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

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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

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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

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## Introduction

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

The two most studied gene variants in exercise genomics (*ACE* I/D and *ACTN3* R577X 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$^\circ$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$^\circ$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$^\circ$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 -20ºC. 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 -20ºC.

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 H$_2$O 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 µL of Genotyping Master Mix, 8 µL $H_2O$, 1 µL assay mix with 1

146     µL 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 µL

153     Genotyping Master Mix, 4.3 µL $H_2O$, 0.5 µL assay mix and 0.2 µL of purified DNA (~9 ng), for

154     samples derived from blood and saliva. For DNA derived from buccal swabs, 5 µL Genotyping

155     Master Mix was combined with 3.5 µL $H_2O$, 0.5 µL assay mix and 1 µL 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 µL of Genotyping

161     Master Mix, 1.55 µL $H_2O$, 0.9 µL of I and D allele-specific probes and 0.38 µL of *ACE* primer

162     111, 112, 113 (sequences below) were combined with 0.5 µL 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 µL $H_2O$ and 2 µL DNA solution (~18 ng DNA) were used. Similarly, in the

165     Northampton laboratory, *ACE* I/D was genotyped by combining 11 µL of Genotyping Master

166     Mix, 2 µL of I and D probes, 2 µL of *ACE* primer 111, 112, 113 and 4 µL 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: 5ˈ-CCCATCCTTTCTCCCATTTCTC-3ˈ
176         Primer ACE112: 5ˈ -AGCTGGAATAAAATTGGCGAAAC-3ˈ
177         Primer ACE113: 5ˈ -CCTCCCAAAGTGCTGGGATTA-3ˈ
178         I Allele specific probe (VIC-ACE100): VIC-5ˈAGGCGTGATACAGTCA-3ˈ-MGB
179         D Allele specific probe (FAM-ACE100): FAM-5ˈTGCTGCCTATACAGTCA-3ˈ-
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 ($\chi^2$) 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 1**A**). 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 (W·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.

326     **Conflicts of interest**

327     No conflicts of interest.

328     **Twitter**

329     Follow the RugbyGene project at @RugbyGeneStudy

**References**

1. **Ahmetov II, Druzhevskaya AM, Lyubaeva EV, Popov DV, Vinogradova OL, and Williams AG.** The dependence of preferred competitive racing distance on muscle fibre type composition and ACTN3 genotype in speed skaters. *Exp Physiol* 96: 1302-1310, 2011.

2. **Alfred T, Ben-Shlomo Y, Cooper R, Hardy R, Cooper C, Deary IJ, Gunnell D, Harris SE, Kumari M, Martin RM, Moran CN, Pitsiladis YP, Ring SM, Sayer AA, Smith GD, Starr JM, Kuh D, and Day IN.** ACTN3 genotype, athletic status, and life course physical capability: meta-analysis of the published literature and findings from nine studies. *Hum Mutat* 32: 1008-1018, 2011.

3. **Beggs AH, Byers TJ, Knoll JH, Boyce FM, Bruns GA, and Kunkel LM.** Cloning and characterization of two human skeletal muscle alpha-actinin genes located on chromosomes 1 and 11. *J Biol Chem* 267: 9281-9288, 1992.

4. **Bell W, Colley JP, Evans WD, Darlington SE, and Cooper SM.** ACTN3 genotypes of rugby union players: distribution, power output and body composition. *Ann Hum Biol* 39: 19-27, 2012.

5. **Bell W, Colley JP, Gwynne JR, Glazier P, Evans WD, and Darlington SE.** ACE ID genotype and leg power in rugby union players. *J Sports Med Phys Fit* 50: 350-355, 2010.

6. **Benjamini Y, and Hochberg Y.** Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal Stat Soc* 57: 289-300, 1995.

7. **Broos S, Malisoux L, Theisen D, Francaux M, Deldicque L, and Thomis MA.** Role of alpha-actinin-3 in contractile properties of human single muscle fibers: a case series study in paraplegics. *PLoS One* 7: 8, 2012.

8. **Cahill N, Lamb K, Worsfold P, Headey R, and Murray S.** The movement characteristics of English premiership rugby union players. *J Sports Sci* 31: 229-237, 2012.

9. **Crewther B, Kilduff L, Cook C, Cunningham D, Bunce P, Bracken R, and Gaviglio C.** Scaling strength and power for body mass differences in rugby union players. *J Sport Med Phys Fit* 52: 27-32, 2012.

10. **Deutsch MU, Kearney GA, and Rehrer NJ.** Time - motion analysis of professional rugby union players during match-play. *J Sports Sci* 25: 461-472, 2007.

11. **Erskine RM, Williams AG, Jones DA, Stewart CE, and Degens H.** The individual and combined influence of ACE and ACTN3 genotypes on muscle phenotypes before and after strength training. *Scand J Med Sci Sport* 24: 642-664, 2013.

12. **Eynon N, Banting LK, Ruiz JR, Cieszczyk P, Dyatlov DA, Maciejewska-Karlowska A, Sawczuk M, Pushkarev VP, Kulikov LM, Pushkarev ED, Femia P, Stepto NK, Bishop DJ, and Lucia A.** ACTN3 R577X polymorphism and team-sport performance: A study involving three European cohorts. *J Sci Med Sport* 17: 102-106, 2013.

13. **Eynon N, Hanson ED, Lucia A, Houweling PJ, Garton F, North KN, and Bishop DJ.** Genes for elite power and sprint performance: ACTN3 leads the way. *Sports Med* 43: 803-817, 2013.

14. **Fuller CW, Taylor AE, Brooks JH, and Kemp SP.** Changes in the stature, body mass and age of English professional rugby players: a 10-year review. *J Sports Sci* 31: 795-802, 2013.

15. **Gilliver SF, Degens H, Rittweger J, Sargeant AJ, and Jones DA.** Variation in the determinants of power of chemically skinned human muscle fibres. *Exp Physiol* 94: 1070-1078, 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, Easteal 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. **Puthucheary 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, Easteal 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) |

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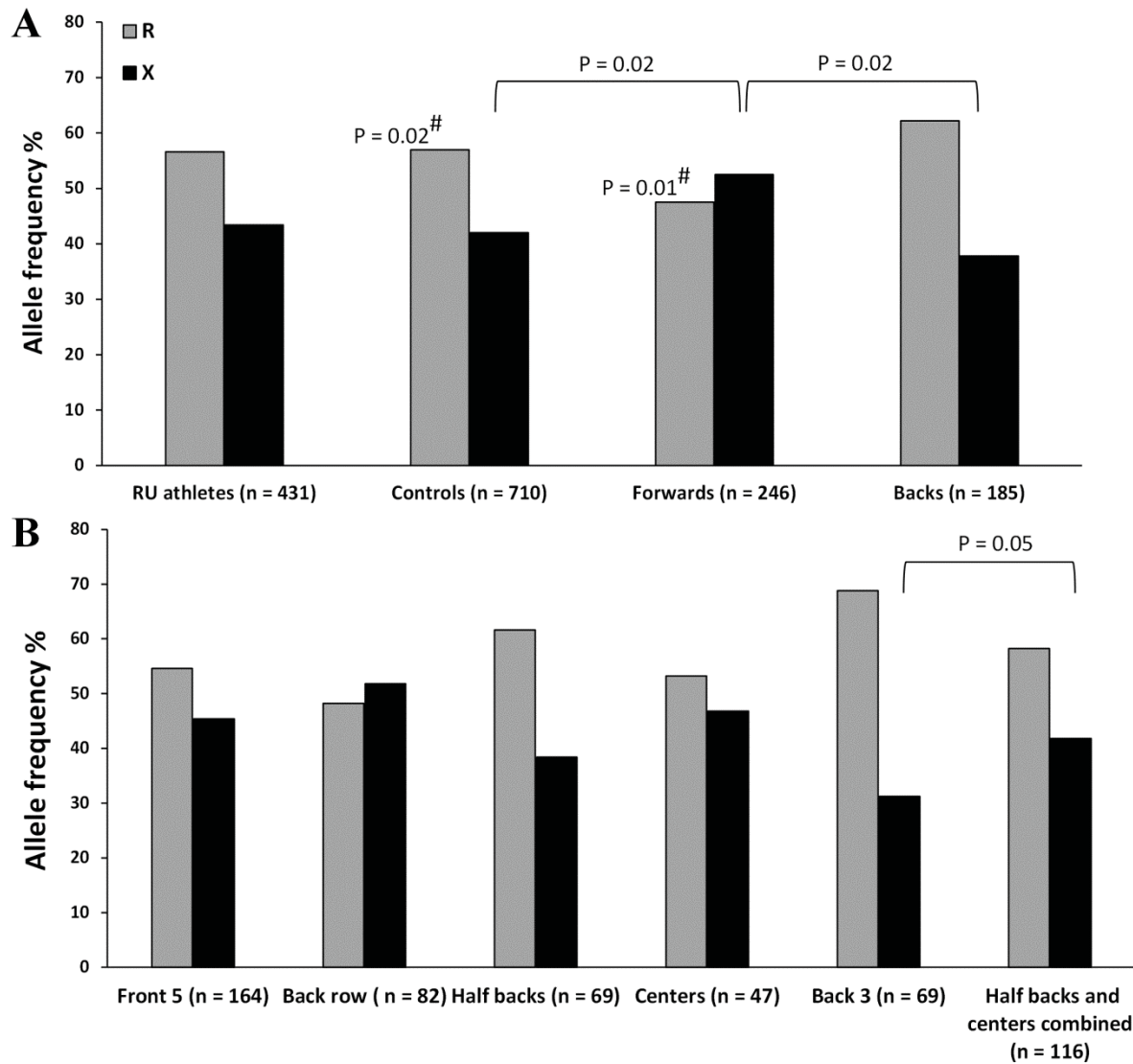
**Figure 1** *ACTN3* allele frequencies. **A,** Allele frequencies of RU athletes and controls, with athletes also divided into playing sub-group (forwards and backs). # Different from the back three. **B,** allele frequencies of RU athletes divided into positional groups with the addition of the 'half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