UNIVERSITY OF STIRLING

Association of Variation in ACTN3, MYOZ2 and MYOZ3 with Complex Quantitative Performance Phenotypes in Lithuanian Athletes and Controls

By

Aidan Quinn Innes, BSc. (Hons)

Supervisors:

Dr Colin Moran

Dr David Lee Hamilton

Research Thesis for the award of M.Phil

School of Sport

University of Stirling

April 8th, 2016

Declaration

I declare that this thesis and the work presented in it are my own and have been generated by myself as the result of my research. It has not been submitted anywhere else for any other award. Where other sources of information have been used, they have been clearly acknowledged and referenced.

Acknowledgements

I would like to thank all of the academic staff from the University of Stirling Health and Exercise Science Research Group who have helped me throughout my studies. Similarly, I would like to thank all of the staff within the School of Sport who awarded me my studentship which has led to the completion of this work. Without their help, this would not have been possible.

Special thanks go to Dr Colin Moran for his supervision during my studies. His support, guidance and advice throughout my studies have not only allowed me to complete this research but have also inspired me to want to continue researching throughout my career.

Further thanks go to all of my family, friends, fellow postgrads and colleagues that have supported me both academically and socially throughout my M.Phil. Without this support, my two years at the University of Stirling would not have been as enjoyable as it has proven to be.

I would like to dedicate this thesis to my father, David Innes, who was an inspirational man in my life and who I miss every day. He has made me the man I am today.

Contents

List of Tables

List of Figures

Abstract

Recent findings have shown that ACTN3 genotype regulates calcineurin signalling and thus muscle performance in mice. The aim of the present study was first to investigate associations of ACTN3 genotype with quantitative performance-related phenotypes in a cohort of native Lithuanian athletes and controls. The second aim was to identify variants within the Calsarcin family that were of a high enough frequency within the European population and investigate variation within these genotypes on performance-related associations. 407 participants (210 athletes, 197 controls: age 22 ± 4 years, BMI 23 ± 2 kg/m^2) performed a range of tests including: anthropometric tests; isokinetic dynamometry; 30 m sprint, counter-movement jump; standing jump; 30 second Wingate test; and a $\dot{V}o_{2MAX}$ test across three separate testing sessions. DNA from venous blood samples was genotyped through standard PCR and RFLP processes. ACTN3 R577X SNP R-allele carriers were faster than XX-homozygotes in a 0-10m stage of a 30m sprint ($p < 0.01$). Variation was identified at a suitable level for Calsarcin-1 (MYOZ2) and Calsarcin-3 (MYOZ3). As Calsarcins tether calcineurin at the sarcomeric z-line, it was hypothesised that any variation within the Calsarcin genes may alter calcineurin signalling and thus athletic performance. MYOZ2 SNP rs9995277 (Calsarcin-1) G-allele was not associated with any performance phenotype. MYOZ3 SNP rs116090320 (Calsarcin-3) G-allele carriers showed significantly increased relative $\dot{V}O_{2MAX}$ ($p = 0.01$) and significantly lower isokinetic upper arm flexion strength at 90 d/sec (N·m, $p < 0.01$) compared to AA-homozygotes. In conclusion, we are the first group to identify functional variation within genes encoding members of the Calsarcin family and have demonstrated for the first time that variation within MYOZ3 affects performance-related phenotypes in humans.

Abbreviations

Endurance sub-group	END
Strength, sprint and power sub-group	SSP
Control sub-group	CON
Knock-out mouse model	KO
Wild-type mouse	WT
Quadriceps	QUAD
Spinalis thoracis	SPN
Extensus digitorum longus	EDL
Soleus	SOL
Myosin heavy chain	MyHC
Lactate dehydrogenase	LDH
Micro-Ribonucleic Acid	miRNA
Polymerase chain reaction	PCR
Restriction fragment-length polymorphism	RFLP
Single Nucleotide Polymorphism	SNP
Deoxyribose nucleic acid	DNA
A-Actinin-3	ACTN3
Myozenin-2	MYOZ2
Myozenin-3	MYOZ3
IKLL	Isokinetic Strength Lower Limb
IKUL	Isokinetic Strength Upper Limb

Introduction

World-class elite athletes exemplify the successful interaction of nature (genetics) versus nurture (environment). The careful orchestration of lifestyle and environmental factors such as training and nutrition are as critical to success as the palette of genetic variation imparted on to the athlete: however, the genetic component of athletic performance remains one of the least understood yet one of the most critical dimensions to consider [1].

Whether there is a genetic component associated with athletic performance and or trainability is no longer questionable. It is widely recognised that physical performance as a phenotype is determined, in part, by the genes an individual has inherited from their parents and is very much a polygenic trait [2,3]. The heritability of exercise-related phenotypic traits are typically ascertained by twin or family studies on a single period of assessment [4]. Heritability expresses the variance explained by genetic factors as a proportion of the total variance for a particular phenotype [5]. Heritability of physical performance-related phenotypes ranges from 31 to 85 % [2]. For aerobic performance alone, the heritability is between 47 % to 74 % [6]. Maximal oxygen uptake ($\dot{V}O_{2MAX}$) is one of the most important indicators that characterises aerobic performance [7]. Baseline $\dot{V}O_{2MAX}$ has been reported to have a maximum heritability of 50 % with changes in $\dot{V}O_{2MAX}$ as a result of training having a maximum heritability of ~47 % [6,7]. For muscular strength, accurate heritability estimates are less clear due to the number of ways in which this complex trait can be measured. Differences in study design for investigating muscular strength place the heritability between 14 % [8] and 96 % [9]. Although the range in heritability estimates may be large, it is

clear that there is a genetic component of exercise and more research aimed at identifying causal genes is required.

This heritability is comprised of the combined influence of variations or polymorphisms in DNA sequences; although heritability studies themselves do not require any DNA, only knowledge of the relationships between the study participants. Polymorphisms in human DNA account for less than 1 % of the entire genome with the remaining 99 % plus of the sequence identical between individuals. That being said, there are still over 12-million potential sites of variation [4]. Due to the nature of heritability studies, it is not possible to investigate the effect of variations in specific genes on given performance phenotypes. For this type of study, DNA must be collected, specific variations assessed and then associated with performance phenotypes. For association studies, athletes are often chosen as they are found at the extremes of human performance [10]. By definition, polymorphisms which effect athletic performance are more likely to be identified in athletes compared to the general population as they would stand to gain the greatest advantage from such polymorphisms making them an ideal cohort to study [4,10]; however, given the relatively small number of athletes, compared to the large number of possible SNP's, identifying suitable SNP's to investigate can be difficult compared to Genome Wide Association Studies which look at a large number of possible SNP's in one go [11]. One of the most common types of genetic variation are single nucleotide polymorphisms (SNPs). A SNP occurs when one nucleotide changes within the sequence of a gene. To date, over 200 SNPs have been identified as having an effect on athletic performance [12]. This type of variation can affect the expression or the function of a particular gene and thus lead to phenotypic variation [13]. The consequences on phenotypic variation will depend on the gene affected by

the SNP; for example, a SNP affecting a gene involved in muscle contraction may affect athletic performance-related phenotypes. One such SNP is one of the most widely researched SNPs and occurs in the muscle structural protein α-Actinin-3.

Skeletal α-Actinin Variants and Roles

The α-Actinin-3 protein (encoded by the *ACTN3* gene) is one of two highly similar (80 % identical) calcium-insensitive, actin binding proteins that are present in skeletal muscle: α-actinin-2 (encoded by *ACTN2*) and α-actinin-3. They diverged from each other in a single mutational event ~300 million years ago [10] but since then have remained highly conserved. This suggests that ACTN3 has a functional role out-with the role of ACTN2 and indeed they are differentially expressed spatially and temporally during embryonic development [14].

The two isoforms, α-Actinin-2 and α-Actinin-3, form a lattice structure anchoring actin containing thin filaments at the sarcomeric Z-line (see Figure 1 below, from [15]).

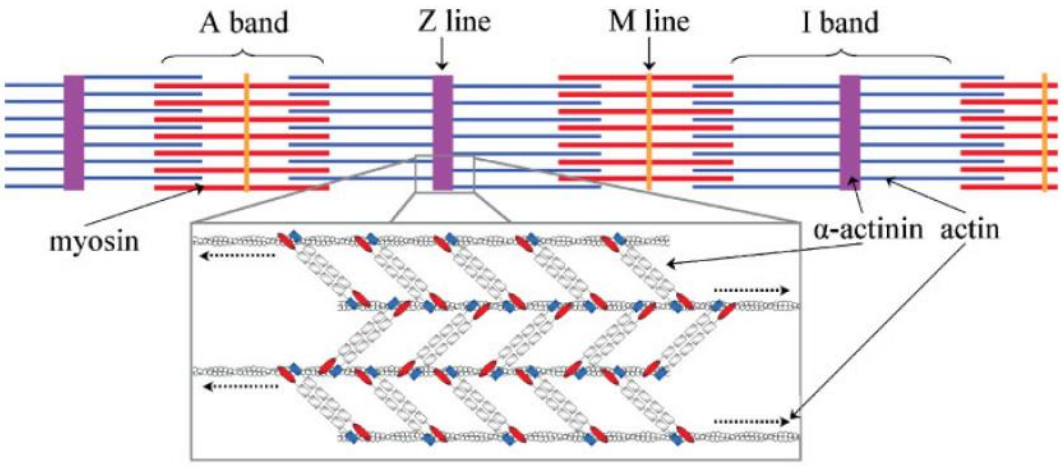


Figure 1, α-Actinin localisation within the sarcomere**.** The sarcomeric α-Actinins are found at the Z-line (purple), where they anchor actin-containing thin filaments (blue) from adjacent sarcomeres. Anti-parallel dimers of α-Actinin cross link actin filaments and stabilise

them against the force generated by the contractile apparatus (dashed arrows indicate direction of force). Figure adapted from [15].

They stabilise the muscle contractile apparatus and contribute to tensile strength during muscle contraction [10,16]. A-Actinin-2 is present in all muscle-types including cardiac and oxidative type-1 skeletal muscle fibres. A-Actinin-3 is found almost solely in glycolytic type-2 skeletal muscle. As well as their structural component, α-Actinins also interact with an array of signalling proteins including metabolic enzymes such as glycogen phosphorylase (GPh) [17] and members of the Calsarcin family (Calsarcin 1, 2 and 3) which bind the calcium-calmodulin-dependant phosphatase calcineurin to the α-Actinins at the sarcomeric z-line [18].

A polymorphism in ACTN3, R577X (rs1815739), determines the presence or absence of functional α-Actinin-3 protein, giving rise to three genotypes; RR, RX and XX. Homozygosity for the 577X stop-codon results in complete deficiency of α-Actinin-3 protein in about 16 % of the global human population [19]. The loss of functional α-Actinin-3 confers no disease phenotype suggesting that the related isoform α-Actinin-2 may somewhat compensate for the loss of α-Actinin-3; however, no up regulation of α-Actinin-2 is observed in ACTN3 XX humans, (Authors' unpublished observations [10]). The frequency of the ACTN3 XX genotype varies greatly, from ~25 % in Asian populations to <1 % in an African Bantu population with European frequency ~18 %. This suggests that ACTN3 genotype may confer differential fitness in humans under certain environmental conditions [10] and that the X-allele was positively selected during recent evolution of modern humans to the Eurasian environment [20]. However, association studies would be required to ascertain any association between ACTN3 R577X genotype and performance-related phenotypes.

Early associations

The prevalence of α-Actinin-3 deficiency in humans was first identified in a cohort of congenital muscular dystrophic (CMD) patients [19]. Initially it was suggested that this deficiency may be a marker for a sub-set of patients with CMD. However, this was followed up and consequently found that the loss of α-Actinin-3 was in fact likely due to homozygosity for a single point mutation, causing a premature stop codon in ACTN3 (R577X). This was not associated with any specific histopathological or clinical phenotype as ACTN3 XX homozygotes were present in both diseased and healthy participants [19,21]

Due to the differing ACTN3 genotype frequencies, Yang *et al.* [10] followed this up, investigating the influence of ACTN3 genotype on factors which influence normal variation in muscle function. Since any effect on muscle function would be most readily observable at the extremes of human performance, they chose elite athletes as their cohort. Given the localisation of α-Actinin-3 in fast-twitch skeletal muscle fibres, they hypothesised and subsequently evidenced that the frequency of the ACTN3 XX genotype would differ according to athletic status thus indicating a potential effect on performance.

They genotyped 426 unrelated Caucasian controls (292 female and 134 males) and 301 elite white athletes from 14 different sports. Of the 301 elite athletes, a sub-set of 107 (72 male and 35 female) were classified as specialist sprint or power competitors and included 32 Olympians (25 male an 7 female); 194 athletes (122 male and 72 female) were classified as specialist endurance athletes and included 18 Olympians (12 male and 6 female). Genotype was not statistically significantly different between the three groups ($p = 0.996$) nor between genders. As a whole, there were no significant difference between the controls and the elite athletes. When athletes were divided between Sprint / Power and

Endurance sub-sets and compared with controls, there was strong evidence of allele frequency variation between groups ($p < 0.001$). Sprint athletes had a lower frequency of the XX genotypes compared to controls (6 % vs. 18 %, $p < 0.001$) and there were no female Sprint / Power Olympians with the XX genotype. The Sprint / Power group also had a higher frequency of the RR genotype (50 % vs. 30 %) and a lower frequency of the RX genotype (45 % vs. 52 %) compared with controls. Allele frequencies between Sprint / Power and Endurance migrated in opposite directions and differed significantly in both genders (male $p < 0.001$, female $p < 0.05$). Yang *et al.* [10] were the first group to investigate such associations with a skeletal muscle structural gene and the first to suggest a performance advantage - between ACTN3 577R allele and power and sprint performances. They also showed an association between the X-allele and endurance performance however this association was weaker and less clear.

ACTN3, Power and Strength Performance in Humans

Since the early work of Yang *et al.* [10], further replication has been undertaken in many other independent athletic and non-athletic cohorts. Similar findings were reported in Finnish elite athletes [22] with the ACTN3 XX genotypes frequency lower in the sprint group (n = 89) compared to the endurance group (n = 52). ACTN3 XX genotype was found to be completely missing in their top sprinters (n = 23) in line with findings in the female top-level sprint group of Yang *et al.* (2003). The ACTN3 RR genotype was conversely found to be higher in the sprint group and lower in the endurance group. In a cohort of 486 Russian power-orientated athletes, the ACTN3 XX genotype was found to be significantly reduced (6.4 % vs. 14.2 % $p < 0.0001$) compared to 1197 controls with a significant linear trend ($p <$

0.0001) between athlete status (highly elite, elite, sub-elite, average, control) and ACTN3 XX genotype frequency (3.4, 4.2, 7.3, 6.7 and 14.2 % respectively) [23]. It could be suggested that these affects may be related to the developmental factors as Moran *et al.* [24] found no association between ACTN3 and sprinting performance in an unselected adolescent female population, in contrast to what would be expected in an elite female sprint population; perhaps due to the training environment and muscle physiology in trained, versus untrained individuals. These association studies paved the way for more in-depth analyses of the ACTN3 R577X genotype and more complex performance-related traits.

Research evolved from simple association studies comparing genotypes between elite athletes versus non-athletic controls to assessing whether ACTN3 genotype is associated with specific performance related phenotypes and whether these findings can be identified in non-athletic cohorts of different ages or genders. In 2005, Clarkson *et al.* [25] were the first to directly investigate ACTN3 genotype and its' association with baseline muscle phenotypes and subsequent changes following a resistance training regimen. The investigation was conducted with men (n = 247) and women (n = 352) and involved 12-weeks of progressive resistance training on elbow flexor / extensor muscles of the non-dominant arm. There was no association of ACTN3 R577X genotype and any muscular phenotype measured in men but, in women, the authors identified that ACTN3 XX-individuals had significantly lower baseline isometric maximal voluntary contraction (MVC) strength compared to ACTN3 RX ($p < 0.01$) and ACTN3 RR ($p < 0.05$) individuals. Women were also found to have a significant association between ACTN3 genotype and 1-repetition max (1RM) response. Conversely to the author's initial hypothesis, and to what would be expected given the functional role of ACTN3 in the transmission of force across the z-line

(Yang *et al.* 2003), the ACTN3 RR women showed a significantly lower ($p < 0.05$) response in absolute 1RM to 12 weeks of training compared to their ACTN3 XX co-participants. The data suggested that women who were homozygous for the ACTN3 X-allele are at a significant disadvantage to those heterozygous or homozygous for the R-allele in terms of baseline strength as measured by MVC (a static test). However, they also demonstrate the largest improvement in 1RM (dynamic test) strength and thus are at an advantage in terms of developing dynamic muscular strength in response to resistance training.

Delmonico *et al.* [26] performed a similar training study to investigate whether the ACTN3 R577X polymorphism would affect knee extensor peak power in response to resistance training in older adults. Their results indicated that the ACTN3 R577X polymorphism influenced the response of quadriceps muscle power to 10-weeks of unilateral knee extensor strength training in older adults. Both men and women as separate groups significantly increased their 1RM ($p < 0.001$); however, the increase was significantly greater in men compared to women ($p < 0.006$). Delmonico and colleagues [26] were the first to demonstrate that increases in knee extensor peak power with strength training are influenced by ACTN3 genotype in both men and women with ACTN3 RR individuals showing a greater peak power response compared to their ACTN3 XX individuals. There were no significant differences in any phenotypic measurement at baseline between ACTN3 genotypes for men but for women, XX homozygotes had a significantly higher peak power ($p < 0.05$) which has not been documented in the literature before. However, the authors acknowledged that their observed genotype frequencies differed from what was expected in Hardy-Weinberg equilibrium ($p = 0.02$).

Moran *et al.* [24] investigated the association of the ACTN3 R577X polymorphism on quantitative body composition and performance phenotypes in adolescent Greeks. This was the earliest study to break down strength, power and endurance performance into specific quantitative measures. They conducted their investigation in adolescents as in certain circumstances, genotype-phenotype associations within complex traits can be more effectively identified in such cohorts due to the confounding effects of the environment having had less time to take effect [24]. They measured: body-mass index (BMI), triceps and subscapular skin-fold thickness (anthropometric data), handgrip strength, sitting basketball throw, vertical jump performance, 40m sprint time, agility run performance (tests of strength and / or power) and shuttle run performance (an endurance test) in each participant. A significant association was found in males (n = 511) for 40m sprint with ACTN3 RR and RX individuals significantly faster ($p < 0.004$) than ACTN3 XX individuals however there was no difference between ACTN3 RR (n = 172) and ACTN3 RX (n = 242) individuals. No other anthropometric, strength, power or endurance phenotype was found to reach statistical significance and no association as found for the 40m sprint in the female (n = 439) participants. Their results demonstrated that ACTN3 genotype influences sprinting ability in an unselected population of non-athletic Caucasians and was similar to the associations found in elite Caucasian sprint athlete status [10]. Due to the lack of association with other strength / power phenotype, involving single muscle contraction events, the authors suggested that the ACTN3 R577X genotype influences the cyclical component of sprinting rather than the strength or power generation.

In 2008, Walsh and colleagues [27] investigated the association of ACTN3 R577X polymorphism in a non-athlete population of differing ages across the lifespan. 454 men and

294 women aged 22-90 years old took part in the study which assessed body composition (via duel energy x-ray absorptiometry, DEXA) and isokinetic strength using an isokinetic dynamometer on the dominant lower limb for both the concentric and eccentric phases at 30 $^{o}/s^{-1}$ (N·m) and 180 $^{o}/s^{-1}$ (N·m). ACTN3 XX homozygotic females displayed significantly lower levels of total body fat-free mass (FFM; p = 0.009) compared with ACTN3 RR homozygous women but not compared to heterozygotes. Female XX homozygotes displayed significantly reduced strength than RR + RX women (p = 0.049) during the shortening (concentric) phase at 30 $^{o}/s^{-1}$ (N·m) when lower limb FFM differences were covaried during the analyses. Results were similar in a sub-analysis of women over 50 years old for the eccentric phase at 30 $^{o}/s^{-1}$ (N·m). No significant results were reported for men with regard to any body composition or strength phenotype measured. The authors concluded that the absence of functional ACTN3 (XX genotype) influenced FFM and knee extensor strength in women but not men.

From 2003, [10] ACTN3 genotype has been associated with strength / power performance related phenotypes. Research has evolved considerably encompassing: association studies with elite athlete status [10, 22, 23, 28] training studies in athletes and older non-athletes [26, 29]; and cross-sectional studies investigating associations with specific performance-related quantitative phenotypes in non-athletic cohorts [24,27]. With regard to strength and power performance, ACTN3 XX genotype appears to be associated with reduced performance, especially in athletes. Non-athletic cohorts have reported mixed results with gender and ethnicity appearing to have a confounding role in associations with genotype and performance. Results from the above studies supports the possibility that although XX homozygosity may be detremental to strength or power-related phenotypes, it

may confer a positive association in endurance-type activities due to increased X-allele frequency in such cohorts. Despite all the studies on the functional role of ACTN3, to understand by which specific mechanisms differing ACTN3 genotypes influence strength, power or endurance performance and or any alterations within skeletal muscle, numerous authors proposed that a mouse model should be generated.

Mouse Model

To characterise the phenotypic consequences of variation in the ACTN3 R577X SNP, MacArthur *et al.* [30] developed an ACTN3 KO mouse line through a targeted deletion of exons 2-7 of ACTN3 in chimeric mice. The KO mice were found to have no detectable α-Actinin-3 protein, assessed by either Immunohistochemistry or Western Blotting mimicking the ACTN3 XX genotype in humans and thus making them a valid candidate for study. The KO mouse line were morphologically similar to their wild-type (WT) littermates. They showed normal sarcomeric function and there was no substantial loss in glycolytic type-2 fibres identified. However, the authors established that a loss of α-Actinin-3 protein appears to be compensated for by an up-regulation in α-Actinin-2, which is expressed in all muscle fibres, with expression shifting from a preference in oxidative fibres to a more uniformed staining in all fibre-types.

Exercise Capacity

Following the ACTN3 genotype frequency findings in elite sprint and endurance athletes published by Yang *et al.* [10], MacArthur *et al.* [30] investigated whether there was any difference in athletic performance between the KO and WT mice. ACTN3 KO and WT

controls were subjected to a modified version of the intrinsic exercise capacity test where they were run on a motorised treadmill at increasing speeds until they reached exhaustion. There was a 33 % average increase in distance run in KO mice compared to WT littermates suggesting that the shift towards oxidative metabolism observed in the muscle of KO mice increases intrinsic endurance performance

MacArthur *et al.* [31] indicated mice deficient in α-Actinin-3 display significantly reduced grip strength compared to their WT littermates with a 7.5 % lower average grip strength in males ($p < 0.007$) and 6 % reduction in female mice ($p = 0.02$). To discern if the reduced grip strength was attributable to a reduction in muscle size, total body and isolated muscle mass was examined. It was found that male KO mice had a 4 % lower total body weight than WT controls ($n = 27$ & 32 respectively $p < 0.001$) with a similar effect found for female mice ($p < 0.01$). Dual-energy x-ray absorptiometry was used to analyse body composition and suggested that the reduced body mass was attributable to a reduction in lean mass ($p < 0.001$) with no significant difference in fat-mass between KO and WT mice. Analysis of a variety of skeletal muscles showed that there was a significant decrease in all fast-twitch muscles in the KO mice compared to WT littermates. The degree of reduction varied dependent upon muscle location with proximal muscles seeing a greater reduction in KO mice than more distal muscles. The postural soleus muscle (slow twitch) of KO mice was significantly heavier ($p < 0.001$) compared to WT mice in contrast with the fast-twitch muscles suggesting a compensatory increase due to the decreased size of surrounding muscles.

The authors eluded from data not shown in their manuscript that the difference in muscle mass between mice-types stems from a reduction in cross-sectional area.

Immunostaining of transverse areas of the quadriceps (QUAD), spinalis thoracis (SPN), extensus digitorum longus (EDL) and soleus (SOL) muscles for myosin heavy chain (MyHC) type-1, 2A, 2X and 2B was used to examine the effects of α-Actinin-3 deficiency on fibre type proportions and sizes. The muscles chosen represented both proximal and distal muscles as well as muscles for which α-Actinin-3 should be both expressed and not expressed (soleus). Total number of fibres' did not differ between WT and KO mice for the EDL and fibre-type proportions was equally similar for the EDL, SOL and SPN muscles indicating that the observed reduction in muscle mass in KO mice is not the result of a reduction in the total fibre number nor a higher proportion of slow fibres', which are smaller in diameter than fast fibres', and thus suggesting a specific reduction in fibre size. Further examination of isolated muscle contractile properties showed the EDL muscles of KO mice displayed a significantly reduced half-relaxation time compared to WT mice ($p < 0.007$) which is consistent with the shift in properties of predominantly fast-twitch EDL fibres' towards a slower, more oxidative type which would be expected to reduce the ability to generate rapid and repetitive forceful contractions required for sprint and power-based performances.

Muscle Metabolism

MacArthur *et al.* [31] found that skeletal muscle samples from the KO mice showed more intense staining for markers of glycolysis and aerobic metabolism: NADH-tetrazolium reductase ($p < 0.02$) and succinate dehydrogenase, compared to WT specimens. Analysis of markers of mitochondrial biogenesis: cytochrome c oxidate and mitochondrial porin were found to be up-regulated in the muscle of KO mice, consistent with increased mitochondrial

density within the muscle fibres. Immunohistochemical analysis of a marker of fast glycolytic fibres, myosin heavy chain 2B, found no significant difference between the KO or WT specimens suggesting that the increased oxidative enzyme activity is not indicative of a change in muscle fibre type but of altered metabolism within the type-2b muscle fibres. To further substantiate their findings, two key enzymes involved in pyruvate metabolism: lactate dehydrogenase (LDH) and citrate synthase, were analysed. Activity of LDH was 16 % lower ($p < 0.001$) and citrate synthase was 22 % higher ($p < 0.05$) in KO mice compared to WT indicating that the loss of α-Actinin-3 results in a significant shift from reliance on the anaerobic lactate pathway to the slower, more efficient aerobic pathway which is usually associated with slow muscle fibres. In a follow-up paper, the authors extended the metabolic analysis to include a palette of enzymes representative of a much broader sample of the metabolic network. As well as the aforementioned activity of citrate synthase and lactate dehydrogenase, they investigated the activity of hexokinase, glyceraldehyde-6-phosphate dehydrogenase and phosphofructokinase, all enzymes of the glycolytic pathway, showing increases of 26 % and 62 % for hexokinase and glyceraldehyde-6-phosphate dehydrogenase respectively but no detectable change in phosphofructokinase activity. Mitochondrial enzyme activity was also found to be increased with citrate synthase, succinate dehydrogenase and cytochrome c oxidase showing activity levels ~25-39 % higher in KO muscle relative to WT. Hydroxyacyl-CoA dehydrogenase and medium chain acyl-CoA, enzymes involved in fatty acid-oxidation, were found to have activity 30-42 % higher in KO muscle suggesting that an absence of α-Actinin-3 results in an increased reliance on β-oxidation of fatty acids. These data suggest that the muscle metabolism within fast fibres of KO mice shift reliance on anaerobic metabolism to the slower, more efficient, aerobic pathway.

Quinlan *et al.* [20] provided further evidence that α-Actinin-3 deficiency alters muscle metabolism and evidenced that this results in an increase in muscle glycogen content in both mice and humans. Given that KO mice display significantly enhanced activity of key enzymes involved in glycolysis and aerobic metabolism [31], and the known link between sarcomeric α-Actinins and GPh, Quinlan and colleagues [20] hypothesised and evidenced that KO mice would show altered glycogen metabolism. They expanded this research to include 26 humans (n = 9 ACTN3 RR; n = 12 ACTN3 RX; n = 5 ACTN3 XX). Muscle biopsies obtained from the quadriceps of the participants showed that muscle glycogen content was significantly increased in XX individuals compared to RX individuals ($p < 0.05$) . There was no significant difference between ACTN3 XX and ACTN3 RR individuals however the authors acknowledged that there was no biometric data or information on exercise status as the biopsies were obtained from people who were undergoing testing but tested negative for malignant hyperthermia. KO mice were similarly found to have increased glycogen levels through a decrease in GPh. GPh levels were 27 % lower in KO mice compared to WT littermates ($p < 0.001$)

The authors explored the validity the KO mouse as a model of ACTN3 XX humans. *In vivo* assessment of reduced strength and lean mass were confirmed *in vitro* by demonstrating reduced force generation capacity in isolated hind-limb muscles, reduced muscle fibre size and altered metabolic activity compared to WT mice. They replicated the association of the XX genotype with enhanced endurance performance first suggested by Yang *et al.* [10] by showing that KO mice have a higher intrinsic endurance capacity than their WT littermates [31] and enhanced recovery from contraction-induced fatigue. Thus, the

ACTN3 KO mouse replicated the reported effects of α-Actinin-3 deficiency in humans providing important mechanistic insights.

ACTN3 and Endurance Performance in Humans

It can be hypothesised that the ACTN3 XX genotype may confer positive benefits to endurance performance in humans. Early work by Yang *et al.* [10] and Niemi & Majamaa [22] was supported by the development of an ACTN3 KO mouse model (MacArthur *et al.* 2008) to aid such a hypothesis. However, the role of the ACTN3 X-allele within endurance performance has not yet been adequately elucidated. An increased frequency of the ACTN3 X-allele within elite endurance cohorts has been documented in several, [10,16,22,32,33] but not all [34–36] studies further complicating any proposed hypothesis, role or mechanism of action.

In 2003, Yang *et al.* [10] demonstrated a trend of the X-allele towards endurance athlete status and thus endurance performance with a significantly increased frequency of the ACTN3 X-allele in female endurance athletes (n = 36) compared to female sprint athletes (n = 25; $p < 0.001$). Eynon *et al.* [32] performed a similar association study comparing ACTN3 genotypes in Israeli top level athletes between a cohort of elite sprinters (n = 81) elite endurance athletes (n = 74) and non-athletic controls (n = 240). Similar to Yang *et al.* (2003), their data suggested an association between ACTN3 R-allele and RR-genotype and elite-level sprint performance with the R-allele and RR genotype percentage distribution significantly higher than endurance athletes (R allele frequency 70 % versus 53 %, $p < 0.001$; RR genotype 52 % versus 18 %, $p < 0.001$) and controls (R-allele frequency 55 %, $p < 0.01$; RR genotype 27.3 %, $p < 0.001$). The ACTN3 XX-genotype was conversely significantly higher in endurance athletes compared to sprinters (34 % versus 13 %, $p = 0.002$) and controls (13 %,

p = 0.002). Their data are in line with previously reported data regarding both ACTN3 alleles and athlete status; however, the authors propose that the X-allele and XX genotype are not 'critical' to endurance performance but likely 'additive' as there was no statistical significance between elite and national level endurance athletes.

In 2012, Eynon *et al.* [33] investigated the ACTN3 R577X polymorphism across three groups of Caucasian elite male European athletes In an attempt to offset controversy amongst the literature arising from between study differences in sample size and ethnicity. Spanish (n = 616; 343, 154 & 119), Polish (n = 571; 354, 112 & 105) and Russian (n = 254; 111, 18 & 125) controls, endurance athletes and power athletes respectively were included in the study. Athletes were of a national or international level and controls were non-athletic individuals. They observed that endurance athletes were significantly more likely to have an ACTN3 XX-genotype rather than an RR-genotype (p = 0.028) when compared to power athletes. Furthermore, world class competitive endurance athletes were significantly more likely to harbour the ACTN3 XX-genotype compared the RR or RX-variants when compared to national level endurance athletes (p = 0.038). Genotype frequency did not differ between national and world-class power athletes. This study was the largest of its kind and comprehensively supported the notion that ACTN3 R577X is associated with elite athletic performance.

In contrast, Moran *et al.* [24] failed to find any such association. The authors' investigated ACTN3 genotype on an adolescent, non-athletic cohort as described previously. They investigated associations with a range of performance and anthropometric tests and found no association was found for shuttle-run test which has previously been validated and found to be a reliable predictor of $\dot{V}O_{2MAX}$ [37]

Similarly, Cięszczyk *et al.* [36] investigated the ACTN3 R577X polymorphism in a cohort of top-level Polish rowers. Their cohort contained 80 national-level competitive rowers (37 elite rowers and 46 sub-elite rowers – no explanation within the literature as to the extra three rowers) and 204 unrelated control participants. Genotype frequency differed significantly between the athletes and control cohorts (RR 53.8 %, RX 38.8 %, XX 7.4 % versus RR 36.3 %, RX 46.1 %, XX 17.6 %; $p < 0.01$). They found no association with ACTN3 X-allele and competitive rowing level. There was a significant association ($p = 0.002$) between the ACTN3 R-allele and rowing which reduced when the cohort was separated into elite ($p = 0.007$) and sub-elite ($p = 0.026$) categories. The authors demonstrated that the ACTN3 X-allele appears to have no benefit to endurance performance in rowers and on the contrary that the R-allele is advantageous with regard to competitive rowing. However, rowing requires a spectrum of performance traits. As well as a high $\dot{V}O_{2MAX}$ 21-30 % of performance in elite level rowing is contributed by anaerobic capacity. Increased muscle mass and strength as well as a low percentage of body fat are also determinants of a successful performance [38]. The R-allele of ACTN3 has previously been associated with muscle mass and strength which may give insights into why this allele has been so strongly associated with the rowers in this cohort.

In conflict with the positive data already explained, Grealy *et al.* [16] found no such association of ACTN3 XX-genotype on performance in their cross-sectional study of 196 Ironman World Championship athletes. Similarly to Eynon *et al.* [33], Grealy and co-authors [16] had a diverse ethnicity within their cohort, including athletes of: European (n = 60), South and North American (n = 6 & 104 respectively) Asian (n = 2) and Oceanasian (n = 23) descent. Allele frequencies were not significantly different between ethnicities and were

similar to those of similar ethnic origin in other literature and ACTN3 genotype was within Hardy-Weinberg equilibrium. The authors elucidated that ACTN3 genotype does not independently influence endurance performance within this cohort and that any such effect on endurance performance is likely to be complex in nature.

Much evidence has been reported regarding the effect of ACTN3 X-allele and XX-genotype on endurance athlete status and or performance following the early work of Yang *et al.* [10]. There are equivocal findings amongst the literature with some elite endurance cohorts eliciting an association [10,32,33] whilst others [16,24,36] fail to do so. A meta-analysis by Alfred *et al.* [39] investigating ACTN3 genotype on athlete status concluded that there was no evidence, above what would be expected through chance, that the X-allele is more common amongst endurance athletes. However, what the consensus of the research does allude to is that investigations into the mechanisms underpinning the role of the ACTN3 X-allele on muscle function and thus endurance exercise are required. Despite the comprehensive characterisation of the ACTN3 KO mouse model [20,30,31] and the aforementioned association studies in humans, the underlying molecular mechanisms that link the absence of a structural protein to the reported endurance phenotype witnessed in the mouse model remains elusive. It is plausible to suggest an as yet unknown epistatic effect may be causal; however, given that ~1-billion people worldwide are homozygous for the ACTN3 X-allele, understanding the role of the X-allele is likely to prove beneficial to the wider field, yet despite numerous studies involving ACTN3 genotype and performance; few papers have explored potential mechanisms on performance.

ACTN3 and Calcineurin Signalling

One of the most significant advances in understanding the potential mechanisms of ACTN3 on performance has been made by the work of Seto and colleagues [40]. They identified one prospective down-stream target of ACTN3, the calcium-calmodulin-dependent phosphatase calcineurin as a potential link between the ACTN3 XX-homozygosity and the suggested associations with endurance performance (Seto *et al.* 2013). It is well known that calcineurin signalling induces activation of the slow myogenic programme and thus plays a critical role in the remodelling of skeletal muscle [41,42] (See Figure 2, adapted from [41]) and has also been shown to confer fatigue resistance in endurance runners [43].

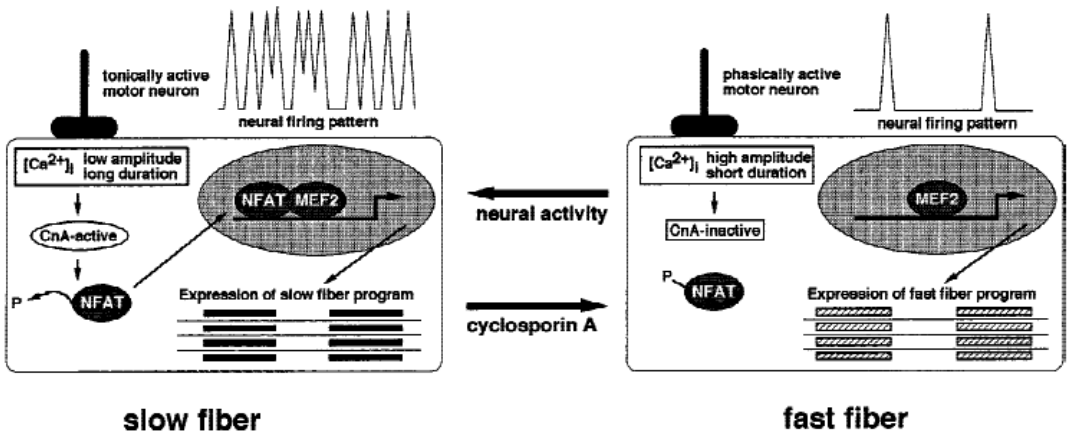


Figure 2, Model of calcineurin-dependent pathway linking particular patters of motor-neuron activity to separate programmes of gene expression that establish phenotypic differences between slow and fast myofibres adapted from [41]. Calcineurin, activated by calcium signalling, causes the translocation of transcription factors to the myocyte nuclei leading to growth and differentiation of myofibres.

Calcineurin is indirectly associated with the sarcomeric α-Actinins due to their mutual binding to Calsarcin, at the sarcomeric Z-line [18,44].

Calsarcins perform a duel role within skeletal muscle; maintaining the structural integrity of the Z-disc by linking α-Actinin, γ-filamin and Telethonin and by tethering the signalling phosphatase calcineurin to α-Actinin [18,44]. There are three members of the Calsarcin family; Calsarcin-1, -2 and -3, encoded by the genes MYOZ2, MYOZ1 and MYOZ3 respectively. Calsarcin-1 is expressed specifically in adult cardiac and oxidative type-1 and type IIa skeletal muscles. Calsarcins -2 and -3 are restricted to expression in the fast fibres of skeletal muscle. Frey *et al.* [45] demonstrated that Calsarcin-2 inhibits calcineurin signalling in *vivo,* suggesting that it possibly modulates exercise performance in mice through the regulation of calcineurin activity. Mice deficient in Calsarcin-2 (KO) displayed enhanced endurance capacity and their muscles exhibited a shift towards slower fibre-properties which mimics the phenotype observed in the ACTN3 KO mouse model generated by MacArthur and colleagues [30].

In 2013, Seto *et al.* [40] provided a mechanistic explanation for the effect of ACTN3 genotype on skeletal muscle. They raised the prospect that the α-Actinins act upstream of Calsarcin as key regulators of muscle fibre phenotypes. They go on to hypothesise that the phenotypic changes observed in α-Actnin-3 deficient fast fibres' are due to increased calcineurin activity which, in turn, is regulated by the differential binding of α-Actinins to Calsarcins.

To identify whether the phenotypic changes observed in α-Actinin-3 deficient muscles were associated with an increase in calcineurin activity, the authors examined the expression of a downstream regulator of calcineurin (RCAN), in ACTN3 KO and WT mice.

Although fold-change in RCAN1 isoform 4 (RCAN1-4) failed to reach significance at baseline ($p = 0.057$); following a single bout of endurance exercise, it was found to be up-regulated 2.9-fold in KO mice ($p = 0.004$; $n = 6$ in each group) compared to WT littermates. This is consistent with potential increase in calcineurin activity due to the absence of α-Actinin-3. To provide further confirmation that calcineurin activity is increased in the KO mouse, Seto and colleagues assayed both total and calcium-independent phosphatase activity. As calcineurin is a calcium-dependent phosphatase, it can be measured by subtracting calcium-independent from total phosphatase activity [40]. The resulting calcineurin activity is consistent with that of RCAN1-4 with a 1.9-fold increase in KO versus WT mice and no difference between calcium-independent phosphatase (data not shown in authors' manuscript). This indicates that, following a single bout of endurance exercise, calcineurin activity is increased, with this increase appearing greater in KO mice compared to WT mice; potentially linking calcineurin activity to the presence or absence of ACTN3 protein.

As sarcomeric α-Actinins and calcineurin share the same N-terminal binding site on Calsarcins [44], Seto and colleagues developed a yeast 2-hybrid assay to investigate binding affinity between Calsarcins 1, 2 and 3 and α-Actinins 2 and 3. They discovered that Calsarcin-2 binds to both sarcomeric α-Actinin isoforms with preference to α-Actinin-2 with binding 13.2-fold higher than for α-Actinin-3. There was no observed growth between Calsarcins-1 (encoded by MYOZ2) and Calsarcin-3 (encoded by MYOZ3) suggesting that interactions with sarcomeric α-Actinins is unique to Calsarcin-2. However, although the yeast 2-hybrid method is the most common method to investigate such protein to protein interactions, it has been estimated that the percentage of false negative results is approximately 45 % [46]. In addition, the protein regions investigated by Seto and colleagues

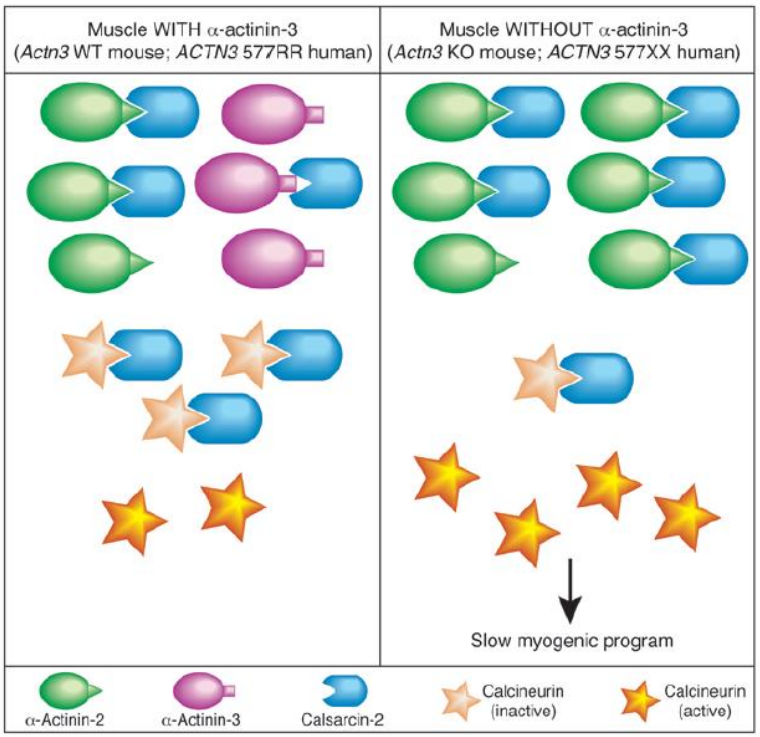
differs across the three Calsarcins. The most significant interaction for Calsarcin-2 was found in the region of 1-90 amino acids. When this was expanded to 1-110 amino acids, the interaction was reduced in Calsarcin-2 and not found in Calsarcin-1 and Calsarcin-3. A further investigation of Calsarcin-2 in the 79-187 amino acid regions failed to distinguish an interaction suggesting that the identified interactions occurred in the 1 to 78 amino acids region. As the authors' have not exhaustively investigated the entirety of all three Calsarcin proteins, it is not possible to rule out that there is no interaction between the α-Actinins and Calsarcin-1 and -3.

Subsequent tagging of Calsarcin-2 and calcineurin with increasing levels of α-Actinin-2 in COS-1 fibroblast like-cells showed an inverse association between the binding of calcineurin with Calsarcin-2 and subsequent levels of α-Actinin-2. This showed that α-Actinin-2 inhibits the binding of calcineurin to Calsarcin-2 thus increases calcineurin activity.

To confirm that their observations are applicable and reproducible in humans, Seto *et al.* (2013) examined muscle biopsies from female humans with ACTN3 577RR (n = 5) and ACTN3 577XX (n = 6) genotype. Consistent with the KO mouse model, ACTN3 577XX humans displayed significantly increased RCAN1-4 levels compared to 577RR humans ($p = 0.004$). There was no investigation of Calsarcin variations and genotypes. This paper evidenced that calcineurin signalling is influenced by performance phenotypes and ACTN3 genotype. Thus it is probable that variations in genes of components of this mechanism are likely to influence performance.

The work of Seto *et al.* [40] was instrumental in providing a mechanistic explanation for the effect of ACTN3 genotype on skeletal muscle. In *vitro* work in cells and yeast 2-hybrid systems and in *vivo* mouse and human trials has shown that α-Actinin-2 competes with

calcineurin for binding with Calsarcin-2. In α-Actinin-3 deficient muscle, α-Actinin-2 out competes calcineurin liberating it from Calsarcin-2 binding (See Figure 3 below, from figure 7 of [40]).



Schematic of sarcomeric α-actinin regulation of calcineurin signaling. α-Actinin-2 is differentially expressed in muscles without α-actinin-3. Higher-affinity binding of calsarcin-2 for α-actinin-2 over α-actinin-3 and competitive binding of α-actinin-2 for calsarcin-2 lead to the increased release of calcineurin from calsarcin-2 inhibition in the absence of α-actinin-3. We propose that increased free (active) calcineurin activates downstream signaling and the slow myogenic program in α-actinin-3–deficient muscle.

Figure 3, Schematic of sarcomeric α-Actinin regulation of calcineurin signalling, adapted from figure 7 of [40].

Activated calcineurin could account for the shift in oxidative properties of fast-twitch fibres towards a slower, more oxidative profile, enhanced baseline and subsequent response to endurance training and concomitant fibre-type shift. To ascertain whether such changes would have an effect on performance in humans, an association study investigating differing genotypes of Calsarcins and ACTN3 with quantitative phenotypes involving athletes and controls is required.

Aims & Hypotheses

The present study intends to follow up the work of Frey *et al.* [45] and Seto *et al.* [40] and investigate possible associations between Calsarcin (MYOZ) and ACTN3 genotypes on quantitative performance phenotypes in a cohort of Lithuanian athletes and controls. This cohort contains quantitative performance information not present in previous studies on athletes. No study has assessed variation in Calsarcin genotypes and subsequent effects on performance-related phenotypes.

It is hypothesise that carriers of the ACTN3 R-allele will have increased performance in power and strength-related phenotypes compared to X-allele homozygotes and that variation in Calsarcin genotypes will relate to performance phenotypes in a manner likely to be similar to that of ACTN3. Therefore, the aims of the present study are: to genotype the cohort for the ACTN3 R577X SNP and investigate associations with strength, power and endurance-related phenotypes; identify suitable SNPs for Calsarcin 1, 2 and 3 (encoded by MYOZ2, 1 and 3 respectively) genes, and finally to genotype the cohort for the identified MYOZ genes and investigate associations with strength, power and endurance-related phenotypes.

Methods

Ethical Approval

Ethical approval for the present study was granted by the Lithuanian State Bioethics Committee and the University of Stirling, School of Sport Research Ethics Committee and abides by the principles outline in the Declaration of Helsinki. Written informed consent was obtained from all participants prior to participation in the study.

Participant Characteristics

Four-hundred and forty-seven native Lithuanian males between the age of 17 and 37 years old were recruited to participate in this study from the Lithuanian Sports University (Kaunas, Lithuania). All participants were required to undertake a series of endurance, strength and power, anatomical and physiological tests. If participants were related, only the participant who competed at the highest competitive level was retained for analyses. All athletes were sub-divided into strength, sprint and power (SSP) and endurance (END) categories depending on their sport. The SSP sub-group included: weightlifters, bodybuilders, gymnasts, short distance swimmers, short distance runners and team sport players (handball, volleyball, basketball and football). Team sport athletes were included within the SSP sub-group as opposed to the END group as their sports require repeated high-intensity bouts of exercise even though they could potentially share similarities with END phenotypes such as improved $\dot{V}O_{2MAX}$. The END sub-group contained long-distance cyclists, modern pentathletes, orienteers, runners, skiers and walkers. If an athlete could not be classified into either group they were excluded from the study. A total of 407 participants (210 athletes, 197 controls) were carried forward into the present study (see Table 1 for participant characteristics). Each athlete sub-group was divided by level of best performance

(Table 2). All athletes trained a minimum of twice per week whereas control participants did not take part in any organised physical activity more than twice per week.

Table 1, Participant Characteristics

				Athlete Sub-groups	
	Overall (N = 407)	**Control (N = 197)**	**Athletes (N = 210)**	**SSP (N = 126)**	**END (N = 84)**
Age (years)	22 ± 4	24 ± 4	21 ± 3	21 ± 3	21 ± 3
Height (cm)	181 ± 6	180 ± 5	181 ± 18	183 ± 24	179 ± 5
Body Mass (kg)	77 ± 10	77 ± 10	77 ± 12	81 ± 12	70 ± 6
BMI	23 ± 2	24 ± 3	23 ± 2	24 ± 2	21 ± 1
Training experience (yrs)	8 ± 4	N/A	8.04 ± 1	8 ± 1	6 ± 1
Training volume (hr/wk)	11 ± 6	N/A	11.4 ± 1	10 ± 1	12 ± 1
Fat Free Mass (kg)	63 ± 7	64 ± 6	66 ± 12	69 ± 14	62 ± 5

Table 2, Highest Competitive Level for SSP and END Athletes

Competitive Level	**SSP**	**END**	**Total**
International Level	8	12	20
National Level	69	52	121
Area Squad	25	14	39
Other	24	6	30

Phenotype Data

Participants were required to attend the laboratory on three non-consecutive days to perform a detailed range of standardised physiological tests which were carried out by trained individuals. Participants attended each day after 12 hours rest; 12 hours fasted on day one – to allow a fasted venous blood sample to be drawn - and two hours fasted on days two and three.

Upon entry to the study, participants were assigned a unique ID code so that all data could be stored in an anonymous manner. An 8 ml venous blood sample was collected from each participant and stored at -85 ^{o}C for DNA extraction at a later date.

The laboratory visits allowed for detailed phenotypic data to be collected including measures of anthropometrics, endurance, strength and power performance. For the present study, the selected tests are detailed below.

For the assessment of strength and power, isokinetic dynamometry (IK) was performed for both extension and flexion of all limbs at 30 $^{o}s^{-1}$ (N·m), 90 $^{o}s^{-1}$ (N·m), and 180 $^{o}s^{-1}$ (N·m). Tests were performed on a Biodex System-3 Pro (Biodex Medical Systems Inc., Shirley, New York). Testing was performed following five minutes of cycling warm-up. Participants were given three attempts at each test with the best attempt recorded. To account for dominance of a given arm or leg, data was combined and presented as upper limb / lower limb, flexion / extension at 30 $^{o}s^{-1}$ (N·m), 90 $^{o}s^{-1}$ (N·m), and 180 $^{o}s^{-1}$ (N·m).

Power was established through counter-movement jump (CMJ); squat jump (SJ), 30-second Wingate test and a 30 m sprint. CMJ and SJ were performed on a tensoplatform (brand unknown) with arms akimbo and knees flexed to 90 degrees. The best of three attempts was taken for each test. 30 m sprint was timed using a laser sensor system (brand unknown). Participants were given two attempts; starting from a static standing position with only feet in contact with the ground. The 30-second Wingate test was performed on a mechanical veloergometer (Monark Exercise AB, Sweden. Model unknown). Participants were given two attempts following a warm-up with the best result retained for analysis. Aerobic capacity was ascertained through a ramp, treadmill, $\dot{V}O_{2MAX}$ test [47] however the model of the treadmill is unknown.

Body fat percentage and body mass data was collected for anthropometric measures. Body fat percentage was ascertained through the Tanita skin-fold calliper assessment of seven sites and calculated according to the Durnim and Womersley Equation

[48]. Body mass was ascertained through Tanita scales however the model is not known (Tanita Europe BV, Amsterdam, The Netherlands).

Participants underwent the anthropometric testing and venous blood sample collection on day 1. Strength and power tests were performed on day 2 and aerobic capacity testing was performed on day 3 although these were non-consecutive days.

DNA Extraction

DNA was extracted from blood samples using the NucleoSpin® Columns (Macherey-Nagel, GmbH & Co, Düren, Germany) and Qiagen buffers (QiAamp® DNA Minikit, Qiagen, Limburg, Netherlands) and stored in 1.5 ml microcentrifuge tubes at -20 ^{o}C. Qiagen (Q) buffers were chosen over Macherey-Nagel (MN) due to prolonged issues surrounding poor to zero DNA yields. A protocol, adapted from both the MN and Q protocols, was developed following the on-going issues with MN buffers. See Appendix 1 for details of the protocol used. The issue was not identified after two months of investigation and as such, Qiagen buffers were chosen. Any sample yielding < 10 ng/µL of DNA was re-extracted. .

Samples were randomly assigned to a well on one of five 96-well plates (STARLAB GmbH, Hamburg, Germany) in a 1-in-10 dilution and stored at -20 ^{o}C. Each plate contained one duplicate sample from all other plates and negative controls ($_{d}H_2O$) for quality control purposes.

Genotyping

ACTN3 Assay Design

Primers were designed using Primer3 Web (version 4.0.0. http://www.primer3.ut.ee/). 1,000 bp Flanking sequences of each identified SNP were inserted and GC-Clamp was set at one. Custom primers were ordered from Integrated DNA Technologies (BVBA Interleuvenlaan, Leuven, Belgium). Primers for ACTN3 are shown in Table 4 and primers for the chosen Calsarcin genes are shown in Table 7.

Restriction enzymes (RE) for each of the identified variants were identified using NEBcutter version 2.0. (http://nc2.neb.com/NEBcutter2/) and are shown in Table 5 for ACTN3 and Table 9 for the chosen Calsarcin genes.

ACTN3 PCR and RFLP conditions

All genes were genotyped using a standard polymerase-chain reaction (PCR) and restriction-fragment-length-polymorphism (RFLP) protocol. See Table 3 for PCR conditions for ACTN3, Table 4 for ACTN3 primer sequences and Table 5 for details of RFLP information.

Table 3, PCR Conditions for ACTN3.

ACTN3		
Temp.	**Time**	
95°C	2.5 minutes	
95°C	45 seconds	35 cycles
53°C	15 seconds	
72°C	25 seconds	
72°C	10 minutes	

Table 4, Primer sequences for ACTN3

Gene	Primer Sequence	Direction
ACTN3	5'-CTGGGCTGGAAGACAGGAG-3' 5'-AGGGTGATGTAGGGATTGGTG-3'	Forward Reverse

Table 5, RFLP Information for ACTN3

Gene	Restriction Enzyme	Digestion Incubation Temp (°C)	Digestion Time (hours)	Agarose Gel Used	Allele Fragment Length
ACTN3	*DdeI*	37	2	Tris-Acetate-EDTA (2 %)	R - 192bp X - 98bp

Calsarcin Bioinformatics

We aimed to identify suitable candidate SNP's from the Calsarcin-1, -2 and -3 genes (encoded by genes MYOZ2, MYOZ1 and MYOZ3 respectively). SNP's for these genes were investigated using the 1,000 Genomes Browser (http://browser.1000genomes.org/index.html/), searched in November 2013.

The work of Seto and colleagues [40] focused on the interaction of ACTN3 and MYOZ1 (Calsarcin-2). They did not investigate the other Calsarcins -1 and -3 (MYOZ2 and MYOZ3 respectively) following results of their yeast-2-hybrid experiments. As previously discussed, the yeast-2-hybrid experiment, albeit the most popular method of investigating protein-protein interactions, is estimated to result in a large percentage of false-negative results [46]. ACTN3 is however known to bind to Calsarcins within the sarcomeric z-line [18]. Given the known associations between ACTN3 and Calsarcins as well as the interaction between Calsarcins and calcineurin signalling and the known role of calcineurin signalling on the

remodelling of skeletal muscle [41] it is possible to suggest that all members of the Calsarcin family are suitable candidates for study.

Stops Gained, Missense Variant and 3' UTR Variants for each gene were analysed as these were most likely to cause functional variation that could result in phenotypic variation. 3' UTR variants were further screened for their likelihood of affecting miRNA binding sites. SNPs with a European minor allele frequency (EMAF) of ≤ 0.05 were excluded as the required affect size for such a rare variant would have been unrealistically large given the size of this cohort.

No variants matching the criteria were identified for Myozenin-1 (Calsarchin-2) and thus the present study cannot further the work of Seto and colleagues [40]. However, two variants were identified for Myozenin-2 (Calsarcin-1; rs62326346 and rs9995277; EMAF 0.31 and 0.27 respectively), both within the 3' UTR and one variant for Myozenin-3 (Calsarcin-3), (rs116090320; EMAF 0.10) within the 3' UTR.

Following identification of SNPs, rs-numbers were input into a microRNA (miRNA) related SNP database (http://www.bioguo.org/miRNASNP2/ - version 2.0; searched in December 2013) to ascertain if the identified SNPs lead to gains or losses in a genes 3'UTR microRNA binding sites and thus theoretical alterations in gene regulation (See Table 6). Micro-RNA's are endogenous 20-25 nucleotide long non-coding ribonucleic acids (RNA's) which are transcribed by the RNA polymerase II and play an important role in regulating gene expression at the protein and messenger-RNA (mRNA) level [49] by imperfectly pairing to the

3’-UTR of target mRNAs [50] They have been shown to both inhibit [51] and activate [52] translation.

Table 6, MYOZ2 and MYOZ3 Associated 3'UTR Variant Micro RNA Binding Sites

				E. Change Kcal/mol			
GENE	**rs No.**	**MiRNA**	**SNP Location**	**Wild T**	**SNP T**	**Effect**	**Entries**
MYOZ2	62326346	2355-5p	chr4:120108263	-24.7	0	LOSS	1
		145-5p		-18.8	-19.7	GAIN	1
		3925-3p		-18.1	-19	GAIN	1
	9995277	30a-5p	chr4. 120108603	-18.6	-8.3	LOSS	1
		30c-5p		-14.8	-7.7	LOSS	1
		30d-5p		-18.6	-8.3	LOSS	1
		30b-5p		-17.1	-7.7	LOSS	1
		30e-5p		-16.4	-8.3	LOSS	1
MYOZ3	116090320	374b-3p	chr5. 150058413	-21.8	-21.9	LOSS	2
		4524a-5p		-22.2	-21.9	LOSS	2
		4524a-5p		-25.2	-24.9	LOSS	2
		4291		0	-24.4	GAIN	2

Previous associations of identified miRNA’s

MYOZ2 rs62326346 was found to have both gained and lost a single miRNA binding site for miRNA-145 and miRNA-2355 respectively. A detailed search of the literature found no association with any human skeletal muscle phenotypes.

MYOZ2 rs9995277 was found to have gained no miRNA binding sites but lost 5 binding sites within the miRNA-30 family (a, b, c, d & e). The miRNA-30 family are found within human skeletal muscle [49] and an inverse association between reduced miRNA-30 family levels and number of superficial slow-twitch muscle fibres has been found in Zebrafish [53].

MYOZ3 rs116090320 was found to have gained miRNA binding sites in MiRNA-4291 and lost binding sites in MiRNA-374b and MiRNA-4524a and b. No data were found to suggest a role of miRNA-4291 or miR-4524 a and b, however miRNA-374b is found in

skeletal muscle and is also found to be up-regulated during myogenic differentiation in CD56+ Myoblasts *in vitro* [49].

Due to data related to possible performance-associated functions, MYOZ2 rs9995277 and MYOZ3 rs116090320 were chosen as the most likely candidates. Thus, the following hypotheses are made. MYOZ2 rs9995277 and MYOZ3 rs116090320 will be associated with phenotypes representative of components of sprinting speed including tests of strength and power as well as relating to endurance related phenotypes as suggested by the potential effects of their downstream miRNA's. As such, both genes were investigated for associations with body mass and body fat percentage as anthropomorphic measures; 30m sprinting time, Wingate relative peak-power and fatigue index as well as counter-movement jump (CMJ) height and static jump height (SJ) as measures of power. Further to this, isokinetic upper and lower limb strength was ascertained as measures of strength. For endurance related associations, relative $\dot{V}O_{2MAX}$ was used.

MYOZ2 and MYOZ3 Assay Design

Primers for both genes were designed in an identical manner as the process for ACTN3. Table 7 details primers used for MYOZ2 and MYOZ3. PCR conditions for each gene are detailed in Table 8. RFLP restriction enzymes were identified using the same online application, NEBcutter version 2.0, as ACTN3 and are displayed in table 9.

Table 7, Primer Sequences for MYOZ2 and MYOZ3

Gene	Primer Sequence	Direction
MYOZ2	5'-CAATTCCTCCCATTTCAATTCAG-3'	Forward
	5'-GTCACCAACTTCCACACTAC-3'	Reverse
MYOZ3	5'-CATGCAACTGCTGCTCTAGG-3'	Forward
	5'-GCAAAAGGAAAATGCAATCG-3'	Reverse

Table 8, PCR Conditions for both MYOZ2 and MYOZ3

Temp.	Time	
95°C	2.5 minutes	
95°C	45 seconds	35 cycles
54.8°C	15 seconds	
72°C	25 seconds	
72°C	10 minutes	

Table 9, RFLP Restriction Enzymes Identified for MYOZ2 and MYOZ3

Gene	Restriction Enzyme	Digestion Incubation Temp (°C)	Digestion Time (hours)	Agarose Gel Used	Uncut Length	Allele Fragment Length
MYOZ2	*NLAIII*	37	1	Tris-Borate-EDTA (3 %)	155	A - 128bp G - 27bp
MYOZ3	*BspCNI*	25	3		182	A - 150bp G - 32bp

Statistics

Hardy-Weinberg equilibrium test (HWE) was performed on each SNP to ascertain if the observed genotype frequencies matched our expected frequencies using a Chi squared analysis. Comparisons between different athletic groupings (SSP and END) were analysed using Chi squared contingency tables. Odds ratios (OR) and 95 % confidence intervals were calculated to estimate the likelihood of an individual being assigned to one of the athletic groups (SSP or END) depending on the genotype of the individual. The significance of the OR was determined by a Chi squared contingency table. These analyses were performed using Microsoft Excel 2013. Significance was accepted as Chi-squared $p < 0.05$.

For quantitative phenotype associations with genotype, phenotype data was tested for normality using the Ryan-Joiner test. Non-normally distributed data was transformed using a Box-Cox transformation to give better approximations of the normal distribution. The data were z-scored by group and one-way ANOVAs were performed to determine associations between genotypes and specific phenotype data.

The Sidak correction for multiple testing was applied to correct for false discovery using the SISA website (http://www.quantitativeskills.com/sisa/). Differences were accepted as significant where $p < 0.012$ determined by Sidak correction unless stated otherwise. Statistical analyses were performed on Minitab 16.0 statistical software (Minitab Ltd, Coventry, United Kingdom). Data were back-transformed for the purposes of display in tables.

Results

Genotyping

For the purposes of quality control and to ensure the reliability of the genotyping, 15 samples were duplicated across the five 96-well plates and a negative control sample (dH_2O) was present at different locations on each plate. Both repeat samples and negative controls matched with 100 % concordance in all three genotypes suggesting that genotyping was accurate and reliable.

ACTN3

Genotype Distribution

Genotyping was over 99 % successful (405/407 samples successfully genotyped). ACTN3 R577X genotype distributions (RR = 36 %, RX = 52 %, XX = 12 %, see Table 10) was consistent with HWE (p = 0.053). Allele frequencies within the whole cohort were: $f_{(R)} =$ 0.62 and $f_{(X)} =$ 0.38. Notably, no XX-genotype was present in the international level strength athletes (Table 11).

Table 10, ACTN3 Allele Frequency within the Cohort. N = 405, Genotype data presented as n (%). Percentage refers to percentage of a given genotype within specific subgroups. Allele frequency (ƒ) given in percentage.

Genotype	SSP	END	Athletes	Control	ALL
RR	48 (38)	31 (37)	79 (38)	68 (35)	147 (36)
RX	62 (49)	46 (55)	108 (51)	101 (52)	209 (52)
XX	16 (13)	7 (8)	23 (11)	26 (13)	49 (12)
R (ƒ)	*63*	*64*	*63*	*61*	*62*
X (ƒ)	*37*	*36*	*37*	*39*	*37*
n =	126	84	210	195	405

Table 11, Athlete Highest Level of Performance and ACTN3 Genotype. Data presented as n (%) where percentage refers to within specific performance level of a specific sub-group. Although there is no significant difference in genotype distribution between sub-groups, it should be noted that there is no incidence of ACTN3 XX genotype in international level SSP athletes.

Highest Level of Performance	SSP (n = 126)			END (n = 84)		
	RR	**RX**	**XX**	**RR**	**RX**	**XX**
International	4 (50)	4 (50)	**0**	3 (25)	8 (67)	1 (8)
National Level	26 (38)	37 (54)	6 (9)	18 (35)	29 (56)	5 (10)
Area Squad	6 (24)	12 (48)	7 (28)	7 (50)	6 (43)	1 (7)
Other athlete	12 (50)	9 (38)	3 (13)	3 (50)	3 (50)	0

Comparisons between different sub-groups were analysed yet no significant genotype differences between any pair wise combinations of sub-groups was detected ($p > 0.05$).

Given the results of the quality control measures and that the data was consistent with HWE; associations with ACTN3 genotype and performance associations were investigated (Table 12).

Associations

ACTN3 is associated with sprinting speed but no other performance phenotype in the present study (Table 12).

Table 12, Association of ACTN3 Genotype and Performance Phenotypes

N = 405 (RR = 147; RX = 209; XX = 49). ***v*** denotes the percentage of the variance explained by the genotype. LCL denotes lower 95 % confidence limit. UCL denotes upper 95 % confidence limit. CMJ = counter-movement jump. IKLL = combined isokinetic strength for both lower limbs. IKUL = combined isokinetic strength for both upper limbs. Significance accepted as $p < 0.012$. Significant results are in ***Bold Italics***. Results of $p < 0.05$ are in *italics.*

	Genotype	Mean (LCL to UCL)
CMJ height (cm)	**RR**	39.099 (39.017 to 39.18)
	RX	39.117 (38.257 to 39.976)
	XX	38.018 (36.25 to 39.786)
		v = 0, ***p*** = 0.529
Static jump height (cm)	**RR**	32.14 (31.27 to 33.011)
	RX	32.328 (31.603 to 33.054)
	XX	30.975 (29.482 to 32.468)
		v = 0.13, ***p*** = 0.289
Body Mass (kg)	**RR**	76.769 (75.027 to 78.512)
	RX	76.836 (75.402 to 78.27)
	XX	78.007 (77.71 to 78.305)
		v = 0, ***p*** = 0.761
IKLL Extension 30 d/sec., N·m	**RR**	129.966 (123.695 to 136.237)
	RX	130.219 (125.009 to 135.429)
	XX	128.263 (118.017 to 138.509)
		v = 0, ***p*** = 0.93
IKLL Flexion 30 d/sec., N·m	**RR**	129.446 (123.29 to 135.601)
	RX	129.699 (124.528 to 134.87)
	XX	132.763 (122.633 to 142.894)
		v = 0, ***p*** = 0.849
IKLL Extension 90 d/sec., N·m	**RR**	131.002 (124.654 to 137.35)
	RX	129.861 (124.574 to 135.148)
	XX	127.359 (116.939 to 137.779)
		v = 0, ***p*** = 0.843
IKLL Flexion 90 d/sec., N·m	**RX**	130.538 (124.306 to 136.771)
	RX	128.186 (122.976 to 133.396)
	XX	135.446 (125.2 to 145.692)
		v = 0.42, ***p*** = 0.457
IKLL Extension 180 d/sec., N·m	**RR**	130.37 (124.06 to 136.68)
	RX	129.729 (124.442 to 135.016)
	XX	127.999 (127.266 to 128.732)
		v = 0, ***p*** = 0.93

IKLL Flexion 180 d/sec., N·m	**RR**	130.757 (124.428 to 137.086)
	RX	128.264 (122.939 to 133.59)
	XX	133.802 (123.401 to 144.202)
		v **=** 0.27, ***p*** **=** 0.616
IKUL Extension 30 d/sec., N·m	**RR**	133.604 (127.411 to 139.798)
	RX	126.667 (121.495 to 131.838)
	XX	133.122 (122.721 to 143.522)
		v **=** 0.33, ***p*** **=** 0.2
IKUL Flexion 30 d/sec., N·m	**RR**	132.036 (128.813 to 135.258)
	RX	128.681 (125.98 to 131.383)
	XX	130.061 (124.736 to 135.387)
		v **=** 0, ***p*** **=** 0.729
IKUL Extension 90 d/sec., N·m	**RR**	134.08 (127.886 to 140.274)
	RX	126.133 (120.903 to 131.362)
	XX	132.227 (121.942 to 142.511)
		v **=** 0.53, ***p*** **=** 0.142
IKUL Flexion 90 d/sec., N·m	**RR**	131.061 (124.713 to 137.409)
	RX	128.829 (123.522 to 134.135)
	XX	132.025 (121.567 to 142.484)
		v **=** 0, ***p*** **=** 0.804
IKUL Extension 180 d/sec., N·m	**RR**	132.637 (126.424 to 138.85)
	RX	127.03 (121.839 to 132.22)
	XX	133.821 (123.518 to 144.125)
		v **=** 0.13, ***p*** **=** 0.291
IKUL Flexion 180 d/sec., N·m	**RR**	130.605 (124.334 to 136.876)
	RX	128.807 (123.539 to 134.074)
	XX	131.313 (120.855 to 141.772)
		v **=** 0, ***p*** **=** 0.87
Wingate Relative Peak Power (w/kg)	**RR**	10.922 (10.757 to 11.087)
	RX	11.108 (10.972 to 11.244)
	XX	10.784 (10.506 to 11.062)
		v **=** 0.93, ***p*** **=** 0.062
Wingate Fatigue Index (%)	**RR**	10.968 (10.802 to 11.134)
	RX	11.049 (10.913 to 11.185)
	XX	10.925 (10.647 to 11.204)
		v **=** 0, ***p*** **=** 0.638
Sprint 0-10m (s)	**RR**	***1.884 (1.862 to 1.893)***
	RX	***1.867 (1.848 to 1.873)***
	XX	***1.916 (1.882 to 1.937)***
		v = 2.25, p = 0.004
Sprint 10-20m (s)	**RR**	1.337 (1.325 to 1.349)
	RX	1.327 (1.317 to 1.337)
	XX	1.347 (1.325 to 1.368)
		v **=** 0.34, ***p*** **=** 0.19
Spring 20-30m (s)	**RR**	1.26 (1.246 to 1.273)

	RX	1.248 (1.237 to 1.259)
	XX	1.266 (1.243 to 1.289)
		v **=** 0.21, ***p*** **=** 0.245
Sprint Total (s)	**RR**	*4.484 (4.448 to 4.521)*
	RX	*4.441 (4.411 to 4.472)*
	XX	*4.524 (4.46 to 4.588)*
		v **=** *1.13,* ***p*** **=** *0.04*
Relative $\dot{V}O_{2MAX}$ (ml/kg/min)	**RR**	53.259 (51.851 to 54.667)
	RX	55.109 (53.884 to 56.334)
	XX	55.448 (53.081 to 57.815)
		v **=** 0.71, ***p*** **=** 0.105
Body Fat (%)	**RR**	14.469 (13.769 to 15.169)
	RX	14.127 (13.535 to 14.719)
	XX	14.824 (13.609 to 16.038)
		v **=** 0, ***p*** **=** 0.541

In the present study, in line with the hypothesis, an association between ACTN3 genotype and performance was identified. There is an association between ACTN3 genotype and sprinting performance as has previously been documented in the literature [10,22,24]. No association was found between ACTN3 genotype and any other strength, power or endurance related phenotype (Table 12); however, ACTN3 is most widely associated with sprinting speed [10,24]. Thus, it is believe that the cohort is of a sufficient quality to investigate associations between new candidate genes and performance that have not previously been investigated in the literature.

MYOZ2

Genotype Distributions

Genotyping was 100 % successful (407/407 samples genotyped). MYOZ2 genotype distributions (AA = 9 %, AG = 45 %, GG = 46 %. See Table 13) were consistent with HWE (p = 0.285) and allele frequencies within the whole cohort were: $f_{(A)} = 0.31$ and $f_{(G)} = 0.69$.

Table 13, MYOZ2 Allele Frequency Within the Cohort (n = 407).

Genotype data presented as n (%). Percentage refers to within specific subgroups. Allele frequency (f) given in percentage.

Genotype	SSP	END	CON	ALL
AA	7 (6)	11 (13)	17 (9)	35 (9)
AG	56 (44)	36 (43)	92 (47)	184 (45)
GG	63 (50)	37 (44)	88 (45)	188 (46)
A (f)	*70*	*58*	*126*	*254*
G (f)	*182*	*110*	*268*	*560*
n =	126	84	197	407

Table 14, MYOZ2 Genotype and Athlete Highest Level of Performance

Data presented as n (%) where percentage refers to within specific performance level of a specific sub-group.

Highest Level of	SSP (n = 126)			END (n = 84)		
Performance	**AA**	**AG**	**GG**	**AA**	**AG**	**GG**
International	1 (12)	2 (25)	5 (63)	0	5 (42)	7 (58)
National Level	4 (6)	32 (46)	33 (48)	7 (14)	21 (40)	24 (46)
Area Squad	1 (4)	10 (40)	14 (56)	3 (21)	6 (43)	5 (36)
Other athlete	1 (4)	12 (50)	11 (46)	1 (16)	4 (67)	1 (17)

No significant genotype differences were detected between any pair wise combinations of sub-groups ($p > 0.05$).

Associations

MYOZ2 is not associated with any performance phenotype tested. (Supplementary Table 1). A sub analysis was performed on each sub-group independently to investigate whether any associations were masked by training stimulus. No association between any sub-group and any performance-related phenotype was identified (Table 15).

Table 15, Independent Sub-group Analysis of MYOZ2 Genotype and Performance Phenotypes. N = 407 (126, 84, 197 for SSP, END and CON sub-groups respectively). Geno refers to Genotype. ***v*** denotes the percentage of the variance explained by the given genotype. LCL denotes lower 95 % confidence limit. UCL denotes upper 95 % confidence limit. IKLL = combined lower-limb isokinetic strength. IKUL = combined upper-limb isokinetic strength. Significance accepted at p < 0.008 for each sub-group as determined by Sidak correction. Results of p < 0.05 marked in *italics*.

		CON Sub-Group	SSP Sub-Group	END Sub-Group
	Geno	**Mean (LCL to UCL)**	**Mean (LCL to UCL)**	**Mean (LCL to UCL)**
CMJ height (cm)	**AA**	38.307 (35.316 to 41.298)	39.726 (35.055 to 44.397)	38.686 (34.854 to 42.517)
	AG	38.839 (37.535 to 40.143)	39.189 (37.508 to 40.869)	40.59 (38.515 to 42.666)
	GG	39.277 (37.96 to 40.593)	38.728 (32.715 to 44.742)	37.601 (35.613 to 39.588)
		v = 0, ***p*** = 0.806	***v*** = 0, ***p*** = 0.881	***v*** = 2.63, ***p*** = 1.32
Static jump height (cm)	**AA**	30.624 (28.073 to 33.176)	31.917 (27.915 to 35.92)	30.559 (27.347 to 33.772)
	AG	31.905 (30.799 to 33.012)	32.41 (30.97 to 33.85)	33.98 (32.239 to 35.72)
	GG	32.604 (31.475 to 33.732)	31.86 (26.708 to 37.012)	30.803 (30.174 to 31.431)
		v = 0.09, ***p*** = 0.34	***v*** = 0, ***p*** = 0.861	***v*** = *6.6*, ***p*** = *0.026*
Body Mass (kg)	**AA**	73.872 (68.868 to 78.875)	74.936 (67.043 to 82.83)	75.993 (69.651 to 82.336)
	AG	78.693 (76.537 to 80.85)	78.125 (75.438 to 80.813)	77.559 (73.958 to 81.161)
	GG	75.715 (73.495 to 77.935)	76.108 (73.421 to 78.796)	76.676 (73.223 to 80.129)
		v = 1.59, ***p*** = 0.08	***v*** = 0, ***p*** = 0.526	***v*** = 0, ***p*** = 0.896
IKLL Extension 30 d/sec., N.m	**AA**	120.22 (102.275 to 138.165)	104.519 (82.947 to 126.092)	133.319 (110.203 to 156.435)
	AG	132.838 (125.274 to 140.402)	130.088 (120.054 to 140.122)	128.117 (114.996 to 141.238)
	GG	127.831 (119.765 to 135.896)	132.346 (127.522 to 137.17)	130.608 (117.893 to 143.324)
		v = 0, ***p*** = 0.382	***v*** = 0.64, ***p*** = 0.262	***v*** = 0, ***p*** = 0.92
IKLL Flexion 30 d/sec., N.m	**AA**	128.326 (110.671 to 145.982)	121.113 (88.928 to 153.298)	136.123 (113.49 to 158.757)
	AG	133.259 (125.811 to 140.707)	133.304 (123.232 to 143.377)	129.149 (116.298 to 142)
	GG	126.718 (118.826 to 134.61)	127.96 (118.351 to 137.569)	128.788 (116.323 to 141.253)

		v = 0, ***p*** = 0.49	***v*** = 0, ***p*** = 0.646	***v*** = 0, ***p*** = 0.847
	AA	118.036 (99.801 to 136.27)	109.428 (76.799 to 142.057)	129.743 (106.279 to 153.206)
IKLL Extension 90 d/sec., N.m	**AG**	134.338 (126.697 to 141.979)	132.498 (127.288 to 137.708)	131.638 (118.305 to 144.972)
	GG	127.297 (120.64 to 133.954)	129.657 (119.913 to 139.401)	128.384 (121.804 to 134.964)
		v = 0.7, ***p*** = 0.194	***v*** = 0, ***p*** = 0.417	***v*** = 0, ***p*** = 0.943
	AA	129.945 (112.058 to 147.832)	119.199 (86.686 to 151.712)	129.911 (106.872 to 152.95)
IKLL Flexion 90 d/sec., N.m	**AG**	134.112 (126.626 to 141.599)	133.234 (123.065 to 143.403)	128.812 (115.73 to 141.895)
	GG	125.171 (117.183 to 133.16)	128.123 (118.417 to 137.829)	130.988 (118.31 to 143.665)
		v = 0.3, ***p*** = 0.281	***v*** = 0, ***p*** = 0.622	***v*** = 0, ***p*** = 0.973
	AA	115.274 (97.213 to 133.335)	113.67 (81.35 to 145.99)	129.441 (106.113 to 152.77)
IKLL Extension 180 d/sec., N.m	**AG**	135.769 (128.205 to 143.332)	132.763 (122.652 to 142.874)	134.044 (120.807 to 147.281)
	GG	126.222 (118.156 to 134.287)	127.833 (118.088 to 137.577)	126.215 (113.383 to 139.047)
		v = 1.91, ***p*** = 0.063	***v*** = 0, ***p*** = 0.491	***v*** = 0, ***p*** = 0.708
	AA	126.461 (108.4 to 144.522)	123.052 (89.805 to 156.298)	128.344 (105.035 to 151.653)
IKLL Flexion 180 d/sec., N.m	**AG**	133.126 (125.563 to 140.69)	131.91 (121.297 to 142.523)	132.353 (119.116 to 145.59)
	GG	126.99 (118.924 to 135.055)	128.29 (118.275 to 138.304)	128.137 (115.324 to 140.949)
		v = 0, ***p*** = 0.515	***v*** = 0, ***p*** = 0.821	***v*** = 0, ***p*** = 0.895
	AA	124.028 (105.89 to 142.166)	97.018 (66.01 to 128.026)	131.108 (108.243 to 153.974)
IKUL Extension 30 d/sec., N.m	**AG**	126.628 (113.603 to 139.652)	133.895 (124.093 to 143.698)	125.004 (109.22 to 140.788)
	GG	134.869 (127.131 to 142.606)	127.678 (118.339 to 137.017)	134.202 (121.621 to 146.783)
		v = 0.29, ***p*** = 0.282	***v*** = 2.78, ***p*** = 0.082	***v*** = 0, ***p*** = 0.607
	AA	124.863 (106.551 to 143.175)	89.761 (57.788 to 121.734)	127.408 (103.925 to 150.891)
IKUL Flexion 30 d/sec., N.m	**AG**	132.559 (124.899 to 140.22)	136.465 (126.643 to 146.286)	130.414 (117.081 to 143.747)
	GG	127.955 (119.773 to 136.136)	127.541 (117.989 to 137.092)	130.241 (117.313 to 143.17)
		v = 0, ***p*** = 0.617	***v*** = *5.04, **p** = 0.021*	***v*** = 0, ***p*** = 0.975
	AA	122.802 (104.703 to 140.901)	99.725 (68.562 to 130.887)	131.144 (119.393 to 142.895)
IKUL Extension 90 d/sec., N.m	**AG**	128.597 (121.013 to 136.18)	134.011 (124.055 to 143.968)	128.878 (115.795 to 141.96)
	GG	132.874 (124.789 to 140.959)	126.706 (117.135 to 136.277)	130.553 (117.875 to 143.23)
		v = 0, ***p*** = 0.544	***v*** = 2.38, ***p*** = 0.106	***v*** = 0, ***p*** = 0.978
	AA	125.892 (107.542 to 144.243)	93.363 (61.12 to 125.606)	124.267 (101.17 to 147.364)
IKUL Flexion 90 d/sec., N.m	**AG**	132.703 (125.023 to 140.382)	134.892 (124.896 to 144.887)	133.514 (120.393 to 146.635)
	GG	127.586 (119.385 to 135.786)	128.869 (119.241 to 138.498)	128.281 (115.565 to 140.997)
		v = 0, ***p*** = 0.608	***v*** = 3.39, ***p*** = 0.056	***v*** = 0, ***p*** = 0.748
	AA	126.528 (108.39 to 144.666)	105.034 (73.312 to 136.756)	122.876 (100.455 to 145.298)
IKUL Extension 180 d/sec., N.m	**AG**	129.376 (121.774 to 136.979)	134.523 (124.605 to 144.441)	133.494 (120.758 to 146.229)
	GG	131.242 (123.138 to 139.346)	126.444 (116.796 to 136.092)	128.722 (116.372 to 141.071)
		v = 0, ***p*** = 0.88	***v*** = 1.48, ***p*** = 0.167	***v*** = 0, ***p*** = 0.7
	AA	128.346 (109.996 to 146.697)	99.086 (67.364 to 130.808)	116.766 (94.055 to 139.477)
IKUL Flexion 180 d/sec., N.m	**AG**	131.255 (123.556 to 138.954)	133.836 (123.919 to 143.754)	136.193 (123.304 to 149.083)
	GG	128.515 (120.256 to 136.774)	127.564 (117.916 to 137.212)	128.037 (115.534 to 140.541)
		v = 0, ***p*** = 0.881	***v*** = 2.18, ***p*** = 0.113	***v*** = 0.35, ***p*** = 0.325
	AA	10.862 (10.392 to 11.333)	11.73 (10.996 to 12.463)	11.025 (10.45 to 11.601)
Wingate Relative Peak Power (w/kg)	**AG**	10.958 (10.753 to 11.162)	10.967 (10.701 to 11.233)	11.252 (10.903 to 11.6)
	GG	11.072 (10.857 to 11.288)	10.94 (10.693 to 11.187)	10.779 (10.461 to 11.097)

		v = 0, ***p*** = 0.631	***v*** = 1.81, ***p*** = 0.129	***v*** = 2.38, ***p*** = 0.153
	AA	10.966 (10.852 to 11.079)	11.067 (10.334 to 11.8)	11.139 (10.551 to 11.727)
Wingate Fatigue Index (%)	**AG**	10.984 (10.779 to 11.188)	11.116 (10.847 to 11.384)	11.092 (10.736 to 11.448)
	GG	11.021 (10.807 to 11.236)	10.887 (10.635 to 11.139)	10.877 (10.554 to 11.201)
		v = 0, ***p*** = 0.96	***v*** = 0, ***p*** = 0.471	***v*** = 0, ***p*** = 0.603
	AA	1.879 (1.868 to 1.89)	1.884 (1.813 to 1.956)	1.866 (1.81 to 1.922)
Sprint 0-10m (s)	**AG**	1.876 (1.857 to 1.896)	1.873 (1.847 to 1.899)	1.863 (1.831 to 1.894)
	GG	1.882 (1.861 to 1.903)	1.884 (1.86 to 1.908)	1.9 (1.868 to 1.931)
		v = 0, ***p*** = 0.926	***v*** = 0, ***p*** = 0.839	***v*** = 1.12, ***p*** = 0.24
	AA	1.352 (1.316 to 1.387)	1.313 (1.258 to 1.369)	1.319 (1.275 to 1.362)
Sprint 10-20m (s)	**AG**	1.335 (1.32 to 1.351)	1.342 (1.322 to 1.362)	1.32 (1.296 to 1.345)
	GG	1.327 (1.311 to 1.343)	1.328 (1.309 to 1.347)	1.351 (1.326 to 1.375)
		v = 0, ***p*** = 0.428	***v*** = 0, ***p*** = 0.477	***v*** = 1.79, ***p*** = 0.184
	AA	1.27 (1.231 to 1.309)	1.229 (1.169 to 1.289)	1.248 (1.2 to 1.296)
Spring 20-30m (s)	**AG**	1.251 (1.234 to 1.268)	1.261 (1.239 to 1.283)	1.249 (1.235 to 1.263)
	GG	1.255 (1.238 to 1.272)	1.252 (1.232 to 1.272)	1.262 (1.249 to 1.276)
		v = 0, ***p*** = 0.67	***v*** = 0, ***p*** = 0.582	***v*** = 0, ***p*** = 0.755
	AA	4.499 (4.391 to 4.607)	4.426 (4.256 to 4.595)	4.428 (4.295 to 4.561)
Sprint Total (s)	**AG**	4.463 (4.416 to 4.51)	4.477 (4.416 to 4.539)	4.429 (4.355 to 4.504)
	GG	4.465 (4.417 to 4.513)	4.463 (4.406 to 4.52)	4.516 (4.442 to 4.589)
		v = 0, ***p*** = 0.833	***v*** = 0, ***p*** = 0.838	***v*** = 1.22, ***p*** = 0.23
	AA	58.076 (54.035 to 62.118)	50.025 (42.724 to 57.325)	52.183 (46.983 to 57.383)
Relative VO2max (ml/kg/min)	**AG**	53.304 (47.912 to 58.696)	54.111 (51.777 to 56.444)	54.855 (51.905 to 57.805)
	GG	54.989 (53.131 to 56.848)	55.239 (53.022 to 57.456)	54.808 (52.066 to 57.549)
		v = 1.69, ***p*** = 0.087	***v*** = 0, ***p*** = 0.376	***v*** = 0, ***p*** = 0.651
	AA	14.332 (12.278 to 16.385)	13.367 (10.169 to 16.566)	14.82 (12.153 to 17.487)
Body Fat (%)	**AG**	14.811 (13.916 to 15.706)	14.921 (13.793 to 16.049)	13.491 (12.086 to 14.896)
	GG	13.827 (12.906 to 14.748)	13.911 (12.839 to 14.983)	15.02 (13.633 to 16.408)
		v = 0.13, ***p*** = 0.328	***v*** = 0, ***p*** = 0.375	***v*** = 0.53, ***p*** = 0.301

MYOZ3

Genotype Distribution

MYOZ3 genotyping was 100 % successful (407/407 samples genotyped). Due to only one G-allele homozygote being identified for MYOZ3, this individual was grouped with all other G-allele carriers and analysis was performed on G-allele carriers and AA-homozygotes (Table 16). MYOZ3 genotype distributions (AA = 84 %, G-allele carriers = 16 %) was consistent with HWE (p = 0.122) and allele frequencies within the whole cohort were: $ƒ_{(A)} = 0.91$ and $ƒ_{(G)} = 0.09$.

Table 16, MYOZ3 Allele Frequency within the Cohort (n = 407).

Genotype data presented as n (%). Percentage refers to within specific subgroups. Allele frequency (ƒ) given in percentage.

Genotype	SSP	END	CON	ALL
AA	107 (85	64 (76)	170 (86)	341 (84)
G	19 (15)	20 (24)	27 (14)	66 (16)
A (ƒ)	*92*	*88*	*93*	*92*
G (ƒ)	*8*	*12*	*7*	*8*
N =	126	84	197	407

Table 17, MYOZ3 Genotype and Athlete Highest Level of Performance.

Data presented as n (%) where percentage refers to within a specific sub-group.

	SSP (n = 126)		END (n = 84)	
Highest Level of Performance	**AA**	**G-Allele Carriers**	**AA**	**G-Allele Carriers**
International	8 (100)	0	8 (67)	4 (33)
National Level	55 (80)	14 (20)	40 (77)	12 (23)
Area Squad	23 (92)	2 (8)	10 (71)	4 (29)
Other athlete	21 (88)	3 (12)	6 (100)	0

No significant genotype differences between any pair wise combinations of sub-groups was detected ($p > 0.05$); however, notably no incidence of G-allele carriers was found within the international level SSP sub-group of athletes.

Associations

MYOZ3 genotype is significantly associated with relative $\dot{V}O_{2MAX}$ and upper-limb isokinetic flexion strength at 90 d/sec (N·m) but no other performance related phenotypes (Table 18).

Table 18, Association of MYOZ3 Genotype and Performance Phenotypes.

N = 407 (AA = 341; G-allele carriers 66). ***v*** denotes the percentage of the variance explained by the genotype. LCL denotes lower 95 % confidence limit. UCL denotes upper 95 % confidence limit. CMJ = countermovement jump. IKLL = combined isokinetic strength for both lower limbs. IKUL = combined isokinetic strength for both upper limbs. Significance accepted as $p < 0.012$. Significant results marked in ***bold italics***. Results of $p < 0.05$ marked in *italics.*

	Genotype	Mean (LCL to UCL)
CMJ height (cm)	**AA**	38.748 (38.077 to 39.419)
	G-allele carriers	40.218 (38.707 to 41.729)
		v **=** 0.51, ***p*** **=** 0.083
Static jump height (cm)	**AA**	*31.812 (31.242 to 32.381)*
	G-allele carriers	*33.602 (32.312 to 34.891)*
		v ***= 1.29,*** ***p*** ***= 0.014***
Body Mass (kg)	**AA**	77.344 (76.217 to 78.47)
	G-Allele Carriers	74.937 (72.472 to 77.401)
		v **=** 0.46, ***p*** **=** 0.094
IKLL Extension 30 d/sec., N·m	**AA**	130.8 (126.728 to 134.871)
	G-allele carriers	125.005 (116.013 to 133.997)
		v **=** 0.09, ***p*** **=** 0.251
IKLL Flexion 30 d/sec., N·m	**AA**	131.052 (129.007 to 133.098)
	G-allele carriers	124.976 (116.081 to 133.872)
		v **=** 0.13, ***p*** **=** 0.224
IKLL Extension 90 d/sec., N·m	**AA**	131.357 (127.228 to 135.486)
	G-allele carriers	123.12 (113.954 to 132.285)

		v **=** 0.42, ***p*** **=** 0.109
	AA	130.594 (126.523 to 134.665)
IKLL Flexion 90 d/sec., N·m	**G-allele carriers**	126.884 (117.853 to 135.914)
		v **=** 0, ***p*** **=** 0.464
	AA	130.53 (126.42 to 134.64)
IKLL Extension 180 d/sec., N·m	**G-allele carriers**	126.164 (117.037 to 135.291)
		v **=** 0, ***p*** **=** 0.394
	AA	130.671 (126.541 to 134.8)
IKLL Flexion 180 d/sec., N·m	**G-allele carriers**	125.886 (116.759 to 135.013)
		v **=** 0, ***p*** **=** 0.351
	AA	130.175 (126.103 to 134.246)
IKUL Extension 30 d/sec., N·m	**G-allele carriers**	127.261 (118.153 to 136.368)
		v **=** 0, ***p*** **=** 0.568
	AA	*131.858 (127.767 to 135.949)*
IKUL Flexion 30 d/sec., N·m	**G-allele carriers**	*120.39 (111.186 to 129.594)*
		v *= 1.05,* ***p*** *= 0.027*
	AA	130.457 (126.385 to 134.528)
IKUL Extension 90 d/sec., N·m	**G-allele carriers**	125.284 (116.138 to 134.43)
		v **=** 0.01, ***p*** **=** 0.313
	AA	***132.296 (128.205 to 136.387)***
IKUL Flexion 90 d/sec., N·m	**G-allele carriers**	***118.281 (109.115 to 127.446)***
		v = 1.71, p = 0.007
	AA	130.62 (126.568 to 134.672)
IKUL Extension 180 d/sec., N·m	**G-allele carriers**	125.229 (116.16 to 134.298)
		v **=** 0.03, ***p*** **=** 0.289
	AA	*131.721 (127.65 to 135.792)*
IKUL Flexion 180 d/sec., N·m	**G-allele carriers**	*119.163 (109.997 to 128.328)*
		v *= 1.34,* ***p*** *= 0.015*
	AA	10.992 (10.884 to 11.1)
Wingate Relative Peak Power (w/kg)	**G-allele carriers**	11.03 (10.791 to 11.269)
		v **=** 0, ***p*** **=** 0.777
	AA	11.03 (10.923 to 11.138)
Wingate Fatigue Index (%)	**G-allele carriers**	10.843 (10.604 to 11.082)
		v **=** 0.25, ***p*** **=** 0.162
	AA	1.879 (1.869 to 1.889)
Sprint 0-10m (s)	**G-allele carriers**	1.881 (1.857 to 1.904)
		v **=** 0, ***p*** **=** 0.898
	AA	1.334 (1.326 to 1.342)
Sprint 10-20m (s)	**G-allele carriers**	1.328 (1.31 to 1.346)
		v **=** 0, ***p*** **=** 0.569
	AA	1.254 (1.237 to 1.272)
Spring 20-30m (s)	**G-allele carriers**	1.257 (1.237 to 1.276)
		v **=** 0, ***p*** **=** 0.831
	AA	4.469 (4.445 to 4.493)
Sprint Total (s)	**G-allele carriers**	4.459 (4.403 to 4.515)

		v = 0, ***p =*** 0.754
	AA	***53.967 (53.042 to 54.892)***
Relative $\dot{V}O_{2MAX}$ (ml/kg/min)	**G-allele carriers**	***56.914 (54.889 to 58.939)***
		v = 1.55, p = 0.01
	AA	14.482 (14.024 to 14.94)
Body Fat (%)	**G-allele carriers**	13.57 (12.524 to 14.616)
		v = 0.36, ***p =*** 0.119

Following identification of significant results pertaining to MYOZ3 genotype and performance phenotypes, an independent analysis of each individual sub-group was performed in an attempt to identify the root of the significant result within the entire cohort (Table 19). Associations similar to the whole cohort analysis were observed within the subgroups; however, due to the multiple testing corrections, no results reached significance ($p < 0.008$).

Table 19, Independent Sub-group Analysis of MYOZ3 Genotype and Performance Phenotypes.

N = 407 (126, 84, 197 for SSP, END and CON respectively). Geno refers to genotype. AA denotes AA-homozygosity. G-AC denotes G-allele carriers. ***v*** denotes the percentage of the variance explained by the given genotype. LCL denotes lower 95 % confidence limit. UCL denotes upper 95 % confidence limit. IKLL = combined lower-limb isokinetic strength. IKUL = combined upper-limb isokinetic strength. Significance accepted at $p < 0.008$ for each sub-group as determined by Sidak correction. Results of $p < 0.05$ marked in *italics*.

		CON Sub-Group	SSP Sub-Group	END Sub-Group
	Geno	**Mean (LCL to UCL)**	**Mean (LCL to UCL)**	**Mean (LCL to UCL)**
CMJ height (cm)	**AA**	38.675 (37.734 to 39.616)	38.933 (37.722 to 40.143)	38.634 (37.06 to 40.208)
	G-AC	41.005 (38.61 to 43.401)	39.295 (36.467 to 42.123)	40.071 (37.325 to 42.817)
		v **=** 1.1, **p** = 0.078	***v*** **=** 0, ***p*** **=** 0.818	***v*** **=** 0, ***p*** **=** 0.377
Static jump height (cm)	**AA**	*31.76 (30.959 to 32.56)*	31.914 (30.867 to 32.962)	31.782 (30.433 to 33.13)
	G-AC	*34.34 (32.299 to 36.382)*	33.124 (30.706 to 35.541)	33.094 (30.741 to 35.447)
		v **=** *2.18,* ***p*** **=** *0.022*	***v*** **=** 0, ***p*** **=** 0.37	***v*** **=** 0, ***p*** **=** 0.377
Body Mass (kg)	**AA**	77.301 (75.697 to 78.905)	77.54 (75.5 to 79.58)	77.129 (74.473 to 79.785)
	G-AC	74.681 (69.486 to 79.876)	73.752 (68.995 to 78.508)	76.394 (71.719 to 81.068)
		v **=** 0.19, ***p*** **=** 0.243	***v*** **=** 0.87, ***p*** **=** 0.154	***v*** **=** 0, ***p*** **=** 0.377
IKLL Extension 30 d/sec., N·m	**AA**	129.26 (123.568 to 134.952)	*133.261 (126.006 to 140.516)*	131.087 (126.108 to 136.065)
	G-AC	131.737 (117.632 to 145.842)	*113.613 (97.095 to 130.13)*	126.583 (109.95 to 143.216)
		v **=** 0, ***p*** **=** 0.75	***v*** **=** *3.13,* ***p*** **=** *0.035*	***v*** **=** 0, ***p*** **=** 0.377
IKLL Flexion 30 d/sec., N·m	**AA**	130.593 (125.016 to 136.169)	132.133 (124.781 to 139.484)	130.541 (120.951 to 140.131)
	G-AC	126.502 (123.067 to 129.936)	119.407 (102.601 to 136.214)	128.164 (111.82 to 144.507)
		v **=** 0, ***p*** **=** 0.592	***v*** **=** 0.76, ***p*** **=** 0.177	***v*** **=** 0, ***p*** **=** 0.377
IKLL Extension 90 d/sec., N·m	**AA**	130.233 (124.463 to 136.002)	*133.538 (126.167 to 140.909)*	130.918 (120.98 to 140.855)
	G-AC	128.057 (113.662 to 142.451)	*111.814 (94.969 to 128.659)*	127.073 (110.17 to 143.976)
		v **=** 0, ***p*** **=** 0.784	***v*** **=** *3.78,* ***p*** **=** *0.022*	***v*** **=** 0, ***p*** **=** 0.377
IKLL Flexion 90 d/sec., N·m	**AA**	130.199 (124.546 to 135.853)	131.424 (123.956 to 138.891)	130.329 (120.584 to 140.073)
	G-AC	128.265 (114.199 to 142.332)	122.887 (105.83 to 139.944)	128.778 (112.183 to 145.372)
		v **=** 0, ***p*** **=** 0.803	***v*** **=** 0, ***p*** **=** 0.371	***v*** **=** 0, ***p*** **=** 0.377
IKLL Extension 180 d/sec., N·m	**AA**	129.646 (123.896 to 135.396)	132.416 (125.046 to 139.787)	129.924 (120.006 to 139.842)
	G-AC	131.692 (117.356 to 146.029)	114.181 (97.413 to 130.949)	129.949 (113.066 to 146.833)
		v **=** 0, ***p*** **=** 0.796	***v*** **=** 2.48, ***p*** **=** 0.054	***v*** **=** 0, ***p*** **=** 0.377
IKLL Flexion 180 d/sec., N·m	**AA**	130.055 (124.362 to 135.747)	131.228 (123.491 to 138.966)	131.549 (121.708 to 141.39)
	G-AC	129.161 (114.979 to 143.344)	121.831 (104.446 to 139.217)	125.244 (108.476 to 142.012)
		v **=** 0, ***p*** **=** 0.909	***v*** **=** 0, ***p*** **=** 0.336	***v*** **=** 0, ***p*** **=** 0.377
IKUL Extension 30 d/sec., N·m	**AA**	128.94 (126.026 to 131.853)	130.848 (123.497 to 138.2)	132.647 (122.98 to 142.314)
	G-AC	136.065 (128.79 to 143.339)	119.601 (102.582 to 136.619)	122.066 (105.626 to 138.506)
		v **=** 0, ***p*** **=** 0.365	***v*** **=** 0.38, ***p*** **=** 0.237	***v*** **=** 0.25, ***p*** **=** 0.377
IKUL Flexion 30 d/sec., N·m	**AA**	130.616 (127.683 to 133.549)	131.895 (124.466 to 139.324)	135.425 (125.797 to 145.054)
	G-AC	125.684 (118.371 to 132.997)	119.405 (101.672 to 137.137)	114.024 (97.565 to 130.483)
		v **=** 0, ***p*** **=** 0.533	***v*** **=** 0.54, ***p*** **=** 0.206	***v*** **=** 5.03, ***p*** **=** 0.377
IKUL Extension 90 d/sec., N·m	**AA**	129.609 (123.898 to 135.321)	130.593 (123.222 to 137.964)	132.709 (123.042 to 142.376)
	G-AC	131.918 (117.717 to 146.12)	118.538 (112.537 to 124.539)	121.886 (105.427 to 138.345)
		v **=** 0, ***p*** **=** 0.768	***v*** **=** 0.5, ***p*** **=** 0.218	***v*** **=** 0.32, ***p*** **=** 0.377
IKUL Flexion 90 d/sec., N·m	**AA**	131.668 (125.938 to 137.399)	131.721 (124.254 to 139.189)	135.135 (125.622 to 144.647)
	G-AC	119.168 (104.889 to 133.447)	120.739 (102.967 to 138.51)	114.865 (98.657 to 131.074)
		v **=** 0.82, ***p*** **=** 0.113	***v*** **=** 0.22, ***p*** **=** 0.267	***v*** **=** 4.53, ***p*** **=** 0.377
IKUL Extension 180 d/sec., N·m	**AA**	129.97 (124.278 to 135.663)	130.465 (123.055 to 137.874)	132.781 (123.326 to 142.236)
	G-AC	129.682 (122.446 to 136.918)	122.385 (105.038 to 139.732)	121.679 (105.586 to 137.772)
		v **=** 0, ***p*** **=** 0.971	***v*** **=** 0, ***p*** **=** 0.404	***v*** **=** 0.49, ***p*** **=** 0.377
IKUL Flexion 180	**AA**	131.129 (125.379 to 136.879)	130.893 (123.561 to 138.226)	134.866 (125.372 to 144.359)

d/sec., N·m	**G-AC**	121.543 (106.956 to 136.131)	119.594 (102.325 to 136.864)	115.643 (99.473 to 131.813)
		v = 0.23, ***p*** = 0.233	***v*** = 0.35, ***p*** = 0.241	***v*** = 3.98, ***p*** = 0.377
Wingate Relative Peak Power (w/kg)	**AA**	11.016 (10.863 to 11.168)	10.975 (10.782 to 11.169)	10.952 (10.695 to 11.209)
	G-AC	10.888 (10.508 to 11.269)	11.117 (10.672 to 11.563)	11.129 (10.696 to 11.563)
		v = 0, ***p*** = 0.543	***v*** = 0, ***p*** = 0.567	***v*** = 0, ***p*** = 0.377
Wingate Fatigue Index (%)	**AA**	*11.056 (10.906 to 11.206)*	10.988 (10.794 to 11.181)	11.028 (10.771 to 11.285)
	G-AC	*10.635 (10.259 to 11.011)*	11.052 (10.607 to 11.498)	10.912 (10.478 to 11.345)
		v *= 1.64,* ***p*** *= 0.043*	***v*** = 0, ***p*** = 0.795	***v*** = 0, ***p*** = 0.377
Sprint 0-10m (s)	**AA**	1.88 (1.865 to 1.894)	1.875 (1.856 to 1.893)	1.881 (1.857 to 1.905)
	G-AC	1.872 (1.835 to 1.909)	1.899 (1.856 to 1.942)	1.872 (1.83 to 1.914)
		v = 0, ***p*** = 0.719	***v*** = 0.01, ***p*** = 0.316	***v*** = 0, ***p*** = 0.377
Sprint 10-20m (s)	**AA**	1.334 (1.323 to 1.346)	1.334 (1.319 to 1.348)	1.331 (1.312 to 1.35)
	G-AC	1.32 (1.291 to 1.349)	1.328 (1.294 to 1.361)	1.337 (1.305 to 1.37)
		v = 0, ***p*** = 0.375	***v*** = 0, ***p*** = 0.752	***v*** = 0, ***p*** = 0.377
Spring 20-30m (s)	**AA**	1.256 (1.243 to 1.268)	1.254 (1.238 to 1.27)	1.248 (1.232 to 1.265)
	G-AC	1.244 (1.213 to 1.275)	1.255 (1.219 to 1.292)	1.272 (1.236 to 1.307)
		v = 0, ***p*** = 0.515	***v*** = 0, ***p*** = 0.952	***v*** = 0.34, ***p*** = 0.377
Sprint Total (s)	**AA**	4.473 (4.44 to 4.507)	4.463 (4.419 to 4.507)	4.461 (4.405 to 4.518)
	G-AC	4.418 (4.328 to 4.509)	4.484 (4.381 to 4.586)	4.482 (4.382 to 4.582)
		v = 0.12, **p** = 0.268	***v*** = 0, ***p*** = 0.722	***v*** = 0, ***p*** = 0.377
Relative $\dot{V}O_{2MAX}$ (ml/kg/min)	**AA**	54.216 (52.974 to 55.457)	53.92 (52.203 to 55.637)	53.403 (51.293 to 55.514)
	G-AC	56.081 (52.756 to 59.406)	57.134 (53.417 to 60.851)	57.788 (54.917 to 60.66)
		v = 0.04, ***p*** = 0.302	***v*** = 1.25, ***p*** = 0.128	***v*** = 3.95, ***p*** = 0.377
Body Fat (%)	**AA**	14.552 (13.895 to 15.209)	14.445 (13.623 to 15.266)	14.36 (13.288 to 15.432)
	G-AC	12.948 (11.296 to 14.599)	13.707 (11.762 to 15.653)	14.246 (12.344 to 16.148)
		v = 1.11, ***p*** = 0.079	***v*** = 0, ***p*** = 0.496	***v*** = 0, ***p*** = 0.377

Discussion

Genetic diversity significantly contributes to performance-related phenotypes. In the present study, the associations of ACTN3, MYOZ2 and MYOZ3 genotypes on performance related phenotypes was investigated in a large Lithuanian cohort of athletes and controls. Unfortunately, due to the size of the cohort and the low EMAF of MYOZ1 (Calsarcin-2) SNPs, this study was unable to further the work of Seto *et al.* [40] regarding Calsarcin-2. However, Calsarcin-1 and Calsarcin-3 (MYOZ2 and MYOZ3) are suitable candidates as they are similarly linked to α-Actinins and are both present in skeletal muscle [18,44] performing similar roles. The large phenotype database encompasses a complex range of strength, power, and endurance, and anthropometrical data which is believed to be more in-depth than in previous literature and presents the unique advantage of investigating the association of these genes with complex quantitative performance-related traits in a spectrum of detail not previously reported.

The present study identified certain associations with two out of the three SNPs and quantitative performance-related phenotypes. The R-allele of ACTN3 is associated with sprinting speed which is in line with previous literature [10,22,24]. The MYOZ2 rs9995277 SNP was found not to be associated with any physiological measure (Supplementary Table 1); however, the MYOZ3 G-allele is associated with increased relative $\dot{V}O_{2MAX}$ and lower isokinetic upper-limb flexion strength at 90 d/sec (N·m) compared to AA-homozygotes.

Genotype distribution for all three genes were consistent with HWE (p = 0.053, 0.285 and 0.122 for ACTN3, MYOZ2 and MYOZ3 respectively). ACTN3 genotype distribution was similar

to findings in previous research [10,24] including a Lithuanian athletic cohort [54]. It is believed by this study that currently no genotype distribution data exists for MYOZ2 and MYOZ3 as this are the first investigation of these genotypes in this context in humans. However, MYOZ2 allele frequencies in the cohort are identical to the EMAF reported in the 1000 Genomes database ($f_{(A)}$ = 0.31) and similar for MYOZ3 (EMAF $f_{(G)}$ 0.10; present study $f_{(G)}$ 0.09) suggesting that the genotyping is accurate and reliable. This is further strengthened with the quality control measures which have been previously described and matched with 100 % concordance. Tables 10, 13 and 16 details the number and frequency percentage of ACTN3, MYOZ2 and MYOZ3 by sub-group respectively. There was no difference in frequency of any allele or genotype between END, SSP, or CON subgroups ($p > 0.05$) which, for ACTN3, is in line with some [28,34,35,55] but not all [10,22,32] literature.

ACTN3

The initial findings demonstrate that ACTN3 R577X genotype influences sprint performance but no other power-related, strength or endurance phenotype (Table 12). The in-depth phenotype data-set allowed the investigation of 30m sprint-time by 10m stages. An association between ACTN3 genotype and 0-10m sprint-time was found but not with total sprint-time as previously reported in other literature [24]. Post-hoc analysis showed that the RR and RX-genotype participants were significantly faster than the XX-homozygotes which is in line with a similar study by Moran *et al.* [24]. Upon correction for sub-group, the SSP group were identified to be the fastest overall, compared to both the END and CON sub-groups ($p < 0.01$). This suggests in-line with other data [10,22,32] that the ACTN3 R-allele is an important performance-related factor in power-orientated sport.

Lack of a lower prevalence of the XX genotype in the SSP sub-group overall is contradictory to what has been reported previously in some strength and or power based cohorts [10,22,29,32,56] but not all [28,30,35,55]. The SSP sub-group does contain athletes which are spread across a range of competitive levels, from non-competitive to Olympians (Table 11), and a multitude of sports, some of which have data suggesting little to no role of ACTN3 genotype on performance [57,58]. This may account for the genotype distribution results obtained; however, it should be noted that there was no case of the ACTN3 XX-genotype in international level SSP athletes (Table 11) which is in line with other data [10,22] and suggests that the ACTN3 R-allele is strongly associated with sprint performance at the most elite level.

Associations with other power-related phenotypes including standing and counter-movement jumps and Wingate test were explored; however, no association was found between ACTN3 and any other power-related phenotype (Table 12). The data is in line with Ruiz *et al.* [57] who investigated the association of ACTN3 R577X genotype on explosive leg-power in elite female volley-ball players. Similar to data in the present study (Table 12), the authors established that ACTN3 appears to have no effect on explosive leg-power, determined by squat and counter-movement jumps by Ruiz *et al.* [57] and in the present study.

Seto *et al.* [40] revealed that in absence of α-Actinin-3 (ACTN3 XX-genotype in humans and ACTN3 KO in mice); α-Actinin-2 out-competes calcineurin for binding with Calsarcin-2 (MYOZ1 gene). The authors postulated that this activated calcineurin could account for the shift in oxidative properties of fast-twitch fibres - of which Calsarcin 1, 2 and 3 are present – leading to an enhanced response to endurance training in ACTN3 KO mice compared to WT littermates. The present study was unable to identify a suitable MYOZ1 (Calsarcin-2)

candidate and thus could not replicate the work of Seto *et al.* [40]; however, we investigated associations between both MYOZ2 and MYOZ3 genotypes and performance-related phenotypes in a sub-set of ACTN3 XX-homozygotes (N = 49, CON = 26; SSP = 16; END = 7). No association was identified with any anthropomorphic, strength, power or endurance-related phenotype (data not shown) suggesting, in line with the yeast-2-hybrid experiment performed by Seto and colleagues [40] that there is possibly no interaction between α-Actinin-3 XX-genotype and Calsarcin-1 and -3.

Findings in the present study are in line with what would be expecting regarding ACTN3 and performance given previous research [10,24,39] and are in line with our hypothesis. This data provides additional evidence that ACTN3 577R allele is strongly associated with sprinting speed [10,22,24,33] but less so for other indices of strength or power in males [25,27]. Similarly the data provides more evidence which suggests no association of ACTN3 577X allele on any endurance-related phenotype [16,24,36]. The quality control measures and ACTN3 results suggest the current cohort was suitable for investigating genotypes which have not previously been investigated in this context in humans.

MYOZ2

The present study is the first to investigate the MYOZ2 rs9995277 gene with complex quantitative performance-related phenotypes in a cohort comprising both athletes and control participants. MYOZ2 rs9995277 (Calsarcin-1) is not associated with any performance phenotype (Supplementary Table 1).

In humans, Calsarcin-1 (MYOZ2) is expressed in cardiac and oxidative type-1 and type-IIa skeletal muscle fibres [18]. Although little research exists pertaining to the role of calsarcin-1 in skeletal muscle, there is much more research based around the association of

Calsarcin-1 and cardiac hypertrophy. Mice lacking Calsarcin-1 have shown increased sensitivity to calcineurin signalling - as would be expected given the role of the Calsarcin in tethering calcineurin. Over activation of calcineurin results in the stimulation of pathological cardiac hypertrophy [59] and increased number of type I, slow twitch muscle fibres in Calsarcin-1 KO mice [60].

Variation in the 3'UTR of MYOZ2 rs9995277 (G → A) causes the loss of miRNA binding sites for the miR-30 family. Similar to Calsarcin-1, miR-30 is present in both cardiac and skeletal muscle [49]. In cardiac muscle, the down-regulation of miR-30 promotes myocardial hypertrophy through excessive autophagy [61]. In skeletal muscle, miR-30a, -c, -d, and –e are found to be significantly up-regulated during myogenic differentiation in CD56+ primary myoblasts and myotubes isolated from healthy humans in a study by Dmitriev and colleagues [49]. The authors confirmed that this miRNA family may target genes involved in: the regulation of protein phosphorylation and kinase activity, cell cycle control, intracellular transport, cytoskeleton organisation, protein ubiquitination, DNA damage response and nucleotide bio-synthesis in human skeletal muscle. In zebra fish, inhibition of the miRNA-30 family increased numbers of superficial slow-muscle fibres during embryonic development [53].

MYOZ2 rs9995277 genotype appears to have no effect on any performance-related phenotype in the participants that comprise the cohort in this study.

MYOZ3

The present study is the first to investigate and subsequently associate MYOZ3 rs116090320 (Calsarcin-3) with complex, quantitative performance-related phenotypes in a large Lithuanian cohort of athletes and controls. The in-depth phenotype database allows the

investigation of associations between genes and performance in a spectrum of detail. G-allele carriers appear to have an increased relative $\dot{V}O_{2MAX}$ ($p = 0.01$) compared to AA-homozygotes but also decreased strength in upper-limb flexion isokinetic dynamometry performed at 90 d/sec (N·m. $p < 0.01$). In the independent sub-group analysis, there appears to be a trend toward G-allele carriers being weaker than AA-homozygotes in a number of isokinetic tests flexion tests of strength, particularly in the END sub-group; however, due to the multiple testing correction applied in this analysis ($p < 0.008$), none of these results reached significance.

Calsarcin-3, encoded by the MYOZ3 gene is exclusively expressed in skeletal muscle [44] with enrichment in fast-type fibres. As Calsarcins tether calcineurin to α-Actinin at the sarcomeric z-lines, any variation within the Calsarcins may alter calcineurin activity and subsequently may affect promote a more oxidative phenotype which has been observed in mouse models and proposed in humans [40,45] although only for Calsarcin-1.

Calcineurin activation has been shown to promote differentiation and fibre type specialisation toward a slow-twitch phenotype and is dependent upon the frequency of motor neuron activity [41,42]. Prolonged periods of tonically active motor neurones stimulate a shift towards the slow-twitch oxidative myofibre phenotypes through changes in intracellular concentrations of several signalling molecules including calcium (Ca^{2+}). As calcineurin is a Ca^{2+}-calmodulin dependent phosphatase, increased levels of Ca^{2+} will lead to calcineurin activation [62].

Activated calcineurin causes the dephosphorylation of number of transcription factors including members of the nuclear family of activate T-cells (NFATs) from the cytoplasm to the nucleus [63] at specific points during myocyte generation and formation and this pathway is known as the Ca^{2+} / Calcineurin / NFAT pathway. During myocyte

development, NFATc3 is translocated into myoblast nuclei, NFATc2 is shuttled into emerging myotube nuclei and NFATc1 moves into mature myoblast nuclei. At this point, molecular or tonic motor nerve activity results in increased calcineurin mediated signalling, altering the phenotype of the skeletal muscle and leading to hypertrophy or fibre type switching toward a slow myogenic profile respectively [62,64].

The MYOZ3 rs116090320 polymorphism results in both losses and gains of miRNA binding sites. MiR-374b and miR-4524b binding sites are lost and there is a gain in binding sites for miR-4291 (Table 6). As the G-allele of MYOZ3 is associated with endurance related phenotypes, even though it is predominantly expressed in fast-type fibres [44], it could be suggested that the role of these miRNA's may alter the function of the Calsarcin-3 protein, increasing calcineurin activity and resulting in fast-twitch fibres eliciting a more oxidative phenotype. If fast-fibres were to elicit a more oxidative phenotype, this may help explain the significantly reduced upper-limb flexion strength observed. A number of isokinetic upper-limb flexion tests appeared to trend toward being lower in G-allele carriers however, only flexion strength at 90 d/sec (N·m) achieved significance. MiR-374b is found in skeletal muscle and up-regulated during myogenic differentiation [49]. Given that the G-allele carriers see a reduction in binding sites for this miRNA, it is postulated that this miRNA may be associated with muscular strength or mass which may explain the observed reduction in upper-limb flexion strength (Table 18). Neither miR-4291 nor miR-4524a have been associated with any skeletal muscle phenotypes however, similarly to miR-374b, affecting their binding sites with MYOZ3 (G-allele carriers) may potentially have an effect on performance and thus the role of these miRNA's warrants further investigation.

The Ca^{2+}-calcineurin-NFAT mechanism [41,64] may explain the MYOZ3 result in the present study. Although it cannot be deduced from the current study, work by Seto *et al.* [40]

suggests that enhanced endurance related phenotypes may be the result of activated calcineurin and a resulting shift in fibre-type properties toward a slower, more oxidative phenotype. The G-allele carriers in this cohort are aerobically superior compared to their AA-homozygotic co-participants regardless of athlete sub-group although they appear to have reduced upper-limb flexion strength.

Moving Forward

These results demonstrate for the first time, the association of a calcineurin-tethering, skeletal muscle specific gene (MYOZ3) with quantitative performance-related phenotypes in humans. The in-depth phenotype data-set has given this study the ability to investigate associations with specific components of otherwise complex performance phenotypes.

Due to the criteria for selecting suitable SNPs and the size of the cohort, the present study was unable to identify a suitable SNP for MYOZ1 (Calsarcin-2) with a minor allele of a suitable frequency (≥ 5 %) at the time of searching. Thus, unfortunately, this study was unable to further the work of Seto and colleagues [40] however, such a study would be beneficial to the wider literature.

The G-allele carriers of MYOZ3 rs116090320 demonstrate an increased relative $\dot{V}O_{2MAX}$ and decrease upper-limb flexion strength. It is proposed that this research is repeated in another cohort to investigate the reproducibility of the current findings. Further to this, investigation of the roles of micro-RNA's associated with MYOZ3 rs116090320 to discern other genes that they may be associated with and their link within skeletal muscle differentiation and proliferation should be conducted.

Limitations

Although this cohort is relatively large, containing both endurance and strength athletes as well as control participants and has what we believe to be the most in-depth exercise-performance related phenotypic dataset, there are limitations. The size of the cohort meant we were unable to identify a suitable MYOZ1 candidate due to the requirement of an EMAF ≥ 5%. The selection criterion has some overlap between control participants and athletes. Although control participants do not take part in any sports, they can train regularly up to twice per week. The athletes were defined as having trained more than a minimum of twice per week. It could be suggested that training in the control sub-group may have impaired the results somewhat. Equally, athletes who did not compete at even area level were included in the cohort and it could be suggested that there may be little difference in performance attributes between these individuals and any control participant who may have trained up to twice per week. Additionally, some athletes were included who participate in sports which have shown little to no association with ACTN3 [57,58].

The testing protocol, although in-depth could perhaps have been spread out farther. Tests of maximal strength, and maximal power were all completed within a single day. It is unknown whether participants were fed between strength and power tests or how long they were given to rest between, which could have affected maximal power results.

Finally, it is noted that the unknown brand and or model types of the testing equipment used means that it would be difficult to exactly repeat this study however it is likely all equipment was of a high standard as the data collection was performed at a national institute for sport.

Conclusion

The current study is the first group to investigate variations within genes that encode the Calsarcin-1 and Calsarcin-3 proteins. We have identified, for the first time, that variation within one of these genes significantly alters performance related phenotypic traits. The in-depth, complex, quantitative phenotypic data-set and large cohort of athletes and control participants has presented the unique ability to investigate associations of genes with specific, measurable, performance-related traits which are associated with more complex performance related phenotypes.

In conclusion, this study provides additional evidence that the ACTN3 R577X SNP, R-allele is associated with sprint performance and that the X-allele is not associated with endurance performance. Variants of the Calsarcin family that are of a high enough frequency within the European ethnicity have been identified and one of these variants is associated with athletic performance. It has been identified that variation within the MYOZ3 rs116090320 SNP – which encodes the Calsarcin-3 protein - significantly affects relative $\dot{V}O_{2MAX}$ and isokinetic upper-limb flexion strength in European males. This study has identified a new family of gene not previously associated with human athletic performance that warrants repetition and further investigation.

Supplementary Table

Supplementary Table 1, Association of MYOZ2 Genotype and Performance Phenotypes.

N = 407 (35, 184 and 188 for AA, AG and GG respectively). ***v*** denotes the percentage of the variance explained by the genotype. LCL denotes lower 95 % confidence limit. UCL denotes upper 95 % confidence limit. CMJ = counter-movement jump. IKLL = combined isokinetic strength for both lower limbs. IKUL = combined isokinetic strength for both upper limbs. Significance accepted as $p < 0.012$.

	Genotype	Mean (LCL to UCL)
CMJ height (cm)	**AA**	38.71 (36.61 to 40.811)
	AG	39.282 (38.36 to 40.203)
	GG	38.76 (37.863 to 39.657)
		v **=** 0, ***p*** **=** 0.705
Static jump height (cm)	**AA**	30.871 (29.077 to 32.665)
	AG	32.454 (31.67 to 33.239)
	GG	31.998 (31.606 to 32.391)
		v **=** 0.16, ***p*** **=** 0.269
Body Mass (kg)	**AA**	74.751 (71.266 to 78.235)
	AG	78.305 (76.775 to 79.835)
	GG	76.038 (74.519 to 77.557)
		v **=** 0.96, ***p*** **=** 0.054
IKLL Extension 30 d/sec., N·m	**AA**	121.913 (109.081 to 134.744)
	AG	131.177 (125.717 to 136.638)
	GG	129.881 (124.382 to 135.38)
		v **=** 0, ***p*** **=** 0.43
IKLL Flexion 30 d/sec., N·m	**AA**	129.678 (116.981 to 142.374)
	AG	132.531 (127.148 to 137.915)
	GG	127.533 (122.092 to 132.975)
		v **=** 0, ***p*** **=** 0.442
IKLL Extension 90 d/sec., N·m	**AA**	120.423 (107.36 to 133.486)
	AG	133.311 (127.793 to 138.83)
	GG	128.291 (122.695 to 133.886)
		v **=** 0.48, ***p*** **=** 0.151
IKLL Flexion 90 d/sec., N·m	**AA**	128.2 (115.33 to 141.071)
	AG	132.903 (127.462 to 138.345)
	GG	127.285 (121.785 to 132.784)
		v **=** 0.03, ***p*** **=** 0.352

IKLL Extension 180 d/sec., N·m	**AA**	119.585 (106.637 to 132.532)
	AG	134.573 (129.093 to 140.053)
	GG	126.747 (121.19 to 132.305)
		v **=** 1.19, ***p*** **=** 0.041
IKLL Flexion 180 d/sec., N·m	**AA**	126.518 (113.493 to 139.543)
	AG	132.637 (127.099 to 138.175)
	GG	127.64 (122.044 to 133.236)
		v **=** 0, ***p*** **=** 0.404
IKUL Extension 30 d/sec., N·m	**AA**	121.955 (109.104 to 134.806)
	AG	128.447 (122.987 to 133.908)
	GG	132.383 (126.865 to 137.902)
		v **=** 0.14, ***p*** **=** 0.288
IKUL Flexion 30 d/sec., N·m	**AA**	120.022 (107.538 to 132.506)
	AG	133.362 (127.862 to 138.861)
	GG	128.264 (122.668 to 133.859)
		v **=** 0.54, ***p*** **=** 0.134
IKUL Extension 90 d/sec., N·m	**AA**	121.77 (108.92 to 134.621)
	AG	130.199 (124.738 to 135.659)
	GG	130.443 (124.905 to 135.981)
		v **=** 0, ***p*** **=** 0.459
IKUL Flexion 90 d/sec., N·m	**AA**	120.121 (107.077 to 133.165)
	AG	133.501 (128.002 to 139)
	GG	128.146 (122.57 to 133.723)
		v **=** 0.58, ***p*** **=** 0.125
IKUL Extension 180 d/sec., N·m	**AA**	121.883 (109.051 to 134.715)
	AG	131.631 (126.209 to 137.053)
	GG	129.192 (123.673 to 134.711)
		v **=** 0, ***p*** **=** 0.38
IKUL Flexion 180 d/sec., N·m	**AA**	119.891 (106.963 to 132.819)
	AG	132.901 (127.44 to 138.361)
	GG	128.107 (122.55 to 133.664)
		v **=** 0.49, ***p*** **=** 0.148
Wingate Relative Peak Power (w/kg)	**AA**	11.087 (10.761 to 11.412)
	AG	11.011 (10.864 to 11.158)
	GG	10.967 (10.822 to 11.113)
		v **=** 0, ***p*** **=** 0.785
Wingate Fatigue Index (%)	**AA**	11.04 (10.714 to 11.366)
	AG	11.042 (10.895 to 11.189)
	GG	10.947 (10.802 to 11.092)
		v **=** 0, ***p*** **=** 0.643
Sprint 0-10m (s)	**AA**	1.876 (1.844 to 1.907)
	AG	1.872 (1.858 to 1.886)
	GG	1.886 (1.872 to 1.899)
		v **=** 0, ***p*** **=** 0.414

Sprint 10-20m (s)	**AA**	1.333 (1.309 to 1.358)
	AG	1.334 (1.323 to 1.345)
	GG	1.331 (1.321 to 1.342)
		v **=** 0, ***p*** **=** 0.953
Spring 20-30m (s)	**AA**	1.254 (1.228 to 1.281)
	AG	1.253 (1.241 to 1.265)
	GG	1.255 (1.243 to 1.267)
		v **=** 0, ***p*** **=** 0.969
Sprint Total (s)	**AA**	4.462 (4.387 to 4.536)
	AG	4.46 (4.427 to 4.494)
	GG	4.474 (4.441 to 4.506)
		v **=** 0, ***p*** **=** 0.847
Relative $\dot{V}O_{2MAX}$ (ml/kg/min)	**AA**	54.876 (51.959 to 57.793)
	AG	53.848 (52.564 to 55.131)
	GG	55.031 (53.772 to 56.289)
		v **=** 0, ***p*** **=** 0.422
Body Fat (%)	**AA**	14.276 (12.828 to 15.724)
	AG	14.582 (13.955 to 15.209)
	GG	14.096 (13.474 to 14.719)
		v **=** 0, ***p*** **=** 0.561

Reference List

1 Bouchard C, Hoffman EP. *Genetic and Molecular Aspects of Sport*. 1st ed. John Wiley & Sons, Ltd, 2011.

2 De Moor MHM, Spector TD, Cherkas LF, Falchi M, Hottenga JJ, Boomsma DI *et al.* Genome-wide linkage scan for athlete status in 700 British female DZ twin pairs. *Twin Res Hum Genet* 2007; **10**: 812–20.

3 Eynon N, Ruiz JR, Oliveira J, Duarte JA, Birk R, Lucia A. Genes and elite athletes: a roadmap for future research. *J Physiol* 2011; **589**: 3063–70.

4 Costa AM, Breitenfeld L, Silva AJ, Pereira A, Izquierdo M, Marques MC. Genetic inheritance effects on endurance and muscle strength: an update. *Sports Med* 2012; **42**: 449–58.

5 Visscher PM, Hill WG, Wray NR. Heritability in the genomics era--concepts and misconceptions. *Nat Rev Genet* 2008; **9**: 255–66.

6 Bouchard C, An P, Rice T, Skinner JS, Wilmore JH, Gagnon J *et al.* Familial aggregation ofV ? o 2 max response to exercise training : results from the HERITAGE Family Study older adults exercise training ˙ O 2 max response to exercise Familial aggregation of V training : results from the HERITAGE Family Study. *Med Sci Sports Exerc* 1999; **30**: 1003–1008.

7 Bouchard C, Lesage R, Lortie G, Simoneau J-A, Hamel P, Boulay M *et al.* Aerobic performance in brothers, dizygotic and monozygotic twins. *Med Sci Sports Exerc* 1986; **18**.

8 Tiainen K, Sipilä S, Alen M, Heikkinen E, Kaprio J, Koskenvuo M *et al.* Heritability of maximal isometric muscle strength in older female twins. *J Appl Physiol* 2004; **96**: 173–80.

9 Huygens W, Thomis MA, Peeters MW, Vlietinck RF, Beunen GP. Determinants and Upper-Limit Heritabilities of Skeletal Muscle Mass and Strength. *Can J Appl Physiol* 2004; **29**: 186–200.

10 Yang N, MacArthur DG, Gulbin JP, Hahn AG, Beggs AH, Easteal S *et al.* ACTN3 genotype is associated with human elite athletic performance. *Am J Hum Genet* 2003; **73**: 627–31.

11 Santos CGM, Pimentel-Coelho PM, Budowle B, Moura-Neto RS, Dornelas-Ribeiro M, Pompeu F *et al.* The heritable path of human physical performance: from single polymorphisms to the 'next generation'. *Scand J Med Sci Sports* 2015.

12 Wolfarth B, Rankinen T, Hagberg JM, Loos RJF, Pérusse L, Roth SM *et al.* Advances in exercise, fitness, and performance genomics in 2013. *Med Sci Sports Exerc* 2014; **46**: 851–9.

13 Shastry BS. SNP alleles in human disease and evolution. *J Hum Genet* 2002; **47**: 0561–0566.

14 Mills M, Yang N, Weinberger R, Vander Woude DL, Beggs a H, Easteal S *et al.* Differential expression of the actin-binding proteins, alpha-actinin-2 and -3, in different species: implications for the evolution of functional redundancy. *Hum Mol Genet* 2001; **10**: 1335–46.

15 MacArthur DG, North KN. A gene for speed? The evolution and function of alpha-actinin-3. *Bioessays* 2004; **26**: 786–95.

16 Grealy R, Smith CLE, Chen T, Hiller D, Haseler LJ, Griffiths LR. The genetics of endurance: frequency of the ACTN3 R577X variant in Ironman World Championship athletes. *J Sci Med Sport* 2013; **16**: 365–71.

17 Chowrashi P, Mittal B, Sanger J, Sanger J. Amorphin is phosphorylase; Phosphorylase is an alpha-actinin-binding protein. *Cell Motil Cytoskeleton* 2002; **53**: 125–135.

18 Frey N, Richardson J a, Olson EN. Calsarcins, a novel family of sarcomeric calcineurin-binding proteins. *Proc Natl Acad Sci U S A* 2000; **97**: 14632–7.

19 North KN. Deficiency of a skeletal muscle isoform of a-actinin (a-actinin-3) in merosin-positive congenital muscular dystrophy. *Neuromuscul Disord* 1996; **6**: 229–235.

20 Quinlan KGR, Seto JT, Turner N, Vandebrouck A, Floetenmeyer M, Macarthur DG *et al.* Alpha-actinin-3 deficiency results in reduced glycogen phosphorylase activity and altered calcium handling in skeletal muscle. *Hum Mol Genet* 2010; **19**: 1335–46.

21 North KN, Yang N, Wattanasirichalgoon D, Mills M, Easteal S, Beggs AH. A common nonsense mutation results in α-actinin-3 deficiency in the gneral population. *Nat Genet* 1999; **21**: 353–354.

22 Niemi A-K, Majamaa K. Mitochondrial DNA and ACTN3 genotypes in Finnish elite endurance and sprint athletes. *Eur J Hum Genet* 2005; **13**: 965–9.

23 Druzhevskaya AM, Ahmetov II, Astratenkova I V, Rogozkin V a. Association of the ACTN3 R577X polymorphism with power athlete status in Russians. *Eur J Appl Physiol* 2008; **103**: 631–4.

24 Moran CN, Yang N, Bailey MES, Tsiokanos A, Jamurtas A, MacArthur DG *et al.* Association analysis of the ACTN3 R577X polymorphism and complex quantitative body composition and performance phenotypes in adolescent Greeks. *Eur J Hum Genet* 2007; **15**: 88–93.

25 Clarkson PM, Devaney JM, Gordish-dressman H, Thompson PD, Hubal MJ, Urso M *et al.* ACTN3 genotype is associated with increases in muscle strength in response to resistance training in women. *J Appl Physiol* 2005; **99**: 154–163.

26 Delmonico MJ, Zmuda JM, Taylor BC, Cauley J a, Harris TB, Manini TM *et al.* Association of the ACTN3 genotype and physical functioning with age in older adults. *J Gerontol A Biol Sci Med Sci* 2008; **63**: 1227–34.

27 Walsh S, Liu D, Metter EJ, Ferrucci L, Roth SM. ACTN3 genotype is associated with muscle phenotypes in women across the adult age span. *J Appl Physiol* 2008; **105**: 1486–91.

28 Scott R a, Irving R, Irwin L, Morrison E, Charlton V, Austin K *et al.* ACTN3 and ACE genotypes in elite Jamaican and US sprinters. *Med Sci Sports Exerc* 2010; **42**: 107–12.

29 Roth SM, Walsh S, Liu D, Metter EJ, Ferrucci L, Hurley BF. The ACTN3 R577X nonsense allele is under-represented in elite-level strength athletes. *Eur J Hum Genet* 2008; **16**: 391–4.

30 MacArthur DG, Seto JT, Raftery JM, Quinlan KG, Huttley GA, Hook JW *et al.* Loss of ACTN3 gene function alters mouse muscle metabolism and shows evidence of positive selection in humans. *Nat Genet* 2007; **39**: 1261–1265.

31 MacArthur DG, Seto JT, Chan S, Quinlan KGR, Raftery JM, Turner N *et al.* An Actn3 knockout mouse provides mechanistic insights into the association between alpha-actinin-3 deficiency and human athletic performance. *Hum Mol Genet* 2008; **17**: 1076–86.

32 Eynon N, Duarte JA, Oliveira J, Sagiv M, Yamin C, Mekel Y *et al.* ACTN3 R577X Polymorphism and Israeli Top-level Athletes. *Int J Sports Med* 2009; **30**: 839–839.

33 Eynon N, Ruiz JR, Femia P, Pushkarev VP, Cieszczyk P, Maciejewska-Karlowska A *et al.* The ACTN3 R577X polymorphism across three groups of elite male European athletes. *PLoS One* 2012; **7**: e43132.

34 Yang N, MacArthur DG, Wolde B, Onywera VO, Boit MK, Lau SYM-A *et al.* The ACTN3 R577X polymorphism in East and West African athletes. *Med Sci Sports Exerc* 2007; **39**: 1985–8.

35 Döring FE, Onur S, Geisen U, Boulay MR, Pérusse L, Rankinen T *et al.* ACTN3 R577X and other polymorphisms are not associated with elite endurance athlete status in the Genathlete study. *J Sports Sci* 2010; **28**: 1355–9.

36 Cięszczyk P, Eider J, Ostanek M, Arczewska A, Leońska-Duniec A, Sawczyn S *et al.* Association of the ACTN3 R577X Polymorphism in Polish Power-Orientated Athletes. *J Hum Kinet* 2011; **28**: 55–61.

37 Leger LA, Lambert J. A maximal multistage 20-m shuttle run test to predict V-O2 max. *Eur J Appl Physiol Occup Physiol* 1982; **49**: 1–12.

38 Mikulie P, Ruzic L. Predicting the 1,000 m rowing ergometer performance in 12 and 13 year old rowers: The basis for selection process. *J Sci Med Sport* 2008; **11**: 218–226.

39 Alfred T, Ben-Shlomo Y, Cooper R, Hardy R, Cooper C, Deary IJ *et al.* ACTN3 genotype, athletic status, and life course physical capability: meta-analysis of the published literature and findings from nine studies. *Hum Mutat* 2011; **32**: 1008–18.

40 Seto JT, Quinlan KGR, Lek M, Zheng XF, Garton F, Macarthur DG *et al.* ACTN3 genotype influences muscle performance through the regulation of calcineurin signaling. *J Clin Invest* 2013; **123**: 4255–4263.

41 Chin ER, Olson EN, Richardson J a., Yang Q, Humphries C, Shelton JM *et al.* A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev* 1998; **12**: 2499–2509.

42 Delling U, Tureckova J, Lim HW, De LJ, Rotwein P, Molkentin JD. A Calcineurin-

NFATc3-Dependent Pathway Regulates Skeletal Muscle Differentiation and Slow Myosin Heavy-Chain Expression A Calcineurin-NFATc3-Dependent Pathway Regulates Skeletal Muscle Differentiation and Slow Myosin Heavy-Chain Expression. *Mol Cell Biol* 2000; **20**: 6600–6611.

43 Jiang LQ, Garcia-Roves PM, de Castro Barbosa T, Zierath JR. Constitutively active calcineurin in skeletal muscle increases endurance performance and mitochondrial respiratory capacity. *Am J Physiol Endocrinol Metab* 2010; **298**: E8–E16.

44 Frey N, Olson EN. Calsarcin-3, a novel skeletal muscle-specific member of the calsarcin family, interacts with multiple Z-disc proteins. *J Biol Chem* 2002; **277**: 13998–4004.

45 Frey N, Frank D, Lippl S, Kuhn C, Kögler H, Barrientos T *et al.* Calsarcin-2 deficiency increases exercise capacity in mice through calcineurin/NFAT activation. *J Clin Invest* 2008; **118**: 3598–608.

46 Walhout AJM, Boulton SJ, Vidal M. Yeast two-hybrid systems and protein interaction mapping projects for yeast and worm. *Yeast* 2000; **17**: 88–94.

47 Poole D, Wilkerson D, Jones A. Validity of criteria for establishing maximal O2 uptake during ramp exercise tests. *Eur J Appl Physiol* 2008; **102**: 403–410.

48 Durnin BYJVG a, Womersley J. Body fat assessed from total body density and its estimation from skinfold thickness : measurements on 481 men and women aged from 16 to 72 years. *Br J Nutr* 1973; **32**: 77–97.

49 Dmitriev P, Barat A, Polesskaya A, O'Connell MJ, Robert T, Dessen P *et al.* Simultaneous miRNA and mRNA transcriptome profiling of human myoblasts reveals a novel set of myogenic differentiation-associated miRNAs and their target genes. *BMC Genomics* 2013; **14**: 265.

50 Filipowicz W, Jaskiewicz L, Kolb FA, Pillai RS. Post-transcriptional gene silencing by siRNAs and miRNAs. *Curr Opin Struct Biol* 2005; **15**: 331–341.

51 Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev* 2006; **20**: 515–524.

52 Vasudevan S, Tong Y, Steitz J. Switching from Repression to Activation: MicroRNAs Can Up-Regulate Translation. *Science (80-)* 2007; **318**: 1931–1934.

53 Ketley A, Warren A, Holmes E, Gering M, Aboobaker a A, Brook JD. The miR-30 microRNA family targets smoothened to regulate hedgehog signalling in zebrafish early muscle development. *PLoS One* 2013; **8**: e65170.

54 Gineviciene V, Pranckeviciene E, Milasius K, Kucinskas V. Gene variants related to the power performance of the Lithuanian athletes. *Cent Eur J Biol* 2011; **6**: 48–57.

55 Paparini A, Ripani M, Giodano GD, Santoni D, Pigozzi F, Romano-Spica V. ACTN3 genotyping by real-time PCR in the Italian population and athletes. *Med Sci Sports Exerc* 2007; **39**: 810–815.

56 Santiago C, Rodríguez-Romo G, Gómez-Gallego F, González-Freire M, Yvert T, Verde Z *et al.* Is there an association between ACTN3 R577X polymorphism and muscle power phenotypes in young, non-athletic adults? *Scand J Med Sci Sports* 2010; **20**: 771–8.

57 Ruiz JR, Fernández del Valle M, Verde Z, Díez-Vega I, Santiago C, Yvert T *et al.* ACTN3 R577X polymorphism does not influence explosive leg muscle power in elite volleyball players. *Scand J Med Sci Sports* 2011; **21**: e34–41.

58 Garatachea N, Verde Z, Santos-Lozano A, Yvert T, Rodriguez-Romo G, Sarasa FJ *et al.* ACTN3 R577X Polymorphism and Explosive Leg-Muscle Power in Elite Basketball Players. *Int J Sports Physiol Perform* 2014; **9**: 226–232.

59 Frank D, Kuhn C, van Eickels M, Gehring D, Hanselmann C, Lippl S *et al.* Calsarcin-1 protects against angiotensin-II induced cardiac hypertrophy. *Circulation* 2007; **116**: 2587–96.

60 Frey N, Barrientos T, Shelton JM, Frank D, Rutten H, Gehring D *et al.* Mice lacking calsarcin-1 are sensitized to calcineurin signalling and show accelerated cardiomyopathy in response to pathological biomechanical stress. *Nat Med* 2004; **10**: 1336–1343.

61 Pan W, Zhong Y, Cheng C, Liu B, Wang L, Li A *et al.* MiR-30 regulated autophagy mediates angiotensin II-induced myocardial hypertrophy. *PloS one2* 2013; **8**: e53950.

62 Crabtree GR. Calcium, Calcineurin and the Control of Transcription. *J Biol Chem* 2001; **276**: 2313–2316.

63 Sakuma K, Yamaguchi A. The functional role of calcineurin in hypertrophy,

regeneration, and disorders of skeletal muscle. *J Biomed Biotechnol* 2010; **2010**: 721219.

64 Shulz RA, Yutzey KE. Calcineurin signalling and NFAT activation in cardiovascular and skeletal muscle development. *Dev Biol* 2004; **266**: 1–16.

Appendix 1

Protocol for Extracting DNA from Whole Blood

This protocol is devised from two existing protocols due to recurring issues with a chaotropic agent found in some of the buffers of the Macherey-Nagel (MN) DNA extraction kit. This protocol is devised using two existing protocols: Macherey-Nagel (MN, Genomic DNA from Tissue User Manual, NucleoSpin® June 2014/Rev .14, Macherey-Nagel GmbH & Co. KG, Düren, Germany) and Qiagen (Q, QIAmp DNA Mini Kit, Qiagen, Hilden, Germany). Samples were extracted in batches of twelve for logistical reasons within the laboratory at the University of Stirling.

Before starting, ensure blood samples are thawed to room temperature; work surface has been disinfected (use both Virkon solution and EtOH sprays) Ensure water bath is set at 70 ^{o}C. Add the Buffer BE bottle (MN) to the water bath to heat up for use later in the protocol.

- In a 1.5 ml Microcentrifuge Tube (MCF), add 25 µL of Proteinase K (MN), 200 µL Whole Blood and 200 µL Buffer AL (Q)
- Vortex for 10-15 s then pulse in a centrifuge to remove liquid from the inside of the lid and ensure all mixture is at the base of the MCF
- Incubate at 70 ^{o}C for 15mins (MN) (70^{o}C because we are using MN Proteinase K)
- Pulse centrifuge to remove liquid from the lid.
- Add 200 µL EtOH, Vortex for 10-15 s, Pulse Centrifuge to remove liquid from lid.
- Add mixture to Neucleospin Columns inside 2 ml Collection Tubes (CT)
- Centrifuge at 6,000 – Max x g (spun at 20,000 x g) for 1 minute. (Speed x g does not affect yield/quantity of DNA, only noise within the laboratory)
- Add Neucleospin Columns to new CT, discard original CT.
- Add 500 µL Buffer AW1 (Q), Centrifuge at 6,000 – max x g (20,000 x g) for 1 minute
- Empty CT and replace
- Add 500 µL Buffer AW2 (Q), Centrifuge at 6,000 – max x g (20,000 x g) for 3 minutes
- Discard CT. Place Spin Column inside 1.5 ml clear MCF tube
- Add 200 µL Buffer BE (MN) (Buffer BE kept in water bath at 70^{o}C according to protocol from MN)
- Centrifuge for 1 minute at 6,000 x g (MN protocol used for MN buffer)
- Discard Spin Column.
- Label and store sample in -20 ^{o}C for analysis of DNA yield, PCR and RFLP in the future.

We spun in our centrifuge at 20,000 x g as it was not particularly noisy however, the speed at which the samples are spun (between 6,000 – max) does not affect yeild or quality of DNA sample.