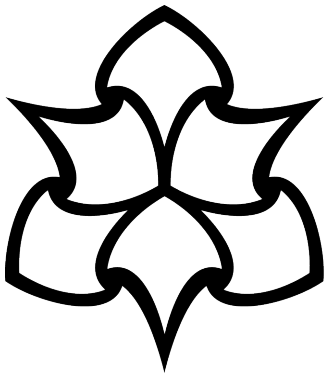

TTN genotype is associated with fascicle length and marathon running performance

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Abstract

Titin provides a molecular blueprint for muscle sarcomere assembly and sarcomere length can vary according to the expression of different titin isoforms, which may influence muscle fascicle length and consequently provide an advantage for running performance. Thus the aim of this study was to investigate if the titin (*TTN*) rs10497520 polymorphism was associated with muscle fascicle length in recreationally active men (RA; $n = 137$) and marathon personal best time in male marathon runners (MR; $n = 141$). Fascicle length of the vastus lateralis was assessed *in vivo* using B-mode ultrasonography at 50% of muscle length in RA. All participants provided either a whole blood, saliva or buccal cell sample, from which DNA was isolated and genotyped using real-time polymerase chain reaction. Vastus lateralis fascicle length was 10.4% longer in CC homozygotes than CT heterozygotes ($p = 0.003$) in RA. In the absence of any TT homozygotes, reflective of the low T-allele frequency within Caucasian populations, it is unclear if fascicle length for this group would have been smaller still. No differences in genotype frequency between the RA and MR groups were observed ($p = 0.500$), although within the MR group the T-allele carriers demonstrated marathon personal best times 2 min 25 s faster than CC homozygotes ($p = 0.020$). These results suggest that the T-allele at rs10497520 in the *TTN* gene is associated with shorter skeletal muscle fascicle length and conveys an advantage for marathon running performance in habitually trained men.

Keywords: Gene polymorphism, muscle architecture, endurance athletes, mechanical efficiency

Introduction

The titin gene (*TTN*) encodes the largest described protein to date, which is the third most abundant protein within the myofilament of human striated muscle (Vikhlyantsev & Podlubnaya 2012). Titin provides a molecular blueprint for the assembly and organisation of the thin and thick filaments during myofibrillogenesis (Chauveau et al. 2014). Seven splice isoform variants of titin exist within human striated muscle, which each differ in size and elasticity (Chauveau et al. 2014; Vikhlyantsev & Podlubnaya 2012).

A missense C>T transition (rs10497520) identified within human *TTN* has been reported to contribute to the variability in the training response of maximal oxygen consumption (VO_{2max}) in previously untrained individuals (Timmons et al. 2010). Within cardiac muscle, titin is suggested to be a key regulator of the Frank-Starling mechanism (Fukuda et al. 2001), and considering the substantial differences in the elasticity of cardiac titin isoforms (Wang et al. 1991), this C>T transition may contribute to the variability within titin isoform expression. Accordingly, differences in the titin isoforms expressed may explain the *TTN*-related increases in stroke volume (Rankinen et al. 2003) and consequently VO_{2max} following endurance exercise training Timmons et al. (2010). Furthermore, if this *TTN* polymorphism influences titin isoform expression in cardiac muscle as speculated, there exists a distinct possibility that a similar influence is occurring within skeletal muscle tissue.

In skeletal muscle, the predominant titin isoform is N2A, of which a smaller (T1) and larger (T2) isovariant exist within humans (Fry et al. 1997). A recent study, which identified a *TTN* mutation that alters isoform splicing in rats, demonstrated an association between isoform size and sarcomere length; with significantly longer resting sarcomere lengths corresponding to the larger mutant titin isoforms (Greaser & Pleitner 2014; Greaser et al. 2008). Assuming the findings of these animal studies can be extrapolated to human populations, it stands to reason that for individuals with an equal number of serial sarcomeres, fascicles would be longer in those expressing more of the larger titin isoforms and vice versa for those expressing more of the smaller titin isoforms. It is important to note,

however, that sarcomere length (like titin isoform expression) is not homogeneous within a muscle (Greaser et al. 2005; Wickiewicz et al. 1983).

If *TTN*-dependent differences in skeletal muscle fascicle length are apparent, variability in muscle functional phenotypes might also be expected. For instance, muscle maximal shortening velocity (V_{max}) is positively correlated with fascicle length (Bodine et al. 1982; Sacks & Roy 1982). Although no direct associations between *TTN* and fascicle length have been reported, the aforementioned C>T transition within the *TTN* gene has been identified as contributing significantly to a genetic predisposition for maximal isokinetic strength at $180°\cdot s^{-1}$ but not $60°\cdot s^{-1}$ (Thomaes et al. 2013), which could indirectly demonstrate that variability in V_{max} is influenced by genotype-dependent differences in fascicle length. Furthermore, enhanced efficiency of stretch-shortening contractions can be expected in individuals possessing shorter muscle fascicles due to the lower metabolic cost of producing a given force. More specifically, shorter fascicles produce the same force per unit cross-sectional area as longer fascicles, but when producing a given force, a smaller volume of muscle is activated in individuals possessing shorter fascicles (Pontzer et al. 2009; Roberts et al. 1998). Accordingly, vastus lateralis and gastrocnemius muscle fascicle length is shorter in elite distance runners than elite sprinters and untrained controls, and longer in elite sprinters than untrained controls (Abe et al. 2000). Shorter fascicles in elite distance runners are likely to contribute to improved mechanical efficiency, whereas the longer fascicles observed in elite sprinters is likely to contribute to enhanced V_{max}. To date, however, it remains unclear whether these differences in the muscle architecture of elite runners are the result of adaptations to training or genetic variation.

Consequently, the present study aimed to investigate if the *TTN* rs10497520 polymorphism was associated with muscle fascicle length in recreationally active men, and to investigate if *TTN* genotype distribution differed between recreationally active men and trained male marathon runners. It was hypothesized that the *TTN* polymorphism would be associated with muscle fascicle length in recreationally active men, and the genotype associated with shorter fascicle length in this population would be overrepresented in trained marathon runners.

Materials and methods

The sample comprised 278 healthy, unrelated Caucasian men who were categorised as either recreationally active [RA; *n* = 137, age 20.6 (2.3) yr, height 1.79 (0.06) m, mass 75.1 (10.1) kg; mean (standard deviation; SD)] or habitually trained marathon runners [MR; *n* = 141, age 34.9 (7.8) yr; height 1.79 (0.07) m, mass 66.5 (6.7) kg]. RA participants had a body mass index (BMI) between 18.5 $kg \cdot m^{-2}$ and 30 $kg \cdot m^{-2}$, self-reported as not having a known musculoskeletal or neurological disorder and had not undertaken any structured training in the preceding 12 months. MR participants comprised Olympic, international and national level marathon runners and had all achieved marathon personal best times under 2 hr 36 mins (range ~2 hr 7 mins to ~2 hr 35 mins). MR participants were primarily recruited from London Marathon competitors during 2013-2015 and regional athletics clubs and organisations. All participants gave written informed consent to participate in this study, which received approval from the Ethics Committee of Manchester Metropolitan University and complied with the Declaration of Helsinki.

Muscle fascicle length of the vastus lateralis (VL) was measured *in vivo* using B-mode ultrasonography (AU5, Esaote, Italy) for each RA participant. VL muscle length of the right limb was measured at rest following identification of the VL origin and insertion, whilst participants were standing upright with knees extended and relaxed (Abe et al. 2000). Whilst in this position, ultrasound scans were taken at 50% of VL muscle length, in the mid-sagittal plane, using a 40 mm wide, 7.5 MHz linear-array probe positioned perpendicular to the skin. Although the knee joint angle during standing does not correspond to that of optimal force production during running (Novacheck 1998; Tsuji et al. 2015), measurement of fascicle length in this position is highly reproducible. Each ultrasound scan was recorded using a 25 Hz sampling frequency in audio video interleave (AVI) format and frame-capture software (Adobe Premiere Elements version 10, Adobe Systems) was used to capture single images for subsequent analysis. The distance between fascicular origin in the lower aponeurosis and insertion in the upper aponeurosis was measured as fascicle length using digitizing software (NIH ImageJ, version 1.44o, National Institute of Health, Bethesda, Maryland). Estimation of fascicle

length was necessary for those fascicles extending beyond the ultrasound field of view, which was achieved by extrapolating the fascicle and aponeuroses (Reeves & Narici 2003). For each participant a minimum of three fascicles were measured and a mean of these was taken as fascicle length. Due to the field-based nature of data collection within MR, it was not possible to obtain measurements of fascicle length in this population.

All participants provided either a blood, saliva or buccal cell sample using the following protocols. For blood sampling, a 5 mL sample was taken from a superficial forearm vein into EDTA tubes (BD Vacutainer Systems, Plymouth, UK) and stored at -20°C. Saliva samples were collected following a minimum 30-minute abstinence from food and drink into Oragene DNA OG-500 collection tubes (DNA Genotek Inc., Ontario, Canada) in accordance with the manufacturer's guidelines and stored at room temperature. Buccal cell samples were collected in duplicate (Whatman Sterile, OmniSwab, GE Healthcare, USA) following a minimum 1-hour abstinence from food and drink. Participants were instructed to brush one OmniSwab collection tip firmly against the inside of the cheek for approximately 30 s and repeat with a second swab on the opposite cheek. Each collection tip was ejected into a 2 mL microcentrifuge tube and stored at -20°C.

The Qiagen QIAcube spin protocol (Qiagen, Crawley, UK), used for the extraction of genomic DNA from whole blood, saliva and buccal cell samples, was completed in accordance with the manufacturer's guidelines and used the buffers contained in the Qiagen DNA Blood Mini Kit. Each participant was genotyped for the *TTN* rs10497520 polymorphism, using real-time PCR on 96-well plates. The 10 μL reaction volume, for genotyping using DNA obtained from whole blood or saliva samples, contained 0.2 μL of participant DNA [9.9 (1.1) ng, amounts determined using ~20% of participant DNA samples], 5 μL of TaqMan genotyping master mix (Applied Biosystems, Paisley, UK), 4.3 μL of nuclease-free H_2O (Qiagen) and 0.5 μL of TaqMan SNP genotyping assay (Applied Biosystems). For DNA samples obtained from buccal cells, the 10 μL reaction volume contained 1 μL of participant DNA [18.6 (4.6) ng], 5 μL of TaqMan genotyping master mix, 3.5 μL of nuclease-free

157 H_2O and 0.5 μL of TaqMan SNP genotyping assay. In the control wells, the DNA sample was
158 replaced by nuclease-free H_2O.
159
160 DNA amplification (StepOnePlus Real-Time PCR System, Applied Biosystems) was completed using
161 the following protocol: an initial 10 min at 95°C followed by 40 cycles of denaturation for 15 s at
162 92°C, primer annealing and extension for 1 min at 60°C and plate read. *TTN* genotype was
163 subsequently determined using StepOnePlus analysis software version 2.3 (Applied Biosystems).
164 Genotypes were called based on reporter dye intensity and visualized using cluster plots. The TaqMan
165 assays included VIC and FAM dyes that for rs1049752 indicated C and T alleles on the forward DNA
166 strand, respectively. Thus, VIC/FAM were interpreted as: 5′- TCCAACTT[C/T]AGGTTCTT -3′. All
167 samples were analysed in duplicate and 100% agreement between all duplicate samples was achieved.
168
169 Genotype frequency of the *TTN* rs10497520 polymorphism was assessed for compliance with Hardy-
170 Weinberg equilibrium using a X^2 test. Due to the low number of TT homozygotes in the whole sample
171 (RA, $n = 0$; MR, $n = 1$), CC homozygotes were compared to T-allele carriers within each sub-group
172 (RA, CC vs. CT; MR, CC vs. CT+TT). Independent samples t-tests were conducted to determine any
173 significant differences in physical characteristics (height, mass, BMI and age) between RA and MR,
174 and according to genotype. Additionally, independent samples t-tests were conducted to identify any
175 genotype differences in fascicle length in RA and marathon personal best time in MR. Pearson's X^2
176 tests were used to compare genotype frequencies between MR and RA. All statistical analyses were
177 performed using SPSS version 21 and alpha was set at 0.05. Data are presented as mean (SD) unless
178 otherwise stated.
179
180 **Results**
181 Genotype frequency of the *TTN* rs10497520 polymorphism was in Hardy-Weinberg equilibrium for
182 the whole sample and both the RA and MR sub-groups (Table 1). MR were older and had lower mass
183 (~9 kg) and BMI than RA (all differences $p \leq 1.0 \times 10^{-13}$), but there was no difference in height ($p =$

184 0.660). Genotype was not associated with mass, BMI or height either within the RA or MR
185 subgroups, nor in the combined sample of 278 participants ($p \geq 0.376$; Table 1).
186
187 In the RA sub-group, VL fascicle length was 10.4% longer in CC homozygotes than in CT
188 heterozygotes ($p = 0.003$; Figure 1). There were no differences in genotype frequency between the
189 RA and MR groups ($X^2 = 1.385$, $p = 0.500$). However, marathon personal best time was significantly
190 lower in T-allele carriers compared to CC homozygotes in the MR group [2:26:28 (0:06:23) vs.
191 2:28:53 (0:05:50); $p = 0.020$; Figure 2].
192
193 **Discussion**
194 The aims of the present study were to investigate whether VL muscle fascicle length was associated
195 with *TTN* rs10497520 genotype in recreationally active Caucasian men, and to identify whether
196 differences in genotype frequency were evident between recreationally active individuals and trained
197 marathon runners. This study is the first to show a genetic influence on muscle architecture;
198 specifically, the results demonstrate that VL muscle fascicle length was significantly longer in *TTN*
199 CC homozygotes compared to CT heterozygotes in RA. This is also the first time marathon
200 performance in trained runners was associated with *TTN* genotype, with T-allele carriers performing
201 significantly better than CC homozygotes.
202
203 Titin acts as a template for myofibrillar protein assembly during sarcomere formation and provides an
204 attachment site for a plethora of myofibrillar proteins to maintain the structural integrity of the
205 sarcomere (Chauveau et al. 2014). This protein is therefore likely to play a key role in the architecture
206 of skeletal muscle, possibly affecting the serial arrangement of sarcomeres and, therefore, the length of
207 muscle fascicles (Greaser & Pleitner 2014; Greaser et al. 2008). Mean VL fascicle length in the RA
208 group [7.1 (1.5) cm] was comparable to some previous reports of VL fascicle length (~7 cm) (Abe et
209 al. 2000; Fukunaga et al. 1997), but less than others (~8 cm and ~9 cm) (Erskine et al. 2009; Reeves et
210 al. 2004). Differences in participant positioning and muscle activation during the measurement of
211 muscle fascicle length are likely to explain the reported differences between the present study and

212 reports elsewhere (Fukunaga et al. 1997). Indeed, VL fascicle length was measured during standing
213 with the knees extended and relaxed in the present study, which was similar to those studies reporting
214 comparable fascicle lengths (Abe et al. 2000; Fukunaga et al. 1997). Those studies observing longer
215 muscle fascicle lengths positioned the knee at 60-90° flexion and obtained measurements during
216 maximal voluntary contraction (Erskine et al. 2009; Reeves et al. 2004).
217
218 The *TTN* genotype and allele frequencies observed in the present study were similar to previous
219 reports in Caucasian populations (www.hapmap.org) (Gibbs et al. 2003). In the present study,
220 individuals homozygous for the major C-allele had longer VL fascicles than heterozygotes, but as no
221 individuals homozygous for the minor T-allele were present in the RA group, reflective of the low
222 frequency of the T-allele within a Caucasian population, it is unclear if the VL fascicles of TT
223 homozygotes would have been smaller still. Future research should attempt to replicate the observed
224 association between *TTN* and fascicle length on larger cohorts that include a sufficient number of TT
225 homozygotes. Based on the T-allele frequency we observed, future studies would require 2000
226 participants to recruit 20 TT homozygotes. A "stress the genotype" approach (Montgomery et al.
227 2002) could help prioritise recruitment of TT homozygotes prior to conducting time-consuming
228 phenotype assessments.
229
230 Nonetheless, it is possible that the presence of the T-allele affects *TTN* splicing thus increasing
231 expression of a smaller titin isoform within the muscle fascicles of heterozygotes. To date, seven
232 different titin splice isoforms have been identified within human striated muscle that each differ in size
233 (Vikhlyantsev & Podlubnaya 2012). Within human skeletal muscle, the predominant titin isoform is
234 N2A, of which two isovariants (T1 and T2) are known to exist (Fry et al. 1997). Thus, it is possible
235 that altered *TTN* splicing, due to the presence of the T-allele, may influence the expression of these
236 N2A isovariants and might explain the current observations. Earlier studies in rat cardiac muscle
237 support these possibilities by demonstrating a link between a *TTN* mutation and alternative isoform
238 splicing (Greaser et al. 2005) and, more recently, *TTN* was associated with both cardiac and skeletal
239 muscle sarcomere length in rats (Greaser & Pleitner 2014; Greaser et al. 2008).

Considering the observed association between *TTN* genotype and VL fascicle length, it was hypothesized that T-allele carriers would be overrepresented in habitually trained marathon runners because shorter fascicles require less energy to produce a given force, which is likely to contribute to improved mechanical efficiency in this population (Pontzer et al. 2009). No difference, however, in *TTN* genotype distribution was observed between the RA and MR groups. Nonetheless, the MR T-allele carriers (those expected to possess shorter fascicles according to our RA data) had marathon personal best times 2 min 25 s faster than MR CC homozygotes. This observation is consistent with previous reports of elite distance runners possessing shorter fascicles than both untrained individuals and elite sprinters (Abe et al. 2000). Thus, possession of the T-allele, whilst not essential for successful marathon running performance, might convey an advantage for marathon running when combined with appropriate training and nutritional regimens as could be expected of the habitually trained runners included in the present study.

Despite observing associations between *TTN* genotype and VL fascicle length in RA and marathon personal best time in MR, it remains unclear whether marathon personal best time was enhanced in the MR T-allele carriers as a consequence of possessing a shorter fascicle length, as this was not directly measured in the MR group. As titin is suggested to be a key regulator of the Frank-Starling mechanism, the influence of *TTN* within cardiac muscle could provide an alternative explanation for the observed association between *TTN* genotype and MR personal best time. *TTN*-related increases in stroke volume following endurance training have been observed previously (Rankinen et al. 2003) and the rs10497520 polymorphism appears to contribute to the training response of VO_{2max} in previously untrained individuals (Timmons et al. 2010). Interestingly, however, Timmons et al. (2010) observed greater gains in VO_{2max} in CC homozygotes (those expected to have longer VL fascicles) than T-allele carriers (those expected to have shorter VL fascicles), with gains experienced by heterozygotes similar to those of TT homozygotes following training. For untrained participants, such as those in Timmons et al., training-induced increases in VO_{2max} are primarily due to increases in cardiac output via increases in stroke volume (Ekblom et al. 1968; Iwasaki et al. 2003) and might be accentuated in

individuals possessing the CC genotype. However, in highly trained athletes with comparable rates of maximal oxygen uptake, as could be expected of the trained MR group, other factors such as lactate threshold and running economy are probably more important in determining performance (Conley & Krahenbuhl 1980). Moreover, improved running economy in individuals possessing lower ratios of titin isoforms (T1/T2) has recently been reported (Pellegrino et al. 2016), although more research is required to investigate whether the rs10497520 T-allele corresponds to lower T1/T2 ratios. Thus, despite a potential pleiotropic influence of *TTN* on both cardiac and skeletal muscle, possession of the T-allele (and consequently shorter VL fascicles) appears more important for marathon performance in trained individuals.

Finally, as RA CC homozygotes possessed longer VL fascicles, an association of this genotype with successful sprint running performance is possible. Longer muscle fascicles are known to contribute to enhanced V_{max} (Bodine et al. 1982; Sacks & Roy 1982), which is an important determinant of sprint performance (Kumagai et al. 2000). Thus, trained sprinters with the CC genotype might possess longer muscle fascicles and enhanced sprint ability compared to trained sprinters carrying the T-allele. Future research should investigate the impact of *TTN* genotype on sprint performance in addition to running economy, mechanical efficiency and V_{max}, to enhance our understanding of these associations.

Conclusion and Perspective

Here we report, for the first time, a genetic influence on human skeletal muscle architecture. The T-allele at the rs10497520 polymorphism in *TTN*, the gene encoding the giant structural protein titin, is associated with shorter VL muscle fascicles in recreationally active men, and faster marathon performance (nearly 2.5 minutes faster) in habitually trained male runners with personal best times of approximately 2.5 hours. Considering shorter muscle fascicles require less energy to produce a given force, the genotype-dependent differences in marathon personal best times may be due to differences in mechanical efficiency between T-allele carriers and CC homozygotes.

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Table 1. *TTN* rs10497520 genotype frequency and physical characteristics for RA and MR participants. Frequency data presented as count (%), all other data presented as mean (SD).

		All	CC	CT	TT	p	X^2
RA	Frequency (%)	137 (100)	110 (80.3)	27 (19.7)	0 (0.0)	0.441	1.637
	Height (m)	1.79 (0.06)	1.79 (0.06)	1.80 (0.07)	-	0.437	
	Mass (kg)	75.3 (10.1)*	75.0 (9.9)	76.3 (11.1)	-	0.376	
	BMI ($kg \cdot m^{-2}$)	23.5 (2.7)*	23.5 (2.7)	23.6 (3.0)	-	0.806	
	Age (yr)	20.7 (2.7)*	20.8 (2.6)	20.6 (3.1)	-	0.768	
	VL fascicle length (cm)	7.1 (1.5)	7.3 (1.6)	6.4 (0.9)		**0.003**	
MR	Frequency (%)	141 (100)	108 (76.6)	32 (22.7)	1 (0.7)	0.756	0.561
	Height (m)	1.79 (0.07)	1.78 (0.07)	1.79 (0.06)	1.82	0.675	
	Mass (kg)	66.6 (6.7)	66.7 (6.8)	66.0 (6.6)	66.0	0.551	
	BMI ($kg \cdot m^{-2}$)	20.9 (1.9)	21.0 (2.0)	20.6 (1.6)	19.9	0.285	
	Age (yr)	34.9 (7.8)	34.3 (6.7)	37.0 (10.6)	31.0	0.196	
	Marathon PB Time (hr:min:s)	2:28:31 (0:06:17)	2:28:53 (0:05:50)	2:26:25 (0:06:12)	2:27:08	**0.020**	
TOTAL	Frequency (%)	278 (100)	218 (78.4)	59 (21.2)	1 (0.4)	0.385	1.908
	Height (m)	1.79 (0.07)	1.79 (0.07)	1.79 (0.06)	1.82	0.415	
	Mass (kg)	70.8 (9.6)	70.9 (9.4)	70.7 (10.4)	66.0	0.834	
	BMI ($kg \cdot m^{-2}$)	22.2 (2.7)	22.2 (2.7)	21.9 (2.7)	19.9	0.452	
	Age (yr)	27.9 (9.2)	27.5 (8.5)	29.5 (11.5)	31.0	0.196	

RA, untrained; MR, habitually trained marathoners; BMI, body mass index; PB, personal best; p relates to two-group analyses (CC vs. CT in RA and CC vs. CT+TT in MR) except for frequency analyses when this includes all genotype groups; * denotes significant difference between RA and MR ($p \leq 1.0 \times 10^{-13}$).

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Figure 1. Comparison of VL fascicle length by *TTN* CC (*n* = 110) and CT (*n* = 27) genotype in RA (*p = 0.003). No TT homozygotes were identified. Columns and error bars are mean and SD.

Figure 2. Comparison of marathon personal best time between *TTN* CC genotype (*n* = 108) and T-allele carriers (*n* = 33) in MR (*p = 0.020). Columns and error bars are mean and SD.