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Variation within three apoptosis associated genes as potential risk factors for Achilles tendinopathy in a British based case-control cohort.

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Abstract

Achilles tendon pathology (ATP) is a degenerative condition which exhibits excessive tenocyte apoptosis. Tumour necrosis factor receptor 1 (TNFR1), caspase-3 (CASP3) and caspase-8 (CASP8) are important regulators of apoptosis. To date, the effect of variation within the genes for TNFR1 and CASP3 as risk factors for ATP have not been described. There is evidence that two single nucleotide polymorphisms (SNPs) within the *CASP8* gene are associated with ATP, but only in populations from the Southern Hemisphere. The primary aim of this study was to determine whether SNPs within the *TNFRSF1A* and *CASP3* genes were associated with ATP in British Caucasians. We additionally sought to determine whether copy number variation (CNV) within the *CASP8* gene was associated with ATP. We recruited 262 (131 ATP cases and 131 asymptomatic controls) Caucasian participants for this genetic association study and used quantitative PCR with chi-squared (χ^2) tests and ANOVA to detect significant associations. We found no association between the *TNFRSF1A* rs4149577 ($p = 0.561$), *CASP3* rs1049253 ($p = 0.643$) and *CASP8* copy number variants ($p = 0.219$) and ATP. Likewise, when we tested potential interactions between gender, genotype and the risk of ATP, we found no association with the variants investigated. In conclusion, the *TNFRSF1A*, *CASP3* and *CASP8* gene variants were not associated with ATP in British Caucasians.

Keywords:

Apoptosis; Genotype; Achilles; tendinopathy:

57 **1. Introduction**

58 The Achilles tendon is prone to damage and rupture[1]. Increased stress on tendons during
59 exercise can cause such damage in both professional and recreational athletes, typically as
60 a result of repetitive mechanical loading[2,3,4]. Achilles tendon pathology (ATP) can manifest as
61 either insertional or noninsertional pathologies[1,5]. Alternatively the pathology can present as
62 partial or complete rupture[1,5] and lead to long-term incapacitation and a reduction in physical
63 activity[1].

64

65 A range of factors, including genetics, have been shown to increase the risk of ATP[4,6-8]. For
66 example, genes that encode proteins with a role in maintaining the integrity of the tendon
67 extracellular matrix (ECM) have been shown to associate with ATP[4,6,8]. Apoptosis is a normal
68 mechanism in tendon healing to remove damaged tenocytes[9], however, relatively little is
69 known about genetic variation within genes involved in apoptosis and the risk of ATP.
70 Previous work in this area has been limited to variants within the *CASP8* (rs384129,
71 rs1045485), *NOS2* (rs2779249) and *NOS3* (rs1799983) genes in two cohorts from the
72 Southern Hemisphere (South Africa and Australia)[10].

73

74 The *TNFRSF1A* gene encodes tumour necrosis factor receptor 1 (TNFR1), a cell receptor
75 that can signal apoptosis in response to the pro-inflammatory cytokine tumour necrosis
76 factor-alpha (TNF-α)[11,12]. TNFR1 mRNA and protein have recently been identified in human
77 Achilles tendon[12] and in cultured tenocytes[13]. Importantly TNFR1 is known to be highly
78 expressed in tenocytes isolated from Achilles tendinosis[12] but the functional or pathologic
79 significance of this is not clear. Single nucleotide polymorphisms (SNPs) within the
80 *TNFRSF1A* gene, especially the rs4149577 variant, have previously been associated with
81 other musculoskeletal [14] and inflammatory diseases[15]. However, the role of this variant as a
82 risk factor for ATP has not been investigated.

83

84 Caspases form a family of proteases that are important in the regulation of apoptosis[16].
85 Caspase-3 can selectively cleave target proteins after aspartate residues in their primary
86 sequence[16,17]. Although apoptosis can be activated through a number of complex pathways,
87 caspase-3 appears to have a critical role in chromatin condensation and DNA
88 fragmentation[18]. The *CASP3* rs1049253 variant resides within the 3' untranslated region
89 (UTR) of the gene and can influence the binding of miR-885-5p to *CASP3* mRNA[19]. Guan *et*
90 *al.* (2013) showed that the CC genotype of this variant influences levels of *CASP3* mRNA
91 expression[19]. Like the *TNFRSF1A* gene, the role of the *CASP3* rs1049253 variant in
92 predisposing to ATP is unknown.
93
94 Another important regulator of apoptosis is caspase-8. Caspase-8, encoded by the *CASP8*
95 gene, can activate downstream effector caspases[11]. It is know to regulate apoptosis of
96 tendon fibroblasts[9]. Regulating the balance between synthesis and degradation is essential
97 in maintaining ECM homeostasis and the removal of damaged tendon fibroblasts during
98 normal tendon turnover is important[9]. However, atypical tenocyte apoptosis has been shown
99 in tendinopathy, with elevated expression levels of *CASP8* observed[10]. Two SNPs within the
100 *CASP8* gene have been associated with ATP[10] but the role of larger scale variation within
101 this gene as a risk factor has not been considered.
102
103 Although our understanding of the role that SNPs play in ATP is growing[4,6,8,10], there has yet
104 to be an investigation into the influence of copy number variation (CNV) as a predisposing
105 factor. Copy number variants (CNVs) are segments of DNA greater than 1kb in size, which
106 show altered copy number (CN) when compared to a reference genome[20]. CNVs can
107 influence phenotypes by altering gene dosage and disrupting coding sequences of DNA[21].
108 Indeed, predisposition to certain diseases appears to be associated with CNV[21,22]. The
109 *CASP8* gene is known to harbour a CN variant that spans intron 11 - intron 12 of the
110 nucleotide sequence (as reported in the Database of Genomic Variants (DGV)
111 (http://projects.tcag.ca/variation/)). With regard to the role of caspase 8 in apoptosis, and as

112 SNPs within this gene have been associated with ATP[10], we considered that CNV within
113 *CASP8* might predispose to ATP. Additionally, for the reasons outlined in the preceding
114 paragraphs, we decided to investigate whether the *TNFRSF1A* rs4149577 and *CASP3*
115 rs1049253 variants were additional risk factors for this pathology.
116
117 **2. Methods**
118 One hundred and thirty one British Caucasian participants diagnosed with ATP and 131
119 asymptomatic British Caucasian controls (CON) were recruited for this genetic association
120 study. ATP participants were recruited through The County Clinic in Northampton, UK.
121 Participants within the CON group (physically active individuals without any history of ATP)
122 were recruited from the East Midlands region of the UK. Cases of Achilles tendinopathy
123 typically presented with gradual progressive pain with early-morning pain/stiffness in the
124 Achilles tendon area. Affected individuals were diagnosed by the clinical author using the
125 criteria published elsewhere[5,23,29] Diagnosis of tendinopathy was objectively confirmed, in
126 most cases, by MRI of the affected Achilles tendon. The ATP group consisted of participants
127 with noninsertional (N=47) or insertional (N=29) tendinopathy along with those presenting
128 with partial or complete Achilles tendon rupture (N=23). We further recruited an additional 29
129 cases with more than one type of tendinopathy (insertional and noninsertional). Three
130 individuals originally recruited as controls later self-reported symptoms of ATP but the
131 specific type of tendinopathy for these was not obtained. Subsequent to initial diagnosis, 15
132 cases with Achilles tendinopathy went on to develop ruptures. As each subpathology
133 (insertional, non-insertional and rupture) was relatively small in number we pooled all cases
134 as having the combined condition of Achilles tendon pathology and this was the main
135 phenotype we chose to investigate. Additional association analysis on each subpathology
136 was, nevertheless, investigated but this was statistically underpowered due to the small
137 sample sizes. All participants completed a physical activity/medical history/injury
138 questionnaire. and gave written, informed consent. The study was approved by the Research
139 Ethics Committee of the University of Northampton, United Kingdom.

140

141 DNA was extracted from 2 mL of saliva collected using ORAGENE-DNA kits (OG-500) and
142 DNA purification was carried out using the prepIT-L2P DNA extraction kit (DNA Genotek Inc.,
143 Ontario, Canada). Quantitative PCR (qPCR) reactions were run on a StepOnePlus Real-
144 Time PCR System (Applied Biosystems, Foster City, California, USA) in 96-well plates, using
145 fluorescence-based TaqMan assays. The genotyping assays for the *TNFRSF1A* rs4149577,
146 *CASP3* rs1049253 and *CASP8* variants were selected from Applied Biosystems. PCR
147 reactions contained probes and primers in a mastermix containing AmpliTaq DNA
148 Polymerase Gold and 10 ng of DNA. The cycling conditions consisted of a holding phase at
149 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 15 s and
150 annealing/extension at 60 °C for 1 min. Quality control for our SNP genotyping consisted of
151 running both within and between plate replicates to assess for reproducibility in genotype call
152 along with the running of 4 no DNA template controls (NTC) in each qPCR run. For the copy
153 number assays, each sample was repeated in quadruplicate along with the inclusion of
154 NTCs.

155

156 For the *TNFRSF1A* rs4149577 and *CASP3* rs1049253 SNPs, the TaqMan Genotyping
157 Assays (C_2645708_10 and C_11683739_10 respectively) contained both FAM and VIC
158 reporter dye labelled probes to discriminate between genotypes, along with ROX dye as the
159 passive reference. Genotypes were called using StepOne Software version 2.1 (Applied
160 Biosystems, Foster City, California, USA). CNV spanning intron 11 - intron 12 of the *CASP8*
161 gene was determined using the TaqMan Copy Number Assay Hs02601709_cn along with
162 the reference assay for RNase P. QPCR was performed as a duplex reaction and all CN
163 data were exported into CopyCaller Software version 2.0 (Applied Biosystems, Foster City,
164 California, USA) for calculation of discrete and continuous CN. CN of the target gene was
165 normalised relative to the reference RNase P gene. We used CopyCaller Software to
166 calculate confidence and z-score quality metrics. CN calls were inspected for reproducibility
167 and accepted with a z-score <1.75. To establish PCR efficiency of both assays (*CASP8* and

RNase P), qPCR was carried out using serial dilutions of genomic DNA. Efficiency (E) was calculated using the equation $E=10^{(-1/m)}-1$ [24].

Data were analysed using IBM SPSS Statistics version 20 (IBM Corp. Armonk, NY). One-way analysis of variance (ANOVA) was used to determine any significant differences between the characteristics (age, height, weight, BMI) of the ATP and CON groups. A Pearson's chi-squared (χ^2) test was used to determine any significant differences between the gender of the ATP and CON groups. A χ^2 or Fisher's exact test was used to analyse for differences in genotype and allele frequency distributions for the *TNFRSF1A* rs4149577 and *CASP3* rs1049253 variants, as well as differences in discrete CN (<2, =2, >2 copies) for the *CASP8* gene in the ATP and CON groups. Differences in continuous CN values between ATP and CON groups were analysed using a non-parametric Mann Whitney *U*-test. Data were also analysed by gender and, where appropriate, comparisons were also made between the ATP subpathologies and CON groups. In all analyses, significance was accepted at $p < 0.05$. Hardy-Weinberg equilibrium (HWE) was established using a HWE calculator (Michael H. Court, 2005-2008) accessed from www.tufts.edu/~mcourt01/Documents/Court lab - HW calculator.xls. $P < 0.05$ was considered to be a deviation from HWE. The statistical power of our SNP analysis was calculated using Quanto version 1.2 (http://hydra.usc.edu/gxe)[25]. Assuming an odds ratio (OR) of 2.2 and a recessive mode of inheritance, our analysis had 80% power to detect associations at the $p < 0.05$ significance level. For our CNV analysis we used sampsize (Version 0.6), available at http://sampsize.sourceforge.net/[26] for a power calculation. With an assumed CN exposure frequency of 20%, without a specified mode of inheritance, our cohort size was sufficient for 80% power.

3. Results

The CON and ATP groups were similarly matched for gender, height, weight and BMI. However, there was a significant difference in age ($p = 0.019$) between the CON (41.3 ±

196 11.3, n = 122) and ATP (45.1 ± 14.2, n = 127) groups. There was a small but significant
197 difference in age between the CON and RUP groups (p=0.036). Full details of the study
198 participant characteristics (including the TEN and RUP subgroups) can be seen in **Table 1**.
199 The FAM labelled TaqMan Copy Number Assay and the VIC labelled Reference Assay
200 RNase P gave PCR efficiencies of 106% and 104% respectively. We found no interaction
201 between the variants investigated and participant characteristics for the entire cohort (data
202 not shown). We also found that none of the variants investigated associated with either the
203 insertional, noinsertional, rupture or mixed pathologies (P>0.05). However, investigating
204 potential associations between the various subpathologies and genotype was not the focus
205 of our study as the N values for each subpathology was small and analysis was statistically
206 underpowered.

207

208 Genotype and minor allele frequency (MAF) distributions for the *TNFRSF1A* rs4149577 and
209 *CASP3* rs1049253 variants, along with HWE p-values, are shown in **Table 2**. For the
210 *TNFRSF1A* rs4149577 variant, the genotype (p = 0.561) and allele (p = 0.335) frequency
211 distributions were not significantly different between the ATP and CON groups. There were
212 no significant differences in genotype or allele frequencies between the male ATP and CON
213 group (p = 0.561 and p = 0.371 respectively), nor between the female ATP and CON group
214 (p = 0.916 and p = 0.680 respectively).

215

216 For the *CASP3* rs1049253 variant, there were no significant differences in genotype (p =
217 0.643) or allele (p = 0.635) frequencies between the ATP and CON groups. There was no
218 significant difference in genotype (p = 0.142) or allele (p = 0.104) frequencies between the
219 female ATP and CON group. There was also no significant difference in genotype (p =
220 0.072) or allele (p = 0.058) frequencies between the male ATP and CON group, although
221 there was a trend towards significance.

222

CN frequency distributions (<2, =2, >2 copies) of the ATP and CON groups for the variant spanning intron 11 - intron 12 of the *CASP8* gene are shown in **Table 3**. At this locus, 4 ATP and 2 CON participants were shown to have < 2 copies, while 10 ATP and 14 CON had > 2 copies. When we compared the distribution of discrete CN between ATP and CON groups, we found no evidence of a significant difference ($p = 0.219$). Furthermore, there was no significant difference in CN between the male ATP and the CON group ($p = 0.703$) nor between the female ATP and CON group ($p = 0.277$). We also found that when we analysed CN data as a continuous variable with a Mann Whitney *U*-test, there were no significant differences between ATP and CON groups. This was also the case in gender specific analyses (data not shown).

4. Discussion

The *TNFRSF1A* rs4149577 variant does not appear to be associated with ATP in a British cohort. TNFR1 has a role in apoptosis and it is expressed at significantly higher levels in tenocytes from Achilles tendinosis compared to controls[12]. However, as we only investigated a single SNP within this gene (*TNFRSF1A* rs4149577), it is possible that other variants within the DNA sequence may modify the risk of ATP. For example, the *TNFRSF1A* rs4149577 variant is an intronic SNP that is in linkage disequilibrium (LD) with a number of other variants, including *TNFRSF1A* rs1800693 and rs4149578 (www.ensembl.org), which have been associated with musculoskeletal and inflammatory diseases[14,15,27].

Nell *et al.* (2012) have previously reported that variants within the *CASP8* gene were associated with Achilles tendinopathy in a South African and Australian cohort. Specifically, they showed that the *CASP8* rs1045485 and rs3834129 SNPs were both associated with Achilles tendinopathy[10]. The copy number variant spanning intron 11 - intron 12 of the *CASP8* gene that we investigated here overlaps the rs1045485 SNP investigated by Nell *et al.* (2012), but it is approximately 50 000 base pairs from the rs3834129 SNP. We found no association between CNV at this locus and ATP, in contrast to Nell *et al.* (2012). This might

251 be related to a number of factors. Firstly, SNPs like the *CASP8* rs1045485 variant may have
252 a more profound impact on the development of ATP, compared to larger-scale variants such
253 as CNV. This assertion is supported by the fact that the *CASP8* rs1045485 SNP is a non-
254 synonymous G>C polymorphism that results in an amino acid change (Asp302His), which
255 may affect interaction with other proteins[9]. In contrast, the functional effect of altered CN at
256 the *CASP8* locus we investigated is presently unknown. Secondly, the association of this
257 region of the *CASP8* gene with ATP might be a population specific effect, as association with
258 the *CASP8* rs1045485 SNP was reported in South African and Australian cohorts[10] and our
259 data were obtained from British Caucasians. With this in mind, we would envisage further
260 studies on this British cohort using the SNPs investigated by Nell et al (2012) to establish
261 whether they specifically associate with ATP. This would confirm or refute any population
262 difference with high resolution. An additional haplotype based association study using these
263 SNPs would also be worthwhile.
264
265 Although we did not find an association between the *CASP3* rs1049253 variant and the ATP
266 group as a whole, we did observe that both genotype and allele frequency distributions
267 approached significance when male CON and male ATP were compared. Furthermore, we
268 did find a quantitative overrepresentation of the CC genotype in males with insertional
269 tendinopathy compared to male controls (data not shown). However, a detailed statistical
270 analysis of this was not possible due to the small number of males in our cohort who
271 presented with this sub- pathology, nevertheless, it does warrant further investigation.
272
273 Our study seems to exclude the *TNFRSF1A* rs4149577, *CASP3* rs1049253 and *CASP8* CN
274 loci from a role in ATP. However, we must be cautious in interpreting these findings as the
275 study has some limitations. Firstly, although our investigation was sufficiently powered to
276 detect relatively large effect sizes given the total sample size of 262, it lacked power to detect
277 associations with more modest effects. Secondly, while we found no association between
278 discrete CN calls and ATP for the *CASP8* locus, we were aware that it has been suggested

279 that rounding CN data into discrete calls might introduce a degree of error[28]. However, to
280 circumvent this possible issue, we also analysed our CN data as a continuous variable and
281 found a concordant lack of association that agreed with the discrete CN findings (data not
282 shown).
283
284 To our knowledge, no previous study has investigated whether the *TNFRSF1A* rs4149577
285 and *CASP3* rs1049253 variants were associated with ATP. Additionally, this was the first
286 study to investigate whether genomic CNV (in this case within the *CASP8* locus) was a
287 possible predisposing factor for ATP. To date, studies on the genetics of ATP have been
288 conducted predominantly in South African and Australian cohorts[10,29-31] Our study was the
289 first to investigate the effect of these variants in British-based individuals.
290
291 In conclusion, the *TNFRSF1A* rs4149577, *CASP3* rs1049253 and *CASP8* variants do not
292 associate with ATP. Although we recommend that this study be repeated in a larger cohort,
293 and where possible using cases where only each subpathology is evident, these preliminary
294 findings exclude three plausible candidate gene variants from a role in a common
295 musculoskeletal pathology.
296
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303 variations (not included in this manuscript) related to risk assessment of Achilles
304 tendinopathy and anterior cruciate ligament injuries.
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408 beta family and the pathogenesis of human Achilles tendon pathology-a genetic association
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411
412
413

414 **Table 1.** General characteristics of the Achilles tendon pathology (ATP), tendinopathy (TEN), rupture (RUP) and control (CON) groups.

	CON (n=131)	ATP (n=131)	p-value	TEN (n=93)	p-value	RUP[b] (n=38)	p-value
Age (years)[a]	41.3 ± 11.3 (122)	45.1 ± 14.2 (127)	**0.019**	44.9 ± 13.8 (90)	**0.036**	45.6 ± 15.4 (37)	0.066
Gender (% male)	62.6 (82)	61.8 (81)	0.899	60.2 (56)	0.718	65.8 (25)	0.719
Height (cm)	175.0 ± 10.5 (121)	172.6 ± 9.6 (128)	0.065	172.4 ± 9.1 (90)	0.058	173.3 ± 10.7 (38)	0.384
Weight (kg)	80.5 ± 19.6 (122)	78.5 ± 15.2 (94)	0.416	77.5 ± 15.2 (64)	0.300	80.4 ± 15.1 (30)	0.996
BMI (kg m^{-2})	25.7 ± 5.1 (122)	26.3 ± 4.1 (94)	0.401	26.0 ± 4.3 (64)	0.688	26.8 ± 3.7 (30)	0.285

415 Values are expressed as means ± SD for study participant characteristics. The total number of participants (n) is in parentheses. cm:
416 centimetres; kg: kilograms; m: metres.
417 [a] Age of CON is age of recruitment, while age of ATP, TEN and RUP is age of initial injury.
418 One-way ANOVA was used to analyse differences in the characteristics of CON vs. ATP, CON vs. TEN and CON vs. RUP groups. Significance
419 was accepted when $p < 0.05$.
420 [b]The RUP group includes those that initially presented with rupture (N=23) and those that later developed ruptures from an initial diagnosis of
421 tendinopathy (N=15).
422

423 **Table 2.** Genotype and allele frequency distributions of the *TNFRSF1A* rs4149577 and
424 *CASP3* rs1049253 variants within cases (ATP) and controls (CON).

	CON	ATP	Male CON	Male ATP	Female CON	Female ATP
TNFRSF1A						
rs4149577						
N	131	131	82	81	49	50
CC	23.7 (31)	21.4 (28)	25.6 (21)	23.5 (19)	20.4 (10)	18.0 (9)
CT	48.9 (64)	45.0 (59)	47.6 (39)	42.0 (34)	51.0 (25)	50.0 (25)
TT	27.5 (36)	33.6 (44)	26.8 (22)	34.6 (28)	28.6 (14)	32.0 (16)
p-value		0.561		0.561		0.916
MAF	48.1 (126)	43.9 (115)	49.4 (81)	44.4 (72)	45.9 (45)	43.0 (43)
p-value		0.335		0.371		0.680
HWE	0.806	0.327	0.660	0.177	0.849	0.888
CASP3						
rs1049253						
N	131	130	82	80	49	50
TT	62.6 (82)	61.5 (80)	68.3 (56)	58.8 (47)	53.1 (26)	66.0 (33)
CT	34.4 (45)	33.1 (43)	30.5 (25)	32.5 (26)	40.8 (20)	34.0 (17)
CC	3.1 (4)	5.4 (7)	1.2 (1)	8.8 (7)	6.1 (3)	0.0 (0)
p-value		0.643		0.072		0.142
MAF	20.2 (53)	21.9 (57)	16.5 (27)	25.0 (40)	26.5 (26)	17.0 (17)
p-value		0.635		0.058		0.104
HWE	0.461	0.700	0.326	0.233	0.742	0.148

425 Values are expressed as a frequency (%) with number of participants (n) in parenthesis.
426 HWE: Hardy-Weinberg equilibrium. MAF: minor allele frequency. Differences in genotype
427 and allele frequencies between CON and ATP were analysed using a Pearson's χ^2 or
428 Fisher's exact test. Significance was accepted when $p < 0.05$.

429

430 **Table 3.** CN frequency distribution within the *CASP8* gene in cases (ATP) and controls

431 (CON).

Group	< 2 CN	= 2 CN	> 2 CN	n	p-value
CON	2	46	14	62	-
ATP	4	70	10	84	0.219
Male CON	1	30	8	39	-
Male ATP	1	44	7	52	0.703
Female CON	1	16	6	23	-
Female ATP	3	26	3	32	0.277

432 Differences in discrete CN between CON and ATP were analysed as described in the

433 Methods section. Significance was accepted when $p < 0.05$.

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