THE INFLUENCE OF ACTN3 R577X GENOTYPE ON PERFORMANCE AND MUSCLE ADAPTATIONS TO A SINGLE BOUT OF EXERCISE

by

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Statement of Originality

I, Ioannis Papadimitriou, declare that the PhD thesis by Publication entitled *"The influence of ACTN3 R577X genotype on performance and muscle adaptations to a single bout of exercise"* is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

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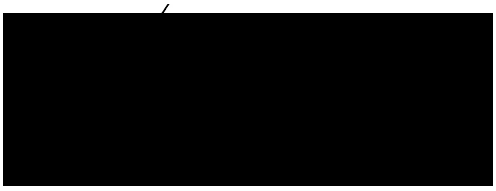

Date

28th August 2017

In Loving Memory

of

Evangelia Taskoudi

6th Nov 1942 – 11th Sep 2016

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1. ABSTRACT

ACTN3 has been labelled as the 'gene for speed' due to the increased frequency of the R allele encoding the α-actinin-3 protein in elite sprint athletes compared to the general population. The results of the first study of this thesis demonstrate that elite athletes who express α-actinin-3 (*ACTN3* RR genotype) have faster sprint times compared to those who do not express α-actinin-3 (*ACTN3* XX genotype). Further analysis indicates that the *ACTN3* genotype accounts for 0.92% in sprint speed amongst elite 200-m athletes. In study two, the same quantitative genetic epidemiological design applied to elite endurance athletes, showed no evidence that a trade-off existed. The endurance athletes with the *ACTN3* XX genotype were no faster than those who express the α-actinin-3 protein. These results added to literature that it is unlikely the *ACTN3* XX genotype to offer an advantage for endurance performance. While *ACTN3* genotype does not appear to influence endurance performance in athletes, studies in mice that completely lack the α-actinin-3 protein suggest the *ACTN3* genotype influences the adaptive response to endurance exercise. Based on these findings, the aim of study 3 was to investigate if *ACTN3* genotype influences exercise-induced changes in the content of genes and proteins associated with mitochondrial biogenesis. At baseline, there was a compensatory greater α-actinin-2 protein content in *ACTN3* XX vs *ACTN3* RR participants ($p=0.018$) but there were no differences in the endurance-related phenotypes measured. There was a main effect of genotype ($p=0.006$), without a significant interaction effect, for RCAN1-4 or significant exercise-induced expression of genes associated with mitochondrial biogenesis. Together, these results suggest that *ACTN3* genotype has a small but significant influence on sprint speed amongst elite sprint athletes. However, loss of α-actinin-3 protein is not associated with higher values for endurance-related phenotypes, endurance performance, or a greater adaptive response to a single session of high-intensity endurance exercise.

2. TABLE OF CONTENT

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LIST OF ABBREVIATIONS:

ACTN2*:* α-actinin-2 gene

ACTN3: α-actinin-3 gene

AIS: Australian Institute of Sport

AMPK: AMP-activated protein kinase

BHAD: 3-hydroxyacyl-CoA dehydrogenase

BMD: Becker muscular dystrophy

cDNA: Complimentary DNA

CaM: Calmodulin Cn Calcineurin

CCO: Cytochrome c oxidase

COX IV: Cytochrome C oxidase (Complex IV) subunit 4

CS: Citrate synthase

DMD: Duchenne muscular dystrophy

DZ: Dizygotic

EDL: Extensor digitorum longus

EF: elongation factor

GXT: Graded exercise test to exhaustion

HIIE: High Intensity Interval Exercise

HIIT: High Intensity Interval Training

HGP: Human Genome Project

KO: Knockout

LDH: Lactate dehydrogenase

MCAD: Medium chain acyl-CoA dehydrogenase

MEF2: Myocyte enhancer factor-2

MZ: Monozygotic

MyHC: Myosin heavy chain

NADH: Nicotinamide adenine dinucleotide

NFAT: Nuclear factor of activated T cells

NHMRC: National Health and Medical Research Council

PAGE: Polyacrylamide gel electrophoresis

PBS : Phosphate-buffered saline

QMT: Quantitative muscle test qPCR Quantitative polymerase chain reaction

RCAN: Regulator of calcineurin

RFLP: (PCR)-restriction fragment length polymorphism

SDH: Succinate dehydrogenase

SDS: Sodium dodecyl sulphate

SNP: Single nucleotide polymorphism

VO_{2max}: Maximal Oxygen uptake

WT: Wild-type

ZASP: Z-line associated structural protein

3. INTRODUCTION

Skeletal muscle is the second most plastic tissue of the body, second only to nervous tissue. As a consequence of this plasticity, exercise training leads to a host of adaptations that contribute to improved skeletal muscle function and health (Pedersen and Saltin, 2006). These adaptations include increases in muscle strength and power, as well as improvements in oxygen consumption and mitochondrial function. The mitochondria are particularly important for health, and mitochondrial dysfunction has been implicated in many chronic diseases such as cardiovascular disease and type-2 diabetes. Given the pivotal role of mitochondria in providing the energy required for muscle metabolism, it is not surprising that mitochondrial adaptations have also been associated with endurance performance (Bishop et al., 2014).

While exercise training is well known to promote skeletal muscle adaptations, a high inter-individual variability in the response to similar exercise training has been consistently reported in the literature. For example, in the pioneering Health, Risk factors, Training and Genetics (HERITAGE) study, Bouchard et al.,(1999) have shown that changes in maximal Oxygen consumption (VO_{2max}) vary markedly in a group of sedentary adults performing similar exercise training. Following 20 weeks of exercise training, Bouchard (2012) reported a mean increase in VO_{2max} of ~ 400 mL/min, which ranged from little or no gain to increases of > 1000 mL/min. Recently, some other researchers have also shown large individual variability for changes in mitochondrial function (respiration) in response to four weeks of high-intensity exercise training (Bishop et al., 2014). This suggests that some people respond well or very well to exercise training ("high-responders"), while others are "low responders" to similar exercise training (Skinner et al., 2001). Identifying and understanding the biological mechanisms behind the individual response to exercise training presents a challenge to scientists, but also has exciting potential implications for "personalized medicine" and the future development of individualised exercise training programs (Hawley et al., 2014).

In the 1970s and 1980s, research into the effect of genetics on responses to exercise and athletic performance was mostly conducted via twin (monozygotic MZ identical vs. dizygotic DZ non-identical) and family studies. When this approach was applied to VO_{2max} using 25 pairs of twins (15 MZ and 10 DZ preadolescent boys), high heritability estimates were reported (e.g., h^2 = 93.4%) (Klissouras, 1971). In other words, genetics could explain as much as 93.4% of the phenotypic variation in VO_{2max}. Furthermore, DZ twins had a significantly higher intrapair difference when compared to MZ twins (Klissouras et al., 1973). Similarly, high heritability estimates were reported in 15 MZ and 16 DZ twins of both sexes (h2 = 96.5%) (Klissouras et al., 1973). However, this approach was unable to identify specific genes that influence individual variability.

The completion of the human genome project (HGP) in 2003 and development of next generation sequencing technologies has facilitated the identification of genetic variants associated with athletic performance and exercise. Current research has shifted from investigating individual candidate genes, to defining specific variants that influence the response to training and identifying the molecular pathways and mechanisms responsible for this fascinating this phenomenon.

One of the most investigated genes with respect to athletic performance and adaptation to exercise training is the *ACTN3* gene (North et al., 1999). This common null polymorphism (R577X) results in replacement of an arginine (R) residue with a premature stop codon (X) at amino acid 577. Homozygosity for the *ACTN3* 577 X variant (*ACTN3* 577XX) is common and results in complete deficiency of α-actinin-3. An estimated 18% of the world population (~1.5 billion people) are completely deficient in α-actinin-3. α-actinin-3 deficiency (*ACTN3* 577XX genotype) has been associated with athletic performance in multiple, unrelated cohorts of athletes worldwide. In case: control studies, the ACTN3 gene have initially been associated with speed because of its higher frequency in elite sprint/power Australian (Yang et al., 2003), Finish (Niemi and Majamaa, 2005), Greek (Papadimitriou et al., 2008), Russian (Druzhevskaya et al., 2008), Israeli (Eynon et al., 2009), Polish (Cięszczyk et al., 2011) and Japanese (Mikami et al., 2013) elite athletes compared to the general population. However, all these associations between the *ACTN3* gene polymorphism and athletic performance were based on athletic status and no studies have quantitatively linked the *ACTN3* genotype with sprinting performance (sprinting times) in elite athletes (AIM 1).

Conversely, some, but not all, of above case: control studies have reported that loss of α-actinin-3 (*ACTN3* XX genotype) is associated with enhanced endurance capacity in elite athletes (Eynon et al., 2009). However, once again lack of quantitative measures of performance in these analyses means that these associations are not based on a measurable trait that is considered a more objective, accurate and reliable measure of performance (AIM 2).

In addition to their well-described role as structural proteins, recent research has suggested **a novel role for α-actinin-3 as an important mediator of cell signalling**. Consequently, a possible mechanism by which α-actinin-3 deficiency might influence endurance performance is by affecting signalling pathways related to slowing of the metabolic and physiological profile of skeletal muscle. In this regard, a study utilising mice bred to completely lack the α-actinin-3 protein (i.e., *Actn3* knockout (KO) mice) reported that the greater association with response to endurance exercise was related to “slowing” of the metabolic and physiological properties of type IIb fast muscle fibres and an enhanced adaptation to endurance training (Seto et al., 2013). Forced changes in the expression of

RCAN1-4 protein (a marker of calcineurin activity) affects cell growth (Lee et al., 2009) and adaptation to endurance training (Emrani et al., 2015). This has been linked to the suppression of calcineurin-nuclear factor of activated T cells signaling by RCAN1-4 (**Figure 15**). Muscles of *Actn3* KO mice showed a 1.3-fold higher baseline RCAN1-4 protein (a marker of calcineurin activity) expression compared with wild type (WT) muscles, suggesting increased calcineurin activity. Direct assays of calcineurin activity confirmed a 1.9-fold increase in calcineurin signalling in exercised KO muscles compared with WT muscles ($p = 0.09$) with a 2.9-fold increase in RCAN1-4 protein content, ($p=0.004$) (Seto et al., 2013). Consistent with the observations in mice, there was also greater protein content of RCAN1-4 in resting muscle samples obtained from *ACTN3* 577XX humans when compared with *ACTN3* 577RR humans (Seto et al., 2013). However, RCAN1-4 content was only measured in resting muscle and it is not known if exercise-induced changes of RCAN1-4 protein content are affected by *ACTN3* genotype in humans. Potential pathways could involve the downstream targets of calcineurin, such as PGC-1α (Handschin et al., 2003).

While the KO mouse model undeniably provided interesting insights into the impact of α-actinin-3 deficiency on exercise responses, there would be massive implications for human health and ageing if we conduct a direct demonstration of the effects of the of α-actinin-3 deficiency on the characteristics of human muscle following exercise. While knocking out genes in humans is ethically off-limits, the high percentage of the population deficient for the α-actinin-3 protein (*ACTN3* XX genotype) serves as a unique naturally occurring “human knock-out” model to investigate functional consequences of α-actinin-3 deficiency on mitochondrial-related pathways in human muscle pre- and post-exercise (AIM 3).

4. AIMS

The aims of this Ph.D thesis are:

A) to assess the association between the *ACTN3* genotype and sprint time (from 100- to 400-m dash) in an epidemiological consortium-based, large cohort of elite sprint athletes from different countries.

B) to assess the association between the *ACTN3* genotype and endurance running time (from 1500 m to Marathon) in an epidemiological consortium-based, large cohort of elite endurance athletes different countries.

C) to identify the influence of *ACTN3* polymorphism on molecular signalling pathways in human skeletal muscle at baseline and in response to a session of High-Intensity Interval Exercise (HIIE) using targeted gene and protein expression.

5. LITERATURE REVIEW

5.2 *The α-actinin family*

A vast array of human phenotypes (e.g., muscle strength, muscle power, endurance, tendon elasticity, and heart size) influence sports performance and adaptation to exercise training; each itself the result of a complex interaction between many anatomical, biochemical and physiological systems (Hawley et al., 2014). These phenotypes are also believed to be influenced by the common inheritance of the more than 20 000 genes which defines each of us as humans. However, substantial variation exists between individual human genomes, including, amongst others, 'replication' of gene sequences (copy number variation, tandem repeats), or changes in individual base pairs (defined as mutations if frequency <1% and Single Nucleotide Polymorphisms (SNPs) if frequency >1%). One of these is the *ACTN3* R577X (rs1815739) SNP. The *ACTN3* gene is located on the long arm of chromosome 11(11q13-q14) and encodes for the α-actinin-3 protein (North et al., 1999). The α-actinins are an ancient family of actin-binding proteins that is believed to have arisen from a single gene prior to the eukaryote-prokaryote divergence (MacArthur and North, 2004). The α-actinin isoforms have been characterised from a large variety of taxa, including protists, invertebrates, birds and mammals (Lek and North, 2010). Interestingly, this diversity is most marked in mammalian cells where four α-actinin encoding genes (α-actinin-1 to -4) produce different protein products, each with a specific expression profile. The sarcomeric α-actinins, α-actinin-2 (encoded by the ACTN2 gene) and α-actinin-3 (encoded by the ACTN3) evolutionarily are the youngest α-actinins, sharing 90% sequence similarity and are primarily expressed in skeletal muscle, (Beggs et al., 1992). Of these, α-actinin-3 appears to have a more specialised role and pattern of expression, being present only in type 2 B/X fast-twitch skeletal muscle fibres. Research into the functional consequences of α-actinin-3 deficiency has been focused on the analysis of engineered knockout (KO) mouse and has begun to unlocked many of the mechanistic aspects of α-actinin-3 deficiency (Eynon et al., 2013).

Chapter 5 of this thesis provides a review of the current literature, explaining the multiple roles of α-actinin-3 protein in skeletal muscle.

5.2.1 The structural role of α-actinins

Evidence suggests that the α-actinins are essential with regard to maintenance of muscle structure, contraction, and connection of the sarcomere to the plasma membrane. α-actinins are found at the Z-line (brown), where it anchors actin-containing thin filaments (black) from adjacent sarcomeres (**Figure 1a**), (Salmikangas et al., 1999).

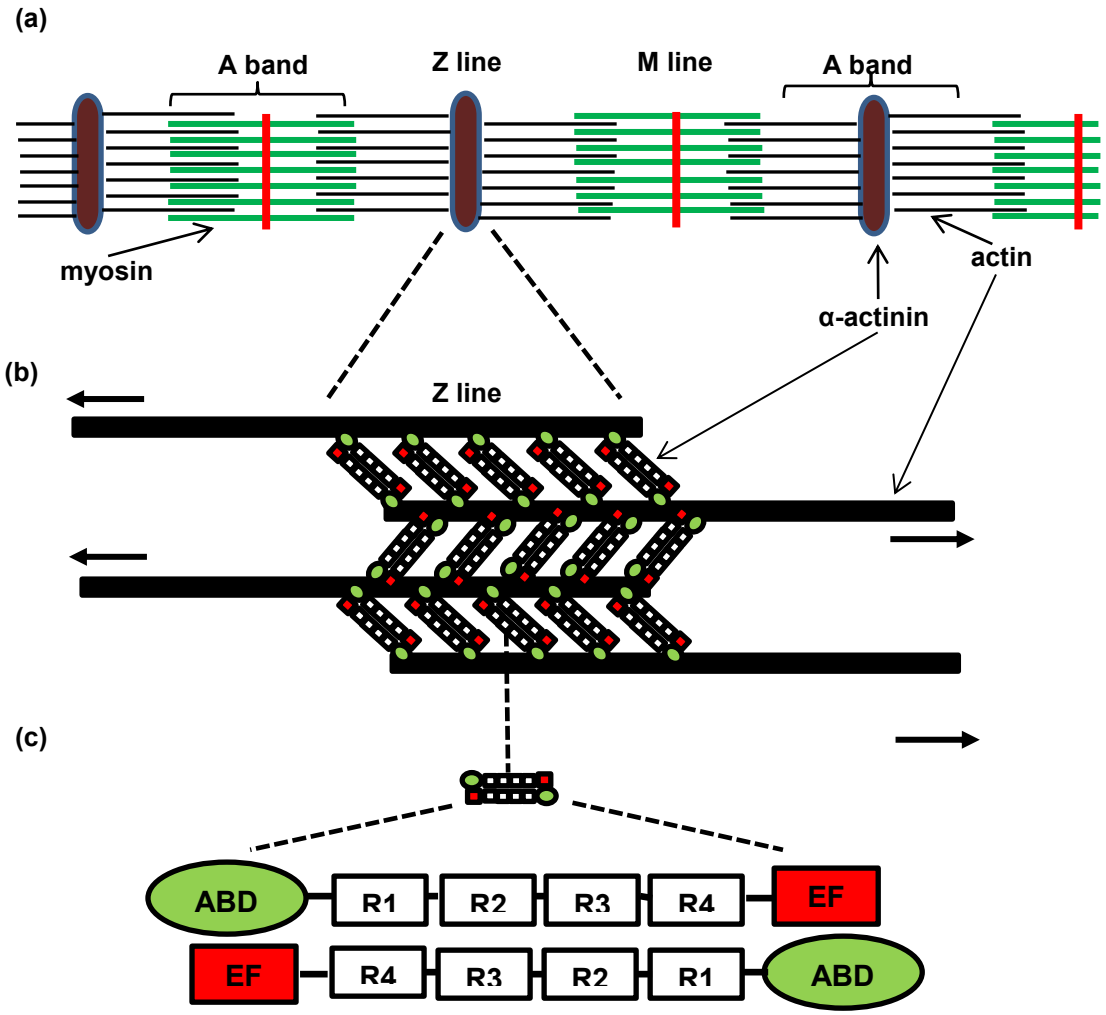

Figure 1 a) *The localization of α-actinins to the sarcomeric Z line, where they cross-link actin thin filaments from adjacent sarcomeres.* ***b)*** *The sarcomeric actinins form head to tail dimers to cross-link actin at the Z-line.* ***c)*** *Their structure consists of an actin-binding domain (ABD), a rod domain with four spectrin repeats (R1-R4) and an EF-hand domain (EF). Adapted from* **(MacArthur and North, 2004)**.

The sarcomeric actinins form head to tail dimers to cross-link actin at the Z-line (**Figure 1b**). α actinins share a similar architecture with a number of functional domains: an N-terminal Actin-Binding Domain (ABD), a C-terminal region and a central rod domain that contains two EF hands (MacArthur and North, 2004). The ABD is connected to the rod

domain via a flexible neck region and contains four spectrin-like repeats (**Figure 1c**). X-ray crystal structures indicated the rod has a twist and each spectrin repeat is bent (Djinović-Carugo et al., 1999). The α-actinin rod domain (R1-4) differs from the other spectrin family molecules by its shorter length, its more rigid helical linkers, and its dimerization (Broderick and Winder, 2002). The flexible ABD allows binding of α-actinins in a variety of conformations between antiparallel and parallel actin filaments (**Figure 1b**). The EF-hand controls binding to calcium regulated by the sarcomeric α-actinins as the EF-hands are incapable of directly binding calcium (Beggs et al., 1992). Among different species, the EF-hand domain and rod domain have a great sequence variation (**Figure 1c**). This can vary the protein-protein interactions, while still maintaining the architecture to allow actin bundles to cross-link in a regulated manner (Djinovic-Carugo et al., 2002). This anti-parallel orientation allows two actin monomers to be bound to each α-actinin dimer via the ABD, a flexible region that allows actin binding to occur in various orientations (**Figure 1b**). In addition, the EF-hand domain contains calmodulin-like domains in close proximity with the linker between the two calponin homology domains in the ABD that help regulate actin binding (Tang et al., 2001).

5.2.2 The functional role of α-actinins

Besides binding to actin filaments, α-actinins associate with a number of cytoskeletal and signaling molecules, cytoplasmic domains of ion channels and transmembrane receptors, rendering its important structural and regulatory roles in cytoskeleton organization and muscle contraction (MacArthur and North, 2004). α-actinins interact with integrin, zyxin, actin, vinculin and filament capping protein CapZ (Papa et al., 1999). Binding of metabolic enzymes to cytoskeletal proteins is a recognised mechanism of enzyme regulation, and tethering the metabolic enzymes at the sarcomeric Z-disk contributes to the local availability of the metabolites required for energy generation. During exercise, glycogen broken down via glycogenolysis while, at rest; stores of glycogen are replenished via glyconeogenisis. α-actinins interact with the metabolic enzymes; aldolase, and fructose-1,6-bisphosphatase (FBPase) on the sarcomeric Z-line. Destabilisation of this FBPase-Z-line interaction causes down-regulation of glyconeogenesis and altered muscle metabolism (Gizak et al., 2003). All α-actinins protein interactions reported in the literature are demonstrated in **Figure 2**.

α-actinins have been shown to be indirectly regulated by calcium levels by forming a signalling complex with calcineurin. Also significant is the direct interaction between the α-actinins and the calsarcins (Faulkner et al., 2000). At the Z-line, calsarcins directly interact with α-actinins to modulate the level of calcineurin activation (Frey et al., 2008). Calcineurin

is a key-signalling molecule in muscle adaptation and has a direct role in fibre type switching (Chin et al., 1998) and muscle remodelling (Olson and Williams, 2000).

Titin, telethonin and muscle LIM protein directly link to α-actinin at the Z-line as well. This complex involves CRP3/Cysteine, which has the ability to translocate to the nucleus to activate myogenic transcription factors (Kadrmas and Beckerle, 2004). In this way, sarcomeric α-actinins not only interact with other proteins on Z-line but also participate in signalling regulation to affect downstream gene expression.

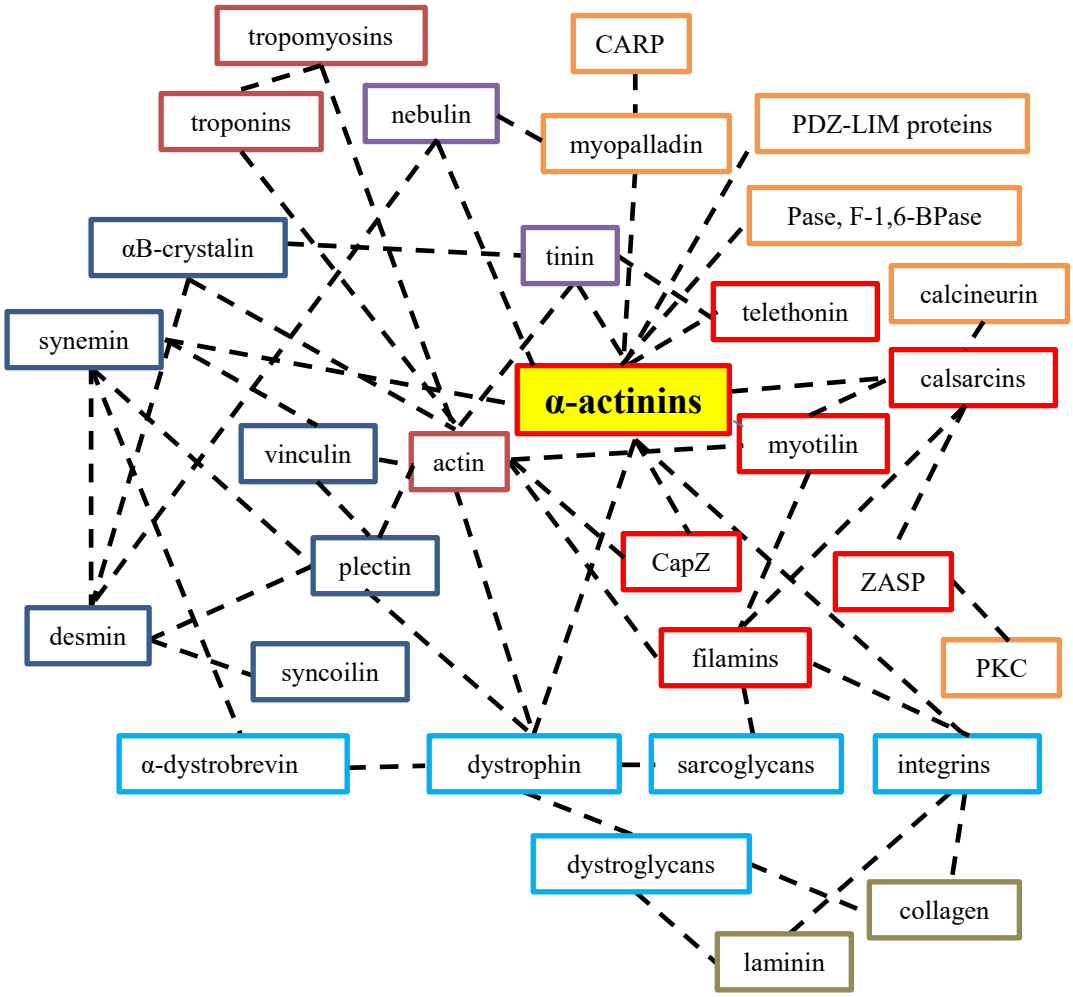

Figure 2 *Summary of α-actinin protein interactions reported in the literature. Proteins are categorised by subcellular localisation as Z line (red), sarcomeric ruler proteins spanning the Z line (purple), thin filament (pink), intermediate filament (dark blue), sarcolemma (muscle membrane; light blue), extracellular matrix (brown) and soluble signalling proteins or metabolic enzymes (orange). Adapted from (MacArthur et al., 2007).*

Given that both α-actinin -2 and -3 isoforms evolved from a common ancestor, show high degree of evolutionary divergence, have high sequence similarity and are co-expressed at the Z-line, it is likely that α-actinin-3 also shares many of these binding partners and participates in similar signalling pathways. However, α-actinin-3 has developed more specialised expression restricted to fast glycolytic skeletal muscle fibres. α-actinin-2 is expressed in the more oxidative muscle fibres and in indefatigable heart. This shows the possibility of functional differences between the two α-actinin isoforms (Lek and North, 2010). Altered balance of sarcomeric α-actinins in the muscle's sarcomere can alter the elastic properties during force generation and eccentric damage. This alteration is also likely to apply to many signalling and metabolic interactions in the muscle. The following sections provide relevance for understanding these differences due to the frequency of α-actinin-3 deficiency in the general population and elite athletes.

5.3 *The α-actinin deficiency*

5.3.1 The ACTN3 R577X polymorphism

Professor Kathryn North was the first to identify a common null polymorphism (registered SNP; rs1815739) in the *ACTN3* gene, which results in replacement of an arginine (R) residue with a premature stop codon (X) at amino acid 577 (North et al., 1999). Remarkably, on average 18% of the world population (~1.5 billion individuals) harbour the *ACTN3* 577XX genotype and consequently are completely deficient in the α-actinin-3 protein; in Australia alone this equates to ~4.3 million individuals (MacArthur and North, 2004b). Importantly, α-actinin-3 deficiency influences skeletal muscle function and metabolism without causing obvious disease (Mills et al., 2001). Earlier studies suggested that lack of *ACTN3* expression is linked to merosin-positive Congenital Muscular Dystrophy (CMD) (North and Beggs, 1996). Evidence from muscle biopsies indicated that ~ 25% of subjects with merosin-positive CMD were α-actinin-3 deficient (*ACTN3* XX genotype in humans), but it was not clear from this data whether or not α-actinin-3 deficiency was due to a mutation(s) in the ACTN3 gene or is a secondary marker. Later biopsy data (Vainzof et al., 1997) from subjects with various forms of muscular dystrophy showed that subjects from the same families and with the identical disease were discordant for α-actinin deficiency, and it was concluded that α-actinin-3 deficiency is only a secondary effect in the various forms of muscular dystrophy. Thus, a deficiency of α-actinin-3 protein doesn't seem to lead to a disease phenotype, as the α-actinin-2 isoform may help compensate. Research indicates that the closely related isoform, α-actinin-2, is able to partially "compensate" for the absence

of α-actinin-3 in human fast-twitch (type IIb) skeletal muscle fibres (Mills et al., 2001). Nonetheless, α-actinin-3 is highly conserved and research has shown that the 577X-allele has undergone strong positive selection during recent human evolution (MacArthur and North, 2004; Friedlander et al., 2013). This suggests that the α-actinins are not functionally redundant and perform specific functions in fast muscle fibres.

5.3.2 The ACTN3 X-allele has undergone positive natural selection

To explore the selective forces that have acted on the *ACTN3* R577X polymorphism during human evolutionary history and understand the varied X-allele frequency distributions observed between different human populations, some researchers examined DNA sequence and long-range linkage disequilibrium (LD) data around the R577X alleles in individuals of European, East Asian and African ancestry using DNA obtained from the International HapMap Project. These analyses found low rates of DNA substitutions and high recombination amongst X-allele-containing haplotypes compared with the R-allele in Europeans and Asians, consistent with strong, recent positive selection of the 577X allele in these populations. It has been hypothesised that the 577X allele provides an advantage to modern humans adapting to the Eurasian environment (MacArthur and North, 2007). This has been explored further and the increase in *ACTN3* 577XX genotype has been correlated with a higher global latitude gradient and reduced species richness (MacArthur and North, 2007), which suggests that environmental variables related to temperature (cold tolerance), and diet (feast/famine) influence the R577X genotype frequencies currently observed worldwide. Therefore, the impact of this common null polymorphism has global relevance via its contribution to variations in muscle function. However, a recent study found low frequency of the X-allele in Siberian populations and these results contradict “cold tolerance” hypothesis of positive selection on X-allele of *ACTN3* gene (Malyarchuk et al., 2018).

5.4 *ACTN3 R577X gene and human performance*

To date, three methodological approaches have been implemented to examine the effect of ACTN3 genotype on muscle function. They include case–control, and cross-sectional studies in humans and mechanistic analyses using the Actn3 KO mouse model. I will now discuss the findings of these three approaches to highlight the role of α-actinin-3 in skeletal muscle in athletes, general population (**Figure 3**).

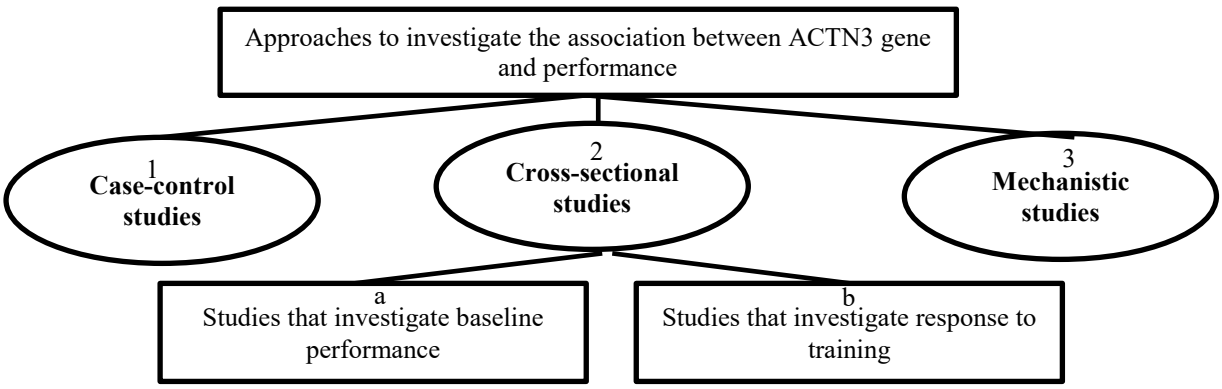

Figure 3 *A schematic of the approaches used to investigate the influence of ACTN3 on muscle performance, to date.*

5.4.1 Case control studies

The majority of human association studies performed to date have been case:control. Case control studies explore the association between the *ACTN3* genotype and athletic performance based on athletic status. This means that the *ACTN3* genotype frequency is compared between cases (athletes) and controls (the general 'non-athletic' population), with the athletic events (either sprint/power-based, endurance-based, or mixed-sports-based) as the main descriptor.

Eighteen case:control studies consisting of sprint/power athletes have been conducted to date (Table 1). These studies show a higher frequency of the *ACTN3* 577RR genotype (and lower frequency of α-actinin-3 deficiency, 577XX) in elite sprint/power athletes (i.e., sprinters, jumpers, and throwers) compared to the control group (non-athletes). This finding was originally observed in Caucasian elite athletes from Australia (Yang et al., 2003). Subsequently, replication in national/international level athlete cohorts from Finland (Niemi and Majamaa, 2005), Greece (Papadimitriou et al., 2008), Russia (Druzhevskaya et al., 2008), Israel (Eynon et al., 2009) and Poland (Cięszczyk et al., 2011) have been reported. A sub analysis of sprint/power cohorts also found that the *ACTN3* RR genotype

has a stronger association with sprinters compared to other track and field events such as jumpers, pole-vaulters, decathletes, and throwers (Papadimitriou et al., 2008) and no Olympic-finalist sprinter has yet been identified with the 577XX genotype (Eynon et al., 2013). Similarly, four Asian sprint/power cohorts from, Taiwan (Chiu et al., 2011), Japan (Mikami et al., 2013; Kikuchi; Nakazato, 2015), Korea (Hong and Jin, 2013; Kim et al., 2014) and China (Yang et al., 2017) have also confirmed the lower *ACTN3* XX frequency in elite sprinters compared to Asian population controls (Table 1).

In contrast, three African ancestry sprint/power cohorts (Jamaican and USA), found low frequencies of the 577XX genotype in both athletes (2-7%) and controls (2-4%) (Scott et al., 2010; Yang et al., 2007). This could be interpreted as the African population being more suited to sprint performance, or that there is no significant effect of *ACTN3* genotype and that other genetic and environmental factors exhibit a greater selection effect (Yang et al., (2003). Taken together, these studies demonstrate that sprint/power oriented athletes, in both genders and across different sports and ethnic backgrounds have lower frequencies of the *ACTN3* 577XX genotype compared to population controls, indicating that α-actinin-3 deficiency is detrimental for optimal fast muscle function at the extremes of sprint/power performance.

Table 1 Case control studies with the ACTN3 R577X polymorphism in elite sprit/power oriented athletes.

Sprint/Power Athletes					Athletes Genotype%			Controls Genotype%				
Country/ Ethnicity	Gender	Sport	N	P	RR	RX	XX	N	RR	RX	XX	Reference
Australian	M	Swimmers, Track cyclists>400, Rowers, Skiers	72	<0.001	53	39	8	134	30	54	16	Yang et al., 2003
	F		35	<0.01	43	57	0	292	30	54	16	
Finnish	M&F	Track & Field athletes	23	<0.03	48	52	0	120	45	46	9	Niemi & Majaama 2006
Greek	M&F	Track & Field - Mainly Sprinters (100m-400m)	73	<0.02	48	36	16	181	26	54	18	Papadimitriou et al., 2008
USA	M&F	Bodybuilders, Powerlifters	75	0.005	31	63	7	876	38	46	16	Roth et al., 2008
Russian	M	Skiers, Gymnasts, Bodybuilders, Skaters, Hockey players, Powerlifters, Footballers, Speed Skaters; Swimmers; Track & Field athletes, Volleyball players, Weightlifters, Wrestlers	363	<0.0001	38	56	6	524	37	47	16	Druzheveskaya et al., 2008
	F		123	0.067	46	48	6	673	37	51	13	
Israeli	M&F	Track & Field athletes- Mainly Sprinters (100m-400m)	55	<0.0001	38	42	20	240	20	62	18	Eynon et al. 2009
Italian	M&F	Artistic Gymnasts	35	0.04	49	49	3	53	32	49	19	Massidda et al., 2009
Polish	M&F	Track & Field athletes, Short distance Swimmers, Weightlifters	178	0.008	40	52	8	254	35	49	15	Cięszczyk et al., 2011
Taiwanese	M	Short distance Swimmers	37	NS	39	48	14	306	32	49	20	Chui et al., 2011
	F		44	<0.05	46	43	11	306	32	49	20	
Japanese	M&F	Wrestlers	52	0.028	27	62	11	333	27	45	28	Kikuchi et al., 2012
Korean	M	Gymnasts, Sprinters, Throwers, Speed Skaters, Weightlifters and Taekwondo athletes	47	NS	21	57	21	361	29	53	18	Hong et al. , 2013
	F		37	0.028	46	51	3	361	32	50	18	
Japanese	M	Track & Field athletes	134	0.002*	25	58	17	649	21	53	26	Mikami et al., 2013
Korean	M&F	Weightlifters, Speed Skaters, Sprinters and Swimmers	121	<0.05	40	48	12	854	30	51	19	Kim et al., 2014
Chinese	M&F	Track & Field athletes, Track Cyclists, Weightlifters	59	<0.001	49	46	5	50	26	40	34	Yang et al., 2017
Nigerian	M&F	Track & Field athletes	62	NS	87	13	0	60	83	17	3	Yang et al., 2007
Jamaican	M&F	Track &Field athletes	86	NS	75	22	3	232	75	22	2	Scoot et al., 2010
USA	M&F	Track& Field athletes	79	NS	70	28	2	126	66	30	4	Scoot et al., 2010

* In Japanese cohort the statistical significant difference was detected in RR+RX sprinters vs. Control group.

An inverse association has been reported in endurance type events, with an over representation of the *ACTN3* XX genotype in some populations of endurance athletes (Yang et al., 2003). While this has been replicated in some elite endurance athlete cohorts (Eynon et al., 2009) other case:control studies (Niemi and Majamaa, 2005; Saunders et al., 2007; Papadimitriou et al., 2008; Ahmetov et al., 2010; Cięszczyk et al., 2011; Mikami et al., 2013; Mägi et al., 2016) have not shown an association between the *ACTN3* R577X genotypes and endurance performance (Table 2).

To date, seven studies have examined the frequency of *ACTN3* genotype in elite endurance athletes. The Australian, Russian, Polish, Korean, Chinese and Estonian studies examined a range of endurance sports, including cycling, rowing, swimming, track & field (>400m) and cross-country skiing. No significant difference in the frequency of *ACTN3* XX genotype was observed in Australian and Chinese elite male endurance athletes, while the subgroup of elite female endurance athletes had an increased *ACTN3* XX genotype frequency compared to the control in both studies (Yang et al., 2003; Shang et al., 2010). However, Russian and Estonian endurance athletes (both males and females) unexpectedly showed a lower *ACTN3* XX genotype frequency compared to control, with none of the 33 highly elite athletes (defined as winners of a world competition) being α-actinin-3 deficient ($P<0.05$), (Ahmetov et al., 2010; Mägi et al., 2016). Furthermore, a Spanish cohort of elite endurance cyclists and runners showed that the frequency of the *ACTN3* XX genotype was also not significantly different from controls (Lucia et al., 2006).

The lack of reproducibility in these findings suggests that the effects of α-actinin-3 deficiency in elite endurance athletes may be small. In addition, these studies also highlight some of the limitations of a case:control approach in determining the effects of candidate genes on performance. Major limitations in the current studies include a relatively low sample size (typically <100 athletes), and limited accountability of performance, training and environment. To overcome these limitations athletes are often grouped together across heterogeneous sport disciplines and events (e.g., sprinters, jumpers, throwers, swimmers, and power/sprint team sport athletes). While this increase in sample size and provides a larger population for analysis, a lack of specificity in performance and events also increases inter-cohort variability. Given that the inherit number of world-class elite athletes available is low, this approach is understandable, but the results are often statistically underpowered.

Table 2 Case control studies with the ACTN3 R577X polymorphism in elite endurance athletes.

Endurance Athletes					Athletes Genotype%			Controls Genotype%				
Country/ Ethnicity	Gender	Sport	P	N	RR	RX	XX	N	RR	RX	XX	Reference
Australian	M	Swimmers, Cyclists, Rowers, Cross-country skiers	NS	118	28	53	19	134	30	54	16	Yang et al., 2003
	F		<0.05	75	20	50	30	292	30	50	20	
Finnish	M&F	Endurance Track & Field athletes	NS	52	50	40	10	1060	43	48	9	Niemi & Majaama 2006
Spanish	M&F	Rowers, Long distance cyclists, Long distance runners	NS	139	27	45	27	103	29	57	14	Lucia et al., 2006
Greek	M&F	Track & Field athletes – Mainly long distance runners	NS	20	50	25	25	181	26	56	18	Papadimitriou et al., 2008
Russian	M	Race walkers, Biathletes, Cyclists, Rowers, Long distance swimmers, Triathletes, Cross-country skiers	NG†	293	40	53	7	532	36	47	17	Ahmetov et al., 2010
	F		NG	163	37	59	4	679	37	50	13	
Israeli	M&F	Track & Field athletes – Mainly long distance runners	<0.006	54	19	46	35	240	20	62	18	Eynon et al. 2009
American, Finnish, German	M	Biathletes, Triathletes, Long distance cyclists, Long distance runners, Long distance rowers	NS	316	29	50	21	304	32	51	18	Doring et al., 2010
Chinese	M	Long distance rowers, Long distance cyclists, Long distance runners and Long distance swimmers	NS	132	37	51	12	450	35	48	17	Shang et al., 2010
	F		<0.05	118	19	60	21	450	35	48	17	
Russian	M&F	Rowers, Speed skaters, Race walkers, Skiers, Long distance swimmers	NS	70	44	56	0	354	35	41	23	Eynon et al. 2012
Polish, Spanish, Russian	M&F	Cyclists, Rowers, Runners	NS	284	37	51	12	808	32	51	18	Eynon et al.,2012
Koreans	M	Badminton Players, Table Tennis Players, Hockey Players and Handball Players	NS	41	46	44	10	188	29	53	18	Hong et al. , 2013
	F		NS	25	24	48	28	173	32	50	18	
Japanese	M&F	Track & Field – Mainly long distance athletes	NS	165	23	54	23	649	21	53	26	Mikami et al., 2013
Estonians	M&F	Cross Country skiers and Biathletes	NG	58	33	58	9	222	76	16	8	Mägi et al., 2016
Chinese	M&F	Long distance runners	NS	44	32	36	32	50	26	40	34	Yang et al., 2017

* In Israeli cohort an Endurance athletes vs. Sprinters statistical significant detected difference (P<0.005) was detected on top of Endurance athletes vs. Controls significant difference.
† In Russian cohort none of the males highly elite endurance athletes had the *ACTN3* 577XX genotype.

To overcome these limitations, meta-analyses of published data have been performed. This type of statistical analyses are applied to separate experiments from independent researchers in the same fields, pooling the data and testing a set hypothesises in a larger population, with greater statistical power. To date, three meta-analyses have been completed to examine the effect of *ACTN3* R577X in athlete populations. The first meta-analyses support the hypothesis that in Caucasians the *ACTN3* RR genotype is more common among sprint/power athletes compared to controls (**Figure 4**), without finding any evidence that the *ACTN3* XX genotype is altered in endurance athlete performance (**Figure 5**) (Alfred et al., 2011). The other two meta-analyses similarly support the role for α-actinin-3 in sprint/power performance, without finding any evidence that the *ACTN3* XX genotype is altered in endurance athlete performance, across multiple athlete cohorts of Caucasian, Asian and African ancestry (Ma et al., 2013; Garton et al., 2018).

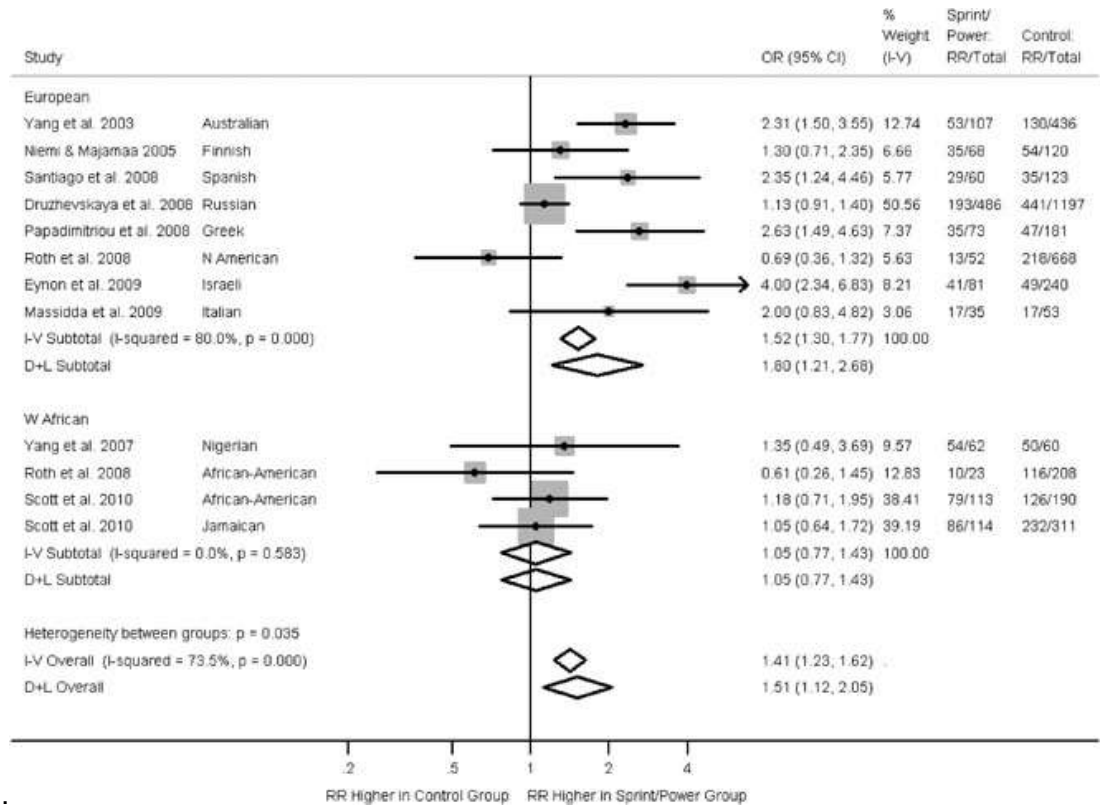

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Figure 4 *Associations between ACTN3 R577X genotype (RR vs. RX + XX) and sprint/power athletic status from the literature. Arrow indicates the confidence interval extends beyond the plot axis. Stratified by ancestral group. Effects are given as odds ratios (OR) and 95% confidence intervals (CI). Points and the horizontal lines represent the study effect sizes and their 95% CIs. Sizes of the squares represent the weights of the studies. Diamonds represent the summary effects and their 95% CIs. I–V: inverse-variance, fixed effect model. D + L: Der Simonian & Laird, random effects model. This figure is used in this thesis with permission from publisher John Wiley & Sons under license number 4291901000444.*

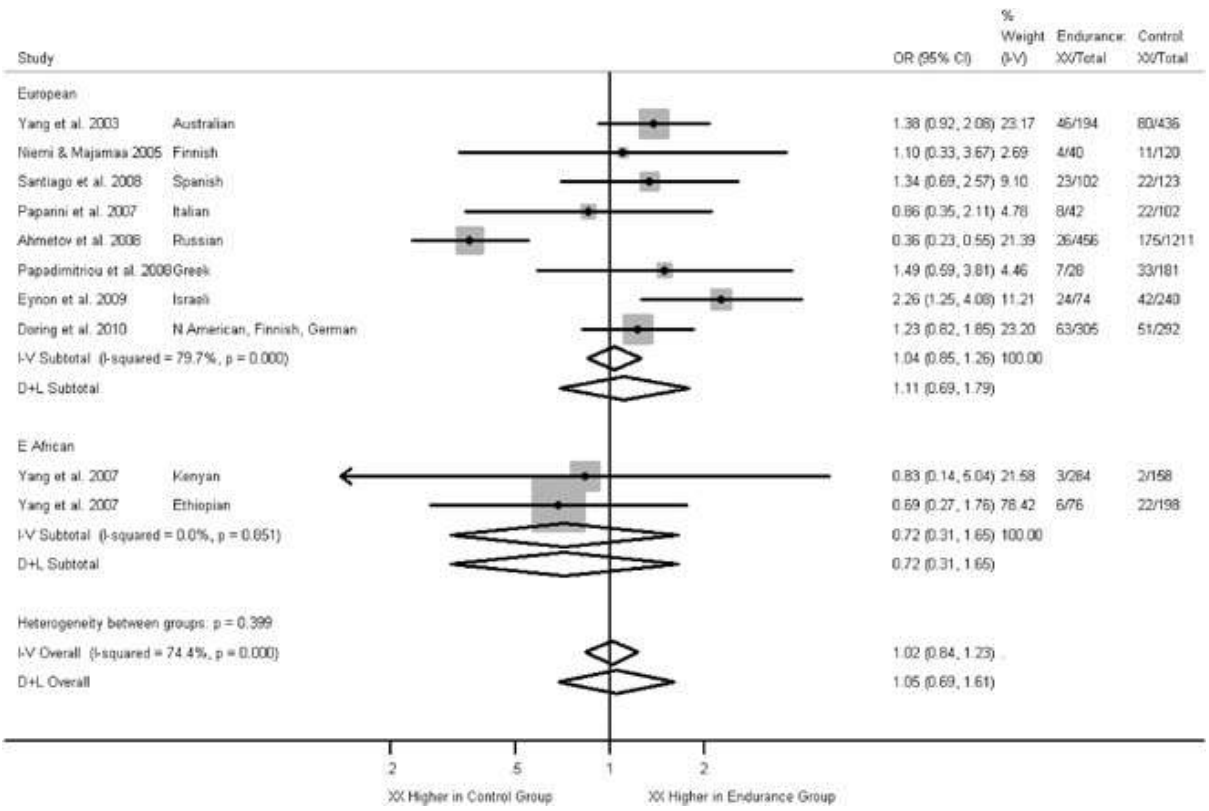


Figure 5 *Associations between ACTN3 R577X genotype (XX vs. RX + RR) and endurance athletic status from the literature. Arrow indicates the confidence interval extends beyond the plot axis. Stratified by ancestral group. Effects are given as odds ratios (OR) and 95% confidence intervals (CI). Points and the horizontal lines represent the study effect sizes and their 95% CIs. Sizes of the squares represent the weights of the studies. Diamonds represent the summary effects and their 95% CIs. I–V: inverse-variance, fixed effect model. D + L: DerSimonian & Laird, random effects model. This figure is used in this thesis with permission from publisher John Wiley & Sons under license number 4291901000444.*

Major limitation of these meta-analysis approaches is that are based on data from previously published case:control studies and the most important of their limitations is the lack of quantitative measures of which means that these associations are not based on athletes measurable traits. This limitation highlights the need for introducing a new approach using the running times of the elite athletes as an improved and more accurate measure of elite performance compared to the definition of “elite performance” based on athletic status that have been used in all studies with elite athletes in the literature so far.

5.4.2 Cross-sectional studies

The effect of *ACTN3* R577X in the general population have been examined using cross-sectional studies based on quantitative performance measures of non-athletes for *ACTN3* R577X summarised in Table 3. One of the first findings to emerge from the investigation on baseline performance in non-athletes is that the *ACTN3* 577 RR genotype shows a strong and positive association for increased muscle strength in adult women (Clarkson et al., 2005). Further to this *ACTN3* R577X is thought to account for ~2.3% of the variability in 60 m sprint time in adolescent boys (n=992), with no effect of *ACTN3* genotype on endurance performance, as assessed by the shuttle run test (Moran et al., 2007).

Another power related exercise that has been used to assess muscle performance is the anaerobic all-out 30 second Wingate cycling test. Norman et al., (2009) found *ACTN3* RR individuals increased their peak power measure on the second trial, while *ACTN3* XX individuals performed similarly to the first. Other studies have used an isokinetic dynamometer to measure strength at varying speeds to recruit fibres in the order slow-to-fast. Isokinetic knee extension strength has been measured in five studies across a range of contraction speeds. At the first testing session no significant differences were reported in the range of 0- 240°/sec (Vincent et al., 2007; Norman et al., 2009; McCauley et al., 2009; Gentil et al., 2011). However, strength at 300°/ (n=90) showed lower torques in *ACTN3* XX individuals ($P < 0.05$) (Vincent et al., 2007) supporting a subtle strength deficit in the fast fibres of *ACTN3* XX in comparison to *ACTN3* RR individuals. Other power related phenotypes that have been used to assess muscle performance are the handgrip strength, the Squat Jump (SJ) and the Counter Movement Jump (CMJ) tests that have demonstrated a subtle (~5 %) speed-power deficit in *ACTN3* XX individuals compared to *ACTN3* RR (Broos et al., 2015).

Table 3 Cross sectional studies with the ACTN3 R577X polymorphism and sprint/power oriented performance characteristics at baseline.

Origin	Tested	Found	Gender Age	N	Reference
UK	40m Sprint; Vertical jump;Throw distance;Grip strength, shuttle run test	40m Sprint	M 11-18	525	Moran et al., 2007
			F 11-18	439	
Belgium	Knee extensor strength;isometric at 45°;Isokinetic at 100, 200 and 300°/s	*ACTN3* XX lower knee torque at 300°	M 18-29	90	Vincent et al., 2007
USA	Isokinetic , concentric & eccentric strength at 30 – 180°/s DEXA for body composition	*ACTN3* XX lower peak torque at all speeds *ACTN3* XX lower total body Fat Free Mass	M 22-90	454	Walsh et al., 2008
			F 22-90	394	
UK	Isometric and isokinetic strength	-	M 18-39	79	MacCauley et al., 2009
Spain	Vertical and Counter Movement Jumps test; 15-30m sprints	-	M 18-2974	217	Santiago et al., 2010
UK	Flexor and extensor isometric strength ;vertical jump and 15m sprint time;	-	F 18-39	62	Gavin & Williams 2010
Russia	Standing long jump; grip strength; BMI	*ACTN3* XX lower body weight	M 11	219	Ahmetov et al., 2012
			F 11	238	
Taiwan	Standing long jump test; 60 s sit-up test; 60 & 800m run	*ACTN3* XX lower number of sit-ups	F 11	170	Chui et al., 2012
China	BMI; hand grip strength; body composition; 100m sprint and 5000m run	*ACTN3* XX lower hand grip strength	M	452	Shang et al., 2012
Belgium	Peak force, Maximal velocity, Peak power, Maximal knee extension torque, Cross-sectional area	*ACTN3* XX had lower bone cross-sectional area ; lower grip strength, SJ & CMJ and knee torque at 300°/s	M	226	Broos et al., 2015

Cross-sectional studies that investigate the response to strength and endurance exercise are limited (Table 4). Clarkson et al., (2005) found that α-actinin-3-deficient females *(ACTN3* XX*)* showed a significantly greater response to strength training for the 1-RM (Repetition Maximum) measurement. Delmonico et al., (2007) found that *ACTN3* XX individuals had lower gains in muscle thickness. However, no associations with 1RM strength or muscle volume were identified, which failed to replicate Clarkson et al., (2005). It should be noted that in both studies, the group with lower initial values showed some improvement. Finally, Vincent et al., (2010) suggests a protective role of α-actinin-3 protein in muscle damage immediately after eccentric training.

Table 4 Cross sectional studies with the ACTN3 R577X polymorphism and power oriented performance improvements in response to (10 -12 weeks) of training.

Origin	Tested	Found		Gender Age	N	Reference
USA	1 RM; Elbow flexor MVC; muscle size MRI	At baseline	*ACTN3* XX had lower MVC	M 18-40	247	Clarcson et al., 2005
		Post training	*ACTN3* XX woman had greater absolute and relative 1 RM gains	F 18-40	355	
USA	Knee extensor concentric peak power	At baseline	*ACTN3* XX lower 1 RM & peak power	M 56-74	71	Delmonico et al., 2007
		Post training	*ACTN3* XX lower gains in peak power	F 64	86	
Belgium*	Eccentric exercise and CK activity	At baseline	This study suggests a protective role of α-actinin-3 protein in muscle damage after eccentric training	M 22	19	Vincent et al., 2010
		Post training				
Brazil	1 RM bench press; knee extensors peak torque (60 °/s) and ultra sound muscle thickness	At baseline	-	M 23-31	141	Gentil et al., 2011
		Post training	*ACTN3* XX had lower gains in muscle thickness			

* In Belgian cohort the protective effect of α-actinin-3 was detected after a single bout of eccentric exercise and the study design did not involve chronic training.

The cross-sectional studies investigating endurance performance at baseline did not show a significant association between *ACTN3* XX genotype and endurance performance (Lucia et al., 2006; Paparini et al., 2007; Doring et al., 2010; Muniesa et al., 2010). While a debate is ongoing on whether the *ACTN3* genotype influences endurance performance, it is evident from the current studies that any effect of *ACTN3* genotype on endurance performance it is possibly small and likely to be affected by differences in environment, training and genetic background/ethnicity. Beyond muscle performance, cross-sectional studies have demonstrated a strong and positive association between the *ACTN3* RR genotype bone mineral density (Min et al., 2016) and bone markers (Levinger et al., 2017) compared to *ACTN3* XX individuals.

In *ACTN3* association studies, it is the elite athletes that represent the most well trained cohorts. Their training is persistent and specific to the nature of their event. Given this fact low volume training sessions, may not be sufficient to induce the manifestation of the functional differences among the R577X genotypes. However, it would be interesting to understand if a relatively lower volume-training program focused on either power or endurance performance may induce different training responses based on *ACTN3* genotype in the general population.

Overall, the associations seen in cross-sectional studies are weaker than the observed case: control analyses reviewed in the previous section. This may suggest that the effects of α-actinin-3 deficiency are less evident in the general population compared to the elite athletes. Given that α-actinin-3 is a variation on normal muscle function and does not cause disease, the observation that *ACTN3* genotype effects are more evident in elite athletes is exciting as they represent an extreme end of the muscle performance spectrum. The subtle differences in healthy muscle from the general population maybe explained by the significant variation in environment, diet, training and ultimately muscle performance seen in the healthy individuals, which represent the general population.

A more comprehensive meta-analysis approach about the *ACTN3* genotype and quantitative measures of performance in non-athletes found no association between *ACTN3* R577X polymorphism and measures of performance such as: grip strength (**Figure 6**) timed get up and go (**Figure 7**), or chair rises (**Figure 8**).

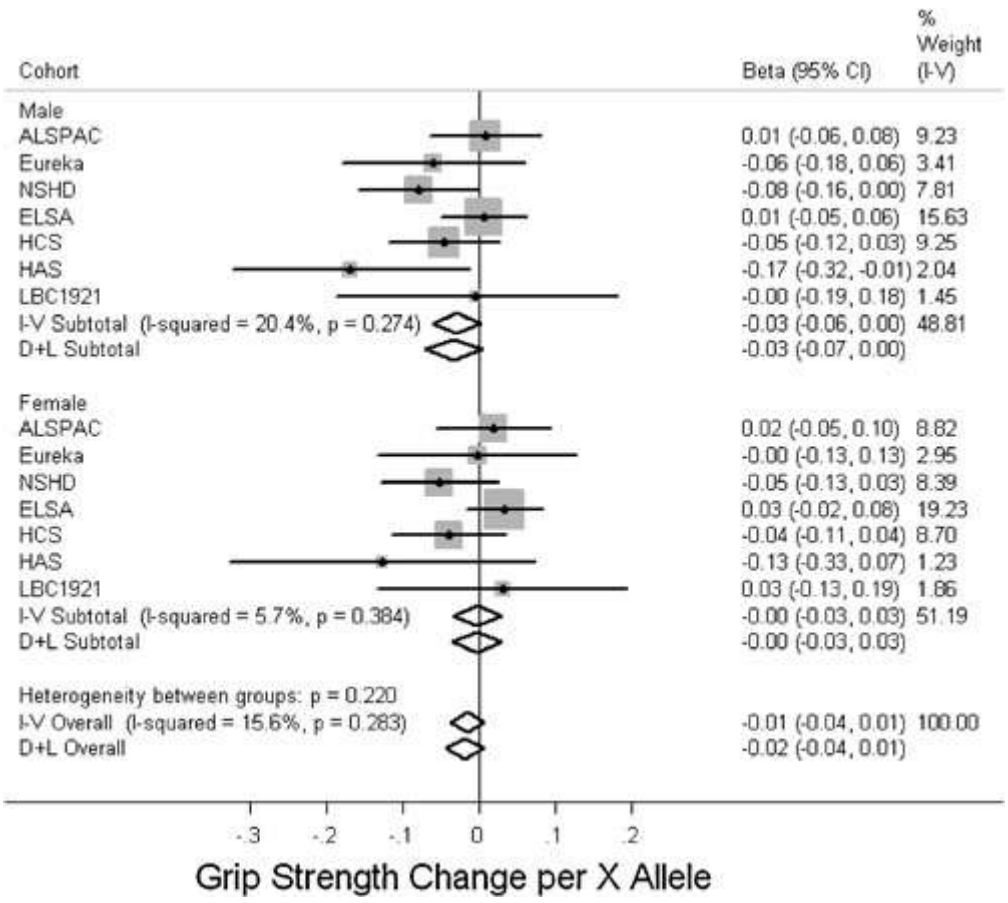


Figure 6 *Associations between ACTN3 R577X genotype and grip strength. Studies ordered by overall median age. Effects are given as per X allele change in grip strength (z-score) and 95% confidence intervals (CI). Points and the horizontal lines represent the study effect sizes and their 95% CIs. Sizes of the squares represent the weights of the studies. Diamonds represent the summary effects and their 95% CIs. I–V: inverse-variance, fixed effect model. D + L: DerSimonian & Laird, random effects model. This figure is used in this thesis with permission from publisher John Wiley & Sons under license number 429190100044.*

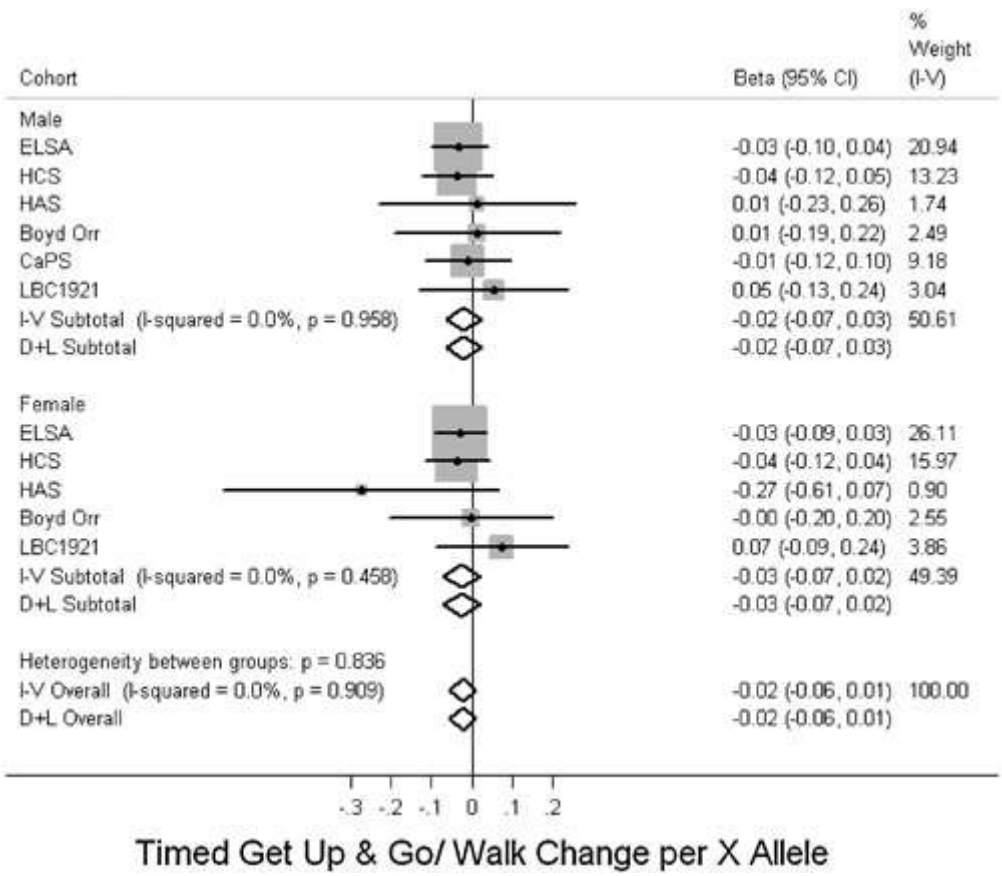


Figure 7 *Associations between ACTN3 R577X genotype and timed get up and go/walk. Studies ordered by overall median age. Effects are given as per X allele change in timed get up and go or walk (z-score) and 95% confidence intervals (CI). Points and the horizontal lines represent the study effect sizes and their 95% CIs. Sizes of the squares represent the weights of the studies. Diamonds represent the summary effects and their 95% CIs. I–V: inverse-variance, fixed effect model. D + L: DerSimonian & Laird, random effects model. This figure is used in this thesis with permission from publisher John Wiley & Sons under license number 4291901000444.*

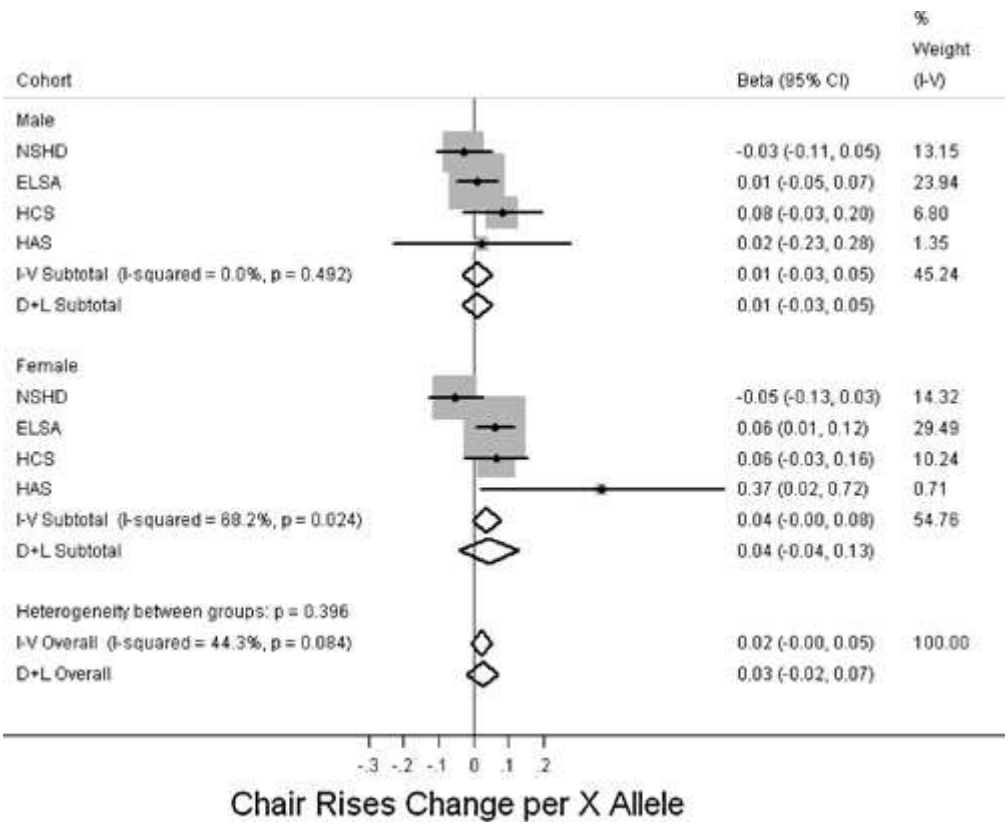


Figure 8 *Associations between ACNT3 R577X genotype and timed chair rises. Studies ordered by overall median age. Effects are given as per X allele change in timed chair rises (z-score) and 95% confidence intervals (CI). Points and the horizontal lines represent the study effect sizes and their 95% CIs. Sizes of the squares represent the weights of the studies. Diamonds represent the summary effects and their 95% CIs. I–V: inverse-variance, fixed effect model. D + L: DerSimonian & Laird, random effects model. This figure is used in this thesis with permission from publisher John Wiley & Sons under license number 4291901000444.*

Shifting our focus from athletic status as a measure of elite athletic performance and the lower volume training approach in non-athletes to reported running times of elite athletes as an improved metric to assess the effect of α-actinin-3 on performance is a new perspective and could have the potential to help scientists to detect the actual influence of *ACTN3* R577X polymorphism on human performance at the high end of the performance spectrum.

5.4.3 Mechanistic studies

Mechanistic studies aim to uncover the mechanisms behind a physiological characteristic (e.g., difference in performance). For this purpose, a genetically-engineered mouse in which researchers have inactivated (knocked out), the *ACTN3* gene was used to reveal the underlying mechanisms behind the performance differences between the *ACTN3* genotypes (Seto et al., 2013). Actn3 KO mice replicate many of the phenotypes described in human α-actinin-3 deficiency; including reduced muscle grip strength (MacArthur et al., 2007). The most significant findings to emerge from this Actn3 KO mouse model is that α-actinin-3 deficiency, leads to a shift in the anaerobic metabolic profile towards a slow-twitch aerobic metabolic phenotype, with increased mitochondrial oxidative enzyme activity (MacArthur et al. 2007). In Actn3 KO mice has been also observed an increase in the rate of calcium (Ca^{2+}) release and absorption by the Sarcoplasmatic Reticulum (SR), with their muscle fibres being more resistant to fatigue due to the slower rate of decline in Ca^{+} release following muscle stimulation (Head et al., 2015). The α-actinins also are known to interact with an array of structural, signalling and metabolic proteins, providing an important platform for protein interactions at the Z-line of fast twitch skeletal muscle (**Figure 2**). Many of these Z-line proteins have been found altered in Actn3 KO muscles, such as myotilin, desmin, PDZ and γ-filamin (Hogarth et al., 2016). Apart from the altered metabolism, Ca^{2+} kinetics and structural alterations, associated with α-actinin-3 deficiency, signalling changes have also been identified in Actn3 KO muscle. The four main biological alternations associated with α-actinn-3 deficiency in Actn3 KO mouse muscle fibres are summarised in **Figure 9** and the main findings of each category that affect mitochondria will be reviewed in the following chapters.

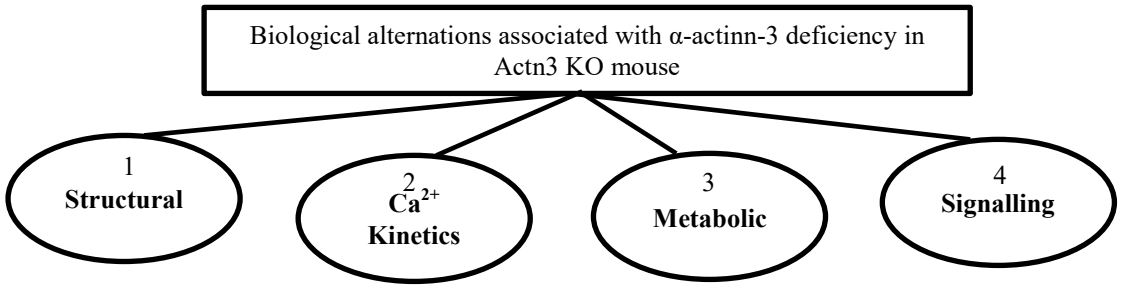


Figure 9 *A schematic of four main biological alternations associated with α-actinn-3 deficiency identified in Actn3 KO mouse*

5.4.3.1 Metabolic effects of α-actinin-3 deficiency

The metabolic profile of the KO mice muscles compared with their WT littermates demonstrated that quadriceps muscles from KO mice display increased activity of the mitochondrial enzyme citrate synthase, and decreased activity of an anaerobic enzyme, lactate dehydrogenase (MacArthur et al., 2007). Two mitochondrial enzymes of the tricarboxylic acid (TCA) cycle (citrate synthase and succinate dehydrogenase) and one electron transport chain enzyme (cytochrome c oxidase) showed 25–39% higher activity levels in KO muscle relative to WT. Two mitochondrial enzymes involved in fatty acid oxidation, hydroxyacyl-CoA dehydrogenase, and medium chain acyl-CoA dehydrogenase, also showed 30–42% higher activity in KO muscle, suggesting an increased reliance on β-oxidation of fatty acids in the absence of α -actinin-3 expression (**Figure 10**).

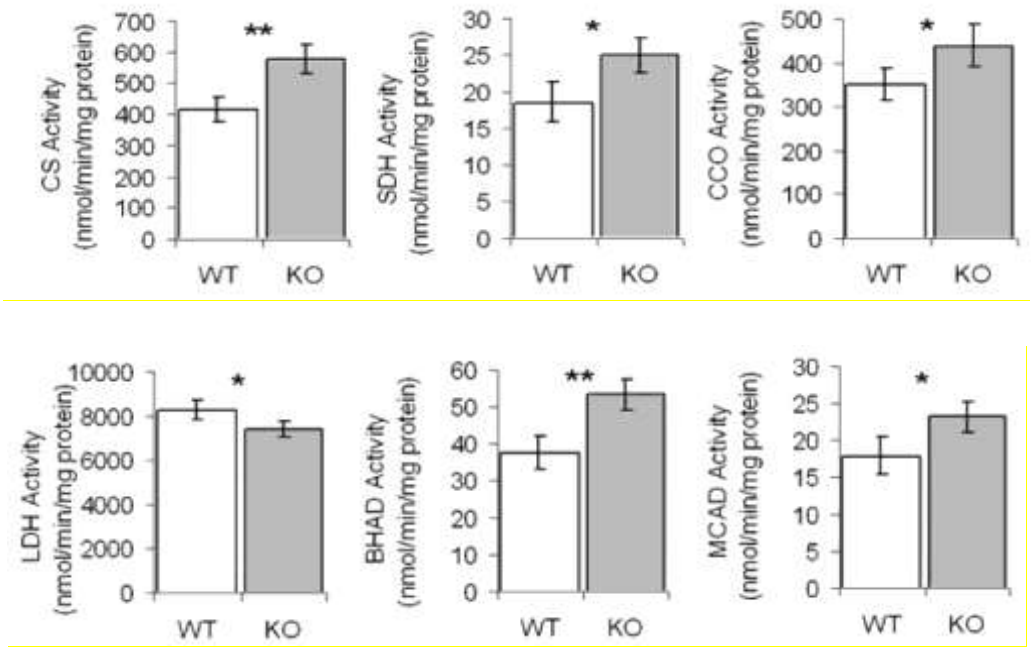


Figure 10 *Increased enzyme activities of citrate synthase (CS), succinate dehydrogenase (SDH), cytochrome c oxidase (CCO), 3-hydroxyacyl-CoA dehydrogenase (BHAD) and medium chain acyl-CoA dehydrogenase and decreased of (MCAD) lactate dehydrogenase (LDH), in KO vs. WT mice skeletal muscle* (MacArthur et al., 2008).

In combination, these data from mice suggest a shift in the muscle metabolism of fast fibres in KO mice away from their traditional reliance on anaerobic metabolism towards the slower but a more efficient aerobic pathway (MacArthur et al., 2007). However, immunohistochemical staining in human fast twitch skeletal muscle fibres has shown no improved aerobic/oxidative pathway in *ACTN3* XX homozygotes (Vincent et al., 2012).

These results contrast with those from Actn3 KO mouse (MacArthur et al., 2008) and may highlight differences in aerobic metabolism that may be exaggerated in the mouse model. Results from KO mice don't always translate to people, and further studies in humans remain essential to confirm the findings observed in mice.

5.4.3.2 Signalling effects: The slow myogenic program

The difference in structural and metabolic properties in α-actinin-3 deficient muscle, without a fast twitch fiber type IIb to IIa shift, suggests that differences in key signalling pathways are responsible for determining fibre properties in the absence of α-actinin-3.

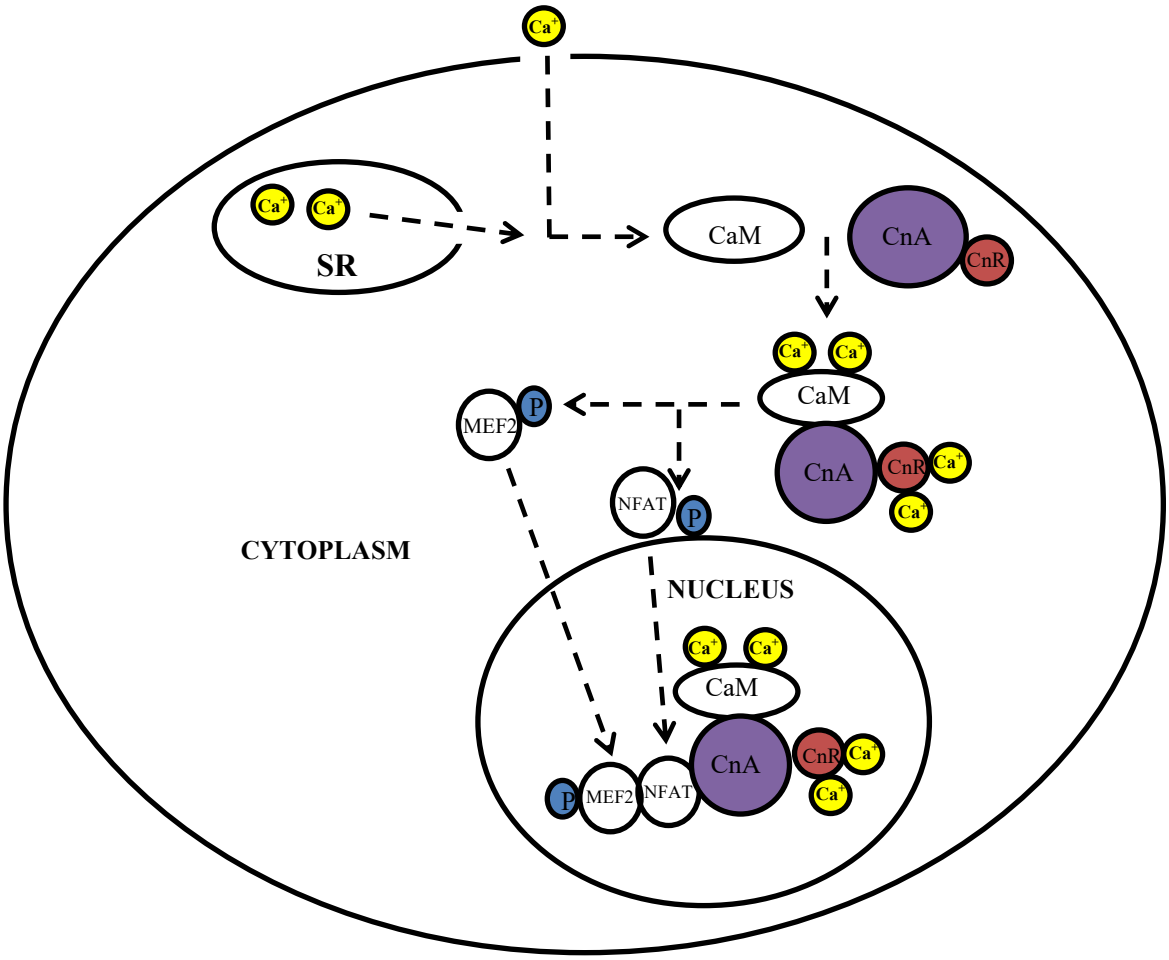


Figure 11 *Calcineurin signaling pathways in skeletal muscle. Elevation of intracellular calcium, leads to calcium binding to calmodulin CaM and regulatory subunit CnR binding domains. The association of calcium-CaM with the CnA–CnR dimer displaces the CnA autoinhibitory domain from its catalytic site, forming an activated phosphatase complex. Activated calcineurin targets substrates, such as NFAT and MEF2 transcription factors, for dephosphorylation activation that enter the nucleus and mediate the induction of specific slow gene myogenic program.*

The molecular mechanisms for the altered calcineurin signalling with α-actinin-3 deficiency appear to be via differential binding of calsarcin-2 to sarcomeric α-actinins. When α-actinin-3 is absent (*ACTN3* 577XX genotype) there is a compensatory increase in α-actinin-2, which binds more tightly to calsarcin-2 (a negative regulator of calcineurin) (Rothermel et al., 2003). Thus, absence of α-actinin-3 protein (and an increase in α-actinin-2 protein) has been shown to release and activates the calcineurin (Seto et al., 2013). Activated calcineurin is able to dephosphorylate many substrates, including NFAT - allowing its translocation to the nucleus (Chin et al., 1998). NFAT interacts with MEF2 in the nucleus to activates the expression of slow myogenic genes (Wu, 2001; Crabtree and Olson, 2002). Direct assays of calcineurin activity confirmed a 1.9-fold increase in calcineurin signaling in exercised KO mice muscles (Seto et al., 2013).

The molecular mechanisms for the altered calcineurin signalling appear to be via differential binding of calsarcin-2 to sarcomeric α-actinins (**Figure 12**). It has been shown that the N-terminal region of calsarcin-2 (amino acids 1-110 and 1-90) binds to both α-actinin-2 and α-actinin-3, but there is preferential binding for α-actinin-2. Calsarcin-2 (a negative regulator of calcineurin) modulates exercise performance in vivo through regulation of calcineurin/NFAT activity and subsequent alteration of the fiber type composition of skeletal muscle (Frey et al., 2008). In Actn3 KO mice however, at baseline, these changes occur without a shift in fibre type[1], only a reduction in the size of fast twitch IIb fibres is observed, such that these fibres become a similar size to their slower IIa counterparts (MacArthur et al., 2008). When α-actinin-3 protein is absent there is a compensatory increase of α-actinin-2 protein, which binds more tightly to calsarcin-2 (Frey et al., 2000); this releases and activates calcineurin (Seto et al., 2013) (**Figure 12**). Hence in KO muscle where only α-actinin-2 protein is expressed, a higher ratio of calsarcin 2 is bound, resulting in a larger pool of free calcineurin and greater induction of the slow myogenic program of genes (**Figure 11 & 12**). Importantly, it has also been shown that the expression of calsarcin-2 does not differ between the muscles of WT and Actn3 KO mice, and hence cannot account for the altered calcineurin activity associated with α-actinin-3 deficiency (Seto et al., 2013). These data, therefore, indicate a novel role for α-actinin-3 as an important mediator of cell signalling that activates the slow myogenic program in α-actinin-3–deficient muscle.

[1] Fast-twitch (type IIb: glycolytic fibres) can be converted into fast-twitch (type IIa: oxidative- glycolytic fibres) or vice versa, through increases in endurance or resistance/power oriented training (Wilson et al., 2012).

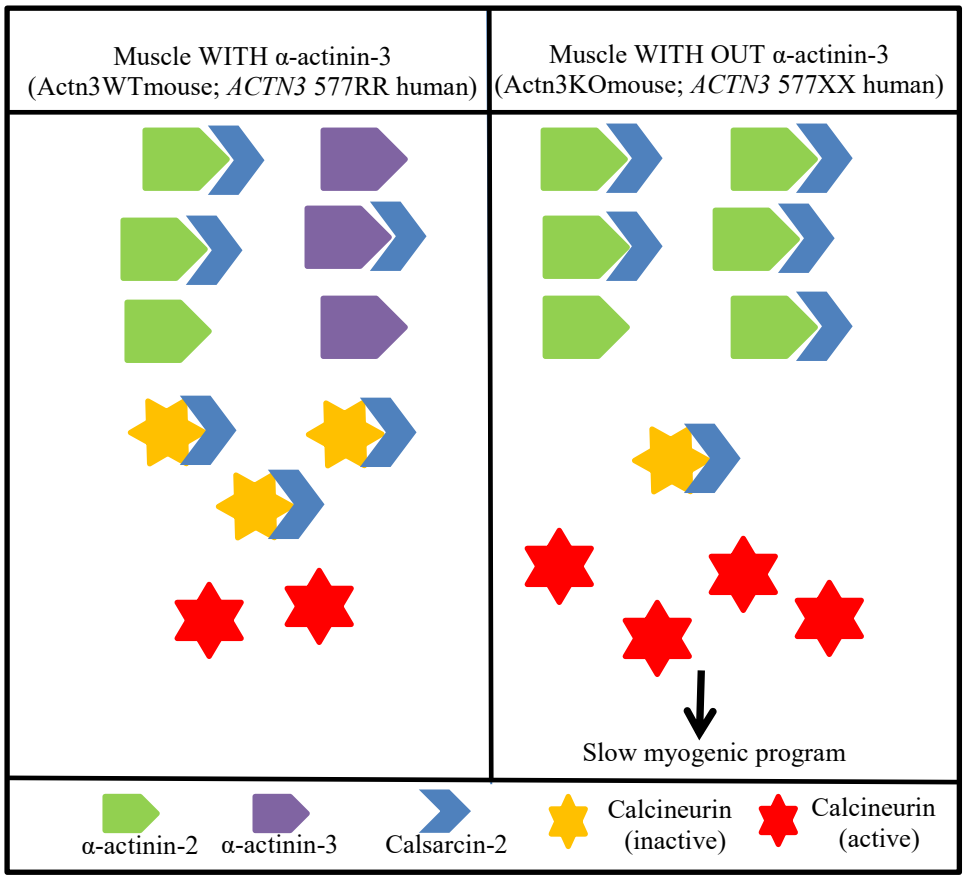


Figure 12 *Schematic of sarcomeric α-actinin regulation of calcineurin signaling. α-actinin-2 is differentially expressed in muscles without α-actinin-3. Higher-affinity binding of calsarcin-2 for α-actinin-2 over α-actinin-3 and competitive binding of α-actinin-2 for calsarcin-2 lead to the increased release of calcineurin from calsarcin-2 inhibition in the absence of α-actinin-3. Seto et al., (2013) proposed that increased free (active) calcineurin activates downstream signaling and the slow myogenic program in α-actinin-3–deficient muscle.*

5.5 *Calcineurin as a regulator of mitochondrial biogenesis*

Whilst calcineurin signalling appears to be the most important factor, there are a number of other factors which combine to give rise to the slow myogenic program. Most obvious is the proliferation of mitochondria. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a transcriptional regulator involved in energy metabolism, in particular controlling mitochondrial biogenesis. PGC-1α is thought to share similar signal molecular pathway to calcineurin in inducing a shift towards slower phenotypes. The genes, proteins and transcriptional factors involved in the activation of the

slow myogenic program related to mitochondrial gene expression in mice have been analysed following conventional continuous exercise protocols. However no studies have been focused on the influence of high intensity interval exercise (HIIE) protocols on genes, proteins and transcriptional factors involved in the activation of the slow myogenic in both mice and humans. HIIE improves oxidative capacity and promotes the expression of nuclear genes encoding mitochondrial proteins (NUGEMPs) involved in mitochondria-to-nucleus communication in muscle cells, equally or even more effective compared to continuous exercise (Chavanelle et al., 2017; MacInnis et al., 2017).

Genes, proteins and transcriptional factors involved in the activation of the slow myogenic program and expressed within 2-3 hours after exercise will be reviewed in the following chapters (**Figure 13)**.

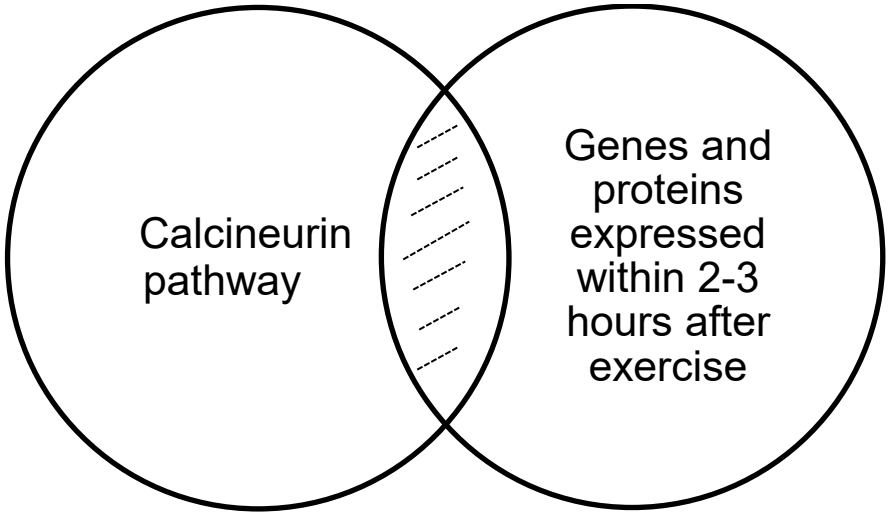


Figure 13 *Schematic representation of genes and proteins that will be the focus of this thesis.*

5.5.1 The role of RCAN 1-4

RCAN 1-4 gene expression shows peak messenger RNA (mRNA) expression at 1-2 h after exercise and a parallel increase in protein expression with a peak at 2 to 4 hours after exercise in rats (Emrani et al., 2015). RCAN1-4 is a downstream target of calcineurin, which is increased upon activation by calcineurin and also acts in a negative feedback loop to further inhibit calcineurin activity (Yang et al., 2000; Rothermel et al., 2003). RCAN 1-4 can be analysed to indirectly indicate calcineurin activity.

Direct assays of calcineurin activity confirmed a 1.9-fold increase in calcineurin signalling in exercised KO muscles compared with WT muscles (p = 0.09) with a parallel a 2.9-fold increase in RCAN1-4 (a marker of calcineurin activity), (p = 0.004), (Seto et al., 2013). Consistent with the observations in mice, there was also greater protein content of RCAN1-4 in resting muscle samples obtained from *ACTN3* 577XX humans when compared with *ACTN3* 577RR humans (Seto et al., 2013). Vincent et al., (2012) comparing *ACTN3* XX n=9 to *ACTN3* 577RR n=10 males found that *RCAN1* mRNA was 2-fold higher in *ACTN3* 577XX males at baseline, although without reaching any statistical significance. Vincent et al., (2012) also found that 1 h post eccentric exercise, the mRNA content of RCAN1-4 increased 33-fold and remained 16-fold higher six hours after exercise compared to the baseline values. Furthermore, muscles of *ACTN3* 577XX humans showed a significant increase in RCAN1-4 protein content, at base-line, compared to humans with *ACTN3* 577RR genotype (**Figure 14**) (Seto et al., 2013). However, the human participants in this study had a large age range, limited sample size and were all females: *ACTN3* RR n = 5 female, age range 27–67 years, mean age 47 years and *ACTN3* XX (α-actinin-3 deficient), n = 6, female, age range 33–77 years, mean age 49 years. At protein level human skeletal muscle fibres of *ACTN3* XX carriers have also shown a significant increase in RCAN1-4 protein content, at baseline, compared to humans with *ACTN3* 577RR genotype (Seto et al., 2013). Up-to-date there are no studies yet investigated the influence of *ACTN3* R577X polymorphism on RCAN 1-4 protein in human skeletal muscle after HIIE, or other types of endurance exercise.

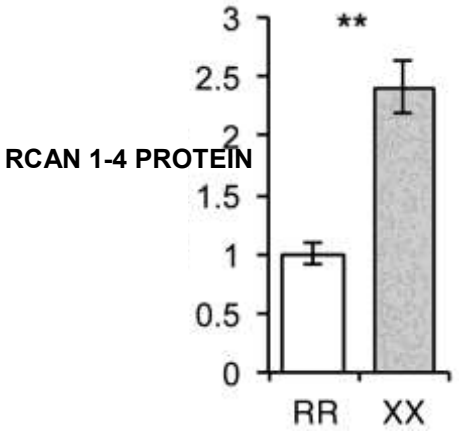


Figure 14 *ACTN3 577XX humans showed a significant difference in RCAN1-4 protein content in muscle, at base-line, compared to humans with ACTN3 577RR genotype (n = 5 and 6 females, respectively; p = 0.004; mean ± SEM; **P < 0.01, Mann-Whitney U test) (Seto et al., 2013)*.

5.5.2 The Cacineurin - PGC1-α interaction

Calcineurin is an enzyme complex comprised of a calcineurin A (CnA) catalytic subunit, calcineurin B (CnR) regulatory subunit and the calcium-binding protein calmodulin (CaM) (**Figure 15**). In skeletal muscle, calcineurin responds to a sustained increase in intracellular Ca^{2+} from both extracellular entry and release from the sarcoplasmic reticulum (SR) (**Figure 15**). The free Ca^{2+} binds to both the CnR regulatory subunit and calmodulin (CaM). CaM changes the conformation of the complex and allows for the catalytic activity of the CnA subunit. Activated calcineurin then dephosphorylates the nuclear factor of activated T-cells (NFAT) and myocyte enhancer factor-2 (MEF2), transcription factors which translocate to the nucleus to influence gene expression (**Figure 15**).

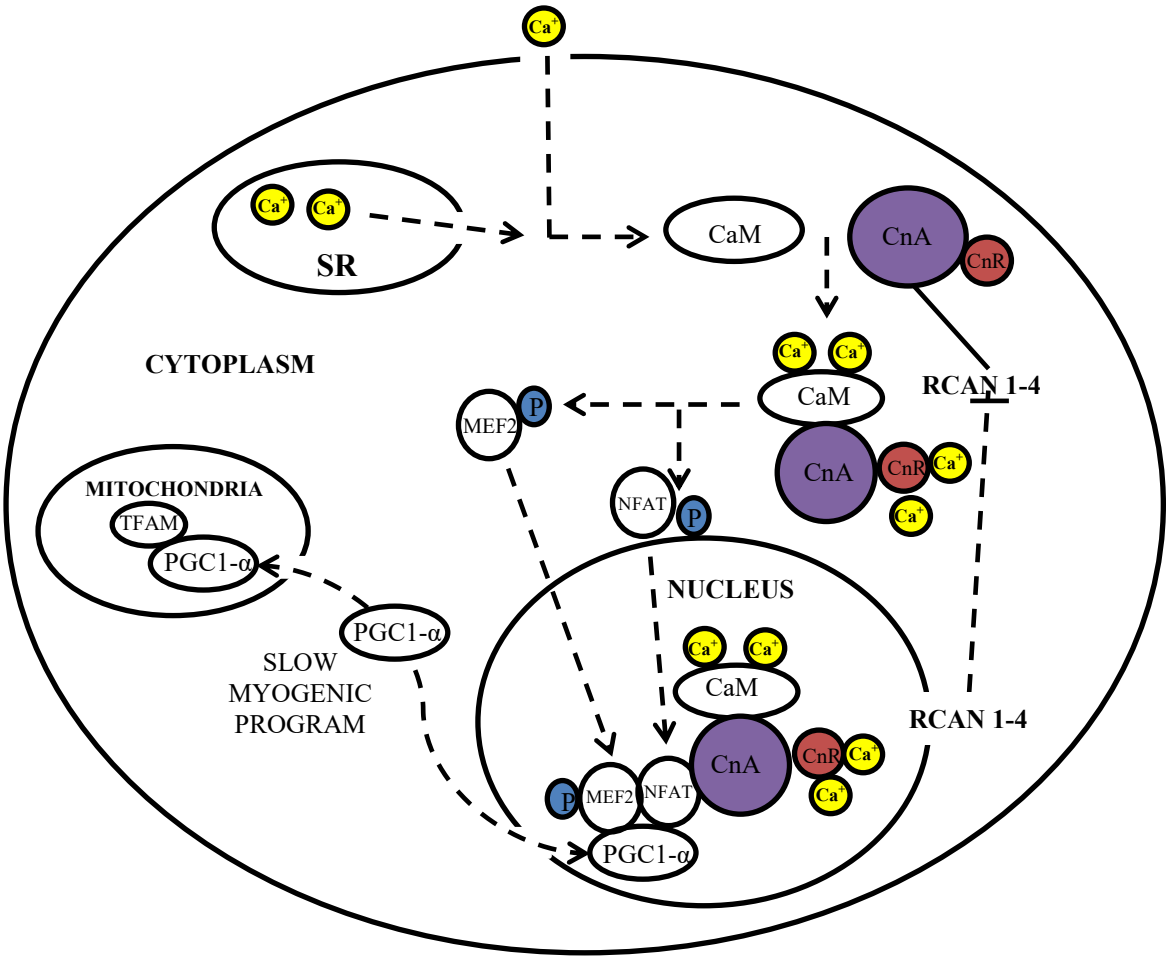


Figure 15 *Regulation of calcineurin-PGC1-α signaling pathway in skeletal muscle. Activated calcineurin dephosphorylates substrates, such as NFAT and MEF2 transcription factors, for activation of the slow gene expression program. A positive feedback loop exists between PGC-1α and MEF2. PGC1-α regulates mitochondrial biogenesis through regulation of mitochondrial transcription factor A (TFAM) (Virbasius and Scarpulla, 1994). RCAN 1-4 as shown in the figure interacts with activated calcineurin as an inhibitor of calcineurin-mediated signaling pathways (Seto et al., 2013).*

PGC-1α is thought to induce MEF2 in this process, suggesting it may share a similar signal transduction pathway to calcineurin in inducing a shift towards slow myogenic program. Indeed, in the nucleus NFAT-activated MEF2, which also binds to and activates the PGC-1α promoter (a master regulator of mitochondrial biogenesis) – predominantly when coactivated by PGC-1α (Handschin et al., 2003; Wu, 2001) (**Figure 15**). Transgenic expression of PGC-1α has been shown to dramatically increase the content of type I muscle fibers in skeletal muscle (Handschin et al., 2003). Additionally, it has been shown that the PGC-1α promoter is regulated by both CaMKIV and CnA activity. CaMKIV activates PGC-1α largely through the binding of CREB to the PGC-1α promoter (Handschin et al., 2003).

PGC-1α is thought to induce MEF2 in this process, suggesting it may share a similar signal transduction pathway to calcineurin in inducing a shift towards a slow myogenic program. Indeed, a positive feedback loop exists between PGC-1α and members of the myocyte enhancer factors 2 (MEF2) family of transcription factors. MEF2s bind to the PGC-1α promoter and activate it, predominantly when coactivated by PGC-1α. MEF2 activity is further stimulated by CnA signaling (Handschin et al., 2003) (**Figure 15**). This demonstrates a unified pathway, integrating key regulators of calcium signaling with the transcriptional switch PGC-1α (**Figure 15**). Calcineurin, interacts with the nuclear receptor PPAR-γ, which permits the interaction of this protein with multiple transcription factors. Calcineurin has subsequently been shown to increase PGC-1α gene transcription. In mice, overexpression of activated calcineurin (CnA*) results in an increase in the mitochondrial proteins of the electron transport system (complexes I to V) (Long et al., 2007), and a 35% increase in resting mitochondrial respiratory capacity (compared with WT mice) (Jiang et al., 2010) with an increase in PGC-1α protein content that has also been observed in the same muscles (Ryder et al., 2003). However, it should be noted that in one study inhibition of Calcineurin has been reported not to prevent the exercise induced increase in mitochondrial biogenesis in rat skeletal muscle (Garcia-Roves, 2006). ***Therefore, the scientific consensus is that activation of calcineurin leads to an increase in the expression of PGC-1α and the induction of nuclear genes encoding mitochondrial proteins, but it remains controversial whether calcineurin plays a role in the stimulation of mitochondrial biogenesis by exercise.*** To date, no study has investigated how the *ACTN3 R577X* genotype in humans regulates mitochondrial gene expression in response to exercise. Potential pathways could involve the downstream targets of calcineurin, such as the PGC-1α.

5.5.3 PGC1-α regulates mitochondrial biogenesis

PGC1-α1 regulates mitochondrial biogenesis through regulation of mitochondrial transcription factor A (*TFAM*) (Virbasius and Scarpulla, 1994) (**Figure 15**). *TFAM* is a protein that is encoded by the *TFAM* gene (McCulloch and Shadel, 2003). This gene encodes a mitochondrial transcription factor that is a key activator of mitochondrial transcription, as well as a participant in mitochondrial genome replication. *TFAM* bends mitochondrial promoter DNA to aid transcription of the mitochondrial genome (McCulloch and Shadel, 2003). *TFAM* plays a critical role in maintaining copy number and structure of mtDNA (Kaufman et al., 2007; Kanki et al., 2004) and is hence crucial for efficient transcription of mtDNA genes such as cytochrome c oxidase subunit 2 (MT-CO2) and other OxPhos proteins (Kaufman et al., 2007).

5.5.4 The two distinct PGC-1α gene promoters

Upregulation of the PGC-1α gene can be driven by two distinct promoter regions (Chinsomboon et al., 2009; Yoshioka et al., 2009). One is located immediately 5′ of exon 1 (proximal promoter), and another (alternative promoter) is located ∼13 kb upstream. PGC-1α (hereafter referred to as PGC-1α1) originates exclusively from the proximal promoter. The other three transcripts (hereafter referred to as PGC-1α2, α3, and α4) result from alternative promoter usage and alternative splicing of the PGC-1α gene (**Figure 16**). A recent study has revealed that what initially was believed to be one single protein, are in fact several variants. PGC-1α4 (from the alternative promoter) might have a role in muscle adaptations following exercise. PGC-1α4 is highly expressed in exercised muscle and the exact role of this variant in response to exercise still remains unknown. Some studies have shown that PGC-1α4 induces IGF1 and represses myostatin, in vitro and in vivo promoting skeletal muscle hypertrophy (Ruas et al., 2012).

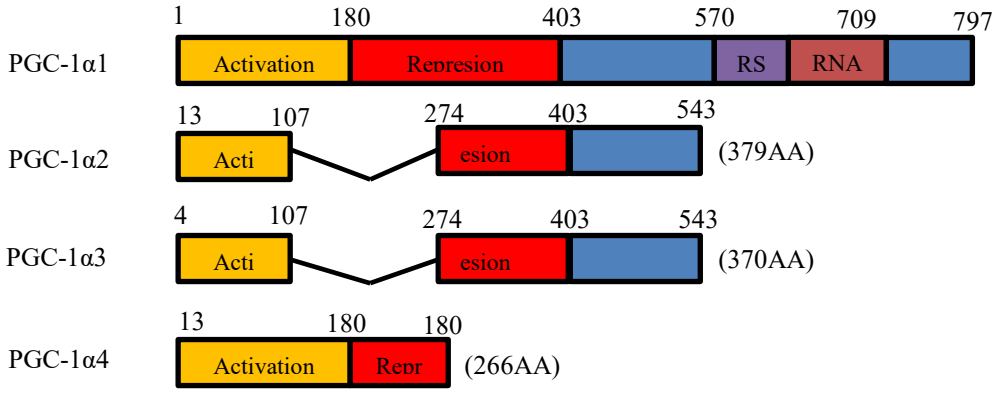


Figure 16 P*GC-1α protein domain. Amino acid numbers refer to mouse PGC-1α (hereafter PGC-1α1). Numbers in brackets indicate the number of amino acids for each isoform. Red boxes indicate new N- and C-terminal amino acid sequences (Ruas et al., 2012).*

5.6 *HIIE vs. Continuous Exercise on mitochondrial adaptations*

High-intensity interval exercise (HIIE) is a form of exercise alternating short periods of intense exercise with less intense recovery periods or even complete rest. HIIE is frequently (mis-)characterized as “anaerobic” exercise. The reality is that the contribution from anaerobic metabolism declines over repeated bouts of intense exercise. This is mainly due to an impaired capacity to break down muscle glycogen. In contrast, aerobic metabolism increases over time and it becomes the dominant energy source (Parolin et al., 1999). HIIE has been also shown to burn more calories than continuous exercise, after the workout during the post-exercise period called “EPOC”, which stands for excess post-exercise oxygen consumption. This is generally about a 2-hour period or more after an exercise bout where the body is restoring itself to pre-exercise levels, and thus using more energy (Bahr et al., 1992; Børsheim; Bahr, 2003).

These effects are suggested to be caused by markedly higher peak workloads during the intervals (Billat, 2001; Daussin et al., 2008). Therefore, aerobic HIIE has become more focus of attention as an alternative endurance training intervention strategy to standard continuous exercise. Recent work has shed new light on the potential for HIIE, which involves a small total volume of exercise, to elicit physiological adaptations that are comparable to traditional continuous exercise protocols despite a smaller total exercise volume and time commitment (Gillen et al., 2016). It has bee recently shown that HIIE was equally or even more effective in improving oxidative capacity (VO_{2max}) (Batacan et al., 2017)

and promoting mitochondrial adaptations (Granata et al., 2015), in muscle compared to continuous exercise (Bishop et al., 2014; Chavanelle et al., 2017; MacInnis et al., 2017).

5.7 *Summary*

The literature suggests that genes are responsible for about half the individual variation in physical performance in the population. Genes also account for half the variation in the response to exercise training. One of the most investigated genes with respect to athletic performance and an adaptation to exercise training is the *ACTN3* gene. This gene encodes α-actinin-3, a sarcomeric protein that is almost exclusively expressed in fast-twitch, type IIb muscle fibres. The common *ACTN3* R577X variant has been associated with sprint athletic performance. Conversely, some but not all, studies have reported that loss of α-actinin-3 (*ACTN3* R577X genotype in humans) is associated with enhanced endurance capacity in elite athletes. However, these associations between *ACTN3* gene and athletic performance are based on athletic status and no studies have quantitatively linked the *ACTN3* genotype with better sprinting performance (sprinting times) or endurance performance (long distance running times) in elite athletes.

ACTN3 gene is associated with response to endurance exercise in mice. This may be related to higher calcineurin activity (Seto et al., 2013), which has been reported to induce skeletal muscle mitochondrial biogenesis (Jiang et al., 2010). To date, however, no study has investigated how the *ACTN3* genotype in humans regulates mitochondrial-related gene and protein expression in response to a session of HIIE exercise that effectively promotes the expression of downstream targets of calcineurin, such as PGC-1α (Little et al., 2011) and other NUGEMPs.

6. STUDY ONE: *ACTN3* GENE & ELITE SPRINT ATHLETIC PEFRORMANCE

Chapter 6 of this thesis received the first prize in 2015 IAFF science competition and is largely based on the following published manuscript which was one of the most influential articles in *BMC Genomics*, according to Altmetric.com for year 2016. The results from this chapter were also discussed in Scientific American, Volume 27, Issue 4 (2016).

Papadimitriou ID, Lucia A, Pitsiladis YP Pushkarev VP, Dyatlov DA, Orekhov EF, Artioli GG, Guilherme JP, Lancha Jr AH, Ginevičienė V, Cieszczyk P, Maciejewska-Karlowska A, Sawczuk M, Muniesa CA, Kouvatsi A, Massidda M, Calò CM, Garton F, Houweling PJ, Wang G, Austin K, Druzhevskaya AM, Astratenkova IV, Ahmetov II, Bishop DJ, North KN, Eynon N. *"ACTN3 R577X and ACE I/D gene variants influence performance in elite sprinters: A multi-cohort study"* BMC Genomics 17: 285, 2016 (Q1 in Genetics & Heredity, Impact Factor=4.0).

6.1 *Abstract*

Earlier studies investigating the contribution of specific genes to sprint performance have been limited by small cohorts from mixed sport disciplines, most lacking quantitative measures of performance. To examine the association between *ACTN3* gene variants and sprint times in elite male and female athletes, 555 personal best 100m, 200m, and 400m times by 346 top Caucasian (189 males and 66 females) and African (91 males) origin sprinters from 10 countries were collected. The sprinters were genotyped for *ACTN3* R577X gene variants. On average, male Caucasian sprinters with the *ACTN3* 577RR variant had faster personal best 200m times than their *ACTN3* 577XX counterparts (21.19±0.53 s vs. 21.86±0.54 s, p=0.016) and no cases of *ACTN3* 577XX were found to have achieved the qualifying time for the 2012 Olympic Games (vs. 12 qualified sprinters with 577RR or 577RX genotypes). Male African sprinters with the *ACTN3* 577RR variant had no faster personal best 200m times than their *ACTN3* 577RX counterparts (20.53±0.64 s vs. 20.98±0.72 s).

Using genetic models, it was found that the *ACTN3* 577R allele dominant model account for 0.92% of 200m performance at the elite level. Although sprint performance relies on many gene variants, environment and other factors, the percentage variance at the elite level explained by *ACTN3* gene is substantial and might be the difference between a world record and only making the semi-finals at the Olympic Games or IAAF World Championships in Athletics.

6.2 *Introduction*

Although the likelihood of becoming an elite sprint/power athlete is likely influenced by genetic factors (Pitsiladis et al., 2013; Eynon et al., 2013), only a handful of genes have been associated with sprint performance. Currently the most promising candidate gene is the *ACTN3,* which encodes the sarcomeric protein α-actinin-3 in skeletal muscle fibres (Yang et al., 2007). The expression of α-actinin-3 protein is almost exclusively restricted to fast, glycolytic, type IIb fibres, which are responsible for producing 'explosive', powerful contraction (Mills et al., 2001). Homozygosity for common null single nucleotide polymorphism (577XX, rs1815739) in the *ACTN3* gene results in complete deficiency of the α-actinin-3 protein in an estimated 18% of humans worldwide (North et al., 1999), and the *ACTN3* RR genotype has been associated with elite sprint/power athletic performance in several independent cohorts of elite athletes (Eynon et al., 2013).

A higher frequency of the *ACTN3* 577RR genotype (and lower frequency of the α-actinin-3 deficient, 577XX genotype) in elite sprint/power athletes (i.e., sprinters, jumpers, and throwers) was originally found in a case (athletes): control (non-athletes) association study with Australian subjects (Yang et al., 2003). This finding was independently replicated in Finnish (Niemi and Majamaa, 2005), Greek (Papadimitriou et al., 2008), Russian (Druzhevskaya et al., 2008), Israeli (Eynon et al., 2009) and Japanese (Mikami et al., 2013) national/international level athletes. No Olympic-finalist sprinter has yet been identified with the 577XX genotype (Yang et al., 2003; Papadimitriou et al., 2008; Eynon et al., 2009). Taken together, these association studies suggest that α-actinin-3 deficiency is detrimental to optimal fast muscle function at the extremes of sprint and power performance. In support of this, mice lacking α-actinin-3 (Actn3 knockout mice, mimic the 577XX genotype in humans) demonstrate a shift in the physiological and metabolic properties of 'fast' glycolytic muscle fibres (type IIb) towards a slower, oxidative muscle phenotype (types I and IIa), which are responsible for postural and endurance activities (MacArthur et al., 2008) and *ACTN3* 577XX humans show lower proportion of fast-twitch muscle fibres (Vincent et al., 2007; Ahmetov et al., 2011), and lower levels of testosterone (Ahmetov et al., 2014).

One of the limitations of most of the above studies investigating the association between the *ACTN3* R577X genotype and sprint/power performance is the grouping together of sprint and power athletes from mixed sport disciplines and events (e.g., sprinters, jumpers, throwers, swimmers, and team sport athletes). This approach, while understandable given the very low number of world-class sprinters, reduces the accuracy of the phenotype. Furthermore, to date, only one report involving world-class sprinters of African ancestry (Scott et al., 2010), has examined the association between *ACTN3* R577X polymorphism and athletic status and found that the lack of positive findings was attributable,

at least partly, to the very low frequency of the *ACTN3* 577XX genotypes in the African population, which almost eliminates the possibility of detecting an association.

I sought to address these limitations and provide deeper insight into the influence of the *ACTN3* R577X variants on sprint performance by using a quantitative collaborative approach and by studying the influence of genotype on actual sprint performance. Therefore, the aim of the present study was to examine the association between the *ACTN3* R577X variants and personal best times for 100m, 200m and 400m personal best times in a performance-homogenous, cohort of elite Australian, Brazilian, Greek, Jamaican, Italian, Polish, Russian, Lithuanian, Spanish and American (United States) sprinters.

6.3 *Methodology*

6.3.1 Participants

A total of 555 personal best 100 m, 200 m and 400 m sprint times of 346 elite pure sprinters from Australia, Brazil, Greece, Jamaica, Italy, Lithuania, Poland, Russia, Spain and the United States were analysed (**Table 5**). The sprinters from Australia, Greece, Poland, Lithuania and Russia were all Caucasians (189 male and 66 female) whereas a total of 91 male Brazilian, Jamaican, Italian, Spanish and US athletes were from African lineage. The sprinters' personal bests (tail wind <2 m/sec when provided) in official competitions were found online (www.iaff.org) or provided by coaches or the athletes themselves and independently corroborated (**Table 5**).

The subjects' personal best times, grouped according to ethnic-background (Caucasians / Africans mixed linage athletes were excluded) and event (100 m, 200 m or 400 m), standardised and expressed relative to the current world records and group bests in the events. Only 'pure' sprinters with times that were within 15% of the current world record or group best of the examined events were included. The following records and group bests were used to calculate the inclusion criterion:

- Male sprinters of African ancestry, 9.58 s in the 100m and 19.9 s in the 200 m - current world record holder: Usain Bolt (JAM), and 43.18 s in the 400 m - current world record holder: Michael Johnson (USA);
- Female sprinters of African ancestry, 10.49 s in the 100 m and 21.34 s in the 200 m - current world record holder: Florence Griffith-Joyner (USA);

- Male Caucasian sprinters, 9.99 s in the 100 m - group best holder: Christophe Lemaitre (FRA), 19.72 s in the 200 m - group best holder: Pietro Mennea (ITA) and 43.45 s in the 400 m - group best holder: Jeremy Wariner (USA);

- Female Caucasian sprinters, 10.77 s in the 100 m – group best holder: Ivet Lalova (BUL), 21.71 s inb the 200 m – group best holder: Marita Koch (GDR) and 47.60 s in the 400 m – world record holder: Marita Koch (GDR).

6.3.2 Genotyping

Genomic DNA was isolated from buccal epithelium, or white blood cells. The Australian, Greek, Italian, Lithuanian, Russian (from St Petersburg) and Spanish sprinters' DNA samples were genotyped using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method as previously described (Mills et al., 2001a). The DNA samples of the Polish, Brazilian, Russian (from Chelyabinsk), Jamaican and US sprinters were genotyped in duplicates using an allelic discrimination assay on a Step One Real-Time PCR instrument (Applied Biosystems, Carlsbad, California, USA) with Taqman® probes. To discriminate *ACTN3* R577X (rs1815739) alleles, TaqMan® Pre-Designed SNP Genotyping Assay was used (assay ID: C_590093_1_), including appropriate primers and fluorescently labeled (FAM and VIC) MGB™ probes to detect the alleles.

6.3.3 Statistical analysis

To compare the sprinters' records between all genotypes the one-way analysis of variance (ANOVA) was used. The Tukey's post-hoc test was used to determine statistical significant difference among the genotype groups. The level of significance was set at 0.05. Using the Simple Interactive Statistical Analysis website (SISA; http://home.clara.net/sisa/) genotype interactions on sprint performance were further assessed using correlation analysis as previously described (Moran et al., 2007).

Briefly, three genetic models (additive model and two dominant models assuming complete dominance of each allele) were tested. The additive genetic model consisted of 0, 0.5 and 1, to represent R allele homozygotes, RX heterozygotes and homozygotes for the X allele, respectively; for the R allele dominant or X allele dominant genetic models, the corresponding values were 0, 0, 1 or 0, 1, 1, respectively. The proportion of the genetic contribution to phenotypic variance explained by each genetic model was estimated by expressing r^2 from the correlation analyses (taken as an estimate of percentage variance explained under the model) as a percentage of the variance explained by genotype effects in

the model-free ANOVAs. This proportion was compared for each model to predict the most accurate model tested.

6.4 *Results*

The personal best male 100m, 200m and 400m sprint times (±SD), according to the *ACTN3* genotype and distribution, are presented in **Table 1**.

Table 5 *The 100-m, 200-m and 400-m best sprint times (average ± SD) in males according to ACTN3 R577X genotype distribution.*

	Caucasians			African Ancestry	
Running event/Genotype	**RR**	**RX**	**XX**	**RR**	**RX**
100m Male	10.55±0.27 (n=35)	10.58±0.33 (n=44)	10.77±0.31 (n=10)	10.26±0.35 (n=22)	10.28±0.30 (n=11)
200m Male	21.19±0.53 (n=35)	21.29±0.61 (n=36)	21.86±0.54* (n=8)	20.53±0.64 (n=23)	20.98±0.72 (n=11)
400m Male	46.90±1.29 (n=44)	47.41±1.43 (n=46)	47.55±1.42 (n=9)	46.49±1.66 (n=18)	47.29±1.69 (n=7)

*200-m (RR vs. RX vs. XX)
P<0.016

ACTN3 genotypes influence 200m personal best time in male athletes

In male Caucasian sprinters a significant association was detected between *ACTN3* genotype and 200m personal best. Using Tukey's Multiple Comparison Test both *ACTN3* 577RR, (-0.66, 95% CI -0.20 to -0.12) and 577RX (-0.56, 95% CI -1.10 to -0.02) individuals were significantly faster than 577XX individuals (P<0.05). The R allele dominant model (RR/RX vs. XX) had the best fit explaining 9.65% of sprint time (P=0.005), compared to the additive (7.28%, P=0.01) and the X allele dominant model (2.77%, P>0.05) in correlation analysis. The percentage of the observed variance (coefficient of determination, r^2) explained by the *ACTN3* genotype using this recessive model was 0.92%. The *ACTN3* RR and *ACTN3* RX groups were not significantly different, indicating the presence of one or two R allele does not have a dose dependant effect on 200m personal best time in elite athletes (**Figure 17**). In elite male African athletes (n=92), there was some evidence for a dose effect

of the *ACTN3* R allele and 200m speed (**Table 5**). Using an unpaired t-test, the *ACTN3* RR individuals had (on average) a faster personal best times than *ACTN3* RX individuals (-0.32, 95% CI, 0.95 to -0.57).

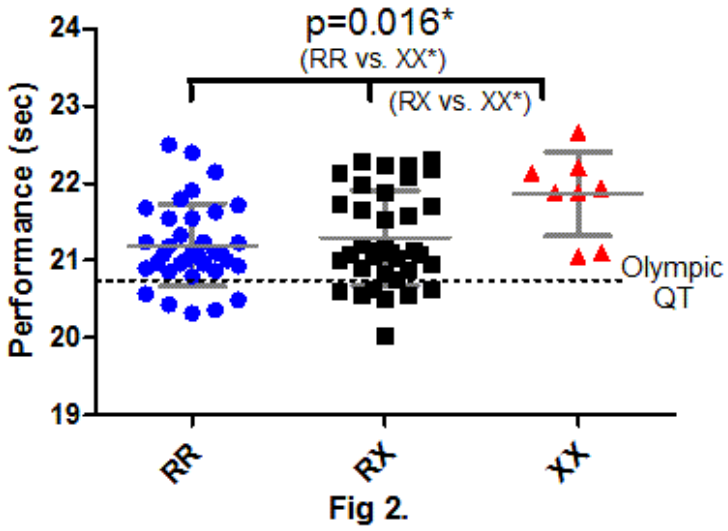


Figure 17 *Individual 200m personal best times (±SD) in male Caucasian sprinters according to their ACTN3 R577X genotype and the qualifying times (QT) for the 2012 Olympic Games (20.65 sec).*

No genotype differences were detected in 100-m and 400-m sprint performance in both Caucasian and African ancestry sprinters

A trend was observed, but not statistically significant differences in best 100m and 400m times across the *ACTN3* R577X (**Table 5**). Caucasian females (n=66) were assessed separately and showed similar associations to males, across the genotypes.

6.5 *Discussion*

In this quantitative assessment of genotype with qualifying time in 346 elite sprinters, It has been shown that it is rare for humans with the α-actinin-3 deficient (*ACTN3* 577XX) genotypes to reach the standard required to compete in the 200m at the IAAF World Championships in Athletics or Olympic Games. From all the male sprinters' personal best times included in this study, there were no cases of 577XX sprinters who were faster than 2012 Olympic qualifying standard in 200m (20.65 sec). This finding suggests that the *ACTN3* 577XX is detrimental for 200m sprint performance.

In this chapter, I have addressed some of the limitations inherent in previous athlete case-control studies regarding the association between *ACTN3* genotypes and elite athletic

performance. First, I have studied ten cohorts of elite sprinters, including the fastest sprinters on earth. Consequently, the number of 'pure' elite sprinters (n=346) in the present study is much larger compared to previous association studies, and demonstrates the benefits of a collaborative approach that has been recommended in the field of exercise genomics (Eynon et al., 2011; Eynon et al., 2012; Pitsiladis et al., 2013).

Second, previous reports have grouped together sprint and power athletes from mixed sports disciplines and events (Yang et al., 2003; Papadimitriou et al., 2008; Eynon et al., 2009; Eynon et al., 2012; Mikami et al., 2013). Here, I have embraced a more stringent approach and included only elite 'pure' sprinters whose main sporting discipline was the 100m, 200m or 400m. Furthermore, I set explicit criteria (100m, 200m and 400mm best personal running times within 15% from the world record) that reflected in the reduction in SD compared to the total sample for each study, and enable me to gain insights into the effect of ACTN3 genotypes on higher possible end of human elite sprinting and endurance performance.

Third, I have analysed quantitative measure with respect to *ACTN3* genotypes to assess the genotype effect impact in both males and females separately. Only one previous study has taken a similar approach with 100m sprinters: a subgroup analysis of male Japanese track and field athletes indicated that those harbouring the *ACTN3* 577XX genotype ran the 100m significantly slower than their 577RX and 577RR counterparts (Mikami et al., 2013). However, this study was limited by its sample size (n=28) and studied only a Japanese cohort.

The analyses from this chapter of the thesis show that the male Caucasian sprinters' personal best times are influenced by *ACTN3* genotypes in an event-specific manner. While similar trends were seen in African and female athletes, it should be highlighted that larger cohorts are urgently needed for adequate genotype-performance assessments. These analyses of male Caucasian performances found that 200m performance (but not 100m and 400m) is influenced by *ACTN3* genotypes. Moreover, it should be noted that at the highest level of performance (i.e., elite sprinters with personal bests faster than the Olympic qualifying times) there was no significant overlap of the times between the *ACTN3* RR and *ACTN3* XX genotypes, suggesting that 'every variable counts' for achieving world-class sprinting performance. Interestingly, these results show that all *ACTN3* XX genotypes not getting into the Olympics, but similarly 83% (59/71) of *ACTN3* RX and *ACTN3* RR athletes do not get into the Olympics as well. This would mean that testing for the R-allele (positive) versus XX genotype (negative) would have a maximal sensitivity (100%-no false negatives) but low specificity (12%) to predict qualifying times for the Olympics with low overall accuracy (25%). These findings, together with the separate results from 200m sprints, support the notion that performance in 200m is influenced by *ACTN3* R577X polymorphism.

Association differences may be related to subtle differences in the physiological performance demands of each event. In the 100m the athlete is required to accelerate for most of the race before reaching their absolute maximum velocity (Morin et al., 2012). In the longer sprints the acceleration phase is relatively shorter and it is the ability to maintain the maximum velocity for a longer time period that is the critical factor for top performance (Hanon and Gajer, 2009)[v]. On the one hand, acceleration involves reaction time, the position of the centre of gravity of the body relative to the blocks, stride frequency and stride length, while on the other hand maintaining absolute maximal velocity requires powerful cyclic muscle contractions and efficient utilisation of the energy systems (mostly lactic and 'alactic' anaerobic systems) that are triggered at different phases of the race (Bret et al., 2013)[v]. Given the genotype-performance associations at longer sprint distances, this suggests their influence may lie in greater effect on muscle's metabolic potential (switch from P/Cr to lactic anaerobic systems) with repeated powerful contractions.

The *Actn3* knockout (KO) mouse has provided a possible explanation for the detrimental effect of α-actinin-3 deficient (577XX genotype) on elite sprinting performance. Mechanistic studies in the *Actn3* KO mouse show that this model mimics *ACTN3 XX* in humans. The wild-type WT mice that express *Actn3* (equivalent to human RR/RX genotypes) prefer the anaerobic system while *Actn3* KO mice prefer the aerobic system. Metabolically, the KO mice have significantly higher activity of oxidative enzymes, and lower activity in enzymes of the anaerobic pathway. In addition, enhanced glycogen accumulation due to lower glycogen phosphorylase activity has been observed (MacArthur and North, 2007; Quinlan et al., 2010). Their fast fibre properties shift towards a slower metabolic profile which has been linked to increases in calcineurin signalling activity (Seto et al., 2013) and altered calcium handling (Head et al., 2015). Overall this shift towards a slower physiological and metabolic profile would be detrimental to sprint performance in *ACTN3* 577XX humans (MacArthur and North, 2007; MacArthur et al., 2008).

This multi-centre study design has enabled me to gain insights into the effect of *ACTN3* genotypes on elite sprinting performance. *ACTN3* R577X polymorphisms modulate specific sprint phenotypes and influence athletic status at the extremes of human performance. I have shown quantitatively for the first time that the *ACTN3* genotypes account for 0.92% in sprint speed amongst elite male 200m athletes. This difference in performance is substantial and can be the difference between a world record and only making the semi-final at the IAAF World Championships in Athletics or Olympic Games. *ACTN3* R577X polymorphism seems to be more influential on 100m and 400m performance. Despite our findings, the predictive value of these tests remains limited. A substantial amount of performance variation remains unaccounted for and further research into both

common and rare variants is still required. With this additional research the findings may have future applications for identifying and coaching talented 200m sprinters.

7. STUDY TWO: *ACTN3* GENE & ELITE ENDURANCE ATHLETIC PERFORMANCE

Chapter 7 of this thesis is largely based on the following published manuscript in *BMC Genomics*.

Ioannis D Papadimitriou, Sarah J. Lockey, Sarah Voisin, Adam Herbert, Fleur Garton, Peter J Houweling, Pawel Cieszczyk, Agnieszka Maciejewska-Karlowska, Marek Sawczuk, Myosotis Massidda, Carla Maria Calò, Druzhevskaya AM, Astratenkova IV, Ildus Ahmetov, Georgina K. Stebbings, Stephen H. Day, Robert M. Erskine, Courtney Kipps, Kathryn N North, Alun Williams, Nir Eynon *"No association between ACTN3 R577X and ACE I/D polymorphisms and endurance running times in 698 Caucasian athletes"* BMC Genomics (2018) 19:13 (Q1 in Genetics & Heredity, Impact Factor=4.0).

7.1 *Abstract*

Earlier studies investigating the association between *ACTN3* R577X genotype and endurance athletic status have been limited by small sample sizes from mixed sport disciplines and a lack of quantitative measures of performance. In chapter 7 I examined the association between *ACTN3* R577X genotype and best personal running times in a large homogeneous cohort of endurance athletes. A total of 1064 personal best 1500 m, 3000 m, 5000 m and marathon running times of 698 male and female Caucasian endurance athletes from six countries (Australia, Greece, Italy, Poland, Russia and UK) were collected. There was no association between *ACTN3* R577X genotype and running performance at any distance in either men or women. Mean (SD) marathon running times (in s) were for men: *ACTN3* RR 9149 (593), RX 9221 (582), XX 9129 (582) p=0.94; for women: *ACTN3* RR 10796 (818), RX 10667 (695), XX 10675 (553) p=0.36. Furthermore, there was no association between these variants and running time for any distance in a sub-analysis of athletes with personal best times within 20% of the world records. Thus, consistent with most case-control studies, this multi-cohort quantitative analysis demonstrates it is unlikely the *ACTN3* XX genotype offer an advantage for competitive endurance running performance in elite athletes.

7.2 *Introduction*

Although the likelihood of becoming an elite athlete is likely influenced by genetic variations across the human genome (Eynon et al., 2013; Pitsiladis et al., 2013), there is currently no evidence for a common genetic profile specific to elite endurance athletes, even when utilising a Genome-Wide Association (GWAS) approach (Rankinen et al., 2016). However, there is considerable evidence suggesting that *ACTN3* R577X gene variants do influence muscle performance and metabolism in humans (Ahmetov et al., 2011; Lee et al., 2016).

A common null polymorphism (rs1815739) was identified in the *ACTN3* gene, which results in the replacement of an arginine (R) residue with a premature stop codon (X) at amino acid 577. Approximately 18% of the world population (~1.5 billion individuals) harbour the *ACTN3* 577XX genotype and consequently are completely deficient in α-actinin-3 protein. Importantly, α-actinin-3 deficiency does not cause any obvious muscle disease (North et al., 1999).

An association between the *ACTN3* R577X genotype and athletic performance was initially found in a cohort of elite Australian athletes (Yang et al., 2003), with a very a low proportion of elite sprint/power athletes harbouring the 577XX genotype. This genotype distribution pattern was quite consistent in other independent cohorts of elite athletes and has since been replicated in Finnish (Niemi and Majamaa, 2005), Greek (Papadimitriou et al., 2008), Russian (Druzhevskaya et al., 2008), Israeli (Eynon et al., 2009), Polish (Cięszczyk et al., 2011) and Japanese (Mikami et al., 2013) athletes.

A tendency for a higher proportion of elite athletes carrying the 577XX genotype was also found in Australian athletes excelling in aerobic activities (Yang et al., 2003), showing some evidence for association of this genotype with endurance performance. While this association was replicated in some cohorts of athletes (Eynon et al., 2009) other studies have shown no association between the *ACTN3* R577X genotypes and endurance athletic status (Niemi and Majamaa, 2005; Papadimitriou et al., 2008; Mikami et al., 2013). Furthermore, a large study with Russian endurance athletes found that the frequency of the XX genotype was lower in endurance athletes than in controls (Ahmetov et al., 2010), demonstrating the conflicting results between the association of this gene variant and endurance athletic performance. In line with this finding, an analysis comparing 50 elite male endurance cyclists and 52 Olympic-level endurance runners with 123 sedentary male controls (Lucia et al., 2006) found no difference in genotype frequencies between controls and either of the two athlete groups. There was also no association between R577X genotypes and a common measure of endurance performance—maximal oxygen uptake

(VO_{2max})—in either of the athlete groups. Cross-sectional studies (Lucia et al., 2006; Paparini et al., 2007; Doring et al., 2010; Muniesa et al., 2010) and a recent metanalysis (Ma et al., 2013) found no association of *ACTN3* XX genotype with endurance performance as well, and debate is ongoing on whether the *ACTN3* gene influences endurance performance. In a different human sporting context, namely the team sport of rugby union, the R allele has recently been associated with success in playing positions reliant on sprinting speed, while the X allele was associated with playing demands allowing relatively short recovery times (Heffernan et al., 2016).

One of the limitations of most of the abovementioned studies investigating the association between the *ACTN3* R577X genotype and athletic status is the grouping of endurance athletes from mixed sport disciplines and events (e.g. middle distance runners, long distance runners, cyclists, swimmers), or analysing team sport athletes from a single sport yet with some variations in physiological demand according to playing position (Heffernan et al., 2016). These approaches, while understandable given the very low number of World-class competitors in a single sport or event, reduce the consistency of the phenotype. Furthermore, those studies only used a simple case-control design based on athletic status without looking at measurable (quantitative) traits within the compared groups (Wang et al., 2016) and no studies have quantitatively linked those genotypes with endurance performance (e.g. running times) in elite athletes.

I sought to address these limitations by providing deeper insight into the possible association between the *ACTN3* R577X variants and endurance performance. In chapter 7 of this thesis, I used the same quantitative approach previously applied in chapter 6 with the elite sprinters (Papadimitriou et al., 2016) that showed that *ACTN3* R577X genotype has a substantial effect at the elite level 200 m sprint performance. The aim of this study was to examine the association between the *ACTN3* R577X variants and personal best running times in 1500 m, 3000 m, 5000 m, 10000 m and marathon in a large cohort of male and female Caucasian endurance runners.

7.3 *Methodology*

7.3.1 Participants

A total of 1064 personal best 1500 m, 3000 m, 5000 m, 10000 m and marathon running times of 698 Caucasian endurance athletes (441 males and 257 females) from Australia (n=14), Greece (n=16), Italy (n=9), Poland (n=60), Russia (n=17) and the UK (n=582), were analysed (**Table 6**). The endurance runners' personal best times in official

competitions were found online (www.iaaf.org) or provided by coaches or the athletes themselves and independently corroborated.

The participants' personal best times were grouped by event (1500 m, 3000 m, 5000 m, 10000 m or marathon). The whole cohort of males and females were analysed separately. Then, a sub-analysis in males was also performed including only the endurance runners with times that were within 20% of the current World record of the examined events, following a similar approach previously used in chapter 6 of this thesis (Papadimitriou et al., 2016). The females were no included in this sub-analysis since the sample size was too low (i.e. n<5 for the XX genotype). The following World records were used as references:

- Male endurance runners. 3:26.00 in the 1500 m - group best holder: Hicham El Guerrouj (MAR), 7:20.67 in the 3000 m - group best holder: Daniel Komen (KEN), 12:37.35 in the 5000 m - group best holder: Kenenisa Bekele (ETH), 26:17.53 in the 10000 m - group best holder: Kenenisa Bekele (KEN), 2:02:57 in the Marathon - group best holder: Dennis Kipruto Kimetto (KEN);

- Female endurance runners. 3:50.07 in the 1500 m - group best holder: Genzebe Dibaba (ETH), 8:06.11 in the 3000 m - group best holder: Junxia Wang (CHN), 14:11.15 in the 5000 m - group best holder: Tirunesh Dibaba (ETH), 29:17.45 in the 10000 m - group best holder: Almaz Ayana (ETH), 2:17:42 in the Marathon - group best holder: Paula Radcliffe (GBR).

7.3.2 Genotyping

In the UK ~70% of the UK, samples were collected as whole blood, ~20% as buccal swabs, ~10% as saliva. Blood was drawn from a superficial forearm vein into an EDTA tube and stored in sterile tubes at -20°C until processing. Saliva samples were collected into Oragene DNA OG-500 collection tubes (DNA Genotek, Ottawa, Ontario, Canada) according to the manufacturer's protocol and stored at room temperature until processing. Sterile buccal swabs (Omni swab; Whatman, Springfield Mill, UK) were rubbed against the buccal mucosa of the cheek for 30 s. Tips were ejected into sterile tubes and stored at -20°C until processing. Genomic DNA was isolated from buccal epithelium, or white blood cells. The Australian, Greek and Italian endurance runners' DNA samples were genotyped using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method as previously described (Mills et al., 2001). In the UK DNA isolation was performed with the QIAamp DNA Blood Mini kit and standard spin column protocol, following the manufacturer's instructions (Qiagen, West Sussex, UK). In brief, 200 µL of whole blood/saliva, or one buccal

swab, was lysed and incubated, the DNA washed, and the eluate containing isolated DNA stored at 4°C. The DNA samples of the British, Polish and Russian endurance runners were genotyped in duplicates using an allelic discrimination assay on a Step One Real-Time PCR instrument (Applied Biosystems, Carlsbad, California, USA) with Taqman® probes. To discriminate *ACTN3* R577X (rs1815739) alleles, TaqMan® Pre-Designed SNP Genotyping Assay was used (assay ID: C_590093_1_), including appropriate primers and fluorescently labeled (FAM and VIC) MGB™ probes to detect the alleles. For the genotyping of the UK samples the StepOnePlus and Chromo4 (Bio-Rad, Hertfordshire, UK) were used.

7.3.3 Statistical analysis

To compare the endurance athletes' running times between *ACTN3* R577X genotypes, the running times were converted to seconds and initially used the one-way analysis of variance (ANOVA). Then a simple linear regression with running time as the dependent variable and genotypes as the independent variable was also applied. Two genetic models were used: the additive model where RR=0, RX=1 and XX=2, and the recessive genetic model where RR=RX=0 and XX=1. Males and females were analysed separately. The level of significance was set at 0.05. All data analyses were conducted with the R statistical software with the lme4 and lrtest packages.

7.4 *Results*

The mean (SD) personal best 1500 m, 3000 m, 5000 m, 10000 m and marathon running times, according to the *ACTN3* genotype and distribution, are presented in **Table 6**.

The ANOVA analyses showed no statistical differences among the 3 three genotypes ($p<0.05$). Further linear regression analysis using an additive or a recessive genetic model also showed no differences of best running times between genotypes ($p<0.05$).

Table 6 *Mean (SD) 1500 m, 3000 m, 5000 m, 10000 m and marathon best running times in (a) males and (b) females in the three ACTN3 R577X genotypes. All running times are expressed in seconds because statistical analyses were performed on times converted to seconds. The last two columns of the table correspond to the p-value of the linear regression, using an additive or a recessive genetic model. The percentage values represent the genotype proportions.*

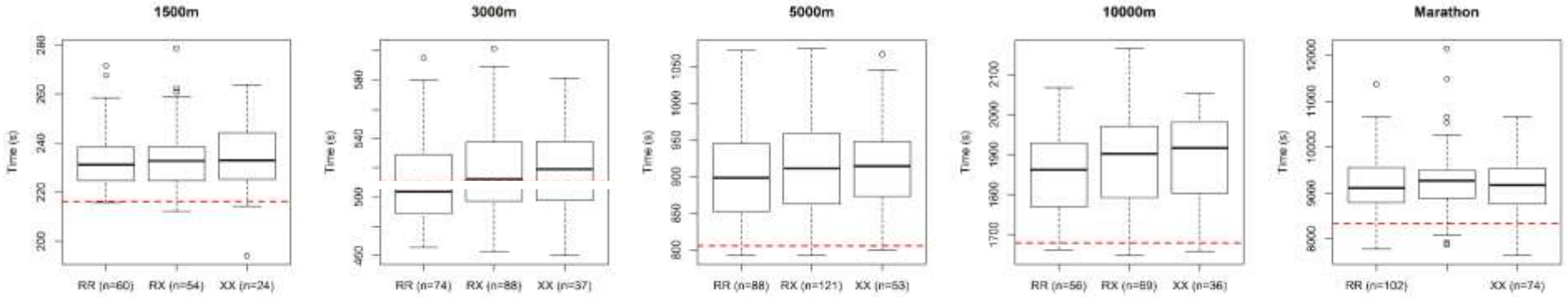
(a)

MALES	RR N=380 34.7%	RX N=492 44.9%	XX N=224 20.4%	Additive (RR=0, RX=1, XX=2)	Recessive (RR = RX = 0, XX=1)
Running time 1500 m (s)	232.6 (10.7) 43.5 %	234.2 (13.2) 39.1%	234.0 (15.1) 17.4%	p=0.54	p=0.81
Running time 3000 m (s)	509.6 (27.5) 37.2%	517.0 (28.0) 44.2%	518.1 (26.9) 18.6%	p=0.08	p=0.37
Running time 5000 m (s)	902.1 (61.7) 33.6%	912.2 (61.8) 46.2%	913.2 (59.5) 20.2%	p=0.25	p=0.58
Running time 10000 m (s)	1860.9 (109.2) 34.8%	1885.7 (125.8) 42.9%	1889.3 (112.1) 22.4%	p=0.22	p=0.51
Running time marathon (s)	9148.6 (593.0) 30.4%	9220.7 (582.1) 47.6%	9129.1 (581.6) 22.0%	p=0.94	p=0.41

(b)

FEMALES	RR N=156 29.6%	RX N=301 57.1%	XX N=70 13.3%	Additive (RR=0, RX=1, XX=2)	Recessive (RR = RX = 0, XX=1)
Running time 1500 m (s)	269.4 (15.4) 34.7%	268.3 (14.0) 55.6%	262.6 (15.4) 9.7%	p=0.35	p=0.30
Running time 3000 m (s)	600.9 (44.7) 30.4%	602.8 (37.3) 61.6%	600.2 (48.5) 8.0%	p=0.93	p=0.89
Running time 5000 m (s)	1028.2 (79.1) 29.2%	1048.9 (97.1) 59.4%	1048.2 (88.9) 11.5%	p=0.39	p=0.83
Running time 10000 m (s)	2067.6 (153.6) 27.7%	2101.0 (159.9) 57.4%	2067.6 (153.6) 14.9%	p=0.61	p=0.91
Running time marathon (s)	10796.4 (818.2) 28%	10667.3 (695.3) 54%	10675.3 (552.8) 18%	p=0.36	p=0.78

(a)



(b)

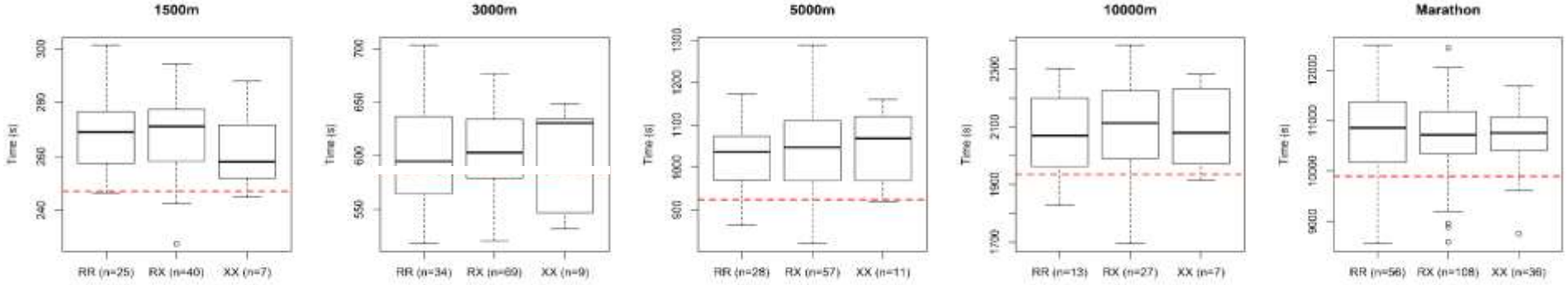


Figure 18 *Individual 1500 m, 3000 m, 5000 m, 10000 m personal best times in (a) male and (b) female endurance athletes according to their ACTN3 R577X genotype. Data are shown as boxplots and time is expressed in seconds. The red dashed line on each plot corresponds to the competition entry standard for the 2016 Olympic Games. 3000 m is not an Olympic event, so there is no analysis performed, this is the reason there are no red dashed line for this event.*

No association between ACTN3* R577X *genotypes and personal best times in the whole cohort

In males and females alike and regardless of the chosen statistical analysis or genetic model, no association between *ACTN3* R577X genotypes and 1500 m, 3000 m, 5000 m, 10000 m or marathon personal best times was found (**Figure 18**, **Table 6**).

Data are shown as boxplots and time is expressed in seconds. The red dashed line on each plot corresponds to the competition entry standard for the 2016 Olympic Games.

No association between ACTN3* R577X *genotype and personal best time in males within 20% of the World record

In males only, I conducted a sub-analysis of the athletes displaying times within 20% of the World record for the corresponding event that reflected in the reduction in SD compared to the total sample to see whether an association with the *ACTN3* R577X variant could be detected at the high end of the performance spectrum. Regardless of the chosen statistical analysis or genetic model, no association between *ACTN3* R577X genotype and 1500 m, 3000 m, 5000 m, 10000 m or marathon personal best time for those athletes within 20% of the World record was found (**Table 7**).

Table 7 *Mean (SD) 1500 m, 5000 m, 10000 m and marathon best running times in males within 20% of the world record in (a) the three ACTN3 R577X genotypes. All running times are expressed in seconds because statistics were performed on times converted to seconds. The last two columns of the table correspond to the p-value of the linear regression, using an additive or a recessive genetic model. The percentage values represent the genotype proportions.*

MALES	RR N=296 37.0%	RX N=340 42.6%	XX N=163 20.4%	Additive (RR=0, RX=1, XX=2)	Recessive (RR = RX = 0, XX=1)
Running time 1500 m (s)	230.8 (7.5) 44.2%	230.0 (8.1) 38.0%	228.7 (11.8) 17.8%	p=0.34	p=0.42
Running time 3000 m (s)	497.0 (16.2) 38.3%	502.2 (16.6) 42.8%	503.1 (18.8) 18.9%	p=0.08	p=0.37
Running time 5000 m (s)	857.5 (29.9) 34.6%	861.2 (30.2) 45.0%	861.7 (31.2) 20.4%	p=0.52	p=0.76
Running time 10000 m (s)	1789.4 (66.0) 36.8%	1776.8 (66.6) 42.4%	1788.7 (70.7) 20.8%	p=0.83	p=0.76
Running time marathon (s)	8502.7 (330.0) 33.3%	8471.5 (302.1) 43.1%	8462.0 (337.8) 23.6%	p=0.64	p=0.76

All running times are expressed in seconds because statistics were performed on times converted to seconds. The last two columns of the table correspond to the p-value of the linear regression, using an additive or a recessive genetic model.

7.5 *Discussion*

The chapter 7 of this thesis shows that world-class sprinters are unlikely to harbour the *ACTN3* XX genotype, and that sprinters with the XX genotype have significantly lower best 100-400 running time compared to their RX and RR counterparts. Here, following a similar approach with the first study (chapter 6) of this thesis (Papadimitriou et al., 2016), I investigated the influence of *ACTN3* R577X polymorphism in endurance performance in a large cohort of elite Caucasian endurance runners. This quantitative assessment of genotype with qualifying time in 1064 personal best times of 698 elite endurance runners suggests that the potential association between *ACTN3* R577X genotype and elite endurance running time is unproven.

In chapter 7 of this thesis, I examined whether a genotype association existed within athletes competing in particular endurance-running events (1500 m, 3000 m, 5000 m, 10000 m and marathon) and in a subset of high-level athletes with a personal-best times within 20% of the World record. I included the specific time cut-off 20% threshold that reflected in the reduction in SD compared to the total sample for each study (**Tables 6 and 7**), and enable me to gain insights into the effect of *ACTN3* genotypes on higher possible end of human elite sprinting and endurance performance. Previous reports have grouped together endurance athletes from mixed endurance sports disciplines and events without quantifying measures of their actual endurance performance (Yang et al., 2003; Papadimitriou et al., 2008; Druzhevskaya et al., 2008; Eynon et al., 2009; Cięszczyk et al., 2011; Mikami et al., 2013). Here, I have embraced a more stringent approach and included only endurance runners whose main sporting discipline was the 1500 m, 3000 m, 5000 m, 10000 m or marathon, including their personal-best running performance. In this manner, I was able to differentiate between events that are estimated to have a different energy reliance on the aerobic system ranging from 77-86% (1500 m), 86-94% (3000 m) whereby it becomes increasingly aerobic up to the Marathon (Duffield et al., 2005). Despite addressing these subtle performance requirement differences, the results from this chapter suggest that *ACTN3* R577X is unlikely to influence Caucasian endurance runners' personal best times in 1500 m, 3000 m, 5000 m, 10000 m and marathon, even at the high end of the performance spectrum.

The *Actn3* KO mouse model mimics the *ACTN3* R577X polymorphism in humans. Metabolically, the KO mice show a higher activity of oxidative enzymes and a lower activity of enzymes involved in the anaerobic pathway (Quinlan et al., 2010). In addition, KO mice show enhanced glycogen accumulation due to lower glycogen phosphorylase activity (MacArthur and North, 2007; Quinlan et al., 2010). Their fast skeletal muscle fibre properties shift towards a slower metabolic profile, which has been linked to an increase in calcineurin signalling activity (Seto et al., 2013) and theoretically can favour endurance performance. Top-level endurance running performance is considered to be predominately based on the metabolic profile of slow-twitch fibres due to the high reliance on the aerobic energy system, which has been hypothesized to favour the 577XX genotype. However, it may also depend on the endurance runner's ability to recruit type IIb fast-twitch myofibres during tactical surges (competitively critical phases requiring increase in pace) or finishing stages of a race (a sprint over a short distance) both of which require an increase in anerobic energy/muscle recruitment (may favour the 577RR genotype). While it is difficult to determine the relative contribution of muscle fibres in performance, it is well understood that murine muscle contains a significantly higher percentage of fast-anaerobic twitch fibres compared to human muscle and any detected effect would be enhanced in this model. Therefore *ACTN3* 577XX

genotype could have only a minimal performance effect without offering any tangible advantage to a competitive α-actinin-3 deficient (*ACTN3* 577XX) endurance runner and/or for 577RX/RR individuals, can be overcome with training (i.e. shift away from IIb to increase 90-95% type I fibre proportions). In this study, I included only Caucasian endurance athletes so I cannot rule out the possibility that an association exists between *ACTN3* R577X genotype and endurance running performance in elite runners from different geographic ancestry. However, most published associations on which my original hypothesis was based involved athletes or other individuals who were Caucasians.

Endurance performance is considered to be a complex trait effected by both genetic and environment (training) (Rankinen and Bouchard, 2008). As recently shown, not only metabolism but also anthropometric and biomechanical factors are important in determining elite performance success (Stebbings et al., 2017). Here I have not found evidence that supports the involvement of these two genes in endurance running performance. Endurance running performance is dependent on extensive training and there is little evidence of either *ACTN3* R577X being associated with training responses of aerobic parameters (Rankinen and Bouchard, 2008; Rankinen et al., 2000; Rankinen et al., 2016).

The multi-centre cohort used in this thesis is larger than any previous such study regarding endurance performance, but a very small effect size could still go undetected using this sample size. It is increasingly recognised that the reality of complex human biology is that inter-individual differences in endurance performance are expected to be influenced by many common and perhaps rare genetic variations; none of which have been discovered at a genome-wide significance level or consistently replicated (GWAS endurance paper) (Rankinen et al., 2016).

This study of quantitative measures of endurance performance in a large homogeneous group of elite endurance runners suggests that the potential association between *ACTN3* R577X genotype and elite endurance running performance should be regarded as unproven. As indicated by the discovery of these variants, large population studies are needed to detect significant proportions of the underlying genetic profile and biology contributing to endurance performance.

8. STUDY THREE: *ACTN3* GENE & RESPONSE TO EXERCISE

Chapter 8 of this thesis is based largely on extensive laboratory analysis of an exercise study that I performed on *ACTN3* XX and RR human muscle. An abstract has been accepted for presentation at the "Targeting Mitochondria" conference in Berlin, 2017 with the following title:

The influence of the ACTN3 R577X *variant in mitochondrial-related molecular pathways at baseline and following a session of high-intensity interval exercise* (2017) **I.D. Papadimitriou,** N. Eynon, X. Yan, J. Kuang, K.N. North. D.J. Bishop.

A paper is also being prepared from this chapter, and is due to be submitted for publication in Nature Communications with a title: *A human knockout model to investigate the influence of the α-actinin-3 protein on exercise-induced mitochondrial adaptations* and the following co-authors order.

I.D. Papadimitriou, N. Eynon, X. Yan, J. Kuang, M. Jacques, F. Munson, S. Voisin , K.N. North, D.J. Bishop

The results from chapter 6 and 7 of this PhD thesis demonstrated that the *ACTN3* R577X genotype is associated with sprinting but not endurance running time in elite athletes. However, there has been limited research with regards to how this variant influences the adaptive response to endurance exercise in humans. In mice it has been shown that α-actinin-3 deficiency results in increased calcineurin activity in exercised muscles, and greater endurance performance following 6 weeks of endurance exercise training. These results provide a mechanistic explanation for the effects of the *ACTN3* genotype on endurance performance in mice. Simultaneously, with the epidemiological approach in elite endurance athletes in chapter 7, a more functional physiological approach in general population in chapter 8 aimed to identify potential molecular pathways influenced by the *ACTN3* gene, by examining exercise-induced changes in genes and proteins following a single session of High-Intensity Interval Exercise (HIIE) in humans.

The research questions are:

I. Is the *ACTN3* genotype (*ACTN3* RR vs. *ACTN3* XX) associated with differences in physiological characteristics and maximal mitochondrial respiration at baseline?

II. Is the *ACTN3* genotype associated with the levels of RCAN 1-4 and PGC-1α protein at baseline, immediately post, and 3 hours after a session of High Intensity Interval Exercise (HIIE)?

III. Is the *ACTN3* genotype associated with differences in the expression of mitochondrial-related genes 3 hours after a session of HIIE?

8.1 *Abstract*

Absence of the α-actinin-3 protein in mice (analogous to the *ACTN3* 577XX genotype in humans) is associated with alteration in endurance-related metabolism and performance. This may be related to higher calcineurin activity, which has been reported to be important for exercise-induced mitochondrial biogenesis. To date, however, no study has investigated how the *ACTN3* genotype regulates mitochondrial-related gene expression in response to a single session of exercise in humans. Potential pathways could involve the downstream targets of calcineurin, such as PGC-1α. The aim of this study was to determine whether the *ACTN3* R577X polymorphism influences exercise-induced changes in mitochondrial-related gene and protein expression in human muscle. More than 100 Caucasian males (18 to 45 y), with moderate physical activity levels and a Body Mass Index (BMI) between 20 and 30, were recruited. Genomic DNA was extracted from leucocytes, and *ACTN3* genotype screening was conducted on these men. Participants were subsequently included in the study if they had either the XX (α-actinin-3 deficient; n=18) or RR (expressing α-actinin-3; n=19) genotype. Following baseline testing, the participants performed one session of High-Intensity Interval Exercise (HIIE), which consisted of 8 x 2-min intervals at individually-determined pre-training LT power and W_{peak} (LT+40% (W_{peak} - LT), interspersed with 1-min rest periods. Muscle samples were collected at rest, immediately after, and 3 h post-exercise to measure the content of mRNA and proteins associated with mitochondrial biogenesis. At baseline, there was a compensatory greater α-actinin-2 protein content in *ACTN3* XX vs *ACTN3* RR participants (p=0.018) with no evidence for significant differences in physiological characteristics, such as VO_{2peak}, the lactate threshold, and ADP stimulated respiration, between genotypes. There was also a main effect of genotype (p=0.006), without a significant interaction effect, for the protein content of RCAN1-4 (a marker of

calcineurin activity). However, there was no significant effect of genotype on the exercise-induced expression of a range of genes associated with mitochondrial biogenesis (e.g *PGC1α, COX4-1, MFN2 C, RCAN1-4* and *VEGF*). These results suggest that loss of α-actinin-3 is not associated with higher baseline values for endurance-related phenotypes, nor a greater adaptive response to a single session of high-intensity endurance exercise.

8.2 *Introduction*

The results from chapter 6 of this thesis indicated that α-actinin-3 deficiency has a detrimental effect on the optimal function of fast-twitch muscle fibres. The *ACTN3* 577XX genotype was also found underrepresented in elite Australian (Yang et al., 2003), Finish (Niemi and Majamaa, 2005), Greek (Papadimitriou et al., 2008), Russian (Druzhevskaya et al., 2008), Israeli (Eynon et al., 2009), Polish (Cięszczyk et al., 2011), and Japanese (Mikami et al., 2013) power-oriented athletes, suggesting that α-actinin-3 deficiency indeed has a detrimental effect on the optimal function of fast-twitch muscle fibres. In contrast chapter 7 suggests it is unlikely the *ACTN3* XX genotype offers an advantage for competitive endurance running performance. There are conflicting findings regarding the influence of the *ACTN3* 577XX genotype on endurance performance. Although some studies have reported that the *ACTN3* 577XX genotype is associated with enhanced endurance status in elite athletes (Eynon et al., 2009), most others have not (Niemi and Majamaa, 2005; Papadimitriou et al., 2008; Ahmetov et al., 2010; Mikami et al., 2013; Ma et al., 2013), which is consistent with the multi-cohort quantitative analysis in the second part of this thesis.

In contrast to human studies, studies in mice bred to completely lack the α-actinin-3 protein (i.e., Actn3 knockout (KO) mice) suggest the *ACTN3* genotype influences the adaptive response to endurance exercise training (Seto et al., 2013). Compared with wild type (WT) mice, after 6 weeks of endurance training Actn3 KO mice had greater endurance exercise performance and faster recovery from fatigue, which was associated with a shift in fast-twitch muscle fibre properties toward a more slow-twitch, oxidative phenotype (MacArthur et al., 2007; MacArthur et al., 2008; Seto et al., 2013). Seto et al. (2013) also reported a 1.9-fold increase in calcineurin activity in exercised KO muscles compared with WT muscles ($p = 0.093$), which was associated with a 2.9-fold increase in RCAN1-4 (a marker of calcineurin activity, $p = 0.004$) (Seto et al., 2013). Consistent with the observations in mice, there was a also greater protein content of RCAN1-4 in resting muscle samples obtained from *ACTN3* 577XX humans when compared with *ACTN3* 577RR humans (Seto et al., 2013). However, RCAN1-4 content was only measured in resting muscle and it is not

known if exercise-induced changes RCAN1-4 protein content are affected by *ACTN3* genotype in humans.

The molecular mechanisms for the altered calcineurin activity with α-actinin-3 deficiency appear to be via differential binding of calsarcin-2 to sarcomeric α-actinins. When α-actinin-3 is absent (*ACTN3* 577XX *genotype*) there is a compensatory increase in α-actinin-2, which binds more tightly to calsarcin-2 (a negative regulator of calcineurin) (Rothermel et al., 2003). Thus, absence of α-actinin-3 protein (and an increase in α-actinin-2 protein) has been hypothesised to increase the release and activation of calcineurin (Seto et al., 2013). Activated calcineurin is able to dephosphorylate many substrates, including NFAT - allowing its translocation to the nucleus (Chin et al., 1998). NFAT interacts with MEF2 in the nucleus to regulate expression of PGC-1α, a key regulator of mitochondrial biogenesis (Wu, 2001). Overexpression of activated calcineurin in mice results in a significant increase in the mitochondrial proteins of the electron transport system (complexes I to V) (Long et al., 2007), a 35% increase in resting mitochondrial respiratory capacity (compared to WT mice) (Jiang et al., 2010), and a significant increase in PGC-1α protein content (Ryder et al., 2003). Altered calcineurin activity therefore provides a plausible biological mechanism for the effects of α-actinin-3 deficiency to promote a more “aerobic” or “endurance” phenotype in mice.

Knocking out the *ACTN3* gene in mice has been an excellent model in which to study the effects of α-actinin-3 deficiency on skeletal muscle metabolism and associated molecular signalling pathways. Nonetheless, results from KO mice don’t always translate to people, and complementary studies in humans remain essential to confirm the findings observed in mice. While knocking out genes in humans is ethically off-limits, the high percentage of the population deficient for the α-actinin-3 protein (i.e., naturally occurring human “knockouts”) affords us the unique opportunity to investigate the consequences of α-actinin-3 deficiency on human skeletal muscle. The aim of the present study was to investigate the role of the α-actinin-3 protein in regulating metabolism and molecular signalling pathways in human skeletal muscle at baseline and in response to a session of High-Intensity Interval Exercise (HIIE). I chose high-intensity interval exercise (HIIE) as it has been reported to be equal or more effective at improving oxidative capacity (VO_{2max}) (Bishop et al., 2014; Batacan et al., 2017), and increasing the expression of nuclear genes encoding mitochondrial proteins (Granada et al., 2015) (NUGEMPs) compared to moderate intensity exercise (MacInnis et al., 2017).

I hypothesised that humans with *ACTN3* XX genotype (i.e., Actn3 human “knockouts”) would have greater endurance performance and higher maximal mitochondrial

respiration at base-line compared to their *ACTN3* RR counterparts. I also hypothesised that *ACTN3* XX humans would have higher mitochondrial-related gene and protein expression following a single session of HIIE, compared to their *ACTN3* RR counterparts.

8.3 *Methodolgy*

8.3.1 Study Design and Participants

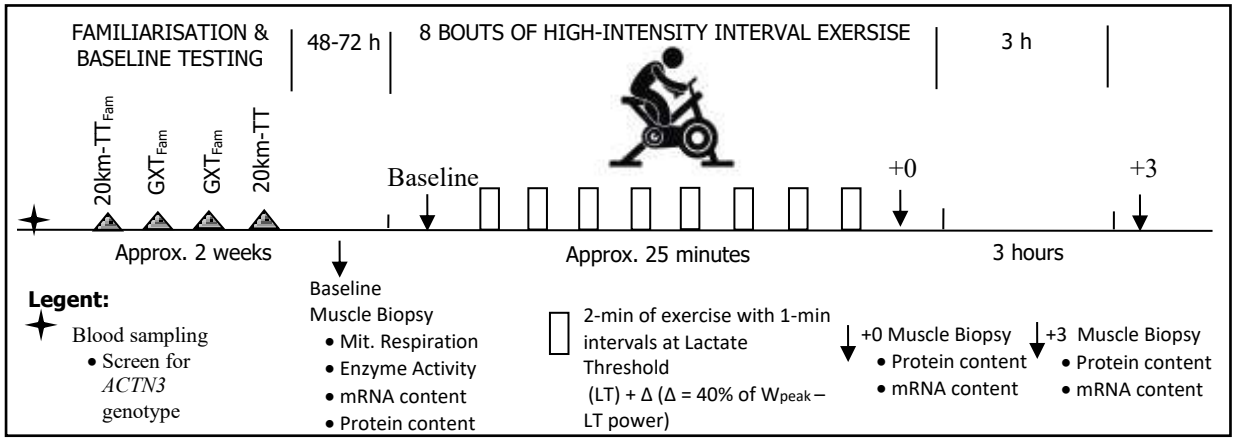


Figure 19 The study flow outline LT: Lactate Threshold, Wpeak: maximum wattage achieved, Δ: Delta difference, LT power: power in Watts achieved at lactate threshold, mRNA: messenger RNA, GXT: graded exercise test, GXTfam: graded exercise test familiarisation, 20k-TT: 20 kilometres times trial, 20k-TTfam: 20 kilometres times trial familiarisation.

More than 100 participants where recruited from the local community. Only the *ACTN3* RR and the *ACTN3* XX participants were included in this study to ensure we compared the two extreme genotypes (i.e., RR vs. RX). Only Caucasians (for ≥ 3 generations) with a Body Mass Index (BMI) between 20 and 30 kg•m^{-2} and body fat < 25% were included in the study to ensure both genetic and body composition homogeneity (Deurenberg et al., 1998). Participants with a past history of the following medical conditions were excluded from the study: possible coronary heart disease, significant chronic or recurrent respiratory condition, significant neuromuscular, major musculoskeletal problems interfering with the ability to cycle, uncontrolled endocrine and metabolic disorders or diabetes requiring insulin and other therapies.

Thirty seven (19 *ACTN3* RR and 18 *ACTN3* XX*)*, moderately-trained (VO_2 peak 35-60 mL•min^{-1}•kg^{-1}), non-obese with a BMI of 26.6±2.6 for RR and a BMI of 24.2 ±2.36 for XX, with a mean age 32.3±7.09 years for RR and 29.4±8.44 years for XX, further analysed.

8.3.2 Genotyping

Genomic DNA was extracted from residual blood samples from the GXT tests using the GeneJET Genomic Whole Blood DNA Purification Kit (#K0781 Thermo Scientific, MA, USA). ACTN3 gene variants were determined using the TaqMan SNP assay (manufacturer details) by Mastercycler® ep realplex2 (Eppendorf, Hamburg, Germany). Genotyping was replicated in another independent institute (The Murdoch Childrens Research Institute, Melbourne) as previously described (Banting et al., 2015), to validate the results.

8.3.3 Pre-training physical activity monitoring

To control for potential differences in habitual physical activity between participants, 1 week prior to commencing the study the participant's activity level was monitored for seven consecutive days. The monitoring of the participants' activity level was performed using an ActiGraph GT3X+ device (ActiGraph LLC, Fort Walton Beach, FL, USA). The GT3X+ activity monitor has been used in many types of research (Aibar et al., 2014) and clinical applications (German National Cohort (GNC) Consortium, 2014). The device includes a micro-electro-mechanical system based tri-axis accelerometer sensor that provides measures of acceleration in three axes, with a composite measure called the vector magnitude (VM =√(x2 + y2 + z2)). The accelerometer has ±6 g range with a sampling rate ranging from 30 Hz to 100 Hz (user selectable) storing the raw, non-filtered/accumulated data, in the units of gravity. The device can sample continuously for between 24 and 32 days depending on the selected sampling frequency.

The ActiGraph GT3X+ device was threaded onto an elastic belt and worn either over or under clothing, whichever was most comfortable for the participant. The device was positioned snugly enough against the body so that it could not flip around. The device was worn with the elastic belt fastened around the waist over the right hip bone all day while the participant was awake. The device was removed only when the participant was in bed at night, swimming and showering. A daily diary assisted in monitoring when the device was being removed, for monitoring any water-based activities as well. The acceleration data were downloaded from the device and processed over a user-specified time sampling interval. Energy expenditure and activity intensity was calculated using algorithms that have been used in similar studies (Freedson et al., 1998).

8.3.4 Nutrition consultation

To ensure similar nutrition and to control for potential nutritional differences between participants, each participant was provided with individualised, pre-packaged meals for the 48 hours prior to muscle biopsies. The energy content of the provided meals was calculated using the Mifflin-St-Jeor equation and the participant's body mass, height, and age (Mifflin et al., 1990). The Foodworks (Xyris) nutritional database was used to determine the nutritional components of the packaged meals and to ensure all nutritional requirements were met with the diet. The content of the diets was constructed based on the current National Health and Medical Research Council (NHMRC) guidelines. To ensure adequate access to carbohydrate energy stores, participants were asked to consume a pre-packaged training meal of high glucose food items (1 to 1.5 g•kg^{-1} BM) 2 hours prior to the commencement of each testing session, according to Australian Institute of Sport (AIS) guidelines (https://www.ausport.gov.au/ais/sports_nutrition/fact_sheets/eating_before_exercise).

Participants were also asked to refrain from alcohol and caffeine during the dietary control period, which was 48 hours prior to the biopsies.

8.3.5 Performance tests

Prior to the start of the exercise intervention phase, all participants completed familiarisation sessions and baseline testing. All visits were separated by a minimum of 48 hours. In addition, participants were required to refrain from exercise, alcohol and caffeine consumption for 24 hours before all tests. The familiarization and baseline testing (**Figure 19**) was consisted of the following:

1. 20-km cycle Time Trial (20K TT) - During the first (familiarization), and third visits (baseline test) participants performed a 20K TT test on a Velotron® cycle ergometer (RacerMate Inc. Seattle, WA, USA). Participants completed a warm-up consisting of 5 minutes of cycling at 60 W. Following a 2-minute rest, participants were then required to complete the 20-km TT in the quickest possible time. Participants were permitted to monitor their progress through completed distance and were provided with verbal encouragement during the test.

2. Graded exercise test to exhaustion (GXT) - During the second (familiarisation), fourth and fifth (baseline testing) visits participants undertook a GXT, for baseline determination of the lactate threshold (LT). This test was performed on an

electronically-braked cycle ergometer (Lode-Excalibur sport, Groningen, the Netherlands) and consisted of 4-min stages separated by 30-s rest periods until exhaustion. The test started at 60, 90 or 120 W (depending on the participant's 20K TT results) and increased by 30 W in each subsequent stage. Capillary blood samples were taken at rest, after each completed stage, and immediately following exhaustion, and were analysed by a YSI 2300 STAT Plus system (Yellow Springs, Ohio, USA). During the GXT the LT was calculated by the modified DMAX method, which is determined by the point on the polynomial regression curve that yields the maximum perpendicular distance to the straight line connecting the first increase in lactate concentration above resting value and the final lactate point (Bishop et al., 1998, Bishop et al., 2000). The average of the two GXT tests was used to customise subsequent exercise intensities if the difference was no more than 5%; otherwise, the highest value was used. Residual blood samples were saved for DNA analysis.

3. VO_{2peak} test - After five minutes of rest, peak oxygen consumption (VO_{2peak}) was measured using a calibrated Quark CPET metabolic system (COSMED, Rome, Italy). Briefly, participants wore the Cosmed face mask and we collected VO_2 data while stationary for 2 minutes while exercising for 3 minutes at 100 W, followed by exercise to exhaustion at 105% of the peak power (W_{peak}) measured during the previous GXT. The VO_{2peak} was considered the highest value in 1 minute obtained during the test. Previous studies have reported the VO_{2peak} measured this way is not different from that derived from a ramp test (Weston et al., 2002). The HIIE phase commenced, and muscle biopsies were collected 48 to 72 hours after the last VO_{2peak} test.

8.3.6 Muscle Biopsies and High-Intensity Interval Exercise (HIIE)

Muscle biopsies were collected from the vastus lateralis muscle of the participants' dominant leg. Following injection of a local anaesthetic (5 mL, 1% Xylocaine), incisions were made and the biopsy needle was inserted. Muscle samples were collected with manual suction applied (Edge et al., 2013). The samples (50 to 200 mg) were then immediately blotted on filter paper to remove excess blood, with a small portion (5 to 15 mg) immediately processed for the determination of mitochondrial respiration in SITU (Boushel et al., 2007); the remaining muscle was snap frozen in liquid nitrogen before being stored at −80 ˚C for subsequent analyses. Following the pre-training baseline muscle biopsy, participants performed a single session of HIIE on an electronically braked cycle ergometer (Velotron®, Racer Mate Inc, Seattle, USA). The session consisted of eight 2-minute intervals performed at an intensity between the individually-determined pre-training LT power and W_{peak}

(LT+40% (W_{peak} - LT), and interspersed with 1-minute recovery periods (work-to-rest ratio of 2:1). All participants were motivated, fully pushed and reached the expected loads during all eight 2-minute intervals. Muscle biopsy samples were also taken immediately after and 3 hours post-HIIE (**Figure 19**).

8.3.7 RNA extraction and gene expression analysis

Total RNA was extracted from approximately 15 mg of frozen muscle. Cellular membranes were dissociated in TRIzol® Reagent (Invitrogen, Melbourne, Australia) through TissueLyser II (Qiagen, Hilden, Germany) for 2 × 1 minute at 30 Hz. The homogenate was centrifuged (13,000 RPM for 15 minutes) and the RNA containing supernatant removed. The homogenate then was combined with chloroform (Sigma-Aldrich, St Louis, USA) and total tissue RNA is then extracted using the TRIzol protocol in accordance with the manufacturer's instructions with the exception of RNA precipitation which will be conducted for a minimum of 2 hours at -20 °C in the presence of 10 µL of 5 M sodium chloride. RNA concentration was quantified spectrophotometrically at 260 nm and purity was checked using the ratio of its absorbance at 260 and 280 nm using a BioSpectrometer (Eppendorf, Hamburg, Germany). First strand cDNA was then generated from 1 µg of template RNA using the commercially available iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, USA) using random hexamers and oligo dTs according to the protocol provided with the iScipt cDNA synthesis kit (Bio-Rad Laboratories, Hercules, USA). cDNA was stored at -20 °C for subsequent analysis. All samples and reverse transcriptase (RT) negative controls were run together to prevent technical variation. Forward and reverse primers for the target and housekeeping genes were designed based on NCBI RefSeq using NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/BLAST/). Specificity of the amplified product was confirmed by melting point dissociation curves generated by the PCR instrument. The mRNA expression of target and housekeeping genes were quantified by quantitative real-time RT-PCR (QuantStudioTM 7 Flex Real-Time PCR System (Life Technologies, Thermo Fisher Scientific, Wilmington, DE, USA), using a 5 µL PCR reaction volume and SYBR® Green chemistry (iTaqTM Universal SYBR® Green Supermix, Bio-Rad, Hercules, CA). All samples were run in duplicate simultaneously with template free controls, using an automated pipetting system (epMotion 5073, Eppendorf, Hamburg, Germany). The following PCR cycling patterns were used: initial denaturation at 95°C for 3 min, 40 cycles of 95°C for 15 s and 60°C for 60 s.

8.3.8 Western blot for protein content

Approximately 10 mg of frozen muscle samples were homogenised in ice-cold RadioImmunoPrecipitation Assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Sodium Deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA with protease/phosphatase inhibitors, 1 mM PMSF, 1 g/mL Aprotinin, 1 g/ml Leupeptin, 1mM Benzamidine, 1 mM Na3VO4, 5 mM Na Pyrophosphate, 1 mM DTT and 1 mM NaF) using a TissueLyser II (Qiagen, Hilden, Germany) for 2 × 1 minute at 30 Hz, and rotated for 1 h at 4 °C. Muscle lysates were stored at -80 °C until further analysis. Total protein content of muscle lysates was determined using the bicinchoninic acid assay.

Protein extracts were loaded on sodium dodecyl sulfate-polyacrylamide gels, separated for 120 minutes at 100 V and subsequently transferred to PolyVinyl DiFluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, USA) using a Bio-Rad blot system for 100 minutes at 100 V. Thereafter, blots were blocked for 60 minutes in 5% milk in tris-buffered saline (TBS) and washed with TBS plus 0.1% Tween at room temperature, followed by incubation with different primary antibodies overnight at 4 ˚C. After washing, the membranes were incubated with the appropriate secondary antibodies for 60 minutes at room temperature and revealed using a chemiluminescent substrate (Bio-Rad Laboratories, Hercules, USA). Light emission was recorded using ChemiDocTM MP System (Bio-Rad Laboratories, Hercules, USA) and quantified by image analysis software (Image Lab, Bio-Rad). Protein content was then normalised to totally protein analysis by TGX stain-free gel (Bio-Rad Laboratories, Hercules, USA) (Eaton et al., 2013).

8.3.9 Mitochondrial Respiration

Immediately after the resting biopsy, muscle fibres were separated gently on ice under a binocular microscope in BIOPS solution (2.77 mM CaK2EGTA, 7.23 mM K2EGTA, 5.77 mM Na2ATP, 6.56 mM MgCl26•H2O, 20 mM Taurine, 15 mM Na2Phosphocreatine, 20 mM Imidazole, 0.5 mM Dithiothreitol, and 50 mM MES at PH7.1), and permeabilised in the same solution with 50 µg/mL of saponin (Sigma-Aldrich, St Louis, USA) for 30 minutes. This was followed by rinsing the muscle fibres for 3 × 7 minutes in mitochondrial respiration medium on ice (0.5 mM EGTA, 3 mM MgCl2•6H2O, 60 mM K-lactobionat, 20 mM Taurine, 10mM KH2PO4, 20 mM Hepes, 110 mM sucrose, and 1 g•L^{-1} bovine serum albumin at pH 7.1). Experiments were performed on washed muscle fibres under continuous stirring using an oxygraph-2k respirometer (Oroboros Instruments, Austria), containing 2 mL of mitochondrial respiration medium with additional substrates at 37 ˚C. The following

substrates were added (final concentration): malate (2 mM) and pyruvate (5 mM) to support electron entry to complex I (CI); MgCl2 (3mM) and ADP (5 mM) to measure Oxidative phosphorylation (OXPHOS) capacity; Succinate (10 mM) to stimulate CI+II-linked respiration and providing convergent electron input into the Q-junction simultaneously (CI+IIP) (Chomistek et al., 2013). A maximal respiratory capacity was reached when these substrates are present in the respirometer chamber. Cytochrome c (10 μM) was used to test the integrity of the outer mitochondrial membrane (Aibar et al., 2014). Electron transfer system capacity (ETS with CI+II-linked substrates, CI+IIE) will be tested by titrating p-trifluoromethoxy phenylhydrazone (FCCP) (steps of 0.5 μM) until maximal noncoupled respiration is reached. Rotenone (0.5 μM) was then added to block the activity of complex I so that electrons can only enter through complex II (CII). Antimycin (3.75 μM) was added to block the activity of complex III and to measure the non-mitochondrial respiration. Different ratios (substrate and coupling control ratios) were calculated from the different titration steps obtained from the protocol use

8.3.10 Statistical analysis

The quantitative genetic association data, mRNA content, protein relative abundance, and maximal mitochondrial respiration data were analysed using linear mixed models, with time point (baseline, post, 3h post) and genotype (RR or XX) as covariates. The interaction between time and genotype was also tested. To test the effect of each covariate (time, genotype or time:genotype), a full model containing all covariates was compared with a null model missing the covariate of interest, using a likelihood ratio test. All data analyses were conducted with the R statistical software with the lme4 and lrtest packages.

8.4 *Results*

8.4.1 Genotyping

Genotyping for the *ACTN3* R577X was performed in duplicate and verified by blotting for *ACTN3* protein (**Figure 20a**). As shown in the representative blots below, the α-actinin-3 protein was present in *ACTN3* RR and not *ACTN3* XX participants. There was also a compensatory greater α-actinin-2 protein content in *ACTN3* XX vs *ACTN3* RR participants

(p=0.018) as shown in **Figure 20b** and demonstrated in representative images of α-actinin-2 in **Figure 20a**.

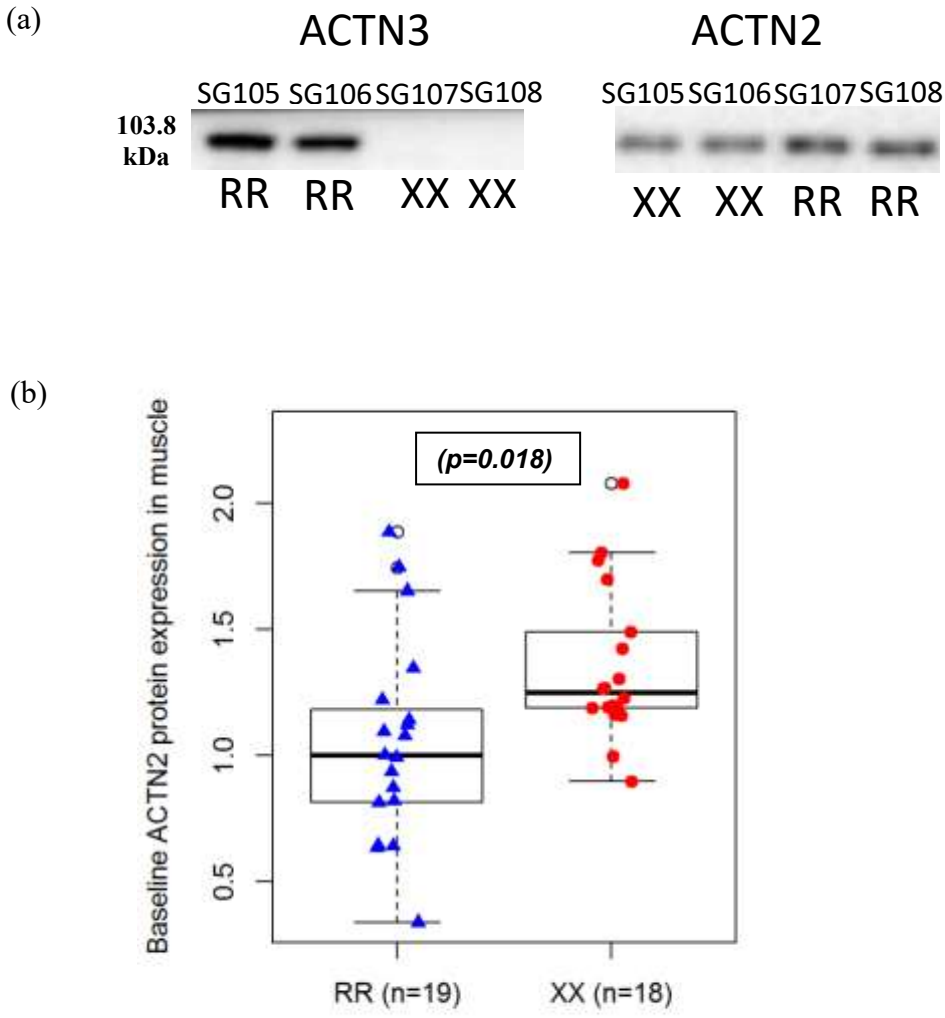


Figure 20 ***a)*** *Representative images of α-actinin-2 and α-actinin-3 protein relative abundance in ACTN3 RR vs. ACTN3 XX participants at baseline. α-actinin-3 protein was present in ACTN3 RR and not in ACTN3 XX participants.* ***b)*** *α-actinin-2 protein relative abundance in ACTN3 RR vs. XX participants at baseline. There was a compensatory greater α-actinin-2 protein content in ACTN3 XX vs ACTN3 RR participants (p=0.018).*

8.4.2 Habitual activity monitoring

There were no significant differences for habitual physical activity measured for seven consecutive days, 1 week prior to commencing the study, between *ACTN3 XX* and *ACTN3 RR* participants 790.22 ±382.51 Kcal for *ACTN3* RR vs. 673.89 ±343.22 Kcal for *ACTN3* XX genotypes, ($p<0.05$).

8.4.3 Baseline Physiological Characteristics

There were no significant differences for the lactate threshold, VO_{2peak}, W_{peak}, Time trial performance, citrate synthase activity or maximal mitochondrial respiration (**Table 8**) between *ACTN3 XX* and *ACTN3 RR* participants at baseline.

Table 8 *Endurance-related phenotypes were measured: Lactate threshold, $VO_{2\ peak}$, W_{peak}, Time trial performance, Mitochondrial respiration, and Citrate synthase activity for each of the ACTN3 genotype (n=37). Values are reported as Mean (SD). No statistical significant difference was demonstrated (p<0.05) for any of these phenotypes between RR and XX genotypes.*

PHENOTYPE	**GENOTYPE**	
	RR (n=19)	**XX** (n=18)
Lactate Threshold (W)	210.4 (52)	196.7 (65.9)
$VO_{2\ peak}$ ($mL \cdot min^{-1} \cdot kg^{-1}$)	43.3 (6.2)	46.9 (6.6)
Time Trial Performance (s)	2246 (311)	2377 (300)
Power Peak (W_{peak})	291.4 (57.6)	272.1 (70.3)
Mitochondrial Respiration (pmol O_2 $/s^{-1}$ ·mg wet weight)$^{-1}$	112.3. (37.3)	111.1 (35.7)
Citrate synthase activity (mol · h^{-1} · kg protein^{-1})	13.9 (3.28)	13.0 (3.96)

8.4.4 The influence of α-actinin-3 deficiency on RCAN 1-4 protein expression following HIIE

There was a main effect of genotype for RCAN 1-4 protein expression (P=0.004), but there was no significant interaction effect (**Figure 21b**).

(a)

(b)

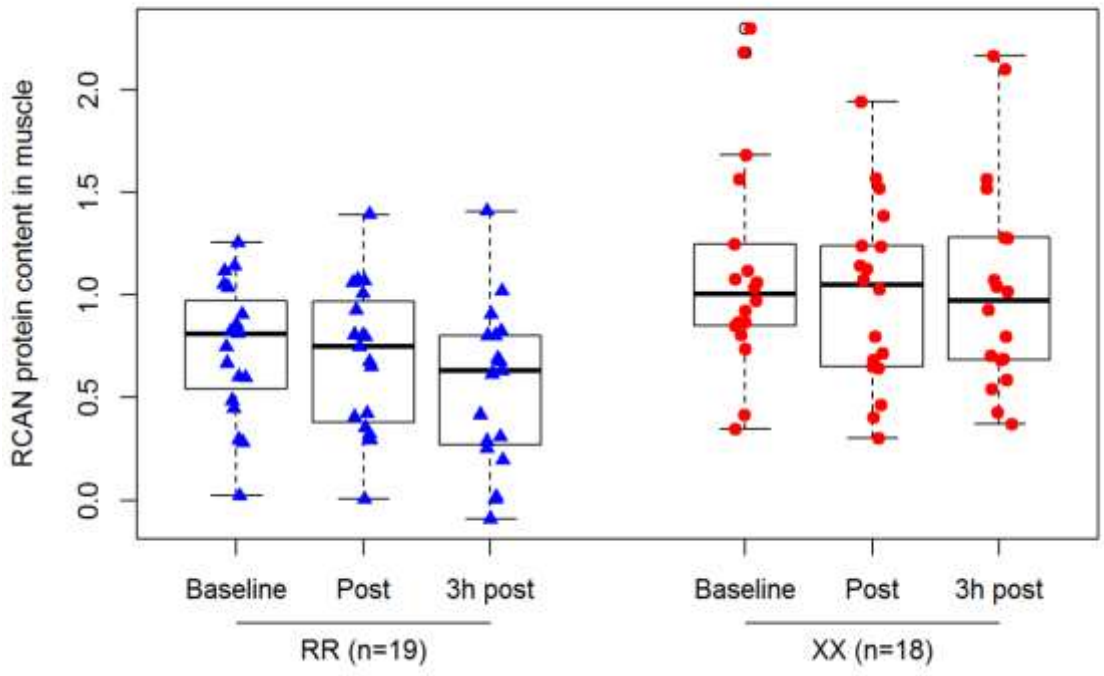


Figure 21 ***a)*** *Representative images of calsarcin-2 protein content in ACTN3 RR vs. ACTN3 XX participants at baseline. Calsarcin-2 protein* content did not differ between muscle samples obtained from *ACTN3 XX* and *ACTN3 RR* humans ***b)*** *A main effect of genotype for RCAN 1-4 (*p<0.05) protein content was demonstrated, but there was no significant interaction effect. Box blot displaying RCAN1-4 relative protein abundance in ACTN3 RR vs. XX participants. The centre rectangle includes the mean and the interquartile range (IQR). The "whiskers" above and below the box show the locations*

of the maximum and minimum (excluding outliners) – ≥0.15 x IQR above the third quartile or below the first quartile and indicated by O.

8.4.5 The influence of α-actinin-3 deficiency on mitochondrial-related gene expression

The results for PGC1α total, PGC1α-1, and PGC1α-4 gene expression are illustrated in **Figure 22**. Although there was a main effect of exercise for all three PGC1α gene transcripts, there was no main effect of genotype and no interaction effect. Gene expression analyses of COX-1, RCAN 1-4, HSP70, VEGF, PDK4, SOD-1, MFN2, SDHB and NUDFB3 immediately post and 3 h post exercise are shown in Table 2. Again, there was no evidence for any difference between ACTN3 XX and ACTN3 RR genotypes, although there were significant time effects ($p>0.05$) for most of the tested genes: COX-1, RCAN 1-4, HSP70, VEGF and PDK4 (Table 9).

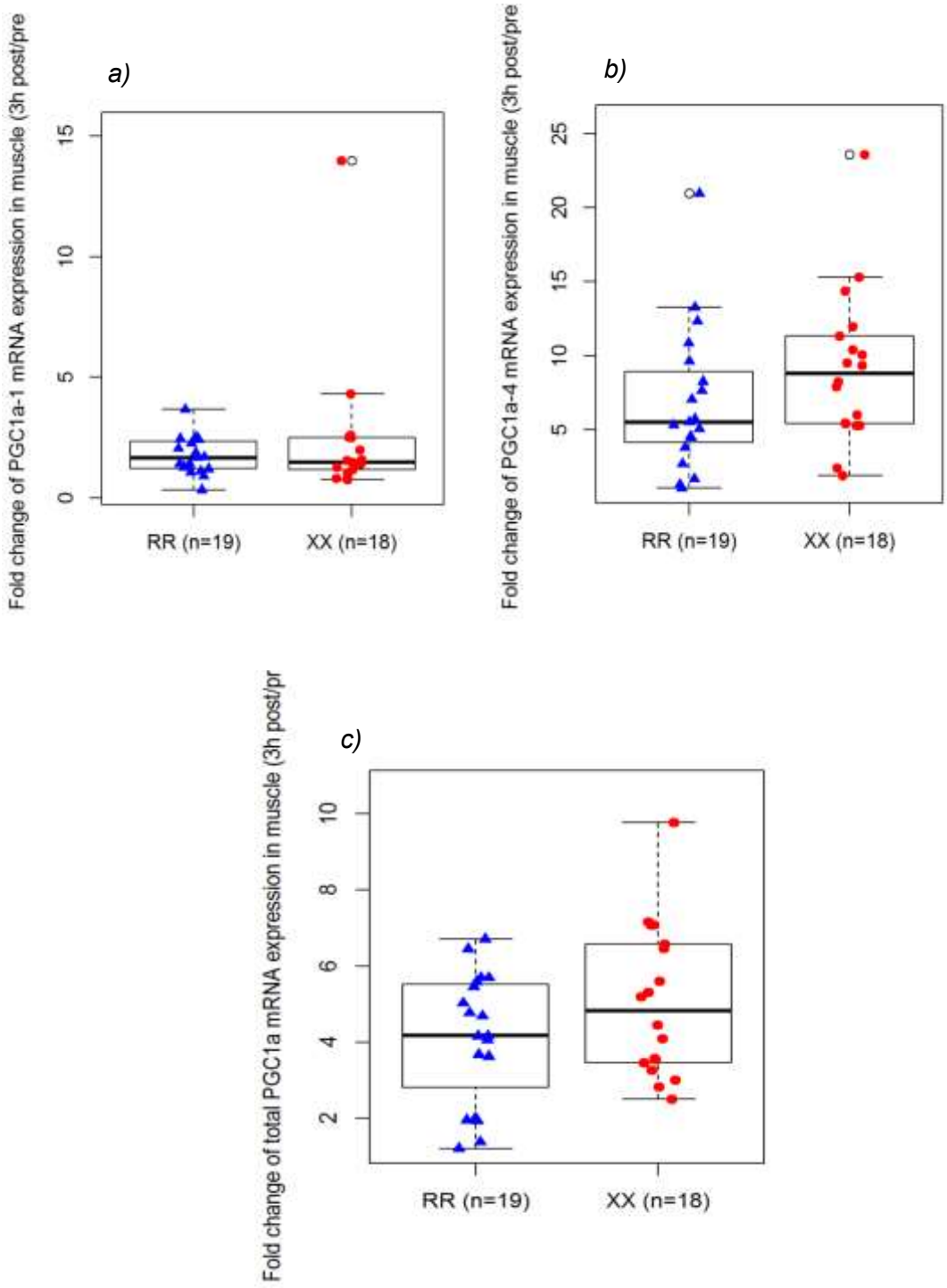


Figure 22 *Fold change from baseline to 3h post exercise for RR and XX participants:* ***a)*** *of PGC1α-1 mRNA expression. P-values: for exercise < 0.05, for genotype = 0.39 and for interaction (exercise: genotype) = 0.58* ***b)*** *PGC1 α-4 mRNA expression. P-values: for exercise < 0.05, for genotype = 0.25 and for interaction (exercise: genotype) = 0.13 and* ***c)*** *total PGC1- α mRNA expression. P-values: for exercise < 0.05, for genotype = 0.76, for interaction (exercise: genotype) = 0.36. Box blot displaying PGC1α-1 relative protein abundance in ACTN3 RR vs. XX participants. The centre rectangle includes the mean and the interquartile range (IQR). The "whiskers" above and below the box show the locations of the maximum and minimum (excluding outliners) – ≥0.15 x IQR above the third quartile or below the first quartile and indicated by O.*

Table 9 *Fold change from baseline to 3h post exercise of mitochondrial gene expression in muscle for COX4-1, MFN2 C, RCAN1-4, SDHB, SOD-1, PDK4, HSP70, VEGF and NUDFB3 (n=37). There were no statistical significant genotype effects for any of these genes (p<0.05). There were significant time effects (p>0.05) for all tested genes, except for SOD-1, NUDFB3, SDHB and MFN2 C genes.*

Gene	ACTN3 Genotype	Pre Exercise	Post Exercise	+3h Exercise
COX4-1	RR n=19	5.11 (3.12)	4.44 (2.82)	4.32 (2.54)
	XX n=18	4.59 (2.04)	4.14 (2.68)	5.08 (2.51)
MFN2 C	RR n=19	3.57 (1.63)	3.21 (1.7)	3.65 (1.78)
	XX n=18	3.66 (1.28)	3.32 (1.57)	4.22 (1.95)
RCAN1-4	RR n=16	0.65 (0.43)	0.71 (0.33)	1.22 (0.91)
	XX n=14	1.02 (0.93)	0.93 (0.70)	1.26 (2.00)
SDHB	RR n=19	1.04 (0.56)	0.84 (0.26)	0.98 (0.46)
	XX n=18	0.74 (0.54)	0.73 (0.526)	0.78 (0.37)
SOD-1	RR n=19	0.97 (0.5)	0.89 (0.23)	0.92 (0.29)
	XX n=18	0.67 (0.41)	0.70 (0.31)	0.68 (0.25)
PDK4	RR n=19	1.59 (1.21)	2.25 (1.57)	9.63 (5.5)
	XX n=18	2.18 (1.61)	4 (3.81)	9.79 (6.53)
HSP70	RR n=19	1.27 (0.52)	2.56 (0.98)	3.55 (2.22)
	XX n=18	1.02 (0.42)	1.99 (1.26)	2.95 (1.68)
VEGF	RR n=19	0.64 (0.20)	0.573 (0.23)	1.19 (0.45)
	XX n=18	0.46 (0.18)	0.64 (0.63)	0.96 (0.23)
NUDFB3	RR n=19	1.48 (0.53)	1.91 (0.58)	1.71 (0.55)
	XX n=18	1.15 (0.66)	1.42 (0.60)	1.39 (0.67)

8.4.6 PGC1-α Protein expression in *ACTN3* RR and XX humans

PGC1-α total protein was significantlly increased after HIIE (p-value for exercise<0.001). However, there was no significant main effect of genotype (p=0.49), and no significant interaction effect (p=0.77) (**Figure 23**).

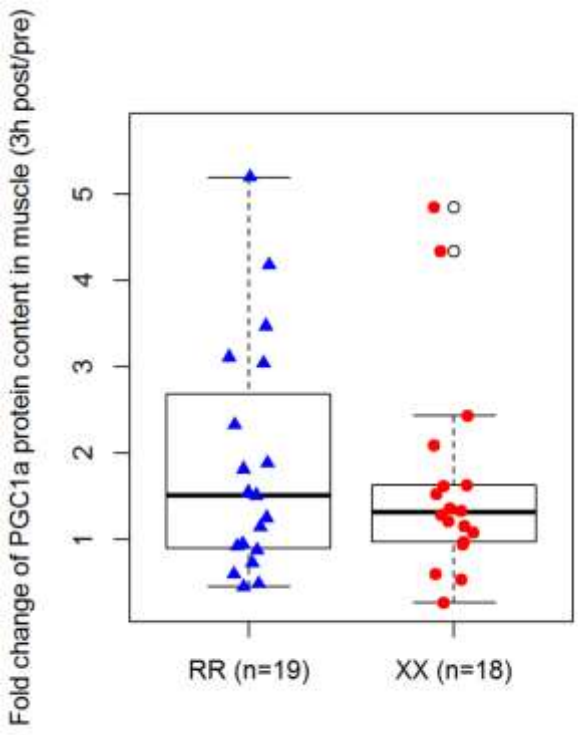


Figure 23 *Fold change from baseline to 3h post exercise for PGC 1-a protein content in the muscle of RR and XX partitipants (3h post exercise/pre exersice). Box blot displaying PGC1α-1 relative protein abundance in ACTN3 RR vs. XX participants. The centre rectangle includes the mean and the interquartile range (IQR). The "whiskers" above and below the box show the locations of the maximum and minimum (excluding outliners) – ≥0.15 x IQR above the third quartile or below the first quartile and indicated by O.*

8.5 *Discussion*

The high percentage of population who are deficient for the α-actinin-3 protein, has allowed me to employ a unique model to investigate the influence of *ACTN3* gene on baseline endurance performance and the molecular response to exercise. Following the initial screening, I have included 19 *ACTN3* RR and 18 *ACTN3* XX participants into the main study. In addition to genotyping in duplicate, I confirmed that the α-actinin-3 protein was present in *ACTN3* RR and not *ACTN3* XX participants. At baseline, absence of the α-actinin-

3 protein was accompanied by a compensatory increase in α-actinin-2 protein content in *ACTN3* XX vs *ACTN3* RR humans. There was however, no evidence for significant differences in physiological characteristics or endurance-related phenotypes between *ACTN3* XX and *ACTN3* RR participants. While there was a main effect of genotype (p=0.006) for RCAN1-4 protein content, there was no significant interaction effect for exercise-induced changes in any of the measured genes associated with mitochondrial biogenesis.

The influence of ACTN3 gene on physiological parameters at baseline

There were no significant differences in the lactate threshold, VO_{2peak}, W_{Peak}, cycling time-trial performance, CS activity, or maximal mitochondrial respiration (**Table 8**), between *ACTN3* XX and *ACTN3* RR humans. These variables were chosen as both VO_{2peak} and the lactate threshold have been reported to play key roles in determining endurance performance (Hawley et al., 2014). Mitochondrial respiration and CS activity were also chosen as they have been associated with endurance performance in humans (Granata et al., 2015). However, the results from chapter 8 indicate there is no influence of the *ACTN3* polymorphism on any of these key endurance phenotypes at baseline. While these results contrast with observations in actn3 KO mice (Seto et al., 2013), these findings are in line with the results from chapter 7 of this thesis, a large case-control study (Ahmetov et al., 2010), cross-sectional studies (Lucia et al., 2006, Paparini et al., 2007; Doring et al., 2010; Muniesa et al., 2010), and meta-analysis (Alfred et al., 2011; Ma et al., 2013; Garton et al., 2018) which also found no evidence that the *ACTN3* polymorphism influences endurance-related phenotypes.

The influence of ACTN3 gene on the protein expression of Calsarcin-2 and RCAN1-4

RCAN1-4 is a downstream target of calcineurin, which is increased upon activation by calcineurin and also acts in a negative feedback loop to further inhibit calcineurin activity (Yang et al., 2000; Rothermel et al., 2003). Consistent with the results in mice (Seto et al., 2013), there was a main effect of genotype for RCAN1-4 protein content (*ACTN3* XX > *ACTN3* RR; P=0.004) (**Figure 21b**). To assess whether the elevated RCAN 1-4 content in *ACTN3* XX humans could be attributed to differential expression of calsarcin-2 (which inhibits calcineurin activity) (Frey et al., 2008), the content of calsarcin-2 expression was also measured. Consistent with observations in mice (Seto et al., 2013), the expression of

calsarcin-2 did not differ between muscle samples obtained from *ACTN3* XX and *ACTN3* RR humans (**Figure 21a**) and therefore cannot account for the increased RCAN1-4 protein content associated with α-actinin-3 deficiency.

In mice, there was no significant difference for the levels of RCAN1-4 protein in KO vs WT mice (1.3 fold difference; P=0.057), but a 2.9-fold greater RCAN1-4 protein content was observed in KO muscle compared with WT muscles 3 days after endurance exercise (p = 0.004) (Seto et al., 2013).

In human muscle, despite the main effect of genotype for RCAN1-4 content, there was no significant interaction effect (p=0.12; **Figure 21b**). This contrasts with findings in mice (Seto et al., (2013), where a 2.9-fold greater increase in RCAN1-4 protein content was observed in exercised KO muscle compared with WT muscles (p = 0.004). Direct assays of calcineurin activity confirmed a 1.9-fold greater calcineurin activity in exercised KO compared with WT muscles, although this difference did not reach statistical significance (p=0.093). Consistent with observations in mice (Seto et al., 2013), in the present study the resting muscles of *ACTN3* 577XX humans had a 2.3 fold greater expression of RCAN1-4 when compared with ACTN3 577RR humans. The effect of ACTN3 genotype on exercise-induced changes in RCAN1-4 has not previously been investigated in humans.

Given that the resting muscle biopsies in this thesis were performed 48 to 72 h after prior endurance exercise (a 20-km TT), this thesis results could also be compared to the after-exercise results reported by Seto et al., (Seto et al., 2013). In this regard, it is also demonstrated a greater RCAN1-4 protein content in the muscle of *ACTN3* 577XX versus *ACTN3* 577RR humans two to three days after exercise (1.3 fold; P=0.004). Despite the main effect of genotype for RCAN1-4 content, there was no significant interaction effect (p=0.12; **Figure 21b**), indicating that neither exercise or genotype affected changes in RCAN1-4 protein content measured immediately post and 3 hours post exercise.

One factor that may have contributed to some of the differences observed between mice and humans is that murine muscle contains a significantly higher percentage of fast-twitch fibres compared to human muscle (Ho-Kim & Rogers, 1992), and the ACTN3 gene is exclusively expressed in fast-twitch skeletal muscles (Chan et al., 2008). The muscles analysed in the study by Seto et al. (Seto et al., 2013) contained less than 1% type I fibres, and therefore any effects of α-actinin-3 protein would have been enhanced in their mouse model. In line with this hypothesis, exhaustive exercise in rats increased RCAN1-4 protein levels in the gastrocnemius, but not the soleus (gastrocnemius contains higher percentage of fast-anaerobic twitch fibres compared to soleus) (Emrani et al., 2015). Recent studies with

single muscle fibres have also observed that the greater maximal unloading velocity in *ACTN3* RR compared to *ACTN3* XX genotypes likely contribute to enhanced whole-muscle performance during high velocity contractions (Broos et al., 2016). Future studies in single muscle fibres might have the potential to shed more light on the influence of *ACTN3* gene on human performance and cell signalling. It is also worth noting that only men were recruited in the present study, while the human muscle samples in the study by Seto et al., (2013) were only from females. It has previously been reported that the effect of *ACTN3* genotype on performance is more pronounced in female compared with male athletes (Yang et al., 2003). Furthermore, the human *ACTN3* XX participants in the study of Seto et al., (2013) that showed increased RCAN1-4 expression compared with the muscles of *ACTN3* RR ($p= 0.004$) were quite old (mean age 49 years, with a very large age range (33–77 years) and a small sample size (n=5), and RCAN1-4 content was only measured in resting muscle.

The influence of ACTN3 R577X polymorphism on mitochondrial-related gene expression in response to exercise

Based on research with mice reporting a significant effect of *ACTN3* genotype on training-induced changes in endurance performance, and exercise-induced changes in RCAN1-4 content, it was hypothesised that *ACTN3* genotype would affect the exercise-induced increase in nuclear genes encoding mitochondrial proteins (NUGEMPs). However, despite a significant exercise-induced increase of all the PGC-1α gene transcripts (**Figure 22**) and PGC-1α protein levels (**Figure 23**) in both genotypes ($p<0.05$), there was no significant main effect for genotype or an interaction effect. Similarly, there was no significant main effect for genotype or an interaction effect for the exercise-induced increase of other nuclear genes encoding mitochondrial proteins (**Table 9**). These finding are consistent with the absence of a significant difference for the exercise-induced elevation of the RCAN 1-4 gene content between *ACTN3* XX and *ACTN3* RR individuals in the present study (**Figure 21**).

Given the previous findings in mice, this thesis contrasting findings for the effect of the ACTN3 genotypes in exercise induced changes in RCAN 1-4 protein content and in changes in genes associated with mitochondrial biogenesis can possibly be attributed to HIIE protocol followed in this study. Future research should examine whether other forms of exercise, such as continuous exercise, have a different effect on this molecular pathway in humans. However, HIIE is found to be equally or even more effective in improving oxidative capacity (VO_{2max}) (Batacan et al., 2017) and promoting mitochondrial adaptations in muscle compared to continuous exercise (MacInnis et al., 2017). This is the main reason HIIE

exercise was chosen over continuous exercise as the ideal type of exercise to investigate the effects of ACTN3 genotype on mitochondrial in this thesis.

In conclusion, the results of the present study showed a compensatory increase in *α-actinin-2* protein content at baseline (p=0.018), with no evidence for a greater adaptive response to high-intensity interval exercise, for the α-actinin-3 human deficient muscle. There were also no significant differences in the physiological characteristics between *ACTN3* XX and *ACTN3* RR participants at baseline. This analysis is the first well powered study in humans to investigate the molecular pathways behind the potential influence of *ACTN3* R577X genotype on the response to a single session of HIIE endurance exercise. These results, combined with the absence of significant differences in baseline endurance characteristics, add to the growing body of literature suggesting *ACTN3* genotype does not have a major influence on endurance performance or the response to endurance training in humans. Despite the absence of significant differences, this study design can still be a useful methodological approach to investigate the role of the loss of function of some proteins on the response to exercise in humans.

9. THESIS OVERALL DISCUSSION

9.1 *Summary*

The three studies in this thesis examined the effects of α-actinin-3 deficiency (*ACTN3* XX genotype in humans) on muscle performance in elite athletes; and exercise-induced changes in the content of genes and proteins associated with mitochondrial biogenesis in non-athletes. In the first study (chapter 6) a quantitative experimental design was utilised in a relatively-large cohort of elite athletes, and the results demonstrated a significant effect for the *ACTN3* genotype on elite sprinting performance; *ACTN3* genotype accounted for 0.92% of the difference in sprint speed amongst elite male 200 m sprinters (Papadimitriou et al., 2016). The second study (chapter 7) with longer distance runners, found no association between the *ACTN3* genotype and endurance performance (Papadimitriou et al., 2018). In the third study (chapter 8), a human knockout model was utilised to investigate the influence of the α-actinin-3 protein on exercise-induced mitochondrial adaptations, and the results demonstrated that the absence of the α-actinin-3 protein was accompanied by a compensatory increase in α-actinin-2 protein content, compared to humans who express the protein (*ACTN3* RR genotype) (p=0.018). There was a main effect of genotype (p=0.006) for the protein content of RCAN1-4 (a marker of calcineurin activity), without an interaction effect. Notably, results from chapter 8 showed that, α-actinin-3 deficiency did not influence key endurance-related phenotypes, such as $VO_{2max,}$ the lactate threshold, or mitochondrial function which is in agreement with the results of chapter 7 of this thesis. These combined findings add to the growing body of literature suggesting the *ACTN3* R577X genotype affects elite sprint performance, but does not influence endurance performance in humans.

9.2 *How do the three studies add to the body of knowledge?*

Two different perspectives have been combined in this thesis to investigate the effect of *ACTN3* genotype on muscle function: a) a genetic-epidemiological (chapter 6 & 7) and a more functional physiological/mechanistic perspective (chapter 8). In the first two studies of this thesis I utilised the genetic-epidemiological approach in such a large-cohort of elite athletes (n=1044, 346 sprinters and 698 endurance athletes). Qualifying standards to compete at a national or international level are not always equal between countries, and an athlete's actual performance in major competitions is a strong indicator of athletic potential. Moran et al. (2007), who quantitatively analysed sprint and endurance performance in Greek adolescent boys (n=992), reported a significant association between the *ACTN3* genotype and sprint performance (the sprint time of RR or RX adolescent boys was significantly faster than their XX counterparts). In contrast, there was no genotype effect on endurance

performance, as assessed by the shuttle run test. Interestingly, in adolescent boys the sprint association was highly specific; no association was detected in other strength/power phenotypes (such as handgrip strength, basketball throw, and vertical jump height). In chapter 6 and 7 a further differentiation was made between *ACTN3* R577X genotype and running events that are estimated to have a distinct energy contribution. While in the 1500-m to Marathon events more than 75% of the energy to contract the muscle is aerobic; the vast majority of the energy used for muscle contraction during 100- to 400-m sprints is coming from the anaerobic system (alactic energy in the 100-m sprint, and increasingly more anaerobic glycolysis up to 400 m (Bret et al., 2013). Despite addressing these subtle performance differences, and involving a large number of elite runners with quantitative performance criteria, consistent with the results from Moran et al. (2007), the results of the second study in the chapter 7 of this thesis suggest that *ACTN3* R577X polymorphism is not likely to influence Caucasian endurance runners' personal best times at the high end of the performance spectrum. However, the analyses with elite sprinters showed that personal best times are significantly influenced by *ACTN3* genotypes in an event-specific manner; the effect of the *ACTN3* genotype on sprinting performance was more pronounced for 200-m sprint performance. These specific association differences may be related to subtle differences in the physiological demands for each event. Interestingly, the results from chapter 6 of this thesis show that all *ACTN3* XX genotypes not getting into the Olympics, but similarly 83% (59/71) of *ACTN3* RX and *ACTN3* RR athletes do not get into the Olympics as well. This would mean that testing for the R-allele (positive) versus XX genotype (negative) would have a maximal sensitivity (100%-no false negatives) but low specificity (12%) to predict qualifying times for the Olympics with low overall accuracy (25%).

A previous cross-sectional study estimated that *ACTN3* R577X accounts for 2.2% of the total variance for muscle strength at baseline (Clarkson, 2005), and the abovementioned study by Moran et al. (2007) suggested that *ACTN3* genotype can explain 2.6% of the total sprint time in non-athletic adolescents. *ACTN3* R577X is not a disease-causing variant, but appears to alter the properties of healthy muscles. This could explain why the effect of the *ACTN3* R577X genotype is more pronounced in elite athletes, who represent the extreme end of the muscle performance spectrum, compared to 'healthy individuals' from the general population who have significant variation in muscle properties and significantly less exposure time to exercise training.

A clear contribution to the body of knowledge arising from this thesis is that *ACTN3* R577X genotype is an important factor influencing sprint performance at the elite level (STUDY 1).

In agreement with this finding are all case:control studies (Table 1) and three meta-analyses (Alfred et al., 2011; Ma et al., 2013; Garton et al., 2018) conducted so far that showed that the *ACTN3* 577XX genotype is underrepresented power-oriented athletes (Yang et al., 2003; Niemi and Majamaa, 2005; Papadimitriou et al., 2008; Druzhevskaya et al., 2008; Eynon et al., 2009; Cięszczyk et al., 2011; Mikami et al., 2013), and collectively these findings support the notion that α-actinin-3 protein when expressed increases sprinting performance, making this one of the more consistently supported associations in the field.

However, there is no evidence to support an influence of *ACTN3* genotype on elite endurance performance (STUDY 2). In line with this, most case:control studies (Table 2) and three meta-analyses (Alfred et al., 2011; Ma et al., 2013; Garton et al., 2018) conducted so far showed that the ACTN3 577XX genotype is not over-represented in endurance athletes. Cross-sectional studies (Lucia et al., 2006) comparing 50 elite male endurance cyclists and 52 Olympic-level endurance runners with 123 sedentary male controls found no significant differences in genotype frequencies between endurance athletes and controls, or evidence for an association of *ACTN3* R577X polymorphism with VO_{2max}. Consistent with these findings, more recent cross-sectional studies also found similar results (Paparini et al., 2007, Doring et al., 2010, Muniesa et al., 2010). In line with these results, results from the third study in this thesis demonstrated no significant differences not only in VO_{2max} but also on other physiological parameters related with endurance performance such as lactate threshold, CS activity and maximal mitochondrial respiration (Table 4). In agreement with this finding are also most of case:control studies conducted so far. A negative association between the α-actinin-3 deficient *ACTN3* XX genotype and endurance performance has been observed in six independent case:control studies (Niemi and Majamaa, 2005; Saunders et al., 2007; Papadimitriou et al., 2008; Ahmetov et al., 2010; Cięszczyk et al., 2011; Mikami et al., 2013) and two meta-analysis (Alfred et al., 2011; Ma et al., 2013). Only some studies have reported that the *ACTN3* 577XX genotype is associated with enhanced endurance status in elite athletes (Eynon et al., 2009).

The result of STUDY 3 in this thesis also provides no evidence for a better response of key mitochondrial genes to high-intensity endurance exercise in α-actinin-3 deficient human muscle.[2]

[2] STUDY 2 and STUDY 3 were run simultaneously hence I could not predict that the outcomes of the second study will not inform the third mechanistic study. That said, several studies in mice from the Kathrine North group, reviewed in chapter 5.4.3 have shown that the metabolic phenotypes are strikingly different between the α-actinin 3 deficient mice and the wild type. For this reason the third study is a proof-of-concept in humans and can be treated as a separate study rather than a continuation of the second study of this thesis.

9.3 *Strengths and Limitations*

Two main, complementary approaches were used in this thesis. In the first approach elite athletic performance (running times) was used as an indicator of athletic performance, and the *ACTN3* genotype was evaluated as a potential performance predictor. The first two studies of this thesis involved elite athletes from similarly defined elite athlete groups, and the use of a wide variety of events allowed the differentiation of events estimated to have very different physiological demands. In the second approach (STUDY 3) non-athletes were selected based on their *ACTN3* genotype, and their adaptive responses to exercise were analysed in the laboratory. In the first approach, extreme and accurate athlete phenotypes were tested. STUDY 3 was complementary to STUDIES 1 and 2, and investigated the potential of non-athletes to adapt to a specific exercise protocol using rigorous baseline and post-exercise assessments in-vivo (VO_{2max}, LT, TT), in vitro (protein and gene expression), and in-situ (mitochondrial respiration). An additional strength of this thesis is the assessment of relatively large cohorts of athletes in a multi-centre, collaborative approach, and therefore increasing the sample size to better enable the detection of genotype: phenotype associations (STUDY 1 and 2); and in STUDY 3 a tightly-controlled cohort (i.e., similar nutrition, accounting for their physical activity levels at base line), with a distinct genotype (XX vs. RR), was investigated. Another overall important strength of this thesis is its design that is not limited to one perspective. The two different methodological perspectives that have been combined in this thesis (a genetic-epidemiological & a physiological-mechanistic perspective) offsetting the weaknesses inherent to using each approach by itself. The epidemiological approach in the first two studies included specific time cut-off thresholds 15% and 20% threshold for each study respectively. These thresholds reflected in SD reductions for both studies and were chosen based on the sample availability for each study and a similar athletic status[3] for the athletes of both studies.

This thesis had limitations as well. Both epidemiological large scale studies are not 'experimental' in nature – experiment: manipulation of independent variable, change in dependent variable. As with any genetic association studies, the *ACTN3* genotype is only one among many other variants that might influence performance and training adaptations, and therefore one should be cautious when interpreting the results. Although the *ACTN3* did appear to have a small influence on sprint performance, it is not recommended to use this or any other genetic variant in genetic testing for young athletes and/or the general population. In third study the bout of HIIE may not have been sufficient to induce the manifestation of the

[3] While the term "elite" has different interpretations, this term was used in this thesis based on the fact that the athletes included had already been labelled as elite in the previous case:control based on their athletic status (Table 1 & 2).

functional differences among the *ACTN3* R577X genotypes. A second limitation is that compared to findings in mice where timing of biopsy was after 3 days, the baseline biopsies in this thesis were taken 48-72hrs after the last pre-tests. If the argument of later rise in RCAN1-4 is followed it could be that RCAN1-4 was already elevated in some subjects, and that pre-test has influenced this thesis results. Furthermore, muscle biopsies, by nature, may result in damaged muscle tissue. Three muscle biopsies were performed in a very short period of time, which may have resulted in repetitive tissue damage possibly led to up/down regulations of tissue repair molecular pathways.

KO mice through generations of selective breeding have established genetically identical mice with the only difference in their genome the examined KO gene deficiency (Doyle et al., 2012) in this case the *ACTN3* gene. The “human knockout” naturally occurring model approach used in the third part of this thesis is limited by the large human genetic variability and this could be the reason for the inconsistent different results between the KO mice and human analyses.

Another limitation is that an elite athlete training is persistent for decades, highly specific to the nature of their event and short duration training sessions, may not be enough to induce the manifestation of the functional differences among the R577X genotypes. Response to exercise is a complex phenotype; it is highly dependent on the baseline state, as well as the type and quality of training administered. Disparities in ages, training status, and training centres make it difficult to control these variables. These make the performance of elite athletes a very accurate indicator of maximal athletic potential, and studies that involve performance of elite athletes are therefore very valuable in understanding the genotype: phenotype relationships. These combined findings add to the growing body of literature suggesting *ACTN3* R577X genotype does not have a major influence on endurance performance in humans.

9.4 *Molecular mechanisms*

Another intriguing area for research is the mechanistic basis for the effect of α-actinin-3 deficiency on muscle function. Studies in mice bred to completely lack the α-actinin-3 protein (i.e., *Actn3* knockout (KO) mice) suggest the loss of α-actinin-3 genotype influences the adaptive response to endurance exercise training (Seto et al., 2013). Compared with wild type (WT) mice, after 4 weeks of endurance training *Actn3* KO mice had greater endurance exercise performance and faster recovery from fatigue, which was associated with a shift in fast-twitch muscle fibre properties toward a more slow-twitch, oxidative phenotype (MacArthur et al., 2007; MacArthur et al., 2008; Seto et al., 2013).

Although much research has been published with *Actn3* KO mouse model, comparatively little is known about the specific role of α-actinin-3 in human skeletal muscle. The only study that has examined human muscle has demonstrated greater protein content of RCAN1-4 (a marker of calcineurin activity) in resting muscle samples obtained from *ACTN3* 577XX humans when compared with *ACTN3* 577RR humans (Seto et al., 2013), consistent with the observations in mice. However, the human participants in this study had a very large age range and a small sample size (n=5), and RCAN1-4 content was only measured in resting muscle. Results from chapter 8 of this thesis showed that absence of the α-actinin-3 protein accompanied by a compensatory increase in α-actinin-2 protein content in *ACTN3* XX vs *ACTN3* RR in humans (p=0.018), consistent with observations in mice. To assess whether the elevated RCAN 1-4 content in *ACTN3* XX humans could be attributed to differential expression of calsarcin-2 which inhibits calcineurin activity (Frey et al., 2008), the content of calsarcin-2 expression was also measured. Also, consistent with observations in mice (Seto et al., 2013), the expression of calsarcin-2 did not differ between muscle samples obtained from *ACTN3* XX and *ACTN3* RR humans (**Figure 21a**), and therefore cannot account for the altered calcineurin activity associated with α-actinin-3 deficiency. However, despite the main effect of genotype for RCAN1-4 protein, there was no significant interaction effect (p=0.12; **Figure 21b**). This contrasts with findings in mice (Seto et al., 2013), where a 2.9-fold increase in RCAN1-4 protein content was observed in exercised KO compared with WT muscles (p = 0.004). The molecular mechanisms for the altered calcineurin signalling with α-actinin-3 deficiency appear to be via differential binding of calsarcin-2 to sarcomeric α-actinins. When α-actinin-3 is absent (*ACTN3* 577XX genotype) there is a compensatory increase in α-actinin-2, which binds more tightly to calsarcin-2 (a negative regulator of calcineurin) (Rothermel et al., 2003). Thus, absence of α-actinin-3 protein and an increase in α-actinin-2 protein in *ACTN3* XX humans found in the third study of this thesis was expected to increase the release and activation of calcineurin (as measured by RCAN 1-4 protein expression), and lead to the activation of slow myogenic program (Seto et al., 2013). However, the absence of any significant interaction effect demonstrates that although all the expected prerequisites at the molecular level for the activation of the slow myogenic program were accomplished; namely:

1) absence α-actinin3 protein in *ACTN3* XX muscle samples,
2) increased compensatory effect of a-acinin2 protein *ACTN3* XX muscle samples
3) no difference in the expression of calsarcin-2 between *ACTN3* XX and *ACTN*3 RR muscle samples.

There was no evidence that there is activation of the slow myogenic program at baseline immediately and 3 h post exercise in *ACTN3* XX humans. However, in mice RCAN 1-4 protein levels were measured 3 days after exhaustive exercise, but here I measured the levels of this protein immediately and 3 h after exercise. Given that our resting muscle biopsies were performed 48 to 72 h after prior endurance exercise (a 20-km TT), this chapter results can be also compared to the after-exercise results reported by Seto et al., (2013). In this regard, It was also observed a greater RCAN1-4 protein content in the muscle of ACTN3 577XX versus ACTN3 577RR humans two to three days after exercise (1.3 fold; P=0.004). Despite the main effect of genotype for RCAN1-4 content, there was no significant interaction effect (p=0.12; **Figure 21b**), indicating that neither exercise or genotype affected changes in RCAN1-4 protein content measured immediately post and 3 hours post exercise. Further research is required to determine whether the non-significant increase in the expression of RCAN1-4 when compared with *ACTN3* 577RR humans in the present study (1.8 fold) might have reached significance after chronic (long-term) exercise training.

Based on previous reports of a significant effect of *ACTN3* genotype on training-induced changes in endurance performance, and exercise-induced changes in RCAN1-4 content, it was hypothesised that *ACTN3* genotype would affect the exercise-induced increase in nuclear genes encoding mitochondrial proteins (NUGEMPs). However, despite a significant, exercise-induced increase of all *PGC-1α* gene transcripts (**Figure 22**) and PGC-1α protein levels (**Figure 23**), as well in both genotypes ($p<0.05$), there was no significant main effect for genotype or an interaction effect (**Figure 22**). Similarly, there was no significant main effect for genotype or an interaction effect for the exercise-induced increase of other nuclear genes encoding mitochondrial proteins (Table 9). These findings are consistent with the absence of a significant difference for the exercise-induced elevation of the RCAN 1-4 gene content between *ACTN3* XX and *ACTN3* RR individuals (Table 9). These mechanistic results, combined with the absence of significant differences in elite athletes, and baseline endurance characteristics, again support the conclusion there is no detectable effect of *ACTN3* genotype on human endurance performance.

Our understanding of the genetic influences on human physical performance is evolving rapidly in the postgenomic era. This thesis has presented evidence that the *ACTN3* R577X polymorphism can influence athletic performance, and identified the specific type of human physical performance that is influenced by this genotype.

9.5 *Future directions*

Much more work remains to be done to answer a number of major questions in the field. Genome-wide approaches (Rankinen et al., 2016; Willems et al., 2017) and consortium-based effort (Pitsiladis et al., 2016) are required for the field to progress, and enhance our understanding of the genes that influence elite performance and the response to exercise.

Improving cohort numbers in current biobanks would pave the way for future next generation sequencing (whole-genome and exome) in large, well defined cohorts of elite athletes will be required to identify new targets associated with performance in both healthy and diseased populations. In regards to the *ACTN3* genotype assessed in this thesis, it is suggested to perform future human studies that could answer the following questions:

1. how does α-actinin-3 deficiency affect the function of skeletal muscle following different endurance exercise protocols ?

2. how does α-actinin-3 deficiency affect the function of skeletal muscle following sprint exercise protocols?

3. can we use the *ACTN3* R577X as an example to discover other gene variants that influence athletic performance?

4. how does the ACTN3 R577X genotype affect the function of skeletal muscle following strength exercise protocols in both healthy and diseased populations?

5. from a population health perspective, what role does ACTN3 gene play in influencing human health, fitness, disease progression?

6. what are the potential applications for “gene doping” to improve performance?

Shifting our research focus from the *ACTN3* gene and the response to endurance exercise, to specific molecular pathways and mechanisms behind the response to strength based types of exercise, could yet yield more interesting and promising insights into the functional mechanisms behind this and other gene variants and muscle mass. Cross-sectional studies and mechanistic investigations into the response to training are currently limited (Yan et al., 2017) and more work remains to be done to identify the molecular pathways and behind the influence of the *ACTN3* gene in response to both endurance and

strength oriented training stimuli. Overall, exercise adaptation is a complex trait with many undiscovered genes likely to influence exercise-related phenotypes. Therefore, future studies assessing the genome-wide genetic contribution to performance are still needed to discover more genetic variants contributing to performance. Understanding both genetic/environmental contributions, and how they differ between individuals, could be beneficial in understanding elite performance, adaptation to training, and muscle function in both healthy and diseased populations (Yan et al., 2016).

Overall, adaptation to exercise and training is a complex trait with many undiscovered genes likely to influence exercise-related phenotypes. Therefore, future studies assessing the genome-wide contribution to performance are still needed. Understanding both genetic/environmental contributions, and how they differ between individuals, could be beneficial in assessing performance, adaptation to training, and muscle function in both healthy and diseased populations.

9.6 *Ethics*

Ethical approval was obtained from Victoria University Ethics Committee
Ethics Application ID: HRE13-223
Application Title : The effect of alpha-actinin-3 deficiency on muscle metabolism and adaptation to exercise training in humans.
Investigator: PROF DAVID BISHOP, NIR EYNON, XU YAN, IOANNIS PAPADIMITRIOU

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