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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Structured Abstract

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

Design: Cross-sectional/longitudinal.

Setting: University.

Participants: Fifty-six males aged 20-42.

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

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

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

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

Keywords:
Myonuclei, Anabolic Androgenic Steroids, Hypertrophy, Fat Free Mass, Muscle Memory, Gene Expression

1.0 Introduction

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

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

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

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

2.0 Methods

2.1 Eligibility and Group Classification.

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

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

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

2.2 Body composition measurements

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

2.3 Muscle Biopsy

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

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

2.4 Staining protocol for fibre CSA, myonuclei and satellite cells

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

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

2.5 Staining protocol for fibre type and fibre CSA.

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

2.6 Section imaging, extraction, and quantification.

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

2.7 Blood Collection

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

2.8 Statistical analysis and data availability.

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

3.0 Ethical Considerations

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

4.0 Results

4.1 Participant sampling and AAS usage

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

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

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

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

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

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

4.2 Demographic and body composition data.

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

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

4.3 Immunohistochemistry.

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

There was a strong positive correlation between myonuclei number and CSA ($r = 0.8$, $p<0.001$) (Figure 3) and 70% of participants with > 4 myonuclei per fibre and a CSA > 8000 μm² had at some point used AAS.

RP1 and RP2 respectively exhibited decreases in fibre CSA between visits (7566 vs 6629 μm² and 7854 vs 5677 μm²) (Figure 2D) whilst their myonuclei per fibre values remained relatively similar between visits (3.5 vs 3.4 and 2.5 vs 2.6) (Figure 2E). RP3 exhibited an increase in fibre CSA (7167 *vs* 7889 μm²) (Figure 2D) and myonuclei per fibre (2.6 *vs* 3.3) (Figure 2E). Satellite cells per fibre decreased for RP2 between visits (0.2 vs 0.1) (Figure 2F) and increased for RP1 (0.2 vs 0.3) (Figure 2F).

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

5.0 Discussion

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

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

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

relative to sampling date, usage history/cycle composition and AAS quality lack control. Self-reported AAS usage can be fallible to recall errors and stated duration of abstinence to supraphysiological doses of testosterone and/or AAS, in previous users and returning participants could not be legitimate. Despite these limitations, obtaining cycle information has some utility as it enables a broad classification between 'high' and 'low' doses as reported cycles from 100 users varied 10-fold in maximum weekly dosage and 100-fold in cumulative cycle dose[42]. Further confounding variables in this study include variances in training histories amongst participants, no control of nutrition of returning participants and no PED testing to confirm AAS abstinence in Group RT. Differing numbers of participants within each group and low numbers in Group PREV also confers an influence on statistical power. In conclusion, with no significant difference in myonuclei per fibre values in past AAS users compared to non-users or controls, this study adds evidence[1-6] that myonuclear permanency may not be the predominant mechanism in the muscle memory phenomenon. Other mechanisms (e.g., an epigenetic memory) may play an important role and more research is required [6 43].Longitudinal data from two participants ceasing AAS usage over a shorter time frame is congruous with myonuclear permanency, but with large differences in AAS usage timelines relative to sampling, further research with diligent AAS record taking is required to investigate these initial case report findings. As comparable hypertrophy[44], compared to control mice, occurred from testosterone administration in a conditionally depleted satellite cell mouse model (thereby no myonuclear accrual can occur), future observational studies regarding AAS and muscle memory via myonuclear permanency should focus on longitudinal sampling before and after usage. This is a more controlled environment than recruiting past users to investigate 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