Is evolutionary loss our gain? The role of *ACTN3* p.Arg577Ter (R577X) genotype in athletic performance, ageing and disease

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Abstract

A common null polymorphism in the *ACTN3* gene (rs1815739:C>T) results in replacement of an arginine (R) with a premature stop codon (X) at amino acid 577 in the fast muscle protein α-actinin-3. The *ACTN3* p.Arg577Ter allele (aka p.R577* or R577X) has undergone positive selection, with an increase in the X allele frequency as modern humans migrated out of Africa into the colder, less species-rich Eurasian climates suggesting that the absence of α-actinin-3 may be beneficial in these conditions. Approximately 1.5 billion people worldwide are completely deficient in α-actinin-3. While the absence of α-actinin-3 influences skeletal muscle function and metabolism this does not result in overt muscle disease. α-Actinin-3 deficiency (*ACTN3* XX genotype) is constantly underrepresented in sprint/power performance athletes. However, recent findings from our group and others suggest that the *ACTN3* R577X genotype plays a role beyond athletic performance with effects observed in ageing, bone health and inherited muscle disorders such as McArdle disease and Duchenne muscle dystrophy. In this review, we provide an update on the current knowledge regarding the influence of *ACTN3* R577X on skeletal muscle function and its potential biological and clinical implications. We also outline future research directions to explore the role of α-actinin-3 in healthy and diseased populations.

Key Words: α-actinin-3, Genetic variant, ACTN3, athletic performance, muscle disease, ageing

1. Introduction

Muscle performance is a complex trait influenced by a myriad of environmental and inherited factors. While exercising, skeletal muscle is challenged to fulfil the mechanical and metabolic demands of contraction, with subsequent increases in its energy expenditure (Hawley, et al., 2014). Environmental effects such as nutrition, physical activity, and ethnicity all influence performance (Pitsiladis, et al., 2016). Genetic differences also influence skeletal muscle's ability to produce and utilise energy during exercise (Yan, et al., 2016) and as such, they might affect training adaptations and athletic performance.

Early twin and family studies suggest that genetics accounts for between 30–80% of the variance in human athletic performance (Bouchard and Rankinen, 2001; Costa, et al., 2012; De Moor, et al., 2007; Guth and Roth, 2013). The completion of the human genome project in 2003 and development of next generation sequencing technologies has facilitated the identification of genetic variants associated with exercise performance. Current research focuses on identification of specific variants influencing the response to training as well as the molecular pathways and mechanisms involved.

The *ACTN3* common null variant (rs1815739:C>T), resulting in the amino acid substitution p.Arg577Ter (p.R577*, commonly referred to in the literature as R577X), was discovered in 1996 (North and Beggs, 1996). R577X has shown relatively consistent association with muscle performance (Eynon, et al., 2013). Research into the functional consequences of α-actinin-3 deficiency has been the focus of our team for the last 2 decades and through studies with both human subjects and an engineered *Actn3* knockout (KO) mouse model we have begun to unlocked many of the mechanistic aspects of α-actinin-3 deficiency (*ACTN3* XX genotype in humans) in healthy and diseased populations (Lee, et al., 2016).

In the current review, we will address the recent reports on the *Actn3* KO mouse and the naturally occurring α-actinin-3 deficient (*ACTN3* 577XX) "human knockouts" to understand the biological influence of this polymorphism on muscle function of elite athletes, healthy individuals, as well as in aged and diseased populations. We will provide an update on the biological mechanisms behind the multiple roles of α-actinin-3 in skeletal muscle and define the effects of α-actinin-3 deficiency from three different research points of view. Firstly, the current human athlete association studies, secondly the *Actn3* KO mouse and finally the implications in ageing and disease. We will compare and discuss the results from these different research methodologies and provide recommendation for future studies based on the exciting findings currently ongoing in this field of research.

2. What is α-actinin-3?

In mammals, the α-actinins are a family of four actin-binding proteins (α-actinin 1 to 4) that evolved from repeated gene duplication events to perform similar roles in different cell types and tissues (Table 1). α-Actinin-2 and -3 (encoded by *ACTN2* and *ACTN3* genes, respectively) are highly conserved (81% identical and 91% similar) and make up the skeletal muscle specific α-actinins (Beggs, et al., 1992). Both α–actinin-2 and -3 are major components of the Z-line within the skeletal muscle contractile apparatus (sarcomeres) and

share a common domain topology with a N-terminal actin-binding domain, a rod domain with four spectrin repeats and a C-terminal region containing calcium-binding EF hand motifs (Figure 1) (MacArthur and North, 2004). α-Actinin-2 is expressed in all human skeletal muscle fibres, whereas α-actinin-3 is only present in fast, glycolytic (Type II) muscle fibres (North and Beggs, 1996). The sarcomeric α-actinins cross-link and stabilise actin thin filaments at the Z-line during muscle contractions as well as interact with a vast network of structural, signalling and metabolic proteins (as reviewed in (Lee, et al., 2016)).

2.1 The *ACTN3* R577X polymorphism

A common null polymorphism in the *ACTN3* gene, R577X, was serendipitously discovered while searching for candidates that caused an inherited muscular dystrophy (North, 2008). Interestingly, the frequency of the *ACTN3* X allele ranges from as low as 10% in Africa, 45% in European, 50% in Asian and more than 70% in the Americas (Amorim, et al., 2015; MacArthur, et al., 2007). Individuals who are homozygous for the X allele (577XX) are completely deficient in α-actinin-3, but this does not cause disease. Homozygosity for the null polymorphism is common. We estimate that 18% of humans, or approximately 1.5 billion people worldwide, are deficient in α-actinin-3 (North, et al., 1999; Yang, et al., 2003). The widespread absence of α-actinin-3 was an unexpected finding and suggests its role in skeletal muscle may be functionally redundant. However, the specialised expression pattern in type II muscle fibres, varied allele frequencies across different populations and DNA sequence differences between *ACTN2* and *ACTN3* support an independent role for α-actinin-3 that cannot be compensated for by the closely related α-actinin-2 protein.

2.2 *ACTN3* 577X allele has undergone strong, recent positive selection

To understand the varied X allele frequency distributions observed between different human populations, we examined *ACTN3* 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 (Project, 2003). 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 (MacArthur, et al., 2007). We and others have suggested that the 577X allele might provide an advantage to modern humans adapting to the Eurasian environment (MacArthur, et al., 2007). This has been supported by recent correlations that link increasing *ACTN3* 577XX frequency with

higher global latitude gradient and reduced species richness (Amorim, et al., 2015; Friedlander, et al., 2013). These findings suggests that environmental variables related to temperature (cold tolerance), and diet (feast/famine) influence the *ACTN3* R577X genotype frequencies currently observed worldwide. Therefore, the impact of this common null polymorphism has global relevance via its potential contribution to early human survival and altered muscle function.

3. *ACTN3* R577X and human muscle performance: the 'speed gene'

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

3.1 Case control studies

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

Eighteen case:control studies comprising sprint/power athletes have been conducted to date (Table 2a). These studies show a higher frequency of the *ACTN3* 577RR genotype (and a 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 (Cieszczyk, et al., 2011) have been reported. A subsequent 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., 2016b; 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 (Kikuchi, et al., 2016; Mikami, et al., 2014), Korea (Hong, 2013; Kim, et al.,

2014) and China (Yang, et al., 2017) have also confirmed the lower *ACTN3* XX frequency in elite sprinters compared to population controls (Table 2a).

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 other genetic or environmental factors are more influential (Yang, et al., 2003). It should also be noted that based on the very low frequency of the *ACTN3* XX genotype in African populations, *ACTN3* R577X genotype may not be informative to elite performance in this particular population. 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.

An inverse association has been reported in endurance type events, with a higher 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 (Ahmetov, et al., 2010; Cieszczyk, et al., 2011; Magi, et al., 2016; Niemi and Majamaa, 2005; Papadimitriou, et al., 2008; Saunders, et al., 2007) have not shown an association between the *ACTN3* R577X genotypes and endurance performance (Table 3a).

The lack of reproducibility in these findings suggests that the effects of α-actinin-3 deficiency in elite endurance athletes may be small, if any. In addition, these studies 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 low sample size (typically <100 athletes), and limited accountability of performance, training and environment. To overcome these limitations, athletes are grouped together across heterogeneous sport disciplines and events (e.g., sprinters, jumpers, throwers, swimmers, and power/sprint team sport athletes). While this increases sample size and provides a larger population for analysis, a lack of specificity in performance and events also increases inter-cohort variability. In addition, there is often a lack of quantitative measure of performance (e.g., event times or speed). Given that the inherent number of world-class elite athletes available is low, this approach is understandable, but the results are often statistically underpowered, highly variable and may not represent the effects on a population level.

We have recently used running time as a performance measure in sprint/power and endurance athletes, which we consider a more objective, accurate and reliable measure of performance. Using a large cohort (n = 346; 555 best personal times) of elite Caucasian athletes with reported running times we identified that *ACTN3* 577RR individuals had significantly faster running times over 200m, compared to XX and that the *ACTN3* genotype accounted for 0.92% of the difference in sprint speed (Papadimitriou, et al., 2016a). However, when the same quantitative approach was used for long distance runners, we found no association between the *ACTN3* XX genotype and endurance performance (Papadimitriou, et al., 2018), a finding consistent with the majority of case:control studies (Table 2a, and b). Shifting our focus from athletic event classification as a measure of performance to reported running times has provided an improved metric to assess the effect of α-actinin-3 on performance in both sprint/power and endurance athletes.

3.2 Cross-sectional studies

The effect of *ACTN3* R577X in the general population has been examined in cross-sectional studies based on quantitative performance measures for *ACTN3* R577X (summarised in Table 3a, and b). 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 with 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). Interestingly this is consistent with quantitative results from elite sprint/power and endurance athletes mentioned in the previous section.

Another power-related exercise test that has been used to assess muscle performance is the anaerobic all-out 30 second Wingate cycling test. Norman et al., (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 across a range of contraction speeds has been measured in five studies. Although no genotype differences were reported in the range of 0–240°/sec (Gentil, et al., 2011; McCauley, et al., 2009; Norman, et al., 2009; Vincent, et al., 2007), strength at 300°/s (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.

Cross-sectional studies that investigate the response to strength and endurance exercise are limited. Clarkson et al., (Clarkson, et al., 2005) found that α-actinin-3-deficient females *(ACTN3 XX)* showed a significantly greater response to strength training for the one-repetition maximum (1-RM) measurement. On the other hand, Delmonico et al., (Delmonico, et al., 2007) found that *ACTN3* XX individuals had lower gains in muscle thickness, with no genotype associations in 1RM strength or muscle volume. Of note, the group with lower initial values showed some improvement in both studies (Clarkson, et al., 2005; Delmonico, et al., 2007).

The studies to date investigating endurance performance at baseline did not show a significant association between *ACTN3* XX genotype and endurance performance (Doring, et al., 2010; Lucia, et al., 2007; Muniesa, et al., 2010; Paparini, et al., 2007). However, it remains a possibility that an effect of *ACTN3* genotype on endurance performance is present but masked by differences in environment, training and genetic background/ethnicity. 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 and the clear associations with *ACTN3* genotype and elite sprint/power athletes, it would be interesting to examine if a relatively lower volume-training program focused on either power or endurance performance may induce a different training response based on *ACTN3* genotype in the general population. While this training approach may not be sufficient to induce a functional difference among the *ACTN3* R577X genotypes, these studies may help decipher the effects of α-actinin-3 in endurance type events.

Overall, the associations seen in cross-sectional studies are weaker than the observed case: control analyses reviewed in the previous section, suggesting that the effects of α-actinin-3 deficiency are less evident in the general population compared to the elite athlete. This observation is consistent with an effect of *ACTN3* genotype at the extremes of muscle performance. Given that *ACTN3* R577X influences muscle strength in elite athletes, case:control studies have also been performed in vulnerable populations to determine if *ACTN3* genotype would also modify muscle function at the other extreme of muscle performance, eg. ageing and in muscle disease. The studies to date relating to this topic will be addressed in greater detail below.

3.3 Mechanistic insights from the mouse model of α-actinin-3 deficiency

In order to explore the effects of α-actinin-3 deficiency in humans, we developed an α-actinin-3 KO mouse model. *Actn3* KO mice replicate many of the phenotypes described in human α-actinin-3 deficiency, including reduced muscle grip strength (Chan, et al., 2008; MacArthur and North, 2007). Unlike humans, mouse α-actinin-2 is not expressed in all muscle fibres (Mills, et al., 2001); however, in *Actn3* KO mice α-actinin-2 is up regulated and ubiquitously expressed in all fibre types, similar to the pattern of expression seen in human *ACTN3* XX muscle. Consequently, α-actinin-2 is the only sarcomeric α-actinin expressed in α-actinin-3 deficient muscle. In *Actn3* KO mouse muscle, the predominant phenotypes include a reduction in fast glycolytic IIb muscle fibre size and a shift in the anaerobic metabolic profile of these fibres towards a slow-twitch aerobic metabolic phenotype, with increased glycogen storage and mitochondrial oxidative enzyme activity (Macarthur, et al., 2008; MacArthur, et al., 2007; Quinlan, et al., 2010). It is thought that this shift in muscle size and metabolic function results in the enhanced endurance running performance, 'slower' fast-twitch muscle fibre characteristics and enhanced recovery from fatigue [7] (Macarthur, et al., 2008; Seto, et al., 2013). A strength of the *Actn3* KO mouse model is that we are able to explore the molecular mechanisms associated with the absence of α-actinin-3.

The α-actinins are known to interact with an array of structural (cytoskeletal and sarcomeric), signalling and metabolic proteins, providing an important platform for protein interactions at the Z-line of fast twitch (Type II) skeletal muscle. Much of the complexities associated with the shift in muscle function and changes in fast-twitch muscle fibre characteristics due to α-actinin-3 deficiency remains to be understood, but to date, changes in structural, metabolic, signalling and calcium handling processes haven been shown to be altered. We will now highlight the current research that explores the molecular effects associated with changes in these four pathways.

The α-actinins are key ***structural*** proteins that cross-link actin (Kuhlman, et al., 1994) and interact with a range of Z-line proteins such as myotilin (Salmikangas, et al., 1999), titin (Ohtsuka, et al., 1997), nebulin (Nave, et al., 1990), dystrophin (Hance, et al., 1999) and β-integrin (Otey, et al., 1990) as highlighted in a recent review in Lee et al., (Lee, et al., 2016). Many of these proteins have been linked to muscle disease (which will be discussed further below), but alterations to the structural protein expression and localisation may provide a

mechanistic link to explain some of the altered contractile properties seen in the absence of α-actinin-3. We have shown that many of these Z-line proteins are upregulated in *Actn3* KO muscles, which includes the Z-band alternatively spliced PDZ motif containing protein (ZASP), myotilin, desmin and γ-filamin. Accumulations of myotilin and desmin in aggregates were also characterised in a subset of *Actn3* KO muscles. These accumulations and increased Z-line protein expression are hallmarks of remodelling in skeletal muscle, suggesting that α-actinin-3 deficient muscles are actively remodelling and may be more susceptible to damage, which has been explored in our *Actn3* KO mice, that show an increased susceptibility to damage following stretch induced muscle damage (Seto, et al., 2011). Similar studies have examined the effects of *ACTN3* R577X in athletes (Myosotis, et al., 2017) and the general population. However, further research is currently ongoing to assess the role of α-actinin-3 in muscle repair and remodelling. While this presents an interesting avenue of inquiry in athletes, the ageing population and those affected by muscle disease, who may be susceptible to increased muscle injury, the precise mechanisms are still under examination.

The shift in contractile properties led us to examine the *metabolic* properties of α-actinin-3 deficient skeletal muscle. Typically, Type II fibres rely on the production of ATP for energy through the anaerobic metabolic pathway, where glucose is converted to lactate via the enzyme lactate dehydrogenase (LDH), while Type I fibres preferentially oxidise pyruvate from glucose through the citric acid cycle, mitochondrial electron transport chain and fatty acid oxidation. Interestingly, *Actn3* KO muscles (which are predominantly Type II fibre rich) show a reduction in LDH activity, while mitochondrial pathways associated are consistently upregulated. This occurs in the absence of change in fibre type proportions or mitochondrial DNA copy, suggesting that the increase in oxidative metabolic enzymes is a direct consequence of α-actinin-3 deficiency in fast, Type II fibres (Macarthur, et al., 2008; MacArthur, et al., 2007).

Fast muscle fibres rely on a ready source of glycogen to supply the energy needed for rapid and forceful muscle contraction. An accumulation of glycogen is a key feature in both α-actinin-3-deficient mice and human muscles. In *Actn3* KO mice, this is due to a reduction in the activity of the muscle isoform of glycogen phosphorylase (Pygm, which catalyses the breakdown of glycogen into glucose-1-phosphate), however this could not been confirmed in human muscle samples (Quinlan, et al., 2010). Lower Pygm activity limits the ability of muscle to catabolise glycogen, leading to its accumulation. Increased glycogen accumulation

may be the mechanistic linchpin for the compensatory shift towards a slower more oxidative metabolism seen in KO mice. It is likely that the combination of changes in structural, signalling and calcium handling proteins. This includes a compensatory increase in α-actinin-2 (a key binding partner to Pygm) which drives many of the complex phenotypes seen in α-actinin-3 deficient muscle.

The changes in structural and metabolic properties in α-actinin-3 deficient muscle, in the absence of a shift in Myosin heavy chain (MyHC) fibre type profile, suggests that differences in key *signalling* pathways are responsible for determining fibre properties in the absence of α-actinin-3. Intriguingly, a calsarcin-2 KO mouse model exhibits a similar phenotype to the *Actn3* KO mouse including greater endurance capacity, and a shift in fast fibre metabolism towards a slower phenotype (Frey, et al., 2008). Calsarcin-2 is specifically expressed in fast-twitch muscle fibres. Its activation inhibits calcineurin, which is a calcium/calmodulin-dependent serine, threonine phosphatase that mediates the transcription of slow, oxidative muscle fiber types. The phenotype observed in calsarcin-2 KO mouse is due to higher activation of calcineurin signalling and a shift in the skeletal muscle profile towards a slower muscle phenotype, similar to that described in the *Actn3* KO mouse (Frey, et al., 2008). We have since shown that calcineurin activity is higher in both mice and humans deficient in α-actinin-3, due to the upregulation of α-actinin-2 and its preferential binding to calsarcin-2 (Seto, et al., 2013). The absence of α-actinin-3 and higher calcineurin activity has now been shown to influence exercise training (Seto, et al., 2013) and response to muscle atrophy (Garton, et al., 2014) and disease (Hogarth, et al., 2017) in *Actn3* KO mice and highlights a key mechanism for altered muscle function and adaptation to physical demands in α-actinin-3 deficient muscle. Overall, a higher calcineurin activity in *Actn3* KO muscle is thought to result in a lower threshold for adaptation to a slower muscle profile (seen in endurance training and immobilisation experiments) and a higher threshold for adaptation to faster muscle properties (seen in denervation experiments), compared to wild-type mice (Garton, et al., 2014; Seto, et al., 2013).

The changes in contractile, metabolic and calcineurin signalling properties in α-actinin-3 deficient muscles led to a further examination of the calcium handling properties of the sarcoplasmic reticulum (SR) in the *Actn3* KO fibres. In both *in vitro* muscle cultures and *ex vivo* muscle fibres, there was a clear increase in the rate of calcium release and absorption by the SR in *Actn3* KO, with KO muscle fibres being more resistant to fatigue due to the slower rate of decline in calcium release following repeated muscle stimulation (Head, et al.,

2015). The increased rate of calcium release can be explained by the increase in levels of the sarcoplasmic reticulum calcium ATPase1 (SERCA1), while increases in the calcium-binding proteins calsequestrin and sarcalumenin, along with SERCA1, facilitate higher calcium uptake by keeping intraluminal free calcium concentrations at low levels.

The increased calcium leak and uptake results in a higher proportion of energy expended, which results in the generation of metabolic heat in the muscle. This change in energy expenditure is thought to be a key contributor to the shift in Type II muscle fibre metabolism, driving the KO muscles towards a more efficient aerobic pathway and generating heat as a by-product of this reaction. We propose that this is a key part of the mechanism for the increase in 577 X allele frequency as modern humans migrated into the colder Eurasian climates, by improving cold acclimatisation in α-actinin-3 deficient humans, which would provide an evolutionary advantage during early human migration (Head, et al., 2015).

4. Is the evolutionary loss of *ACTN3* our gain? The role of α-actinin-3 in ageing, and muscle disease

4.1 *ACTN3* R577X influence the ageing process

While the absence of α-actinin-3 does not cause disease, there is increasing evidence to show that *ACTN3* genotype is associated with morbidity in people who are frail. Centenarians (i.e., people over 100+ years of age) are a model for successful ageing as they have usually postponed (if not avoided) the development of major chronic diseases and their lifespan is 10–15 years longer than that of the average age. Caucasian (Spanish) centenarians have an unusually high frequency of the null *ACTN3* XX genotype compared to the control population (XX: 23.7% *vs* XX:15.9%, P=0.011), suggesting that α-actinin-3 deficiency may provide a certain survival advantage with age (Fiuza-Luces, et al., 2011). Deschamps et al., reported that young healthy individuals (both males and females; age=23 ± 4.2 years) with the *ACTN3* RR genotype exhibited higher resting systolic and diastolic blood pressure compared to individuals with the XX genotype. This implies that centenarians with the XX genotype may be less predisposed (i.e., more protected) to chronic diseases associated with high blood pressure, such as hypertension (Deschamps, et al., 2015). Several studies have now examined the impact of *ACTN3* R577X on skeletal muscle traits and function in the ageing population, as reviewed recently in Pickering et al., (Pickering and Kiely, 2018). With some exceptions, the consensus from human and mouse longevity studies is that the effect of *ACTN3* genotype on physical performance measures is life-long, with α-actinin-3 deficiency

associated with lower muscle mass and strength and higher sarcopenia risk. However, the greater proportion of α-actinin-3 deficient Centenarians suggests that there is more to this effect than just altered muscle mass and strength. The effect of *ACTN3* genotype on resistance training response in the elderly is being scrutinised as a way to identify at-risk groups and develop personalise strategies for sarcopenia prevention and treatment with exercise. It may be that the improved response to endurance exercise provides a protective effect against age-related frailty in these populations, as we now know that the absence of α-actinin-3 influences how skeletal muscle adapts to exercise (Seto, et al., 2013).

In addition, we found that *Actn3* KO mice exhibit lower levels of muscle and fast IIb fibre atrophy in response to denervation and immobilisation, suggesting that α-actinin-3 deficiency also protects against muscle wasting (Garton, et al., 2014). KO muscles show a reduced threshold for a fast-to-slow fibre cross section area (CSA) shift (with immobilisation) and an increase in the threshold for a slow-to-fast CSA shift (with denervation) compared to WT, along with shifts in muscle metabolism and calcineurin signalling consistent with maintenance of a "slower" muscle phenotype following both stimuli. Taken together, our mouse studies identified a counter point in muscle adaptation to competing energy demands and atrophy stimuli which suggests that the absence of α-actinin-3 results in improved muscle ageing and reduced muscle wasting response. Further work is required to define the role of α-actinin-3 in muscle protein synthesis and degradation pathways to determine how these alterations in metabolic demand and adaptation may influence sarcopenia and the ageing response in *ACTN3* XX humans.

4.2 *ACTN3* R577X is a disease-modifier of inherited muscle disorders

Mutations in genes known to interact with the sarcomeric α-actinins have been implicated in the cause of inherited muscle disorders (such as nemaline myopathy, intranuclear rod myopathy, McArdle disease and Duchenne muscular dystrophy) as well as dilated cardiomyopathy, as reviewed in Houweling and North (Houweling and North, 2009). Dominant missense mutations in many of these proteins are also up-regulated in *Actn3* KO muscle, such as myotilin, desmin, αβ-crystallin, γ-filamin, and ZASP (Seto, et al., 2011) which are also associated with inherited forms of myofibrillar myopathy. These disorders are characterised by the breakdown of the Z-line leading to accumulation of degradation products and destabilisation of the sarcomere. There is often substantial variation in the onset and severity of muscle weakness and disease progression in many of these inherited muscle

disorders, which is thought to be the result of modifier variants independent of the pathogenic mutation. Since *ACTN3* R577X is common, influences muscle function in elite athletes and the general population and interacts with a wide range of key structural and metabolic proteins – we hypothesised that *ACTN3* genotype may be a disease modifier of various inherited muscle disorders.

We and others have begun to examine the role of *ACTN3* R577X in the progression and development of various muscle disorders. The *ACTN3* X allele has now been linked with increased likelihood of developing inflammatory myopathies (Sandoval-Garcia, et al., 2012) and earlier onset of Pompe disease (De Filippi, et al., 2014). *ACTN3* genotype has also been correlated with survival in patients with congestive heart failure, where patients with the X allele have 1.72 times higher mortality than their peers with *ACTN3* 577RR genotype (P=0.01) (Bernardez-Pereira, et al., 2014). These observations suggest that *ACTN3* genotype may play a role in modifying disease severity and age of onset for a number of clinical conditions.

4.2.2 *ACTN3* genotype and McArdle disease

McArdle disease (glycogen storage disease type V) is a metabolic disorder characterised by an accumulation of glycogen in skeletal muscle of affect individuals due to deficiency of PYGM. Phenotypic variability is high and includes exercise intolerance (in the form of early fatigue, myalgia and contractures) of varying severity, often leading to rhabdomyolysis (Lucia, et al., 2007). The *ACTN3* 577X allele has been shown to provide a protective effect in women affected by McArdle disease, with female α-actinin-3 deficient patients showing a higher exercise capacity compared with their RX or RR counterparts. While this provides support for a modifying role of *ACTN3,* it is not yet clear whether the effects are modified by the altered metabolic, structural or physiological properties of α-actinin-3 deficiency. It is likely that the improved metabolic efficiency of α-actinin-3 deficiency modifies disease progression but the precise mechanism for this improvement remains unclear and will be an interesting area of future analysis.

4.2.3 *ACTN3* genotype and Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is a X-linked inherited muscle disease caused by mutations in the dystrophin gene, characterised by progressive muscle degeneration resulting

in increasing muscle weakness, which ultimately culminates in respiratory and cardiac failure. Patients are typically diagnosed at an early age, become wheel-chair bound and succumb to the disease in their late 20's to 30's. The current therapy for DMD is chronic corticosteroid (prednisone or deflazacort) (Moxley, et al., 2005) that slows muscle weakness and disease progression. There is considerable inter-patient variability in DMD onset and progression (Desguerre, et al., 2009), and this is thought to arise from the presence of modifier genes that are independent from the causative mutations.

In collaboration with the Cooperative International Neuromuscular Research Group consortium, we examined 61 young, ambulant patients with DMD to determine if *ACTN3* R577X modifies their clinical measures (Hogarth, et al., 2017). Consistent with our hypothesis, patients who were α-actinin-3 deficient showed reduced muscle strength and took longer to walk 10 m. Utilising a double knockout (DKO) mouse model that was deficient in both dystrophin (*mdx*) and α-actinin-3 we were able to explore the mechanisms responsible for this change in muscle strength and performance. Similar to *ACTN3* 577XX patients, *Actn3/mdx* DKO mice showed reduced muscle strength. However, 12-month-old DKO mice, which represent a more progressive disease model, showed a reduction in eccentric induced muscle damage, suggesting that α-actinin-3 deficiency protects the dystrophic muscle from contraction-induced muscle damage and may slow disease progression. Moreover, the muscle fibres from these 12-month-old *Actn3/mdx* DKO mice exhibited a reduction in fibre branching complexity. Fibre branching is a well-documented phenomenon in regenerating skeletal muscle and a hallmark of damage in DMD. Mechanistically *Actn3/*mdx DKO muscles showed no differences in utrophin (a dystrophin homologue that when upregulated can compensate for the absence of dystrophin), compared to *mdx*. However, DKO muscles show increased calcineurin activity and oxidative muscle metabolism, which suggests that the "slower" muscle characteristics associated with α-actinin-3 deficiency may provide a protective effect in dystrophic muscle.

The findings from our muscular dystrophy studies have implications for clinical management of DMD patients. Stratification of clinical trials by *ACTN3* genotype may reduce the variation often seen in small cohorts by decreasing the variability often seen in key outcome measures (muscle strength and 10-meter walk times) for DMD clinical trials. It is also possible that accounting for *ACTN3* genotype may inform clinical progression and aid in the development of personalised treatment plans for patients with DMD in the future.

5. Perspectives and recommendations for future research

5.1 Advancing genetic association studies - identify novel markers of performance and applying this to health and disease

Candidate gene studies have dominated the sports and performance research area in the recent past and *ACTN3* is one of only an estimated 200 different genetic variants reported to influence athletic performance. However, genome wide association studies (GWAS) and next generation sequencing (whole-genome and exome) in large, well defined cohorts of elite athletes will be required to both validate existing association studies and identify new targets associated with performance (endurance and sprint/power) and training.

It is important to emphasise that observed associations between genetic variants and a defined phenotype do not necessarily equate causation. Following the discovery and replication phase, it is critical that mechanistic and functional studies are performed to establish how these variants act at the molecular level (Eynon, et al., 2017). Studies on the *ACTN3* R577X in both mouse and human models, as we have outlined in this review, have undergone all of the above-mentioned stages, and illustrate empirically how a common genetic variant results in the observed phenotype.

Many questions remain that need to be addressed in future studies including:

1. How does the *ACTN3* R577X genotype affect the function of skeletal muscle following different endurance or strength training protocols in healthy and diseased populations?

2. Does *ACTN3* R577X genotype influence skeletal muscle response to damage and repair following injury?

3. What role does *ACTN3* R577X genotype play in human health and fitness in chronic diseases like obesity, cardiovascular disease and Type 2 diabetes mellitus?

Cross-sectional studies and mechanistic investigations into the response to these questions are currently limited. The recently established Gene SMART (Skeletal Muscle Response to Training) study, which is part of the international Athlome consortium (Pitsiladis, et al.,

2016) is an example of tightly controlled exercise training study collecting a variety of physical phenotypes as well as muscle biopsies and blood from healthy moderately trained individuals (Yan, et al., 2017). These studies have the potential to elucidate the influence of *ACTN3* R577X on human muscle function in a well-defined and tightly controlled experimental setting which has yet to be achieved. From a population health perspective, shifting our research focus to look more broadly at the effects of this common null polymorphism in both health and disease will potentially identify pathways responsible for altering our response to both training (endurance and strength-based exercise), muscle mass (associated with performance) and the progression of various diseases. The culmination of this research will provide further evidence for a precision-based approach to assess and treat the extremes of muscle performance from athletes, the general population, in ageing and those affected by skeletal muscle disorders.

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Figure Legends:

Figure 1: Localisation and structure of the sarcomeric α-actinins in skeletal muscle The sarcomeric α-actinins are found at the Z-line (purple), where they anchor actin-containing thin filaments (blue) from adjacent sarcomeres. The sarcomeric α-actinins form head to tail dimers to cross-link actin at the Z-line. 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 [2] and [1].

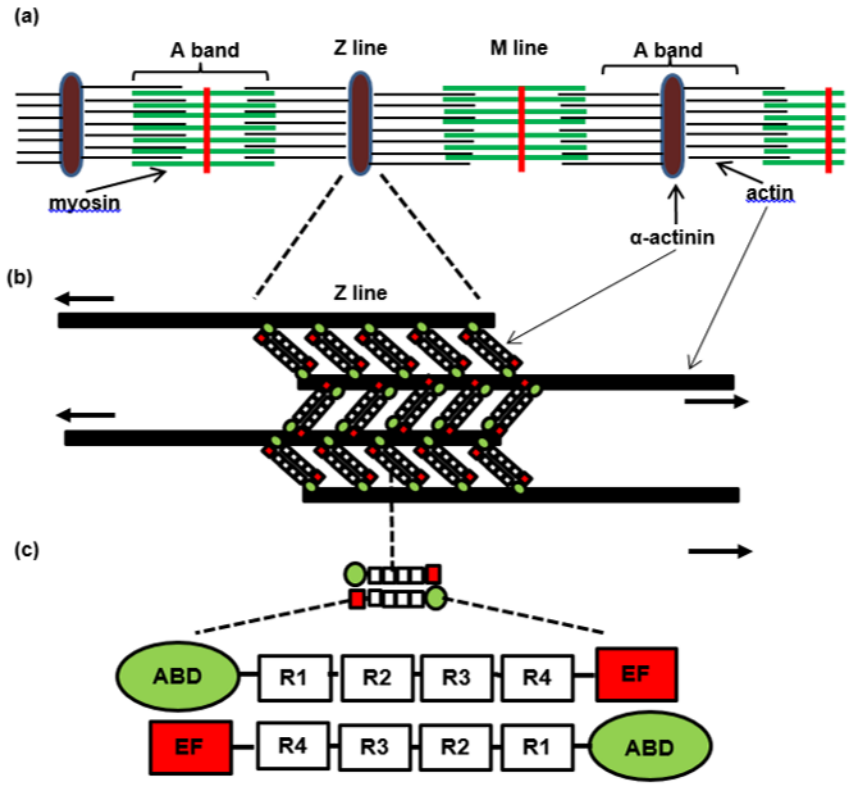
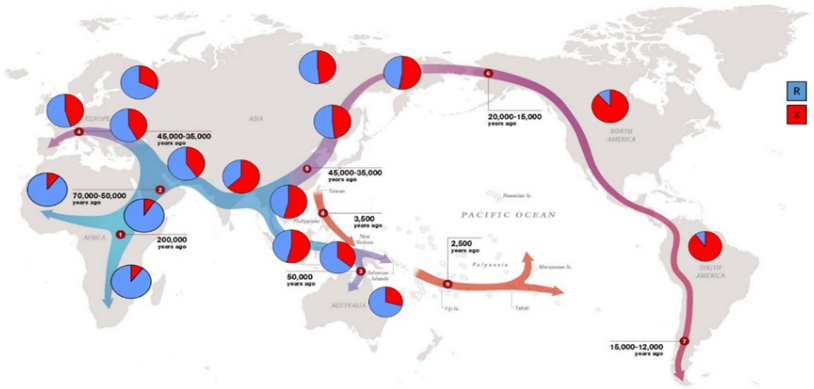


Figure 2: Human migration map with global frequencies of ACTN3 R and X alleles in native populations. World map and migration data Curtesy of National Geographic, from the Genographic Project. Arrows depict current knowledge of human migration out of Africa ~70-50 thousand years ago. (Allele frequencies adapted from MacArthur et al 2007, map from National Geographic)

Figure 2.



ACTN3 rs1815739:C>T

Table 1. The α-actinin isoforms, location and function.

Gene	Chromosome Location	Tissue	Number of Isoforms	Function
ACTN1	14q24	Ubiquitous	3	Calcium sensitive, focal adhesions, even distribution along the actin stress fibres in motile cells
ACTN2	1q42-q43	Skeletal, cardiac and smooth muscle, brown adipose tissue and brain	2	Calcium-insensitive, contractile apparatus, the Z-line
ACTN3	11q13.1	Skeletal muscle, bone, brown adipose tissue and brain	1	Calcium-insensitive, contractile apparatus, the Z-line
ACTN4	19q13	Ubiquitous, high levels in Kidney	4	Calcium-sensitive, focal adhesions and stress fibres, promotes cell motility, unique functions in kidney tissue

Table 2 a. Case control studies with the *ACTN3* R577X polymorphism in sprit/power oriented athletes and **b.** Case control studies with the *ACTN3* R577X polymorphism in endurance athletes.

a.

Sprint/Power Athletes					Athletes Genotype%			Controls Genotype%				
Country/ Ethnicity	Gender	Sport	P	N	RR	RX	XX	N	RR	RX	XX	Reference
Australian	M	Short distance Swimmers, Track cyclists>400, Rowers<2000m, Short distance Skiers	<0.001	72	53	39	8	134	30	54	16	Yang et al., 2003
	F		<0.01	35	43	57	0	292	30	54	16	
Finnish	M&F	Power oriented Track & Field athletes	<0.03	23	48	52	0	120	45	46	9	Niemi & Majaama 2006
Greek	M&F	Power oriented Track & Field - Mainly Sprinters (100m-400m)	<0.02	73	48	36	16	181	26	54	18	Papadimitriou et al., 2008
USA	M&F	Bodybuilders, Powerlifters	0.005	75	31	63	7	876	38	46	16	Roth et al., 2008
Russian	M	Speed Skiers, Gymnasts, Bodybuilders , Hockey players, Powerlifters, Footballers, Speed Skaters; Swimmers; Sprint Track & Field athletes, Volleyball players, Weightlifters, Wrestlers	<0.0001	363	38	56	6	524	37	47	16	Druzheveskaya et al., 2008
	F		0.067	123	46	48	6	673	37	51	13	
Israeli	M&F	Power oriented Track & Field athletes- Mainly Sprinters (100m-400m)	<0.0001	55	38	42	20	240	20	62	18	Eynon et al. 2009
Italian	M&F	Artistic Gymnasts	0.04	35	49	49	3	53	32	49	19	Massidda at al., 2009

Polish	M&F	Power oriented Track & Field athletes, Short distance Swimmers, Weightlifters	0.008	178	40	52	8	254	35	49	15	Cięszczyk et al., 2011
Taiwanese	M	Short distance Swimmers	NS	37	39	48	14	306	32	49	20	Chui et al., 2011
	F		<0.05	44	46	43	11	306	32	49	20	
Japanese	M&F	Wrestlers	0.028	52	27	62	11	333	27	45	28	Kikuchi et al., 2012
Korean	M	Gymnasts, Sprinters, Throwers, Speed Skaters, Weightlifters and Taekwondo athletes	NS	47	21	57	21	361	29	53	18	Hong et al. , 2013
	F		0.028	37	46	51	3	361	32	50	18	
Japanese	M	Power oriented Track & Field athletes - Mainly Sprinters (100m-400m)	0.002*	134	25	58	17	649	21	53	26	Mikami et al., 2013
Korean	M&F	Weightlifters, Speed Skaters, Sprinters and Short distance Swimmers	<0.05	121	40	48	12	854	30	51	19	Kim et al., 2014
Chinese	M&F	Power oriented Track & Field athletes, Track Cyclists, Weightlifters	<0.001	59	49	46	5	50	26	40	34	Yang et al., 2017
Nigerian	M&F	Power oriented Track & Field athletes	NS	62	87	13	0	60	83	17	3	Yang et al., 2007
Jamaican	M&F	Power oriented Track &Field athletes	NS	86	75	22	3	232	75	22	2	Scoot et al., 2010
USA	M&F	Power oriented Track& Field athletes	NS	79	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.

Endurance Athletes					Athletes Genotype%			Controls Genotype%				
Country/ Ethnicity	Gender	Sport	P	N	RR	RX	XX	N	RR	RX	XX	Reference
Australian	M	Long distance Swimmers, Endurance Cyclists, Rowers>2000m, 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	Long distance Rowers, Long distance cyclists, Long distance runners	NS	139	27	45	27	103	29	57	14	Lucia et al., 2006
Greek	M&F	Endurance 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, Endurance Cyclists, Long distance 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	Endurance 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	Long distance Rowers, Speed skaters, Race endurance walkers, Cross country Skiers, Long distance swimmers	NS	70	44	56	0	354	35	41	23	Eynon et al. 2012
Polish, Spanish, Russian	M&F	Long distance Cyclists,Long distance Rowers, Long distance 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	Endurance 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.

b. † In Russian cohort none of the males highly elite endurance athletes had the *ACTN3* 577XX genotype.

Table 3 a. Cross-sectional studies with the ACTN3 R577X polymorphism and power oriented performance characteristics in untrained, non-athlete populations.

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

a.

Origin	Tested	Found	Gender Age	N	Reference
UK	40m Sprint; Vertical jump; Throw distance; Grip strength, shuttle run test	*ACTN3* XX slower 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	No significant genotype differences	M 18-39	79	MacCauley et al., 2009
Spain	Vertical and Counter Movement Jumps test; 15-30m sprints	No significant genotype differences	M 18-2974	217	Santiago et al., 2010
UK	Flexor and extensor isometric strength ;vertical jump and 15m sprint time;	No significant genotype differences	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

b.

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	
Brazil	1 RM bench press; knee extensors peak torque (60 °/s) and ultra sound muscle thickness	At baseline	No significant genotype differences	M 23-31	141	Gentil et al., 2011
		Post training	*ACTN3* XX had lower gains in muscle thickness			