Anabolic Androgenic Steroid doping in Weightlifting and the Summer Olympic Games alongside their impact on muscle memory and the human transcriptome.

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Dedicated to my family and the participants who took part.

Abstract

Anabolic Androgenic Steroid (AAS) doping pervasiveness, identified retrospectively through International Olympic Committee (IOC) re-tests of the 2004-2012 summer Olympic Games (OG) threatened weightlifting's place at the 2024 OG. Despite this, analysing doping practices in weightlifting and an investigation of IOC re-test efficacy, across all summer OG sports, is outstanding. AAS induce human hypertrophy via increasing myonuclei, and mice data suggests myonuclei permanency causing a "memory" of exposure and long-term advantage. However, limited human data exists on past AAS users and there is no longitudinal data post AAS exposure. Furthermore, RNA-Seq has yet to be conducted on human samples exposed to AAS. Chapter 1 outlines an introduction and Chapter 2 methodologies. Chapter 3 provides results of analysing weightlifting doping practices from 2008-2019 and identified continental differences in detected substances. Chapter 4 analysed doping that impacted medal results for the 1968-2012 summer OG and showed most doping (74% of medals impacted by doping) was identified retrospectively, either from events prior to OG (17%) or IOC re-tests of 2004-2012 (57%). Chapter 5 describes the males recruited for cross-sectional observational research on AAS and myonuclear permanency and Chapter 6 their transcriptome data. Fifty-six men aged 20-42 years were recruited: Non–resistance-trained (C), resistance-trained (RT), RT currently using AAS (RT-AS), of which if AAS usage ceased for ≥18 weeks resampled as Returning Participants (RP) or RT previously using AAS (PREV). There were no significant differences between C (n = 5), RT (n = 15), RT-AS (n = 17), and PREV (n = 6) for trapezius myonuclei per fibre data. Three of 5 returning participants (RP1-3) were sampled longitudinally. Fibre cross-sectional area decreased for RP1 and RP2 between visits, whilst myonuclei per fibre remained similar, congruent with the memory mechanism. However, these values increased for RP3 and self-declared AAS regimens varied. For RNA-Seq, RT-AS was divided to participants who ceased exposure ≤2 or ≥10 weeks prior to sampling. For validation, RNA-Seq was conducted twice but cross-comparison of whole blood datasets showed no differential expression between RP time points or comparisons of RT-AS≤2 to other groups. In both muscle datasets, nine differentially expressed genes overlapped with RT-AS≤2 *vs* RT and RT-AS≤2 *vs* C, but were not differentially expressed with RT *vs* C, possibly suggesting they are from acute doping alone, but differential training routines is a confounder. This thesis identified geographical differences in weightlifting doping, demonstrated retrospective doping testing efficacy and contributed data on AAS regarding muscle memory and the human transcriptome.

List of Related Published Work

* Indicates co-primary authorship

Kolliari-Turner, A., Oliver, B., Lima, G. *et al.,* Doping practices in international weightlifting: analysis of sanctioned athletes/support personnel from 2008 to 2019 and retesting of samples from the 2008 and 2012 Olympic Games. *Sports Med - Open* 7, 4 (2021). https://doi.org/10.1186/s40798-020-00293-4

Kolliari-Turner, A., Lima, G., Hamilton, B. *et al.,* Analysis of Anti-Doping Rule Violations That Have Impacted Medal Results at the Summer Olympic Games 1968–2012. *Sports Med* 51, 2221–2229 (2021). https://doi.org/10.1007/s40279-021-01463-4

Lima, G*., **Kolliari-Turner, A*.,** Wang, G. *et al.,* 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. *Clin J Sport Med* (2022). https://doi:10.1097/JSM.0000000000001037

Kolliari-Turner, A. Implications of muscle memory from testosterone exposure on transgender athletes and previous dopers, and study design constraints in humans. Comments on Cross Talk 52: Myonuclei are/are not lost with ageing and atrophy. *J Physiol* 0.0 (2022) pp 1–9. https://doi.org/10.1113/JP282380. This comment is supplied within the Supporting Information: https://physoc.onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1113%2FJP282380&file=tjp15011-sup-0001-Comments.pdf.

Kolliari-Turner, A., Lima, G., Wang, G. et al. An observational human study investigating the effect of anabolic androgenic steroid use on the transcriptome of skeletal muscle and whole blood using RNA-Seq. *BMC Med Genomics* 16, 94 (2023). https://doi.org/10.1186/s12920-023-01512-z

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Acronyms

5α-diol - 5α-androstane-3α,17β-diol

5β-diol - 5β-androstane-3α,17β-diol

A - Androsterone

AAF - Adverse Analytical Finding

AAS - Anabolic Androgenic Steroid

ABP - Athlete Biological Passport

ACSM - American College of Sports Medicine

ADRV - Anti-Doping Rule Violation

ATPF - Atypical Passport Finding

BER - Balanced Error Rate

BMP - Bone Morphogenetic Protein

ChIP-Seq - Chromatin Immunoprecipitation assays with Sequencing

CHRDL1 - Chordin-Like 1 Protein

CSA - Cross Sectional Area

CNS - Central Nervous System

DBS - Dried Blood Spots

DGE - Differential Gene Expression

EDL - Extensor Digitorum Longus

Etio - Etiocholanolone

FDR - False Discovery Rate

FFM - Fat Free Mass

GC-MS - Gas Chromatography and Mass Spectrometry

GFP - Green Fluorescent Protein

GO - Gene Ontology

GO BP - GO Biological Processes

GO MF - GO Molecular Functions

GSEA - Gene Set Enrichment Analysis

HUNADO - The Hungarian Anti-Doping Group

IF - International Federation

IOC - International Olympic Committee

IRMS - Isotope Ratio Mass Spectrometry

ITA - International Testing Agency

IWF - International Weightlifting Federation

KEGG - Kyoto Encyclopaedia of Genes and Genomes

LTM - Long-Term Metabolites

MDS - Multidimensional scaling

MF - Member Federation

MGI - MGI Tech Co., Ltd which is a subsidiary of the BGI Group (formally Beijing Genomics Institute).

MMAAS - Muscle Memory Anabolic Androgenic Steroid

OOC - Out of Competition

OPLS-DA - Orthogonal Projections to Latent Structures Discriminant Analysis

OSF- Open Science Framework

PLS-DA - Partial Least Squares Discriminant Analysis

PoWeR – Progressive Weighted Wheel Running

rHuEPO – Recombinant Human Erythropoietin

T/E Ratio – Testosterone/Epitestosterone Ratio

TE – Testosterone Enanthate

TU – Testosterone Undecanoate

UGT – Uridine glucuronosyltransferase enzymes

UoB – University of Brighton

VIP - Variable Importance of Projection

WADA - World Anti-Doping Agency

Statement of Candidate Contribution

For Chapter 3 and 4 regarding research into the doping practices of weightlifters and doping at the Summer Olympic Games I was solely responsible for the curation and analysis of this data.

The MMAAS Project was highly collaborative and co-contributors are listed within the publications that have arisen from this research. My supervisory team (particularly Dr Guan Wang) obtained ethical approval for The MMAAS Project in 2016 and once I enrolled as a PhD student on 1st November 2017, I took control of any required ethical addendums and extensions. I was responsible for recruiting ~80% of the participants and for all logistical arrangements regarding participant sampling and visits to the laboratory. I was responsible for the ordering of all equipment and consumables throughout The MMAAS Project. I consented and interviewed all participants. A medical team conducted the muscle biopsy and blood samples were taken by this medical team and other certified individuals, including myself. I registered all samples with the Human Tissue Authority database at the University of Brighton and managed this biobank of samples and was responsible when audits took place. The laboratory work of the immunohistochemistry analysis of muscle samples from The MMAAS Project was shared with Giscard Lima, PhD. Image counting services were provided by MyoAnalytics LLC, as described in the associated publication. Descriptive data, body composition and immunohistochemistry data were co-analysed by Giscard and I. Giscard and I shared RNA extraction of the whole blood samples. I extracted the RNA from all muscle samples. I placed the extracted RNA from the whole blood and muscle samples on the Agilent 2100 Bioanalyzer to determine RIN values. I created all RNA libraries of the whole blood and muscle samples. For sequencing that was conducted at the University of Brighton, I placed the RNA libraries onto the sequencer. For sequencing that was conducted at MGI, Latvia, MGI staff (notably Cynthia Potter) placed RNA libraries onto the sequencer. I conducted all bioinformatic data analysis of the RNA-Seq data from the whole blood and muscle samples.

COVID Impact Statement

The UK entered the 1st COVID lockdown on 26th March 2020. This cancelled a planned weekend of participant sampling on 5th April 2020. Effectively from this point, because of social distancing and travel restrictions, it was not possible to recruit further participants for the observational human study. Fortunately, the immunohistochemical analysis of the muscle samples was concluded by November 2019 and the initial plan was to sample more participants to add to this dataset, but logistically this was no longer feasible. No RNA-Seq laboratory work had been conducted at this timepoint (March 2020) for transcriptomic data analysis. Participant numbers were therefore limited to those sampled prior to 2020 (56 participants visiting the laboratory for sampling, with 5 returning participants visiting for a second visit post AAS exposure). Our initial plan was to recruit a minimum of 15 returning participants, COVID curtailed this plan.

The university laboratories were closed from the end of March 2020 until the end of August 2020. From September 2020 – December 2020 I was able to return to campus, even with the second national lockdown of November 2020, and extract RNA from all the whole blood samples and sequence these RNA libraries on the MGI DNBSEQ-G400 sequencer. At the beginning of January 2021, the UK entered the third national lockdown. I was able to work in the laboratory, however I had to wait until 15th March 2021 for muscle RNA extraction kits to arrive due to the high national demand for RNA extraction reagents (SARS-CoV-2 is an RNA virus). Typically, these kits would arrive within 5 days. Additionally, due to this burgeoning demand in COVID related sequencing and intense market competition, MGI informed us that their main competitor Illumina had sued them for patent infringement within the UK. This was related to the biochemical reaction of sequencing nucleotides with fluorescent dyes. MGI appealed and the hearing date was initially set for January 2022.

I managed to conclude all muscle RNA extractions by early September 2021. The sequencer was serviced on 21st September 2021, and I started the two required muscle sequencing runs the following week. Unfortunately, the sequencer developed a fluidics fault, likely related to having been left idle since December 2020 due to COVID related delays preventing its usage for other research items planned by the group. While running the sequencer, on what would conclude in these failed runs, all sequencing reagents were exhausted. MGI serviced the sequencer again in November 2021 and MGI initiated sending us complementary reagents due to those lost in the failed sequencing runs. These arrived on 17th December 2021 and

coincidentally on this exact same date the MGI *vs* Illumina appeal took place. The appeal was unanimously dismissed and with immediate effect all MGI sequencing reagents become prohibited to use within the UK. This prevented me from concluding the RNA-Seq experiment of the muscle samples, as not all samples had been sequenced.

In January 2022 I had to help MGI physically remove the sequencer from campus and pour all reagents away. Due to this situation MGI agreed that complementary sequencing of the muscle samples could be concluded within their Latvian headquarters. As a further gesture of goodwill, the muscle samples would be sequenced twice, and the blood samples would be sequenced again. I sent all libraries to Latvia on 16th February 2022 and received the full 1TB raw dataset on 14th April 2022. Finally, at this point I could start the required bioinformatic analysis and I had a complete dataset.

Overall, the COVID pandemic, with the social distancing restrictions and other corollary effects had a substantial impact on my PhD journey.

Acknowledgements

Firstly, I would like to thank all the participants who took part in The MMAAS Project. Many of them travelled long distances to take part in this study and I will always be grateful for their willingness to volunteer without remuneration. I need to also thank the online content creators that either shared the advertisement for recruitment or invited me as a guest on their podcast platform to discuss the study, without their help I would not have recruited so many participants. These include Stronger by Science (Greg Nuckols & Eric Trexler), Iron Culture (Eric Helms & Omar Isuf), Think Big Bodybuilding Media (Scott McNally, Dave Crossland & Dr Scott Stevenson), Revive Stronger (Steve Hall & Pascal Flor), Danny Lennon, David Nolan, Mike Israetel, Tom Maw, Dr Dean St. Mart, Alex Pearson-Jones, Ben Newbould, Joe Jeffery and the testosterone Facebook group (particularly Al Benson and Dr Mike Scally). I must also thank the local gyms who allowed my advert to be placed within their facility. I must also thank the medical team (Vasileios, Jonathan, and Paulette) who conducted the muscle biopsies in The MMAAS Project (on the weekends during their days off) as without them it would not have been possible to investigate these research questions. A similar thank you to all my Brighton colleagues who helped on all 9 sampling weekends.

Many academic colleagues have helped me in this PhD journey and are listed as co-authors or within the acknowledgements in the publications that have come from the studies within this thesis. I must thank Darren Chambers for initially showing me how to freeze muscle samples and Brian Oliver for our conversations on doping in weightlifting. Most notably I need to thank Giscard Lima for the shared work we conducted in Melbourne in November 2019 on the immunohistochemistry work related to The MMAAS Project. A special thank you must also go to Cynthia Potter from MGI. Unfortunately, before I could finish sequencing all muscle samples from The MMAAS Project MGI sequencing reagents become prohibited to use within the UK after Illumina successfully won a patent infringement suit against BGI. Cynthia very kindly accepted our RNA libraries in Latvia and placed them onto the sequencer for us and enabled me to obtain the complete dataset. I must also thank Garrett Ash for providing me access to the Yale School of Medicine Farnam HPC cluster. Without this I would not have been able to conduct the required bioinformatic analysis.

Finally, my supervisory team (Yannis Pitsiladis, Guan Wang & Fergus Guppy) have provided continued support throughout this five-and-a-half-year journey, and I am thankful for their mentorship and guidance.

Author Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed: Alexander Kolliari-Turner

Dated: 22nd June 2023

1. Introduction.

1.1. A mechanistic introduction to Testosterone and AAS.

Androgens are sex hormones that promote the development and maintenance of male sexual characteristics [1]. Androgens increase skeletal muscle mass and strength (anabolic effects) and initiate the development of secondary male sexual characteristics, including axillary hair growth, lowering of the voice, an increase in sebaceous gland activity and increased libido (androgenic effects) [2]. Testosterone is the principle secreted androgen in cisgender men, and it affects many physiological systems such as: the musculoskeletal system, the cardiovascular system, the reproductive system, and the haematological system [2]. The effects of androgens are modulated at a cellular level by the steroid-converting enzymes within the particular target tissue, such as 5α-reductase which can convert testosterone to dihydrotestosterone and aromatase which can convert testosterone to oestradiol [3].

Since the 1950's pharmaceutical companies have been developing synthetic analogues of testosterone with the aim of treating patients in a "catabolic state" [4]. By structurally modifying testosterone and its derivatives the objective has been to enhance anabolic effects, dissociate unwanted androgenic effects and create both oral and parenteral preparations. Although the anabolic effects of androgens can be enhanced, it has not been possible to remove their androgenic effects and therefore a more accurate term for anabolic steroids is anabolic-androgenic steroids (AAS) [4]. Complete dissociation of anabolic and androgenic effects has not been possible because the single hormonal Androgen Receptor (AR) mediates the androgenic and anabolic effects of testosterone [5]. Therefore, to enhance anabolic effects and reduce androgenic effects differences in androgen metabolism by steroid converting enzymes inside muscle and other tissues must be exploited.

The effects of androgens are mediated through the AR, an intracellular receptor belonging to the nuclear receptor superfamily [6]. This subclass of receptors consists of a DNA-binding domain, a ligand-binding domain and at least two transcriptional activation domains and they elicit a "classical" or genomic mode of action by interacting with DNA and modulating transcription [6]. Apart from binding with the androgen, the ligand-binding domain also functions in dimer formation and mediates transcriptional activation. The DNA-binding domain targets the receptor to specific DNA sequences known as androgen response elements [7]. In the absence of androgens the AR exists inside the cytoplasm as an inactive oligomeric complex with the molecular chaperone heat-shock protein 90, p23 and co-chaperones utilizing the tetratricopeptide repeat motifs [8]. Androgens are relatively small molecules and can diffuse passively into cells. Once inside cells that contain the AR the androgen will bind

to the ligand-binding domain, causing dissociation of the receptor-Hsp90 complex and the resulting allosteric change creates an active receptor that is translocated to the nucleus. Activated receptors interact as homodimers with the androgen response elements on chromatin. After DNA attachment coregulators are recruited and a transcription initiation complex forms with RNA polymerase II. These coregulators can be either positive or negative regulatory proteins, referred to as either coactivators or corepressors [8]. Coactivator and corepressor complexes are required for nuclear receptor mediated transcriptional regulation. A liganded AR will recruit coactivators, resulting in gene activation, gene transcription, translation and a subsequent alteration in cell function, growth, or differentiation. AAS bind to the AR with different affinities, but there is a large discrepancy between what is known about the *in vivo* activities of AAS compared to their *in vitro* activity, when additionally factoring in possible differences in the bioavailability and clearance of these steroids which is influenced by their affinity to sex hormone-binding globulin in blood circulation [3]. As noted in [3] oxymetholone and stanozolol have low relative binding affinity compared with 17α-methyltestosterone in *in vitro* study, but conversely these steroids have a relatively high myotropic activity compared with 17α-methyltestosterone when administered to the castrated rat, with further results for other AAS additionally presented in [3].

The process of a steroid receptor translocating from the cytoplasm to the nucleus typically takes at least 30 to 60 minutes [9]. In contrast, other regulatory actions of steroids are manifested within seconds to a few minutes. These time periods are too short to be due to changes at the genomic level and are therefore termed non-genomic or rapid actions, to distinguish them from the classical steroid hormone regulation of gene expression. Much of the physiological importance of the non-genomic actions of androgens is still to be elucidated, but several mechanisms seem to be involved, including a membrane-bound sex hormone-binding globulin receptor, a putative G-protein-coupled receptor that androgens directly bind with and stimulation of nonreceptor tyrosine kinase c-SRC [10]. The role of the non-genomic actions of androgens at physiological concentrations in skeletal muscle growth is not known, or to what extent the non-genomic effects are evoked by the administration of AAS [3, 11].

1.2. The performance enhancing effects of supraphysiological Testosterone from human administration studies.

Although athletes have been using AAS and citing performance enhancing benefits since the mid-1950's, with a possibility that athletes managed to experiment with AAS prior to this time, because artificial testosterone was first synthesised in 1935, early research did not corroborate the ergogenic effects experienced by athletes [2] and the academic community decried their ergogenic effects citing a lack of evidence. In 1977 the American College of Sports Medicine (ACSM) published a position stand [12] that cited AAS as ineffective, not subsequently revising their position until 1987 [13]. There are numerous reasons as to why early studies failed to show the ergogenic effects of AAS. These included poor study designs (e.g., non-randomized, non-double-blind, non-placebo controlled trials), under administration of androgens (e.g., a lower dose typically prescribed for androgen deficiency, which is far exceeded by athletes), included untrained subjects, whose initial neurological adaptations to resistance training and low loads may mask potential hypertrophic increases from androgens and failed to examine dietary interventions such as increased protein intake coinciding with androgen use [2]. For studies to truly show the reported ergogenic effects of AAS they need to follow real life AAS dosages and practices followed by athletes [14]. However, this results in an ethical challenge for researchers as it is ethically questionable to expose healthy humans, to potentially hazardous drugs in supratherapeutic dosages, over long periods of time, for the single purpose of improving sports performance. Therefore, it must be kept in mind that the handful of studies of high scientific quality (randomised, double blind-placebo controlled studies with standardised training and nutrition regimes) only scratch the surface of what happens in AAS users [14]. In most well-designed studies the duration and dosages of AAS administration are far below daily practices in the field and they do not investigate polydrug regimes. For example, the highest dosage of Testosterone Enanthate (TE) administered in a study investigating athletic performance was 600mg·wk^{-1} over 20 weeks [15]. In the first publicly released book for steroid usage in powerlifting, it has been reported that world class athletes uses 2g·wk^{-1} of TE and 1g·wk^{-1} of Trenbolone Acetate for 6 months continuously and oral steroids are used as competition approaches [16].

In a seminal study in 1996 Bhasin *et al.*, [17] administered 600mg·wk^{-1} of TE over 10 weeks to investigate if supraphysiologic doses of testosterone, administered alone or in conjunction with a standardized program of strength training, could increase fat-free mass, muscle size and strength in normal healthy men. This placebo controlled, double blinded study,

ameliorated the inadequacies of previous AAS administration studies and it is regarded as the first study to rigorously investigate the effects of supraphysiologic doses of testosterone. The combination of strength training and testosterone produced greater increases in muscle size, strength and fat free mass (FFM) than were achieved with either intervention alone.

In subsequent studies Bhasin and colleagues demonstrated that the effects of testosterone are dose dependent in regards to gains in FFM, muscle size and strength, in both young and old men, despite the fact that participants were asked not to undertake strength training or moderate-to-heavy endurance exercise during this research [18, 19]. Older men are therefore as responsive as younger men to testosterones anabolic effects, but they have higher frequencies of adverse effects (haematocrit of greater than 54%, leg edema and prostate events) [18].

Muscle biopsies of the *vastus lateralis* from these studies demonstrated that testosterone induces muscle hypertrophy of both Type I and Type II muscle fibres, but their relative proportions do not change significantly after treatment in any group, indicating that testosterone does not influence fibre type transitions [18, 19]. Testosterone was shown to dose dependently increase satellite cell number and myonuclear number and muscle fibre CSA was significantly correlated with myonuclear number [20, 21]. Specifically, in young men the change in myonuclear number per millimetre of muscle fibre, compared to baseline, was greater in men receiving the 600-mg dose (19 ± 3 *vs* 7 ± 1) than in those receiving the 125-mg (9 ± 1 *vs* 8 ± 1) or the 300-mg (13 ± 2 *vs* 9 ± 2) dose of TE with this change from baseline being statistically different for the 300-mg and 600-mg groups (21). Average serum total testosterone significantly increased for the 300-mg (724 ± 124 ng/dl) and 600-mg groups (1,822 ± 492 ng/dl) with serum free testosterone also being statistically higher compared to baseline in both groups (raw change from baseline data is not presented) [19]. Although raw myonuclear data is not provided for older men a similar pattern is observed with significant increases from baseline in myonuclear number in the 125-mg, 300-mg, and 600-mg dose groups, with the magnitude of this difference to baseline increasing with TE dose [20]. It was therefore concluded for both young and old men that testosterone induced hypertrophy is associated with an increase in satellite cell number and a proportionate increase in myonuclear number [20, 21].

Despite these human studies showing AAS induced hypertrophy occurs from myonuclear accrual, a recent murine model has shown that myonuclear accrual is not required for

testosterone induced hypertrophy [22]. In this study, comparable hypertrophy occurred from testosterone administration in a conditionally depleted satellite cell mouse model, a model where no myonuclear accrual can occur, when compared with control mice, demonstrating that myonuclear accrual is not mandatory for AAS induced hypertrophy. Given this finding, and that testosterone is known to promote muscle anabolism by stimulating fractional muscle protein synthesis, inhibiting muscle protein degradation, and by increasing the efficiency of amino acid reuse by the skeletal muscle [23-25] additional mechanisms exist in which comparable hypertrophy can occur without myonuclear accrual. Intramuscular injections of as little of 200mg of TE, after 5-days, has been shown to induce a twofold increase in net protein synthesis via increased re-utilization of intracellular amino acids in skeletal muscle [26]. This twofold increase was observed even though total testosterone concentrations were in the upper physiological range (953 ± 283 ng/dl) at this 5-day time point, but these concentrations were statistically higher than baseline (425 ± 99 ng/dl) [26]. 200mg of TE a week, for 4-weeks, through increased translational capacity, rather than efficiency, has also been shown to drive lean mass increases in response to TE administration in an energy deficit [27], demonstrating the hypertrophic potency of AAS. The hypertrophic effect of AAS on protein synthesis is also further complicated by AAS restoring muscle hypertrophy in castrated mice limb muscles to sham levels even when rapamycin is administered to inhibit mTOR [28], further demonstrating that more research is needed on how AAS elicit hypertrophic benefit.

1.3. The role of myonuclei in hypertrophy and evidence supporting myonuclei as a substrate for "muscle memory".

Muscle fibres are by volume the largest cells in the mammalian body and constitute one of the few syncytia in the mammalian body [29]. The syncytial nature of muscle fibres is probably related to the lack of a long-distance transport system for proteins within these large cells [30]. Myonuclei have been demonstrated to be positioned optimally to minimise transport distances within each fibre [29] and perturbations in the position of nuclei leads to impaired muscle function [31]. Since the 19th century it has been suggested that a nucleus can serve only a certain volume of cytoplasm [32]. In muscle, each nucleus is surrounded by synthetic machinery that seems to remain localised [33] and it is believed that each nucleus serves a certain domain because it has been shown that proteins are localised to the site of expression both *in vitro* and *in vivo* [33, 34].

In its strictest version, this nuclear domain theory has implied that a nucleus supports a constant volume of cytoplasm and therefore the number of nuclei should increase proportionally as fibre cross sectional area increases during hypertrophy [35]. Support for this concept has come from observations that under normal physiological conditions the number of myonuclei per muscle fibre is proportional to muscle fibre size in both animal [29, 36-38] and human muscle [19, 39]. However, multiple studies have demonstrated that the myonuclear domain size is not fixed and varies with the oxidative capacity of the fibre and the stage of maturational growth/age [35, 38, 40-45]. Deviations in myonuclear domain size are also observed when the muscle adapts to altered functional demands during atrophy and hypertrophy. Despite these observations suggesting that the myonuclear domain size is not fixed, some researchers conclude it would be too radical to abandon this concept entirely because in normal situations a relationship between fibre size and myonuclear number does exit [35].

Within skeletal muscle there is a continuous process of production and subsequent degradation of muscle proteins and the balance between protein synthesis and breakdown determines whether hypertrophy or atrophy of muscle mass occurs [46]. Total muscle protein synthesis is a product of the number of myonuclei and synthesis per nucleus. Therefore, the increase in muscle proteins during muscle hypertrophy can be achieved by either increasing RNA and protein synthesis from the existing myonuclei or by maintaining the same level of RNA and protein synthesis from each myonucleus and adding new nuclei to the fibres. Since adult muscle myonuclei are unable to divide, the new nuclei to be incorporated by the fibre must originate from outside the fibre and satellite cells, the myogenic stem cells, are the major donors of new nuclei [47]. However, it should be noted that this dogma of myonuclei being post-mitotic has recently been challenged [48] with new data suggesting that myonuclei can synthesise DNA through endoreplication resulting in polyploidy, thereby increasing myonuclear transcriptional capacity without increases in myonuclear number. Further research is needed to investigate what role myonuclei endoreplication and polyploidy plays in hypertrophic processes [48]. Once activated, satellite cells exit from their quiescent state, proliferate, differentiate and fuse into pre-existing myofibres to create new myonuclei, or they will return to the basal quiescent state and subsequently maintain a pool of satellite cells that can be activated during repair, re-generation or hypertrophy [49].

Generally, it is accepted that myonuclei are added under many hypertrophic conditions and that the increase in the number of myonuclei precedes the radial growth of the fibre [36, 39,

50-55]. This has been demonstrated with both ^{3}H-thymidine labelling and *in vivo* imaging [52] and therefore the myonuclei domain is temporarily decreased during the initial growth phase. Although this time course suggests that the accretion of myonuclei is causally related to the build-up of muscle mass, it has been debated if the addition of myonuclei is obligatory for hypertrophic growth. If myonuclei accrual is not obligatory, it would have implications on the purported muscle memory mechanism of myonuclear permanency, as if no myonuclei are accumulated, the primary structure required for this memory would be absent in the first instance.

Historically, studies investigating the role of satellite cells in hypertrophy and re-growth have attempted to prevent satellite cell activity by blocking DNA synthesis using γ-irradiation or chemical agents [56-59]. From these studies it has been suggested that satellite cell proliferation and myonuclei accretion is a necessary step for mounting a robust growth response. However, these approaches have been criticised because of their lack of cellular specificity and the fact that irradiation might have affected other growth mechanisms, in addition to ablating satellite cells [60]. After comprehensive debate in 2007 [61, 62] it was concluded that limitations in current methodology made it difficult to reach final conclusions on whether an increase in the number of myonuclei is required for hypertrophy [63].

Hypertrophy, to some extent, is possible without myonuclei accretion [64-67]. Significant increases in myonuclei content have been reported in studies where muscle fibres hypertrophied by more than 26% [36, 50, 68], but not in studies in which fibres hypertrophied by 6.8-15.5% [69]. In addition, fibre CSA has been shown to increase by 6.7% after 30 days and by 17% after 90 days of resistance training, without the addition of new myonuclei [66]. In addition, after a 4-month resistance training programme in humans, it was observed that participants who responded with only moderate (9.7 ± 2.4%) hypertrophy the number of myonuclei did not increase and the myonuclei domain size remained below 2,000μm [65]. From these observations it has been suggested that moderate changes in skeletal muscle fibres can be achieved without the addition of new myonuclei, which indicates that existing myonuclei are able to support a certain level of muscle fibre hypertrophy [66].

Regarding hypertrophy without myonuclei accretion it has been proposed that there is a "ceiling" of myonuclear domain size beyond which additional hypertrophy can only be realised by the addition of new myonuclei [65]. This limit has been defined as hypertrophy above a certain percentage threshold (e.g. 17-36% [70]) or as an absolute myonuclear

cytoplasmic domain volume, that when exceeded, triggers recruitment of myonuclei [65]. The exact nature of the bottleneck governing the "ceiling" of myonuclear domain size is not known, but Gundersen (2016) [30] has suggested that inherent rate limiting factors in ribosome biogenesis may have a role to play. rRNA accounts for 70% of all transcription, and most steps in the assembly of the ribosomal subunits take place in the nucleolus, where rRNAs are transcribed as large precursors, which undergo extensive nucleotide modification [71]. In addition, more than 200 non-ribosomal, resident nucleolar proteins are required to process and modify the rRNAs and to aid their assembly with the ~80 ribosomal proteins. These processes require efficient trafficking across the nuclear membrane and such high throughput processes in the nucleus might represent the steps that limit hypertrophic growth and thus act as a bottle neck for polysome formation and subsequent protein synthesis if the number of myonuclei are not sufficiently high [30].

After the conclusion that limitations in current methodology made it difficult to reach final conclusions on whether an increase in the number of myonuclei is required for hypertrophy [63], a series of studies using transgenic models that display large fibres without a corresponding increase in the number of myonuclei, has resulted in a rejuvenation of this debate. Mice that overexpress the protein Ski [72], Akt [73], junB [74] and myostatin-null mice [75] all have displayed hypertrophied fibres without a concomitant increase in the number of myonuclei. However, for Ski and myostatin the hypertrophy is not fully functional, as specific force is reduced [75-77]. Although it was not measured, the same is probably true for junB, as it might also act by inhibiting myostatin [74]. Akt mice seemed to have normal specific force after 3 weeks of overexpression [73] but it is unclear whether this condition is sustainable over longer periods [47]. The novel Pax7-DTA mouse strain, where >90% of satellite cells can be conditionally ablated, similarly showed normal specific force as well as normal Ca^{2+} sensitivity and rate of cross-bridge cycling at the single fibre level [78]. The specific depletion of satellite cells did not prevent or blunt the hypertrophic response in terms of muscle mass or fibre CSA and the increase in fibre CSA resulted in significant expansion of the myonuclear domain, resulting in the conclusion that satellite cells are not necessary for hypertrophy [78]. This data thereby suggests that alternative hypertrophic pathways, not involving satellite cells and myonuclear accrual are compensating for this comparable hypertrophic response. However, both fibre hyperplasia and regeneration were significantly blunted following satellite cell depletion, indicating a distinct requirement for satellite cells during these processes [78].

However, these results have recently been questioned in a study that involved the same genetic model, but overload hypertrophy was used as opposed to synergistic ablation to induce muscle growth [79]. In this study overload hypertrophy was prevented in the satellite cell-deficient mice, in both the plantaris and the extensor digitorum longus (EDL) muscles and the authors attributed the previous findings to a reliance on muscle mass as a proxy for fibre hypertrophy, and to the inclusion of a significant number of regenerating fibres in the analysis. Due to these methodological discrepancies Egner *et al.*, (2016) [79] concluded that fully functional, sustainable, *de novo* hypertrophy without satellite cell depletion, remains to be unequivocally demonstrated. Further research has been conducted in which a different genetic model was used to investigate the importance of satellite cells in overload-hypertrophy [80]. In this study, muscle progenitors were rendered fusion-incompetent through genetic deletion of myomaker, a muscle specific membrane protein that is essential for myoblast fusion, and a complete reduction in overload-hypertrophy was observed. In response to increased muscle workload, myomaker was shown to be upregulated in activated satellite cells and was required for fusion with a myofibre. In the absence of satellite cell derived myomaker, increases in myofibre size were diminished and therefore myomaker mediated fusion is required for load-induced hypertrophy of skeletal muscle [80]. Through the generation of fusion defective satellite cells this study provides independent evidence for the necessity of satellite cells for any major muscle hypertrophy and that Egner *et al*., (2016) [79] could be correct in their conclusion.

To counteract problems associated with measuring myonuclear loss during atrophy three modes of atrophy: denervation, nerve impulse block, and mechanical unloading, were utilised to investigate nuclear loss via direct observation of nuclei by *in vivo* time lapse imaging of single fibres [81]. Nuclei from single fibres were labelled with green fluorescent protein using somatic gene transfer by electroporation or intracellular injection and then followed during the atrophying condition by re-exposing the muscle and re-imaging the same fibre segments. Interestingly in all three conditions atrophy was not accompanied by loss of myonuclei as measured by *in vivo* time lapse imaging of single fibres. Furthermore, muscle cross-sections were immunostained with dystrophin to identify the muscle fibre cortex, Hoechst dye 33342 to identify nuclei and TUNEL to identify DNA fragmentation associated with apoptosis. To guarantee the identification of myonuclei a strict set of identification criteria was during the analysis of cross sections. Nuclei with their geometric centre outside of the dystrophin ring were classified as satellite cells or stromal cells, whereas nuclei that

had their geometric centre inside the dystrophin ring were classified as myonuclei. Of the approximately 27,000 myonuclei that were screened 3–21 days after denervation, only 2 were TUNEL positive in the EDL and none of the 5,500 myonuclei screened on sections from nerve impulse block were TUNEL positive confirming that apoptosis of myonuclei is extremely rare following inactivity [81].

This finding led Bruusgaard *et al.,* (2010) [52] to investigate if the myonuclei that are acquired during hypertrophy are lost during subsequent detraining. Following 14 days of overload by partial synergist ablation the number of myonuclei within the EDL increased by 37% and muscle CSA by 35%. The average cytoplasmic volume per nucleus for each fibre (i.e., the nuclear domain) was statistically lower by 16% at 6–10 days after overload compared to 0-3 days after overload, but at 12-21 days after overload was not statistically different from that of controls [52]. Subsequent denervation of the EDL for 14 days resulted in a decrease of CSA by 40%. Despite this atrophy, myonuclei were not lost. Similar results regarding myonuclear permanency were also found in muscles overloaded for 14 days and denervated for 3 months. In this longer experiment the number of nuclei were maintained despite median CSA being reduced to 23% of the values after overload. It was therefore concluded that overload results in a lasting increased number of nuclei that are not lost, despite subsequent disuse extending over a large part of the mouse lifespan [52].

This led Bruusgaard *et al.,* (2010) [52] to suggest a new model of muscle nuclei acquisition during hypertrophy and retention during atrophy. They suggest that previously untrained fibres are small with few nuclei and they acquire new nuclei through a “first training route” resulting in a large fibre with many nuclei. They appreciate that resident myonuclei have the ability to increase muscle fibre CSA by increasing synthetic capacity per nucleus, but the extent of this hypertrophy is limited because of the limitations in the synthetic capacity of each nucleus to support the expanding larger domain size, due to the expanding diffusion distance. During the “first training route” satellite cells donate their nuclei to the muscle fibre and these new myonuclei are causally related to the subsequent fibre enlargement. The acquisition of these myonuclei precedes hypertrophy and thus the myonuclear domain is temporarily decreased during the growth phase. Additionally, they regard that increases in myonuclei number is the major cause of hypertrophy.

Upon detraining these fibres maintain an elevated number of nuclei where this “advantage could manifest itself as resistance toward detraining-related atrophy and a more efficient

response to retraining" [51]. Bruusgard *et al.*, (2010) [51] suggest there is no compelling evidence that nuclei are ever lost from intact muscle fibres and so the observed atrophy during detraining is due to rates of protein degradation outweighing rates of protein synthesis. After atrophy this results in a small fibre with a large number of myonuclei. Upon retraining a gain in size can be achieved by a moderate increase in the protein synthesis rate of each of these resident myonuclei, skipping the step of adding newly formed nuclei during the "first training route". Therefore, these lasting elevated numbers of myonuclei serve as a cellular biological substrate for "muscle memory". As an extension of the muscle memory model first proposed in Bruusgaard *et al*., (2010) [52], Gundersen (2016) [30] suggests that the number of myonuclei not only reflects the current size of the fibre as implied by the ceiling hypothesis, but also the history of the fibre. Current data might fit a "peak pegging" hypothesis, where the number of myonuclei found in a fibre represents the largest size the fibre has been in its history, and new myonuclei are only added if the fibre grows beyond that size. The Gundersen group has also shown that myonuclear memory occurs in juvenile mice who are subjected to climbing training before reaching full sexual maturity [82].

The phenomenon of muscle memory, where previous strength training makes regaining muscle mass in later life easier, even after long intervening periods of inactivity and mass loss, has been commonly observed in society. For example older individuals (mean age 72.5yrs) who had strength trained for 2 years, but subsequently stopped for 3 years, still had 9-24% higher 1-RM values above baseline for the leg press, arm curl, and bench press, with the authors of this study proposing that "neural adaptations were likely responsible for the relative preservation of dynamic strength" in these subjects [83]. Additionally, during 30–32 wk of detraining, a group of women lost a considerable part of the extra strength obtained by 20 wk of previous training but regained the strength after only 6 wk of retraining [84]. Previous to work on myonuclei retention [52, 85] there was no known cellular memory in muscle, and the long lasting effects of previous training were solely attributed to motor learning in the CNS [86]. Egner *et al.,* (2013) [85] propose that this cellular memory mechanism has an evolutionary importance because it would allow for individuals performing seasonal strength-demanding tasks to regain strength quickly without having to maintain a large permanent muscle mass. Van Der Meer *et al.,* (2011) [35] have also suggested that the maintenance of myonuclei may be an appropriate strategy as the the breakdown of myonuclei is an energy requiring process. Myonuclei maintenance enables a muscle to be "ready" to respond to a stimuli that induce hypertrophy. Whilst muscle proteins

might be rapidly broken down during the commencement of atrophy the machinery and structures required to rapidly regain muscle mass are preserved. This machinery may be required in the initial stages of atrophy to cope with the dramatic changes in gene expression and synthesis of enzymes that are involved in protein breakdown.

1.3.1. Murine research on Testosterone and muscle memory and implications for human sport.

In 2013 Egner and collegues [85] conducted a study to investigate myonuclei accretion via AAS exposure, within the aforementioned muscle memory model proposed by Bruusgaard *et al*., (2010) [52], to determine the potential long term benefits of AAS exposure, after usage has stopped. Female mice had either a testosterone propionate or sham pellet implanted subcutaneuosly into their necks for 14 days and both the EDL and soleus muscles were overloaded via synergistic ablation. There were 4 experimental groups (Sham, Steroids, Sham+Overload & Steroids+Overload). In the soleus muscle steroid treatment alone increased the number of myonuclei by 66% compared to treatment with sham pellets. Overload alone in the sham group increased the number of myonuclei by 51%, whereas when overload and steroids were combined, myonuclei increased by 92% and similar results were observed in the EDL [85]. Changes in fibre CSA mirrored these results, within the soleus muscle steroid treatment alone increased fibre CSA by 77% compared to treatment with sham pellets, whilst overload alone increased fibre CSA by 48%. When steroids and overload were combined, a 118% increase in CSA was observed, and thus the effect of the two treatments was almost additive. Similar to the results of myonuclei accretion, qualitiatively similar, but less dramatic results were observed in the EDL. The ordering of fibre CSA expansion in these groups mirrors the effects of resistance exercise and testosterone administration in humans [17].

The pellets were subsequently removed to investigate if these extra myonuclei could aid in hypertrophy after drug removal. Three weeks after pellet removal the number of myonuclei was not significantly reduced in the soleus muscle and remained on average 42% higher than in the sham group. However, fibre CSA reduced dramatically in the steroid group and was indistinguishable from the sham treated group. Overload was then introduced for 14 days and the steroid group displayed a hypertrophy of 44% which was statistically higher than the 17% hypertrophy observed in the sham group. *In vivo* single fibre observations in the EDL supported this *ex vivo* analysis of the soleus. Three weeks after pellet removal fibre CSA was

indistinguishable between steroid and sham treated groups, whilst the number of myonuclei did not significantly change in any of these groups with the steroid group maintaining a 20% higher number of myonuclei. When overload was introduced at this time, fibre CSA increased significantly more (42%) in the steroid group compared to the sham group (21%).

In order to demonstrate a longer term memory overload was introduced 3 months after pellet removal. Three months constitues ~12% of the mice lifespan and would correspond to approximatley a decade in humans. At this time there was no difference in fibre CSA between groups but the number of nuclei was 28% higher in the steroid group compared to the sham group. When overload was introduced in the EDL fibre CSA significantly increased by 31% in the steroid group during the first 6 days, while in the sham group a nonsignificant increase of 6% was observed. Between day 6 and 14 both groups experienced parallel increases in fibre CSA, but fibre CSA was still 20% higher in the steroid group after 14 days of overload. This "re-training" route seems to allow for faster growth than the first training route.

The concept of retained myonuclei and muscle memory has large implications for anti-doping because anabolic steroids have been shown to increase the number of myonuclei in human skeletal muscle (see section 1.2.). Therefore the benefits of anabolic steroids could be long lasting, if not permanent, and this may have consequences for the exclusion time after a doping offence [52]. Egner *et al.,* (2013) [85] demonstrated that the memory effect lasts for 3 months in mice which is >10% of the animal's lifespan. The age of myonuclei in humans and therefore the length of this potential muscle memory effect has been harder to measure. However Spalding *et al*., (2005) [87] demonstrated that the level of ^{14}C in genomic DNA closely parallels atmospheric levels and because of increases in ^{14}C following nuclear weapon testing in the 1950-60's this can be used as a metric to measure the age of cells within the human body. Intercostal muscle tissue was harvested from two individuals (age 37–38 years) and the ^{14}C content in genomic DNA indicated an average age of the nuclei of 15.1 years [87]. It is possible that this is a low-end estimate of the possible lifespan of human myonuclei [30]. The measurement was only taken from two young individuals who have been growing for a significant part of their life and thus creating new myonuclei; and new myonuclei might have been created during relatively recent strength exercise. Human brain cortical neurons appeared to be as old as the individual [87]. However, in contrast to the brain, muscle tissue is subjected to repair after damage with new nuclei from satellite cells. How much damage and repair normally accumulates over decades is, however, unknown. Thus, with current

knowledge it is hard to be more precise than estimating the lifespan of myonuclei, and hence the muscle memory to span somewhere between 15 years and a human lifetime [30]. However, the position of the World Anti-Doping Agency (WADA) is that no regulatory action should be taken before the muscle memory phenomenon is confirmed in humans. If confirmed in humans, the possibility of a long term muscle memory induced by a brief steroid exposure raises serious questions about the possibility of policing drug-free sport and would infer that life bans would be an appropriate course of action to maintain the integrity of drug free sport [30].

1.4. Evidence opposing myonuclei as a substrate for "muscle memory".

According to the concept of myonuclei domain size skeletal muscle atrophy should be associated with a proportional decrease in the number of myonuclei (via apoptosis) to maintain the myonuclei domain size [35]. Numerous studies, over a wide range of conditions resulting in atrophy, have suggested that myonuclei are lost. These include denervating neuromuscular disorders [88] and experimental models of denervation, abolishment of nerve electrical activity and mechanical muscle unloading [89-91]. However, most of the studies that suggest nuclei are lost analyse muscle cross-sections via light microscopy and they do not use staining methods that can critically differentiate between myonuclei, satellite cell nuclei and stromal cell nuclei [92]. Additionally, evidence on lost myonuclei is also based on finding the molecular markers of apoptosis in muscle homogenates [93], but this metric of apoptosis is not specific to myonuclei [92]. Overall, this shows the methodological difficulties of studying myonuclear permanency, with authors who support permanency arguing that in vivo imaging is one of the few ways currently available to study permanency, with this method demonstrating the long-lasting mature of myonuclei [81].

Experiments investigating unloading induced atrophy inside previously overloaded quail muscles have suggested that newly added nuclei during hypertrophy are particularly prone to apoptosis during subsequent disuse [94]. BrdU and TUNEL staining demonstrated that nuclei that had undergone mitosis during the hypertrophy phase were particularly prone to apoptosis, suggesting that hypertrophy introduces a less stable population of myonuclei [94]. However, these findings have been questioned because no distinction was made between myonuclei and other nuclei within the muscle tissue [52]. Additionally, only one apoptotic

nucleus was shown within the dystrophin ring and the frequency of such confirmed myonuclei was not reported [52].

More modern studies that have considered these methodological difficulties have been undertaken [95-100]. Subsequently, due to their new and conflicting data the proposed mechanism of myonuclei permanency for the muscle memory phenomenon has recently been placed into question and is now not universally accepted, like it once was [30]. For example, an animal model involving progressive weighted wheel running (PoWeR) demonstrated that the myonuclei within the plantaris muscle that accumulated following 8 weeks of training were lost after 3 months of detraining and the muscle reverted to an untrained phenotype [95]. An additional study of 6-months of detraining after 8 weeks of PoWeR showed that myonuclei returned to untrained levels in both the plantaris and gastrocnemius, with these myonuclei being longer and in the gastrocnemius miR-1, which represses pro-growth processes, levels remaining lowered [100]. This led to the proposal of a new muscle memory model in which elevated myonuclei could potentially serve as one component of muscle memory of previous hypertrophy in the short term (e.g. <3 months in mice) but that alternate epigenetic mechanisms such as, myonuclear DNA methylation, histone modifications, and miRNA expression, could explain muscle memory in the long term after myonuclear number has stabilised [100]. Further research is required to investigate these claims [100] but there is evidence to support the role of epigenetic mechanisms, with a human study involving resistance training finding evidence of an epigenetic memory of hypertrophy [101].

Another study of 26 healthy older adults was conducted in which 24 weeks of supervised resistance training was performed [99]. The participants experienced significant increases in myonuclei number and CSA of their type II muscle fibres in their vastus lateralis, with no significant differences observed for type I fibres [99]. After one year of detraining, myonuclei levels returned to baseline and were significantly lower than post training and these changes were mirrored by a decrease in fibre CSA [99]. A resistance training, detraining and retraining study in humans tested the muscle memory mechanism but only 7 of the 19 participants demonstrated an increase in myonuclei number post training [102] and reanalysis of this publicly available data [96] demonstrated, contrary to the muscle memory phenomenon, a significant reduction in myonuclei number with detraining in 6 of these 7 individuals [96].

Given these conflicting studies on myonuclei permanency, a literature review concluded that current evidence provides no consensus on the existence of muscle memory by myonuclear permanency [103]. Furthermore, a systematic review and meta-analysis of the body of literature also did not support the concept of skeletal muscle memory based on myonuclear permanency [104]. A recent crosstalk [105] has further highlighted the contrasting views on myonuclear permanency, with some authors detailing that the selective destruction of myonuclei within a syncytium, with diffusible apoptotic enzymes, is too difficult to fathom as there is no obvious mechanism for selectively targeting individual nuclei within a common cytoplasm [106]. With a rebuttal detailing that transcript and protein compartmentalization does occur in myofibres, contrasting authors argued that the unique cellular architecture of skeletal myofibres would necessitate a similarly unique mechanism to selectively target and remove myonuclei [107]. This yet to be identified mechanism, for authors who support myonuclear permanency, adds weight to their argument that myonuclei are permanent and that selective destruction in a syncytium is unfeasible [108]. Authors that question permanency, state that novel technologies such as genome-editing that enable myonuclei labelling, will allow investigation into myonuclei turnover by seeing how much these genetic corrections get 'diluted' over time via the fusion of non-edited satellite cells [105]. If a dilution of labelled myonuclei did occur, it would question the concept of permanency and possibly suggest that an unknown mechanism does exist to remove-and-replace entire myonuclei within the muscle fibre syncytium [105]. This mechanism could be targeted to ensure low numbers of myonuclei are removed, or non-targeted with myonuclei being removed randomly, but this happens only to a small number of myonuclei so that even if some myonuclei are removed, some are still being maintained to preserve essential transcription.

The last words on this debate from Kirby and Dupont-Versteegden, who question permanency, concluded that short-term disuse studies should no longer be the primary model to study myonuclear loss and that longer term studies should be implemented, ideally with modern technologies, that can investigate permanency on a longer time scale, including techniques (labelling/epigenetic) that can measure myonuclear "age" [105]. The last words from supporters of permanency, Schwartz and Gundersen, are that both an unequivocal demonstration of myonuclear loss and identification of the underlying mechanism that could enable this selective destruction needs to be shown to counteract the purported difficulty of selective apoptosis in a syncytium. Overall given the lack of consensus on the myonuclear

permanency debate it has been suggested that more research, particularly in humans, is warranted to experimentally test the muscle memory mechanism [103, 104].

1.5. Previous observational studies on AAS hypertrophy in strength trained athletes.

The powerful myotrophic effects of testosterone and AAS have also been identified in a variety of studies using a population of elite-level powerlifters [109-111]. Eriksson [111] recruited nine powerlifters who self-reported the use of high doses of AAS for a period of 9.0 ± 3.3 years. At the time of the biopsies the mean weekly AAS dosage was 938 ± 527mg. Ten other powerlifters, who had never used AAS, were also recruited and the powerlifting performances of both groups of athletes were comparable to some of the best powerlifters in the world [109]. Muscle biopsies obtained from the trapezius muscle and vastus lateralis demonstrated that the mean fibre area of both Type I and IIA fibres in the steroid using powerlifters was significantly larger than in the non-steroid using powerlifters. Seven male powerlifters who had previously used anabolic steroids were also recruited, along with a sedentary control group comprised of 6 participants [111]. The previous anabolic steroid users had ceased usage for more than one year (mean 8.1 ± 3.2 years) and they had previously used anabolic steroids for a period of 4.5 ± 0.5 years. At the time of the muscle biopsies three of these previous users had stopped all forms of physical exercise and the other four were still performing strength training but with various degree of intensity. Interestingly, in both the vastus lateralis and the trapezius muscles, the mean fibre area (all fibre types) in the previous steroid users was significantly smaller than in the current steroid users, but not smaller than in the previously described clean powerlifter group [111]. The previous users had significantly larger fibre areas than the control group in both muscles.

In the vastus lateralis, the previous users had significantly fewer myonuclei per fibre compared to the current users, but numbers were comparable to non-steroid using powerlifters and still higher than the control group. Whereas in the trapezius muscle, the previous users had significantly more myonuclei per fibre than all other groups and the previous users had significantly more myonuclei in their trapezius muscle compared to their vastus lateralis muscle. In the vastus lateralis there the only significant difference in the size of the nuclear domains was between clean powerlifters and controls. However, in the trapezius, the previous users had smaller nuclear domains than all other groups. Furthermore,

the previous users had smaller nuclear domains in their trapezius muscle fibres compared to their vastus lateralis muscle fibres.

In the current users, the non-steroid using powerlifters and the control group the proportion of AR containing myonuclei per fibre cross-section was significantly higher in the trapezius compared to the vastus lateralis [112]. In the vastus lateralis there were no differences in the proportion of AR containing myonuclei between these groups. However, in the trapezius muscle the proportion of AR containing myonuclei was significantly higher in the non-steroid using powerlifters compared to controls and in the current users compared to both these other groups.

If assuming muscle fibre cross sectional area in the previous users when they were using anabolic steroids was comparable to the current users, fibre cross sectional area must have decreased after cessation of usage. However, the fibre cross sectional area in the previous users is still comparable to that of active elite trained powerlifters who have not used anabolic steroids. The high number of myonuclei per fibre in the trapezius muscle in the PREV group is one possible mechanism for the large fibre areas in this group despite the lack of high intensity training. This means that after steroid usage the prevention of muscle fibre atrophy is potentially mediated by the high amount of myonuclei in the trapezius muscle. These findings also potentially indicate that the myonuclei incorporated during strength training and AAS usage do not undergo atrophy, even with a decreased demand in training volume and subsequent decrease in the demand for protein synthesis.

Eriksson's study (2006) [111] did not reveal if the observed morphological changes within skeletal muscle were accompanied by improvement in muscle strength and body composition [113]. Yu *et al.*, (2014) [113] conducted a study to investigate the long-term effects of AAS administration on muscle strength and morphology and if a relationship exists between these variables and AAS dosage through time. Seventeen strength training elite athletes were recruited: 10 were current uses of AAS (Doped group; age 41.1 ± 8.0 years) and 7 reported never using AAS (Clean group; age 29.1 ± 6.2 years). The Doped group consisted of a mixture of bodybuilders and strongmen competitors whereas the Clean group consisted of powerlifters only. Subsequently the mode of training differed between groups with the Doped group using both 1–4 repetitions/set and 8–12 repetitions/set, while the Clean group used mainly 1–4 repetitions/set. However, all subjects reported that they had trained regularly between 4–6 times per week for at least five years.

Reported AAS dosages were higher in Yu *et al.* (2014) [113] compared to Eriksson (2006) [111], the average testosterone weekly dosage used was 850mg and 875mg, over the past 5 years and more than 5 years ago, respectively. Four of the 10 participants reported using over 1000mg·wk^{-1} of testosterone over both time frames with one participant using 2000mg·wk^{-1} in the past 5 years and 2500mg·wk^{-1} more than 5 years ago [113]. There was no difference in fibre type proportions amongst the Doped and Clean groups, similar results have also been shown elsewhere [19]. In contrast to these other studies there was no difference in mean fibre area of either Type I or Type IIA muscle fibres between groups [113]. However, the Doped group did have a 15% larger mean fibre area and a larger variation in fibre area was observed with the Doped group having Type I fibre areas of 3,734 - 15,208μm^2, whereas in the Clean group Type I fibre areas were 4,408 - 6,139μm^2, with this large fibre area discrepancy in the Doped group likely due to variation in AAS exposure. The Doped group had significantly more nuclei per Type I fibre, but not per Type II fibre and when fibre area was taken into consideration this significant difference disappeared. The Doped group had significantly more lean leg mass than the clean group, but the clean group had significantly higher maximal squat force and significantly higher maximal squat force per lean leg mass. Additionally, the clean group also had significantly higher maximal squat force per mean fibre area, as well as significantly higher maximal squat force per mean Type I & Type IIA fibre area. Within the doped group AAS dose was significantly correlated with lean body mass, mean fibre area and nuclei per fibre area. Furthermore, maximal squat force relative to muscle mass, maximal squat force relative to fibre area, capillary density, and nuclei density were the most important variables in separating Doped athletes from Clean athletes in a multivariate statistics model [113]. The interesting finding that the Doped group had higher lean leg mass, but lower leg strength than the Clean group could be explained by the training regimens of the participants as the Clean group were powerlifters who compete in the specific event of a 1RM squat, whereas the Doped group were bodybuilders and strongmen who do not compete with this metric of leg strength.

Proteomic analysis of muscle samples from the Doped and Clean groups from Yu *et al.* (2014) [113] was published in 2020 [114] and clear separation of Doped and Clean groups occurred with the same multivariate statistics model [114]. Liquid chromatography followed by tandem spectrometry identified 14 protein spots, representing nine different proteins, of significant difference in relative quantity between Doped and Clean groups [114]. According to their major functions, the nine proteins could be briefly classified into four different

categories, metabolism, motility and contractile, cell protection, and transport/storage proteins [114]. The authors suggest that possible a proteomic method applied to muscle tissue could aid doping detection, particularly for gene-doping, but also acknowledge that age and training differences between the groups could have affected these results and so they should be interpreted cautiously [114].

Another observational study [115] recruited current (n = 7) and past (n = 11) AAS users. Past AAS users comprised of two cohorts one which ceased AAS use 3-months prior to sampling and another which stopped using AAS within the last two years after having used AAS in supraphysiological doses (≥500mg per week) for ≥6 months [115]. The past and current users respectively had AAS exposure times of 144.1 ± 125.5 weeks and 127.7 ± 68.0 weeks. For the vastus lateralis no significant differences were observed for myonuclei per muscle fibre for Type I and II fibres between current and past AAS users and resistance training non-AAS users [115]. However, a significant difference in myonuclear domain between resistance training non-AAS users (1587.4 μm^2 ± 181.4 μm^2) and former AAS users (1431.0 μm^2 ± 197.4 μm^2) was observed for type II muscle fibres (p = 0.0438), potentially suggesting elevated myonuclear density in former users.

1.6. A brief history of AAS doping detection in weightlifting and Summer Olympic sports and the formation of WADA.

Several anecdotal reports suggest that Californian bodybuilders in the late 1940s and early 1950s began experimenting with the use of testosterone preparations [116] and the first systematic use of testosterone in Olympic sports was reportedly in 1952 by Soviet Weightlifters [117]. In 1954, at the World Weightlifting Championships, the US Olympic Weightlifting team physician, Dr John Ziegler, was told by his Soviet counterpart that Soviet weightlifters were using testosterone preparations and this could explain their exceptional performances at the 1952 Olympics and the 1954 World Championships [117]. After the completion of the world championships, Dr Zeigler returned to the U.S. and immediately began experimenting with testosterone use, but he came concerned with the potential side effects. This led him to search for a drug that enhanced anabolic effects but reduced androgenic side effects. In 1958, the first U.S. manufactured androgen Dianabol (Metandienone) was approved by the U.S. Food and Drug Administration, and this provided a potential solution to the problems Dr Zeigler was experiencing. Dr Zeigler noted that weightlifters using Dianabol experienced large increases in performance [116]. News of the

effectiveness of Dianabol as an ergogenic aid spread to other strength and power-based sports, including track and field and American football [117]. By the 1964 Olympics it is believed that steroid usage was widespread [118]. By 1966 the German Democratic Republic (GDR) began using dehydrochloromethyltestosterone (Oral-Turinabol) in a state-sponsored doping program to enhance their athletes' performances at the 1968 Games [2]. In 1967 the International Olympic Committee (IOC) established a medical commission that aimed to develop a list of prohibited substances and methods [119]. The IOC also adopted a medical code that encompassed 3 principles: (a) protection of the health of the athlete, (b) respect for both medical and sports ethics, and (c) equality for all competing athletes [119]. However, in the 1968 Olympics all the U.S. Olympic Weightlifters reportedly admitted using steroids and reports suggest that one third of the U.S. track and field team had used steroids in the lead up to the games [117, 120]. During these years the IOC failed to include androgens on the banned substance list [2] because the medical community suggested that androgens were ineffective at increasing performance, as exemplified by the 1977 ACSM position stand stating that AAS were not performance enhancing [2, 12]. This resulted in a large credibility gap between athletes and the medical community that resulted in many athletes developing a distrust for medical doctors. Additionally, there was no reliable or valid test for AAS during this time so the IOC could not police their usage if incorporated onto their list of prohibited substances. In 1973 the IOC adopted both radioimmunoassay and gas chromatography and mass spectrometry (GC-MS) techniques to detect orally active alkylated steroids [4, 117]. A significant problem of these initial testing procedures was that very few laboratories in the world possessed all the equipment and computer data to do IOC level testing [117]. The new testing procedure was first used on a trial run basis at the Commonwealth Games in Auckland, New Zealand, in February 1974. No sanctions were imposed and the participating athletes were not identified, but nine of the 55 samples tested contained oral steroids [117]. The IOC Medical Commission reacted quickly to these results and two months later AAS were introduced as a banned class of compounds in the Anti-Doping Code [4]. The first Olympic use of the new test came in 1976 at the Montreal Games, and only eight athletes out of 275 tested were found positive for oral steroids (seven weightlifters and one discus thrower). It would be easy to assume from such a low percentage of steroid positives that the athletes had simply decided to come "clean" to the 1976 Games, however, despite the IOC's best intentions, they had left a way to get around anti-doping tests because there were still some compounds that enhanced athletic performance but for which the IOC had not developed a test [117]. For example, it has been reported than many athletes reverted from

using oral steroids, such as Dianabol, to injectable testosterone esters because no test existed for testosterone at that time [117]. However, in 1980, Professor Manfred Donike the head of the IOC approved drug testing laboratory in Cologne, West Germany, developed a method for detecting testosterone use by comparing the testosterone (T) to epitestosterone (E) ratio (T/E ratio) [4]. Research based testing on samples collected from the 1980 Moscow Games showed that ~20% of all athletes tested and 7.1% of female athletes tested had a T/E ratio of >6:1, which is suggestive of doping, and this test was used by the IOC from the 1984 Games onwards, although these athletes from the 1980 Games were not sanctioned [2]. For further discussion on the detection of endogenous steroids please see section 1.8.

In the 1988 Olympic Games in Seoul Canadian sprinter Ben Johnson had his world record 100m performance annulled and his gold medal rescinded after testing positive for the AAS stanozolol [2]. This scandal sensitized the sporting world to the need of Out Of Competition (OOC) testing for detecting AAS abuse [121] and the International Association of Athletics Federations (now World Athletics) introduced this in 1989, but most other international federations (IF) were initially slow in their adoption of OOC. The fall of the Berlin Wall in 1989 resulted in the full extent of state sponsored GDR doping being revealed in the 1990's [122]. It is believed that as many as 10,000 athletes were part of the programme, some as young as twelve, and were administered a variety of performance enhancing drugs (PEDs) by scientists, doctors and coaches who described the Oral-Turinabol pills they were administering as "vitamins" to maintain the secrecy of the regime [122]. The Ben Johnson scandal and revelations of the GDR system changed attitudes to doping within sport in the 1990's and it was acknowledged that doping was a threat to the reputation of the Olympic movement and sport in general [121]. However, in the 1990's due to the uncoordinated way in which IF and the IOC policed anti-doping there were inconsistencies in which rule violation penalties were applied across sports [121]. There was no harmonisation in the lists of prohibited substances across IFs or how athletes were managed who required the use of banned substances for therapeutic reasons and large discrepancies in the level of OOC testing across IFs [121]. For example, at the 1996 Atlanta Olympic Games the stimulant Bromantan was detected in the urine of four athletes, including two medallists, all of whom had their originally disqualified results overturned on appeal on the basis that the drug was not a stimulant but used to boost the immune system [123-125]. The IOC-MC would subsequently prohibit the usage of Bromantan [123] and it still remains a prohibited substance [126]. In 1999 the IOC convened the World Conference of Doping in Sport in Lausanne and this

conference served as the foundation of an international anti-doping initiative, which resulted in the formation of WADA in 2001 [2]. The immediate challenge for WADA was generating a set of universally excepted rules, known as the WADA Code, that contained international standards for laboratories, testing procedures, substances contained on prohibited lists, and mechanisms and rules for therapeutic exemptions [2]. The IOC compelled the Olympic federations to adopt the Code and stated those who did not by the opening of the 2004 Athens Games would not be allowed to have their sport on the Olympic program [121]. Consequently, all federations adopted the Code, and it went into effect on January 1st 2004 [121, 127].

1.7. WADA & IOC policy for sample retesting to retrospectively catch doping athletes.

In anticipation that anti-doping analytical techniques will improve in the future and to deter doping, the IOC financed the shipment and long-term storage of all anti-doping samples collected at Olympic venues from 2004 onwards, with the initial statute of limitations for a retrospective Anti-Doping Rule Violations (ADRV) from sample re-analysis being set at 8 years and later extended to 10 years in the 2015 WADA Code [127, 128]. Anti-doping authorities can retest samples at any point during this window of time as a function of the implementation of new methods or instruments in WADA accredited laboratories allowing the detection of prohibited substances or their metabolites at a much lower concentration or for a larger detection window [129]. Critics of reallocating Olympic medals via the retrospective re-analysis of samples, say this reduces live sport to “meaningless spectacles” as until the retesting is concluded (which could be 10 years later) as the initial results are provisional as neither the athletes nor spectators know who the real medal winners are [130].

Between 2004 and 2008 WADA, the pharmaceutical industry and the Lausanne anti-doping laboratory put resources together to create an enzyme-linked immunosorbent assay for a third generation Erythropoietin (EPO) called Continuous EPO Receptor Activator (CERA) [128]. This test was made ready before CERA was available on the market due to the high likelihood of it being utilised as a doping substance [128, 131]. The first re-analysis of Olympic samples was conducted 6-months after the 2008 Beijing Olympic Games [129] in which all serum samples collected during these Games were retested with this new test for CERA [131]. Six athletes, including two medallists, tested positive [125]. The next major re-testing of Olympic samples occurred after advances in the sensitivity of chromatographic/mass spectrometric techniques [132]. These advances enabled

improvements in the detection window of exogenous AAS via the discovery of the long-term metabolites (LTMs) for compounds such as metandienone [133], oxandrolone [134], dehydrochloromethyltestosterone [135, 136] and stanozolol [137]. The IOC used these improved analytical methods to initiate the first targeted retrospective re-analysis of urine samples collected at the 2004 Athens Games in 2012 [128].

Prior to the Rio Olympic Games in 2016 the IOC initiated a re-analysis programme that utilised these improved analytical methods on samples collected during the Beijing 2008 and London 2012 Olympic Games and by March 2016, the targeted re-analysis of hundreds of samples was already underway [138]. The IOC has not disclosed the exact test distribution plan for the retesting of these samples (e.g., exact numbers of which sports/nations were retested) as they regard this as "*useful information for cheaters - the more unpredictable testing is, the more effective the deterrence*" [139]. However, the IOC notes that the selection of samples for re-analysis was made in consultation with WADA and IFs after a risk analysis and it focused on sports and groups of athletes with a higher risk of doping and who were successful [139]. Selection was also dependent on the number of samples collected, the number of athletes at the Games in each group and had the aim of preventing athletes who cheated in these Games from competing in Rio 2016 [139]. Additionally, after receiving the completed WADA Independent Person Report in December 2016 the IOC mandated the examination of all collected samples from Russian athletes during the London 2012 Games following findings of a systematic and centralised cover up and manipulation of the doping control process around this time [140]. Four-thousand eight hundred anti-doping tests were carried out during Beijing 2008, where 10,948 athletes were present and after the conclusion of the 8-year statute of limitations 1,053 samples had been selected for re-analysis [139]. Five-thousand anti-doping tests were carried out during London 2012, where 10,568 athletes were present and by 2017 the IOC stated that 492 samples were selected for re-analysis [139].

1.7.1 The subsequent doping crisis in weightlifting.

By June 2017 a targeted reanalysis of samples collected from the Beijing 2008 and London 2012 Olympic Games, in which a total of 515 weightlifters competed, had resulted in thirty weightlifters having their medals rescinded as they had retrospectively been identified to have committed an ADRV [141, 142]. At this time Thomas Bach the IOC President said weightlifting had "*a massive doping problem*" [143] and the IOC Executive Board instructed

the International Weightlifting Federation (IWF) to demonstrate by December 2017 that it had addressed, or had put in place plans to address, the serious incidence of doping if the sport was to be considered for inclusion in the 2024 Olympic Games [144]. This targeted reanalysis of weightlifting samples took advantage of improvements in the detection window of exogenous AAS via the discovery of LTMs [132] for compounds such as metandienone [133], dehydrochloromethyltestosterone [135] and stanozolol [137].

In response, the IWF created two new independent commissions to advise on anti-doping policy changes which respectively became the Clean Sport and Sport Programme Commissions [145, 146]. Additionally, the IWF started a series of actions to combat doping and in 2017 announced one-year suspensions for nine Member Federations (MFs) found to have had three or more ADRVs from the retesting of samples taken at the 2008 and 2012 Olympic Games [147]. The IWF also enforced a new qualification system for the Tokyo 2020 Olympic Games [148] and each athlete must compete in a minimum of six eligible events that occur within defined time frames to increase the likelihood of being tested in-competition prior to the Olympic Games. This will include at least one event between 1 October 2020 and 30 April 2021 to account for the coronavirus pandemic delaying the Olympic Games [148, 149]. The IWF also announced limitations on MFs for participants per country for the 2020 Olympic Games based on the MFs doping record since the start date of the 2008 Olympic Games and the end of the 2020 qualification period [148]. MFs that had 20 or more ADRVs would be able to send only one male and one female athlete in total; MFs that recorded 10-19 ADRVs would be eligible to send two male and two female athletes; and MFs with less than ten ADRVs would be eligible to send four male and four female athletes [148]. The IWF also signed an agreement with the International Testing Agency (ITA) to take responsibility for key areas of its anti-doping programme and once this partnership was finalised the IOC lifted the conditional status of weightlifting for the 2024 Olympic Games, citing the positive steps taken by the IWF to combat doping [150, 151]. However, the IOC still reserves it's right to review weightlifting's place on the 2024 Olympic Games Programme, due to the recent revelations of anti-doping corruption in the sport [152].

The Hungarian Anti-Doping Group (HUNADO), who carried out a large proportion of the anti-doping tests requested by the IWF in the last decade, and both the IWF and ex-President Tamás Aján, who's tenure started in 2000, have had recent accusations of anti-doping corruption with irregularities in OOC testing, urine sample manipulation and the

disappearance of positive doping results [153] which eventually resulted in Ján's resignation in April 2020 [154]. While an independent report concluded that HUNADO had acted in accordance with WADA standards [155], it also concluded that former President Tamás Ján had breached confidential information for the planned dates of OOC testing potentially leaking this information to certain nations or athletes [155]. The IWF also deliberately delayed notifying 18 Azerbaijani athletes of their ADRVs, thus enabling them to win medals at international competitions in 2013 [155]. The report also identified that 21 Turkish weightlifters provided samples resulting in Adverse Analytical Findings (AAFs) during OOC tests but they were not followed through appropriately as although the IWF president was notified of these AAFs the athletes continued competing and winning medals [155]. These cases, plus 41 hidden cases and 10 possible other cases where the AAFs have not been followed through have been forwarded onto WADA for further investigation [155]. The investigative team also found evidence that an additional 130 samples had been taken but not processed [156]. This was information absent from the original report due to insufficient time to investigate prior to the report deadline [156]. Due to the WADA and the ITA investigation currently being open on this case it is not publicly known how many ADRVs these unprocessed samples relate to, with WADA "m*onitoring this closely to ensure no case is left unprocessed*" [156].

1.8. Detecting endogenous steroid doping.

The misuse of endogenous AAS is detected via alterations in the urinary steroid profile. The main parameters of the urinary steroid profile are the concentrations and ratios of the glucuronidated testosterone metabolites androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (5α-diol) and 5β-androstane-3α,17β-diol (5β-diol), in addition to glucuronidated epitestosterone (which originates from the biosynthesis of testosterone) and glucuronidated testosterone [132]. A test based on whether a urine concentration of testosterone exceeds the upper limit of a reference range would be insensitive because of the large observed interindividual and intraindividual urinary concentrations associated with single-pass urine collection [4]. However, intake of testosterone and other endogenous steroids causes a characteristic change in the urinary profile of the aforementioned steroids [157]. In 1983 the IOC adopted a test for the detection of testosterone on the GC-MS determined ratio of testosterone to its 17α-epimer epitestosterone, following glucuronide hydrolysis [158]. E is only a minor product of the metabolism of testosterone and does not

increase after testosterone administration and therefore because supraphysiological doses of testosterone will cause an increase in the excretion rate of testosterone the T/E ratio will increase [157]. In healthy men and women, the mean T/E ratio approximates 1:1 [4]. Initially the IOC stated that a T/E ratio of greater than 6.0 constituted a doping violation unless there was evidence that this ratio was due to a physiological or pathological condition such as low epitestosterone secretion, enzyme deficiencies or an androgen producing tumour [157] which can make it physiologically possible for athletes to chronically have a T/E ratio of greater than 6.0 and thus represent "*natural biological outliers*" [4]. Once a T/E ratio greater than 6 is recorded a pathological investigation should be conducted, in addition to reviewal of all previous T/E ratios, to confirm that these natural confounding variables are not the cause of an elevated ratio. If previous T/E ratio data is not available, the athlete should be tested unannounced at least once a month for 3 months [4]. This is because if an athlete was administering testosterone and subsequently stops their T/E ratio will decrease and revert to baseline, whereas for the rare athlete with a chronically elevated T/E ratio this would remain chronically elevated [159]. The T/E detection limit has been subsequently decreased and according to guidance by WADA in 2004, urine samples should now be submitted to isotope ratio mass spectrometry (IRMS) if the T/E ratio is greater or equal to 4.0 and testosterone metabolites, epitestosterone, and dehydroepiandrosterone concentrations are greater than fixed cut-off concentrations [157]. The IRMS test is regarded as providing definitive proof of endogenous steroid doping. Testosterone and endogenous steroids used in pharmaceutical preparations are generally synthesised from stigmasterol, which is obtained from soya beans [4]. Subsequently these pharmaceutical preparations have a lower ^{13}C content compared to their endogenous homologues and urinary steroids with a low $^{13}C/^{12}C$ ratio are determined to have originated from pharmaceutical sources. The cost and analytical facilities required to conduct the IRMS test preclude any routine use of this methodology and therefore its major use is to conform suspected doping in samples with T/E ratios equal or greater than 4.

The role of genetic variation in enzymes that are responsible for the excretion of AAS in the urine has recently been discovered as a confounding variable of the T/E ratio [160]. Uridine glucuronosyltransferase (UGT) enzymes are the main enzymes for the inactivation and elimination of steroid hormones via conjugation with glucuronic acid [161]. UGT2B17 has been shown to be the main enzyme in testosterone glucuronidation activity *in vitro* and *in vivo* [162, 163]. A deletion polymorphism in this gene has been identified and *del/del* individuals have been shown to have no or negligible amounts of testosterone in their urine

[163]. Additionally, *del/del* individuals are 7 times more common in a Korean than Swedish population sample and the T/E ratio was 0.15 (0.08–0.68) in Koreans compared to 1.8 (1.0–2.6) in Swedes at baseline [163]. Individuals with different UGT2B17 polymorphisms (*del/del, ins/del and ins/ins*) were then subjected to a single intramuscular injection of 500mg TE and the urinary excretion of testosterone glucuronide and the T/E ratio over 15 days was calculated [160]. Testosterone glucuronide excretion rate was highly dependent on UGT2B17 genotype with a 20-fold higher average maximum increase in the *ins/ins* group compared to the *del/del* group. Furthermore, of the *del/del* subjects 40% of subjects never reached the T/E ratio of 4.0 on any of the 15 days and their baseline T/E ratios never exceeded 0.4. In the *ins/ins* group 14% had baseline T/E ratios above four, resulting in false-positive T/E ratios. These results indicate the inadequacy of using the same population cut-off T/E ratio for all individuals independent of the UGT2B17 genotype. By using a differentiated cut-off for the *del/del* (1.0) and other genotypes (6.0) the sensitivity in the *del/del* increased substantially (increase in sensitivity from 6 to 53% on day 2 and from 59 and 29% to 100% on days 6 and 11 respectively) and the false positives in the *ins/ins* group were eliminated.

Given that T/E ratios vary widely amongst populations of individuals, but individual T/E ratios do not deviate from the mean value by more than 30%, longitudinal monitoring of the T/E ratio with Bayesian statistics has been suggested as a tool to identify testosterone doping [164]. This Bayesian model sequentially compares newly collected T/E ratios against previous readings from the same individual and gradually evolves from population-derived limits when $n = 0$ to individual-based cut-off thresholds when n is large and has subsequently become integrated within the steroidal module of the Athlete Biological Passport (ABP) as of January 1st 2014 [164, 165]. T/E, T/A, A/Etio, and 5α-diol/5β-diol ratios are monitored longitudinally and an atypical passport finding (ATPF) is declared if a sample value falls outside of the individual limits as defined by the specificity of the adaptive Bayesian model. For example, for an athlete who is not deficient in the UGT2B17 gene with a specificity of 99%, as defined by the adaptive Bayesian model, the initial T/E ratio limits are 0.24-6.88, meaning that only one individual out of a population of 100 male adult athletes not deficient in the gene UGT2B17 should present a value out of this range on average [166]. With a first test result equal to 0.94 the reference range for the second test becomes 0.34-2.44, meaning that only one individual out of a group of 100 male adult athletes not deficient in the UGT2B17 gene, who have shown an initial value of 0.94, must fall out of this range on average [166]. The Bayesian model continues this process iteratively. After an ATPF is

declared the Athlete Passport Management Unit is responsible for contacting an expert to anonymously review it [128]. This expert will examine the longitudinal profile and determine if the ATPF is an example of extreme variation and thus considered normal, or it is suspicious. If no clear doping specific pattern can be identified at this stage the expert can request for more samples to be collected to further investigate the T/E ratio or for an IRMS test to be conducted. As a confirmation procedure the sample can also be re-analysed. If doping is suspected two further experts will anonymously and independently review the data without access to the first expert's comments. Once all aspects have been considered, such as pathologies and sample quality, if the three experts unanimously agree that doping has taken place a formal ATPF is declared and the sporting body for the athlete is informed [128]. Altogether, close cooperation between testing authorities, sample collection authorities and testing laboratories is required to ensure prompt transfer of information and adequate timing of targeted testing to allow the steroidal module of the ABP to be efficient [167]. Due to the reduction in T/E ratio in UGT2B17 *del/del* individuals, if the T/E ratio was not monitored longitudinally within individual set limits, it would be possible for these individuals to take testosterone and never go above population defined limits [128]. For example, 8 urine samples collected from an elite *del/del* athlete gave an average T/E ratio of 0.12 (CV=37%) [128]. Prior reliance on the population defined T/E ratio of 4 would have enabled this athlete to dope with testosterone and not be caught. However, within the adaptive model of the ABP a T/E ratio of greater than 0.44 would result in a ATPF starting the cascade of events described previously to confirm if doping has taken place. Furthermore, it has been demonstrated that UGT2B17 *del/del* individuals challenged with 125, 250 and 500mg of TE never exceeded the T/E ratio of 4 [168]. These athletes could therefore dope with testosterone and not produce atypical urine findings. Once T/E readings were incorporated into the steroidal module of the ABP and ATPF were further investigated with IRMS testing all three doses could be detected with a high degree of specificity [168].

Polymorphisms in UGT2B17 genotypes are not the only confounding variable that influences the individual T/E steroid profile [167]. Both endogenous and exogenous confounding variables can create fluctuations in the urinary steroid profile that must be considered to differentiate natural biological fluctuations from doping. In addition to genetic polymorphisms other endogenous confounding variables include: age, as the T/E ratio is naturally elevated during puberty; gender, as females have lower urinary concentrations of T and its metabolites; circadian variations, as T has been shown to exhibit natural fluctuations

through the year and pregnancy because E glucuronide concentrations are elevated in the first trimester and there is a general decrease in androgen concentrations [167]. Exogenous confounding variables include: hormonal contraceptives, because females using these excrete 40% less E than non-users [169], medications, such as 5α-reductase inhibitors that suppress the formation of dihydrotestosterone from T [166], ethanol consumption, because alcohol can decrease A and Etio concentrations up to 10% of the basal levels and less significantly increase T excretion resulting in a slight rise of the T/E ratio; masking agents, such as liposomes which have been shown to reduce the analytical recovery of glucuronide metabolites [170] and bacterial contamination with enzyme activity linked to microorganisms leading to a rise or a drop of endogenous steroid concentrations [166]. However, manipulation of the urinary steroid profile via each of these exogenous factors can be confirmed via further analysis of urinary metabolites [167]. Furthermore, steroid analyses conducted in different antidoping laboratories also revealed that in some cases, the steroidal passports were flagged due to non-standardized analytical procedures [128]. This subsequently resulted in WADA compiling a new technical document (TD2014EAAS) which outlines mandatory operational procedures for laboratories analysing the urinary steroid profile for the ABP [171].

There are multiple benefits to adopting longitudinal monitoring of a urinary steroid profile in the form of the steroidal module of the ABP [166]. Firstly, longitudinal monitoring of the urinary steroid profile represents a transition in anti-doping testing away from population defined thresholds that, due to confounding factors, are not able to detect doping, towards better defined individual limits that have a higher sensitivity to doping. Secondly, once a T/E ratio is discovered to fall out of the individual reference range an athlete can be targeted for the IRMS test with better efficiency than only conducting IRMS tests when the T/E ratio falls above 4. Thirdly, it is probable that the markers within the steroidal module of the ABP are already sensitive to future generations of doping substances, for example, to all future substances that may increase testosterone concentrations.

1.9. The potential for “omic” technologies to aid doping detection.

The next generation “omic” approach to detect doping is based on the premise that doping methods will cause profound and, therefore, detectable changes in the ways genes are expressed and thereby generate a unique “omic signature” of exposure to a specific doping

practice [172]. This “omic signature” is thought to be difficult to mask and deemed to have the potential to significantly improve the reliability and extend the window of detection of doping tests [172]. For example, both high [173] and low dose [174] recombinant human erythropoietin (rHuEPO) administration studies have shown a whole blood transcriptional signature that has a more prolonged detection window for rHuEPO doping compared to traditional methods [174] that is not confounded by exercise [174] or altitude exposure [174, 175], with this transcriptional signature shown across two microarray platforms (Affymetrix and Illumina) and two RNA-Seq platforms (Illumina and MGI) [176]. Whole blood stored in K_2EDTA tubes, which lack RNA preservative, still yielded RNA of sufficiently high quantity, purity, and integrity for transcriptomic analysis with no impact on genes previously identified in rHuEPO administration studies, potentially indicating that transcriptomic analysis could be integrated into the current haematological anti-doping system, by utilising remaining/excess blood from routine testing [177].

Broadly speaking the omic technologies can be differentiated into targeted and untargeted approaches. Targeted methods aim to quantify single elected factors, such the expression of single RNAs, proteins or metabolites. Untargeted methods screen for large amounts of differentially expressed factors at once [178]. For example, within transcriptomics quantitative real-time polymerase chain reaction technology (qRT-PCR) can detect changes in the expression of single genes in a targeted manner. The original “gold standard” technology for untargeted screening of the transcriptome was cDNA microarray technology. This methodology allows for the analysis of all identified genes in one experiment, but the sequence of these genes must be known. Another problem is the high background level of information owing to cross-hybridisation and the limited dynamic range of detection owing to both background and saturation of signals. This high background level and limited range of detection leads to less sensitivity and thus the expression of low abundant genes is difficult to determine. [179]. The development of novel high-throughput DNA sequencing methods has provided a new untargeted method, termed RNA-Sequencing (RNA-Seq), for both mapping and quantifying transcriptomes. Unlike microarrays, RNA-Seq can detect changes in expression of all regulated genes, irrespective of sequence knowledge, as sequences can be aligned to a reference genome or assembled *de novo* if no reference genome exists. Additionally, there is no upper limit of quantification, a high dynamic range of expression levels is possible, there is nearly no background signal and the technology is sensitive enough

to detect changes in “one single RNA molecule” enabling the *de novo* detection of new mRNA’s or splice variants of expressed genes [179].

1.9.1 Previous studies on AAS transcriptomic signatures to aid doping detection.

Research into using RNA biomarkers to aid the detection of anabolic agents has mainly been conducted in the realm of animal husbandry where these agents are used to increase weight gain and feed efficiency [180]. The use of anabolic agents is approved in some countries such as Mexico, Canada, Australia, South Africa and the USA. However, it has been proven that hormonal residues of anabolic agents are present in meat, and these have potential adverse health effects for the consumer [181, 182]. Within the European Union (EU) the use of anabolic agents in animal husbandry and the importation of meat derived from cattle given these substances has been forbidden since 1988. Within the EU controlled programme residues of anabolic agents are detected with immunoassays or GC-MS. This is problematic because with these methods newly designed drugs cannot be identified until their chemical structure is identified [178]. Additionally, these techniques cannot measure the application of low concentration “hormone cocktails”. The components within these cocktails have additive effects and show comparable physiological effects like a single drug applied in high concentration, but they cannot be measured using the usual techniques because of their low concentrations [178]. Therefore, it is necessary to develop new sensitive screening methods to detect a broad range of substances independent of their structure and concentration and RNA biomarkers have been researched to facilitate this aim [178].

Of particular note was a study investigating the effect of trenbolone acetate plus estradiol on gene expression in liver samples from Nguni heifers with RNA-Seq [183]. RNA-Seq gene expression analysis resulted in 9331 significantly regulated transcripts between control and treatment groups. Forty of these significantly regulated genes were selected as candidate genes to be validated by qRT-PCR. Of these 40 candidate genes 20 were significantly regulated, with fold regulation values from 0.19 up to 82.36, with 9 genes being significantly down-regulated and 11 genes significantly up-regulated. By using Principal Component Analysis (PCA) on these 20 significantly regulated genes a clear separation between treatment and control groups could be obtained. After the potential of these 20 genes to act as biomarkers, further experiments were conducted to verify these candidate biomarkers in crossbreed boars, who were also treated with trenbolone acetate plus estradiol and in Holstein Friesian calves who were treated with a combination of estradiol benzoate, testosterone

decanoate, and testosterone cypionate via pour on (i.e., topical administration), either once or three times [183]. Fourteen genes could be successfully quantified in boars, with 4 of these genes showing a significant regulation between control and treatment groups, with fold changes between 0.04 and 5.04. PCA analysis on these 14 genes showed a clear separation between both groups. For the calves treated with pour on hormone mixtures only one gene was significantly regulated (fold change of 2.27) in the group treated once with the hormone mix and three genes showed significant regulation in the three times treated group, with fold changes between 0.51 and 3.02. PCA analysis conducted on these 4 regulated genes demonstrated that the three times treatment group separates from the once time treated group and controls, but there is incomplete separated between control animals and calves treated with the hormone mix only once. Analysis of weight gain across these 3 groups demonstrated that weight gain was significantly higher in the three times treated group compared to controls, whereas there was no difference in weight gain between the one-time treated group and controls. This indicates that only one treatment via pour on did not have a significant anabolic effect, possibly explaining why PCA analysis could not fully separate this group from the controls. The results obtained in Nguni heifers and boars treated with trenbolone acetate plus estradiol represent the first RNA biomarker candidates for the detection of this substance cocktail. In calves treated with a hormone cocktail consisting of androgens and estrogens, 4 of these 20 candidate genes could be verified as biomarker candidates. The fact that biomarkers verified in cattle and calves could be verified in pigs leads to the hypothesis that gene expression biomarkers are independent of species, breed, sex, reproductive state and age. However, for the verification of these genes and for the identification of future potential biomarkers, more trials with other anabolic substances and different species are necessary. Furthermore, a higher number of untreated samples covering a wide range of influencing factors such as age, immune status and housing condition will also be needed for validation. Despite these complications it was speculated that the gene expression biomarkers identified in these animals could act as potential biomarkers for the detection of AAS doping in human sports [183].

One of the few human studies that investigated the potential of an RNA signature to detect AAS doping used circulating miRNAs [184]. In this 5-week study, plasma was collected during the first week to give a collection of control samples from the participants. In the second week two transdermal patches that delivered 2.4mg/24hr of testosterone were administered and after a washout period of 2-weeks two 40mg testosterone undecanoate (TU)

tablets were ingested by each participant in week 5 [184]. 372 miRNAs were profiled by a qRT-PCR array in samples obtained before (0h) and at various time points (12hr and 24hr) after oral and transdermal testosterone administration. miR-122 was the only miRNA that increased in abundance under both conditions with an average increase of 2.3-fold in both conditions. After 24hrs the levels of miR-122 were significantly higher in both conditions relative to 0h and the control. The expression of miR-122 remained constant throughout the day during the control period, supporting the idea that the peak of miR-122 at 24 hrs was due to testosterone administration and not circadian rhythm. The current urinary approach of monitoring elevated T/E ratios has a detection window of 2-12hrs for low dose oral testosterone, so the discovery of significantly elevated levels of a biomarker after 24 hours is promising for enhancing the detection of this doping strategy. Due to high interindividual variability circulating miR-122 levels were investigated within subjects by longitudinally monitoring levels and seeing if they surpassed a subject-based threshold of the mean miR-122 level + 3 standard deviations of the corresponding values collected in the control phase. In both UGT2B17 *ins/ins* and *ins/del* individuals longitudinal monitoring of plasma miR-122 levels with this subject based threshold was more sensitive after oral than transdermal testosterone administration. Interestingly serum free testosterone was shown to significantly increase during 8-24 hours after transdermal testosterone administration compared to baseline, whereas oral ingestion of testosterone did not affect serum testosterone which exhibited similar kinetics to the control period.

These findings give support for miR-122 as a novel transcriptomic biomarker for low-dose testosterone abuse and the researchers note some advantages of using this RNA biomarker [184]. Firstly, in contrast to classical urinary biomarkers such as the T/E ratio circulating miR-122 is independent of UGT2B17 genotype. Secondly, the detection window is longer than the individual monitoring of urinary metabolites, especially in the context of oral testosterone administration, which is between 2hrs and 12hrs but miR-122 was significantly elevated after 25hrs [184]. Despite these advantages a single biomarker is not sufficiently powerful and circulating miR-122 should therefore be combined with the numerous other biomarkers that are already used within the steroidal module of the ABP to increase the sensitivity of testosterone screening. Furthermore, a notable confounding factor to using circulating miR-122 as a biomarker for testosterone abuse is that this is a liver specific miRNA that is known to increase in the circulation after drug induced liver injury (e.g., from acetaminophen) [185]. Thereby, it could be very difficult from circulating miR-122 levels

alone to determine that an elevation from circulating miR-122 is exclusively from testosterone abuse. An androgen response element is present in the promotor region of miR-122 [186] and so it can be hypothesized that supraphysiological levels of testosterone are inducing enhanced miR-122 transcription rates and subsequent miR-122 secretion. However, an alternative explanation is that because testosterone is metabolized by the liver and subjected to first-pass metabolism an increased activity of miR-122 could occur resulting in enhanced miR-122 secretion.

1.10. Thesis Aims.

1.10.1. Doping Practices in Weightlifting.

Given the doping crisis in weightlifting noted in section 1.7.1 the aim of analysing the doping practices of international weightlifters is to aid the sport fight doping as its ongoing commitment to clean sport is required to allow the sport to be on the 2024 Olympic Games Programme [152]. Even though WADA and ITA investigations are still open regarding identifying the full extent in which AAFs have been hidden by the ex-IWF president, an analysis of salient prohibited substances noted in sanction data by geographical location can still build a clearer picture of doping practices. This will aid governing bodies and anti-doping authorities in identifying regions with higher rates of doping for improved targeted testing and educational programmes. Furthermore, an analysis of the retrospectively identified ADRVs from the retesting of samples from the Beijing 2008 & London 2012 Olympic Games has yet to be conducted. This study aimed to additionally analyse this dataset to further investigate doping practices in weightlifters as the doping identified by this retesting programme was a key event in the IOC reviewing weightlifting's place at the 2024 Olympic Games.

1.10.2. AAS doping at the Summer Olympic Games and the success of retrospective doping identification via sample re-analysis.

As noted in section 1.7. since the original 2004 WADA code it has been IOC policy that Olympic samples are placed into long term storage for potential re-analysis. The 8-year statute of limitations for sample re-analysis from London 2012 concluded in August 2020 finalising the IOC retesting programme of samples collected during the 2004, 2008 and 2012 summer Olympic Games. A study has not yet been conducted to investigate the effectiveness

of identifying doping in Olympic medallists from long-term sample storage and re-analysis. Thereby, this study aimed to collate all summer Olympic medal winning results impacted by doping across 1968 – 2012 and classify if the doping was identified retrospectively or not, to determine the effectiveness of this storage and retesting programme.

1.10.3. The influence of AAS on "muscle memory" in humans – The MMAAS Project.

As noted in section 1.3. and section 1.4. due to conflicting data, current evidence provides no consensus on the existence of muscle memory by myonuclear permanency (in humans or animal models), and more research is needed to test this hypothesis. Although other studies [111,115] have investigated myonuclei values in cohorts of past AAS users to investigate if they are elevated compared to current users and/or non-AAS users there is a lack of longitudinal data after AAS cessation on myonuclei number in humans. The Muscle Memory Anabolic Androgenic Steroid (MMAAS) Project aimed to recruit past AAS users, to further build on this literature base, and to longitudinally monitor current AAS users after the cessation of usage to further investigate AAS cessation on muscle fibre parameters pertinent to myonuclear permanency in humans. Control groups of resistance trained non-AAS users and non-resistance trained males were also recruited for comparative purposes. The MMAAS Project is used as a term in this thesis to describe this cohort of recruited participants.

1.10.4. The influence of AAS on the transcriptome of whole blood and skeletal muscle in humans.

As noted in section 1.9.1. RNA-Seq has shown some preliminary promise to aid doping detection in animal husbandry [183] and circulating miR-122 might be a testosterone biomarker in humans [184]. Furthermore, given that rHuEPO administration studies [173, 174] have shown a cross-platform [176] whole blood transcriptional signature to rHuEPO doping and that AAS usage is known to stimulate erythropoiesis directly and EPO synthesis in the kidney [187] it is possible that AAS might generate a human whole blood transcriptional signature. However, RNA-Seq has yet to be conducted on whole blood samples from AAS users and controls and thereby this study aimed to conduct RNA-Seq on whole blood samples collected in The MMAAS Project. RNA-Seq has also not been conducted on muscle samples from AAS users and thereby this also aimed to conduct RNA-Seq on the muscle samples collected in The MMAAS Project. This will further our

knowledge of the genomic mode of action of AAS in hypertrophic pathways and complement previous research on this cohort of participants on muscle fibre parameters related to hypertrophy and muscle memory. A transcriptional signature of AAS doping in muscle may also be identified.

2. Materials and Methodologies.

2.1. Data acquisition and entry of IWF Sanction data and ADRVs from 2008 & 2012 Olympic Games retesting.

Data from 2008 to 2019 were obtained from the IWF Sanction List publicly available on the IWF website [188] in February 2020. For weightlifters who had announced retrospective ADRVs from AAFs noted in the retesting of samples from either the 2008 or 2012 Olympic Games, data were obtained from the IWF Sanction List [188] and other publicly available web pages: IOC "Fight Against Doping" Press Releases [189], IOC [190, 191] and IWF Event Results Pages [192, 193] and IWF Anti-Doping News Archives [194] in mid-May 2020. All detected substance names were made uniform and identified to the parent compound which generated the noted metabolite.

For classification of substances as a marker indicating endogenous AAS (EAAS) usage WADA technical documents were utilised [195]. These state that EAAS administration can cause alterations in the markers of the urinary steroid profile which is comprised of: androsterone, etiocholanolone, 5α-androstane-3α,17β-diol (5αAdiol), 5β-androstane-3α,17β-diol (5βAdiol), testosterone and epitestosterone. Additionally, the administration of testosterone or its precursors, androstenediol, androstenedione, dehydroepiandrosterone or a testosterone metabolite, dihydrotestosterone, or a masking agent such as epitestosterone are proven to alter one or more of the parameters of the urinary steroid profile [196] and therefore any mention of a component of the urinary steroid profile or these substances was denoted as a marker of EAAS usage.

Each sanction was classified based on: (1) the IWF Continental Federation (Africa, Asia, Europe, Oceania, Pan America), and (2) the category of the detected substance/prohibited method as described by the 2019 WADA Prohibited List [197]. Three sanctions were omitted from any analyses that involved comparisons of, or counts of, detected substances/prohibited methods because this information was absent or only the article number that was violated by the Anti-Doping Policy of the IWF was stated.

2.1.1. Statistical analysis of IWF Sanction data.

Fishers exact test was used to investigate if four IWF Continental Federations (Europe, Africa, Asia, Pan America) had differences in the detection of exogenous AAS metabolites, markers indicating EAAS usage and other substance metabolites, in a 4x3 matrix, from the sanctions between 2008-2019 obtained from the IWF Sanction List publicly available on the IWF website [188] in February 2020. An adjusted alpha level of 0.05 was used with the Benjamini–Hochberg [198] false discovery rate method for multiple comparisons. Data analysis was conducted using R version 3.6.3 [199] using the tidyverse [200], data.table [201] rcompanion [202] choroplethr [203] and choroplethrMaps [204] packages. The data files and R code used in this study have been made publicly available online [205].

2.2. Data acquisition and entry of ADRVs identified from IOC re-analysis of samples from the 2004, 2008 & 2012 Olympic Games.

Data on athletes retrospectively identified to have committed an ADRV at the 2004, 2008 and 2012 Olympic Games, via the IOC's targeted re-analysis of samples, were obtained from publicly available data published by the IOC on April 28th 2020 [206], the Athletics Integrity Unit (AIU) list of Provisional Suspensions in Force [207] (last updated on 16th July 2020), the AIU Global List of Ineligible Persons [208] (last updated 28th July 2020) and the International Weightlifting Federation's Public Disclosures of 8th October 2019 [209] 10th and 20th January 2020 [210, 211] to include all known announced ADRVs from IOC retesting.

2.2.1. Data acquisition and entry of other ADRVs that impacted the 1968 – 2012 summer Olympic Games.

Data on other ADRVs that impacted the 1968 – 2012 summer Olympic Games was obtained from a publicly available list of Doping Irregularities at the Olympics curated by Olympic historians on olympedia.org [125] of which data entry ceased on 9th July 2020. News reports of press releases [212, 213] were used to confirm the timing of the identified cause of one sanction as it was not clear on olympedia.org. ADRVs that were overturned on appeal were excluded. If an athlete competed in a team sport this was counted as a single performance and as a single medal won (if applicable) and teammate medals that may have additionally been rescinded because of doping were not counted. Reasons for the ADRVs were classified as

described in Table 1, with the classifications of substances defined by their location in the 2020 WADA Prohibited list [214] or their closest categorisation therein. ADRVs were classified if they occurred at the Olympic Games, prior to an Olympic Games and if they were identified retrospectively (either by IOC retests or by other investigations).

2.2.2. Data analysis of ADRVs that impacted the 1968 – 2012 summer Olympic Games & ADRVs identified from IOC re-analysis of samples from the 2004, 2008 & 2012 Olympic Games.

Data analysis was conducted in Microsoft Excel and in R version 3.6.3 using the tidyverse [215], choroplethr [216] and choroplethrMaps [217] packages. The data files and R code used in this study have been made publicly available online [218].

2.3. Ethical Approval for The MMAAS Project.

The MMAAS Project was ethically approved by the University of Brighton Tier 2 College Research Ethics Committee (SSCREC2016-28). Participants were recruited via word of mouth and internet advertisements and did not receive remuneration for sampling with only travel expenses reimbursed. Participants provided written informed consent and potential complications of enrolment were explained beforehand. All applicable samples were registered with the University of Brighton Human Tissue Authority online database.

2.4. Sample Collection and body composition measurements.

Participants were instructed to not resistance train 48-hours prior to the biopsy and they ate normally before visiting the laboratory due to the medical advice that participants could feel dizziness during the biopsy, in which they were seated upright on a chair with no backing. Three millilitres 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, with participants iteratively sampled, in the order of consenting, at regular intervals throughout a working day. Immediately after collection the tube was shaken vigorously for 10 seconds, incubated at room temperature for 3 hours and stored at −80°C.

Immediately after whole blood was collected into the Tempus™ Blood RNA Tube a 10 mL whole blood sample was also collected into a BD® serum tube (Becton, Dickinson and Company, Wokingham, Berkshire, UK) for serum extraction and a 10 mL whole blood sample was collected into a BD® EDTA (K2) (Becton, Dickinson and Company, Wokingham, Berkshire, UK) tube for plasma extraction. Immediately after collection, the plasma EDTA tube was inverted 180° and back 8-10 times and stored upright at 4°C. Within 4 hours of blood collection, the EDTA tube was centrifuged at ≤1300 g for 10 minutes. The plasma layer was aliquoted into 3 x 1.5 mL TubeOne® Microcentrifuge Tubes with 1 mL per aliquot. Immediately after collection, the serum tube was stored upright at room temperature for an hour to allow clotting. If centrifugation was not possible immediately, the serum tube was stored upright at 4°C. Within 4 hours of blood collection the serum tube was centrifuged and aliquoted using the same aforementioned protocol for the EDTA tube. All serum and plasma aliquots were stored at −80°C. Although serum and plasma were collected from participants these samples were not analysed in this thesis.

Subjects additionally donated 2 mL of saliva into either an Oragene® RE-100 or CP-190 RNA tube (DNA Genotek, Ottawa, Ontario, Canada). There was no difference between the reagents or collection protocol/storage for these tubes except for their shape as manufacturing of the RE-100 tube was discontinued in October 2018. The subject then donated 2 mL of saliva into an Oragene® DNA OG-500 tube (DNA Genotek, Ottawa, Ontario, Canada). Saliva samples were subsequently stored at −80°C. Upon the subject's first visit, saliva was donated for both RNA and DNA preservation whereas during a second visit, subjects only donated saliva for RNA preservation as saliva for DNA preservation had already been collected. 60 mL of urine was collected into Sterilin™ Urine Bottles (Thermo Fisher Scientific, Loughborough, Leicestershire, UK). 1 mL aliquots were then taken from this bottle and stored in 3 x 1.5 mL TubeOne® Microcentrifuge Tubes (Star Lab, Milton Keynes, Buckinghamshire, UK). Both these aliquots and the urine bottle were stored at −80°C. Although saliva and urine were collected from the participants these samples were not analysed in this thesis.

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 [111, 112, 219]. 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.

Body composition was assessed via Bioelectrical Impedance with the Tanita® BC-420MA two hours prior to the muscle biopsy. Participants were instructed to not consume food and drink two hours prior to body composition assessment.

2.5. Participant Eligibility and Group Classification.

2.5.1. Group Classification for IHC data analysis.

Participants were male, aged 20-42 and within four groups, according to their self-reported resistance training and AAS usage history (Table 2). 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) [220] returns close to baseline 5-6 months post exposure. Returning participants (RP) could conduct Post Cycle Therapy (PCT) [221, 222].

One-year withdrawal from AAS to denote past users from current users has been used in a previous study investigating the effect of AAS on muscle fibre parameters in this demographic [111]. Supraphysiological dosages of testosterone were defined as self-usage of intramuscular injections >100mg/week based on clinical recommendations of testosterone replacement therapy (TRT) [223, 224].

Self-reported AAS cycles, other Performance Enhancing Drugs (PEDs) and PCT protocols are available as Supplementary Data online (see Supplementary Data File 1 on OSF [225]). If a range were stated because an exact dosage or time frame could not be recalled the median was used in AAS exposure calculations.

2.5.2. Group Classification for RNA-Seq data analysis.

For RNA-Seq data analysis participants in RT-AS and PREV were subsequently subdivided to those where last self-declared AAS exposure was less than or equal to two weeks prior to sample collection (RT-AS≤2) and those where self-declared last AAS exposure was 10 or more weeks prior to sample collection (RT-AS≥10). Partly, this division was made because the process of a steroid receptor translocating from the cytoplasm to the nucleus typically takes at least 30 – 60 minutes [3]. Thereby, time since last exposure is a pertinent variable to classify participants when investigating potential differences in gene expression. Therefore, broadly speaking, participants who have ceased AAS exposure nearby to sampling should be in a different category than those who ceased AAS exposure further away from sampling. Additionally, due to the observational nature of this research, where no control was possible on time since last AAS exposure for the participants, some flexibility on group classifications is needed to reflect the self-declared last AAS exposure time frames of the participants. The self-declared last AAS exposure time frames of the participants who had samples analysed with RNA-seq is available in Results section 6.3.1. Given these time frames, the classification of RT-AS≤2 and RT-AS≥10 seemed the most appropriate middle ground to not divide the AAS using participants into groupings that were too small, (which would result in too many group comparisons), or into groupings that are too large/disproportionally balanced and still allow for this broad division of those who ceased AAS usage relatively closer to sampling, compared to those who ceased AAS usage further away from sampling. For comparative purposes, in addition to the classification RT-AS≤2 and RT-AS≥10, participants in RT-AS & PREV were joined as one group and classified as Doped, similar to how AAS using participants were classified in [113].

2.6. IHC staining protocols, section imaging, extraction, and quantification.

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.

The fibre typing protocol utilised previous published methodologies [226] and the recommendation to identify pure MyHC IIX fibres, as detailed in antibodies for IHC fibre typing in Table 2 of [226], where a BA.D5 antibody is used to identify MyHC type I fibres, a BF-35 antibody is used to identify MyHC type I & IIA (enabling the detection of pure MyHC type IIX, which always remain unstained) with the automated detection software MyoVision (227) then able to distinguish type I & IIA fibres based on BA.D5 staining and/or BF-35 staining. Slides were washed in PBS and incubated overnight at 4°C inside a humidifying slide chamber with a primary antibody cocktail consisting of MyHC type I BA.D5 IgG2b

(1:100, Concentrate, DHSB), BF-35 IgG1 (1:100, Concentrate, DHSB) and Rb α-Laminin (for fibre borders) (L9393,1:100, Sigma-Aldrich) diluted in 2.5% BSA (Sigma-Aldrich). The following day sections were washed in PBS and incubated in a secondary antibody cocktail for 60 minutes at room temperature consisting of Gt α-Ms IgG2b, AF647 (1:250, A-21242, Invitrogen™), Gt α-Ms IgG1, Alexa Fluor 488 (1:250, A21121, ThermoFisher Scientific) and AMCA Gt α-Rb IgG (1:100, Cl-1000, Vector Laboratories) diluted in 1 x PBS. Sections were then washed in PBS, fixed for 5 minutes in methanol, washed in PBS and mounted with Vectashield (H-1000, Vector Laboratories) or Immu-Mount (9990402, Shandon™, ThermoFisher Scientific) and stored at 4°C.

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 [227]. Fibre outlines, MyHC types, nuclei, and Pax7-positive nuclei were detected and MyoVision calculates 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. Statistical analysis of age, height, weight, body composition and immunohistochemistry data.

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 [228] using the tidyverse package [215]. 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 [225].

2.8. RNA extraction, purification, library preparation and RNA-Seq.

Blood and muscle samples were randomly sorted prior to RNA extraction and library preparation. Total RNA was extracted from whole blood using the Tempus™ Spin RNA Isolation Kit according to manufacturer instructions (Life Technologies, Carlsbad, CA, USA). Total RNA >200 nt was extracted from muscle samples using the Qiagen® RNeasy Fibrous Tissue Mini Kit with TissueRuptor II Disposable Probes. Muscle total RNA samples were then digested using DNase I (New England Biolabs, Ipswich, MA, USA) and purified using RNAClean XP beads (Beckman Coulter, Indianapolis, IN, USA). After extraction, all RNA samples were stored at −80°C until further analysis.

RNA quantity and quality were assessed using a Nanodrop® ND-2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). RIN value was assessed using an Agilent® 2100 Bioanalyzer with an Agilent® RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, United States).

rRNA was depleted from 200 ng of total whole blood RNA or purified total muscle RNA with RIN ≥7 using an MGIEasy rRNA Depletion Kit. dsDNA libraries (with conditions for a 250-bp Insert Size) were created from the rRNA-depleted eluate using an MGIEasy RNA Directional Library Prep Set. dsDNA library quantity was assessed using a Thermo Fisher Scientific Qubit® dsDNA High Sensitivity Assay Kit and a Qubit® Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States). The quality of the fragment size distribution of the dsDNA library was assessed by visual inspection of electropherograms created using an Agilent® DNA 1000 Kit on an Agilent® 2100 Bioanalyzer. Only dsDNA libraries with satisfactory fragment size distributions were carried forward onto the next steps, and dsDNA libraries were recreated for any samples with aberrant electropherograms or low concentrations. dsDNA libraries were circularized and converted into ssDNA libraries using an MGIEasy Circularization Kit. ssDNA library concentration was assessed using a Thermo Fisher Scientific Qubit® ssDNA Assay Kit and a Qubit® Fluorometer. DNA nanoballs (DNBs) were prepared from ssDNA library pools, with a 40-fmol ssDNA library for each reaction, using an MGI DNBSEQ-G400RS High-throughput Sequencing Set for blood samples and for muscle samples either this kit or a CoolMPS High-throughput Sequencing Set. DNB concentration was assessed using a Qubit® ssDNA Assay Kit (Thermo Fisher Scientific) and a Qubit® Fluorometer (Thermo Fisher Scientific). DNB preparations >8 ng/μL were loaded onto flow cells using an MGIDL-200H Portable DNB Loader, with

muscle and whole blood samples distributed over two flow cells each, with six to eight samples in each lane based on their order of RNA extraction and library preparation. The flow cells were placed on an MGI DNBSEQ-G400 sequencer and subjected to PE100 sequencing with standard chemistry reagents for blood samples and either standard chemistry reagents or CoolMPS chemistry reagents for muscle samples. Two flow cells of whole blood samples were sequenced at the UoB, School of Sport and Health Sciences campus in Eastbourne. For validation purposes these same libraries were re-sequenced in MGI's research hub in Latvia. Two flow cells of muscle samples were sequenced with standard chemistry reagents in MGI's research hub in Latvia and for validation purposes these same libraries were re-sequenced with CoolMPS chemistry reagents in MGI's research hub in Latvia.

2.9. Bioinformatic analysis of RNA-Seq data.

Raw sequences were examined by FastQC [229] version 0.11.9 for basic quality checks (e.g., per base sequence quality, per base N content, duplicate sequences and adapter content). RSeQC [230] version 4.0.0 and the function read_duplication.py was also utilised for further quality checks. FastQ Screen [231] version 0.15.0 was used for detecting sample swaps and/or sample contamination utilising Bowtie 2 [232] version 2.4.2 for alignment to reference genome assemblies with pre-built Bowtie 2 genome indices [233] for Human (GRCh38 no-alt analysis set), Mouse (GRCm39) and Rat (Rnor6.0). MultiQC [234] was used to summarize FastQC, FastQ Screen and compatible RSeQC analysis reports. FastQC per base sequence quality scores, interactive MultiQC reports for FastQ Screen and RSeQC are available on OSF [225].

HISAT2 [235] version 2.2.1 was used for alignment of reads to the reference genome assembly GRCh38.p5 using the Ensemble 84 annotation as the publicly available grch38_tran pre-built HISAT2 index [236, 237] was utilised. For HISAT2 alignment '--dta' was utilised and '--rna-strandness RF' was stated as RSeQC infer_experiment.py showed a directional, first strand library. Galaxy [238] was used to convert the Homo_sapiens.GRCh38.84.gtf.gz file to the BED12 file format for RSeQC infer_experiment.py. RSeQC read_distribution.py was used for read distribution analysis also utilized this BED12 file and SAM files generated from HISAT2. RSeQC split_bam.py was used to estimate how many reads originated from rRNA utilising the publicly available hg38_rRNA.bed file [239] from RSeQC. Salmon [240]

version 1.7.0 was used for transcript quantification in mapping-based mode utilising the publicly available hg38 full decoy-aware salmon index [241] with --validateMappings, --seqBias and --gcBias flags switched on. Using Bioconductor version 3.14 and R [228] version 4.1.2 in Rstudio version 2022.2.0.443 the package "tximport" [242] was used for summarising transcript-level estimates to gene names based on the Ensembl release 105 [243] annotation and transcript IDs with undefined gene names were removed. Data was normalised by the calcNormFactors function in edgeR [244], explored with multidimensional scaling (MDS) and PCA Plots and if deemed appropriate, based on aberrant positioning, outlying samples were removed and data re-normalised. Data was then filtered by group for group comparisons or by visit for paired sample comparisons in returning participants. For group comparisons only first visit data from RP1-5 was utilised. For group comparisons the minimum number of counts per sample matched the smallest group size and for paired sample comparisons for returning participants, the minimum number of counts per sample matched the total number of samples in the dataset. Data was then re-normalized, experimental designs were then modelled (i.e., group comparisons (of C, RT, RT-AS≤2 & RT-AS≥10 and separately C, RT & Doped) or returning participant paired sample comparisons), dispersion estimates were calculated and then the quasi-likelihood approach was used to fit generalised linear models to the data. Group contrasts or paired sample contrasts were made, and DGE testing was conducted. The function topTags was used to select the most differentially expressed genes using a false discovery rate (FDR) < 0.05 and a fold change of 1.2.

Partial least squares discriminant analysis (PLS-DA) was performed on group comparison datasets using the mixOmics [245] package in R version 4.1.2. Post filtered, and post re-normalized Counts-per-million (CPM) data, generated by edgeR (i.e., the same data used in group comparison DGE testing), was used for PLS-DA, with cross-validation repeated 50 times. The Balanced Error Rate (BER) and the max.dist numerical output was noted as an indicator of overall performance. To investigate the importance of a gene, a cut-off of variable importance of projection (VIP) score >1 was used, as per the recommendations of mixOmics [245], in addition to if edgeR and topTags determined the gene to be differentially expressed.

Gene Set Enrichment Analysis (GSEA) was conducted in R using the Bioconductor package GSEABase [246] version 1.56.0 and fry [247] by examining the Molecular Signatures Database [248, 249] (MSigDB) v7.5.1 Hallmark (containing 50 gene sets) [250], Gene

Ontology [251, 252] (C5; BP: subset of GO biological processes containing 7,658 gene sets & MF: subset of GO molecular functions containing 1,738 gene sets), KEGG [253] pathway (186 gene sets) and Reactome [254] pathway (1615 gene sets) collections. A gene set/pathway was noted as differentially expressed if FDR < 0.05. For each comparison, lists of differentially expressed genes and gene sets/pathways were exported into InteractiVenn [255] to identify overlaps between different sequencing locations (blood samples, UoB & MGI) or sequencing chemistries (muscle samples, standard & CoolMPS).

Gene and sample clustering was performed within pheatmap [256]. Firstly, normalised counts per million were log transformed for the top 30 most significantly differentially expressed genes by FDR for the group comparison noted. This matrix was inputted into pheatmap [256]; rows and columns were clustered with a complete clustering method, Pearson correlation was used as the distance measure and scale = "row" was applied.

Group/returning participant information matrices used in edgeR for DGE analysis, raw salmon count matrices (quant files) and R code used for DGE analysis and GSEA analysis are publicly available on OSF [225]. Raw FASTQ files have not been made publicly available due to the remote possiblity of participant anonymity being compromised.

3. Doping Practices in International Weightlifting.

3.1. Introduction.

As discussed and introduced in section 1.7.1 weightlifting has been described by IOC president Thomas Bach as having "a massive doping problem" [257] due to the high prevalency of Olympic medal reallocations from the 2008 & 2012 Olympic Games from sample re-testing. The sport has had to implement numerous policy changes to show a commitment to anti-doping to ensure that it remains on the Olympic programme for Paris 2024 and beyond. As discussed in section 1.10.1 this study aimed to analyse prohibited substances noted in sanction data by geographical location to build a clearer picture of doping practices. This data will aid governing bodies and anti-doping authorities in identifying regions with higher rates of doping for improved targeted testing and educational programmes. Furthermore, an analysis of the ADRVs identified from sample re-testing of the 2008 & 2012 Olympic Games has yet to be conducted and this study additionally aimed to investigate this dataset due its importance in influencing the long-term future of the sport at the Olympic Games.

3.2. Methods.

The methods of this study are noted in section 2.1 and section 2.1.1.

3.3. Results.

3.3.1. The most frequently detected substances.

Five hundred and sixty-five Sanctioned Athletes/Athlete Support Personnel, across 83 different MF, were recorded between 2008 and 2019 (Figure 1). Five hundred and sixty-two of these sanctions had a named prohibited substance/prohibited method noted. Five hundred and fifty-nine of these sanctions occurred due to the detection of prohibited substances, with only three sanctions occurring due to the use of prohibited methods (n = 2 urine substitution, n = 1 blood substitution). Of these 559 sanctions, 51 different substances were detected, from 10 different categories within the WADA Prohibited List, with exogenous AAS metabolites and markers indicating EAAS usage accounting for 82% of detected substances (Figure 2).

Three hundred and ninety-six sanctions occurred from an in-competition (IC) test and 167 from an OOC test with two sanctions testing location undefined. From the ten most detected

substances, six substances, dehydrochloromethyltestosterone (89%), markers indicating EAAS usage (76%), metenolone (100%), methylhexanamine (100%), methyltestosterone (71%) and nandrolone (86%) showed a higher instance of detection IC (Figure 2).

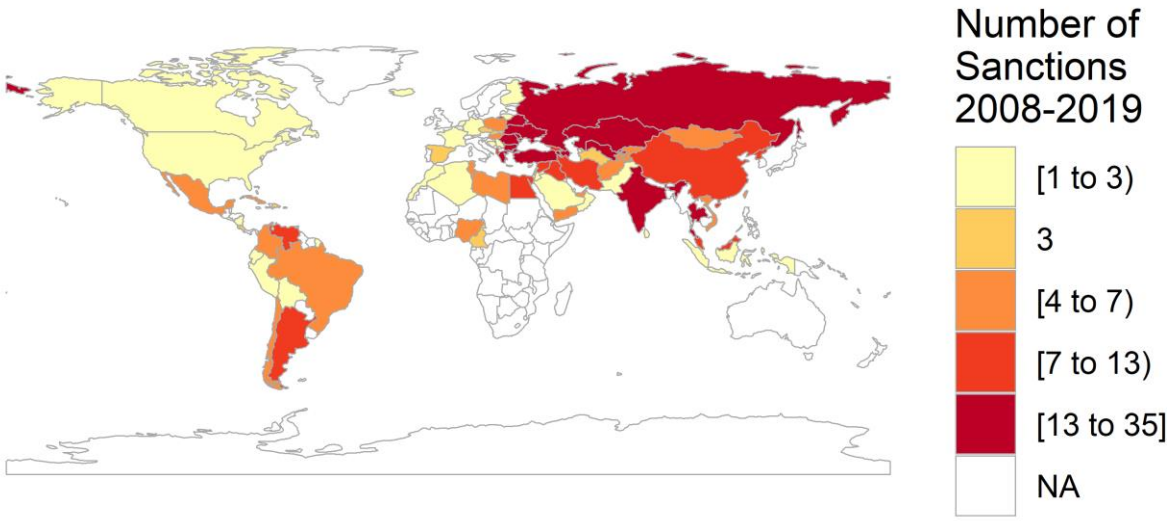


Figure 1. The number of sanctions recorded from the IWF Sanction List between 2008-2019 when it was accessed in February 2020 and their geographical location [188]. NA indicates zero recorded sanctions. Five hundred and sixty-five sanctions were recorded but 553 were used for the creation of this Figure as the following MF were not present in the country.map dataset in the choroplethrMaps package in R: Puerto Rico (*n* = 3), Mauritius (*n* = 2), Palestine (*n* = 2), Seychelles (*n* = 2), Aruba (*n* = 1), Barbados (*n* = 1) and Bahrain (*n* = 1) [203].

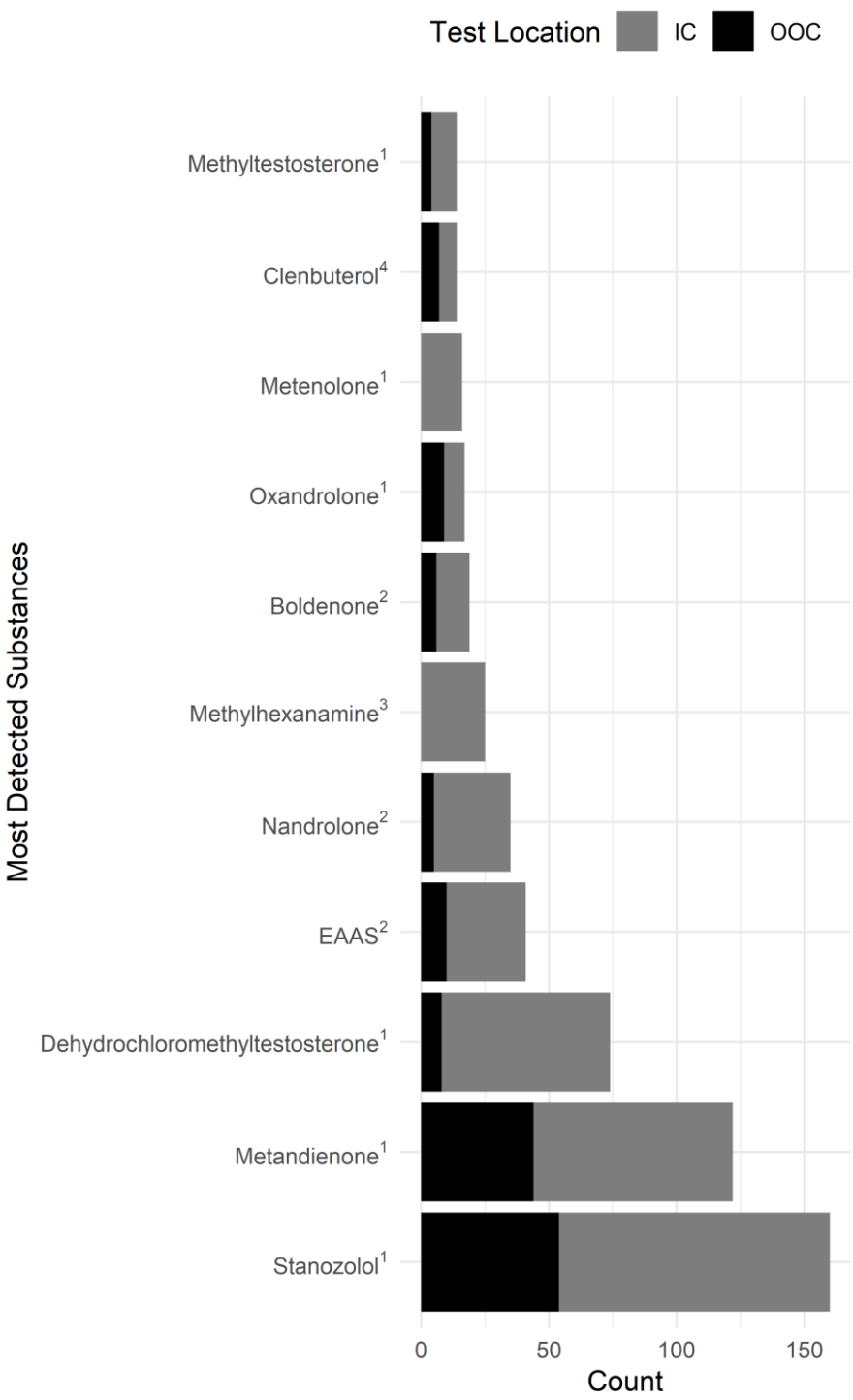


Figure 2. The 10 most detected substances from the IWF Sanction List between 2008 to 2019 and if their detection occurred in-competition (IC) or out-of-competition (OOC) with superscript numbers classifying substances based on the WADA 2019 Prohibited List [188]: exogenous Anabolic Androgenic Steroid (AAS)[1]; markers indicating endogenous AAS usage (EAAS)[2]; Specified Stimulants[3]; Other Anabolic Agents[4] [197]. Clenbuterol and Methyltestosterone are tied in 10th place with 14 occasions of detection each. One data point for Methandienone was omitted as the testing location was not defined.

3.3.2. Prohibited substance usage and Continental Federation.

Of the 565 Sanctioned Athletes/Athlete Support Personnel counted 199 were from Asia, 267 from Europe, 34 from Africa, and 65 from Pan America. There were no sanctions from Oceania. From the 562 sanctions that had the available data, the proportion of detected substances that were classified as exogenous AAS, markers of EAAS usage (i.e. the most detected substances) and all other substance category types varied by IWF Continental Federation ($p < 0.001$). The proportions of these detected substance types was significantly different between Asia (70%, 15%, 15%) and Pan America (37%, 30%, 33%) ($p < 0.001$), Asia and Africa (50%, 17%, 33%) ($p = 0.039$), Europe (74%, 11%, 15%) and Pan America ($p < 0.001$) and Europe and Africa ($p = 0.015$) with no differences between Asia and Europe or Pan America and Africa, highlighting regional differences in detected prohibited substances.

3.3.3. Prohibited substance usage and nation.

For the 10 nations with the highest number of sanctions, when looking at the 10 most detected substances, each nation had at least one substance that accounted for more than one third of all detected substances as follows: Azerbaijan ($n = 35$ sanctions) (metandienone 38%), Kazakhstan ($n = 35$) (stanozolol 51%), Russia ($n = 32$) (dehydrochloromethyltestosterone 52%), Bulgaria ($n = 30$) (metandienone 42% and stanozolol 45%), Belarus ($n = 23$) (stanozolol 44%), Armenia ($n = 22$) (stanozolol 38%), Ukraine ($n = 19$) (dehydrochloromethyltestosterone 40% and stanozolol 40%), Romania ($n = 18$) (stanozolol 60%), Thailand ($n = 18$) (metandienone 50% and EAAS 50%) and Moldova ($n = 17$) (dehydrochloromethyltestosterone 37%) (Figure 3).

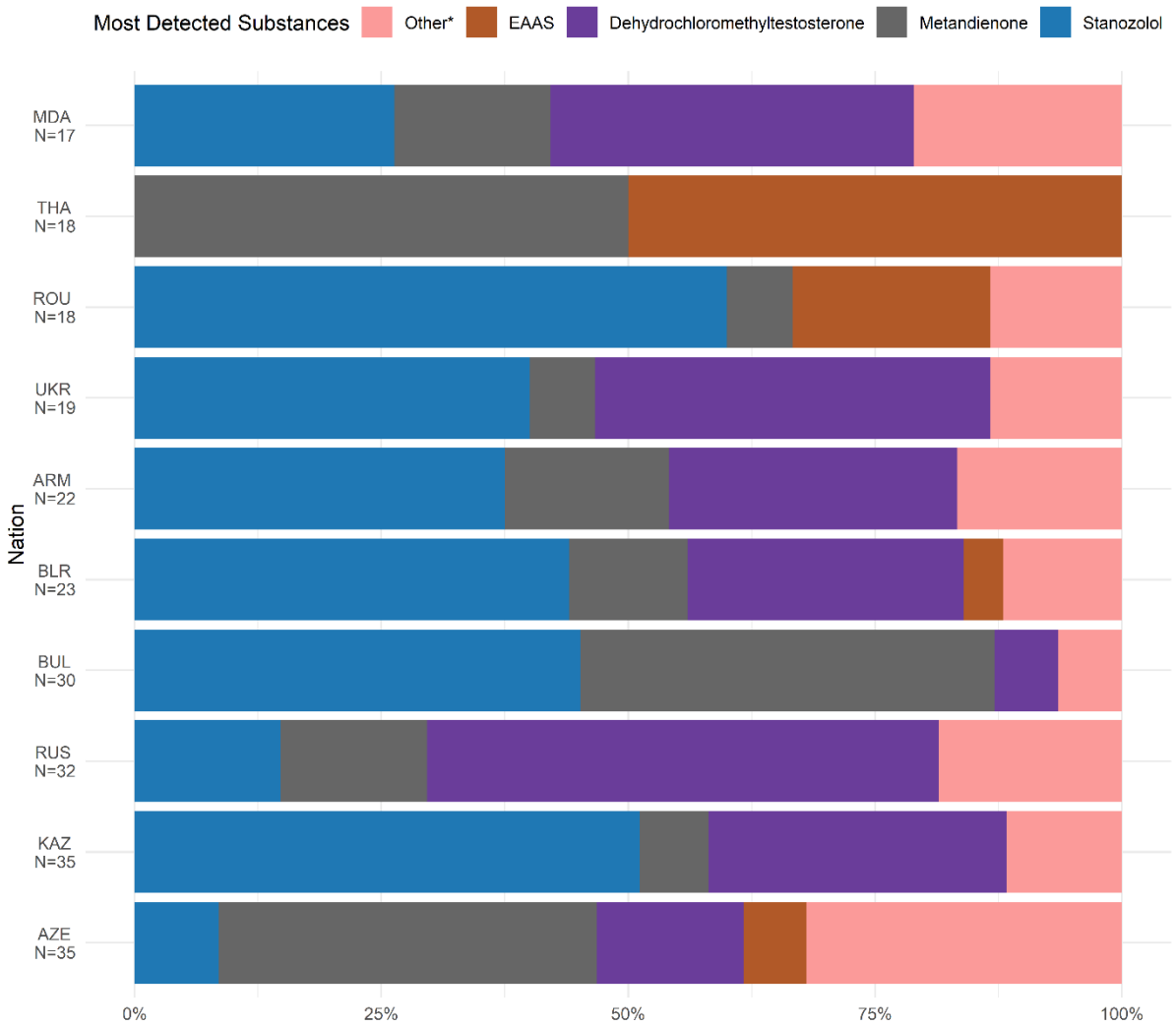


Figure 3. The 10 nations with the highest number of sanctions, from the IWF Sanction List between 2008 to 2019 and for the 10 most detected substances the percentage of times they were detected [188]. Other* denotes either methyltestosterone, clenbuterol, metenolone, oxandrolone, boldenone, methylhexanamine or nandrolone. EAAS; markers indicating endogenous AAS usage. AZE; Azerbaijan, KAZ; Kazakhstan, RUS; Russia, BUL; Bulgaria, BLR; Belarus, ARM; Armenia, UKR; Ukraine, ROU; Romania, THA; Thailand, MDA; Moldova.

3.3.4. Most Affected Nations of 2008 and 2012 Olympic Games Retesting.

Sixty-one weightlifters, from 13 different countries, were retrospectively announced to have committed an ADRV for prohibited substances from the Beijing 2008 (n = 25) and London 2012 (n = 36) Olympic Games. Sixteen of these weightlifters (64%) from Beijing 2008 were medallists (4 Gold, 5 Silver and 7 Bronze). For Beijing 2008 Kazakhstan and Azerbaijan had more athletes generate a retrospective ADRV than those who did not and for Belarus, Ukraine, Russia, and Kazakhstan more medals were won by athletes who generated a retrospective ADRV than those who have not (Figure 4). Eighteen of these weightlifters (50%) from London 2012 were medallists (5 Gold, 5 Silver and 8 Bronze). For London 2012 Russia, Kazakhstan, Belarus, Azerbaijan, and Armenia had more athletes generate a retrospective ADRV than those who have not and for both Romania and Moldova all athletes that competed generated a retrospective ADRV. All medallists from Ukraine, Kazakhstan, Belarus, Romania, Azerbaijan, Armenia, and Moldova generated retrospective ADRVs and for Russia twice as many medals were won by athletes who generated a retrospective ADRV (Figure 4).

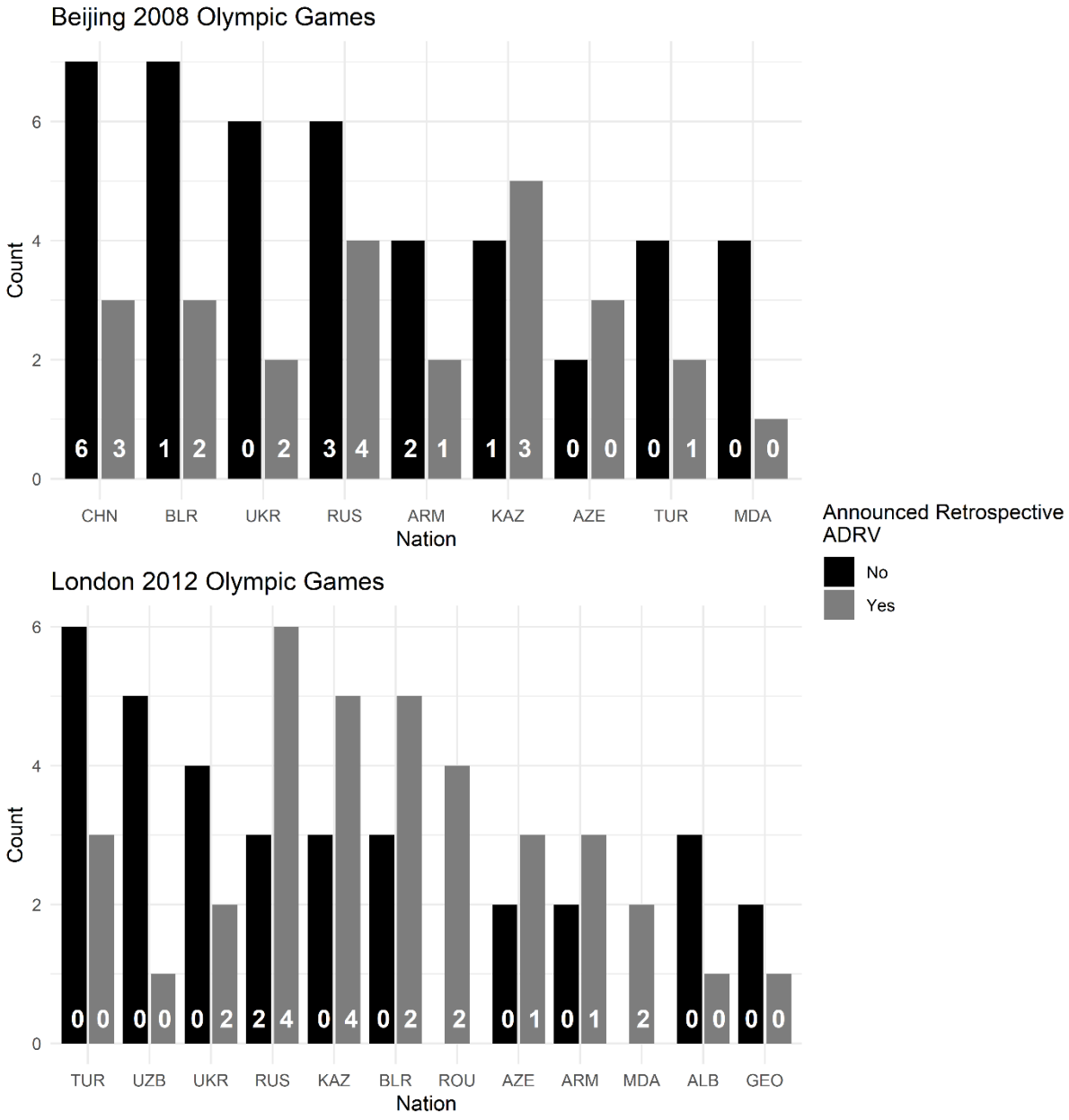


Figure 4. The number of weightlifters that competed from each nation announced to have given retrospective Anti-Doping Rule Violations (ADRVs) via Adverse Analytical Findings (AAFs) from the re-testing of samples collected at the Beijing 2008 and London 2012 Olympic Games. Numbers inside bars show the number of original medallists. Weightlifters with announced retrospective ADRVs who did not start are included. In Beijing 2008 one athlete from UKR produced an AAF from an in-competition sample and is excluded in these counts. CHN; China, BLR; Belarus, UKR; Ukraine, RUS; Russia, ARM; Armenia, KAZ; Kazakhstan, AZE; Azerbaijan, TUR; Turkey, MDA; Moldova, UZB; Uzbekistan, UKR; Ukraine, ROU; Romania, ALB; Albania, GEO; Georgia.

3.3.5. Most Affected Categories of 2008 and 2012 Olympic Games Retesting.

From Beijing 2008 five weight categories, (Men's u94kg, Women's u48kg, Women's u69kg, Women's u75kg, and Women's 75kg+) and from London 2012 three weight categories, (Women's u53kg, Women's u63kg and Women's u69kg) had two medallists produce retrospective ADRVs from AAFs. In two instances from London 2012 (Men's u94kg and Women's u75kg) all medal winners produced retrospective ADRVs from AAFs, with the Men's u94kg category being the worst affected with eight athletes generating retrospective ADRVs from AAFs, six of whom originally placed in the top 10.

3.3.6. Detected Substances from 2008 and 2012 Olympic Games Retesting.

In total, across both the Beijing 2008 and London 2012 Olympic Games, 94 prohibited substances were detected in the re-tested samples with dehydrochloromethyltestosterone and stanozolol accounting for 83% of all detected substances. The majority of retrospective ADRVs (58 of 61) were caused by the detection of one of these two substances with exogenous AAS accounting for 94% of all detected substances. Across both Olympic Games, for the 10 nations with the highest number of announced retrospective ADRVs the proportions of detected substances are shown in Figure 5. For each nation there is at least one substance that makes up ≥40% of all detected substances as follows: Kazakhstan (*n* = 10 ADRVs) (Stanozolol 67%), Russia (*n* = 10) (dehydrochloromethyltestosterone 71%), Belarus (*n* = 8) (dehydrochloromethyltestosterone 44%, stanozolol 44%), Azerbaijan (*n* = 6) (dehydrochloromethyltestosterone 67%), Armenia (*n* = 5) (dehydrochloromethyltestosterone 50%, stanozolol 50%), Turkey (*n* = 5) (stanozolol 71%), Romania (*n* = 4) (metenolone 40%, stanozolol 40%), Ukraine (*n* = 4) (dehydrochloromethyltestosterone 100%), China (*n* = 3) (Growth Hormone-Releasing Peptide-2 75%) and Moldova (*n* = 3) (dehydrochloromethyltestosterone 67%).

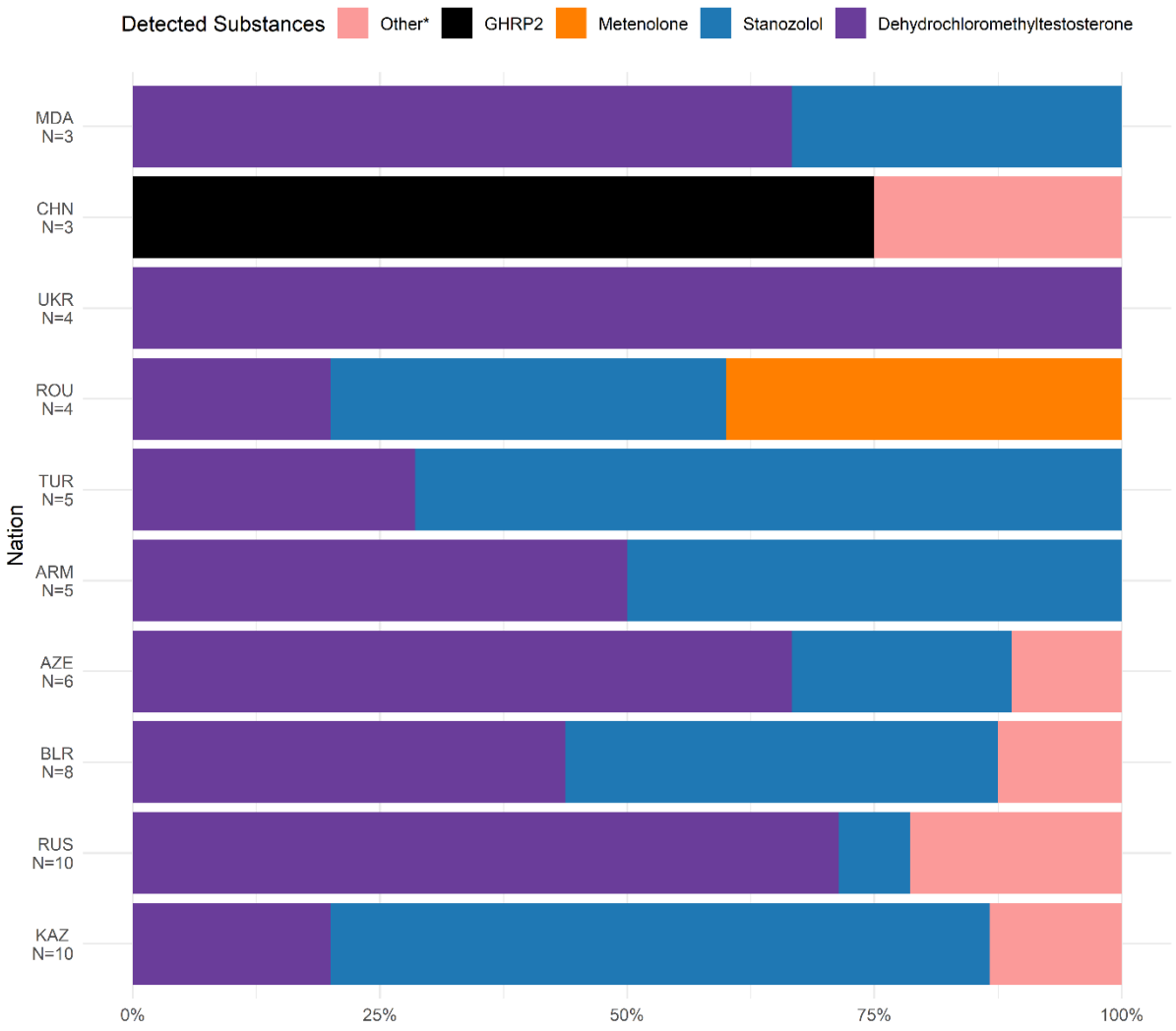


Figure 5. The 10 nations with the highest number of announced retrospective Anti-Doping Rule Violations via Adverse Analytical Findings from both the Beijing 2008 and London 2012 Olympic Games and the percentages of detected substances identified. Other* denotes either drostanolone, erythropoietin, oxandrolone, sibutramine or tamoxifen. GHRP2: Growth Hormone-Releasing Peptide-2. KAZ; Kazakhstan, RUS; Russia, BLR; Belarus, AZE; Azerbaijan, ARM; Armenia, TUR; Turkey, ROU; Romania, UKR; Ukraine, CHN; China, MDA; Moldova.

3.4. Discussion.

The time period of this analysis (2008 – 2019) has seen the highest number of sanctions in weightlifting's history. It has also seen an independent investigation into allegations of anti-doping corruption finding the IWF president to have breached the confidentiality of the planned timing of OOC sample collection, potentially giving advanced notice of OOC testing to individual countries or athletes [155]. The president also delayed the announcement of ADRVs from 18 Azerbaijani weightlifters and AAFs from 21 Turkish weightlifters were not followed through appropriately, enabling them to win medals in international events. Investigations by WADA and the ITA are still pending on an additional "41 hidden cases and 10 possible other cases where the AAFs have not been followed through" [155] and on 130 unprocessed samples (in which the number of AAFs is unknown) [156]. Despite these ongoing investigations this study intended to build a clearer picture of doping practices of weightlifters and how these practices varied across the IWF Continental Federations, based on known sanction data, to enhance future doping detection and to investigate the re-testing of samples collected at the 2008 and 2012 Olympic Games.

Over an 11-year period exogenous AAS metabolites and markers indicating EAAS usage accounted for 82% of detected substances from the IWF Sanction List. As previously discussed in section 1.2. the effects of AAS on increasing skeletal muscle mass and strength have been well documented [15, 17, 19-21, 258] and these ergogenic benefits are a likely reason for their preference of usage by doping athletes who compete in a strength sport. Europe generated the highest number of sanctions (n = 267) followed by Asia (n = 199), Pan America (n = 65) and Africa (n = 34), with no sanctions from Oceania recorded. During this time frame weightlifting has been most popular in Europe and Asia, but globally popularity has been expanding with the senior 2019 Oceania [259], African [260] and Pan American [261] Championships respectively hosting 129, 112 and 187 weightlifters whilst the senior 2019 European [262] and Asian [263] Championships respectively hosted 322 and 214 weightlifters.

The proportions of detected exogenous AAS metabolites, markers indicating EAAS usage and all other substance category types varied by IWF Continental Federation. Europe and Asia both respectively showed statistically different (p<0.05) proportions of detection for these three substance types compared to both Africa and Pan America with exogenous AAS showing the largest difference in the proportion of substance types detected. The most

detected exogenous AAS were stanozolol, metandienone and dehydrochloromethyltestosterone. At the national level there is also differences in the detection of substances as when looking at the 10 most detected substances, from the 10 nations with the highest numbers of sanctions, there is at least one substance overrepresented that accounts for 38-60% of detected substances in these countries (Figure 3). The cultural preference of certain doping substances at the regional/national level is likely heavily confounded by regional/national drug availability (legally or illicitly). Nationally the continued usage of certain substances could be decentralized (e.g., athletes independent choice based on availability) or centralized (i.e., state-sponsored doping). Making inferences on the cause of substance over-representation from substance detection data is not possible but identifying these patterns is useful knowledge for anti-doping authorities as they could use this data for targeted educational programmes to elicit the change required to change these patterns. These patterns corroborate with the notion from the independent report into Anti-Doping corruption into weightlifting that although the ex-president "interfered with the IWF Anti-Doping Commission, *the real problem is the culture of doping that exists in the sport*." [155]. Additionally, these geographic differences in doping practices could better inform targeted testing applied by anti-doping authorities and targeted investigations into other ways of identifying ADRVs such as trafficking, aiding/abetting and complicity. For effective doping control international sporting authorities should have anti-doping programmes that frequently conduct unannounced OOC testing, across all regions of the globe, to catch doping athletes who intend for prohibited metabolites to clear their urine prior to anticipated IC tests. With Europe and Asia showing the highest number of sanctions and highest prevalence in the usage of exogenous AAS an extra emphasis on OOC testing in these regions may be warranted in weightlifting as these substances are likely to be used in training prior to competition where anticipated testing occurs. Additionally, if athletes from these regions now consider that the likelihood of getting caught using exogenous AAS is now high due to their high detection prevalence they may now start to use other PEDs with shorter detection windows meaning that OOC testing is even more important.

The decision of the IOC to store athletes' samples collected from Olympic Games for 10 years has proven particularly fruitful for catching doping medallists in weightlifting. This analysis of re-tested samples has shown that the intention of doping athletes who ceased the usage of exogenous AAS prior to the 2008 and 2012 Olympic Games with the aim of diagnostic metabolites clearing their urine prior to an anticipated test was successful (with the

known metabolites and detection science at the time of these Olympic Games). However, once the LTM's for exogenous AAS were discovered via improvements in highly sensitive detection methods employing chromatographic/ mass spectrometric techniques [132] a doubling of the detection window [133, 137] occurred for some exogenous AAS and subsequently 34 medallists were caught doping retrospectively when these samples were reanalysed. From the sixty-one retrospective ADRVs identified via re-testing the exogenous AAS dehydrochloromethyltestosterone and stanozolol accounted for 83% of detected substances with 95% of announced athlete ADRVs noting at least one of these substances. These findings should send a strong deterrent to prospective doping athletes that, due to LTM discovery, the detection window of these substances has substantially improved and the doping practices of athletes in the runup to the 2008 and 2012 Games may not be possible anymore for future competitions. The IOC has announced that the ITA has planned the "most comprehensive pre-Games testing programme ever conducted" for Tokyo 2020 and that $5 million, spread over 10 years, will be allocated to a comprehensive long-term storage programme [264, 265], potentially acting as a stronger deterrent to prospective doping weightlifters. However, long-term storage is not standard across Continental Games, with the IF having to fund the cost of long-term storage [266]. Based on the success shown with weightlifting, the IWF and other IF should further their investment in long term sample storage at Continental Games and other important international competitions. Other categories of PEDs that may currently have shorter detection windows may be used instead of exogenous AAS by weightlifters, or other athletes in strength/power sports (e.g. EAAS), due to this improved detectability in exogenous AAS, but these shorter detection windows could improve in the future (e.g. by advances in the steroidal module of the ABP) and thus long term sample storage would enable a re-analysis of samples with improved detectability.

This analysis of doping practices, over a period of 11 years, has shown avenues that may enhance the future detection of doping weightlifters. For example, with Europe and Asia producing the highest numbers of sanctioned weightlifters, as well as the highest prevalence in the detection of exogenous AAS, higher rates of targeted OOC testing in these regions may be warranted, both in the instance that these substances continued to be used or if a transition is made to substances with shorter detection windows. Educational programmes on anti-doping may also be required to change the behaviour in nations with the highest number of sanctions especially focussing on detected substances that are overrepresented in their doping weightlifters. Lastly, the prevalence of retrospectively identified doping at the Beijing and

London Olympic Games shows that the long-term storage of weightlifting samples should continue, with the aim of increasing this practice at additional competitions to the Olympic Games, as anti-doping science continues to improve its detection methods.

4. AAS Doping at the Summer Olympic Games.

4.1. Introduction.

As discussed in section 1.6. and section 1.7., doping has been formally identified at every Summer Olympic Games except for the 1980 Moscow Games, although there are allegations of sample swapping [267] and a research based analysis of testosterone doping did also identify likely doping in ~20% of all athletes tested [2]. The first WADA code in 2004 stipulated that samples could be stored for long-term re-analysis to retrospectively catch doping and the IOC has financed this for samples collected at Olympic venues since 2004. As discussed in section 1.10.2. the 8-year statute of limitations for sample re-analysis from London 2012 concluded in August 2020 finalising the IOC retesting programme of samples collected during the 2004, 2008 and 2012 summer Olympic Games. A study has yet to been conducted to investigate how many ADRVs & medals were reallocated from this re-testing programme. Thereby, this study aimed to determine the effectiveness of this storage and retesting programme.

4.2. Methods.

The methods of this study are noted in section 2.2., 2.2.1. and 2.2.2. At the time of compiling the data for this study the re-analysis of samples removed from the former Moscow laboratory by WADA's Intelligence and Investigations team in December 2014 and April 2019 is still on-going and the associated "Operation Laboratory Information Management System (LIMS)" probe into institutionalized doping in Russia has not been concluded [268]. Due to this pending investigation which could involve samples collected at the winter Sochi Olympics 2014, this study only investigated the impact of doping on medal winning results of the summer Olympic Games and not winter Olympic Games.

4.3. Results.

4.3.1. IOC Retests of Athens 2004, Beijing 2008, and London 2012.

One-hundred and forty-two athletes were retrospectively identified to have committed ADRVs at the Athens 2004 (n = 5), Beijing 2008 (n = 65) and London 2012 (n = 72) Olympic Games from the targeted re-analysis of samples by the IOC. In London 2012 one of these athletes was deceased when this retrospective ADRV was discovered and so no

proceedings could be filed and two athletes in London 2012 were also retrospectively identified to have committed an additional ADRV prior to the Games. Metabolites of exogenous AAS were present in 90% of these samples with dehydrochloromethyltestosterone and stanozolol accounting for 79% of all detected substances (Table 3). Of the eight sports affected the highest number of athletes caught doping in these re-tests competed in athletics (n = 64) and weightlifting (n = 62) which combined accounted for 89% of the total (Table 4). Twenty-five nations were affected and the five nations with the highest number of affected athletes (Russia (n = 41), Belarus (n = 22), Ukraine (n = 14), Kazakhstan (n = 13) and Turkey (n = 8)) accounted for 69% of the total (Figure 6).

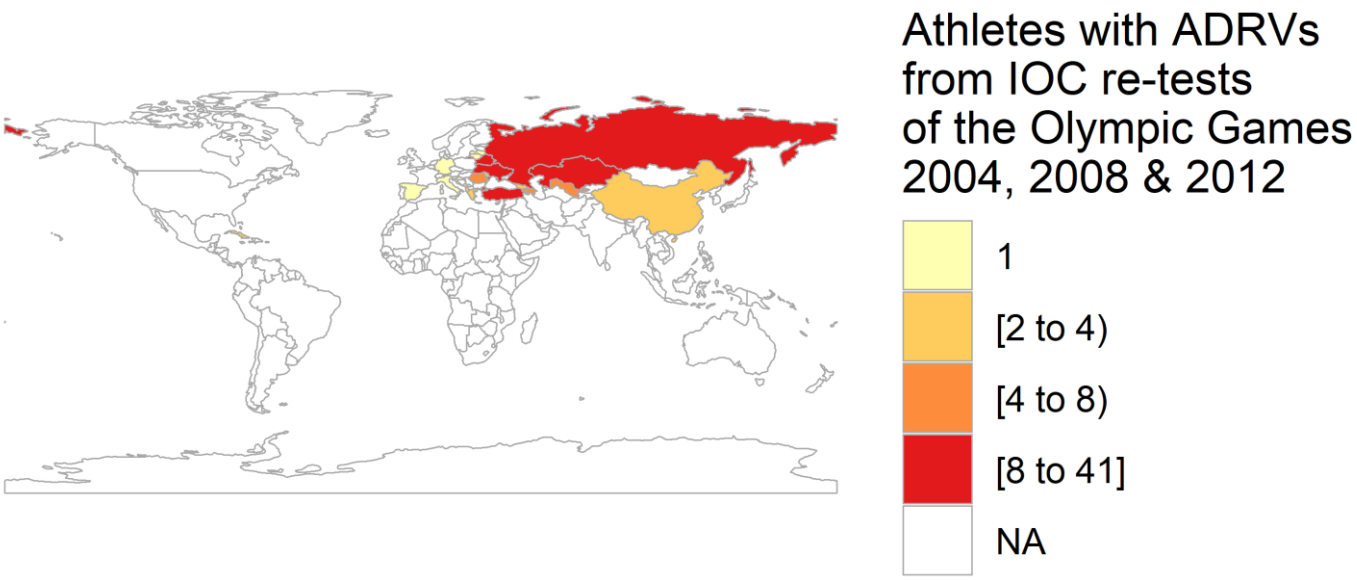


Figure 6. The athletes (n = 142) from the twenty-five nations who generated Anti-Doping Rule Violations (ADRVs) from IOC re-tests of the 2004, 2008 and 2012 Olympic Games. NA indicates zero recorded ADRVs.

4.3.2. Summer Olympic Games medals impacted by doping 1968 – 2012.

From 1968 to 2012 one-hundred and thirty-four summer Olympic medal-winning performances (Gold 43, Silver 47, and Bronze 44) have been impacted by an ADRV. The Sydney 2000 (Gold 8, Silver 1, Bronze 5), Athens 2004 (Gold 8, Silver 2, Bronze 5), Beijing 2008 (Gold 9, Silver 22, Bronze 19) and London 2012 Games (Gold 12, Silver 17, Bronze 11) account for 89% of the total number of impacted medals (Table 5). For only thirty-five medals (26% of the total number of impacted medals) the associated doping violation was identified at the time of the Games (Table 5). Doping violations that have been identified retrospectively, either occurring prior to the Games in which the medal was won and then impacting the subsequent Olympic result (Gold 10, Silver 7, Bronze 6) or occurring during the 2004, 2008 or 2012 Games but identified retrospectively by IOC re-tests (Gold 18, Silver 31, Bronze 27 - including one Gold medal that involves both scenarios) account for the majority (74%) of impacted medal-winning results (Table 5). The seventy-six medals associated with ARDVs from IOC re-tests of the 2004, 2008 and 2012 Games account for 57% of the total number of impacted medals. For these seventy-six medals it took a mean of 6.8 ± 2.0 years for the announcement of these ADRVs relative to the end of their respective Games. Weightlifting (Gold 9, Silver 10, Bronze 16) and athletics (Gold 7, Silver 12, Bronze 10) were the most affected sports and accounted for 84% of medals associated with ADRVs from these IOC re-tests. The number of medals impacted by ADRVs that have been identified retrospectively *vs* those not classified as retrospective cases is greater in Sydney 2000 (8 *vs* 6), Beijing 2008 (46 *vs* 4) and London 2012 (38 *vs* 2) (Table 5). From 1968 – 2012 for all medal-winning results impacted by ADRVs the detection of AAS account for 67% of all ADRVs (Table 6). From 1968 – 2012 of the twelve sports with medal results impacted because of ADRVs, athletics (Gold 21, Silver 21, Bronze 16) and weightlifting (Gold 14, Silver 14, Bronze 19) have been the most affected and account for 78% of the total number of impacted medals.

4.4. Discussion.

Athletes have been caught using prohibited substances at every summer Olympic Games in which testing has occurred except for at the 1980 Moscow Games. However, later unofficial research-based analysis suggested that ~20% of all athletes tested were likely doping with testosterone yet no test existed at the time [2] and there are reports from a retired KGB Lieutenant and a retired ex-Soviet Union medallist that urine swapping occurred at the 1980

Games “and that’s how the samples were clean” [267]. This analysis from 1968 – 2012 shows that for the majority (74%) of Olympic medals that have been impacted by doping violations, these doping violations have been identified retrospectively. The IOC’s targeted re-analysis of samples collected at the 2004, 2008 and 2012 Olympic Games accounted for 57% of all medals impacted by doping violations. It took a mean of 6.8 ± 2.0 years for these IOC re-tests that impacted medal results to be announced relative to the end of the Games in which the medal was originally won. Metabolites of exogenous AAS were present in 90% of the positive samples re-analysed by the IOC in 2004, 2008 and 2012 with dehydrochloromethyltestosterone and stanozolol accounting for 79% of all detected substances. The majority (89%) of the 142 athletes retrospectively charged with ADRVs from the IOC re-tests of the 2004, 2008 and 2012 Olympic Games competed in athletics (n = 64) and weightlifting (n = 62). Additionally, of twenty-five affected nations the five nations (Russia (n = 41), Belarus (n = 22), Ukraine (n = 14), Kazakhstan (n = 13) and Turkey (n = 8)) with the highest number of affected athletes accounted for 69% of the total number of athletes. These two findings, in conjunction with high levels of detection for long term metabolites for exogenous AAS, suggest that the prevalency of OOC doping with AAS is higher in certain sports and regions than others. At the time of competition these athletes had timed the clearance of prohibited metabolites from their system so that the available detection science would not catch them. These athletes may have been caught doping in real time prior to the Games if sufficient levels of OOC testing had been conducted across all geographic areas.

It takes time to research and develop new reliable and effective drug tests. When the WADA Code was implemented in 2004 long-term sample storage and re-analysis was envisaged to act as a deterrent to doping [127]. This is because even if athletes managed to beat tests whilst competing, they still risk getting caught doping years later. However, considering that athletes knew since 2004 that sample re-analysis with improved technologies was possible and that 6-months after Beijing 2008 two Olympic medallists were caught via this practice, twenty-eight medallists still got caught doping retrospectively at London 2012. This had led to some authors to suggest that the deterrence effect of long-term sample storage is limited, otherwise there would not have seen so many retrospective doping incidents [130].

The IOC will only reallocate a medal once all remedies of appeal are exhausted and all proceedings are closed, which can take a considerable amount of time (in some cases years)

after the retrospective ADRV is announced [269]. Dopers are requested by the IOC to return their medals so they can be given to the rightful winners, but they are not always so forthcoming and the IOC maintains a stock of blank medals for reallocations if the originals can't be acquired in time of the new planned medal ceremony [269]. Critics of the retrospective reallocation of Olympic medals years after the original event do acknowledge that it delivers sporting justice if enough athlete samples are stored and re-tested [130]. However, they also argue that any economic benefits from winning Olympic medals acquired from culprits in the years post victory are impossible to re-allocate and the athletes' experience of medal re-allocation years later can never replace a podium celebration after victory [130]. The IOC has improved their medal re-allocation protocols and in May 2018 approved six options for athletes to receive their medal(s): at the next edition of the Olympic Games; at the Youth Olympic Games; at the IOC headquarters or The Olympic Museum; at an event of their IF; at an event of their National Olympic Committee; or a private ceremony [270, 271]. Previously, there are reports of an athlete [272] nine years after the original event, being given his rightful Olympic gold medal in the food court of on airport by an official of their National Olympic Committee; a stark contrast to hearing their national anthem playing in a stadium filled with tens of thousands of people.

Start-up funding from the IOC in 2015 enabled the creation of the ITA who's overarching goal is to make anti-doping testing independent from sports organisations to prevent conflicts of interest [273]. The ITA has planned the "most comprehensive pre-Games testing programme ever conducted" for Tokyo 2020 and $5 million, spread over 10 years, will be allocated to a comprehensive long-term storage programme of these pre-Games' samples in addition to the regular long-term storage of samples collected during the Tokyo Games [274, 275]. This was announced prior to the coronavirus pandemic which has delayed the Tokyo Games to 2021 [276]. Globally, anti-doping testing has been greatly reduced during the coronavirus pandemic, (e.g., the United Kingdom Anti-Doping Agency between April and June 2020 carried out only 126 tests compared to 2,212 in the same quarter in 2019 [277]) making the long-term storage of pre-Games samples even more important for Tokyo as this lack of testing could have been an opportunity for a "doping-holiday" [278]. The IOC has also discussed the possibility of samples being collected in Tokyo for novel testing technologies/matrices, such as Dried Blood Spots (DBS) and gene expression ("omic") analysis, with the expectation that the long-term storage of samples with new methods will strengthen deterrence so that the cheats "never feel safe, anytime or anywhere" [273]. The

collection of capillary blood on DBS cards [279] and the collection of venous blood in RNA preservative for gene expression ("omic") analysis [172] and other currently unknown advances in anti-doping science may be complementary matrices/methodologies for future drug detection.

This study has shown that for the summer Olympic Games 1968 – 2012, long-term sample storage and re-analysis with improved technologies has caught more doping medallists than the testing technology available at the time of sample collection. The disproportionate representation of athletes from certain sports and nations charged with ADRVs from the IOC re-testing of the 2004, 2008 and 2012 Olympic Games suggests that future levels of pre-Olympic OOC testing should increase in these areas. It is therefore welcomed news [274] that the ITA is planning "the most comprehensive pre-Games testing programme ever conducted" for Tokyo 2021 that additionally includes the long-term storage of samples collected pre-Games. Educational programmes on anti-doping will also assist in changing this disproportionate presentation. Long-term storage is not standard across Continental Games, with IF having to fund the cost of long-term storage with WADA encouraging this practice to extend to Continental Games and other competitions [266]. Given these findings more IF should be encouraged to further their investment in long-term sample storage at Continental Games and other important international competitions to enhance future doping detection and to deliver sporting justice. Furthermore, given the success of sample re-testing with novel methods, it should be encouraged that long-term sample storage additionally incorporates the specific requirements of novel testing technologies/matrices, even if at the time of collection these methodologies are not fully validated for doping detection. During the 10-year statute of limitations [127] in which sample re-analysis can happen, further research on these technologies will occur and once validated they could be applied to this biobank of samples and may complement doping detection.

5. An observational human study investigating the effect of AAS on muscle memory in humans (The MMAAS Project).

5.1. Introduction.

As discussed in section 1.1. & 1.2. human testosterone administration studies [15, 17, 18, 20, 21] have shown that supraphysiological testosterone exposure is performance enhancing and causes a dose dependent increase in myonuclei in young and older men. As discussed in section 1.3. & section 1.3.1., myonuclei have been hypothesised to be a substrate for muscle memory [30] and a mice study has shown residual myonuclei from testosterone exposure are long lasting and result in enhanced rates of muscle re-growth suggesting a long term benefit from exposure [85]. However, as discussed in section 1.4. a body of evidence has emerged that suggests myonuclei are not permanent, with a review of the literature concluding there is currently no consensus on this topic due to conflicting evidence [103]. Furthermore, a systematic review and meta-analysis [104] of the literature base has concluded myonuclei are not permanent, although there are still a limited number of studies on this debated topic. As discussed in section 1.10.3 this study (The MMAAS Project) aimed to build on the literature base of previous studies [111,115] that have looked at myonuclei values in past AAS users and for the first time longitudinally monitor current AAS users post exposure to investigate AAS cessation on muscle fibre parameters pertinent to myonuclear permanency.

5.2. Methods.

The methods of this study are noted in section 2.3., 2.4., 2.5.1., 2.6. and 2.7.

5.3. Results.

5.3.1. Participant sampling and AAS usage.

Fifty-six participants visited the laboratory and consented (Table 8). 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 (Table 8). 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, with AAS usage as defined in Table 2, 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 (see Supplementary Data File 1 on OSF [225]).

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.

5.3.2. 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 7). Two participants within RT, respectively aged 25 and 42, did not have height or body composition measurements due to withdrawing from the study after blood collection and equipment unavailability (Table 7). Participants within Group C (20.5 ± 6.6 %) had a higher body fat percentage than Group RT (15.4 ± 4.4%, p = 0.015) and Group RT-AS (14.4 ± 3.4%, p = 0.004) (Table 7). Participants within Group RT-AS had greater FFM (77.0 ± 7.0 kg) than Group C (69.6 ± 12.1 kg, p = 0.036) and RT (71.4 ± 8.8 kg, p = 0.044) (Table 7). Mass, Body Fat (%) and FFM for RP1-5 are presented in Figure 7A, B

& C. FFM of RP2, RP3, RP4 and RP5 decreased by 3.9 - 4.7kg between visits. FFM of RP1 decreased by 0.9kg between visits.

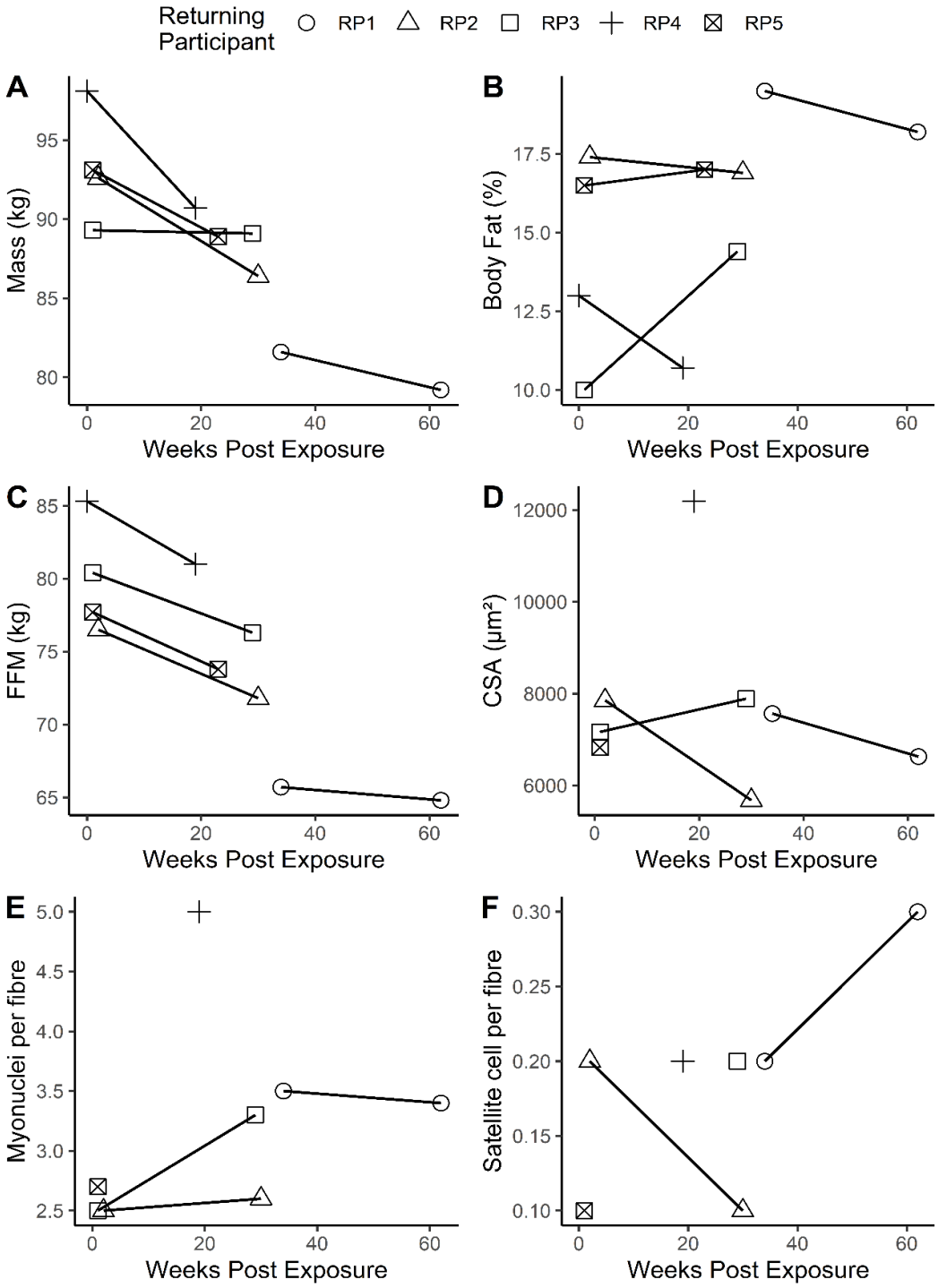


Figure 7. Body composition and Immunohistochemistry (IHC) data for first and second visits for returning participants (RP) within Group RT-AS (Resistance Trained Current AAS users) using the Tanita® Body Composition Analyzer BC-420MA (Bioelectrical Impedance) (*n* = 5). FFM: Fat Free Mass. IHC data in Panel D, E & F from RP4 is from second visit only.

5.3.3. Immunohistochemistry.

Mean fibre CSA was highest in RT-AS (8160 ± 1769μm²) (Figure 8A) 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 8B). 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 8C).

There was a strong positive correlation between myonuclei number and CSA ($r = 0.8$, $p<0.001$) (Figure 9) 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 7D) whilst their myonuclei per fibre values remained relatively similar between visits (3.5 *vs* 3.4 and 2.5 *vs* 2.6) (Figure 7E). RP3 exhibited an increase in fibre CSA (7167 *vs* 7889 μm²) (Figure 7D) and myonuclei per fibre (2.6 *vs* 3.3) (Figure E). Satellite cells per fibre decreased for RP2 between visits (0.2 *vs* 0.1) (Figure 7F) and increased for RP1 (0.2 *vs* 0.3) (Figure 7F).

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 9). 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 9). Type IIx CSA was significantly lower in RT than RT-AS ($p = 0.032$) (Table 9). Myonuclei per Type I and II fibres were not significantly different between groups (Table 9).

For illustrative purposes the appendix contains IHC images from Participant 24 (Group C).

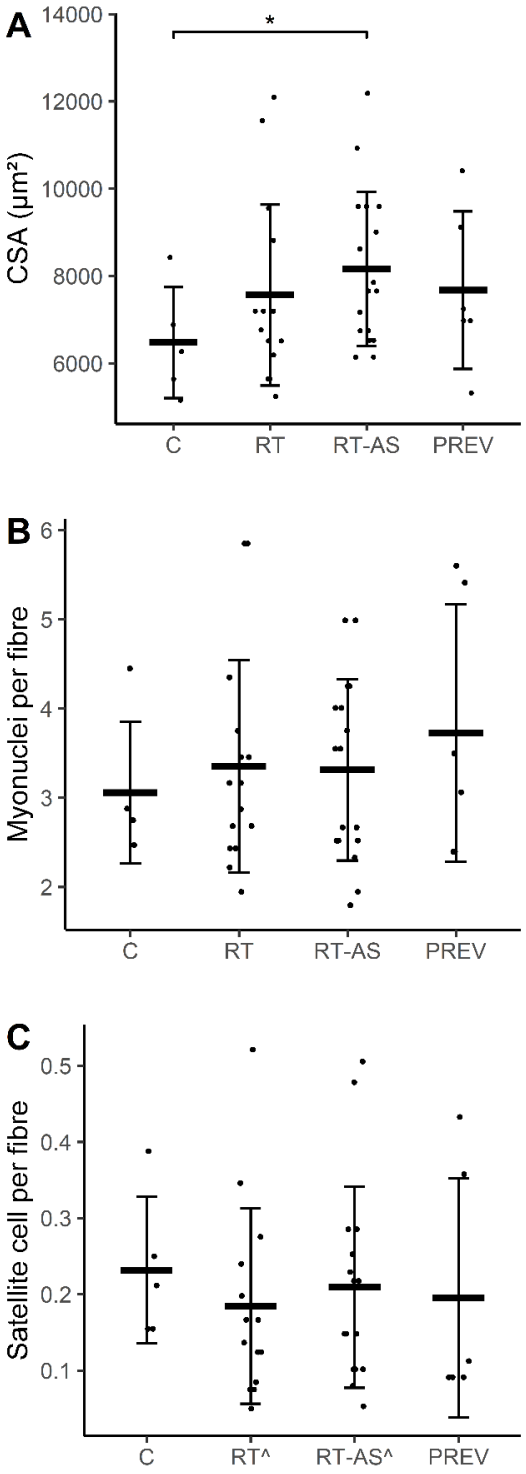


Figure 8. Muscle fibre CSA (A), Myonuclei per fibre (B) and Satellite cells per fibre (C) data from participants (n = 43) first sampling visit (including the single sample collected from Returning Participant 4 which occurred on his second visit). C: Control (n = 5); RT: Resistance Trained (n = 15); 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).

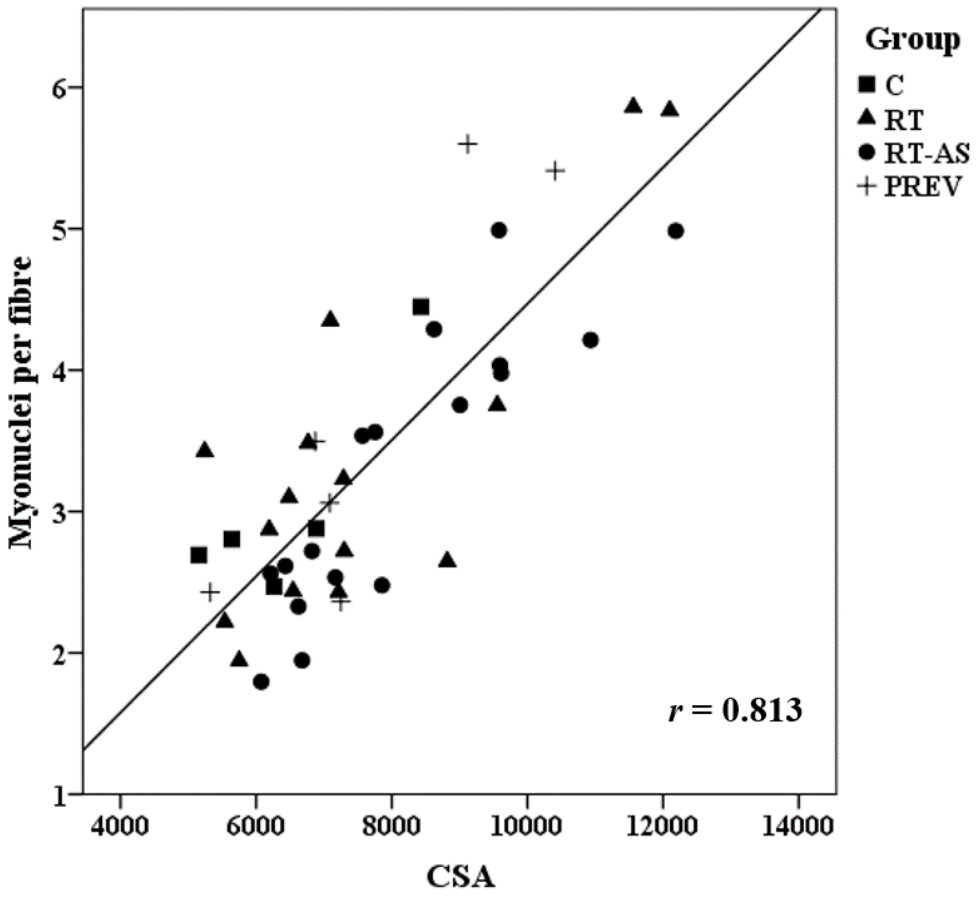


Figure 9. The correlation between Myonuclei per fibre and muscle fibre CSA from participants (*n* = 43) first sampling visit detailed in Chapter 3 & 4 (including the single sample collected from Returning Participant 4 which occurred on his second visit). C: Control (*n* = 5); RT: Resistance Trained (*n* = 15); RT-AS: Resistance Trained Current AAS users (*n* = 17); PREV: Previous AAS users (*n* = 6). Equation of the line, format y=mx + c: Myonuclei per fibre = 0.0005*(CSA) – 0.3499

5.4. 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 [220] and older men [281]. 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 [30, 52, 81] 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 [115] 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[113], 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 [197] 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 [282] the only ethically feasible way to study high dose/sustained AAS usage is via observational research [113]. 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 [283]. 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 [95-100] 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 [100, 101]. 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 [22], 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.

6. RNA-Seq of whole blood and skeletal muscle samples from The MMAAS Project.

6.1. Introduction.

As discussed in section 1.1. and 1.2. AAS increase FFM, muscle size and strength in men [15,17,18] with the majority of hypertrophic effects mediated through the AR which induces a genomic mode of action by modulating transcription [3,11]. As discussed in section 1.9. the "omic" technologies have shown promise for anti-doping purposes, particularly for rHuEPO [172] and a transcriptomic signature has been used to distinguish AAS doped and non-doped livestock [183]. As noted in section 1.10.4. RNA-Seq has yet to be conducted on whole blood samples collected from AAS users to investigate a transcriptomic signature of doping and so this study aimed to close this knowledge gap by conducting RNA-Seq on the whole blood samples collected in The MMAAS Project. Similarly, as noted in section 1.10.4. RNA-Seq has not been conducted on muscle samples from AAS users and so this study aimed to also conduct RNA-Seq on muscle samples collected in The MMAAS Project. It is possible that a transcriptomic signature of doping in muscle may be identified. Furthermore, this transcriptomic muscle data will further our knowledge of AAS induced gene transcription in hypertrophic pathways and completement the IHC research on muscle fibre parameters.

6.2. Methods.

The methods of this study are noted in section 2.5.2. and section 2.8.- 2.9.

6.3. Results.

6.3.1. Participant Sampling and AAS usage.

Fifty-five participants were sampled on first laboratory visit (Table 10a & 10b). Of those participants within RT-AS≤2, ten declared using AAS the week of sampling, four declared ceasing usage 1-week prior and one 2-weeks prior with an average last recorded weekly AAS dosage of 489 ± 319mg and range of 175mg – 1,300mg. Of those participants within RT-AS≥10, the number of weeks since last self-declared AAS exposure ranged from 10-347 weeks, with one participant declaring ceasing usage 10 weeks ago and the remaining ten declaring ceasing usage ≥34 weeks ago with an average last recorded weekly AAS dosage of 424 ± 226mg and range of 175mg – 700mg.

Returning Participant AAS regimens are described in section 5.3.1. Due to the differing timescale of cessation relative to sampling for RP1 compared to RP2-5, RP1 was excluded from returning participant comparisons.

6.3.2. RNA Quantity, Purity, and Integrity.

For all whole blood samples (*n* = 60, Table 10a,b) extracted total RNA was of sufficient concentration (103.1 ± 33.70 ng/μL), purity (A_{260}/A_{280} 2.09 ± 0.02; A_{260}/A_{230} 2.35 ± 0.1) and integrity (RIN 8.88 ± 0.57) for the thresholds stated in the used RNA library preparation protocols and thereby were subjected to RNA-Seq. After DNase I digestion four muscle samples (C=2, RT=2) did not have sufficient RNA yield for RNA library preparation. The remaining muscle samples (*n* = 52, Table 10a,b) had RNA that was of sufficient concentration (46.16 ± 19.59 ng/μL), purity (A_{260}/A_{280} 2.05 ± 0.05; A_{260}/A_{230} 1.81 ± 0.16) and integrity (RIN 8.34 ± 0.5) for the thresholds stated in the used RNA library preparation protocols and thereby were subjected to RNA-Seq.

6.3.3. RNA-Seq Quality Control.

FastQC showed that Mean Sequence Quality Scores were high (>30) for all samples (data available on OSF [225]). Fast Q Screen showed that sample swaps to those containing other species had not occurred in any sequencing dataset (data available on OSF [225]). Six blood samples (P04, P13, P15, P36, P41, P43), in both MGI and UoB sequencing datasets (data available on OSF [225]), showed abnormally high sequence duplication levels with overrepresented sequences matching known DNA oligos [284] used for rRNA depletion. This is caused by pipetting errors in the initial steps of library preparation of these samples in which an insufficient quantity of DNase enzyme was added that would digest added DNA oligos used for rRNA depletion. Four of these samples (P04, P15, P41, P43), that had the highest levels of reads mapping to other intergenic regions with RSeQC, were further deemed as outliers based on an MDS & PCA Plots (Appendix Figure 1a & 1b) of all blood samples (*n* = 55) sequenced at UoB used in RT-AS≤2 & RT-AS≥10 group comparisons and were subsequently removed from all further downstream analysis, including, for consistency, C, RT & Doped group comparisons (Table 10a,b). MDS & PCA Plots (Appendix Figure 2a & 2b) of all blood samples (*n* = 55) sequenced at MGI used in RT-AS≤2 & RT-AS≥10 group

comparisons showed, compared to the UoB dataset, a cluster of 8 samples sequenced on Flow Cell Lane B1 (P13, P17, P20, P26, P32, P44, P49, RP5 Visit 1). From this finding and the notion lane position was a randomised order these samples were removed as outliers and were therefore subsequently removed from all further downstream analysis, including C, RT & Doped group comparisons (Table 10a,b) as the corresponding PCA Plot (Appendix Figure 2c) also showed them as outliers. The subsequent MDS & PCA plots (Appendix Figure 3a & 3b) of remaining blood samples (n = 47) sequenced at MGI used in RT-AS≤2 & RT-AS≥10 group comparisons were similar to the UoB MDS & PCA Plots (Appendix Figure 1a & 1b) and for consistency between UoB and MGI datasets the four samples (P04, P15, P41, P43) with aberrant library preparation were also removed from further downstream analysis, including C, RT & Doped group comparisons, from the whole blood MGI dataset as outliers (Table 10a,b) with the PCA plot of Group C, RT and Doped comparisons shown in Appendix Figure 3c.

MDS & PCA plots of blood samples (n = 8) of RP2-5 1st and 2nd visits from the UoB and MGI datasets (Appendix Figure 4a,b & 5a,b) showed that RP5 Visit 1, like in the MGI RT-AS≤2 & RT-AS≥10 group comparison data set, was an outlier and so due to paired sample analysis RP5 was removed from further downstream analysis in the MGI returning participant dataset (Table 10a,b). The subsequent MDS plot (Appendix Figure 6a & 6b) of the MGI dataset of RP2-4 (n = 6) 1st and 2nd visits were similar to the UoB dataset of all (n = 8) RP samples (Appendix Figure 4a & 4b).

No muscle samples were excluded from downstream analysis (Table 10a,b). No abnormally high levels of sequence duplication levels were observed, and no overrepresented sequences matched DNA oligos used for rRNA depletion. MDS & PCA Plots of the standard and CoolMPS chemistry sequencing datasets, used in RT-AS≤2 & RT-AS≥10 group comparisons (Appendix Figure 7a,b & 8a,b) and RP Visit comparisons (Appendix Figure 9a,b & 10a,b), showed no obvious outliers and were similar. The same can be said for PCA Plots when Group C, RT & Doped group comparisons were used (Appendix Figure 7c & 8c). The RSeQC function split_bam.py showed that all blood and muscle samples had zero reads originating from rRNA showing that rRNA depletion was successful.

6.3.4. Read Mapping, Read Distribution, and transcript quantification.

Genome mapping using HISAT2 [235] respectively showed average overall alignment rates of 96.6 ± 1.2% and 95.4 ± 1.9% for whole blood samples sequenced at UoB (*n* = 55) and MGI (*n* = 46) and 95.3 ± 1.7% and 98.4 ± 0.4% for muscle (*n* = 51) samples sequenced with standard and CoolMPS reagents that were used in downstream DGE/GSEA analyses.

RSeQC [230] showed that a lower proportion of reads in whole blood samples sequenced at UoB (*n* = 55, 50.3 ± 6.4%) and MGI (*n* = 46, 50.6 ± 6.0%) used in downstream DGE/GSEA analyses mapped to exons compared to muscle (*n* = 51) samples sequenced with standard and CoolMPS reagents used in downstream DGE/GSEA analyses (66.8 ± 2.8% and 66.6 ± 2.8%). Correspondingly, a higher proportion of reads in whole blood samples sequenced at UoB (*n* = 55, 26.4 ± 4.5%) and MGI (*n* = 46, 26.2 ± 4.7%) used in downstream DGE/GSEA analyses mapped to introns compared to muscle (*n* = 51) samples used in downstream DGE/GSEA analyses sequenced with standard and CoolMPS reagents (17.1 ± 2.7% and 17.2 ± 2.6%) with these differences likely related to differences in the RNA extraction protocols used (extracting total RNA in whole blood *vs* extracting total RNA >200 nucleotides in length in muscle).

For whole blood samples sequenced at UoB (*n* = 55) and MGI (*n* = 46) used in downstream DGE/GSEA analyses the average number of processed reads (59.5 ± 15.4 million and 59.9 ± 11.5 million) and mapped reads (55.4 ± 6.9% and 55.6 ± 6.4%) used by Salmon was lower than in muscle samples (*n* = 51) sequenced with standard (69.3 ± 12.2 million, 70.2 ± 3.8%) and CoolMPS (68.2 ± 11.6 million, 70.5 ± 3.7%) reagents.

The number of genes available for DGE analysis, across the four analysed datasets, was 14,353 – 16,687 (Table 11a - d) following the stated count filtering criteria after Salmon transcript-level estimates were summarised to genes.

The biological coefficient of variation (BCV) for whole blood samples sequenced at UoB and MGI is greater (0.26) than the 0.01 threshold stated in the edgeR user manual [285] as an acceptable amount of variation for technical replicates and thereby they have not been merged as one dataset and instead have been used to cross-validate each other. As standard reagent and CoolMPS reagent sequencing chemistries differ [286] these two muscle datasets cannot be merged as technical replicates and so have been used to cross-validate each other. The cross-comparison of RNA libraries that have been sequenced twice was thereby the methodology used to verify identified differentially expressed genes/pathways, as opposed to

the additional time and financial demands of using an alternative laboratory method (e.g., qPCR).

6.3.5. Differential Gene Expression Analysis – Returning Participants.

For returning participant visit comparisons no differentially expressed genes in whole blood were identified in the UoB (n = 8) sequencing dataset, although 11 differentially expressed genes were identified in the MGI (n = 6) sequencing dataset and so none overlapped between sequencing datasets (Table 12a & 12b). Returning participants clustered by participant in MDS & PCA plots (Appendix Figure 4a,b & 6a,b).

Of the six genes identified as differentially expressed, across both muscle sequencing datasets (Table 12e & 12f), from returning participants first and second visits (n = 6), only one of these genes was differentially expressed in both datasets (Table 13) with *CHRDL1* being upregulated in Visit 2. Returning participants clustered by participant in an MDS & PCA plots (Appendix Figure 9a,b & 10a,b). PLS-DA cannot be conducted on any returning participant data as mixOmics [245], at the time of writing, does not support longitudinal/time-course data analysis.

6.3.6. Differential Gene Expression Analysis – Group Comparisons.

Both blood sequencing datasets, using RT-AS≤2 & RT-AS≥10 group comparisons, subjected to DGE analysis (Table 10a) did not show clear-cut group clustering in MDS, PCA & PLS-DA Plots (Appendix Figure 11a,b,c & 12a,b,c). For PLS-DA, the BER varied from 0.77 – 0.81.

Cross comparison of DGE analysis results of both blood sequencing datasets, using RT-AS≤2 & RT-AS≥10 group comparisons, (Table 12a & 12b) only identified two genes as differentially expressed in both datasets, with *MTND1P23* downregulated when RT was compared to C and *IGLV3-10* upregulated when RT-AS≥10 was compared to RT. For both genes VIP was >1.

Similarly, both blood sequencing datasets using C, RT & Doped group comparisons, subjected to DGE analysis (Table 10b) did not show clear-cut group clustering in PCA or

PLS-DA Plots (Appendix Figure 11d,e & 12d,e). For PLS-DA, the BER varied from 0.65 – 0.68.

Cross comparison of DGE analysis results of both blood sequencing datasets, using C, RT & Doped group comparisons, (Table 12c & 12d) identified that a small number of genes were differentially expressed in both datasets with *MTND1P23* downregulated when RT was compared to C, with *MTND1P23*, *IGLV2-8* and *C4BPA* downregulated when Doped was compared to C, but no overlapping genes were differentially expressed when Doped was compared to RT. For all three of these differentially expressed genes VIP was >1.

Both muscle sequencing datasets, using RT-AS≤2 & RT-AS≥10 group comparisons subjected to DGE analysis (Table 10a) did not show clear-cut group clustering in MDS, PCA or PLS-DA Plots (Appendix Figure 7a,b,c & 8a,b,c). For PLS-DA, the BER varied from 0.56 – 0.69.

When both muscle datasets (Table 12e & 12f) were cross compared, for validation purposes, using RT-AS≤2 & RT-AS≥10 group comparisons, each group comparison had differentially expressed genes, except for when RT-AS≥10 was compared to RT when no differences were present (Table 13). The greatest number of differentially expressed genes occurred when RT-AS≤2 was compared to Group RT in which 68 genes were upregulated and 37 downregulated (Table 13). Figure 10 shows, for genes that overlap across both muscle sequencing datasets, a Venn Diagram of all five group comparisons that had differentially expressed genes. Nine differentially expressed genes (*ABCA7*, *ARHGEF17*, *BOK*, *FILIP1L*, *LDAF1*, *RBL1*, *RPIA*, *SDC4*, *ZFP36L1*), overlapped with RT-AS≤2 *vs* RT and RT-AS≤2 *vs* C, but were not differentially expressed with RT *vs* C, possibly suggesting they are from acute doping alone (Figure 10). For all nine genes VIP was >1.

Similarly, both muscle sequencing datasets, using C, RT and Doped group comparisons subjected to DGE analysis (Table 10b) did not show clear-cut group clustering in PCA or PLS-DA Plots (Appendix Figure 7d,e & 8d,e). For PLS-DA, the BER varied from 0.56 – 0.64.

When both muscle datasets (Table 12g & 12h) were cross compared, for validation purposes, using C, RT and Doped group comparisons, only the comparisons RT *vs* C and Doped *vs* C had differentially expressed genes, with no differentially expressed genes for Doped *vs* RT in both datasets. For RT *vs* C the overlapping differentially expressed genes across both datasets were: an upregulation of *NAP1L4* and *CARS1* and a downregulation of *VMO1* and for Doped

vs C: an upregulation of *NAP1L4* and *CARS1* and a downregulation of *PATJ* and *GUSBP1* (Table 12g & 12h). For all three genes VIP was >1.

Lists of all genes and associated log Fold Change and FDR values for blood (UoB & MGI datasets) and muscle samples (standard & CoolMPS datasets) for returning participant and group comparisons that were analysed in DGE analysis are publicly available on OSF [225]. After cross comparison of the muscle samples sequenced in standard chemistry reagents and CoolMPS reagents a list of all overlapping significantly differentially expressed genes (FDR < 0.05 and a 1.2- fold change) for all group comparisons is also available on OSF [225].

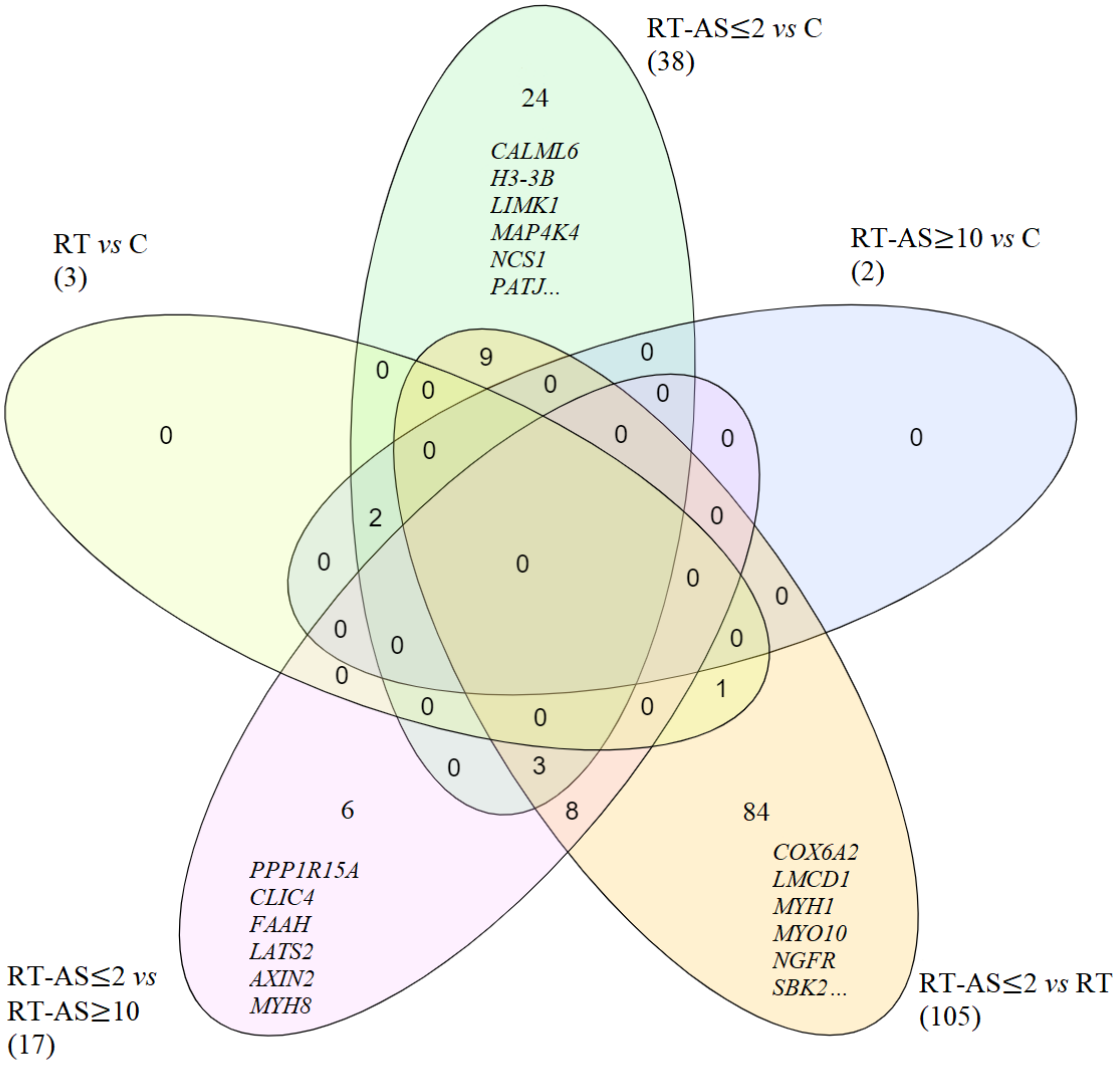


Figure 10. A Venn Diagram of Differentially Expressed Genes (DEG) that overlapped between the standard and CoolMPS sequencing datasets of the muscle samples with group comparisons involving C, RT, RT-AS≤2 & RT-AS≥10. Numbers in brackets indicate the total number of DEG for that comparison. C: non-resistance trained control group (*n* = 5); RT: Resistance Trained control group (*n* = 17); RT-AS≤2: Resistance Trained participant who self-declared AAS exposure ceased ≤2 weeks before sampling (*n* = 15); RT-AS≥10: Resistance Trained participant who self-declared AAS exposure ceased ≥10 weeks before sampling (*n* = 11).

6.3.7. Gene Set Enrichment Analysis – Returning Participants.

Blood samples from the UoB (*n* = 8) and MGI (*n* = 6) sequencing datasets for returning participants between visits, did not show any differences in the gene sets or pathways tested.

Both muscle sequencing datasets, for returning participants (*n* = 3, RP2-4) did identify a low number of differences in the gene sets and pathways tested. The standard chemistry dataset had one GO BP gene set differentially expressed and the CoolMPS dataset had two Reactome pathways differentially expressed (Table 14a & 14b). However, cross comparison of the two muscle sequencing datasets, for validation purposes, showed that for comparisons between visits for returning participants no differences in the gene sets or pathways tested overlapped between sequencing datasets (Table 15).

6.3.8. Hierarchical clustering.

Respectively, hierarchical clustering of muscle samples and the top 30 most significantly differentially expressed genes by FDR, with a minimum 1.2-fold change, for the group comparison RT-AS≤2 (*n* = 15) *vs* RT (*n* = 17) for standard (Figure 11) and CoolMPS (Figure 12) datasets showed that samples within RT-AS≤2 tended to cluster together with a subset of genes being down and upregulated.

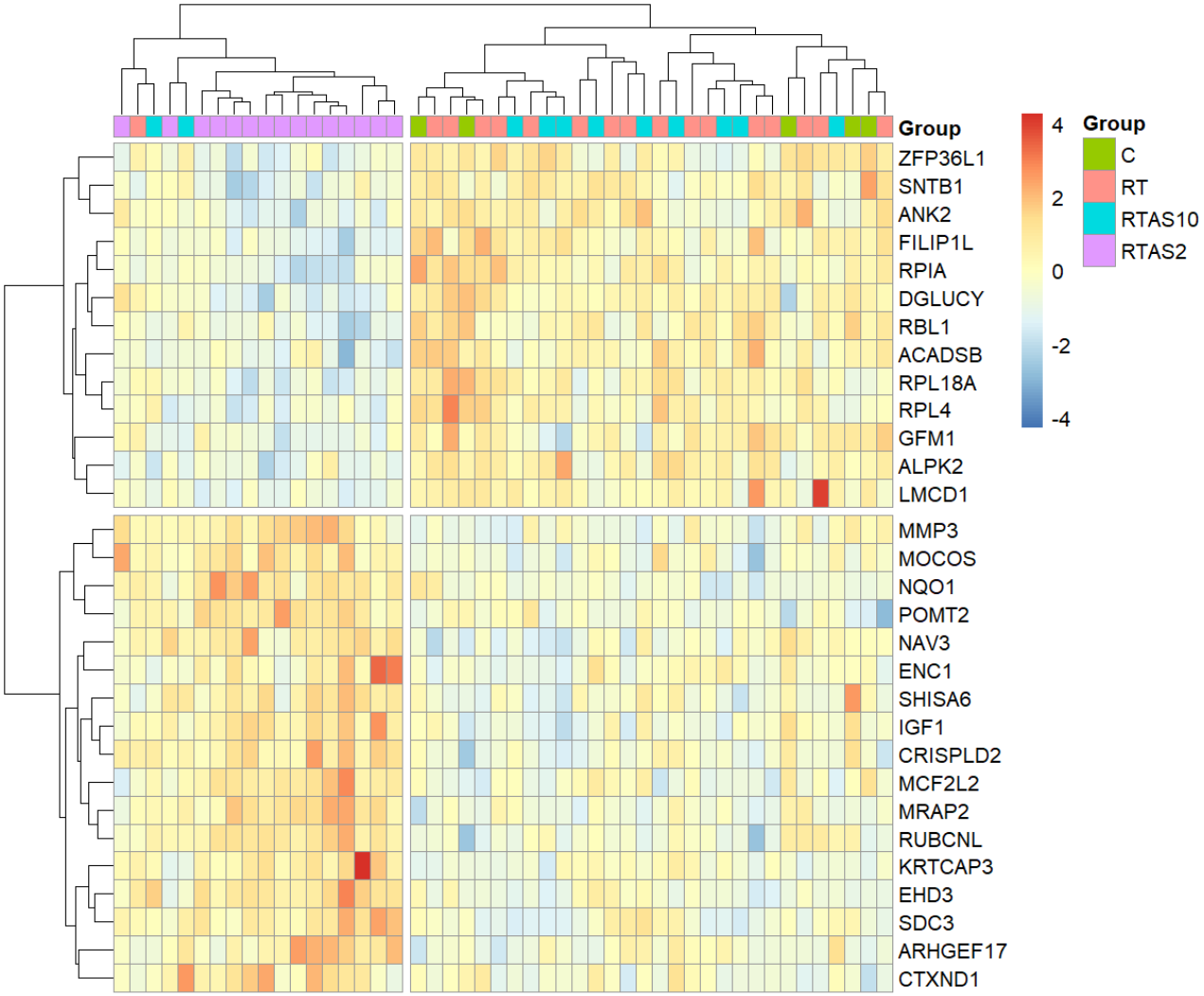


Figure 11. A heatmap of the top 30 most differentially expressed genes by FDR, with a minimum 1.2-fold change, from the group comparison RT-AS≤2 *vs* RT for all muscle samples subjected to DGE analysis sequenced with standard chemistry reagents at MGI, Latvia. C: non-resistance trained control group (*n* = 5); RT: Resistance Trained control group (*n* = 17); RT-AS≤2: Resistance Trained participant who self-declared AAS exposure ceased ≤2 weeks before sampling (*n* = 15); RT-AS≥10: Resistance Trained participant who self-declared AAS exposure ceased ≥10 weeks before sampling (*n* = 11); DGE: differential gene expression.

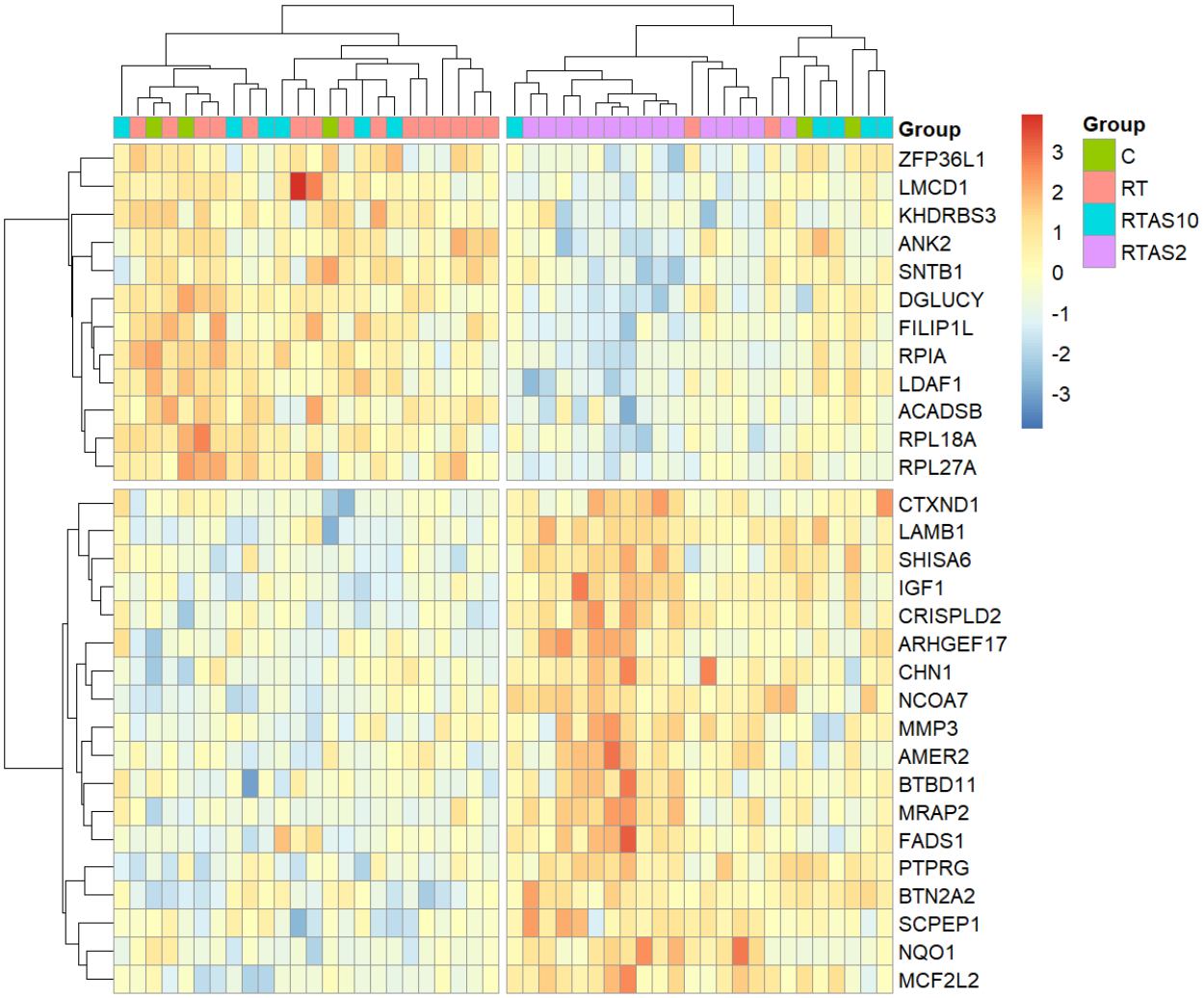


Figure 12. A heatmap of the top 30 most differentially expressed genes by FDR, with a minimum 1.2-fold change, from the group comparison RT-AS≤2 *vs* RT for all muscle samples subjected to DGE analysis sequenced with CoolMPS reagents at MGI, Latvia. C: non-resistance trained control group (*n* = 5); RT: Resistance Trained control group (*n* = 17); RT-AS≤2: Resistance Trained participant who self-declared AAS exposure ceased ≤2 weeks before sampling (*n* = 15); RT-AS≥10: Resistance Trained participant who self-declared AAS exposure ceased ≥10 weeks before sampling (*n* = 11); DGE: differential gene expression.

6.3.9. Gene Set Enrichment Analysis – Group Comparisons.

For blood samples (*n* = 51; C=7, RT=20, RT-AS≤2=14, RT-AS≥10=10) sequenced at UoB with standard chemistry reagents subjected to GSEA, only two GO BP gene sets showed differences when RT-AS≤2 was compared to C and one GO MF gene set when RT-AS≤2 was compared to RT-AS≥10 with no other group comparisons showing differences in the tested gene sets or pathways. For blood samples (*n* = 43; C=6, RT=17, RT-AS≤2=12, RT-AS≥10=8) sequenced at MGI with standard chemistry reagents subjected to GSEA no group comparison showed differences in any of the tested gene sets or pathways. Thereby, no gene sets or pathways were differentially expressed in both the UoB and MGI sequencing datasets when cross compared.

Overlapping differentially expressed gene sets/pathways from cross comparison of the standard and CoolMPS sequencing datasets, for validation purposes, of the muscle samples (*n* = 48; C=5, RT=17, RT-AS≤2=15, RT-AS≥10=11) are shown in Table 15. The greatest number of differentially expressed gene sets/pathways occurred when RT-AS≤2 was compared to RT. All seventy-four (Table 15) of the differentially expressed Reactome pathways in this comparison were unique and eleven of twelve of the differentially expressed KEGG pathways for this comparison were unique. Venn Diagrams for RT-AS≤2 compared to C, RT and RT-AS≥10 for GO BP and GO MF gene sets are shown in Figure 13 and 14.

Lists of all gene sets and pathways and associated FDR values for blood (UoB & MGI datasets) and muscle samples (standard & CoolMPS datasets) for returning participant and group comparisons that were subjected to GSEA are available on OSF [225]. Lists of GO BP and GO MF gene sets and KEGG and Reactome pathways that have overlapping expression data with FDR <0.05 based on cross comparison of the standard and CoolMPS datasets for the muscle samples are available on OSF [225].

GSEA was not conducted on whole blood or muscle sequencing datasets for group comparisons of C, RT & Doped because of no overlapping differentially expressed genes of Doped *vs* RT when both whole blood and both muscle datasets were cross compared for validation purposes (see section 6.3.6).

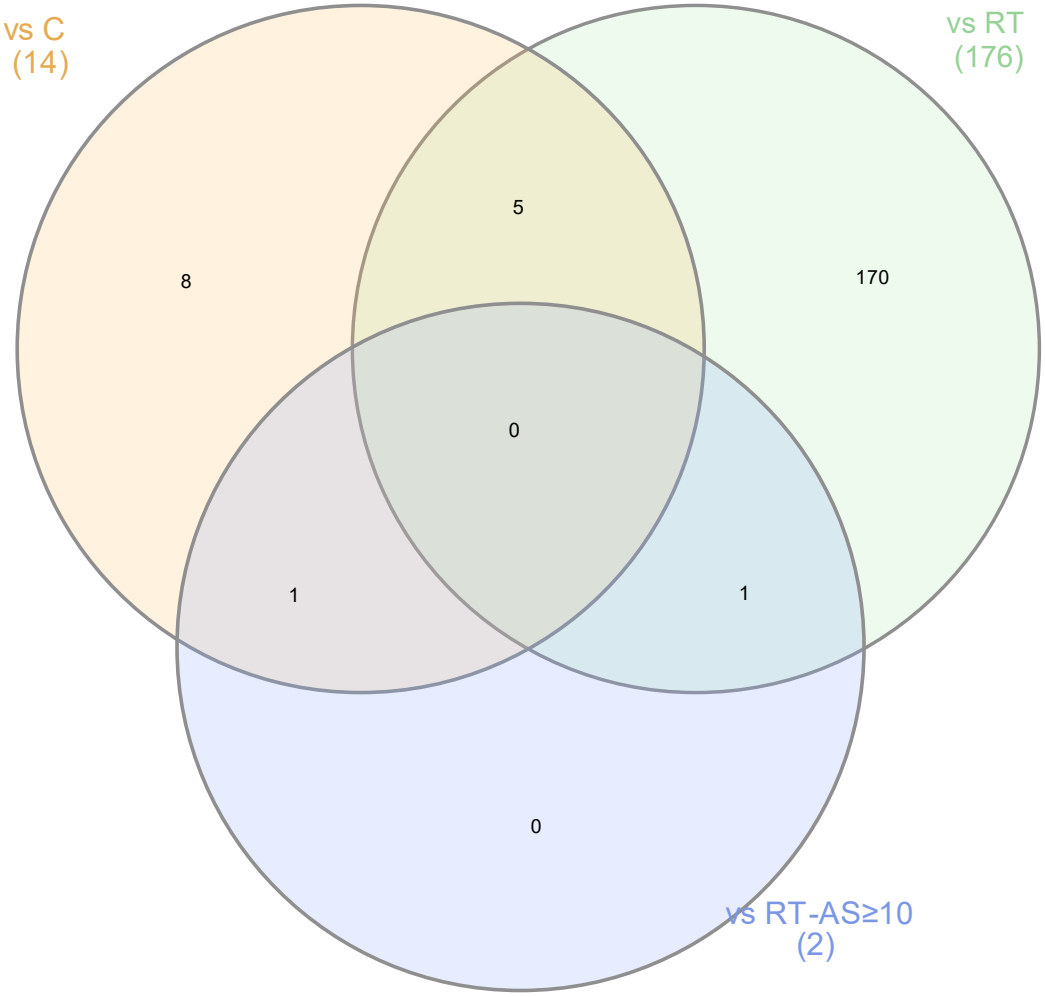


Figure 13. A Venn Diagram of GO BP gene sets that overlapped between the standard and CoolMPS sequencing datasets of the muscle samples, showing comparisons of RT-AS≤2 (*n* = 15) to C (*n* = 5), RT (*n* = 17) and RT-AS≥10 (*n* = 11), which were the only comparisons that had differences in GO BP gene sets. C: non-resistance trained control group; RT: Resistance Trained control group; RT-AS≤2: Resistance Trained participant who self-declared AAS exposure ceased ≤2 weeks before sampling; RT-AS≥10: Resistance Trained participant who self-declared AAS exposure ceased ≥10 weeks before sampling.

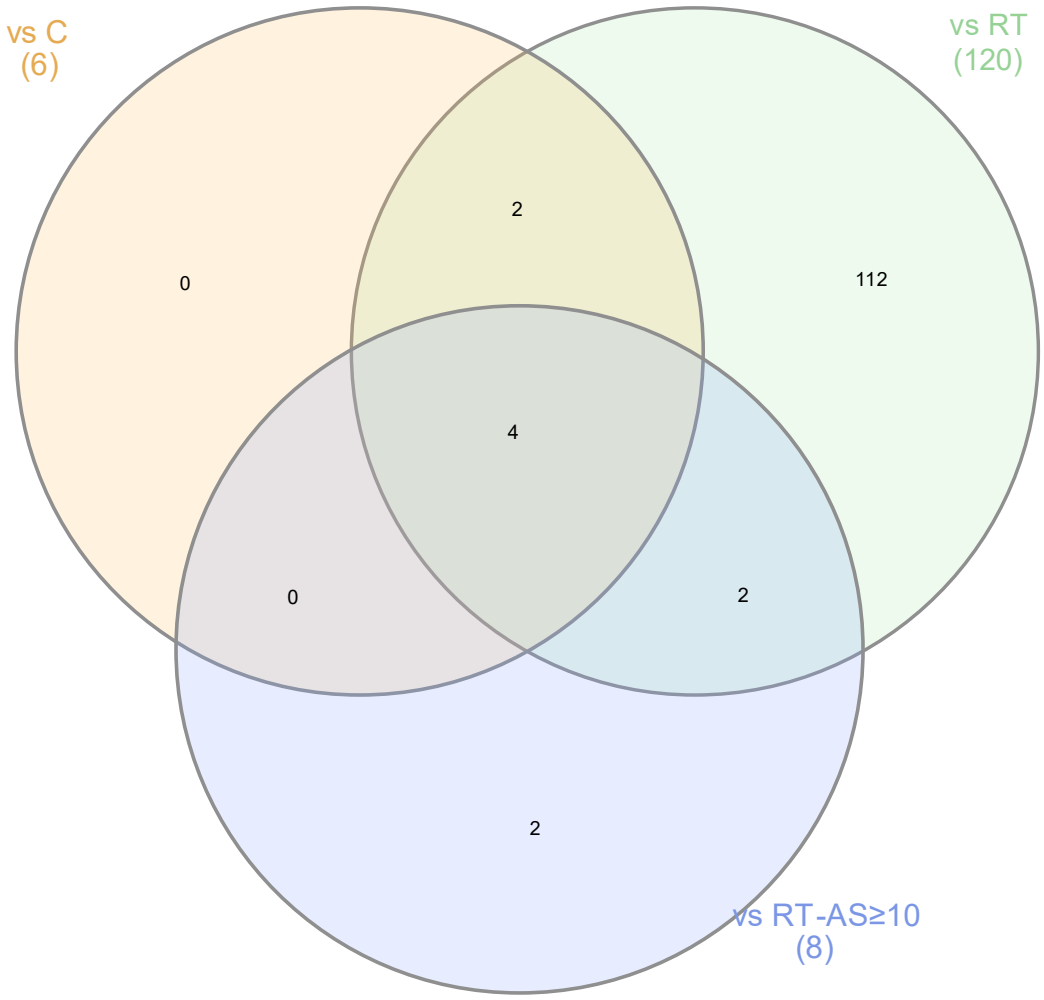


Figure 14. A Venn Diagram of GO MF gene sets that overlapped between the standard and CoolMPS sequencing datasets of the muscle samples, showing comparisons of RT-AS≤2 (*n* = 15) to C (*n* = 5), RT (*n* = 17) and RT-AS≥10 (*n* = 11), which were the only comparisons that had differences in GO MF gene sets. C: non-resistance trained control group; RT: Resistance Trained control group; RT-AS≤2: Resistance Trained participant who self-declared AAS exposure ceased ≤2 weeks before sampling; RT-AS≥10: Resistance Trained participant who self-declared AAS exposure ceased ≥10 weeks before sampling.

6.4. Discussion.

Returning AAS users (n = 4) who self-declared AAS cessation ≤ two weeks prior to first sampling and returned 19 – 28 weeks later, did not show differences in gene expression in whole blood between time points when all samples were analysed (Table 12a). Re-sequencing of these RNA libraries and removal of one sample for aberrant sequencing quality control values unique to this re-sequencing dataset did show some differences in gene expression between time points (Table 12b), but this reduction in statistical power from lower participant numbers could have influenced this dataset specific result. Both returning participant whole blood sequencing datasets did not show any differences in the gene sets or pathways tested between timepoints.

For validation purposes, cross-comparison of both whole blood sequencing datasets of group comparisons showed that RT-AS≤2 did not differ in gene expression to C, RT, and RT-AS≥10. Both sequencing datasets did show RT-AS≥10 did have had one gene (*IGLV3-10*) upregulated when compared to RT (Table 12a & 12b). However, it would be difficult to conclude that this is a lingering whole blood transcriptional biomarker of AAS usage given there was no differences in this gene in users who ceased AAS exposure at an earlier time frame, or controls and because this gene codes for a variable domain of an immunoglobulin light chain it is likely to be impacted heavily by the immune response to pathogens/exogenous factors. Using the group comparisons, C, RT & Doped, cross comparing both whole blood sequencing datasets for gene verification/validation purposes, showed that no overlapping genes were differentially expressed when Doped was compared to RT. For validation purposes cross-comparison of both whole blood sequencing datasets for group comparisons involving C, RT, RT-AS≤2 and RT-AS≥10 also did not reveal any differentially expressed gene sets or pathways.

For the two muscle sequencing datasets, cross-comparison of the paired sample analysis of returning participants post AAS exposure demonstrated that one gene (*CHRDL1*) was upregulated in visit two in both datasets (Table 13). *CHRDL1* encodes for the Chordin-Like 1 (CHRDL1) protein which is a known antagonist of bone morphogenetic protein (BMP), and BMP signalling is known to play a key role in muscle development, hypertrophy and regeneration [287]. In adult mice, in the absence of injury, increasing BMP expression, or BMP receptor activity, is known to induce hypertrophy via activation of mTOR signalling [288]. Furthermore, inhibition of BMP signalling causes muscle atrophy and abolishes the

hypertrophic phenotype of myostatin-deficient mice, with BMP signalling being regarded as a fundamental hypertrophic signal in mice [289]. These three returning participants (RP2-4) that showed an upregulation of *CHRDL1* in their second visit, also showed a 4.4 ± 0.3 kg loss of FFM (Figure 7C) as measured with bioelectrical impedance, a finding which corroborates with CHRDL1 as an antagonist of BMP and BMP inhibition causing atrophy. Furthermore, RP2 also exhibited a decrease in muscle fibre CSA between visits (7854 *vs* 5677 μm^2), whereas for RP3 an increase in CSA (7167 *vs* 7889 μm^2) was observed (Figure 7D) and RP4 did not have a sample stored for immunohistochemistry on first sample visit (Table 8). When cross-compared, none of the tested gene sets or pathways showed differences in both muscle sequencing datasets for comparisons of returning participant visits.

Cross-comparison, for validation purposes, of both muscle sequencing datasets using C, RT, RT-AS≤2 and RT-AS≥10, showed that the greatest number of differentially expressed genes occurred when RT-AS≤2 was compared to other groups (Table 13). Comparing RT *vs* C (the effect of training), RT-AS≤2 *vs* C (the effect of training and acute AAS usage) and RT-AS≤2 *vs* RT (the effect of acute AAS usage), showed that nine differentially expressed genes (*ABCA7*, *ARHGEF17*, *BOK*, *FILIP1L*, *LDAF1*, *RBL1*, *RPIA*, *SDC4*, *ZFP36L1*) overlap (Figure 10) between RT-AS≤2 *vs* C and RT-AS≤2 *vs* RT, but were not differentially expressed in RT *vs* C, potentially indicating this is caused by acute AAS usage and not from training alone. Amongst these genes, associated with performance benefit would be a downregulation of *RBL1*, with reduced expression of this transcriptional corepressor being associated with mitochondrial biogenesis, typically stimulated by exercise [290]. However, contradictory to performance/hypertrophic benefit amongst these genes was downregulation of *SDC4*, a proteoglycan known to be crucial for muscle differentiation [291] that may act as a reservoir for promyostatin [292], subsequently inhibiting the formation of active myostatin, with reduced expression being associated with elevated levels of myostatin [292]. Additionally, *BOK*, a pro-apoptotic member of the BCL-2 family, was upregulated, with this family of proteins being upregulated in denervation-induced muscle atrophy [293] and *ZFP36L1* was downregulated, with reduced expression being associated with reduced skeletal muscle mass and reduced satellite cell numbers [294].

Although two genes (*NAP1L4* and *CARS1*) were differentially expressed when RT-AS≥10 was compared to C (Table 13) in both muscle sequencing datasets, these two genes were not unique to this comparison and were also differentially expressed in RT *vs* C and RT-AS≤2 *vs* C (Figure 10) indicating they are unlikely to be unique markers of long-term steroid usage

and more likely due to resistance training alone as they were also not differentially expressed when RT-AS≤2 was compared to RT.

For both muscle sequencing datasets using the group comparisons C, RT & Doped there were no differentially expressed genes for Doped *vs* RT.

Hierarchical clustering and heatmaps of muscle samples using the top 30 most significantly differentially expressed genes by FDR, with a minimum 1.2-fold-change, for the group comparison RT-AS≤2 to RT showed that most samples within RT-AS≤2 clustered together in both sequencing datasets (Figure 11 & 12). However, previous research in animal husbandry has shown that similar hierarchical clustering methods using 20 differentially expressed genes in liver samples can fully distinguish boars and calves treated with AAS with no cross-group clustering [183]. Using an Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) model [114], proteomic analysis of human vastus lateralis muscle samples from 10 current AAS users, who had used large AAS doses (>800mg) for 5-15 years, showed a clear separation from 7 non-AAS using resistance trained controls. Liquid chromatography followed by tandem spectrometry identified 14 protein spots (representing nine different proteins) of significant difference in relative quantity between the doped and clean groups [114]. However, analysis of the RNA-Seq data from both muscle datasets in this study did not identify any of the genes that correspond to these nine proteins as differentially expressed in any comparison (see data files on OSF [225]). The participants in this present study having much lower AAS exposure regimens could have contributed to this discrepancy, in addition to the different muscle biopsy location.

Of note, was that in both muscle sequencing datasets *IGF1* was significantly upregulated when RT-AS≤2 was compared to RT and RT-AS≥10, but no difference was observed when RT-AS≤2 was compared to C, but the relatively lower participant numbers in group C compared to these other groups may have played a role in this finding with a subsequent reduction in statistical power. The insulin-like growth factor 1-Akt/protein kinase B (IGF1-Akt/PKB) pathway is known to play a major role in the regulation of skeletal muscle growth [295]. Binding of IGF1 to its receptor results in the activation of Akt which both stimulates protein synthesis via mTOR and glycogen synthase kinase 3β (GSK3β) and also inhibits protein degradation by repressing transcription factors of the FoxO family [296]. It should be noted that the hypertrophic effect of AAS on protein synthesis is further complicated by the fact that AAS can restore muscle hypertrophy in castrated mice limb muscles to sham levels

even when rapamycin is administered to inhibit mTOR [28], indicating that AAS can induce hypertrophy in a rapamycin insensitive manner.

Cross-comparison of both muscle sequencing datasets, using C, RT, RT-AS≤2 and RT-AS≥10, showed that no gene sets or pathways were differentially expressed when RT was compared to C, with differences only observed when RT-AS≤2 was compared to C, RT and RT-AS≥10 with no differences between returning participants visits (Table 15). Notably, eight GO BP gene sets (Figure 13), were uniquely differentially expressed for the comparison RT-AS≤2 *vs* C, including a down regulation of a gene set that reduces the activity of intracellular steroid hormone receptor signalling pathways, corroborating with the known AR pathway for AAS induced transcription. For GO MF gene sets, none were uniquely differentially expressed for the comparison RT-AS≤2 *vs* C (Figure 14). Two KEGG Pathways were differentially expressed when RT-AS≥10 was compared to RT, but no KEGG Pathways were differentially expressed when RT-AS≥10 was compared to C, making it unlikely that these are long-term markers of AAS usage (Table 15).

This study has numerous methodological limitations owing to its observational nature, with an observational method of research being the main ethically feasible way to study sustained AAS exposure. Many of these are initially detailed in section 5.4. as they are also limitations to IHC data analysis. Particularly pertinent to transcriptomic data analysis is AAS regimens and date of cessation differing between participants, self-reported AAS cycles being fallible to recall errors, reported time frames of AAS abstinence being inaccurate and AAS quality being unknown. The dosage exposure results in this study therefore only serve as estimates, but as discussed in section 5.4. there is some merit in obtaining this information as reported AAS cycles are known to vary 10-fold in maximum weekly dosage and 100-fold in cumulative cycle dose [283]. Budget constraints prevented AAS purity testing.

It is also common for AAS users to undergo a “blast and cruise” usage pattern [297] in which AAS exposure peaks (the “blast”) but then never drops to genuine physiological levels of testosterone where users “cruise” on above physiological testosterone levels instead of AAS cessation or using true TRT. Finding AAS users who did not partake in a “blast and cruise” usage pattern, which heavily confounds AAS cessation post initial exposure, was difficult and contributed to the low number of returning participants. Group C also had the lowest number of recruited participants; however this was a difficult group to recruit as individuals who do not resistance train were not as interested to partake in a study that did not offer

remuneration, unlike those in Group RT and RT-AS. Future studies investigating the impact of AAS usage on gene expression should focus on sampling higher numbers of AAS users longitudinally, ideally pre, during and post AAS exposure, as paired-sample analysis reduces the impact of confounding variables. AAS samples could also be collected and tested for purity. Although the muscle RNA-Seq datasets have been crossed compared for validation purposes, future studies could focus on AAS administration to cell culture lines to further validate these differentially expressed genes.

In conclusion, although the observational nature of this study would have impacted its findings, given that no differentially expressed genes were identified in whole blood in both sequencing datasets when RT-AS≤2 was compared to RT or C, this current data suggests that it seems unlikely that a whole blood transcriptional signature could be used to identify AAS doping. However, in muscle, AAS exposure had a greater impact on gene expression, with differential expression in genes known to impact hypertrophic processes. Furthermore, the majority of current AAS users clustered together in muscle gene expression profiles, showing that a subset of genes seems to be both up- and downregulated from AAS exposure, with this finding potentially contributing to furthering our understanding of AAS induced hypertrophic processes.

7. Discussion and Conclusion.

7.1. How identifying the doping practices of weightlifters may help the sport combat doping.

Numerous doping patterns have emerged from the analysis of the time frame (2008 – 2019) that has seen the highest number of doping sanctions in weightlifting's history. The main limitation of this study is that an independent report into IWF corruption led by Professor Richard H. McLaren [155] has identified instances, as detailed in section 1.7.1., in which anti-doping policy has not been followed through correctly. This means that the IWF sanction data utilised in this study did not include doping cases that were under ITA investigation following this report and thereby this dataset did not represent the full sanction list identified during this time frame as these new pending cases had not yet concluded. Despite these ongoing investigations this study intended to build a clearer picture of doping practices of weightlifters and how these practices varied across the IWF Continental Federations, based on known sanction data, to enhance future doping detection and to investigate the re-testing of samples collected at the 2008 and 2012 Olympic Games.

Notably this study found that over this 11-year period exogenous AAS metabolites and markers indicating EAAS usage accounted for 82% of detected substances from the IWF Sanction List. Although this is from identified doping substances, this shows a preference for doping weightlifters to use exogenous anabolic steroids or endogenous steroids as doping substances. Doping substances also varied by IWF Continental Federation, with Europe and Asia showing statistically different ($p < 0.05$) proportions of detected exogenous AAS metabolites, markers indicating EAAS usage and all other substance category types compared to both Africa and Pan America. Exogenous AAS showed the largest difference in the proportion of substance types detected, possibly indicating a preference of this substance category in doping European and Asian weightlifters. Furthermore, when looking at the national level there is also differences in the detection of substances. When looking at the 10 most detected substances, from the 10 nations with the highest number of sanctions, there is at least one substance overrepresented that accounts for 38-60% of detected substances in these countries (Figure 3). It is not possible from doping detection data to make inferences on the causes of this overrepresentation, however, identifying that this pattern exists is useful for anti-doping authorities as they could use this data for targeted educational programmes to elicit the change required to change these patterns. Additionally, identifying a preference for certain substances in certain geographic regions can better enable targeted testing to catch dopers prior to competition. With Europe and Asia showing the highest number of sanctions

and highest prevalence in the usage of exogenous AAS an extra emphasis on OOC testing in these regions may be warranted in weightlifting as these substances are likely to be used in training prior to competition where anticipated testing occurs.

Given this preference for exogenous AAS as doping substances in weightlifters it has transpired that long term sample storage has proven effective at retrospectively catching doping weightlifters at the 2008 & 2012 Olympic games. From the sixty-one-retrospective weightlifting ADRVs identified via re-testing the exogenous AAS dehydrochloromethyltestosterone and stanozolol accounted for 83% of detected substances with 95% of announced ADRVs noting at least one of these substances. These findings should send a strong deterrent to prospective doping weightlifters that, due to LMT discovery, the detection window of these substances has substantially improved, and the doping practices used in the runup to the 2008 and 2012 Games may not be possible anymore for future competitions. The implications of high rates of identified retrospective doping from sample re-analysis is further discussed in section 7.2.

7.2. Implications of high rates of Olympic doping medallists detected from retrospective analyses.

The 8-year statute of limitations for sample re-analysis from London 2012 concluded in August 2020 finalising the IOC retesting programme of samples collected during the 2004, 2008 and 2012 summer Olympic Games. Despite this financial investment of long-term sample storage and re-analysis a study has not yet been conducted to investigate the effectiveness of identifying doping in Olympic medallists from this practice. Most notably, this analysis from 1968 – 2012 shows that for the majority (74%) of Olympic medals that have been impacted by doping violations, these doping violations have been identified retrospectively. The IOC's targeted re-analysis of samples collected at the 2004, 2008 and 2012 Olympic Games accounted for 57% of all medals impacted by doping violations. It took a mean of 6.8 ± 2.0 years for these IOC re-tests that impacted medal results to be announced relative to the end of the Games in which the medal was originally won. The IOC has financed the shipment, storage and re-analysis of samples collected at Olympic venues from 2004 onwards. However, long-term storage is not standard across other major competitions, with IFs having to fund the cost of long-term storage with WADA encouraging this practice to extend to Continental Games and other competitions like World Championships [266].

Given the high rates of doping Olympic medallists identified from long term sample storage and re-analysis it would be pertinent for IFs to invest into this practice at major competitions such as World Championships and Continental Games. LTMs of exogenous AAS were present in 90% of the positive samples re-analysed by the IOC in 2004, 2008 and 2012 with dehydrochloromethyltestosterone and stanozolol accounting for 79% of all detected substances. These LTMs are detected via the analysis of urine, which is a routine matrix collected in anti-doping drug testing and so was a stored sample matrix by default. However, the next advancements in anti-doping science might not utilise urine or other routine sample matrices. The IOC has also discussed the possibility of samples being collected in Tokyo 2020 for novel testing technologies/matrices, such as DBS and gene expression analysis, [273]. The collection of capillary blood on DBS cards [279] and the collection of venous blood in RNA preservative for gene expression analysis [172] and other currently unknown advances in anti-doping science may need different matrices/methodologies for future drug detection. Thereby, it should be considered that samples could be stored in the matrices that the next generation of doping tests may use. During the 10-year statute of limitations in which sample re-analysis can happen, further research on these technologies will occur and once validated they could be applied to this biobank of samples and may complement doping detection.

As detailed in section 4.4. the IOC has implemented a process to enable medal reallocations with the rightful winner deciding where they receive the medal. Despite the success of the retrospective reallocation of medals, there is criticism of this methodology to catch dopers. Some scholars have noted this has the potential to reduce live sport to "meaningless spectacles" as until the re-testing is concluded (which could be 10 years later) the initial results are provisional as neither the athletes nor spectators know who the real medal winners are [130]. Furthermore, any monetary gain from sponsors/societal notoriety that could directly occur in real time after winning a medal is lost and likely never fully recovered to the rightful medal recipient after reallocation. As detailed in section 4.4. there are also instances in which prior to the IOC medal reallocation protocols rightful Olympic medallists were receiving reallocated medals in airports and had no opportunity of standing on a podium and listening to their national anthem in front of a crowd. When the first WADA code was implemented in 2004 allowing for long-term sample storage and the retrospective identification of doping, it was envisaged that this practice would act as a doping deterrent [127]. This is because even if an athlete managed to beat the doping test at the time of the

competition, they could still get caught years later with improved technologies and retrospectively have their medal rescinded. However, the timeline of the retrospective identification of doping does not reflect that this deterrent effect has been fully realised. For example, considering that athletes knew since 2004 that sample re-analysis with improved technologies was possible and that 6-months after Beijing 2008 two Olympic medallists were caught via this practice, twenty-eight medallists still got caught doping retrospectively at London 2012. This had led to some authors to suggest that the deterrence effect of long-term sample storage is limited, otherwise there would not have seen so many retrospective doping incidents [130]. Overall, given this historical timeline, the deterrence effect of this practice is unlikely to extend to all doping attempts. It is more likely that when a specific doping substance is retrospectively identified with improved technologies, this specific doping substance would then no longer be used by prospective dopers as they realise this substance (or methodology) is now more easily detectable. For example, when CERA was identified 6-months after the Beijing 2008 Games in six athletes it was not detected in real time or retrospectively at London 2012, potentially suggesting that prospective dopers from 2009 onwards were deterred from using this specific doping substance when they knew testing for it had improved.

7.3. Body composition, myonuclei and immunohistochemistry data from The MMAAS Project & study limitations.

As discussed in section 1.2. testosterone administration studies have shown FFM can be gained from supraphysiological doses of testosterone. However, there is limited data regarding how much FFM can be lost after AAS exposure [220, 281]. 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 this previous research that LBM decreases post AAS exposure. Although RP1 and RP2 exhibited decrements in CSA whilst myonuclei per fibre values remained relatively similar between visits, which is a pattern consistent with the myonuclear permanency model of muscle memory, the number of participants is low, their AAS cycle timelines differ, this is observational data and as such this data should be viewed as initial case reports. RP3 had increases in fibre CSA and myonuclei per fibre values post AAS exposure, suggesting that localised hypertrophy had occurred in trapezius muscle fibres, even if FFM had decreased.

Myonuclei per fibre (Figure 8B) and Myonuclei per Type I and per Type II trapezius muscle fibres (Table 9) were not statistically different between any groups. These findings potentially add to the evidence [95-100] 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 [100, 101]. However, it should be noted that the total time length of AAS exposure in group PREV was low compared to another study (discussed below). Given that testosterone can induce comparable hypertrophy in mice that lack satellite cells, compared to controls, there is the possibility that the AAS dosage burden (and congruent training regimen) of the past users was not substantive enough to ensure substantive myonuclei accrual could occur.

As noted in section 1.5. a previous observational study [111] did find significantly elevated myonuclei values in the trapezius muscle in the cohort of previous AAS users. In Eriksson's study the previous AAS users (n = 7) had withdrawn from AAS and other substances for more than one year (8.1 ± 3.2 years) and had previously used AAS for 4.5 ± 0.5 years. Our cohort of previous users had withdrawn from AAS for more than one year (3.5 ± 2.2 years) and previously used AAS for 3-192 weeks (median of 12). Eriksson's cohort of previous users had higher myonuclei per fibre values (7.0 ± 1.3) compared to our previous user cohort (3.7 ± 1.4). At the time of the muscle biopsies, Eriksson reported that 3 participants within this previous user group had stopped all forms of exercise while the other 4 continued strength training to some degree. This observation may explain why previous users in our cohort had larger muscle fibre CSA (7677.0 ± 1804.4 μm²) than the participants in Eriksson's study (6807 ± 1467 μm²) even though myonuclei levels were reduced in our cohort.

There are many limitations to The MMAAS Project. As noted above, the total time of AAS exposure of group PREV was relatively low and may not have been prolonged enough to ensure substantive myonuclei accrual at the time of exposure. Looking at a cohort of past AAS users and seeing if myonuclei values are still elevated is a proxy measurement for investigating myonuclear permanency because biopsies pre and immediately post AAS exposure were not taken to ensure myonuclei were accumulated. The longitudinal monitoring of participants post AAS exposure is a more controlled environment, but small numbers of participants were recruited, and they varied in their time of AAS cessation relative to dates of sampling. The COVID pandemic greatly impacted our ability to recruit more past AAS users and longitudinally monitor current AAS users through time.

Participants were interviewed by me regarding their AAS usage. 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. Stated dosages in The MMAAS Project therefore only serve as AAS exposure estimates. However, there is still some benefit to asking participants on their AAS usage history as it enables a broad classification between 'high' and 'low' doses. Report cycles from 100 users varied 10-fold in maximum weekly dosage and 100-fold in cumulative cycle dose [283]. Thereby, even if the exact cycle cannot be recalled fully, a broad understanding of the rough dosages used does have some merit. Lastly, only one participant declared to me that he had used exclusively pharmaceutical AAS in his usage journey. The remaining participants were using AAS made in underground laboratories which typically lack quality control testing that ensures the stated label compound and dosage is correct. A recent study of underground made AAS has shown that of the 272 samples analysed only 35 (13%) solely contained the declared AAS indicated on the label [298]. Furthermore, in this study 60 (22%) of all received AAS samples were a duplicate (i.e., different subjects delivering the same AAS subtype from the same brand), and in only 20 (33%), the analysis result was the same [298]. No AAS testing was conducted in The MMAAS Project, due to budget constraints, but this would have likely produced similar results indicative of the poor quality of underground made AAS.

It is also common for AAS users to undergo a "blast and cruise" usage pattern [297] in which AAS exposure peaks (the "blast") but then never drops to genuine physiological levels of testosterone where users "cruise" on above physiological testosterone levels instead of AAS cessation or using true TRT. Finding AAS users who did not partake in a "blast and cruise" usage pattern, which heavily confounds AAS cessation post initial exposure, was difficult and contributed to the low number of returning participants. Group C also had the lowest number of recruited participants; however, this was a difficult group to recruit as individuals who do not resistance train were not as interested to partake in a study that did not offer remuneration, unlike those in Group RT and RT-AS.

Further confounding variables in this study include variances in training histories amongst participants, no control of nutrition of returning participants and no AAS 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.

7.4. The impact of AAS on the transcriptome of whole blood and skeletal muscle from The MMAAS Project data.

As discussed in section 1.1. most of the hypertrophic effects of AAS are mediated through the AR [5] which induces a genomic mode of action by modulating transcription [6]. As discussed in section 1.9. the "omic" technologies have shown promise for anti-doping purposes, particularly for rHuEPO [172] and an AAS transcriptomic doping signature has been identified in livestock [183]. However, as noted in section 1.10.4. RNA-Seq has yet to be conducted on whole blood samples collected from AAS users to investigate a transcriptomic signature of doping. Although the observational nature of The MMAAS Project and the associated limitations, noted in section 7.3., would have impacted its findings, given that no differentially expressed genes were identified in whole blood in both sequencing datasets when RT-AS≤2 was compared to RT or C, or when Doped was compared to RT, this current data suggests that it seems unlikely that a whole blood transcriptional signature could be used to identify AAS doping. Low numbers of differentially expressed genes in whole blood was a surprising and disappointing result, given that, as discussed in section 1.9., rHuEPO has shown a whole blood RNA signature of doping [173, 174, 175, 176] and that AAS have been hypothesised, albeit mainly in animal husbandry, to be detectable with transcriptomic signatures [180]. At the time of writing, the exact number of genes/binding sites under AR regulation in whole blood, due to the inability to find ChIP-Seq data in this tissue type, is difficult to confirm. However, in primary human muscle cells ChIP-on-Chip analysis has identified 32,518 potential AR-binding sites throughout the genome that are enriched upon androgen treatment [280]. Sequence analysis of these regions indicated that approximately 90% possess a consensus androgen response element or half-site [280].

RNA-Seq has not been conducted on muscle samples from AAS users and so this study aimed to also conduct RNA-Seq on muscle samples collected in The MMAAS Project to close this knowledge gap. Of note is that in both muscle sequencing datasets, one gene (*CHRDL1*) which has atrophying potential, was upregulated in the second visit of returning participants (RP2-4), with these same participants also showing a 4.4 ± 0.3 kg loss of FFM (Figure 7C) as measured with bioelectrical impedance. Although the upregulation of one gene that has atrophying potential would be unlikely to be solely sufficient for causing the observed losses in FFM, this is still an intriguing finding. With CHRDL1 acting as an antagonist of BMP and BMP inhibition causing atrophy [287] the BMP pathway could be

investigated by future transcriptomic studies researching atrophy post AAS exposure. In both muscle sequencing datasets, nine differentially expressed genes (*ABCA7, ARHGEF17, BOK, FILIP1L, LDAF1, RBL1, RPIA, SDC4, ZFP36L1*), overlapped with RT-AS≤2 *vs* RT and RT-AS≤2 *vs* C, but were not differentially expressed with RT *vs* C, possibly suggesting they are from acute doping alone. The confounding variable of different training regimens between RT-AS≤2 and RT could be pertinent to causing these nine differentially expressed genes, however, most participants in both groups were recreational lifters. As discussed in section 6.4. amongst these genes associated with a performance benefit is a downregulation of *RBL1*, with reduced expression of this transcriptional corepressor being associated with mitochondrial biogenesis, typically stimulated by exercise [290]. However, amongst these genes are also pathways that are contradictory to performance/hypertrophic benefit. These include downregulation of SDC4, which is associated with elevated myostatin [292], an upregulation of BOK, which is associated with muscle atrophy [293] and a downregulation of ZFP36L1 which is associated with reduced skeletal muscle mass [294]. Thereby some of these identified differentially expressed genes have produced conflicting findings to the known enhances in hypertrophy that occur from AAS exposure. In both muscle sequencing datasets, there were no differentially expressed genes when RT was compared to Doped.

No genes seemed to be differentially expressed in muscle after the long-term cessation of AAS, (i.e., when investigating group comparisons that involved RT-AS≥10) whereas a previous study has found long term proteomic changes associated with nine proteins [114]. None of the genes associated with these nine proteins were found to be differentially expressed in any comparison. Differences in AAS exposure regimens, with the self-declared AAS regimens being much higher in this proteomic research [114] and the different site of the muscle biopsy (vastus lateralis [114]) could have contributed to this discrepancy.

7.5. Future Directions.

As discussed in section 1.3. and section 1.4. there is evidence for and against the muscle memory mechanism of myonuclear permanency and with no known mechanism for the selective degradation of myonuclei within a syncytium, this topic is actively being debated [105, 106]. Of note, is that the key murine testosterone administration study [85] (discussed in section 1.3.1.) that forms a core piece of evidence for myonuclei permanency, has not been replicated. This study has been used as a key piece of evidence for those who rebut that

myonuclei can be lost [108]. Given the central place this study holds in the argument for permanency, a replication study would be sensible to be undertaken. A further complication is that a genetically modified murine model, where satellite cells can be conditionally depleted meaning myonuclei accrual cannot occur, has shown comparable hypertrophy occurs after testosterone exposure between control and modified mice [22]. Therefore, myonuclear accrual is not mandatory for AAS induced hypertrophy. Future studies in humans or animal models must ensure that samples are collected pre and immediate post AAS exposure to ensure myonuclei were accumulated from the administered AAS, further longitudinal sampling efforts can then investigate if these myonuclei are longer-lasting. Recent groundbreaking revelations [48] that myonuclei in mice can synthesise DNA via endoreplication causing polyploidy need to be investigated further regarding what impact AAS exposure would have on this novel pathway for enhanced transcriptional output.

The most controlled and ecologically valid experimental design to investigate if myonuclei accumulated from AAS exposure in humans are permanent would be a randomised double-blind placebo controlled AAS administration study with pre-exposure, immediately post exposure and further longitudinal sampling points. However, any form of AAS administration is not without the risk of side effects to participants [14]. Randomised double-blind AAS administration studies have occurred, as discussed in section 1.2., with a dose escalating testosterone administration study in young men [15, 19] being of note, but the longitudinal monitoring of participants post AAS exposure has been lacking. Although administration studies do have to be ethically challenged, this study in young men did find that 300mg/week of TE for 20 weeks was enough to cause a significant increase in myonuclear number per millimetre of muscle fibre and significant increases in leg press strength and quadriceps muscle volume, without any resistance training [15, 19]. Therefore, future administration studies may which to use 300mg/week of TE as an administration regimen. However, participants did experience an increase in haemoglobin ($p = 0.0639$) and a decrease in plasma HDL cholesterol ($p = 0.0690$) and so some side effects on cardiovascular health were noted [15].

Given the ethical difficulties of administration studies, observational studies are the next viable alternatives, with longitudinal monitoring providing the greatest control over confounding variables. AAS testing could also occur to control for unknown AAS purity which is one of the main limiting factors of this type of observational study. As discussed in the Thesis Aims (section 1.10.3.) for The MMAAS Project, there is a lack of longitudinal

observational data after the cessation of AAS exposure on metrics pertinent to myonuclear permanency. The MMAAS Project aimed to close this knowledge gap but the number of longitudinally monitored participants who had muscle biopsies was low (n = 3) and they differed in AAS cessation date relative to sampling date. Social distancing restrictions from the COVID-19 pandemic greatly hindered our opportunity to recruit and longitudinally monitor more participants. In general, as discussed in section 7.3. it was also difficult to recruit AAS users who did not partake a "blast and cruise" AAS usage pattern [297] and self-declared intentions for full AAS cessation, further hindering longitudinal participant monitoring. However, one large observational prospective cohort study, pre COVID-19 pandemic, has managed to monitor 100 men at four time points: 1) before initiating an AAS cycle, 2) at the end of the cycle, 3) three months post cycle and 4) one year after the start of the cycle [283]. However, this study did not conduct muscle biopsies or measure body composition changes and was focussed on the impacts of AAS on adverse health outcomes. If these measurements were taken, this study design would have enabled for a thorough investigation into the influence of AAS on muscle memory and the associated changes in FFM. Future studies with this sampling schedule and scale of participant enrolment may consider having a muscle biopsy arm to the research project to enable muscle memory research questions to be investigated, even if muscle memory muscle research is not the original purpose of the project. The other setting in which muscle memory questions related to testosterone exposure could be investigated would be when gender-affirming treatment is used to indefinitely supress testosterone in transwomen. Although this experimental context does not involve supraphysiological exposure to AAS, as would in doping instances, it still could enable a favourable research environment to investigate if myonuclei are lost after a prolonged decrease in testosterone exposure and would further advance the evidence base on how/if transwomen can fairly be integrated into female sport [299].

The MMAAS Project focused on the proposed myonuclear permanency mechanism of muscle memory, but a human resistance training study has shown that an epigenetic muscle memory exists [101]. Thereby, given the AR binds to DNA and regulations transcription, it is feasible that a possible epigenetic memory of AAS exposure may exist, yet data is lacking on the influence of AAS on the epigenome. Long last changes in the proteome [114] of past AAS users, as discussed in section 1.5., also gives weight to the possibility of an epigenetic memory of AAS exposure. However, The MMAAS Project did not find a long-lasting transcriptional change in past AAS users, although as discussed in section 7.3. study design

constraints could have impacted this finding, with the low dose exposure of past AAS users being of particular importance in this regard. A longitudinal study [300] monitoring transgender individuals undergoing gender-affirming treatment does plan to investigate the epigenome, which will provide some data to this question, but more research is needed on the influence of supraphysiological doses of AAS on the epigenome.

RNA-seq of whole blood samples collected in The MMAAS Project has not identified a transcriptomic signature of doping. However, The MMAAS Project study limitations (noted in section 7.3.) could have impacted this finding and more well controlled studies (such as administration studies) may still find whole blood transcriptomic differences. Although the muscle RNA-Seq datasets have been crossed compared for validation purposes, future studies could focus on AAS administration to cell culture lines to further investigate/validate identified differentially expressed genes. Future studies into the impact of AAS on the skeletal muscle transcriptome should also adhere to the suggestions of research into myonuclei permanency (i.e., if ethically possible, conduct longitudinal administration studies, or recruit higher numbers of returning participants in observational research and conduct AAS purity testing) as paired sample analysis is a more controlled environment than group comparisons. Unlike in the MMAAS Project, future studies should also consider conducting some form of performance testing, like in [113], to further investigate their performance enhancing benefits and if AAS exposed participants have greater performance parameters than recruited non-users and controls.

Serum and plasma samples were also collected from participants recruited in the MMAAS Project and a future avenue of research could be to investigate if an AAS metabolomic doping signature could be identified, as a rHuEPO metabolomic doping signature has recently been identified [301].

7.6. Final Conclusion.

This thesis has investigated doping practices in international weightlifting and identified geographic differences that may enable targeted educational and testing programmes to enhance anti-doping efforts. A novel analysis of doping medallists at the Summer Olympic Games has identified that the majority were identified retrospectively. Long-term sample storage should therefore continue at Olympic and non-Olympic events and additionally incorporate novel collection technologies/sample matrices as anti-doping methodologies

evolve and improve. A cohort of past AAS users did not have elevated myonuclei per fibre values compared to non-resistance trained or resistance trained controls and current AAS users, although reported AAS usage was lower than previous research. A small number of case reports in which current AAS users were monitored longitudinally may support myonuclei permanency in the medium term, but AAS exposure regimens varied widely, participant numbers are low and one returning participant exhibited increases in myonuclei and fibre CSA. Future research into the realm of AAS and muscle memory should focus on the longitudinal sampling of participants, involve diligent record keeping of AAS exposure regimens and additionally investigate the potential of an epigenetic memory and not just the purported muscle memory mechanism of myonuclei permanency. A whole blood gene expression signature of AAS exposure could not be identified and so it is unlikely that this methodology could enhance AAS anti-doping testing. RNA-Seq of muscle samples has identified numerous differentially expressed genes/pathways in current AAS users (who ceased AAS ≤2 weeks prior to sampling) when compared to other groups, but differences in training regimens and unknown AAS purity are limitations to this finding. Some of these differentially expressed genes corroborate with a performance benefit and so possibly contribute to the understanding of AAS induced hypertrophy, however some of these genes are associated with atrophying processes producing conflicting findings. For returning participants post AAS exposure only one gene was differentially expressed in visit two and this was associated with an atrophying pathway corroborating with observed FFM losses. No genes seemed to be differentially expressed in muscle after the long-term cessation of AAS, whereas a previous study has found long term proteomic changes. Future studies into the impact of AAS on the muscle transcriptome should focus on either conducting ethically feasible AAS administration studies or recruiting higher numbers of returning participants in observational research with AAS purity testing, to ensure exposure classifications are accurate and enable paired sample analysis which is a more controlled environment than group comparisons. AAS administration to cell culture lines could also be conducted to further validate any identified differentially expressed genes.

Tables

Table 1. Classifications and examples of ADRVs within the study described in Chapter 3. Substance classifications were defined by their location in the 2020 WADA Prohibited list or their closest categorisation therein [214].

Classifications of ADRVs	**Examples**
AAS	AAF for the detection of AAS e.g., Testosterone, metandienone, nandrolone, oxandrolone, stanozolol, dehydrochloromethyltestosterone, metenolone.
Stimulants	AAF for the detection of stimulants e.g., sibutramine, methylhexaneamine, ephedrine.
Other substances	AAF for the detection of the following: Diuretics and masking agents (e.g., furosemide). Other anabolic agents (e.g., clenbuterol). Beta-blockers (e.g., propranolol). Substances used in equestrian doping (e.g., capsaicin). Ethanol. Hormone and metabolic modulators (e.g., tamoxifen). Peptide hormones, growth factors, related substances, and mimetics (e.g., Growth Hormone Releasing Peptides).
ABP Violations	A violation of the ABP due to abnormal athlete data.
Other specific cases	Revelations of athlete involvement with an organised doping regime but specific substances used at the relevant Games are not fully elucidated (e.g., confessed or known involvement in the BALCO scandal). Confessions of doping. Refusal to submit urine or urine tampering. Doping identified retroactively at a prior Olympics causing result disqualification at a later Olympics. Combinations of these reasons and any of the previously mentioned classifications.

ADRV: Anti-Doping Rule Violation; AAS: Anabolic Androgenic Steroids; AAF: Adverse Analytical Finding; ABP: Athlete Biological Passport; BALCO: Bay Area Laboratory Co-operative

Table 2. Group allocation criteria for The MMAAS Project.

Group	Criteria
C	1) A control group comprised of non-resistance trained healthy males.
RT	1) History of resistance training ≥8 hours a week 2) Self-reported as never using any PEDs.
RT-AS	1) History of resistance training ≥8 hours a week 2) Self-reported to using exogenous AAS (i.e., synthetic AAS), supraphysiological dosages of injectable testosterone (>100mg/week) or a closely related AAS compound (i.e., AAS pro-hormones or SARMs) <52 weeks prior to their first sampling date.
PREV	1) History of resistance training ≥8 hours a week 2) Self-reported to ceasing usage of exogenous AAS (i.e., synthetic AAS), supraphysiological dosages of injectable testosterone (>100mg/week) or a closely related AAS compound (i.e., AAS pro-hormones or SARMs) ≥52 weeks prior to their sampling date and not exceeded any clinical recommendations for TRT [223, 224] during this period of absence.

AAS: Anabolic androgenic steroids; PED: Performance Enhancing Drugs; SARMs: Selective Androgen Receptor Modulators; TRT: Testosterone Replacement Therapy

Table 3. Counts of detected prohibited substances (or their metabolites) from athletes (*n* = 142) who generated an Anti-Doping Rule Violation from the IOC re-testing of samples from the 2004, 2008 and 2012 Olympic Games.

Games	**Count of Detected Prohibited Substances (or their metabolites) from the IOC re-tests of samples collected at the 2004 – 2012 summer Olympic Games**			
	DHCMT	Stanozolol	Other exogenous AAS*	Other substances**
2004 Athens	-	-	4	1
2008 Beijing	41	22	6	15
2012 London	59	28	11	4
Total	100	50	21	20

*Denotes either: oxandrolone, metenolone, methandienone, drostanolone, 1-androsterone or clostebol. **Denotes either: EPO; CERA, Growth Hormone-Releasing Peptide-2, acetazolamide, methylhexaneamine, tamoxifen, clenbuterol, ipamorelin, Athlete Biological Passport Violation or sibutramine. DHCMT: dehydrochloromethyltestosterone; AAS: Anabolic Androgenic Steroid; EPO erythropoietin; CERA (Continuous EPO Receptor Activator).

Table 4. The distribution of sports of athletes (*n* = 142) who generated an Anti-Doping Rule Violation from IOC re-testing of samples from the 2004, 2008 and 2012 Olympic Games.

Sport	**Olympic Games**			
	2004 Athens	2008 Beijing	2012 London	**Total**
Athletics	4	31	29	64
Weightlifting	1	25	36	62
Freestyle wrestling	-	4	3	7
Cycling	-	2	1	3
Greco-Roman wrestling	-	3	-	3
Boxing	-	-	1	1
Canoe Sprint	-	-	1	1
Swimming	-	-	1	1

Table 5. For the Summer Olympic Games 1968 – 2012 all medals impacted by an Anti-Doping Rule Violation (ADRV) are shown, alongside when this ADRV occurred and when it was identified.

Games	**Olympic Medals Impacted by an ADRV**			
	ADRV occurred at the Games & identified during the Games	ADRV occurred at the Games & identified retrospectively by IOC re-tests	ADRV occurred prior to the Games & identified retrospectively	Combination*
1968 Mexico City	1	-	-	-
1972 Munich	4	-	-	-
1976 Montréal	3	-	-	-
1980 Moscow	-	-	-	-
1984 Los Angeles	2	-	-	-
1988 Seoul	5	-	-	-
1992 Barcelona	-	-	-	-
1996 Atlanta	-	-	-	-
2000 Sydney	6	-	8	-
2004 Athens	8	5	2	-
2008 Beijing	4	43	3	-
2012 London	2	27	10	1
Total	**35**	**75**	**23**	**1**

*Denotes a combination of an ADRV occurring at the Games and being identified by retrospective IOC-retesting and an ADRV also occurring prior to the Games and being identified retrospectively by another testing initiative.

Table 6. Counts for the reason of Anti-Doping Rule Violations (ADRVs) that have impacted Olympic medal-winning results (*n* = 134) for the summer Olympic Games 1968 – 2012.

Games	**Counts for the reasons of ADRVs that have Impacted Summer Olympic Medal Winning Results 1968 – 2012**				
	AAS	Stimulants	ABP Violation	Other Substances*	Other Specific Cases**
1968 Mexico City	-	-	-	1	-
1972 Munich	1	3	-	-	-
1976 Montréal	3	-	-	-	-
1980 Moscow	-	-	-	-	-
1984 Los Angeles	2	-	-	-	-
1988 Seoul	2	-	-	3	-
1992 Barcelona	-	-	-	-	-
1996 Atlanta	-	-	-	-	-
2000 Sydney	3	1	-	3	7
2004 Athens	7	1	-	4	5
2008 Beijing	53	2	-	12	-
2012 London	41	1	6	2	3
Total	**112**	**8**	**6**	**25**	**15**

*Denotes either: diuretics and masking agents, other anabolic agents, beta-blockers, substances used in equestrian doping, ethanol, hormone and metabolic modulators, peptide hormones, growth factors, related substances and mimetics as defined, if applicable, by these substances locations in the 2020 Wada Prohibited list [214] and as defined in Table 1.** Denotes either: revelations of athlete involvement with an organised doping regime but specific substances used at the relevant Games are not fully elucidated (e.g. confessed or known involvement in the Bay Area Laboratory Co-operative scandal), confessions of doping, refusal to submit urine or urine tampering, doping identified retroactively at a prior Olympics causing result disqualification at a later Olympics and combinations of these reasons and any of the previously mentioned classifications as defined in Table 1. AAS; Anabolic Androgenic Steroid. ABP; Athlete Biological Passport.

Table 7. Descriptive data and body composition comparisons between groups using the Tanita® Body Composition Analyzer BC-420MA (Bioelectrical Impedance).

	Group			
	C (n = 7)	**RT** (n = 21) [$]	**RT-AS** (n = 19)	**PREV** (n = 7)
Age (years)	29.9 ± 3.8	25.5 ± 4.8*	26.4 ± 5.7	30.1 ± 5.2[#]
Height (cm)	182.9 ± 10.8	177.2 ± 7.4	180.9 ± 6.7	175.0 ± 6.0*
Mass (kg)	89.0 ± 22.8	84.6 ± 11.5	89.4 ± 7.4	87.3 ± 16.4
Fat Mass (%)	20.5 ± 6.6	15.4 ± 4.4*	14.5 ± 3.3*	15.8 ± 8.1
FFM (kg)	69.6 ± 12.1	71.4 ± 8.8	77.0 ± 7.0*,[#]	72.7 ± 10.3

C: Control; RT: Resistance Trained; RT-AS: Resistance Trained Current AAS users; PREV: Previous AAS users; FFM: Fat Free Mass; * statistically different from C; [#] statistically different from RT; $p \leq 0.05$. [$] Two participants within RT, respectively aged 25 and 42, did not have height or body composition measurements due to withdrawing from the study after blood collection and equipment unavailability.

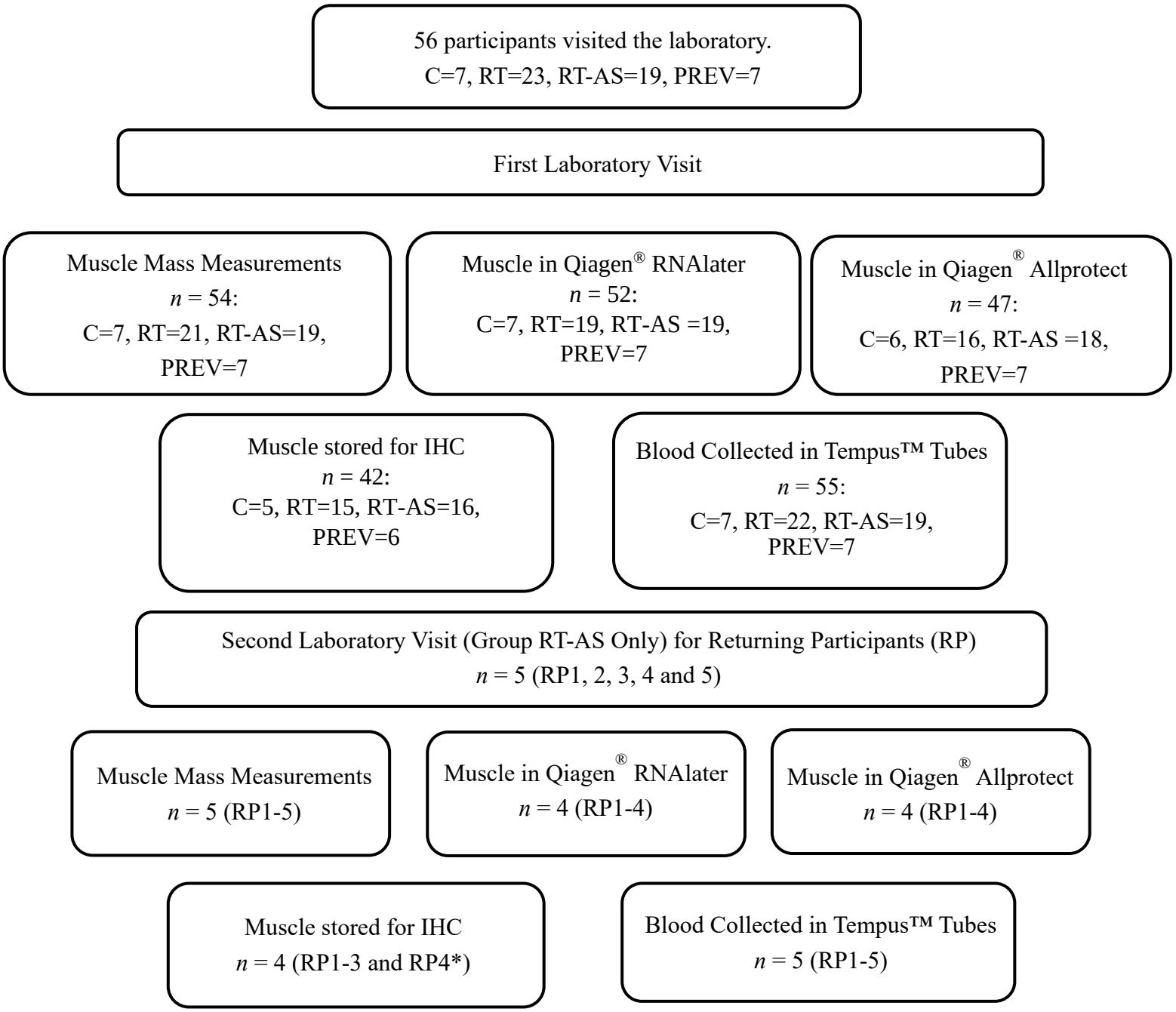


Table 8. Participant recruitment from different sampling steps across The MMAAS Project. C=Control Group, RT=Resistance Trained Group, RT-AS=Resistance Trained Currently using AAS Group, PREV=Past AAS using Group, IHC = Immunohistochemistry. RP4* had muscle stored for IHC on his second visit only.

Table 9. Immunohistochemistry data from participants first sampling visit (n = 40), including the only sample collected from RP4 during his second visit, with two missing participants due to different image extraction settings in MyoVision (C=1, RT=1). The fibre type staining protocol was not conducted on one participant in Group PREV.

Group	Type I (%)	Type Iia (%)	Type Iix (%)	Type I CSA (µm²)	Type Iia CSA (µm²)	Type Iix CSA (µm²)	Myonuclei per Type I	Myonuclei per Type II
C (n = 4)	58.2 ± 8.0	36.3 ± 5.8	5.5 ± 3.8	7657 ± 2248	6209 ± 2106$^{\#,\$}$	3509 ± 1005	3.3 ± 0.8	3.2 ± 1.0
RT (n = 14)	64.9 ± 12.0	32.4 ± 12.0	2.7 ± 1.8	7324 ± 2649	9023 ± 2995$^{*,\&}$	3283 ± 1603$^{\&}$	3.1 ± 1.2	4.0 ± 1.4
RT-AS (n = 17)	66.1 ± 13.1	30.6 ± 12.6	3.3 ± 2.1	7089 ± 1711	9117 ± 2523$^{*,\&}$	4303 ± 1533$^{\#}$	3.0 ± 1.0	4.0 ± 1.3
PREV (n = 5)	60.3 ± 15.0	34.5 ± 10.1	5.2 ± 7.4	6477 ± 1265	6744 ± 2468$^{\#,\$}$	4330 ± 811$^{\&}$	3.4 ± 1.0	4.2 ± 2.2

C: Control; RT: Resistance Trained; RT-AS: Resistance Trained Current AAS users; PREV: Previous AAS users; * statistically different from C; $^{\#}$ statistically different from RT; $^{\$}$ statistically different from RT-AS; $p \leq 0.05$; $^{\&}$ data is from $n = n$-1 participants as one sample presented 0% fibre Type Iix.

Table 10a. Participant sampling and sample inclusion for transcriptomic analysis, for all returning participant comparisons and group comparisons involving RT-AS≤2 & RT-AS≥10.

Sampled on First Laboratory Visit, *n* = 55: C=7, RT=22, RT-AS≤2=15, RT-AS≥10=11		**Sampled on Second Laboratory Visit, *n* = 5:** RP1, 2, 3, 4 & 5	
Whole Blood, *n* = 55: C=7, RT=22, RT-AS≤2=15, RT-AS≥10=11	Muscle, *n* = 52: C=7, RT=19, RT-AS≤2=15, RT-AS≥10=11	Whole Blood, *n* = 5: RP1-5	Muscle, *n* = 4: RP1-4
Sequenced: **UoB & MGI** Whole Blood, *n* = 55: C=7, RT=22, RT-AS≤2=15, RT-AS≥10=11	**Sequenced:** **CoolMPS & MGI** Muscle, *n* = 48 C=5, RT=17, RT-AS≤2=15, RT-AS≥10=11	**Sequenced:** **UoB & MGI** Whole Blood, *n* = 5: RP1-5	**Sequenced:** **CoolMPS & MGI** Muscle, *n* = 4: RP1-4
DGE & GSEA: **Group Comparison** **UoB dataset:** Whole Blood, *n* = 51 C=7, RT=20, RT-AS≤2=14, RT-AS≥10=10	**DGE & GSEA:** **Group Comparison** **CoolMPS dataset:** Muscle, *n* = 48 C=5, RT=17, RT-AS≤2=15, RT-AS≥10=11	**DGE & GSEA:** **RP 2nd Visit** **UoB dataset:** Whole Blood, *n* = 4: RP2-5	**DGE & GSEA:** **RP 2nd Visit** **CoolMPS dataset:** Muscle, *n* = 3 RP2-4
DGE & GSEA: **Group Comparisons** **MGI dataset:** Whole Blood, *n* = 43 C=6, RT=17, RT-AS≤2=12, RT-AS≥10=8	**DGE & GSEA:** **Group Comparison** **MGI dataset:** Muscle, *n* = 48 C=5, RT=17, RT-AS≤2=15, RT-AS≥10=11	**DGE & GSEA:** **RP 2nd Visit** **MGI dataset:** Whole Blood, *n* = 3: RP2-4	**DGE & GSEA:** **RP 2nd Visit** **MGI dataset:** Muscle, *n* = 3 RP2-4

Table 10b. Participant sampling and sample inclusion for transcriptomic analysis, for all group comparisons involving the classification of AAS exposed participants classified as Doped.

Sampled on First Laboratory Visit, *n* = 55: C=7, RT=22, Doped=26	
Whole Blood, *n* = 55: C=7, RT=22, Doped=26	Muscle, *n* = 52: C=7, RT=19, Doped=26
Sequenced: **UoB & MGI** Whole Blood, *n* = 55: C=7, RT=22, Doped=26	**Sequenced:** **CoolMPS & MGI** Muscle, *n* = 48 C=5, RT=17, Doped=26
DGE: **Group Comparison** **UoB dataset:** Whole Blood, *n* = 51 C=7, RT=20, Doped=24	**DGE:** **Group Comparison** **CoolMPS dataset:** Muscle, *n* = 48 C=5, RT=17, Doped=26
DGE: **Group Comparisons** **MGI dataset:** Whole Blood, *n* = 43 C=6, RT=17, Doped=20	**DGE:** **Group Comparison** **MGI dataset:** Muscle, *n* = 48 C=5, RT=17, Doped=26

RP Visit 1 is included in their corresponding RT-AS or Doped cohort. C = Control; RT = Resistance Trained; RT-AS≤2 = Resistance Trained participant who self-declared AAS exposure ceased ≤2 weeks before sampling; RT-AS≥10 = Resistance Trained participant who self-declared AAS exposure ceased ≥10 weeks before sampling; Doped = All participants who at some point had AAS exposure; RP = Returning Participant; DGE: differential gene expression; GSEA: Gene Set Enrichment Analysis; UoB: indicates samples sequenced at the University of Brighton utilising standard chemistry reagents; MGI: indicates samples sequenced at MGI, Latvia, utilising standard chemistry reagents; CoolMPS: indicates samples sequenced at MGI, Latvia, utilising CoolMPS chemistry reagents.

Table 11a. The number of genes identified for DGE from whole blood samples sequenced with standard chemistry reagents at UoB.

Tissue	**Comparison**		
Blood	RP2-5: Visit 2 (*n* = 4), Visit 1 (*n* = 4)	Group: C=7, RT=20, RT-AS≤2=14, RT-AS≥10=10	Group: C=7, RT=20, Doped=24
Low-expressed genes	14,636	13,637	13,637
Genes available for DGE analysis	14,353	15,352	15,352

Table 11b. The number of genes identified for DGE from whole blood samples sequenced with standard chemistry reagents at MGI.

Tissue	**Comparison**		
Blood	RP2-4: Visit 2 (*n* = 3), Visit 1 (*n* = 3)	Group: C=6, RT=17, RT-AS≤2=12, RT-AS≥10=8	Group: C=6, RT=17, Doped=20
Low-expressed genes	14,314	13,374	13,374
Genes available for DGE analysis	14,675	15,615	15,615

Table 11c. The number of genes identified for DGE from muscle samples sequenced with standard reagents at MGI.

Tissue	**Comparison**		
Muscle	RP2-4: Visit 2 (*n* = 3), Visit 1 (*n* = 3)	Group: C=5, RT=17, RT-AS≤2=15, RT-AS≥10=11	Group: C=5, RT=17, Doped=26
Low-expressed genes	13,639	12,302	12,302
Genes available for DGE analysis	15,350	16,687	16,687

Table 11d. The number of genes identified for DGE from muscle samples sequenced with CoolMPS reagents at MGI.

Tissue	**Comparison**		
Muscle	RP2-4: Visit 2 (*n* = 3), Visit 1 (*n* = 3)	Group: C=5, RT=17, RT-AS≤2=15, RT-AS≥10=11	Group: C=5, RT=17, Doped=26
Low-expressed genes	13,645	12,332	12,332
Genes available for DGE analysis	15,344	16,657	16,657

DGE: differential gene expression; UoB: University of Brighton; RP: Returning Participant; RT-AS≤2: Resistance Trained participant who self-declared AAS exposure ceased ≤2 weeks before sampling; RT-AS≥10: Resistance Trained participant who self-declared AAS exposure ceased ≥10 weeks before sampling; Doped: All participants who declared they have used AAS at some point.

Table 12a. The number of differentially expressed genes across returning participant comparisons and group comparisons involving RT-AS≤2 & RT-AS≥10 for blood samples sequenced with standard chemistry reagents at UoB subjected to DGE analysis.

Comparison							
RP2-5 Visit 2 (*n* = 4) *vs* Visit 1 (*n* = 4)	RT (*n* = 20) *vs* C (*n* = 7)	RT-AS≤2 (*n* = 14) *vs* C (*n* = 7)	RT-AS≥10 (*n* = 10) *vs* C (*n* = 7)	RT-AS≤2 (*n* = 14) *vs* RT (*n* = 20)	RT-AS≥10 (*n* = 10) *vs* RT (*n* = 20)	RT-AS≤2 (*n* = 14) *vs* RT-AS≥10 (*n* = 10)	Up/Down regulation
0	0	0	0	0	1	0	Up
0	1	0	0	0	0	1	Down

Table 12b. The number of differentially expressed genes across returning participant comparisons and group comparisons involving RT-AS≤2 & RT-AS≥10 for blood samples sequenced with standard chemistry reagents at MGI subjected to DGE analysis.

Comparison							
RP2-4 Visit 2 (*n* = 3) *vs* Visit 1 (*n* = 3)	RT (*n* = 17) *vs* C (*n* = 6)	RT-AS≤2 (*n* = 12) *vs* C (*n* = 6)	RT-AS≥10 (*n* = 8) *vs* C (*n* = 6)	RT-AS≤2 (*n* = 12) *vs* RT (*n* = 17)	RT-AS≥10 (*n* = 8) *vs* RT (*n* = 17)	RT-AS≤2 (*n* = 12) *vs* RT-AS≥10 (*n* = 8)	Up/Down regulation
1	0	0	0	0	1	0	Up
10	2	1	0	0	0	0	Down

Table 12c. The number of differentially expressed genes across group comparisons involving C, RT & Doped for blood samples sequenced with standard chemistry reagents at UoB subjected to DGE analysis.

Comparison			
RT (*n* = 20) *vs* C (*n* = 7)	Doped (*n* = 24) *vs* C (*n* = 7)	Doped (*n* = 24) *vs* RT (*n* = 20)	Up/Down regulation
0	0	0	Up
1	3	1	Down

Table 12d. The number of differentially expressed genes across group comparisons involving C, RT & Doped for blood samples sequenced with standard chemistry reagents at MGI subjected to DGE analysis.

Comparison			
RT (*n* = 17) *vs* C (*n* = 6)	Doped (*n* = 20) *vs* C (*n* = 6)	Doped (*n* = 20) *vs* RT (*n* = 17)	Up/Down regulation
0	0	0	Up
3	5	0	Down

Table 12e. The number of differentially expressed genes across different returning participant comparisons and group comparisons involving RT-AS≤2 & RT-AS≥10 for muscle samples sequenced with standard reagents at MGI subjected to DGE analysis.

Comparison							
RP2-4 Visit 2 (*n* = 3) *vs* Visit 1 (*n* = 3)	RT (*n* = 17) *vs* C (*n* = 5)	RT-AS≤2 (*n* = 15) *vs* C (*n* = 5)	RT-AS≥10 (*n* = 11) *vs* C (*n* = 5)	RT-AS≤2 (*n* = 15) *vs* RT (*n* = 17)	RT-AS≥10 (*n* = 11) *vs* RT (*n* = 17)	RT-AS≤2 (*n* = 15) *vs* RT-AS≥10 (*n* = 11)	Up/Down regulation
2	2	34	2	120	0	12	Up
0	3	28	0	52	0	7	Down

Table 12f. The number of differentially expressed genes across different returning participant comparisons and group comparisons involving RT-AS≤2 & RT-AS≥10 for muscle samples sequenced with CoolMPS reagents at MGI subjected to DGE analysis.

Comparison							
RP2-4 Visit 2 (*n* = 3) *vs* Visit 1 (*n* = 3)	RT (*n* = 17) *vs* C (*n* = 5)	RT-AS≤2 (*n* = 15) *vs* C (*n* = 5)	RT-AS≥10 (*n* = 11) *vs* C (*n* = 5)	RT-AS≤2 (*n* = 15) *vs* RT (*n* = 17)	RT-AS≥10 (*n* = 11) *vs* RT (*n* = 17)	RT-AS≤2 (*n* = 15) *vs* RT-AS≥10 (*n* = 11)	Up/Down regulation
4	2	38	2	94	0	21	Up
0	2	41	0	50	0	17	Down

Genes with a false discovery rate (FDR) < 0.05 and fold change of 1.2 were reported as differentially expressed. DGE: differential gene expression; UoB: University of Brighton; RP: Returning Participant; C: non-resistance trained control group; RT: Resistance Trained control group RT-AS≤2: Resistance Trained participant who self-declared AAS exposure ceased ≤2 weeks before sampling; RT-AS≥10: Resistance Trained participant who self-declared AAS exposure ceased ≥10 weeks before sampling.

Table 12g. The number of differentially expressed genes across group comparisons involving C, RT & Doped for muscle samples sequenced with standard reagents at MGI subjected to DGE analysis.

Comparison			
RT (*n* = 17) *vs* C (*n* = 5)	Doped (*n* = 26) *vs* C (*n* = 5)	Doped (*n* = 26) *vs* RT (*n* = 17)	Up/Down regulation
2	2	0	Up
3	3	0	Down

Table 12h. The number of differentially expressed genes across group comparisons involving C, RT & Doped for muscle samples sequenced with CoolMPS reagents at MGI subjected to DGE analysis.

Comparison			
RT (*n* = 17) *vs* C (*n* = 5)	Doped (*n* = 26) *vs* C (*n* = 5)	Doped (*n* = 26) *vs* RT (*n* = 17)	Up/Down regulation
2	4	0	Up
2	7	0	Down

Table 13. The number of differentially expressed genes that overlap for muscle samples sequenced with standard and CoolMPS reagents at MGI subjected to DGE analysis across different group comparisons.

Comparison							
RP2-4 Visit 2 (*n* = 3) *vs* Visit 1 (*n* = 3)	RT (*n* = 17) *vs* C (*n* = 5)	RT-AS≤2 (*n* = 15) *vs* C (*n* = 5)	RT-AS≥10 (*n* = 11) *vs* C (*n* = 5)	RT-AS≤2 (*n* = 15) *vs* RT (*n* = 17)	RT-AS≥10 (*n* = 11) *vs* RT (*n* = 17)	RT-AS≤2 (*n* = 15) *vs* RT-AS≥10 (*n* = 11)	Up/Down regulation
1	2	18	2	68	0	11	Up
0	1	20	0	37	0	6	Down

Genes with a false discovery rate (FDR) < 0.05 and fold change of 1.2 were reported as differentially expressed. DGE: differential gene expression; UoB: University of Brighton; RP: Returning Participant; C: non-resistance trained control group; RT: Resistance Trained control group RT-AS≤2: Resistance Trained participant who self-declared AAS exposure ceased ≤2 weeks before sampling; RT-AS≥10: Resistance Trained participant who self-declared AAS exposure ceased ≥10 weeks before sampling.

Table 14a. The number of differentially expressed gene sets or pathways across different group comparisons for muscle samples sequenced with standard reagents at MGI subjected to GSEA analysis.

Comparison							
RP2-4 Visit 2 (*n* = 3) *vs* Visit 1 (*n* = 3)	RT (*n* = 17) *vs* C (*n* = 5)	RT-AS≤2 (*n* = 15) *vs* C (*n* = 5)	RT-AS≥10 (*n* = 11) *vs* C (*n* = 5)	RT-AS≤2 (*n* = 15) *vs* RT (*n* = 17)	RT-AS≥10 (*n* = 11) *vs* RT (*n* = 17)	RT-AS≤2 (*n* = 15) *vs* RT-AS≥10 (*n* = 11)	Gene Set or Pathway
0	0	0	0	0	0	0	Hallmark
0	0	1	0	15	2	0	KEGG
0	0	4	0	119	0	3	Reactome
1	2	49	0	439	0	17	GO BP
0	1	18	0	220	0	23	GO MF

Table 14b. The number of differentially expressed gene sets or pathways across different group comparisons for muscle samples sequenced with CoolMPS reagents at MGI subjected to GSEA analysis.

Comparison							
RP2-4 Visit 2 (*n* = 3) *vs* Visit 1 (*n* = 3)	RT (*n* = 17) *vs* C (*n* = 5)	RT-AS≤2 (*n* = 15) *vs* C (*n* = 5)	RT-AS≥10 (*n* = 11) *vs* C (*n* = 5)	RT-AS≤2 (*n* = 15) *vs* RT (*n* = 17)	RT-AS≥10 (*n* = 11) *vs* RT (*n* = 17)	RT-AS≤2 (*n* = 15) *vs* RT-AS≥10 (*n* = 11)	Gene Set or Pathway
0	0	0	0	0	3	0	Hallmark
0	0	2	0	14	4	0	KEGG
2	0	2	0	89	0	1	Reactome
0	0	18	0	224	0	4	GO BP
0	0	9	1	136	0	8	GO MF

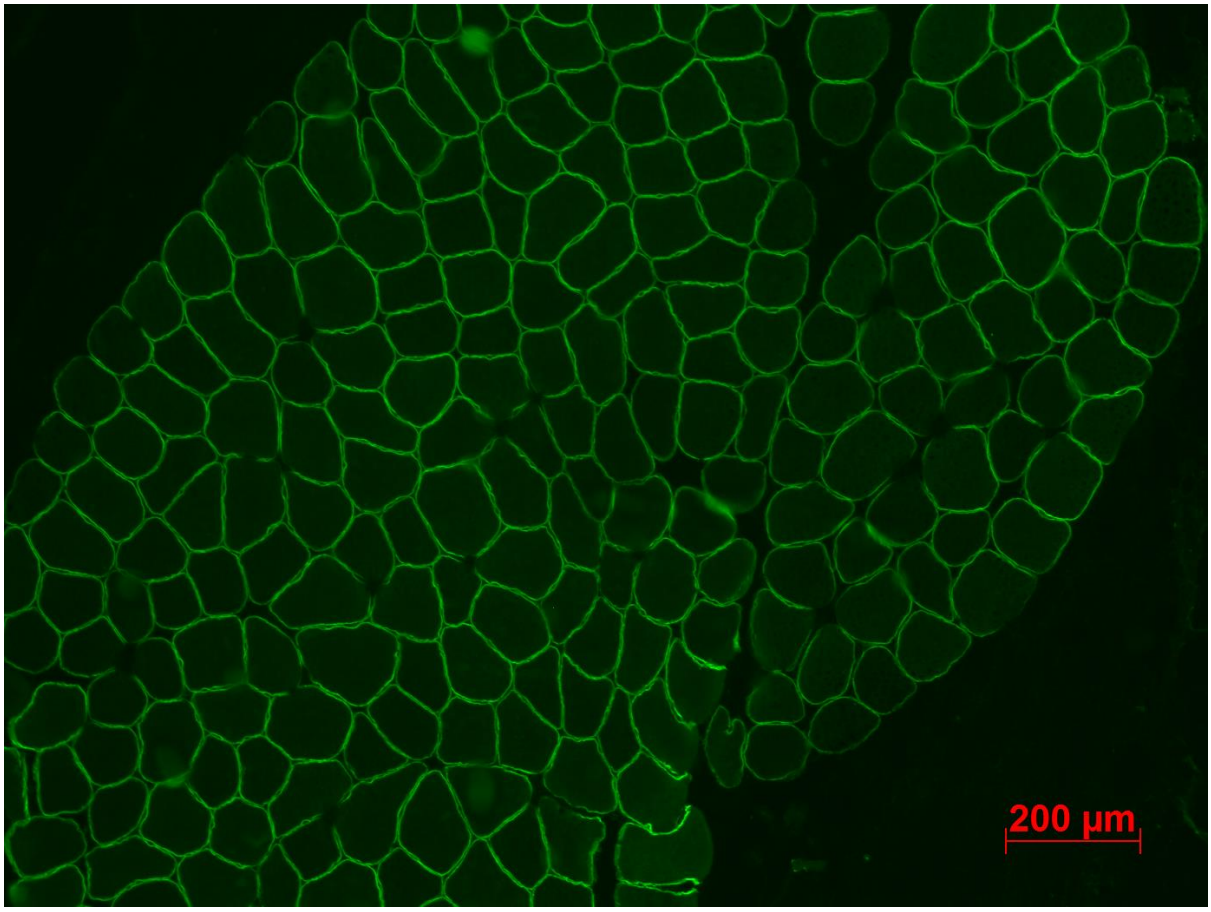
Gene sets or pathways with a false discovery rate (FDR) < 0.05 were reported as differentially expressed. GSEA: Gene Set Enrichment Analysis; UoB: University of Brighton; RP: Returning Participant; C: non-resistance trained control group; RT: Resistance Trained control group; RT-AS≤2: Resistance Trained participant who self-declared AAS exposure ceased ≤2 weeks before sampling; RT-AS≥10: Resistance Trained participant who self-declared AAS exposure ceased ≥10 weeks before sampling; GO BP: subset of GO biological processes; GO MF: subset of GO molecular functions.

Table 15. The number of overlapping differentially expressed gene sets or pathways for muscle samples sequenced with standard reagents and CoolMPS reagents at MGI subjected to GSEA analysis across different group comparisons.

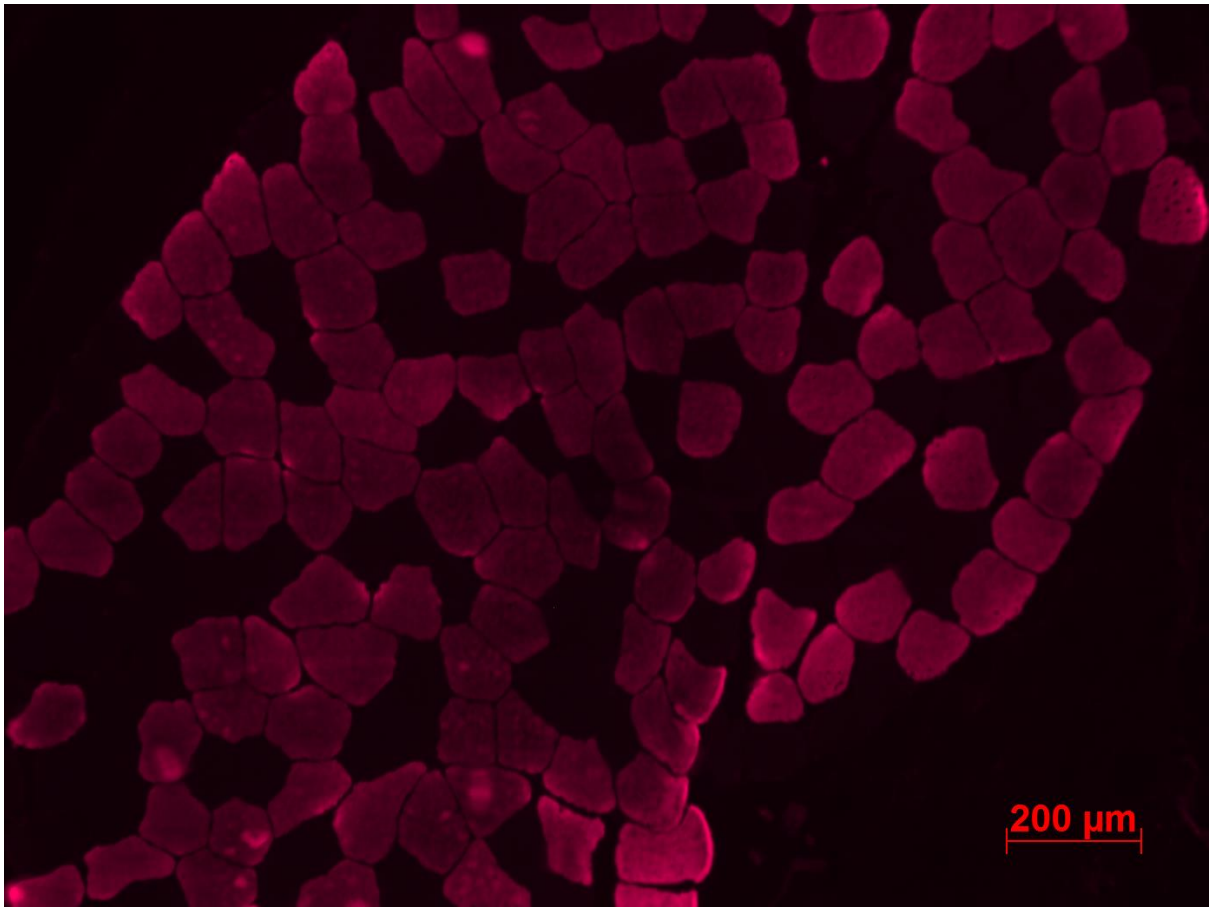
Comparison							
RP2-4 Visit 2 (*n* = 3) *vs* Visit 1 (*n* = 3)	RT (*n* = 17) *vs* C (*n* = 5)	RT-AS≤2 (*n* = 15) *vs* C (*n* = 5)	RT-AS≥10 (*n* = 11) *vs* C (*n* = 5)	RT-AS≤2 (*n* = 15) *vs* RT (*n* = 17)	RT-AS≥10 (*n* = 11) *vs* RT (*n* = 17)	RT-AS≤2 (*n* = 15) *vs* RT-AS≥10 (*n* = 11)	Gene Set or Pathway
0	0	0	0	0	0	0	Hallmark
0	0	1	0	12	2	0	KEGG
0	0	0	0	74	0	1	Reactome
0	0	14	0	176	0	2	GO BP
0	0	6	0	120	0	8	GO MF

Gene sets or pathways with a false discovery rate (FDR) < 0.05 were reported as differentially expressed. GSEA: Gene Set Enrichment Analysis; UoB: University of Brighton; RP: Returning Participant; C: non-resistance trained control group; RT: Resistance Trained control group; RT-AS≤2: Resistance Trained participant who self-declared AAS exposure ceased ≤2 weeks before sampling; RT-AS≥10: Resistance Trained participant who self-declared AAS exposure ceased ≥10 weeks before sampling; GO BP: subset of GO biological processes; GO MF: subset of GO molecular functio

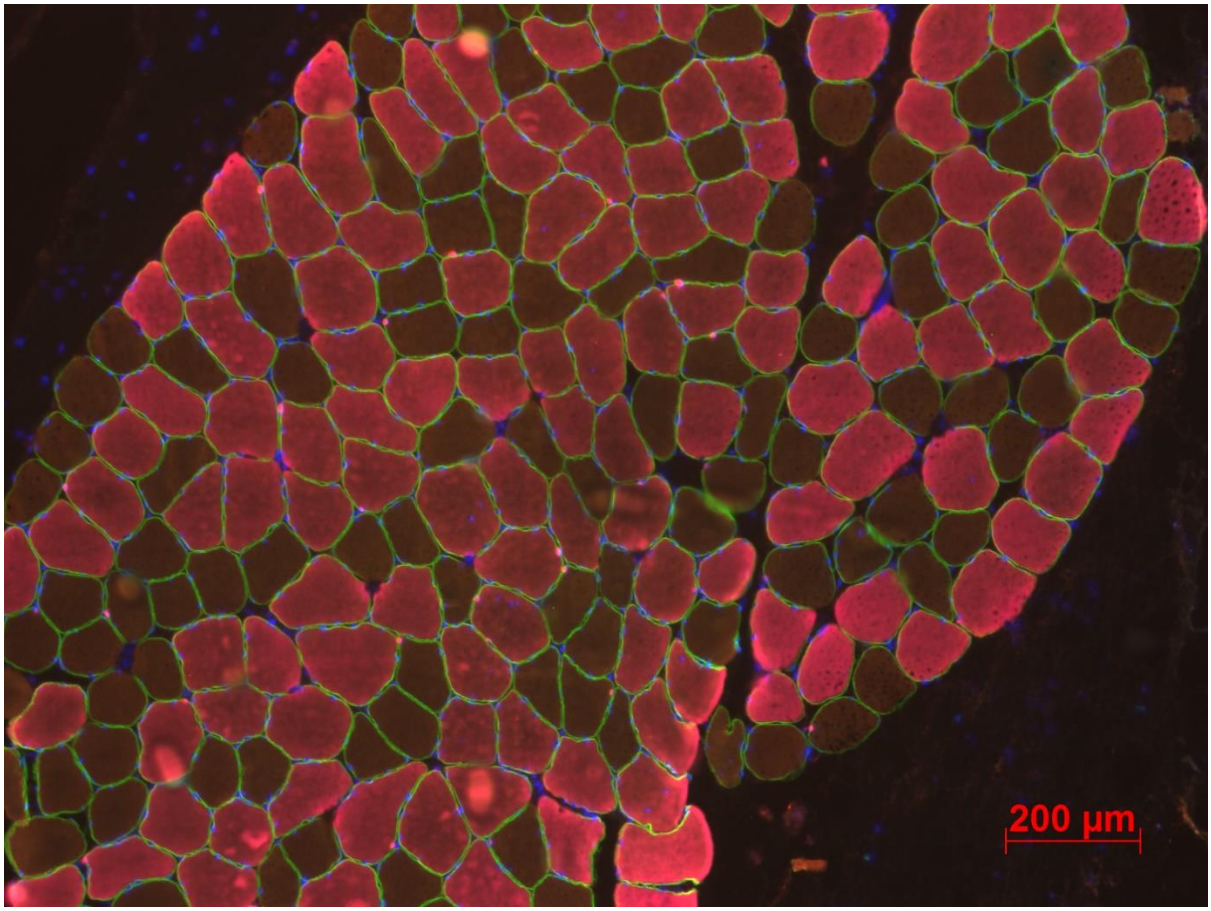
Appendix



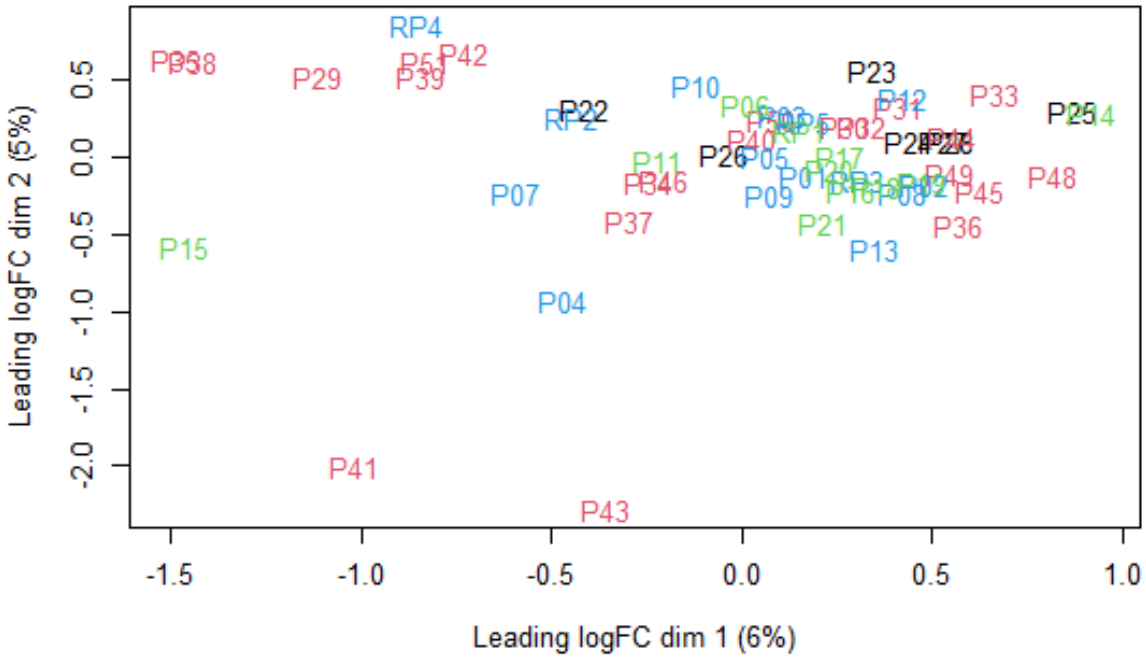
Appendix Image 1: For illustrative purposes, an IHC image from a muscle section of Participant 24 (Group C) is shown with the single channel extracted to show Dystrophin staining for fibre borders visualised in green.



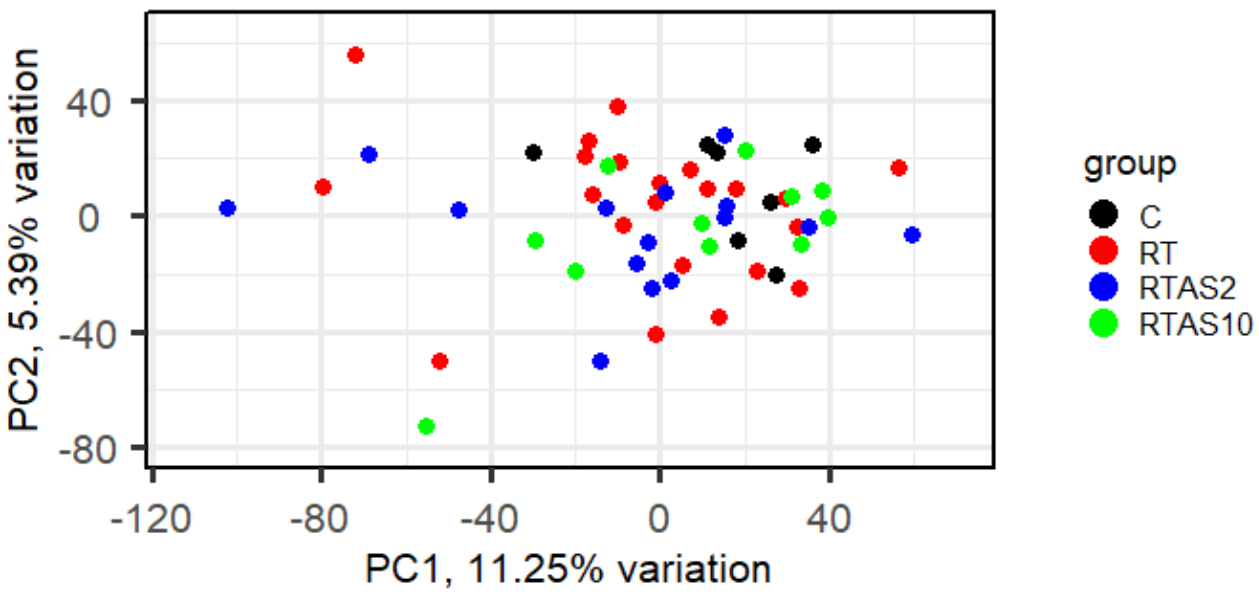
Appendix Image 2: For illustrative purposes, an IHC image from a muscle section of Participant 24 (Group C) is shown with the single channel extracted to show MyHC type I fibre staining visualised in purple/red.



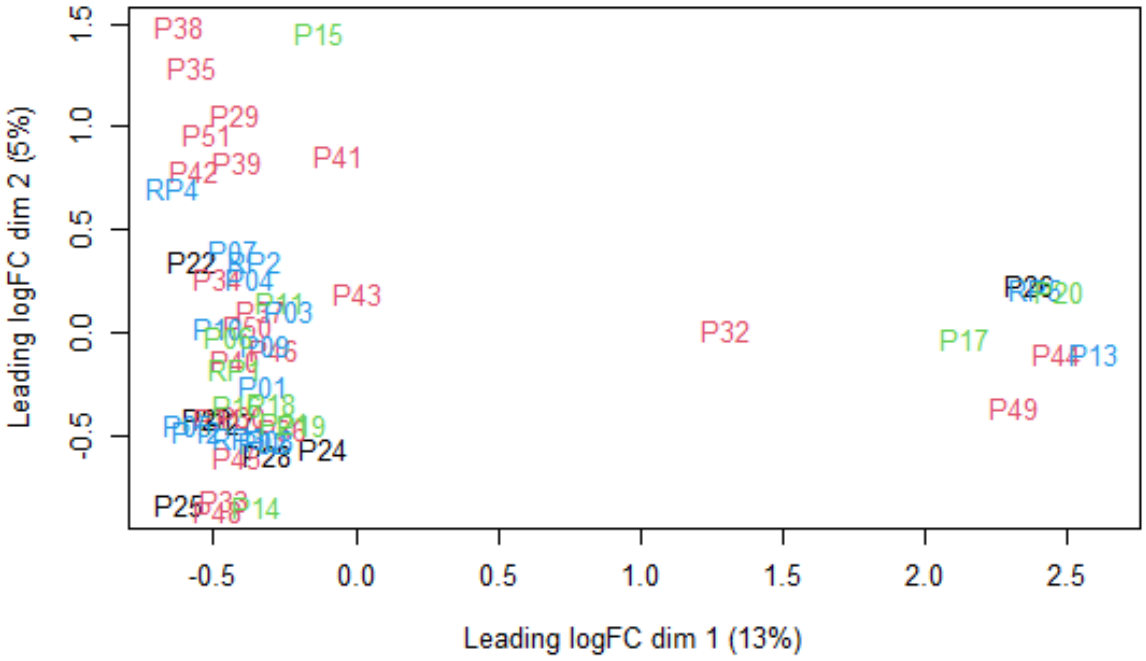
Appendix Image 3: For illustrative purposes, an IHC image from a muscle section of Participant 24 (Group C) is shown, with all image channels overlayed. Dystrophin staining for fibre borders (visualised in green), MyHC type I fibre staining (visualised as fibres filled with red), nuclei (visualised in blue) and Pax7-positive cells (i.e., satellite cells, visualised in pink, with white arrows highlighting some representative examples). Computationally, MyoVision will only count nuclei as myonuclei whose geometric centre resides inside the dystrophin ring [227].



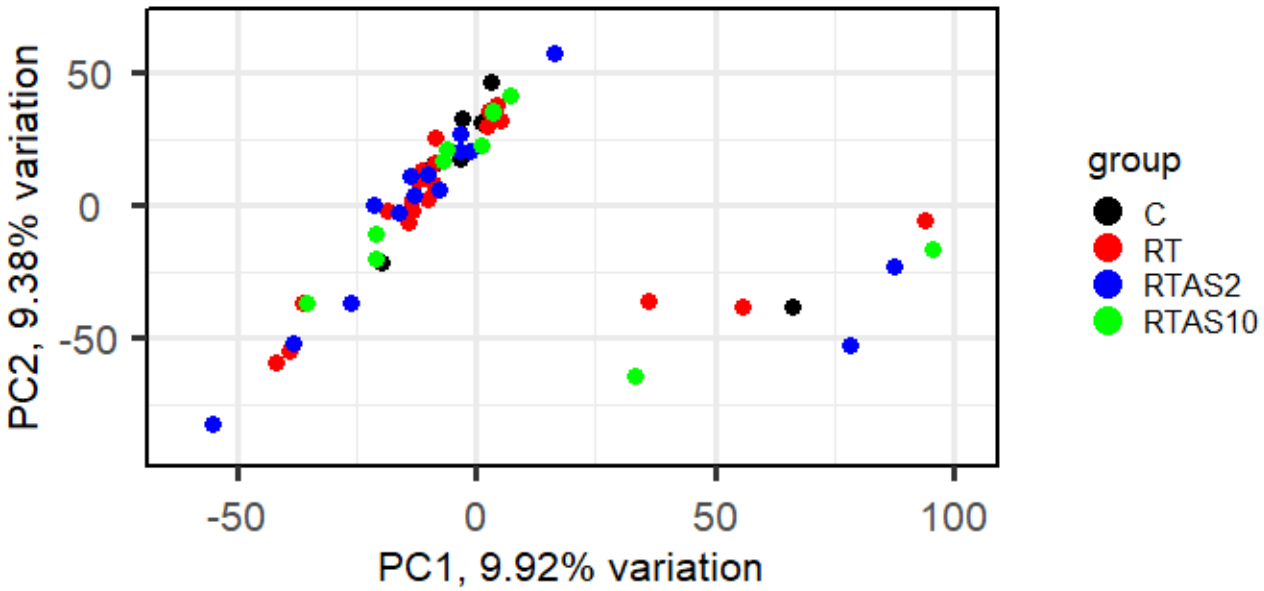
Appendix Figure 1a. MDS Plot of blood samples (*n* = 55) to be used in group comparisons sequenced at the University of Brighton with standard chemistry reagents from Group C (black, *n* = 7), Group RT (red, *n* = 22), Group RT-AS≤2 (blue, *n* = 15) and Group RT-AS≥10 (green, *n* = 11).



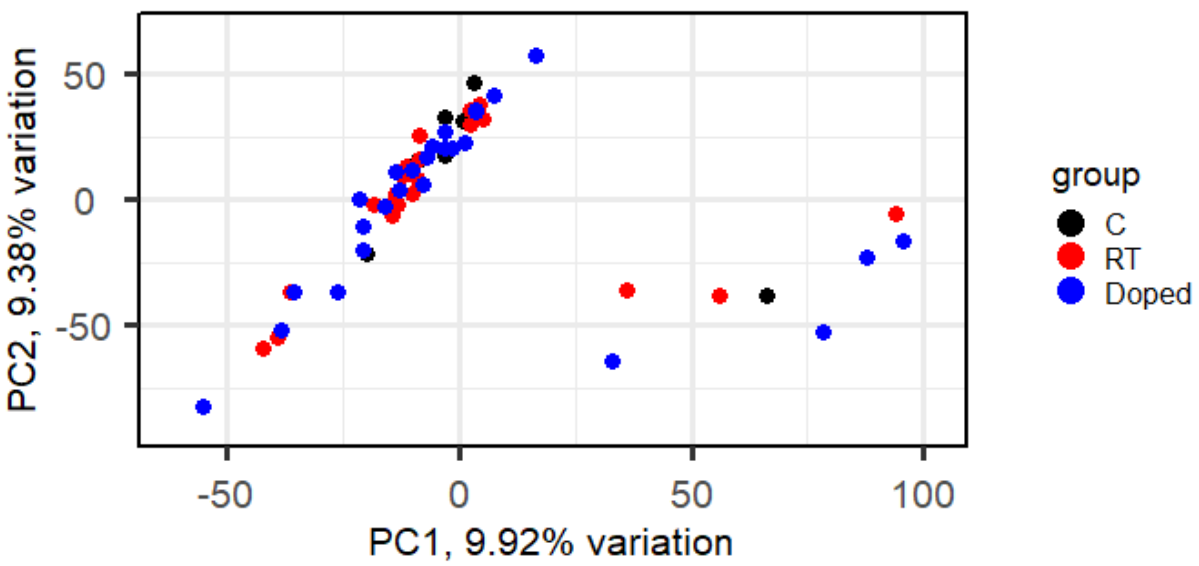
Appendix Figure 1b. PCA Plot of blood samples (*n* = 55) to be used in group comparisons sequenced at the University of Brighton with standard chemistry reagents from Group C (*n* = 7), Group RT (*n* = 22), Group RT-AS≤2 (*n* = 15) and Group RT-AS≥10 (*n* = 11).



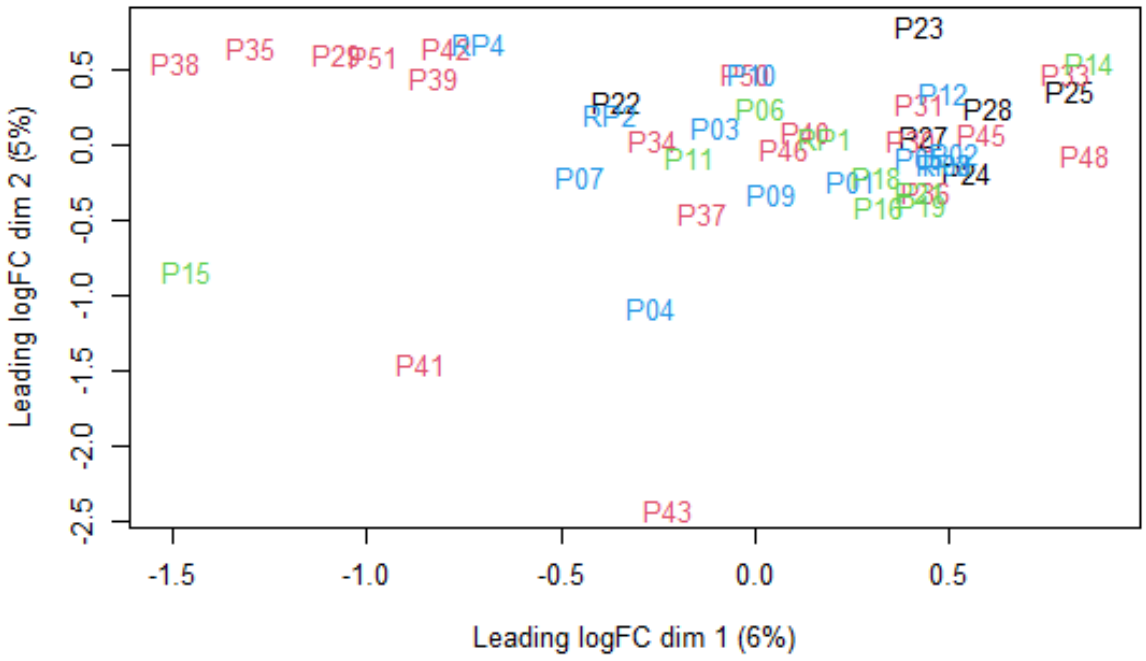
Appendix Figure 2a. MDS Plot of blood samples (*n* = 55) to be used in group comparisons sequenced at MGI with standard chemistry reagents from Group C (black, *n* = 7), Group RT (red, *n* = 22), Group RT-AS≤2 (blue, *n* = 15) and Group RT-AS≥10 (green, *n* = 11).



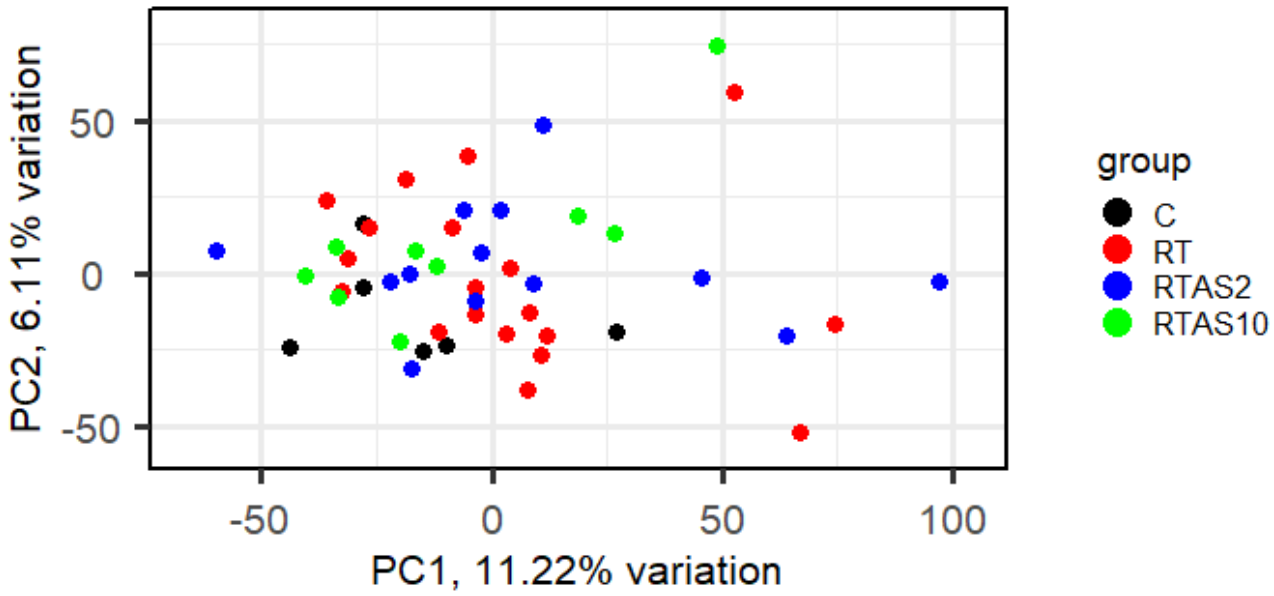
Appendix Figure 2b. PCA Plot of blood samples (*n* = 55) to be used in group comparisons sequenced at MGI with standard chemistry reagents from Group C (*n* = 7), Group RT (*n* = 22), Group RT-AS≤2 (*n* = 15) and Group RT-AS≥10 (*n* = 11).



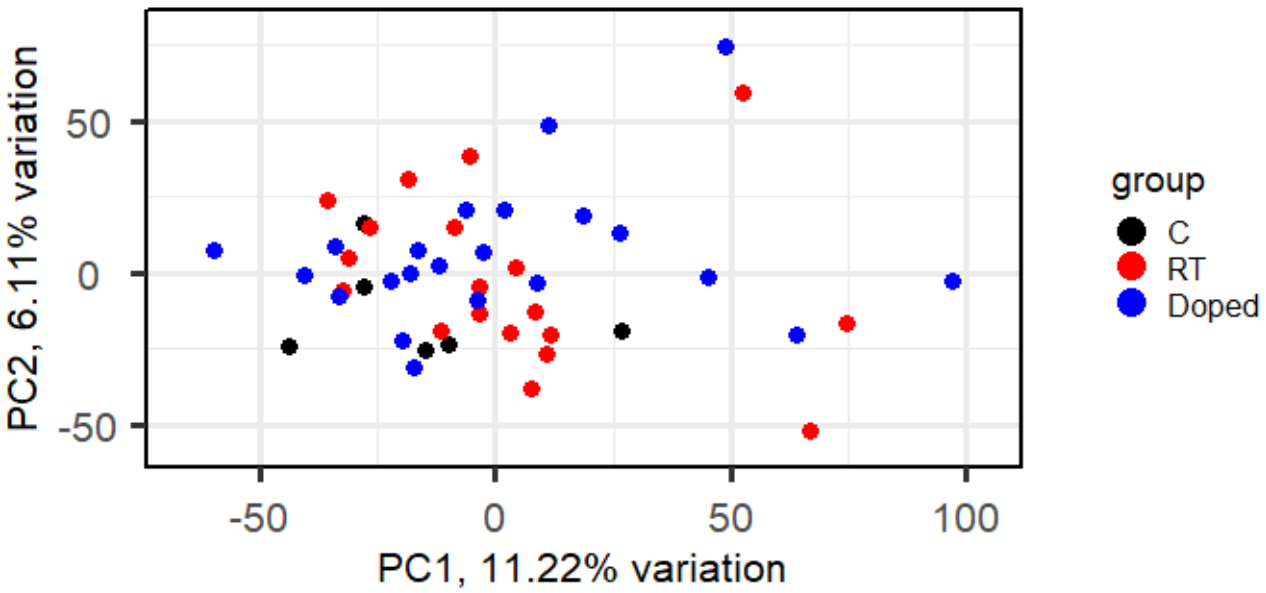
Appendix Figure 2c. PCA Plot of blood samples (*n* = 55) to be used in group comparisons sequenced at MGI with standard chemistry reagents from Group C (*n* = 7), Group RT (*n* = 22) and Group Doped (*n* = 26).



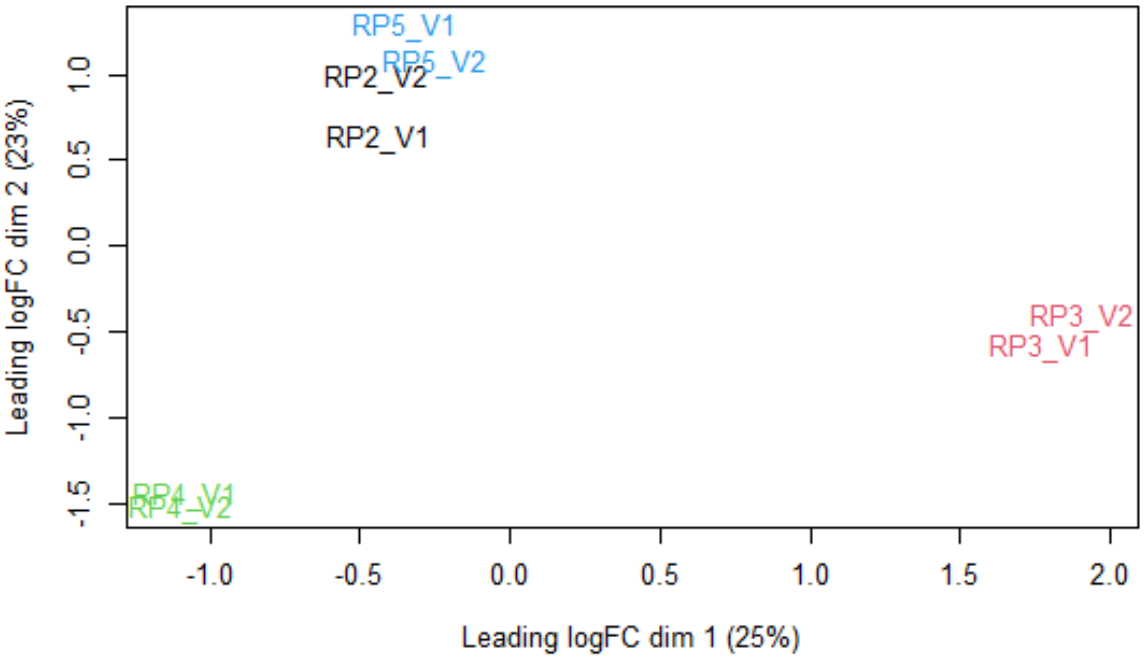
Appendix Figure 3a. MDS Plot of blood samples (*n* = 47) to be used in group comparisons sequenced at MGI with standard chemistry reagents after samples (*n* = 8) sequenced on Flow Cell B Lane 1 were removed as outliers: Group C (black, n = 6), Group RT (red, *n* = 19), Group RT-AS≤ 2 (blue, *n* = 13) and Group RT-AS≥10 (green, *n* = 9).



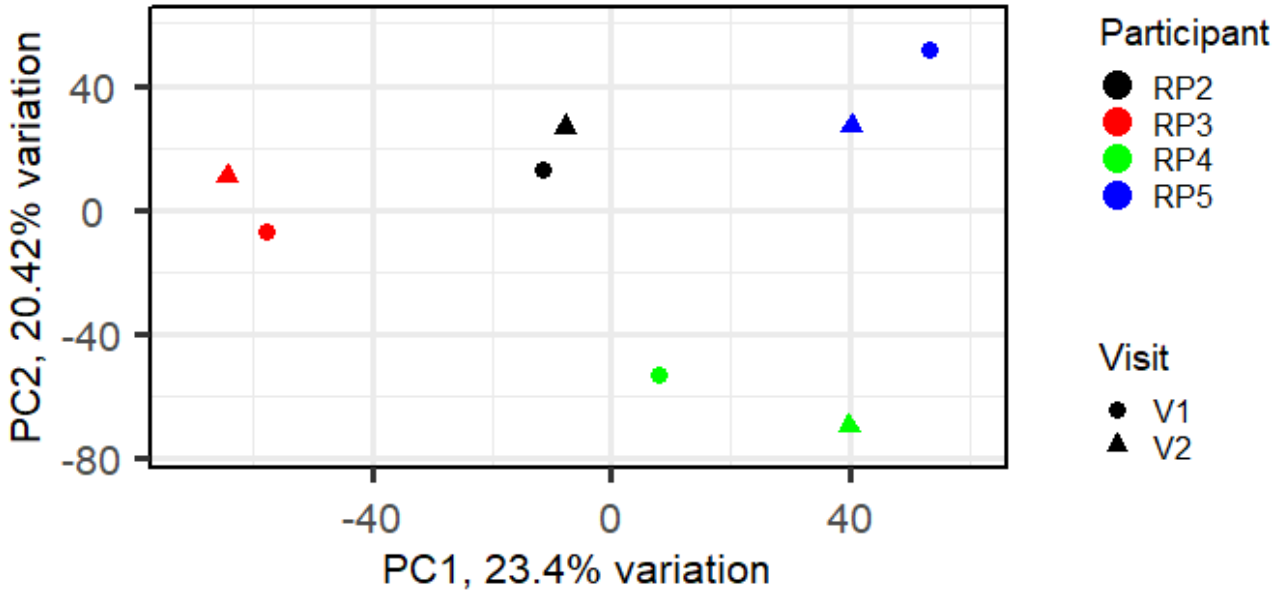
Appendix Figure 3b. PCA Plot of blood samples (*n* = 47) to be used in group comparisons sequenced at MGI with standard chemistry reagents after samples (*n* = 8) sequenced on Flow Cell B Lane 1 were removed as outliers: Group C (*n* = 6), Group RT (*n* = 19), Group RT-AS≤2 (*n* = 13) and Group RT-AS≥10 (*n* = 9).



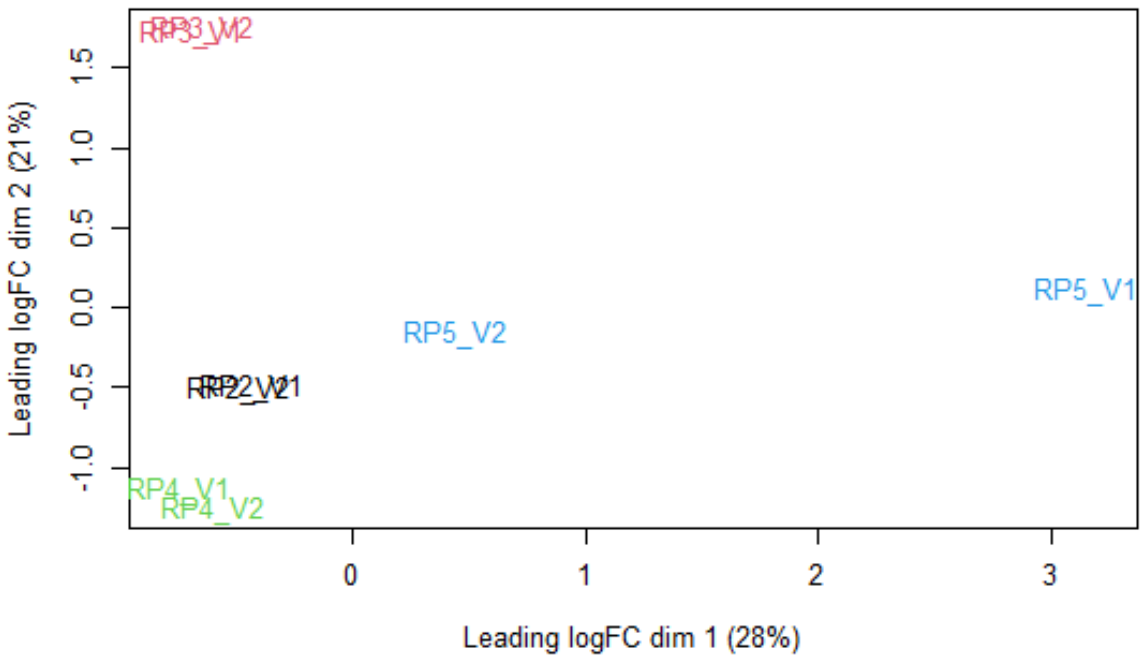
Appendix Figure 3c. PCA Plot of blood samples (*n* = 47) to be used in group comparisons sequenced at MGI with standard chemistry reagents after samples (*n* = 8) sequenced on Flow Cell B Lane 1 were removed as outliers: Group C (*n* = 6), Group RT (*n* = 19) and Group Doped (*n* = 22).



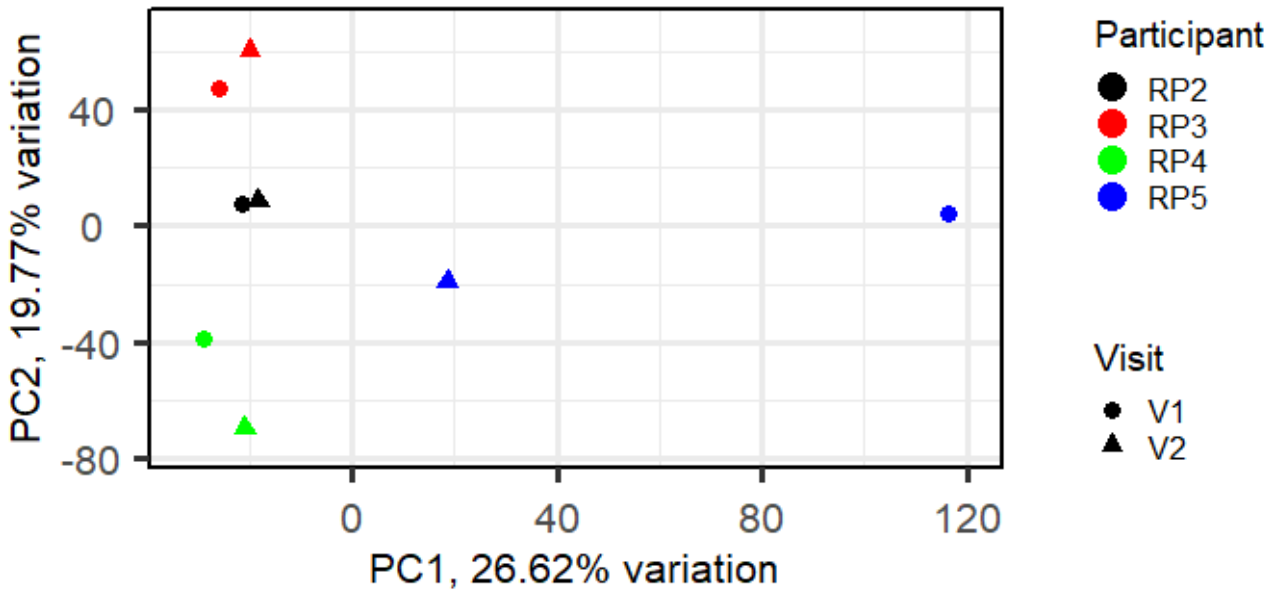
Appendix Figure 4a. MDS Plot of blood samples (*n* = 8) sequenced at the University of Brighton with standard chemistry reagents from Returning Participant (RP2-5) first and second visits (_V1 or _V2).



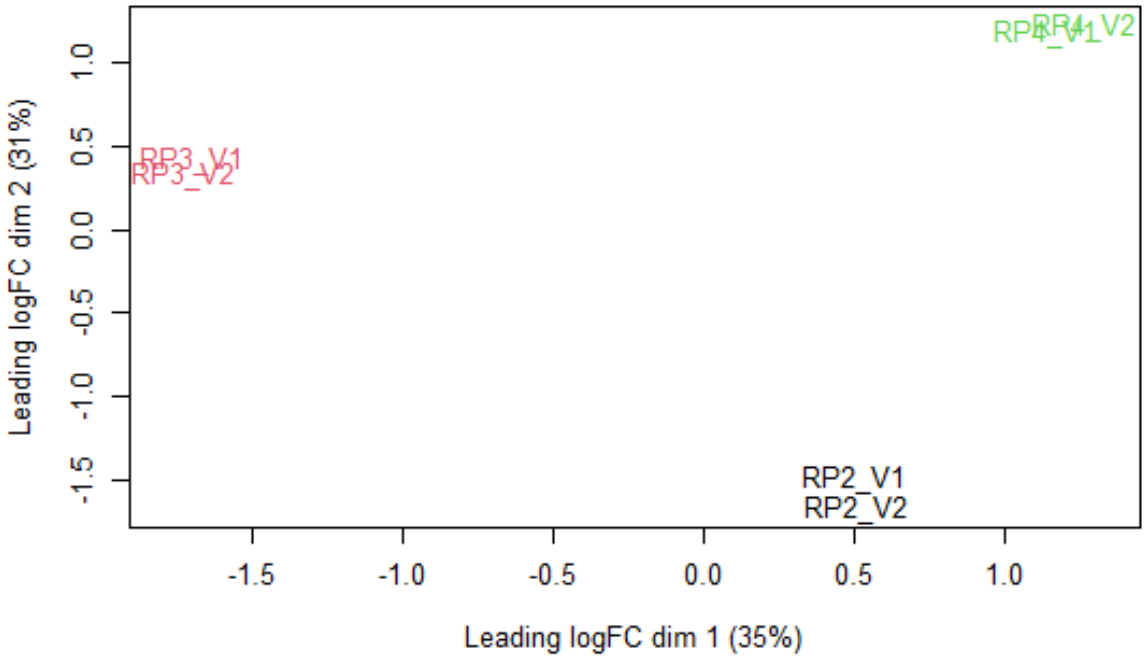
Appendix Figure 4b. PCA Plot of blood samples (*n* = 8) sequenced at the University of Brighton with standard chemistry reagents from Returning Participant (RP2-5) first and second visits (V1 or V2).



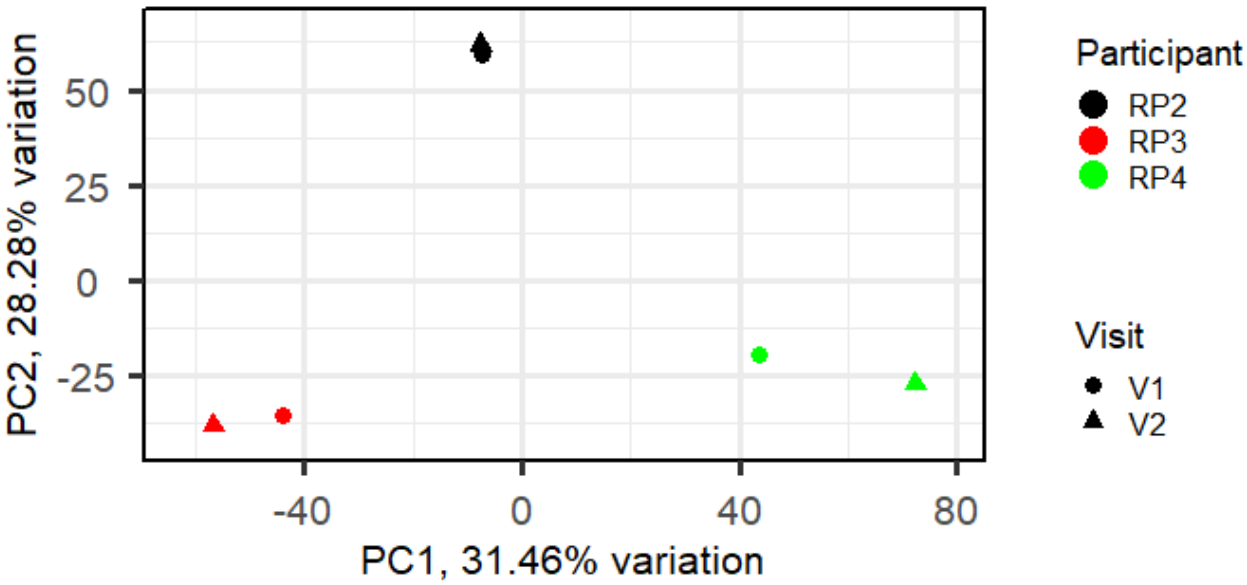
Appendix Figure 5a. MDS Plot of blood samples (*n* = 8) sequenced at MGI with standard chemistry reagents from Returning Participant (RP2-5) first and second visits (_V1 or _V2).



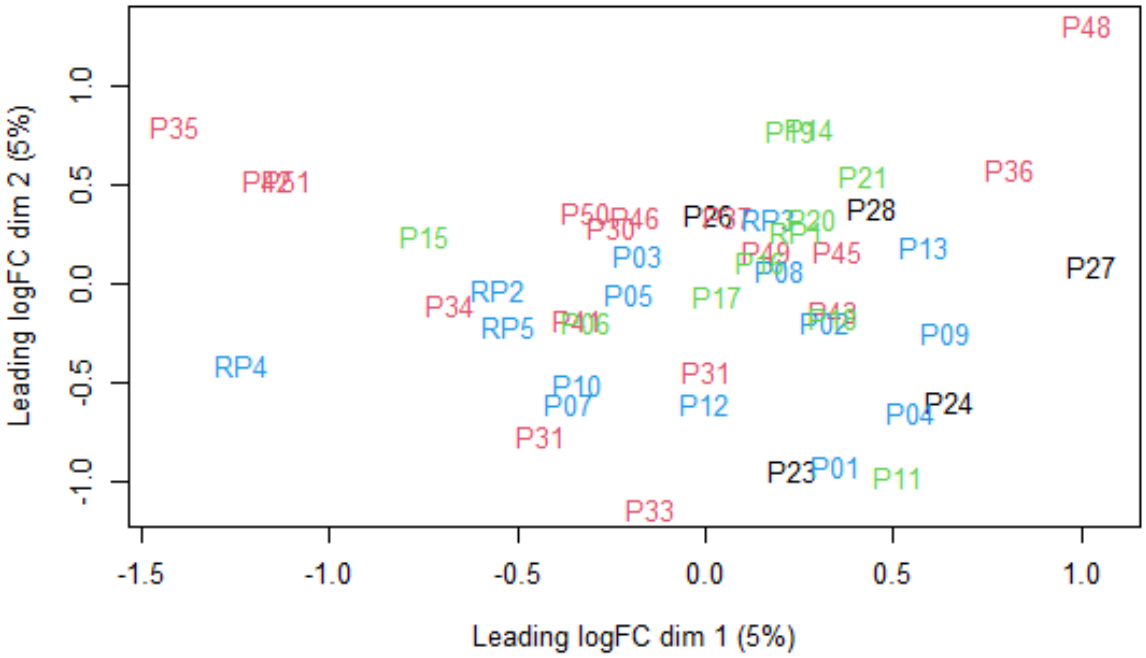
Appendix Figure 5b. PCA Plot of blood samples (*n* = 8) sequenced at MGI with standard chemistry reagents from Returning Participant (RP2-5) first and second visits (V1 or V2).



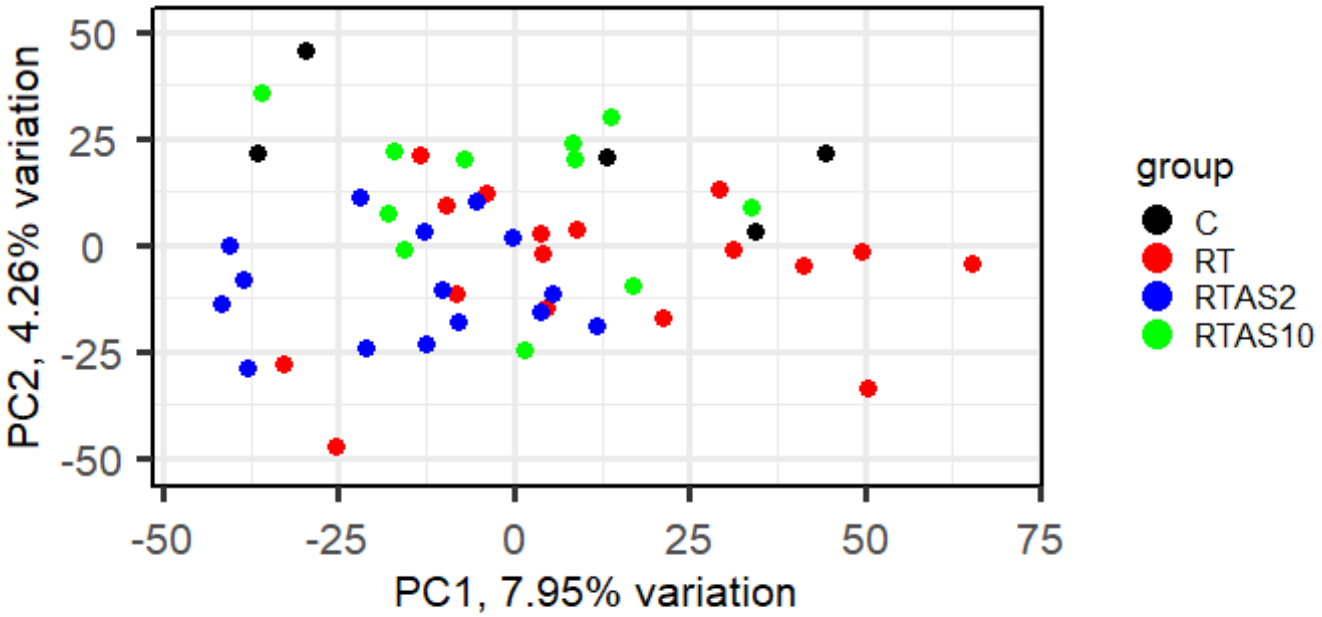
Appendix Figure 6a. MDS Plot of blood samples (*n* = 6) sequenced at MGI with standard chemistry reagents that underwent differential gene expression analysis from Returning Participant (RP2-4) first and second visits (_V1 or _V2).



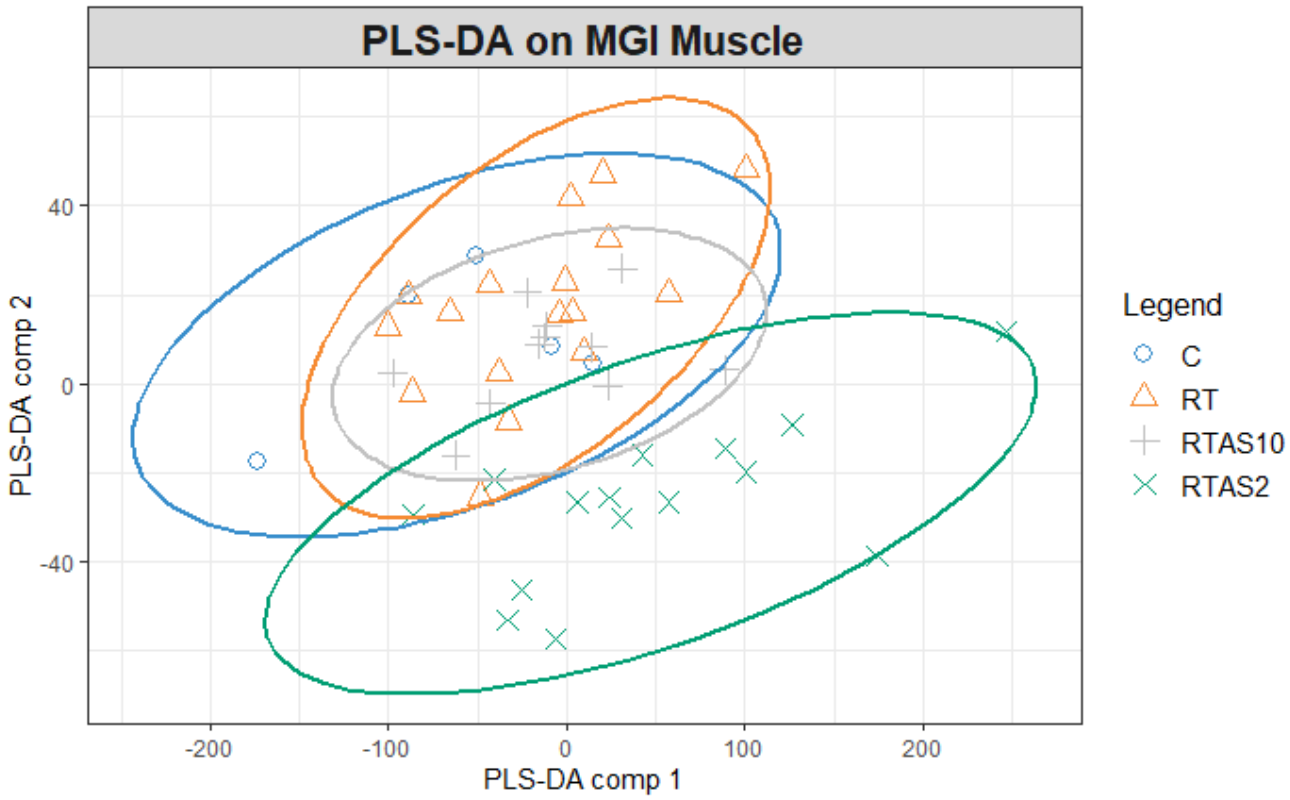
Appendix Figure 6b. PCA Plot of blood samples (*n* = 6) sequenced at MGI with standard chemistry reagents that underwent differential gene expression analysis from Returning Participant (RP2-4) first and second visits (V1 or V2).



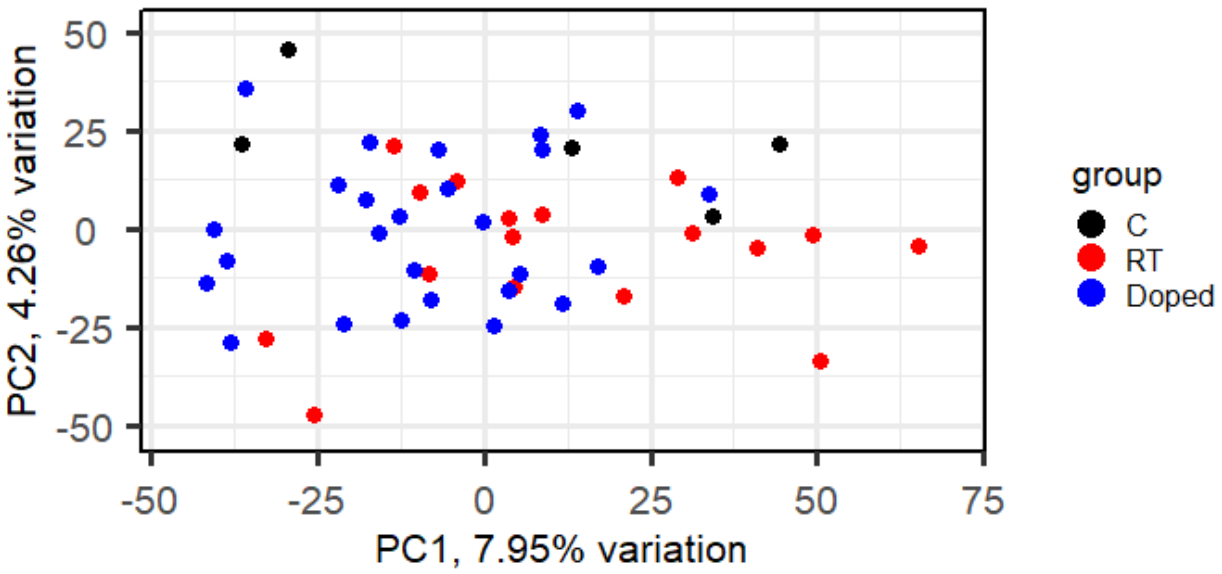
Appendix Figure 7a. MDS Plot of muscle samples (*n* = 48) to be used in group comparisons sequenced with MGI standard chemistry reagents at MGI from Group C (black, *n* = 5), Group RT (red, *n* = 17), Group RT-AS≤2 (blue, *n* = 15) and Group RT-AS≥10 (green, *n* = 11) subjected to Differential Gene Expression analysis.



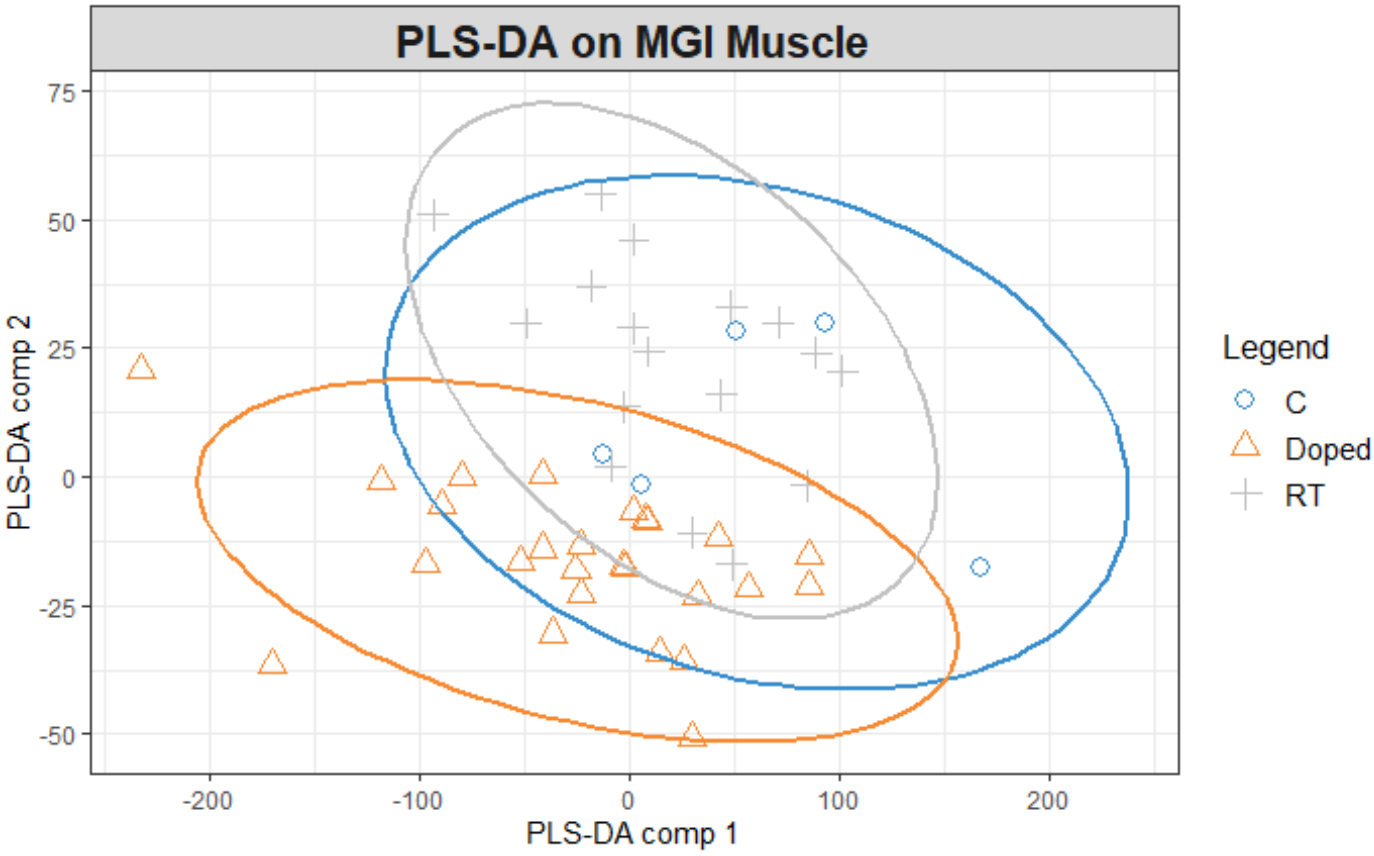
Appendix Figure 7b. PCA Plot of muscle samples (*n* = 48) to be used in group comparisons sequenced with MGI standard chemistry reagents at MGI from Group C (*n* = 5), Group RT (*n* = 17), Group RT-AS≤2 (*n* = 15) and Group RT-AS≥10 (*n* = 11) subjected to Differential Gene Expression analysis.



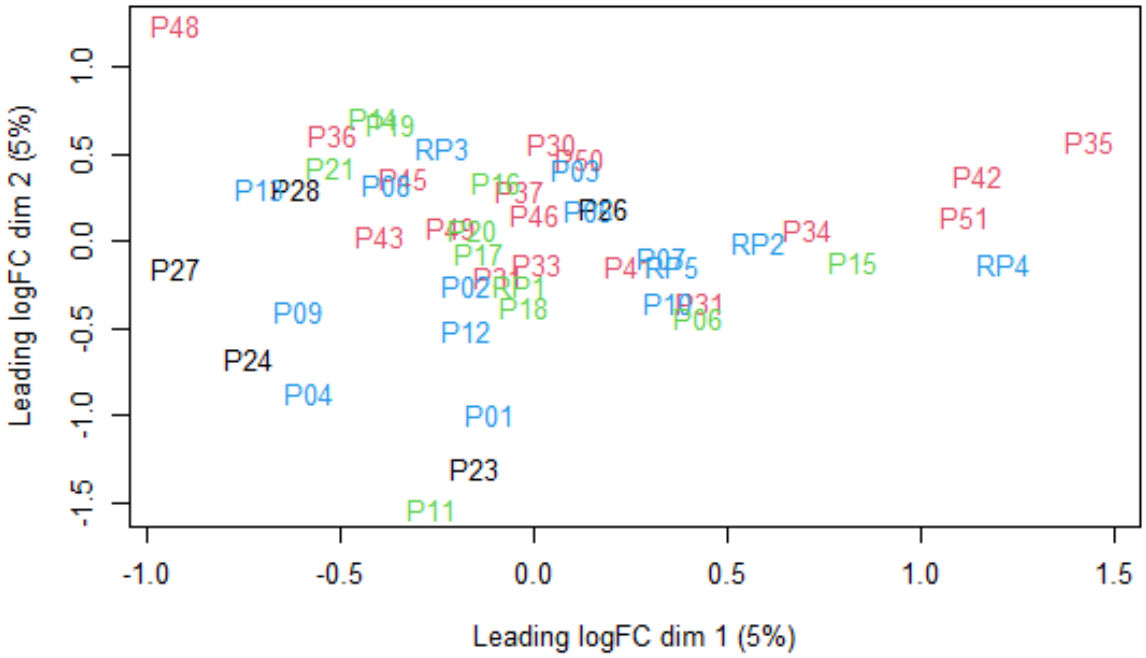
Appendix Figure 7c. PLS-DA Plot of muscle samples (*n* = 48) to be used in group comparisons sequenced with MGI standard chemistry reagents at MGI from Group C (*n* = 5), Group RT (*n* = 17), Group RT-AS≤2 (*n* = 15) and Group RT-AS≥10 (*n* = 11) subjected to Differential Gene Expression analysis.



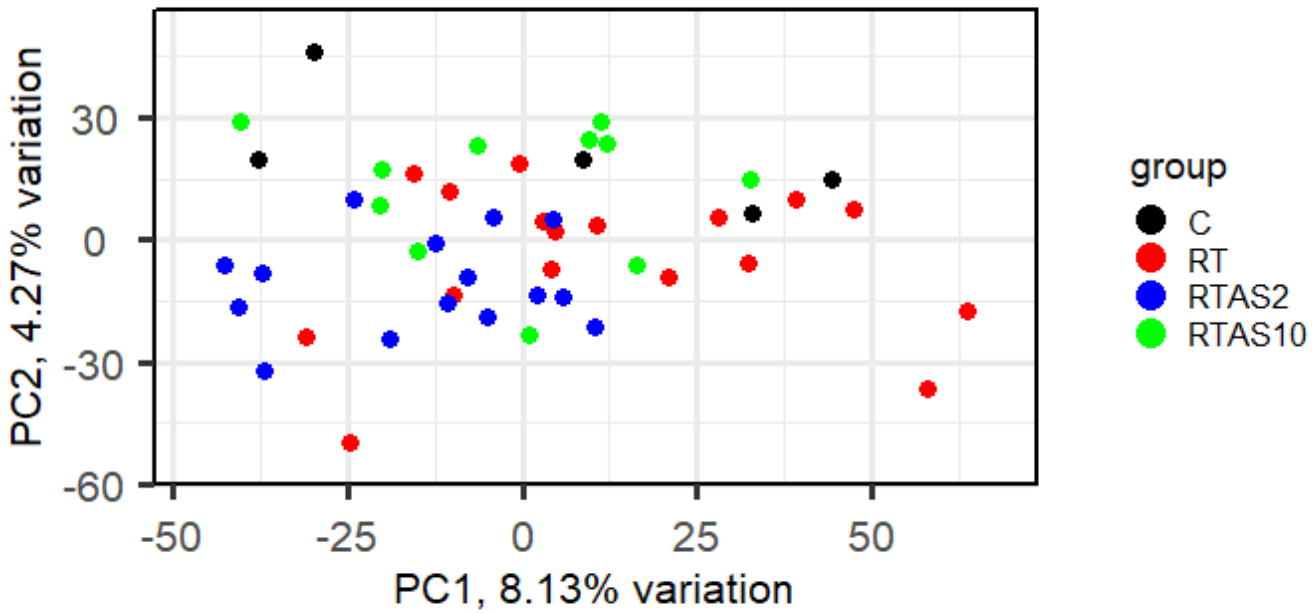
Appendix Figure 7d. PCA Plot of muscle samples (*n* = 48) to be used in group comparisons sequenced with MGI standard chemistry reagents at MGI from Group C (*n* = 5), Group RT (*n* = 17) and Group Doped (*n* = 26) subjected to Differential Gene Expression analysis.



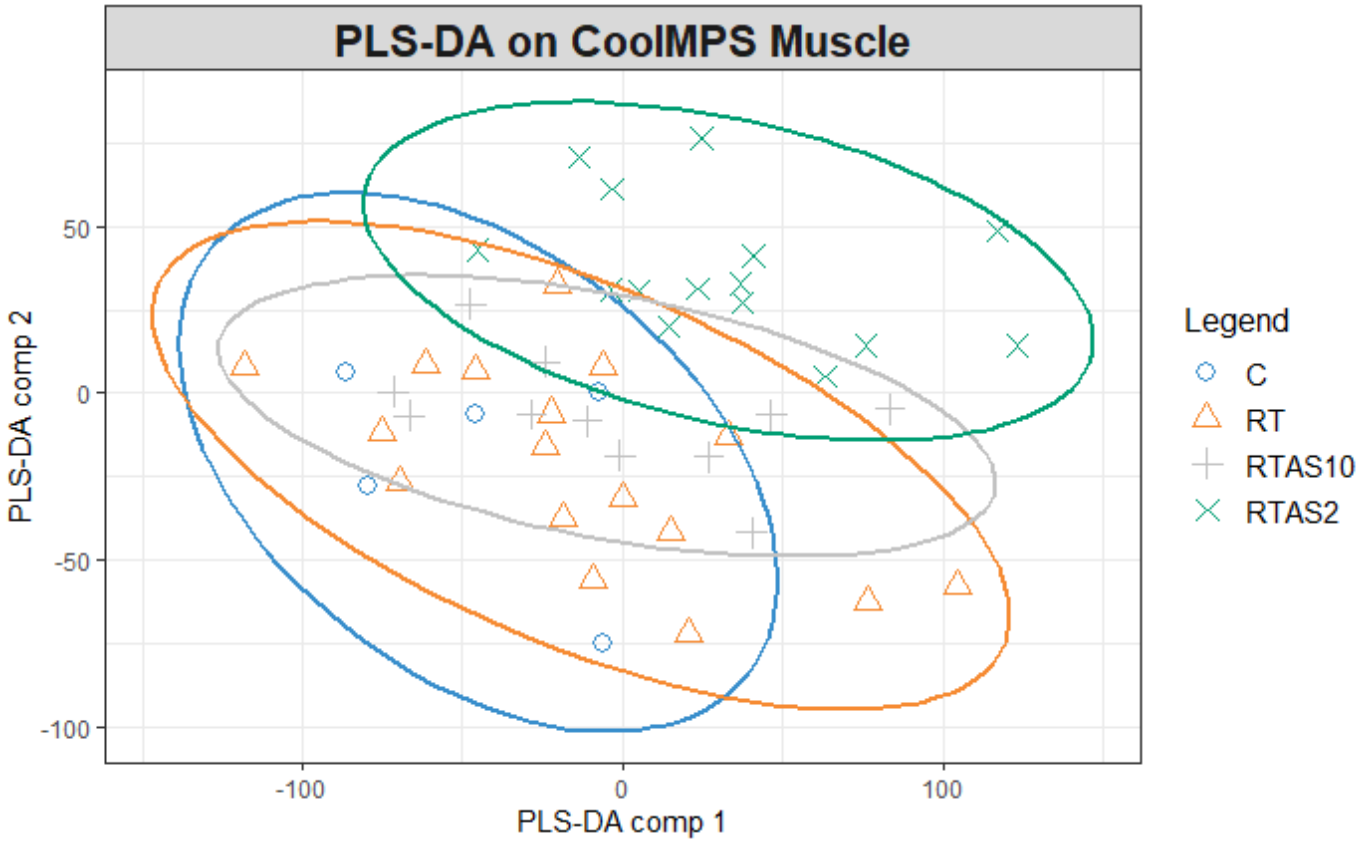
Appendix Figure 7e. PLS-DA Plot of muscle samples (*n* = 48) to be used in group comparisons sequenced with MGI standard chemistry reagents at MGI from Group C (*n* = 5), Group RT (*n* = 17) and Group Doped (*n* = 26) subjected to Differential Gene Expression analysis.



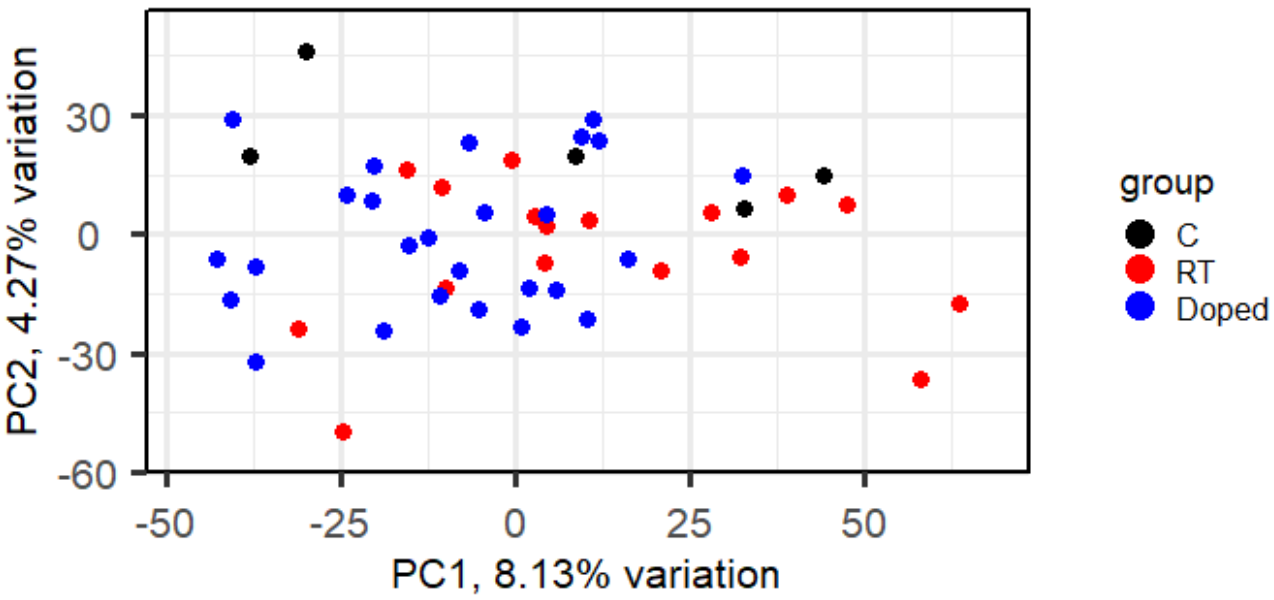
Appendix Figure 8a. MDS Plot of muscle samples (*n* = 48) to be used in group comparisons sequenced with CoolMPS reagents at MGI from Group C (black, *n* = 5), Group RT (red, *n* = 17), Group RT-AS≤2 (blue, *n* = 15) and Group RT-AS≥10 (green, *n* = 11) subjected to Differential Gene Expression analysis.



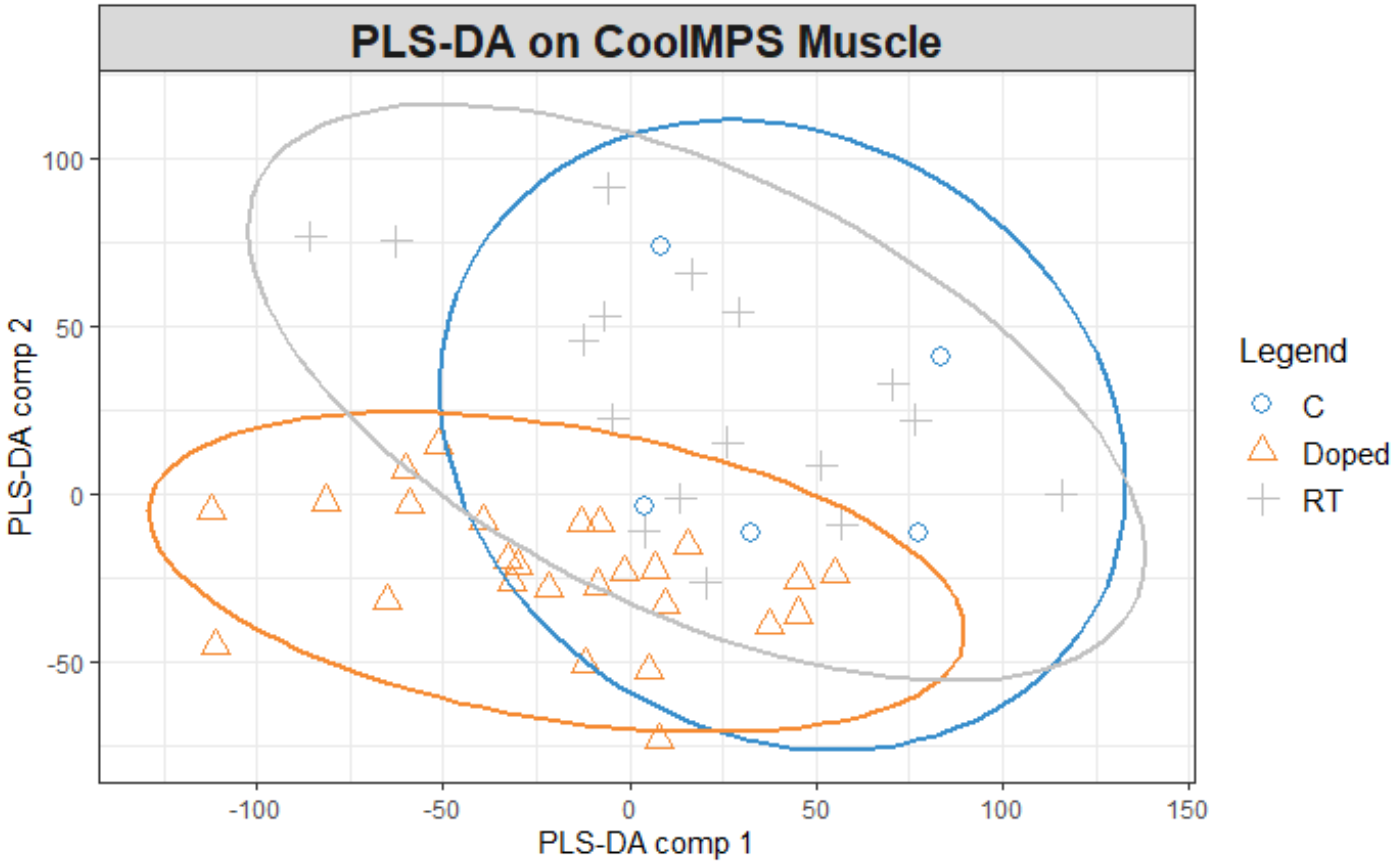
Appendix Figure 8b. PCA Plot of muscle samples (*n* = 48) to be used in group comparisons sequenced with CoolMPS reagents at MGI from Group C (*n* = 5), Group RT (*n* = 17), Group RT-AS≤2 (*n* = 15) and Group RT-AS≥10 (*n* = 11) subjected to Differential Gene Expression analysis.



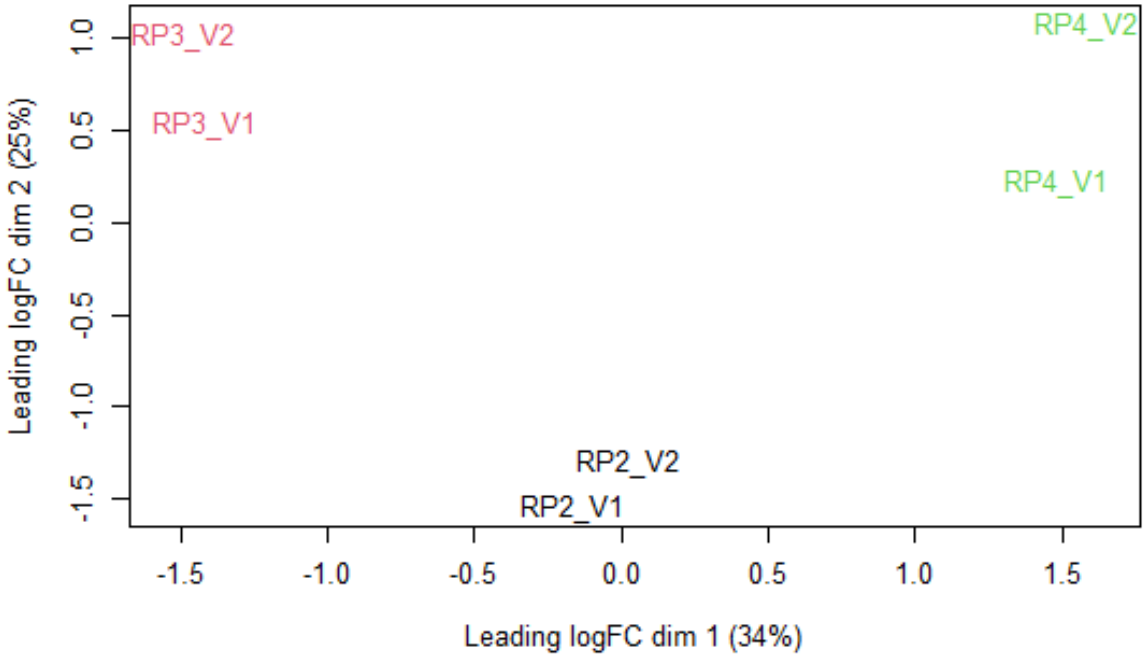
Appendix Figure 8c. PLS-DA Plot of muscle samples (*n* = 48) to be used in group comparisons sequenced with CoolMPS reagents at MGI from Group C (*n* = 5), Group RT (*n* = 17), Group RT-AS≤2 (*n* = 15) and Group RT-AS≥10 (*n* = 11) subjected to Differential Gene Expression analysis.



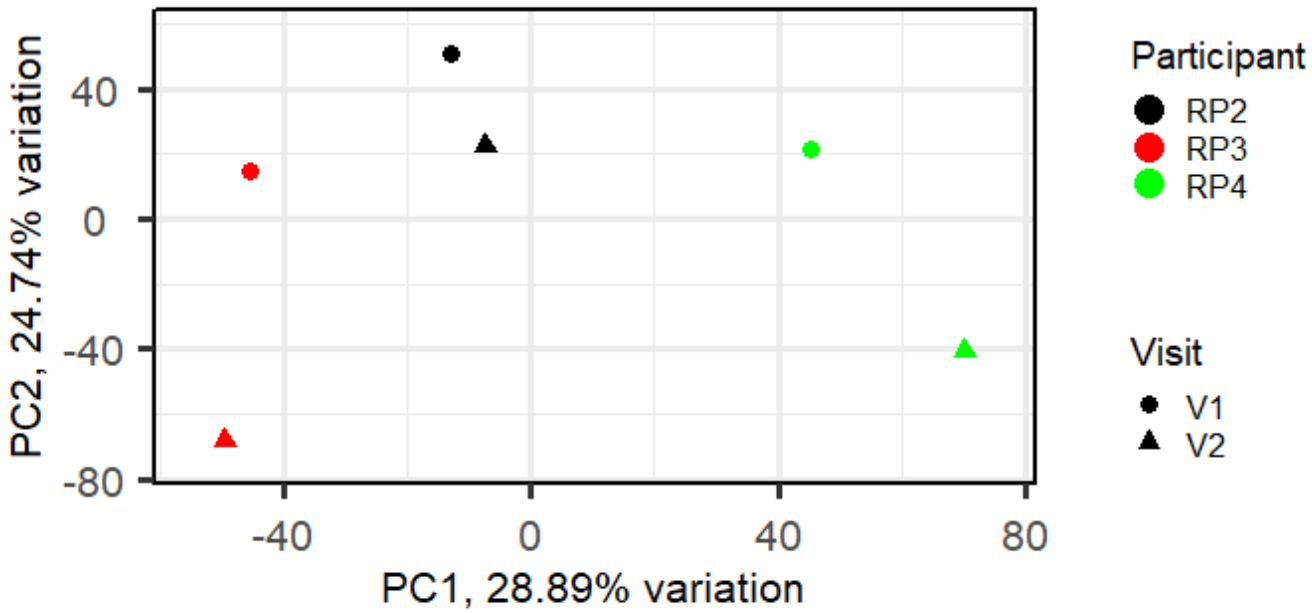
Appendix Figure 8d. PCA Plot of muscle samples (*n* = 48) to be used in group comparisons sequenced with CoolMPS reagents at MGI from Group C (*n* = 5), Group RT (*n* = 17) and Group Doped (*n* = 26) subjected to Differential Gene Expression analysis.



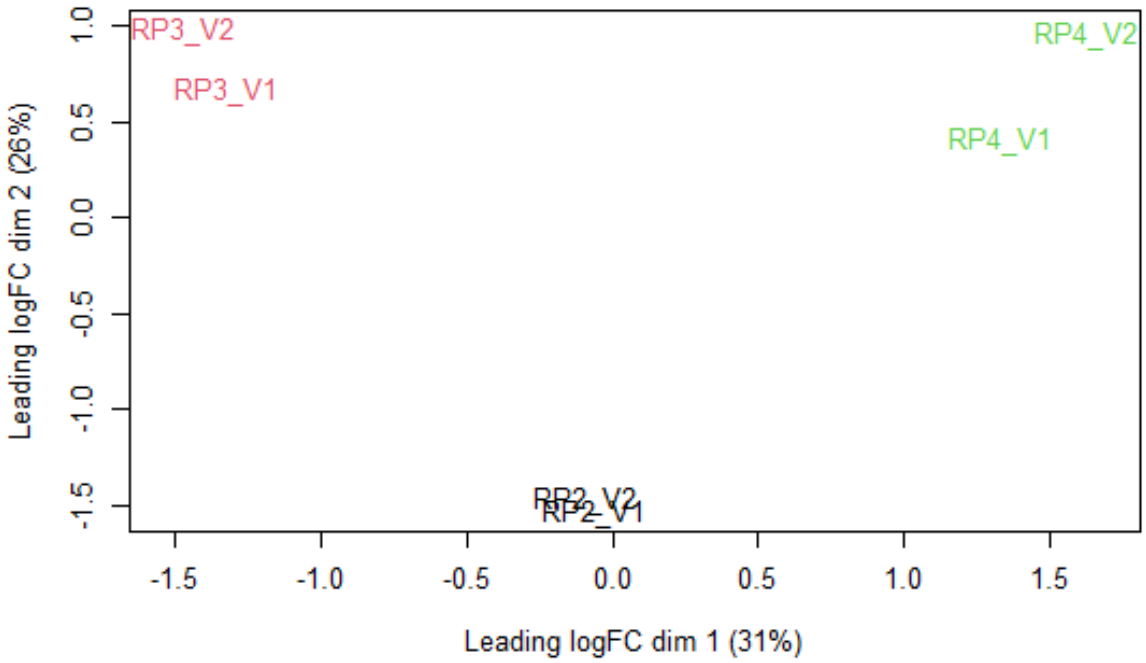
Appendix Figure 8e. PLS-DA Plot of muscle samples (*n* = 48) to be used in group comparisons sequenced with CoolMPS reagents at MGI from Group C (*n* = 5), Group RT (*n* = 17) and Group Doped (*n* = 26) subjected to Differential Gene Expression analysis.



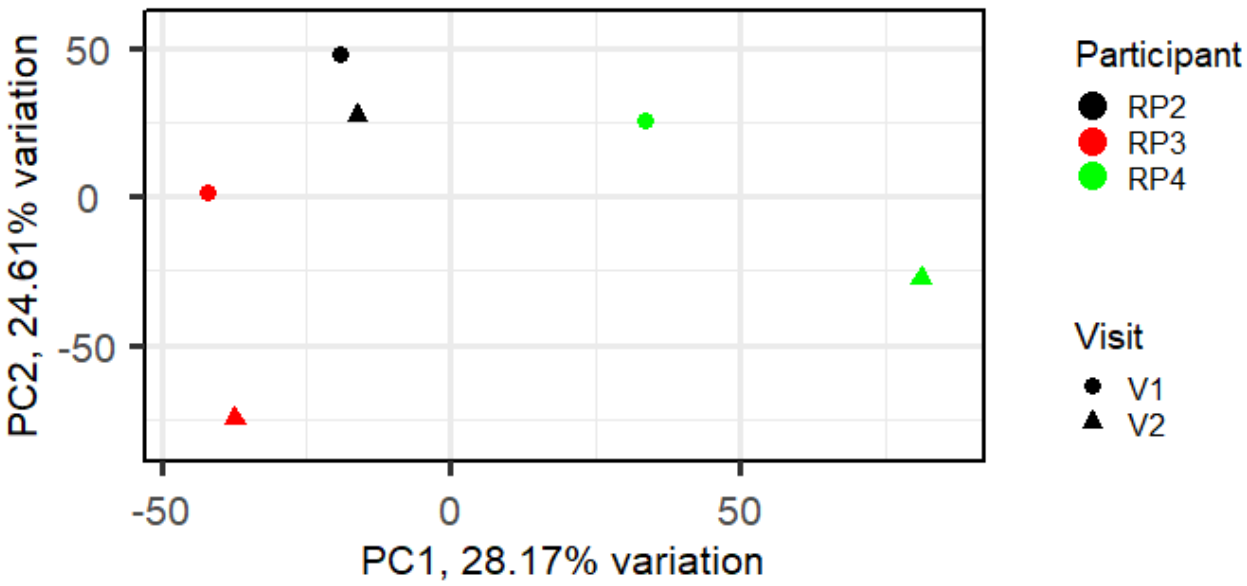
Appendix Figure 9a. MDS Plot of muscle samples (*n* = 6) sequenced with Standard MGI reagents at MGI from Returning Participant (RP2-4) first and second visits (_V1 or _V2).



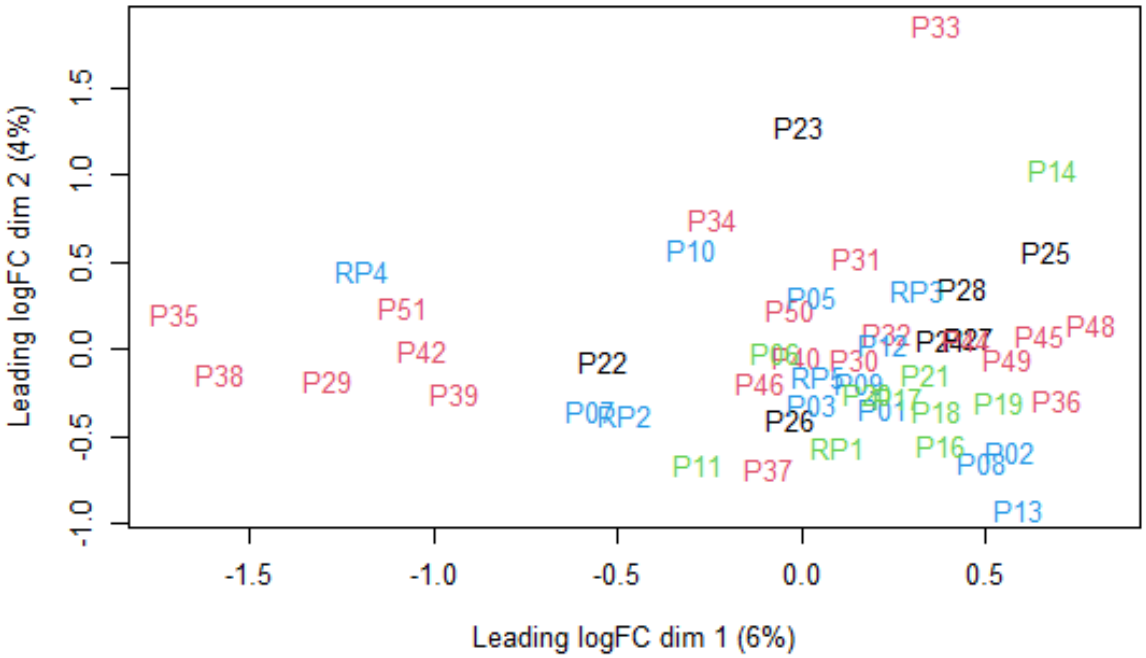
Appendix Figure 9b. PCA Plot of muscle samples (*n* = 6) sequenced with Standard MGI reagents at MGI from Returning Participant (RP2-4) first and second visits (V1 or V2).



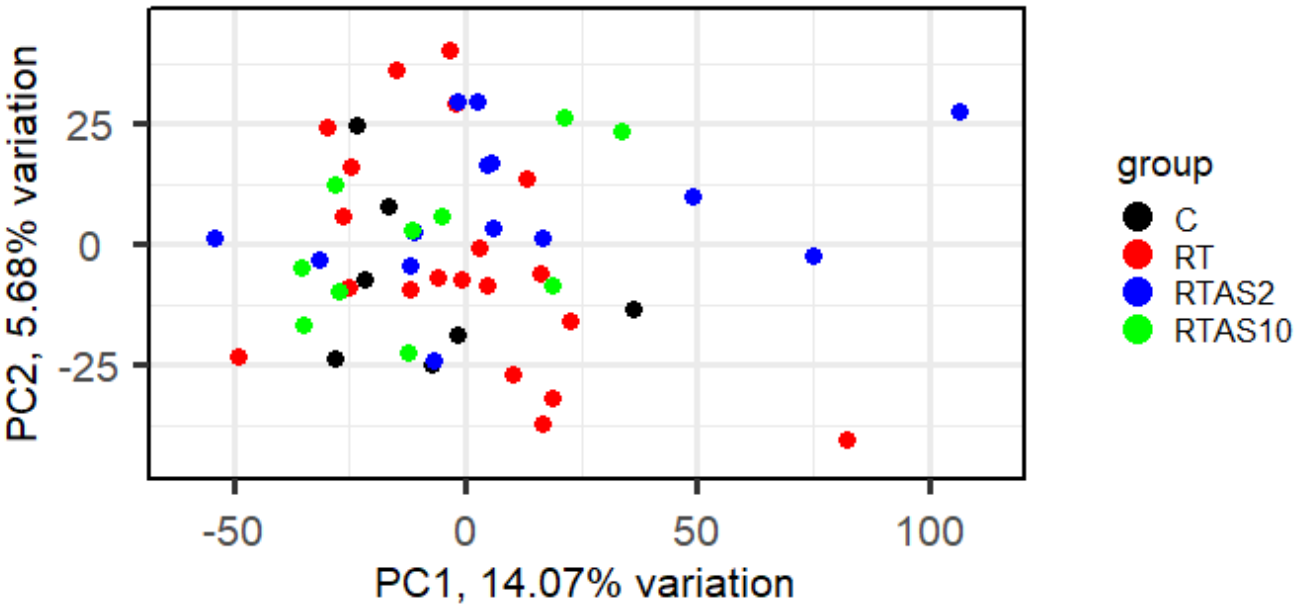
Appendix Figure 10a. MDS Plot of muscle samples (*n* = 6) sequenced with CoolMPS reagents at MGI from Returning Participant (RP2-4) first and second visits (_V1 or _V2).



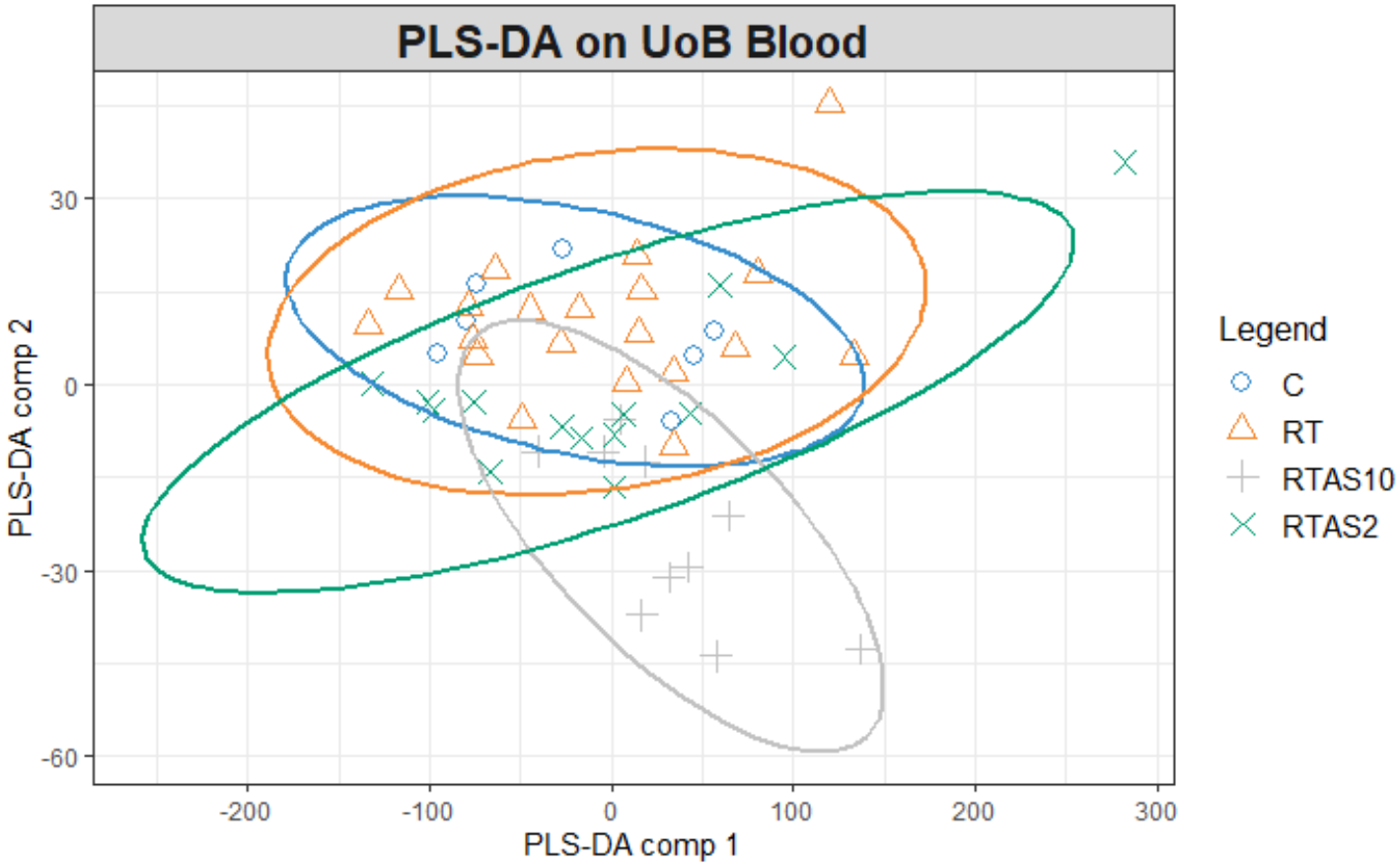
Appendix Figure 10b. PCA Plot of muscle samples (*n* = 6) sequenced with CoolMPS reagents at MGI from Returning Participant (RP2-4) first and second visits (V1 or V2).



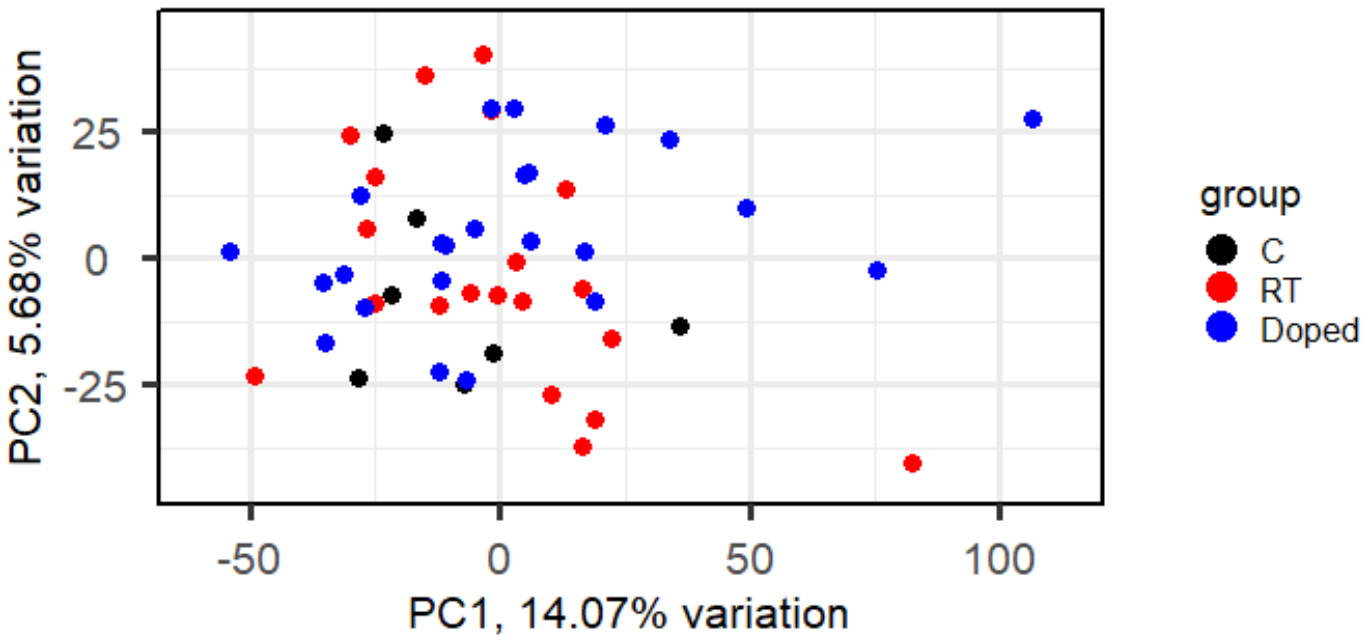
Appendix Figure 11a. MDS Plot of blood samples (*n* = 51) sequenced at the University of Brighton with standard chemistry reagents that underwent Differential Gene Expression analysis. Group C (black, *n* = 7), Group RT (red, *n* = 20), Group RT-AS≤2 (blue, *n* = 14) and Group RT-AS≥10 (green, *n* = 10).



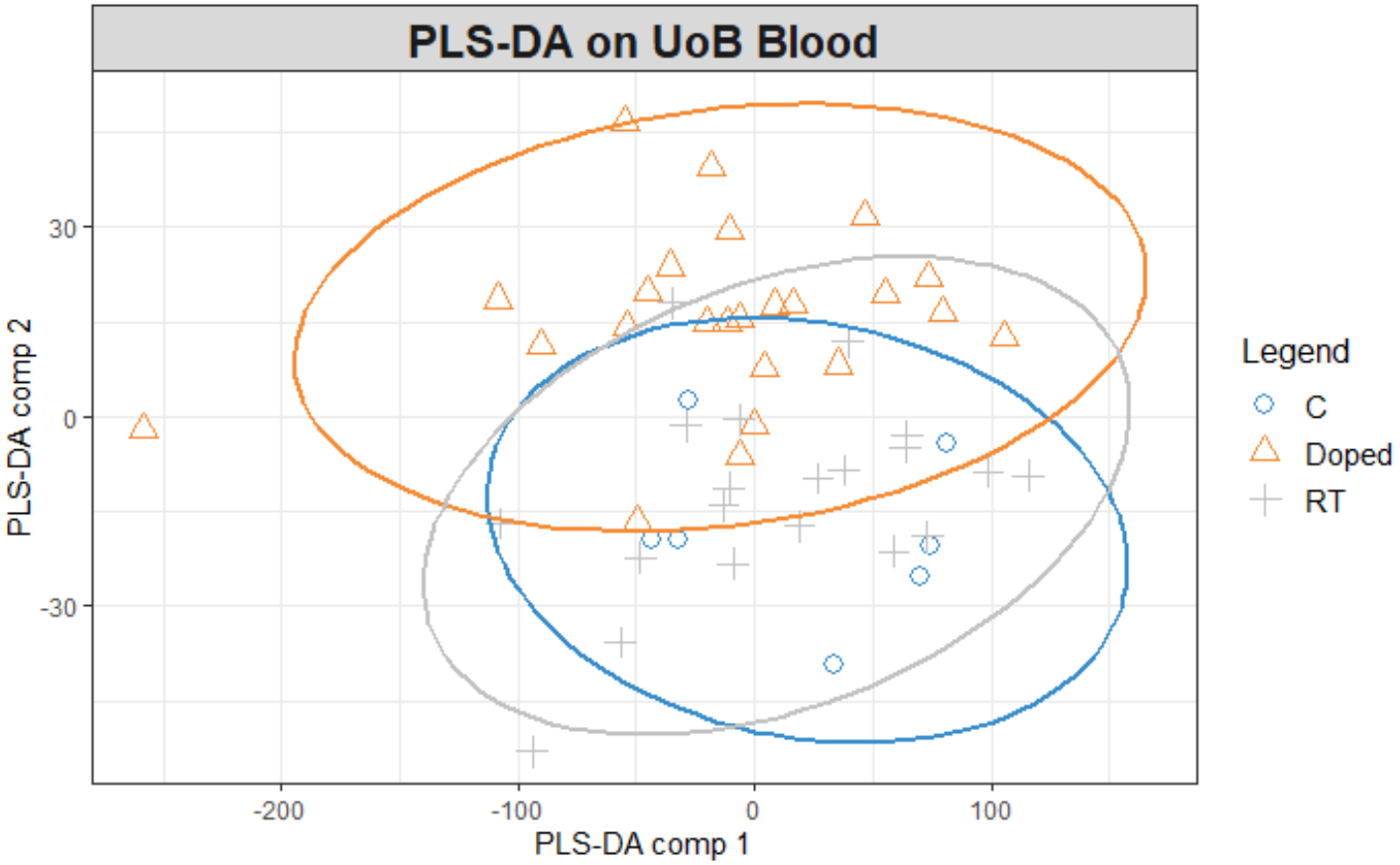
Appendix Figure 11b. PCA Plot of blood samples (*n* = 51) sequenced at the University of Brighton with standard chemistry reagents that underwent Differential Gene Expression analysis. Group C (*n* = 7), Group RT (*n* = 20), Group RT-AS≤2 (*n* = 14) and Group RT-AS≥10 (*n* = 10).



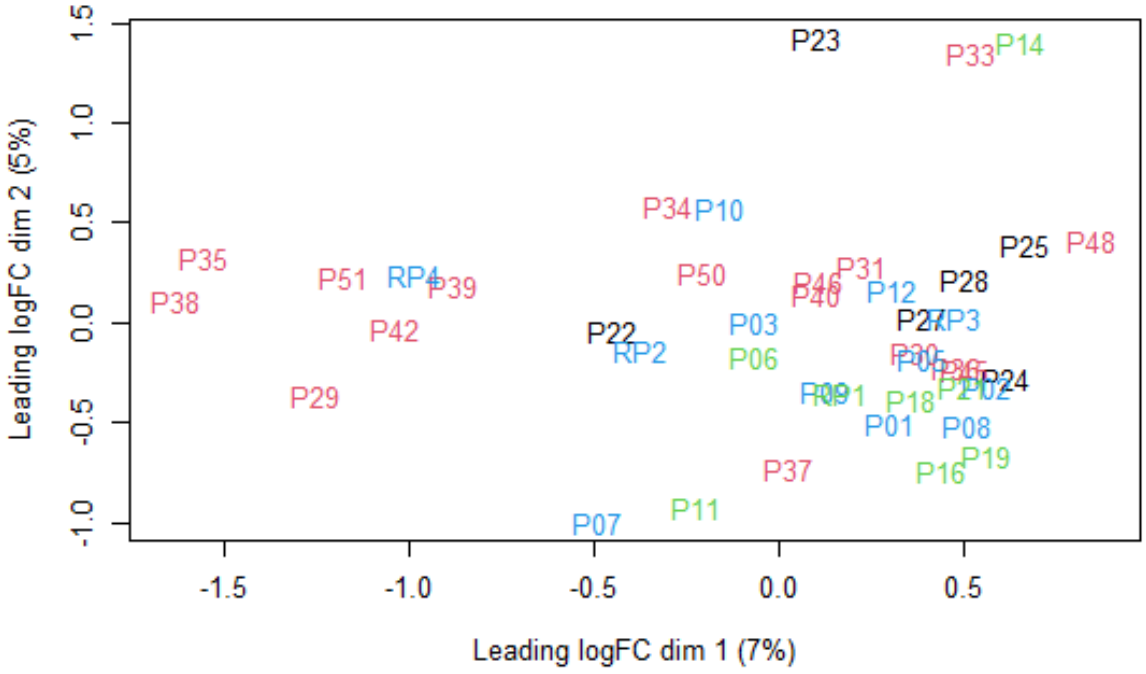
Appendix Figure 11c. PLS-DA Plot of blood samples (*n* = 51) sequenced at the University of Brighton with standard chemistry reagents that underwent Differential Gene Expression analysis. Group C (*n* = 7), Group RT (*n* = 20), Group RT-AS ≤ 2 (*n* = 14) and Group RT-AS ≥ 10 (*n* = 10).



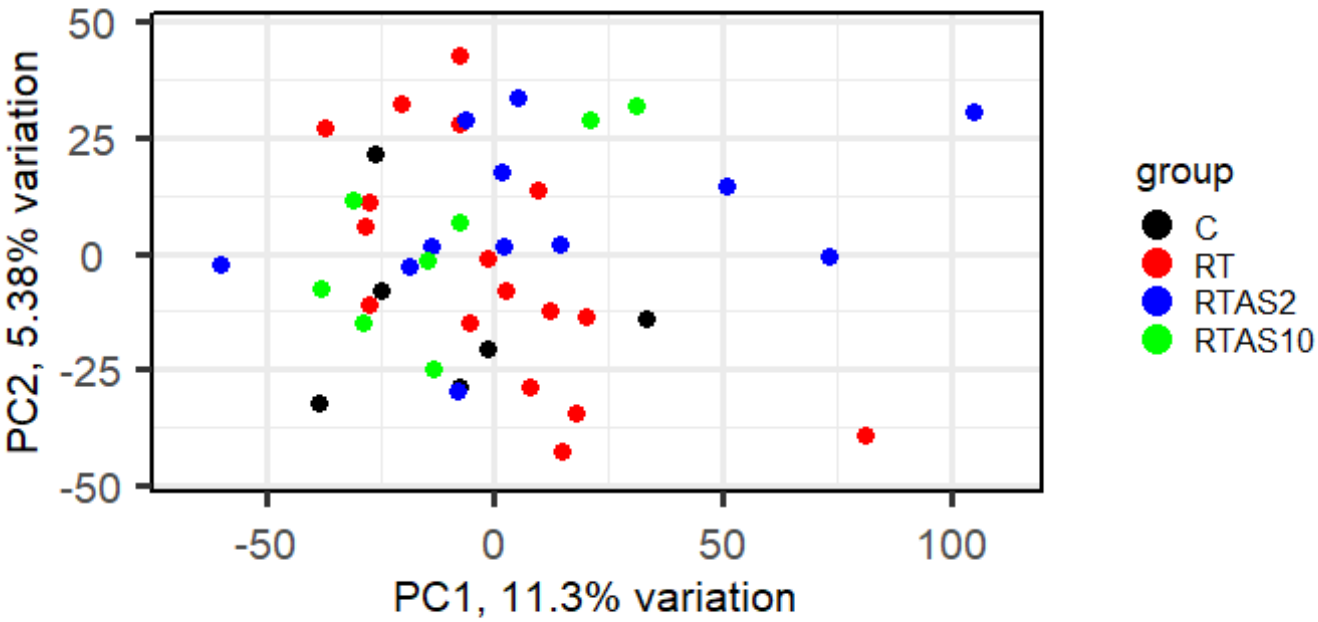
Appendix Figure 11d. PCA Plot of blood samples (*n* = 51) sequenced at the University of Brighton with standard chemistry reagents that underwent Differential Gene Expression analysis, with participants classified as: Group C (*n* = 7), Group RT (*n* = 20) and Group Doped (*n* = 24).



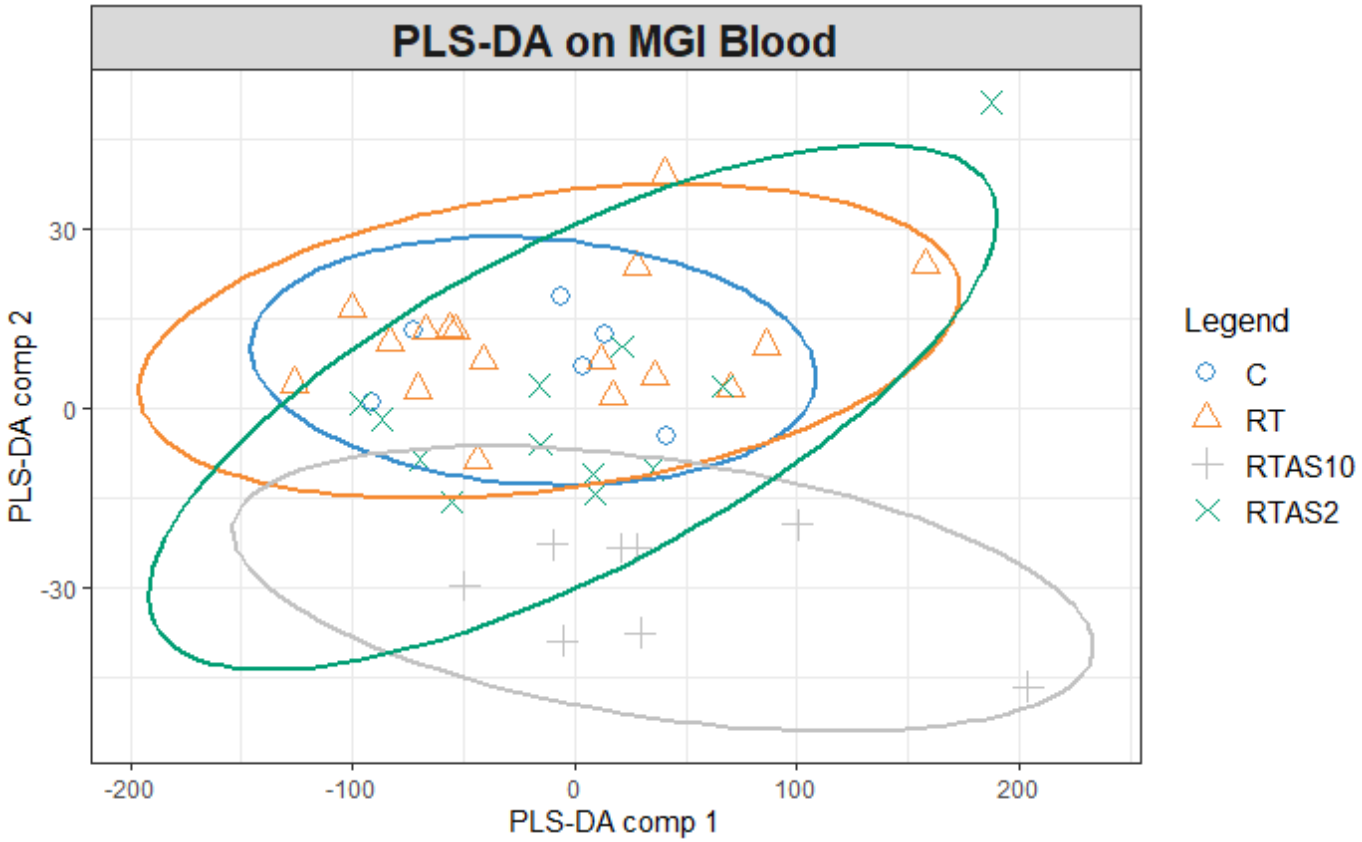
Appendix Figure 11e. PLS-DA Plot of blood samples (*n* = 51) sequenced at the University of Brighton with standard chemistry reagents that underwent Differential Gene Expression analysis, with participants classified as: Group C (*n* = 7), Group RT (*n* = 20) and Group Doped (*n* = 24).



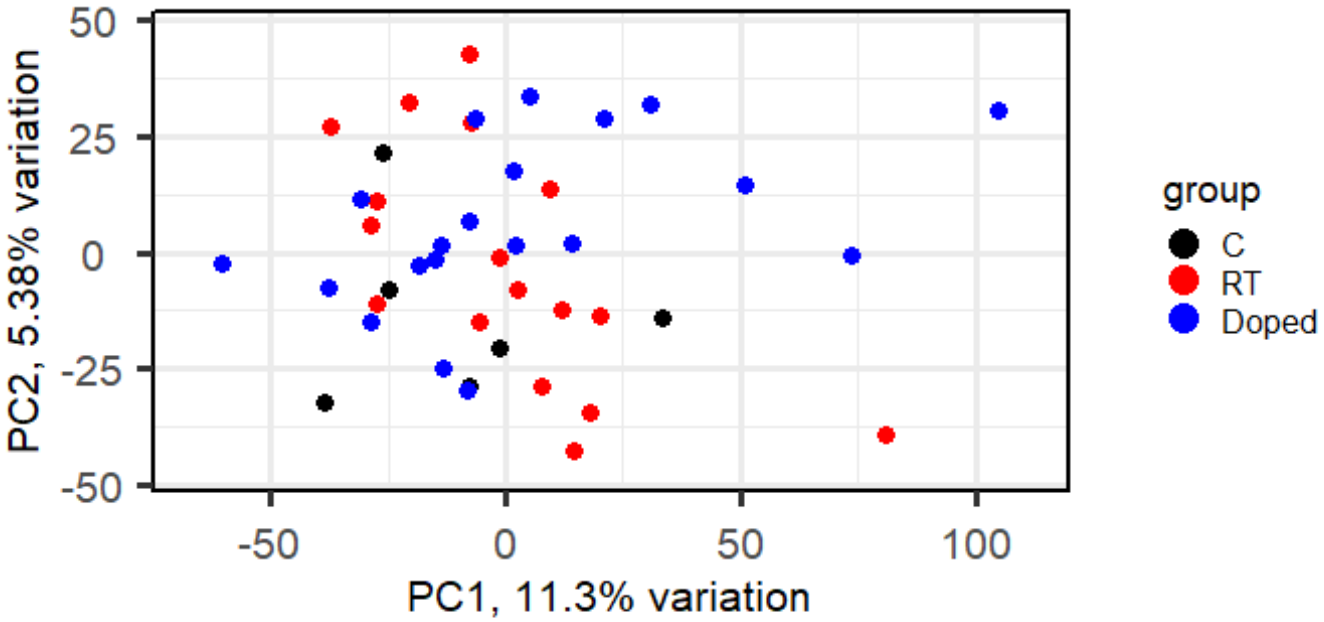
Appendix Figure 12a. MDS Plot of blood samples (*n* = 43) sequenced at MGI with standard chemistry reagents that underwent Differential Gene Expression analysis: Group C (black, *n* = 6), Group RT (red, *n* = 17), Group RT-AS≤2 (blue, *n* = 12) and Group RT-AS≥10 (green, *n* = 8).



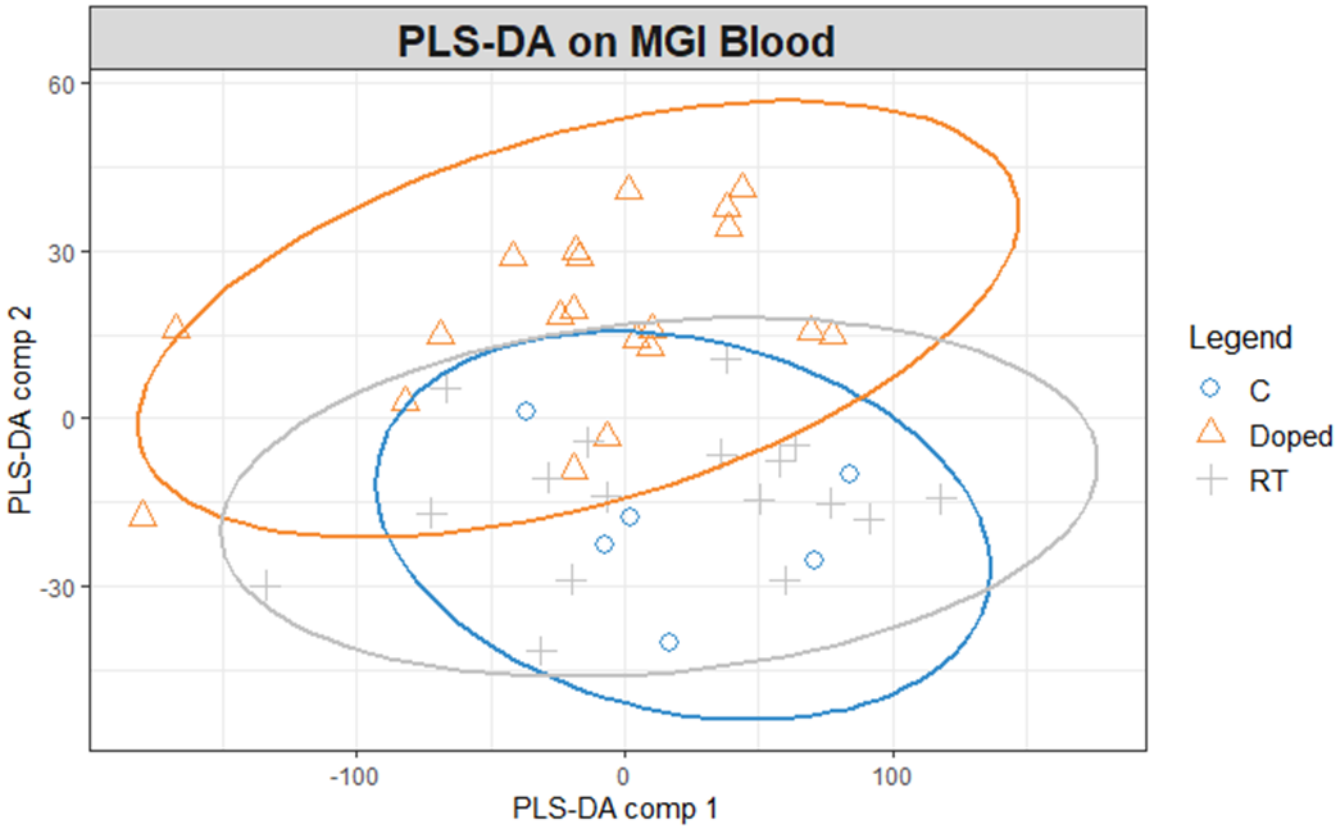
Appendix Figure 12b. PCA Plot of blood samples (*n* = 43) sequenced at MGI with standard chemistry reagents that underwent Differential Gene Expression analysis: Group C (*n* = 6), Group RT (*n* = 17), Group RT-AS≤2 (*n* = 12) and Group RT-AS≥10 (*n* = 8).



Appendix Figure 12c. PLS-DA Plot of blood samples (*n* = 43) sequenced at MGI with standard chemistry reagents that underwent Differential Gene Expression analysis: Group C (*n* = 6), Group RT (*n* = 17), Group RT-AS≤2 (*n* = 12) and Group RT-AS≥10 (*n* = 8).



Appendix Figure 12d. PCA Plot of blood samples (*n* = 43) sequenced at MGI with standard chemistry reagents that underwent Differential Gene Expression analysis: Group C (*n* = 6), Group RT (*n* = 17) and Group Doped (*n* = 20).



Appendix Figure 12e. PLS-DA Plot of blood samples (*n* = 43) sequenced at MGI with standard chemistry reagents that underwent Differential Gene Expression analysis: Group C (*n* = 6), Group RT (*n* = 17) and Group Doped (*n* = 20).

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