Relationship of anabolic hormones with motor unit characteristics in quadriceps muscle in healthy and frail ageing men.

Agnieszka Swiecicka[1,a], Mathew Piasecki[2,a], Daniel Stashuk[3], David Jones[4],Frederick Wu[1], Jamie McPhee[4,b], Martin K Rutter[1,5,b]

[1] Division of Endocrinology, Diabetes and Gastroenterology, School of Medical Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, U.K.

[2] Clinical, Metabolic and Molecular Physiology, MRC-Versus Arthritis Centre for Musculoskeletal Ageing Research, and National Institute of Health Research (NIHR), Nottingham Biomedical Research Centre, University of Nottingham, UK.

[3] Department of Systems Design Engineering, University of Waterloo, Ontario, Canada.

[4] Department of Sport and Exercise Sciences, Faculty of Science and Engineering. Manchester Metropolitan University

[5] Manchester Diabetes Centre, Manchester University NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, U.K.

[a,b] Authors contributed equally to this work

Address correspondence to: Professor Jamie McPhee, Department of Sport and Exercise Sciences, Faculty of Science and Engineering. Manchester Metropolitan University, Manchester, UK, +44 (161) 2475675, j.s.mcphee@mmu.ac.uk

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Short title

Anabolic hormones and motor unit characteristics in men

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Abstract

Context: Anabolic hormones are important factors in maintaining muscle mass for ageing men, but their role in overall motor unit structure and function is unclear.

Objective: To determine associations of anabolic and reproductive hormone levels with motor unit characteristics in quadriceps muscle in older healthy and frail men.

Design: Observational cohort study of community dwelling men.

Participants: Healthy and frail men > 65 years old.

Intervention: None.

Outcome measure: Quantitative assessments of electromyography-derived motor unit potential size (MUP) and compound muscle action potential size (CMAP) of *vastus lateralis* muscle.

Results: We studied 98 men (mean±SD: age 73±6 years; BMI 25.7±4.0 kg/m^2; diabetes 11%) of whom 45% were prefrail and 18% frail. After adjusting for age, BMI and prevalent diabetes, higher total and free testosterone levels were significantly related to larger CMAP (total testosterone: β (95% CI): 0.3 (0.08, 0.53); free testosterone: 0.34 (0.13, 0.56)). Exploratory analysis showed the relationship between free testosterone and CMAP was stronger in frail rather than robust men. In univariate analyses, estradiol was associated with CMAP size (0.37 (0.16, 0.57)); and vitamin D was associated with MUP size (0.22 (0.01, 0.43)) but these relationships were no longer significant after adjusting for potential confounders.

Conclusion: Our data highlight the associations between androgen levels and the electrophysiological characteristics of older men, particularly in the frail. Clinical trials involving administration of androgens will help to elucidate the potential benefits of intervention on neuromuscular function and/or frailty status.

Introduction

Adverse outcomes associated with frailty, including reduced mobility and falls, might be linked to underlying sarcopenia, characterized by low muscle mass and related physical dysfunction. Lower cross sectional area and total number of muscle fibers, both features of sarcopenia, have been linked with impaired anabolic signalling, increased levels of pro-inflammatory cytokines and declining numbers of motor units(1–4). Whilst anabolic hormones are considered key factors in maintaining muscle fiber cross sectional area through the effects on muscle protein turnover, their role in the broader neuromuscular system, including motor unit structure and function, during healthy ageing and frailty is less clear.

A motor unit includes a single alpha motor neuron and all the skeletal muscle fibers it innervates. Activation of individual motor units during movements ensures the precise matching of muscle forces to meet the task requirements. Various methods of electromyography (EMG) have been utilised to study human motor unit (MU) characteristics. Intramuscular EMG (iEMG) employing needle electrodes is able to provide detailed information on the structure and function of MUs via the measurement of consecutive action potentials during voluntary contractions in a 'localized' fashion (5–7). Similarly, involuntary electrically stimulated contractions provide a more 'global' view of the electrophysiological characteristics of muscle via skin surface measures(8).

The declining numbers of motor units with advancing age(9,10) may cause denervation of muscle fibers and constrain the ability of the central nervous system to control voluntary movements. By way of compensation to preserve muscle function, some denervated fibers can be reinnervated by axonal branching from neighbouring motor neurons(11). This remodelling process leads to an increase in the size of surviving motor units in older adults compared with young, but contributes to fiber atrophy and fiber losses when reinnervation fails(12)(13). The underlying regulation of motor unit remodelling in sarcopenia and frailty remains poorly understood, but may be associated with hormonal changes during ageing, particularly declines in anabolic hormone levels.

The neuromuscular protective effects of androgens have been studied in animal models in which male castration led to motor unit dendrites' atrophy, which was reversed by testosterone administration(14,15). Similarly, when compared with controls, testosterone therapy attenuated atrophy of motor neuron dendrites and muscle fibers in female rats with spinal cord injury(16). Exogenous testosterone accelerated regeneration of facial(17) and sciatic nerves(18) post injury and in humans, testosterone treatment protected neuron cultures from cell death caused by testosterone deprivation(19). More

recent studies, however, suggest that dihydrotestosterone might be a more potent anabolic hormone in mammalian skeletal muscle, exerting effects on force in slow and fast twitch fibres alike(20). Dehydroepiandrosterone sulphate (DHEA-S) is a weak androgen with neuroprotective and anti-apoptotic properties, which are independent of any anabolic effects exerted after conversion to testosterone. Both *in vivo* and *in vitro* models suggest that DHEA-S promotes neurogenesis, neuronal survival, and prevents neurotoxicity due to its anti-glucocorticoid effects(21). None of these properties, however, have been studied in the context of the peripheral nervous system and motor neuron preservation in humans.

Similarly, cumulative evidence indicates that estradiol has a neuroprotective role in the central nervous system(22). However, animal and human research also suggests neuroprotective effects of estradiol on spinal motoneurons, where the Akt anti-apoptotic signalling pathway is regulated by estradiol(23,24).

The anabolic role of vitamin D in the muscle has previously been studied in health, sarcopenia and frailty predominantly in the context of muscle protein turnover(25,26). Despite the fact that low levels of vitamin D have been linked to impaired balance and frequent falls, no studies to date have investigated whether vitamin D levels are related to neuronal control of muscle function and motor unit health.

Given the possible roles of these anabolic factors for human neuromuscular function and the lack of data at the whole motor unit level in healthy ageing and frailty, we aimed to determine the association between anabolic hormone levels and motor unit characteristics in quadriceps muscles in older men from the general population.

Subjects and methods

Participants

A total of 114 men aged 65-90 years were recruited from the Greater Manchester area between 2014-2017. Participants were recruited from local universities' databases, National Health Service general practices and secondary care, including outpatient departments, day hospitals and community physiotherapy centres. All participants provided written informed consent. The study was also open to the general public through poster and newspaper advertisements. A full list of the selection criteria is included in the Supplemental Material(27).

Ethical approval for the study was obtained from the National Research Ethics Service Committee Northwest (15/NW/0426).

Assessments

Questionnaires

Each participant provided details of lifestyle, medical history and medications taken. The men also completed the Geriatric Depression Scale questionnaire (GDS)(28) and the Physical Activity Scale for the Elderly (PASE) questionnaire(29).

Anthropometry measures

Body mass (kg) and height (m) were measured and total body composition assessed by dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy Advance, version EnCore 10.50.086). Appendicular lean mass with appendicular bone mineral content (BMC) removed was normalized to height to establish sarcopenia status(12). Appendicular lean mass strongly correlates with total body lean mass in our unpublished data of 168 older males ($r = 0.88$, $p<0.001$).

Assessment of physical function, activity and frailty

Physical function was assessed objectively by Short Physical Performance Battery testing which included assessment of four-meter walking speed, standing balance and 5 chair stands. The "Timed up and go" (TUG) was also performed, where participants were invited to stand from a seated position and walk 3 meters forward around a cone as quickly as possible, returning to their original seating position. Time from the command "Go" until the participant returned to their original seated position was recorded.

Grip strength was measured using a handgrip dynamometer (JAMAR). Participants were invited to squeeze the handle as hard as possible for around 3 seconds and the maximum contraction force (in kg) was recorded. This was repeated 2 times for each hand, alternating between the right and left with 30 seconds rest between trials. Maximal voluntary isometric contraction of the knee extensors was assessed with the participants leg fastened to a force transducer 30cm below the centre of the knee joint, with hips and knees flexed at 90^0. Participants performed a standardised warm-up of several contractions, after which they were asked to perform a maximal effort which lasted approximately 2-3 seconds. This was repeated a further two times separated by short rest intervals, and the highest of the values was accepted as the MVC.

Frailty was characterized by the two commonly used approaches: the frailty phenotype (FP) and frailty index (FI). Frailty phenotype was adapted from the Cardiovascular Health Study (CHS) based on five criteria: sarcopenia, exhaustion, slowness, weakness and low activity(30). The variables used to construct FP and the

population-specific cut-off points are presented in the Supplemental Table 1, alongside the original CHS criteria(27). Individuals with one or more of these criteria were classed as frail and those with none were classed as robust.

The FI is comprised of 37 health deficits (symptoms and signs, functional impairments), which are known to accumulate with age and are associated with adverse health outcomes. The FI was created using a standardized procedure(31). Continuous variables were dichotomized based on the distribution of participants' scores; cut-off points were set at the worst performing 10^{th} centile. Individuals with over 20% of missing data on relevant deficits were excluded from the analysis. The details of the variables used to create an FI and specific cut-off points are described in Supplemental Table 2(27).

Hormone measurements

A fasting venous blood sample was used for all hormone measurements. All samples were collected between 08:30 – 09:30 AM. A validated liquid chromatography-mass spectrometry system was used to analyze total testosterone (T, intra- and interassay coefficients of variation (CVs): 1.4% and 8.3%), estradiol (E2; CVs: 5.4% and 3.1%), dihydrotestosterone (DHT; CVs: 8.3%) vitamin D (CVs: 6.2% and 5.1%) and DHEA-S (CVs: 1.9% and 3.1%). Free T (fT) levels were derived from total T, SHBG (analyzed using chemiluminescence), and albumin (measured by bromcresol purple) concentrations using Vermeulen's formula(32).

Electromyography

The EMG data were collected from around the motor point of the vastus lateralis (VL). The parameters of interest were the supramaximal compound muscle action potential (CMAP) and motor unit potential (MUP).

The CMAP represents the sum of the electrophysiological signal from all motor units detectable by the recording electrode when simultaneously activated at the same time using supramaximal stimulation of the peripheral nerve. It has been used clinically to track disease progression in spinal muscular atrophy and amyotrophic lateral sclerosis(33,34). The CMAP was recorded at the VL motor point by surface EMG after percutaneous femoral nerve stimulation.

Motor unit potential (MUP) represent the sum of electrophysiological signals as action potentials propagate along the sarcolemma of individual muscle fibers of a single motor unit. MUPs were recorded using a 25 mm intramuscular needle electrode inserted

into the VL muscle at the motor point to a depth of ~1–2 cm. The participant then performed a sustained voluntary isometric contraction at 25% of their maximal effort and held it for 12–15 s. In between contractions, the needle was repositioned using combinations of 180 degrees needle rotations and needle withdrawals of ~5 mm to obtain a minimum of six recordings from spatially distinct areas. The details of the EMG technique used, data recording and analysis are described in the Supplemental Material(27).

Statistical analysis

Descriptive statistics are presented as the mean ± standard deviation (SD) or n (%), and statistical significance of between-group differences was assessed using analysis of variance.

Linear regression models determined relationships between predictors (hormone level) and outcome (MUP or CMAP). Each predictor as well as CMAP was considered as an untransformed value standardized as a Z score [(raw score – mean)/standard deviation] to allow comparison of results between predictors. Motor unit potential area, in view of significant skewing, was log-transformed before being standardized as Z score to meet the linear regression assumptions.

Models were adjusted for age, body mass index (BMI), diabetes and alcohol excess as these correlated with the predictors and therefore were potential confounders. The analyses where estradiol was a predictor were further adjusted for total testosterone - the main precursor of E2 production in men. The results of these analyses were displayed as standardized coefficients (beta) with 95% confidence intervals.

In an exploratory analysis, we introduced an interaction term (hormone x frailty phenotype or hormone x frailty index) as well as a FP or FI variable, as appropriate, to the fully adjusted models to assess whether the relationships between hormone levels and EMG parameter values varies in health in relation to the level of frailty.

All analyses were performed using STATA 13 SE software (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP).

Results

Out of 114 men who participated in the study, 98 men had complete data on MUP and CMAP and were included in the analysis. The mean age of men was 73 years and mean BMI 25.7 kg/m^2 (Table 1). Sixteen percent were current smokers and 39% consumed

more than 14 units of alcohol per week. Cardiovascular disease was present in 19% of participants and diabetes in 11%.

We assessed relationships between hormone predictors and clinical variables (age, BMI, diabetes, smoking and alcohol excess) that could potentially confound relationships between hormone levels and EMG parameters (Table 2).

These data indicated that age, BMI, diabetes and alcohol excess might be potential confounders and therefore we included these as covariates in subsequent models.

In unadjusted analysis T, free T, DHT and E2 were positively related to CMAP size: one standard deviation (SD) higher level of T, free T, DHT and E2 were associated with larger CMAPs normalized as standard deviation units [total T: β (95% CI): 0.48 (0.29, 0.68); free T: 0.48 (0.29, 0.67); DHT: 0.37 (0.16, 0.57); E2: 0.37 (0.16, 0.57)]. After adjusting for age, BMI, alcohol excess and prevalent diabetes, only total and free T remained significantly related to CMAP (Table 3).

In unadjusted analysis one SD higher level of free T and vitamin D was associated with larger mean MUP. However, these associations were no longer statistically significant after BMI adjustment (Table 3).

Exploratory analysis suggested the relationship between free T and CMAP was much stronger in frail men compared to the robust ((β (95%CI): 0.82 (0.05, 1.60), p for interaction of 0.038)), as assessed by frailty phenotype (Figure 1).

The relationship between free T and CMAP was greater with increasing frailty levels as assessed by the frailty index ((β (95%CI): 1.54 (0.02, 3.06), p for interaction of 0.047; Figure 2)).

This secondary analysis also suggested a positive relationship between DHEA-S and CMAP in prefrail ((β (95%CI): 0.58 (0.15, 1.00), p=0.009 for interaction) and frail men (β (95%CI): 0.54 (0.03, 1.05), p=0.039 for interaction); Figure 3)).

When we explored associations between physical function and EMG parameters, Timed up and go (TUG) was negatively related to MUP size, and the association was partially attenuated after adjusting for lean muscle mass in keeping with a partial mediation model (Supplemental Table 3)(27). TUG was negatively linked with CMAP and the associations appeared largely independent of lean muscle mass (Supplemental Table 3)(27). Knee extensor maximum voluntary contraction (KEMVC) was associated with MUP size and this association appeared to be explained by lean muscle mass (Supplemental Table 3)(27). We did not observe a significant relationship between knee extensor MVC and CMAP.

Finally, we performed an analysis assessing the potential influence of selection bias. Compared to the 16 men (14%) who were excluded because of incomplete data, the

98 men in the study cohort were less likely to be frail and were less likely to have sarcopenia, weakness, respiratory disease, diabetes and arthritis (Supplemental Table 4)(27). Therefore, the strength of relationships described above may be conservative estimates of what would have been obtained in the original cohort.

Discussion

Main findings

Our study presents several novel findings: Firstly, testosterone levels were positively associated with skeletal muscle electrophysiological characteristics as assessed by CMAP in unadjusted models and also models adjusted for BMI, age and prevalent diabetes. We have also observed a similar trend for DHT, however adjustment for diabetes attenuated the DHT-CMAP relationship. Secondly, we showed that estradiol was related to muscle CMAP, but this relationship was rendered non-significant after adjusting for total testosterone levels indicating that the effect is likely to be testosterone-related. Thirdly, we showed that vitamin D was positively related to motor unit potential size in unadjusted models, but this effect was no longer significant after adjusting for BMI. Finally, the significance of free T and DHEA-S relationships with muscle contractility (assessed by CMAP) appeared to be greater in frail men. These novel human observations may have important implications for future clinical research and clinical care.

Prior studies and mechanistic insights

Data from our previous work, and that from other groups, indicate that muscle strength and the number of motor units declines progressively from old (˜66yrs) to very old (˜82yrs)(35) age and that CMAP and MUP size differ according to sarcopenic and frailty status(12,36).

Whilst these past studies demonstrate important links between motor units, muscle function and health status in older age, they do not provide more detailed underpinning mechanisms that may identify causes of motor unit changes with advancing age. The pathophysiological mechanisms linking neuromuscular health with frailty in humans remain largely unknown. Although our study is the first to investigate associations between hormone levels and motor unit function of older adults, others have previously suggested such a link may exist based on the observed age-related decline in

anabolic hormones occurring in parallel with development of physical impairments, sarcopenia and frailty(37).

The role of testosterone in neuromuscular function has largely been investigated in the context of its effects on muscle mass. In both animal and human studies, testosterone has been shown to increase skeletal muscle size by, in part, increasing the number of muscle satellite cells to support muscle fiber hypertrophy(38).

There is a paucity of research into the role of testosterone in motor unit health and remodelling processes. Available evidence comes from animal models of motor neuron injury. Byers *et al*, in an experimental model of spinal cord injury, found that testosterone treatment of female rats prevented atrophy of motor neuron dendrites and muscle fibers(16). Similarly, castration of adult male rodents led to motor neuron atrophy which was, almost completely, reverted by testosterone administration(14,15). In other rodent studies, exogenous testosterone accelerated regeneration of injured facial and sciatic nerves(17,18,39).

The effects of testosterone are not uniform across the nervous system owing to reduced expression of androgen receptors in typical somatic motor neurons, such as those of the quadriceps, compared to the cranial motor neurons. Nonetheless, rodent research data suggest that testosterone has a neuroprotective role in the L2 spinal segment(40–42).

Gonadal hormones regulate the brain-derived neurotrophic factor (BDNF) receptor, trkB(43). Work by Osborne suggests androgen-mediated expression of the BDNF receptor could help maintain motor neurons(41).

In humans, an age-related decline in testosterone levels has been linked to the loss of muscle mass and sarcopenia; interestingly, the magnitude of a concomitant decline in muscle strength and neuromuscular coordination appears to be far greater than expected from the degree of the muscle mass loss only(44).

Similarly, testosterone-induced muscle hypertrophy in healthy older men does not necessarily translate into significant gains in muscle function and improved physical performance, raising questions about the previously unexplored role of testosterone in neuronal control of muscle function.

We showed that low free testosterone, which is a biologically active fraction of circulating testosterone, is associated with impaired muscle electrophysiology assessed by maximal CMAP. The maximal CMAP size, whilst not dependant on total size of larger muscle groups(7) depends on the volume of contractile material within the recording range of the electrodes, and is proportional to the total size of the motor units activated minus any attenuation of the signal as it reaches the recording electrode(8), such as

subcutaneous fat thickness which did not differ here, as we and others have previously reported (7,45). Smaller CMAPs in older age have been reported for a number of muscles(46). In clinical practice, the CMAP remains a useful parameter to monitor progression of neuromuscular disorders such as motor neuron disease(34). Interestingly, the relationship of free T with CMAP size was greater with increasing levels of frailty. Although this is an observational study that cannot determine causality, the differing relationships by frailty status, could lead us to speculate that testosterone supplementation might improve neuromuscular function to a greater extent in frail rather than non-frail elderly populations. This idea is largely in keeping with the results of Testosterone Trials in which testosterone replacement in relatively healthy men resulted in very small gains in objectively-assessed and self-perceived physical function(47–50).

Contrary to the relationship with CMAP, there was no observed relationship between T and MUP size, which may be explained by the non-linear trajectory of MUP size with increasing age. Expansion of the MU occurs as a compensatory process to minimise fibre loss, via reinnervation of denervated fibres, and a failure of this process contributes to sarcopenia, evidenced by larger MUPs in healthy old when compared to young, which are smaller again in older people with sarcopenia(12). It is therefore apparent that MUP size increases up to a certain ill-defined point when reinnervation is out-paced by denervation, fails to expand and proceeds to become smaller.

Our findings suggest that vitamin D may play a role in successful reinnervation occurring with ageing as evidenced by association of low vitamin D with smaller motor unit potentials. Adjusting for BMI attenuated the relationship between vitamin D and MUP size, and whilst we might have been underpowered to detect significant associations on multiple adjustments, the unadjusted model might still provide valuable insights into mechanisms linking vitamin D and sarcopenia. For example, it is possible that BMI is on the causal pathway linking vitamin D with MUP size, which could explain the effect of statistical adjustment.

In experimental models, treatment with vitamin D has been shown to induce nerve growth factor synthesis (involved in peripheral nerve recovery post injury), reduce demyelination and induce axonal regeneration in a spinal cord compression and peroneal nerve injury model(51–53). Certainly the evidence from randomized placebo-controlled trials suggests that vitamin D replacement not only results in improved lower limb muscle mass and strength but also neuromuscular control and balance(54),(55).

Whereas some of the effects of vitamin D deficiency on muscle are thought to be, in part, mediated by raised pro-inflammatory cytokines levels(26,56) and direct activation of the IGF-1 receptor(57,58), vitamin D deficiency has previously been linked

to altered muscle innervation(59) which is further supported by our findings .

Strengths and limitations

Our study has a number of strengths. Our cohort is representative of older community-dwelling men and although the sample size may appear small, it is relatively large for an invasive study in elderly and frail participants. To our knowledge, it is the first study in humans to relate hormone levels to motor unit size and muscle electrophysiological characteristics assessed by intramuscular and surface electromyography. However, we did not measure calcium or PTH levels which may have helped in the interpretation of the vitamin D data. We have also not performed nerve conduction studies, which could have helped in the interpretation of study findings to identify whether deficits exist in motor neuron axons. Our work was limited to men, so the generalizability to women is unknown.

Clinical and research implications

Interventions preventing age-related motor unit loss are largely unknown. We have previously shown that even lifelong exercise does not attenuate this process and the muscles of master athletes show a similar loss to those of normally-active older men, however the older athletes appear to be more successful at reinnervation(13,60). The associations of free testosterone and vitamin D with neuromuscular parameters suggest that both hormones might contribute to preservation of muscle fibers and successful reinnervation.

Whether intervention with these anabolic hormones could prevent motor unit loss or improves reinnervation remains unclear. We recommend additional *in vivo* studies and clinical trials before there is any change in clinical practice. Testosterone replacement in frail hypogonadal men resulted in improvements in physical function but larger trials in this group of people are lacking(61). Moreover, the greater significance of relationships in frailer men suggests that hormonal manipulation aimed at improving muscle function might be of particular benefit in the frail.

In conclusion, we have shown that testosterone was positively associated with the volume of excitable muscle tissue as assessed by CMAP. We have also shown, in univariate analysis, that vitamin D was related to motor unit size. These cross-sectional hypothesis generating data suggest that it may be appropriate to design clinical trials to assess the impact of androgen therapy on neuromuscular decline in frail older men.

Figure Legends:

Figure 1. Adjusted prediction of CMAP by free testosterone and frailty phenotype.

Figure 2. Adjusted probability of CMAP by free testosterone and frailty index.

Figure 3. Adjusted probability of CMAP by DHEA-S and frailty phenotype.

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