The MMAAS Project: An observational human study investigating the effect of Anabolic Androgenic Steroid use on gene expression and the molecular mechanism of Muscle Memory.

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115 **Structured Abstract**

116 **Objective**: It remains unknown if myonuclei remain elevated post Anabolic Androgenic
117 Steroid (AAS) usage in humans. Limited data exists on AAS induced changes in gene
118 expression.

119 **Design**: Cross-sectional/longitudinal.

120 **Setting**: University.

121 **Participants**: Fifty-six males aged 20-42.

122 **Independent Variables**: Non-resistance trained (C) or resistance trained (RT), RT-currently
123 using AAS (RT-AS), of which if AAS usage ceased for ≥18 weeks re-sampled as Returning
124 Participants (RP) or RT-previously using AAS (PREV).

125 **Main Outcome Measures**: Myonuclei per fibre and cross-sectional area (CSA) of trapezius
126 muscle fibres.

127 **Results**: There were no significant differences between C (n=5), RT (n=15), RT-AS (n=17)
128 and PREV (n=6) for myonuclei per fibre. Three of five returning participants (RP1-3) were
129 biopsied twice. Prior to visit one RP1 ceased AAS usage 34 weeks before, RP2 and RP3 ceased
130 AAS usage ≤2 weeks before and all had 28 weeks between visits. Fibre CSA decreased for
131 RP1 and RP2 between visits (7566 vs 6629 μm^2; 7854 vs 5677 μm^2) whilst myonuclei per fibre
132 remained similar (3.5 vs 3.4; 2.5 vs 2.6). Respectively these values increased for RP3 between
133 visits (7167 vs 7889 μm^2; 2.6 vs 3.3).

134 **Conclusions**: This cohort of past AAS users did not have elevated myonuclei per fibre values,
135 unlike previous research, but reported AAS usage was much lower. Training and AAS usage
136 history also varied widely amongst participants. Comparable myonuclei per fibre numbers
137 despite decrements in fibre CSA post exposure adheres with the muscle memory mechanism
138 but there is variation in usage relative to sampling date and low numbers of returning
139 participants.

140

141 **Keywords:**
142 Myonuclei, Anabolic Androgenic Steroids, Hypertrophy, Fat Free Mass, Muscle Memory,
143 Gene Expression
144

145 ## 1.0 Introduction

146 Due to conflicting data[1-7], current evidence provides no consensus[8] on the existence of muscle
147 memory by myonuclear permanency and more research is required to test this hypothesis[9-11].
148 Testosterone administration studies report dose-dependent increases in myonuclei number and
149 muscle fibre cross-sectional area (CSA) in young and elderly men[12-14]; alongside performance
150 enhancing effects in young men[15] and women[16]. However, there is a lack of longitudinal data
151 on the cessation of Anabolic Androgenic Steroid (AAS) usage on myonuclei number in
152 humans, which has implications on doping ban length in sport. In mice, although fibre CSA
153 has been shown to return to control levels 3-months post testosterone exposure, accumulated
154 myonuclei have been shown to be long lasting as the number of myonuclei remains 28% higher
155 in steroid treated mice compared to controls[17]. An observational study recruiting elite
156 powerlifters who used AAS for 4.5 ± 0.5 years but ceased usage for 8.1 ± 3.2 years[18] (Group
157 PREV, *n*=7) found significantly elevated myonuclei per fibre values in the trapezius muscle
158 (7.0 ± 1.3) compared to current AAS users (PAS, *n*=9) (5.2 ± 0.5), non-users (P, *n*=10) (4.3 ±
159 0.4) and untrained controls (U, *n*=6), potentially suggesting a retained advantage from AAS
160 usage.

161 The effects of AAS are mediated through the Androgen Receptor (AR) which modulates
162 transcription[19]. In the aforementioned study in elite powerlifters, within groups, the proportion
163 of AR containing myonuclei was significantly higher in the trapezius muscle compared to the
164 vastus lateralis for PAS, P and U and comparing groups significantly higher in P vs U and PAS

165 vs P and U[20]. This potentially indicates this muscle as superior to investigate the AR genomic
166 mode of action. The AR is expressed in whole blood[21] and thereby RNA biomarkers could aid
167 in detecting AAS doping, as similarly shown with blood doping[22-24]. However, there are limited
168 human studies[25] investigating AAS induced changes in gene expression[26].

169 Given these findings, this study aimed to longitudinally monitor current AAS users after the
170 cessation of usage and recruit past users and store muscle samples for immunohistochemical
171 (IHC) analysis and whole blood and muscle for gene expression analysis.

172 2.0 Methods

173 *2.1 Eligibility and Group Classification.*

174 Participants were male, aged 20-42 and within four groups, according to their self-reported
175 resistance training and AAS usage history (Table 1). Participants were excluded if their
176 demographics fell outside these groupings or if medical history contraindicated collection
177 procedures. Participants within RT-AS self-reporting to cease all AAS usage after their first
178 visit were re-invited for sampling if abstinence lasted for ≥18 weeks, as a previous testosterone
179 administration study in young healthy men showed that Lean Body Mass (LBM)[27] returns close
180 to baseline 5-6 months post exposure. Returning participants (RP) could conduct Post Cycle
181 Therapy (PCT)[28 29].

182 One-year withdrawal from AAS to denote past users from current users has been used
183 previously[18]. Supraphysiological dosages of testosterone were defined as self-usage of
184 intramuscular injections >100mg/week based on clinical recommendations of testosterone
185 replacement therapy (TRT) [30 31].

186 Self-reported AAS cycles, other Performance Enhancing Drugs (PEDs) and PCT protocols are
187 presented in Supplementary Digital Content Table 1. If a range were stated because an exact
188 dosage or time frame could not be recalled the median was used in AAS exposure calculations.

189 *2.2 Body composition measurements*

190 Body composition was assessed via Bioelectrical Impedance with the Tanita® BC-420MA.

191 *2.3 Muscle Biopsy*

192 All muscle biopsies were performed by an experienced Consultant Musculoskeletal
193 Radiologist. If a participant verbalised significant discomfort, the procedure was abandoned
194 immediately, and all sampling stopped. The upper part of the trapezius muscle (descending I)
195 was the chosen site of the muscle biopsy, as detailed in previous research[18 20 32]. The non-
196 dominant hand was initially examined with ultrasound (Siemens Acuson S3000™) to exclude
197 any potential coexisting pathology. A skin mark was placed at the entry point (the posterior
198 aspect of the shoulder), the area was covered with a sterile drape and sterilized with a 3 mL
199 ChloraPrep® applicator twice. The skin and overlying fascia were infiltrated with low-volume
200 local anaesthetic (Lidocaine 50 mg/5 mL) and a small skin incision was performed using a
201 sterile scalpel. Using direct ultrasound visualisation four tissue samples were collected with a
202 single use sterile 12-gauge BARD Magnum® Disposable Core Biopsy Needle via an 11-gauge
203 coaxial needle. In an alternative manner these samples were fully immersed in either Qiagen®
204 RNAlater RNA Stabilization Reagent (76106) or Qiagen® Allprotect Tissue Reagent (76405)
205 inside separate tubes, completing the first part of the biopsy. The skin incision point was
206 enlarged using a sterile scalpel and a sterile 6- or 8-gauge University College Hospital (UCH)
207 needle was inserted under ultrasound guidance. The UCH needle was rotated and closed (with
208 suction applied) four times, concluding the biopsy.

209 Muscle removed from the UCH needle was placed on a disposable freezing mould, its
210 orientation was assessed via a dissecting microscope, covered in Tissue-Tek® O.C.T.™ (Agar
211 Scientific) and immediately frozen in isopentane and transferred to -80°C for long-term
212 storage. Samples inside Qiagen® preservative were placed at 2-8°C and kept overnight after
213 being transferred to -80°C for long-term storage.

214 *2.4 Staining protocol for fibre CSA, myonuclei and satellite cells*

215 Frozen muscle sections (8 µm) were cut on a Leica CM3050S cryostat at -20°C, collected on
216 charged slides, air-dried for ≥2 hours and stored at -30°C. Muscle slides were fixed in acetone
217 for 3 minutes at -20°C. Sections were washed three times in phosphate-buffered saline (PBS)
218 for three minutes, placed inside a humidifying slide chamber with 0.5 cm of water and then
219 endogenous peroxidases were blocked for 7 minutes with 3% hydrogen peroxide in PBS at
220 room temperature. Slides were washed in PBS and blocked for 1 hour in 2.5% Bovine Serum
221 Albumin (BSA) at room temperature. Sections were incubated with a primary antibody cocktail
222 consisting of 1) Pax7 mouse (Ms) IgG1 for satellite cell identification (1:100, Concentrate,
223 Developmental Studies Hybridoma Bank (DHSB)), 2) MyHC type I BA.D5 IgG2b for an initial
224 assessment of fibre typing (1:75, Concentrate, DHSB) and 3) Rabbit (Rb) anti(α)-Dystrophin
225 for fibre borders (1:100, ab15277, Abcam) in 2.5% BSA and left overnight at 4°C inside a
226 humidifying slide chamber. The following day sections were washed in PBS and then left for
227 90 minutes at room temperature with goat (Gt) α-Ms IgG1 biotinylated secondary antibody
228 (1:1000, 115-065-205, Jackson ImmunoResearch). Sections were washed in PBS and
229 incubated for 1 hour at room temperature in a secondary antibody cocktail consisting of
230 Streptavidin, horseradish peroxidase conjugate (SA-HRP, 1:500, S-911, Invitrogen™), Gt α-
231 Rb IgG (H+L) AF488 (1:250, A-11034, Invitrogen™) and Gt α-Ms IgG2b AF647 (1:250, A-
232 21242, Invitrogen™). Sections were washed in PBS and left for 20 minutes at room

233 temperature with SuperBoost™ Tyramide Signal Amplification Alexa Fluor™ 594 (1:500,
234 B40957, ThermoFisher Scientific) in PBS, washed with PBS again and left for 10 minutes at
235 room temperature with DAPI (1:10,000, D1306, ThermoFisher Scientific) in PBS. Sections
236 were washed in PBS and mounted with Vectashield (H-1000, Vector Laboratories) or Immu-
237 Mount (9990402, ThermoFisher Scientific) and stored at 4°C.

238 *2.5 Staining protocol for fibre type and fibre CSA*.

239 The fibre typing protocol has been published elsewhere[33]. We utilised the recommendation to
240 identify pure MyHC IIX fibres.

241 *2.6 Section imaging, extraction, and quantification*.

242 Initial imaging of sections was performed on a Zeiss Imager M1 AX10 microscope using
243 associated Zeiss software. Sections deemed of sufficient quality were stored at 4°C for further
244 analysis. Sections were imaged using a digital fluorescent slide-scanner (MetaSystems V-Slide
245 Scanner) at 20X magnification. Images were visualised with MetaViewer V2.0.121, extracted
246 as individual channels and imported into MyoVision[34]. Fibre outlines, MyHC types, nuclei,
247 and Pax7-positive nuclei were detected and used to calculate fibre CSA, myonuclei/fibre,
248 satellite cell/fibre, MyHC type I, IIA, IIX proportions, and fibre type specific values. Regions
249 containing damage, longitudinal fibres or defects in staining were excluded.

250 *2.7 Blood Collection*

251 3 mL of whole blood was collected into a Tempus™ Blood RNA Tube (Life Technologies) by
252 a phlebotomist from an antecubital vein utilising a closed vacuette system a few hours prior to
253 the biopsy. Immediately after collection the tube was shaken vigorously for 10 seconds,
254 incubated at room temperature for 3 hours and stored at −80°C.

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256 *2.8 Statistical analysis and data availability.*

257 Data are presented as mean ± standard deviation unless otherwise stated. Statistical analyses
258 for age, height, weight (hereby collectively referred to as descriptive data), body composition
259 and IHC data was conducted using SPSS (v.23) with alpha level set at 0.05. Dot plots were
260 made using R version 3.6.3[35] using the tidyerse package[36]. For comparisons between groups
261 for descriptive, body composition and IHC data, only the first visit values from RT-AS were
262 used (except for RP4 who was only sampled on his second visit). A Generalized Linear Model
263 with both Linear and Gamma distributions was applied, and Akaike's Information Criterion
264 was used to select the best fitting model. For the descriptive data variables, body composition
265 measurements and IHC data, the four levels of Group were used as a predictor. The least
266 significant difference (LSD) was set as the adjustment for multiple comparisons. Pearson's
267 correlation coefficient (*r*) evaluated the correlation between myonuclei number and CSA. Raw
268 data and R code are publicly available[37].

269 **3.0 Ethical Considerations**

270 This study was ethically approved by the University of Brighton Research Ethics Committee
271 (SSCREC2016-28). Participants were recruited via word of mouth and internet advertisements,
272 provided written informed consent with potential complications discussed beforehand and did
273 not receive remuneration.

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279 **4.0 Results**

280 *4.1 Participant sampling and AAS usage*

281 Fifty-six participants visited the laboratory and consented (Figure 1). Five participants within
282 RT-AS returned for a second laboratory visit post exposure (RP1-5), four of these participants
283 (RP2, RP3, RP4 and RP5) finished exposure ≤2 weeks prior to their first visit and had 28, 28,
284 19 and 22 weeks, respectively between visits. The last recorded weekly dose of AAS used was
285 505 ± 236mg for 7.8 ± 1.8 weeks for RP2-5. RP1 used 700mg of AAS for 10 weeks, his first
286 visit was 34 weeks after exposure, and his second visit 28 weeks later.

287 Forty-three participants had at least one sample preserved for IHC (C=5, RT=15, RT-AS=17,
288 PREV=6), this includes samples from all participants first visit and the single sample collected
289 from RP4 from his second visit (Figure 1). Of those biopsied for IHC, for RT, most participants
290 were recreational lifters (*n*=13), with two participants competing in local powerlifting
291 competitions. For RT-AS, most participants were recreational lifters (*n*=13). Two had
292 competed in Men's Physique competitions and two were powerlifters, with one competing at
293 national level and the other international level. PREV were all recreational lifters.

294 For participants within RT-AS (*n*=17) who were biopsied with samples preserved for IHC, the
295 last self-reported weekly average exposure to AAS was 487 ± 304mg, lasting for 3-63 weeks
296 (median = 8) with 12 participants ceasing usage ≤2 weeks prior to sampling and 5 participants
297 respectively ceasing usage 10, 19, 34, 38, and 50 weeks prior.

298 The average lifelong length of AAS usage for participants within RT-AS (*n*=17) who were
299 biopsied with samples preserved for IHC was 1.27 ± 1.07 years. Participants within PREV
300 (*n*=6) biopsied with samples preserved for IHC previously used AAS for 3-192 weeks (median
301 of 12) had withdrawn from AAS, as defined in Table 1, for more than one year (3.5 ± 2.2 years).

302 RP1, RP3 and RP4 self-reported only using PCT compounds and no other PEDs between visits.
303 RP2 and RP5, respectively, self-reported using Ibutamoren and Clenbuterol between visits
304 (Supplementary Digital Content Table 1).

305 All returning participants kept to the same number of days training during the interval between
306 visits, however, RP3 refrained from training for a 6-week period during his 28-week interval
307 between visits (weeks 13-19) due to flu-like symptoms.

308 *4.2 Demographic and body composition data.*

309 Age, height, and weight measurements were collected from 54 participants (C=7, RT=21, RT-
310 AS=19, PREV=7) (Table 2).

311 Mass, Body Fat (%) and FFM for RP1-5 are presented in Figure 2A, B & C. FFM of RP2, RP3,
312 RP4 and RP5 decreased by 3.9 - 4.7kg between visits. FFM of RP1 decreased by 0.9kg.

313 *4.3 Immunohistochemistry.*

314 Mean fibre CSA was highest in RT-AS (8160 ± 1769µm²) (Figure 4) and this was significantly
315 higher compared to C (6477 ± 1271µm², p=0.028) but there were no significant differences
316 between the other groups (RT= 7563 ± 2072 µm², p=0.325; PREV= 7677 ± 1804 µm²,
317 p=0.550). Compared to PREV (3.7 ± 1.4) there were no significant differences between any
318 groups for myonuclei per fibre (C= 3.1 ± 0.8, p=0.285; RT= 3.4 ± 1.2, p=0.486; RT-AS= 3.3
319 ± 1.0, p=0.432) (Figure 4). Satellite cell per fibre data was omitted from one participant within
320 RT and from RP3 first visit due to being considered outliers (i.e., lower than 0.05, which would
321 be considered abnormally low for these populations). Average satellite cells per fibre were
322 similar between groups (C= 0.2 ± 0.1, RT= 0.2 ± 0.1, RT-AS= 0.2 ± 0.1 and PREV= 0.2 ± 0.2)
323 (Figure 4).

324 There was a strong positive correlation between myonuclei number and CSA ($r = 0.8$, $p<0.001$)
325 (Figure 3) and 70% of participants with > 4 myonuclei per fibre and a CSA > 8000 μm^2 had at
326 some point used AAS.

327 RP1 and RP2 respectively exhibited decreases in fibre CSA between visits (7566 vs 6629 μm^2
328 and 7854 vs 5677 μm^2) (Figure 2D) whilst their myonuclei per fibre values remained relatively
329 similar between visits (3.5 vs 3.4 and 2.5 vs 2.6) (Figure 2E). RP3 exhibited an increase in fibre
330 CSA (7167 *vs* 7889 μm^2) (Figure 2D) and myonuclei per fibre (2.6 *vs* 3.3) (Figure 2E). Satellite
331 cells per fibre decreased for RP2 between visits (0.2 vs 0.1) (Figure 2F) and increased for RP1
332 (0.2 vs 0.3) (Figure 2F).

333 For the first visit of 40 participants (C=4, RT=14, RT-AS=17, PREV=5) including the only
334 sample collected from RP4 during his second visit, there were no significant differences in
335 fibre type percentages between groups (Table 3). Data from two participants is missing (C=1,
336 RT=1) due to different image extraction settings in MyoVision and another (PREV=1) was not
337 stained with the Fibre Type staining protocol. CSA of Type IIa fibres was significantly higher
338 in RT and RT-AS than C (p=0.011 and p=0.007) and PREV (p=0.037 and p=0.025) (Table 3).
339 Type IIx CSA was significantly lower in RT than RT-AS (p=0.032) (Table 3). Myonuclei per
340 Type I and II fibres were not significantly different between groups (Table 3).

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347 **5.0 Discussion**

348 Of nineteen current AAS users recruited, only six verbalised intensions for complete removal
349 of AAS for ≥18 weeks post usage and only five were sampled on a second visit. A 3.9 - 4.7 kg
350 decrease in FFM from four returning participants who all ceased AAS usage ≤2 weeks prior to
351 their first visit with 19-28 weeks between visits corroborates with previous research showing
352 that LBM decreases post AAS usage in young[27] and older men[38]. RP1 and RP2 exhibited
353 decrements in CSA whilst myonuclei per fibre values remained relatively similar between
354 visits. Although this pattern is consistent with the myonuclear permanency model of muscle
355 memory[9-11] limited conclusions can be drawn from a low number of participants in an
356 observational study and this data should be viewed as initial longitudinal case reports.

357 An observational study[39] that recruited current (*n*=7) and past (*n*=11) AAS users, found a
358 significant difference in myonuclear domain between resistance training non-AAS (*n*=17)
359 users (1587.4 μm^2 ± 181.4 μm^2) and past AAS users (1431.0 μm^2 ± 197.4 μm^2) for Type II
360 vastus lateralis muscle fibres ($p = 0.0438$), but like this present study, did not find significant
361 differences in myonuclei per fibre values between groups. Another observational study[40],
362 recruiting current AAS users with 5-15 years of usage (*n*=10), and resistance trained non-AAS
363 users (*n*=7), did show significantly higher nuclei per Type I fibres in the vastus lateralis (2.20
364 ± 0.11 *vs* 1.83 ± 0.13, $p = 0.04$), but when compensated for fibre area, no difference, like in
365 this present study, was observed in nuclei per fibre for any fibre type between groups. However,
366 a previous observational study[18] in which previous AAS users had an extensive history of
367 usage, did find significantly elevated myonuclei per fibre values in the trapezius muscle.

368 It can be argued that due to known AAS side effects[41] the only ethically feasible way to study
369 high dose/sustained AAS usage is via observational research[40]. This results in many innate
370 limitations regarding purported AAS usage as pertinent variables such as: cessation date

371 relative to sampling date, usage history/cycle composition and AAS quality lack control. Self-
372 reported AAS usage can be fallible to recall errors and stated duration of abstinence to
373 supraphysiological doses of testosterone and/or AAS, in previous users and returning
374 participants could not be legitimate. Despite these limitations, obtaining cycle information has
375 some utility as it enables a broad classification between 'high' and 'low' doses as reported
376 cycles from 100 users varied 10-fold in maximum weekly dosage and 100-fold in cumulative
377 cycle dose[42]. Further confounding variables in this study include variances in training histories
378 amongst participants, no control of nutrition of returning participants and no PED testing to
379 confirm AAS abstinence in Group RT. Differing numbers of participants within each group
380 and low numbers in Group PREV also confers an influence on statistical power.

381 In conclusion, with no significant difference in myonuclei per fibre values in past AAS users
382 compared to non-users or controls, this study adds evidence[1-6] that myonuclear permanency
383 may not be the predominant mechanism in the muscle memory phenomenon. Other
384 mechanisms (e.g., an epigenetic memory) may play an important role and more research is
385 required [6 43].Longitudinal data from two participants ceasing AAS usage over a shorter time
386 frame is congruous with myonuclear permanency, but with large differences in AAS usage
387 timelines relative to sampling, further research with diligent AAS record taking is required to
388 investigate these initial case report findings. As comparable hypertrophy[44], compared to control
389 mice, occurred from testosterone administration in a conditionally depleted satellite cell mouse
390 model (thereby no myonuclear accrual can occur), future observational studies regarding AAS
391 and muscle memory via myonuclear permanency should focus on longitudinal sampling before
392 and after usage. This is a more controlled environment than recruiting past users to investigate
393 by proxy if myonuclei per fibre values remain elevated.

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7.0 Figure Legends

Figure 1. Participant recruitment from different sampling steps across the study. C=Control Group, RT=Resistance Trained Group, RT-AS=Resistance Trained Currently using AAS Group, PREV=Past AAS using Group. IHC = Immunohistochemistry. *RP4 had muscle stored for IHC on his second visit only.

Figure 2. Body composition and Immunohistochemistry (IHC) data for first and second visits for returning participants (RP) within Group RT-AS (Resistance Trained Current AAS users) using the Tanita® Body Composition Analyzer BC-420MA (Bioelectrical Impedance) ($n = 5$). FFM: Fat Free Mass. IHC data in Panel D, E & F from RP4 is from second visit only.

Figure 3: The correlation between Myonuclei per fibre and muscle fibre CSA from participants ($n = 43$) first sampling visit (including the single sample collected from Returning Participant 4 which occurred on his second visit). C: Control ($n = 5$); RT: Resistance Trained ($n = 15$); RT-AS: Resistance Trained Current AAS users ($n = 17$); PREV: Previous AAS users ($n = 6$).

Figure 4. Muscle fibre CSA (A), Myonuclei per fibre (B) and Satellite cells per fibre (C) data from participants ($n = 43$) first sampling visit (including the single sample collected from Returning Participant 4 which occurred on his second visit). C: Control ($n = 5$); RT: Resistance Trained ($n = 15$); RT-AS: Resistance Trained Current AAS users ($n = 17$); PREV: Previous AAS users ($n = 6$). Brackets with * indicate $p \leq 0.05$. ^ data is from n = (n - 1) participants as one data point has been excluded due to being an outlier (i.e. lower than 0.05).

List of Supplemental Digital Content

Supplementary Digital Content Table 1.pdf