compared with backs (work to rest ratios
264 1:7.4 and 1:21.8, respectively; 10) indicates that greater fatigue resistance would be particularly
265 beneficial for forwards. Moreover, the greater calcineurin activity in XX homozygote humans
266 and approximately threefold increase in calcineurin activity and distance run after endurance
267 training in KO mice (29), further support the notion that forwards would benefit from a greater
268 fatigue resistance, especially with exposure to extensive training. These data are consistent with
269 our observation that forwards exhibit higher XX genotype and lower R allele frequencies than
270 backs and controls (Table 1).

271
272 When considering many sports simultaneously, team sport athlete status showed no association
273 with *ACTN3* R577X genotype (12). However, due to a relatively small number of athletes (205)
274 with mixed status (56.6% elite) from a range of sports (ice hockey, handball, soccer, etc.), that is
275 perhaps not surprising. While combining cohorts from different sports can boost sample size and
276 theoretically increase statistical power, if an association does not exist in all sports, or even in all
277 athletes within a particular sport due to positional differences, one would be less likely to detect
278 an association. The positional differences identified within the present study demonstrate the
279 value of studying a large sample from a single sport and, in the absence of detailed physiological
280 data (often difficult to obtain from large numbers of elite athletes), provides a viable alternative
281 for future genetic research involving team sport athletes.

282

283 *ACE I/D*

284 The current study reports no difference between rugby athletes and controls or any positional
285 subgroups for *ACE* I/D. This lack of association contrasts with a recent meta-analysis where the
286 *ACE* I allele was associated with physical performance (23). However, Ma et al. also reported
287 that specialized distance/endurance athletes showed the strongest association with the I allele
288 (OR 1.35). Given the mixed metabolic nature of rugby, a comparable association in the present
289 study was less likely. Furthermore, the importance of *ACE* I/D remains controversial in the
290 literature, with no associations reported in other isolated team sports such as elite European
291 soccer (16) and non-elite RU (5). These prior data, in conjunction with our current findings in a
292 larger study that also considers playing position, suggest that *ACE* I/D plays little role in
293 performance of team sport athletes. *ACE* I/D genotype-athlete phenotype associations are more
294 likely to exist in specialized endurance athletes (26).

295

296 *Effect size and future applications*

297 Odds ratios were calculated to estimate the likelihood that individuals with the advantageous
298 genotype/allele become an elite RU athlete in a specific position. The *ACTN3* XX genotype was
299 almost twice (OR=1.77) as common in forwards than backs, which suggests α -actinin-3 deficient
300 individuals are more suited to forward play. Furthermore, forwards were over three times
301 (OR=3.46) more likely to be XX genotype than the back three athletes, while the remaining
302 backs (centers and halves) were over twice as likely to show the α -actinin-3 deficient genotype
303 than the back three (OR=2.59). These data suggest the *ACTN3* R577X polymorphism shows
304 potential to contribute to position-specific player profiling within RU when combined with other
305 genetic and physiological data in the future. In contrast, the *ACE* I/D polymorphism (OR ~1)
306 does not show equivalent potential in rugby.

307

308 While the present cohort size is large compared to previous single sport genetic analyses, when
309 the cohort was subdivided into playing position, the numbers were reduced so enlargement of
310 our cohort and replication would be welcome. Accordingly we continue to recruit elite RU and
311 RL players in the RugbyGene project, so will steadily become better able to investigate genetic
312 aspects of specific demands within rugby. To conclude, the present study revealed position-
313 specific genetic variation in elite RU athletes for *ACTN3* R577X. The R allele was an advantage
314 for backs, particularly the back three. Moreover, the current results do not support *ACE* I/D as a
315 genetic marker for rugby performance, showing no differences between athletes and controls or
316 positional subgroups. This study demonstrates the value of single sport cohorts and the need for
317 large sample sizes when conducting gene association studies in sport. Future objectives of the
318 RugbyGene project within the broader Athlome project include investigating whether genetic
319 variants associated with excellence in other sports are similarly associated in the multifaceted
320 sport of rugby.

321 **Acknowledgements**

322 The authors wish to thank all athletes, their respective scientific support staff and the control
323 participants for their time and willingness to participate in this research. We would like to thank
324 Louis El Khoury for his technical assistance at the University of Northampton and Ben Davies for
325 assistance in participant recruitment.

326 **Conflicts of interest**

327 No conflicts of interest.

328 **Twitter**

329 Follow the RugbyGene project at @RugbyGeneStudy

330 **References**

- 331 1. **Ahmetov II, Druzhevskaya AM, Lyubaeva EV, Popov DV, Vinogradova OL, and**
332 **Williams AG.** The dependence of preferred competitive racing distance on muscle fibre type
333 composition and ACTN3 genotype in speed skaters. *Exp Physiol* 96: 1302-1310, 2011.
- 334 2. **Alfred T, Ben-Shlomo Y, Cooper R, Hardy R, Cooper C, Deary IJ, Gunnell D, Harris**
335 **SE, Kumari M, Martin RM, Moran CN, Pitsiladis YP, Ring SM, Sayer AA, Smith GD,**
336 **Starr JM, Kuh D, and Day IN.** ACTN3 genotype, athletic status, and life course physical
337 capability: meta-analysis of the published literature and findings from nine studies. *Hum Mutat*
338 32: 1008-1018, 2011.
- 339 3. **Beggs AH, Byers TJ, Knoll JH, Boyce FM, Bruns GA, and Kunkel LM.** Cloning and
340 characterization of two human skeletal muscle alpha-actinin genes located on chromosomes 1
341 and 11. *J Biol Chem* 267: 9281-9288, 1992.
- 342 4. **Bell W, Colley JP, Evans WD, Darlington SE, and Cooper SM.** ACTN3 genotypes of
343 rugby union players: distribution, power output and body composition. *Ann Hum Biol* 39: 19-27,
344 2012.
- 345 5. **Bell W, Colley JP, Gwynne JR, Glazier P, Evans WD, and Darlington SE.** ACE ID
346 genotype and leg power in rugby union players. *J Sports Med Phys Fit* 50: 350-355, 2010.
- 347 6. **Benjamini Y, and Hochberg Y.** Controlling the false discovery rate: a practical and powerful
348 approach to multiple testing. *J Royal Stat Soc* 57: 289-300, 1995.
- 349 7. **Broos S, Malisoux L, Theisen D, Francaux M, Deldicque L, and Thomis MA.** Role of
350 alpha-actinin-3 in contractile properties of human single muscle fibers: a case series study in
351 paraplegics. *PLoS One* 7: 8, 2012.
- 352 8. **Cahill N, Lamb K, Worsfold P, Headey R, and Murray S.** The movement characteristics of
353 English premiership rugby union players. *J Sports Sci* 31: 229-237, 2012.
- 354 9. **Crewther B, Kilduff L, Cook C, Cunningham D, Bunce P, Bracken R, and Gaviglio C.**
355 Scaling strength and power for body mass differences in rugby union players. *J Sport Med Phys*
356 *Fit* 52: 27-32, 2012.
- 357 10. **Deutsch MU, Kearney GA, and Rehrer NJ.** Time - motion analysis of professional rugby
358 union players during match-play. *J Sports Sci* 25: 461-472, 2007.
- 359 11. **Erskine RM, Williams AG, Jones DA, Stewart CE, and Degens H.** The individual and
360 combined influence of ACE and ACTN3 genotypes on muscle phenotypes before and after
361 strength training. *Scand J Med Sci Sport* 24: 642-664, 2013.
- 362 12. **Eynon N, Banting LK, Ruiz JR, Cieszczyk P, Dyatlov DA, Maciejewska-Karlowska A,**
363 **Sawczuk M, Pushkarev VP, Kulikov LM, Pushkarev ED, Femia P, Stepto NK, Bishop DJ,**
364 **and Lucia A.** ACTN3 R577X polymorphism and team-sport performance: A study involving
365 three European cohorts. *J Sci Med Sport* 17: 102-106, 2013.
- 366 13. **Eynon N, Hanson ED, Lucia A, Houweling PJ, Garton F, North KN, and Bishop DJ.**
367 Genes for elite power and sprint performance: ACTN3 leads the way. *Sports Med* 43: 803-817,
368 2013.
- 369 14. **Fuller CW, Taylor AE, Brooks JH, and Kemp SP.** Changes in the stature, body mass and
370 age of English professional rugby players: a 10-year review. *J Sports Sci* 31: 795-802, 2013.
- 371 15. **Gilliver SF, Degens H, Rittweger J, Sargeant AJ, and Jones DA.** Variation in the
372 determinants of power of chemically skinned human muscle fibres. *Exp Physiol* 94: 1070-1078,
373 2009.

- 374 16. **Gineviciene V, Jakaitiene A, Tubelis L, and Kucinskas V.** Variation in the ACE,
375 PPARGC1A and PPARA genes in Lithuanian football players. *Eur J Sport Sci* 14: S289-S295,
376 2014.
- 377 17. **Gómez-Gallego F, Santiago C, González-Freire M, Muniesa CA, Del Valle MF, Perez**
378 **M, Foster C, and Lucia A.** Endurance performance: genes or gene combinations? *Int J Sports*
379 *Med* 30: 66-72, 2009.
- 380 18. **Heffernan SM, Kilduff LP, Day SH, Pitsiladis YP, and Williams AG.** Genomics in rugby
381 union: A review and future prospects. *Eur J Sport Sci* 1-9, 2015.
- 382 19. **Jardine MA, Wiggins TM, Myburgh KH, and Noakes TD.** Physiological characteristics
383 of rugby players including muscle glycogen content and muscle fibre composition. *S Afr Med J*
384 73: 529-532, 1988.
- 385 20. **Johnston RD, Gabbett TJ, and Jenkins DG.** Applied sport science of rugby league. *Sports*
386 *Med* 44: 1087-1100, 2014.
- 387 21. **Koch W, Latz W, Eichinger M, Ganser C, Schömig A, and Kastrati A.** Genotyping of the
388 angiotensin I-converting enzyme gene insertion/deletion polymorphism by the TaqMan method.
389 *Clin Chem* 51: 1547-1549, 2005.
- 390 22. **Lahiri DK, and Nurnberger JI.** A rapid non-enzymatic method for the preparation of
391 HMW DNA from blood for RFLP studies. *Nucleic Acids Res* 19: 5444-5445, 1991.
- 392 23. **Ma F, Yang Y, Li X, Zhou F, Gao C, Li M, and Gao L.** The association of sport
393 performance with ACE and ACTN3 genetic polymorphisms: A systematic review and meta-
394 analysis. *PLoS One* 8: e54685, 2013.
- 395 24. **Mills M, Yang N, Weinberger R, Vander Woude DL, Beggs AH, Eastale S, and North**
396 **K.** Differential expression of the actin-binding proteins, α -actinin-2 and-3, in different species:
397 implications for the evolution of functional redundancy. *Hum Mol Genet* 10: 1335-1346, 2001.
- 398 25. **Niemi A-K, and Majamaa K.** Mitochondrial DNA and ACTN3 genotypes in Finnish elite
399 endurance and sprint athletes. *Eur J Hum Genet* 13: 965-969, 2005.
- 400 26. **Puthuchery Z, Skipworth JRA, Rawal J, Loosemore M, Van Someren K, and**
401 **Montgomery HE.** The ACE gene and human performance. *Sport Med* 41: 433-448, 2011.
- 402 27. **Roberts SP, Trewartha G, Higgitt RJ, El-Abd J, and Stokes KA.** The physical demands
403 of elite English rugby union. *J Sport Sci* 26: 825-833, 2008.
- 404 28. **Seto JT, Chan S, Turner N, MacArthur DG, Raftery JM, Berman YD, Quinlan KG,**
405 **Cooney GJ, Head S, Yang N, and North KN.** The effect of alpha-actinin-3 deficiency on
406 muscle aging. *Exp Gerontol* 46: 292-302, 2011.
- 407 29. **Seto JT, Quinlan KGR, Lek M, Zheng XF, Garton F, MacArthur DG, Hogarth MW,**
408 **Houweling PJ, Gregorevic P, Turner N, Cooney GJ, Yang N, and North KN.** ACTN3
409 genotype influences muscle performance through the regulation of calcineurin signaling. *J Clin*
410 *Invest* 123: 4255-4263, 2013.
- 411 30. **Vincent B, De Bock K, Ramaekers M, Van den Eede E, Van Leemputte M, Hespel P,**
412 **and Thomis MA.** ACTN3 (R577X) genotype is associated with fiber type distribution. *Physiol*
413 *Genomics* 32: 58-63, 2007.
- 414 31. **Yang N, MacArthur DG, Gulbin JP, Hahn AG, Beggs AH, Eastale S, and North K.**
415 ACTN3 genotype is associated with human elite athletic performance. *Am J Hum Gen* 73: 627-
416 631, 2003.
- 417

418 **Table 1** Genotype and allele distribution of controls and athletes divided into positional sub-groups (for RU only), presented as genotype/allele
 419 counts followed by percentage in parentheses. RL, rugby league and RU, rugby union. * Different from forwards. # Different from the Back 3.

Genotype	All athletes	RL athletes	RU athletes	Controls	Forwards	Front 5	Back row	Backs	Half Backs	Centers	Back 3
ACE											
II	108 (21.4)	18 (21.7)	92 (21.5)	113 (19.8)	49 (20.0)	36 (22.1)	13 (15.9)	43 (23.6)	14 (20.3)	14 (31.1)	15 (22.1)
ID	251 (49.7)	39 (47.0)	214 (50.1)	286 (50.0)	129 (52.7)	86 (52.8)	43 (52.4)	85 (46.7)	33 (47.8)	17 (37.8)	35 (51.5)
DD	146 (28.9)	26 (31.3)	121 (28.3)	172 (30.2)	67 (27.3)	41 (25.2)	26 (31.7)	54 (29.7)	22 (31.9)	14 (31.1)	18 (26.5)
Total	505	83	427	572	245	163	82	182	69	45	68
I allele	467 (46.3)	75 (45.2)	398 (46.6)	512 (44.7)	227 (46.3)	158 (48.5)	69 (42.1)	171 (47.0)	61 (44.2)	45 (50.0)	65 (47.8)
D allele	543 (53.7)	91 (54.8)	456 (53.4)	630 (55.3)	263 (53.7)	168 (51.5)	95 (57.9)	193 (53.0)	77 (55.8)	45 (50.0)	71 (52.2)
ACTN3											
XX	104 (20.5)	15 (18.1)	90 (20.9)	130 (18.3)	61 (24.8)	39 (23.8)	22 (26.8)	29 (15.7)	12 (17.4)	11 (23.4)	*6 (8.7)
RX	234 (46.2)	45 (54.2)	194 (45.0)	337 (47.5)	112 (45.5)	71 (43.3)	41 (50.0)	82 (44.3)	29 (42.0)	22 (46.8)	31 (44.9)
RR	169 (33.3)	23 (27.7)	147 (34.1)	#243 (34.2)	#73 (29.7)	54 (32.9)	19 (23.2)	74 (40.0)	28 (40.6)	14 (29.8)	32 (46.4)
Total	507	83	431	710	246	164	82	185	69	47	69
X allele	442 (43.5)	75 (45.2)	374 (43.4)	*597 (42.0)	234 (47.6)	149 (45.4)	85 (51.8)	*140 (37.8)	53 (38.4)	44 (46.8)	43 (31.2)
R allele	572 (56.5)	91 (54.8)	488 (56.6)	#823 (58.0)	258 (52.4)	179 (54.6)	79 (48.2)	230 (62.2)	85 (61.6)	50 (53.2)	*95 (68.8)

420

421

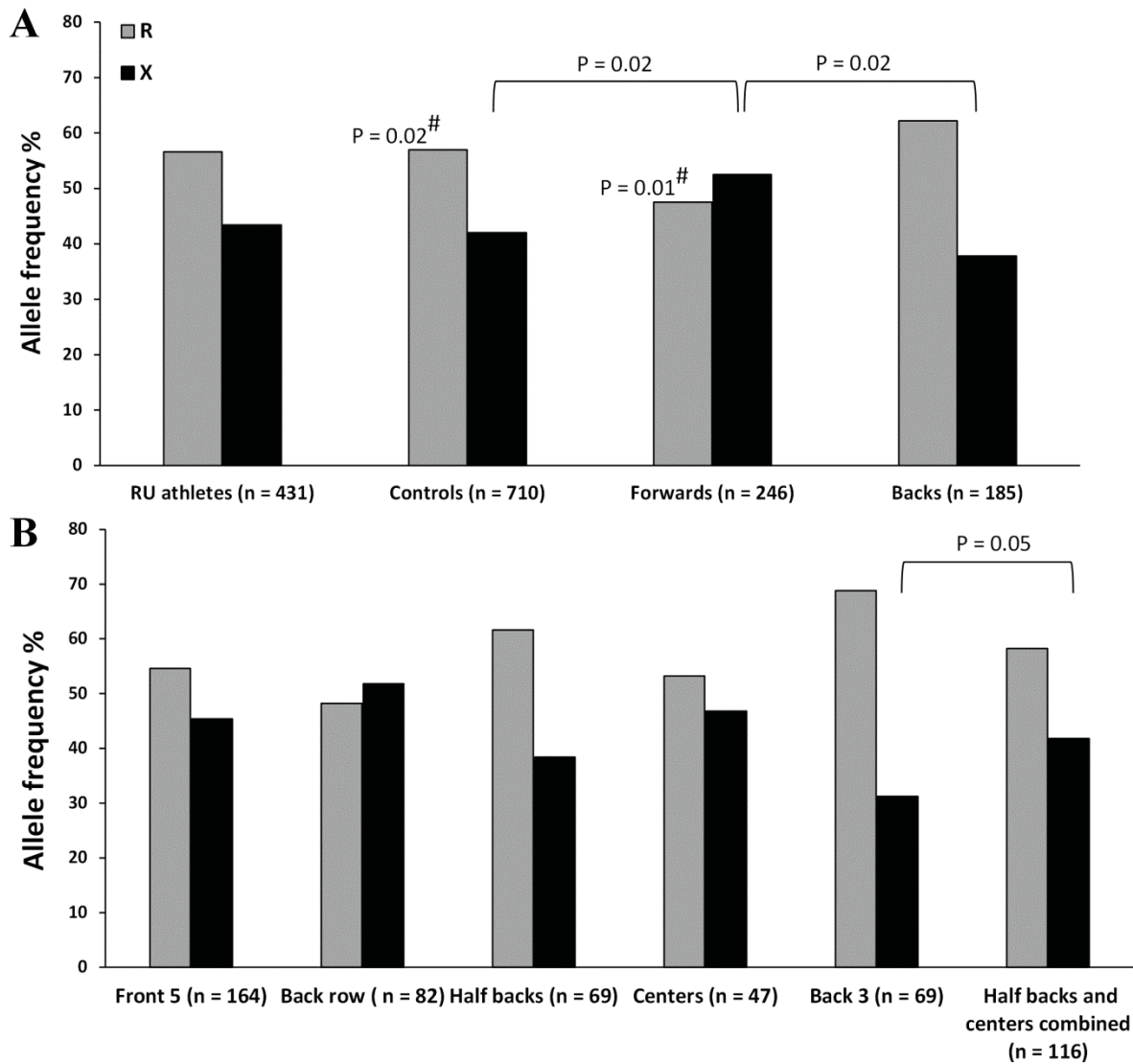


Figure 1 *ACTN3* allele frequencies. **A**, Allele frequencies of RU athletes and controls, with athletes also divided into playing sub-group (forwards and backs). # Different from the back three. **B**, allele frequencies of RU athletes divided into positional groups with the addition of the 'half backs and centers combined' group. Statistical analysis between these positional groups only compared the back three with the half backs and centers combined.