

***MMP3* and *TIMP2* gene variants as predisposing factors for Achilles tendon pathologies: Attempted replication study in a British case-control cohort.**

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ABSTRACT

Variants within the *MMP3* (rs679620) and *TIMP2* (rs4789932) genes have been associated with the risk of Achilles tendon pathology (ATP) in populations from South Africa and Australia. This study aimed to determine whether these variants were associated with the risk of ATP in British Caucasians. We recruited 118 cases with ATP, including a subset of 25 individuals with Achilles tendon rupture (RUP) and 131 controls. DNA samples were isolated from saliva and genotyped using qPCR. For the *TIMP2* rs4789932 variant we found a significant ($p=0.038$) difference in the genotype distribution frequency between males with ATP (CC, 39.4%; CT, 43.7%; TT, 16.9%) compared to male controls (CC, 20.7%; CT, 59.8%; TT, 19.5%). We also observed a difference in the *TIMP2* rs4789932 genotype distribution between males with rupture compared to male controls ($p=0.038$). The *MMP3* rs679620 GG genotype was found to be overrepresented in the Achilles tendon rupture (RUP) group (AA, 24.0%; AG, 32.0%; GG, 44.0%) compared to controls (AA, 26.7%; AG, 54.2%; GG, 19.1%). In conclusion, the CT genotype of the *TIMP2* rs4789932 variant was associated with lower risk of ATP in males. Furthermore, while we revealed differences for both variants in genotype distribution between the RUP and control groups, the sample size of the RUP group was small and confirmation would be required in additional cohorts. Finally, although both the *TIMP2* rs4789932 and *MMP3* rs679620 variants tentatively associated with ATP, there were differences in the direction of association compared to earlier work.

INTRODUCTION

Achilles tendon pathology (ATP) is an umbrella term that refers to both Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP). ATP is a debilitating condition and most cases are related to overuse injury caused by chronic or acute exposure to repetitive micro-traumas (Kvist 1994). Several intrinsic and extrinsic risk factors for ATP have been reported, and an increasing number of association studies have demonstrated that genetic variants can predispose to the condition (Raleigh and Collins 2012).

The extracellular matrix (ECM) within a tendon consists of collagen fibrils, proteoglycans, glycoproteins and glycosaminoglycans (Sharma and Maffulli 2005). In addition to structural proteins, non-structural proteins and enzymes contribute towards homeostasis by constantly remodelling the ECM (Magra and Maffulli 2005). MMP3 belongs to the family of metalloproteinases (MMPs) which are known for their role in controlling the ECM integrity by catalytically degrading structural proteins including laminin, fibronectin, and various types of collagen (Somerville *et al.* 2003; Birkedal-Hansen *et al.* 1993). MMP3 is known to stimulate the activity of other MMPs which eventually contribute towards the catalysis of other proteins within the ECM (Toth *et al.* 2003). However, *MMP3* mRNA levels have been consistently shown to be lower in ATP samples when compared to the controls (Ireland *et al.* 2001; Alfredson *et al.* 2003). Tissue inhibitors of metalloproteases (TIMPs) are known for their role in inhibiting the activity of metalloproteinases by interacting with the MMP active site (Young *et al.* 2002). Although TIMP2 does not inhibit the activity of MMP3, it inhibits the activation of MMP2 which works in parallel with MMP3 to activate MMP9 (Toth *et al.* 2003). Therefore, by limiting the activity of MMP2, TIMP2 is considered to indirectly reduce the impact of MMP3 activity.

Recent work has shown that the rs679620 single nucleotide polymorphism (SNP) within the *MMP3* gene and the rs478992 SNP within the *TIMP2* gene were risk factors for ATP in a South African (Raleigh *et al.* 2009) and a combined South African and Australian cohort respectively (El Khoury *et al.* 2013). However, as genetic predisposition to multifactorial phenotypes is often population specific, we wished to ascertain whether these polymorphisms were also risk factors in a recently recruited British population.

MATERIALS AND METHODS

One hundred and eighteen (47 females and 71 males) British Caucasian participants diagnosed with Achilles tendon pathology (ATP) and 131 (49 females and 82 males) asymptomatic British Caucasian controls (CON) were recruited for this case-control genetic association study. All case participants were recruited from The County Clinic, Northampton, UK between 2011 and 2014. A suitable control population of physically active individuals with regular exposure to lower limb exercise (minimum 2 hrs/wk of high intensity exercise) and no history of Achilles tendon pathology, were recruited from sports clubs within the East Midlands area. All participants gave written informed consent, according to the Declaration of Helsinki, and completed a medical and injury history questionnaire. All participants with ATP were diagnosed by the medical professional and co-author WJR based on the clinical criteria reported in previous studies (Mokone *et al.* 2005; September *et al.* 2009). For patients with tendon ruptures, the clinical diagnosis was corroborated in most cases (88.0%, n=22) by either imaging (60.0%, n=15) and/or surgical observation (52.0%, n=13). Our recruitment and experimental protocols were approved by the University of Northampton Research Ethics Committee.

The ATP group consisted of 93 (39 females and 54 males) participants with chronic Achilles tendinopathy (TEN), and 25 (8 females and 17 males) participants with

Achilles tendon rupture (RUP). Patients with chronic Achilles tendinopathy (TEN) were diagnosed as suffering from either non-insertional (n=51, 54.8%), insertional tendinopathy (n=28, 30.1%), or both (n=14, 15.0%). Achilles ruptures were categorised as full or partial ruptures. For the purpose of analysis, participants diagnosed with Achilles tendinopathy who later sustained an Achilles rupture (n=12), were included in the RUP group. At the time of recruitment participants in the ATP group were generally healthy with the exception of 18 (15.2%) individuals who were on medication for different cardiovascular conditions. We also report that 34 (28.8%) ATP cases reported a history of tendinopathy in a tendon other than the Achilles tendon, 46 (38.9%) reported a history of ligament injury.

Saliva samples were collected from each participant using Oragene™ OG-500 tubes (DNA Genotek, Ontario, Canada). DNA was extracted according to the manufacturer's protocol using a PT-L2P lysis solution. DNA samples were genotyped for the *MMP3* rs679620 and *TIMP2* rs4789932 gene variants using fluorescence-based TaqMan® Custom SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). The qPCR reactions contained probes and primers and PCR mastermix containing AmpliTaq DNA Polymerase Gold (Applied Biosystems, Foster City, CA, USA) in a total reaction volume of 6 µL. PCR was performed on an Applied Biosystems StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA, USA). Genotypes were determined following end point fluorescence analysis using Applied Biosystems StepOnePlus™ real-time PCR software Version 2.1. Rox was used as a passive reference and each PCR run included a number of both positive (sample repeats) and negative (water) controls.

Data were analysed using SPSS Version 20 (IBM SPSS, IBM Corp, Somers, NY, USA) statistical program. A chi-squared (χ^2) analysis was used to determine whether significant genotype and/or allele distribution differences existed between CON and

ATP groups. An independent t-test was used to establish if any significant differences existed between the participants' characteristics of the ATP and CON groups and an analysis of variance was used to identify differences in participants' characteristics when grouped into genotypes. In all analyses significance was accepted when $p < 0.05$. No adjustment for multiple testing was made due to the rationale described in our previous studies (Posthumus et al. 2011). Hardy-Weinberg equilibrium was determined using Michael H. Court's (2005–2008) online calculator (www.tufts.edu). We used Quanto software (version 1.2) for power calculations and found that, for our entire cohort, the sample size was sufficient to detect associations with approximately 80% power at the $p < 0.05$ level with odds ratios of 2.2 and above.

RESULTS

The CON and ATP groups were matched for age, height, weight, and body mass index (BMI). However, when the data was stratified according to gender, we found a difference in height ($p = 0.023$) between the males ATP and CON groups. Males in the CON group were taller than those in the ATP group (table 1). However, height was not a confounding variable in our analysis as genotypes at both of the loci tested did not associate with height.

The allele and genotype distribution frequencies for the *MMP3* and *TIMP2* variants are shown in table 2 and 3 respectively. We found a significantly ($p = 0.021$) different genotype distribution frequency of the *MMP3* rs679620 variant between the CON (AA, 26.7%; AG, 54.2%; GG, 19.1%) and the RUP (AA, 24.0%; AG, 32.0%; GG, 44.0%) groups. The GG genotype was significantly over-represented among the RUP group ($p = 0.009$; OR=3.33; 95% CI 1.35 – 8.21), whereas the AG genotype was significantly over-represented among the CON group ($p = 0.046$; OR=2.51; 95% CI 1.01 – 6.23). However, the number of patients who presented with ruptures was small and, as such, this result would require confirmation in additional cohorts. In

addition, we found a significantly different ($p=0.038$) genotype distribution for the *TIMP2* rs4789932 variant in male participants between the CON (CC, 20.7%; CT, 59.8%; TT, 19.5%) and ATP (CC, 39.4%; CT, 43.7%; TT, 16.9%) groups. The CT genotype was significantly over-represented within the male CON group ($p=0.004$; OR=2.75; 95% CI 1.38 – 5.44), whereas the CC genotype was over represented in the ATP group ($p=0.012$; OR=2.49; 95% CI 1.22 – 5.09). A similar trend in distribution was found between the male CON and RUP groups ($p=0.038$).

There was no significant genotypic distribution difference for the *TIMP2* rs4789932 variant between the CON and ATP groups within female participants ($p=0.639$), or in the combined male plus female group ($p=0.364$). Furthermore, we report no genotypic distribution differences for the *MMP3* rs679620 variant between the CON and ATP groups ($p=0.413$), or between the CON and the TEN group ($p=0.564$) (table 2). We also report no allelic distribution difference for either gene variants between the CON and ATP groups (Table 2 and 3). All the genotype distribution groups included in this study were in Hardy-Weinberg equilibrium (HWE).

DISCUSSION

We have primarily found a significant association ($p=0.021$) between the *MMP3* rs679620 variant and Achilles tendon rupture, with the GG genotype being over represented in the RUP group compared to the CON group. In an earlier study we found that the GG genotype was a risk factor for Achilles tendinopathy but not Achilles tendon rupture in South Africans Caucasians (Raleigh *et al.* 2009). The G allele of the rs679620 locus is known to increase *MMP3* expression (Foster *et al.* 2012), as it is in high linkage disequilibrium (LD) with the 5A allele of the rs3025058 variant (Chen *et al.* 2012). The 5A allele has been reported to up-regulate *MMP3* mRNA levels (Ye 2006). Accordingly, over-expression of the *MMP3* gene could increase the risk of tendon degeneration since *MMP3* protein plays a role in the

breakdown of ECM substrates such as proteoglycan, decorin, and laminin (Birkedal-Hansen *et al.* 1993). Therefore, an accelerated or excessive MMP3 mediated destruction of some of these ECM components might predispose to tendon rupture.

We have also shown that the *TIMP2* rs4789932 variant is associated with the risk of ATP in British males. Specifically, the CC genotype was over represented in the ATP and RUP groups compared to an over representation of the CT genotype in the CON group. This finding differs with an earlier study in which the CC genotype was over represented among controls and the CT genotype associated with ATP cases in a combined Australian and South African Caucasian population (El Khoury *et al.* 2013). However, although the direction of the association found in this study (CC genotype as risk factor) was opposite to that reported in the earlier work (CC genotype as protective factor), the data might indicate that a complex mechanism underlies the involvement of this locus in the predisposition to ATP. Indeed it has recently been noted that reverse effects in genetic association studies may reveal additional information about a susceptibility locus that was previously unknown. For example, minor alterations in allele frequency between populations, differences in interacting loci, gene-gene, and gene-environment interactions may all contribute to a reverse effect, even in populations that are of the same ethnicity but drawn from different geographic locations (Greene *et al.* 2009). The reversal effect could also be due to a difference in LD between the associated and the causative variant between different populations of the same ethnicity (Ferreiros-Vidal *et al.* 2007). Furthermore, it is possible that genes in individuals from different regions undergo local adaptation following geographical and environmental isolation (Tiffin & Ross-Ibarra 2014; Sexton *et al.* 2014). This can sometimes be explained by the concept of conditional neutrality where an allele can be neutral in certain locations and risk related in a completely different setting (Fournier-Level *et al.* 2011). Such effects have been reported in

systemic lupus erythematosus, where the PD1.3 A allele of the *PDCD1* gene associates with risk related characteristics in some European Caucasian populations but it has protective characteristics in others (Ferreiros-Vidal et al. 2007; Ferreiros-Vidal et al. 2004). With relevance to this, the association described in this study was found in British Caucasian males whereas our previous work was conducted in a Caucasian population of both genders recruited from Australia and South Africa (El Khoury et al. 2013).

At present we cannot specifically explain why the *TIMP2* rs4789932 variant influences the risk of ATP or Achilles tendon rupture but it is noteworthy that a number of studies have found that *TIMP2* expression is altered in tendon tissue under a variety of conditions. For example, Jones et al. (2006) have shown that *TIMP2* expression is reduced in human degenerate Achilles tendon compared to healthy tissue. Furthermore ageing was found to significantly reduce the expression level of *TIMP2* in rabbit patellar tendons (Thornton et al. 2013). Hence, a reduction in *TIMP2* levels might contribute to tendon degradation since lower levels have been speculated to disrupt the *TIMP/MMP* balance and adversely alter ECM homeostasis (Pasternak et al. 2010). Interestingly, *TIMP2* expression in human ruptured Achilles tendon has been found to increase relative to non-rupture tendon samples (Karousou et al. 2008) and a study in rodents showed that overuse of the supraspinatus tendon was accompanied by an initial decrease in *TIMP2* levels followed by a subsequent rise (Attia et al. 2013). This latter observation may well explain the observed decrease in *TIMP2* during tendon degradation which apparently takes place at the pre-rupture stage (Jones et al. 2006). Consequently, a rupture may occur at an advanced stage of tendinopathy and thus might be accompanied by an increase in *TIMP2* levels.

In summary, this study follows the work previously conducted in South African and Australian Caucasian populations (Raleigh *et al.* 2009; El Khoury *et al.* 2013). These earlier studies showed that both the *MMP3* rs679620 and the *TIMP2* rs4789932 variants associated with ATP in the South African cohort (*MMP3* rs679620 variant) (Raleigh *et al.* 2009) and when both populations were combined (*TIMP2* rs4789932 variant) (El Khoury *et al.* 2013). Our present study in British Caucasians reveals that the association of the *TIMP2* rs4789932 variant with ATP and Achilles tendon rupture is only apparent in males. Furthermore, we found that it was carriage of the CC genotype that inferred risk as opposed to the CT genotype in the combined South African and Australian cohorts which inferred risk of ATP but not Achilles tendon rupture. Finally, for the *MMP3* rs679620 variant, we found a tentative association between the GG genotype with Achilles tendon rupture but not with tendinopathy *per se*.

To conclude, although this study did not directly replicate the direction of associations we found in earlier work (Raleigh *et al.* 2009; El Khoury *et al.* 2013) it was confirmed that the same loci seemed to modify the risk of Achilles tendon pathology or rupture in some way. This may well reflect the fact that the current work was conducted in a population recruited from a different geographic region compared to our earlier work. In terms of limitations, we must treat these data as tentative findings and remember that confidence in our data would be greater if the work was repeated in a larger cohort. A specific limitation was that sample size was reduced when we conducted gender specific analysis and when we stratified the ATP cohort into the separate sub groups (tendinopathy or rupture). This would result in a reduction in statistical power. Finally, these findings suggest that the risk of ATP phenotypes, associated with the loci investigated, are modified by both gender and geographical location.

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Tables

Table 1. General biological characteristics of the British Achilles tendon pathology (ATP) group and the asymptomatic control (CON) group, as well as the gender sub-groupings

				Female			Males		
	CON (n=131)	ATP (n=118)	P-value	CON (n=49)	ATP (n=47)	P-value	CON (n=82)	ATP (n=71)	P-value
Age	41.7 ± 11.6 (124)	43.7 ± 13.8 (117)	0.225	44.3 ± 14.4 (48)	43.3 ± 13.2 (46)	0.691	40.0 ± 11.5 (76)	43.9 ± 14.4 (71)	0.071
Gender (Male)	62.6 (82)	60.2 (71)	0.695						
Height (cm)	174.9 ± 10.4 (123)	172.9 ± 9.2 (115)	0.122	165.5 ± 7.1 (48)	165.0 ± 5.6 (46)	0.731	180.9 ± 7.2 (75)	178.2 ± 7.1 (69)	0.023
Weight (kg)	80.1 ± 19.6 (124)	78.7 ± 15.6 (86)	0.544	63.7 ± 10.9 (48)	67.6 ± 11.0 (35)	0.111	90.6 ± 16.4 (76)	86.3 ± 13.7 (51)	0.126
BMI (kg/m²)	25.9 ± 4.5 (123)	26.3 ± 4.1 (86)	0.543	23.2 ± 3.5 (48)	24.9 ± 4.7 (35)	0.061	27.6 ± 4.4 (75)	27.2 ± 3.3 (51)	0.558

Values are expressed as mean ± SD or a frequency (%). The total number of participants (n). The maximum number (n) of participants in each category is also indicated at the top of each column.

^a age of the ATP, TEN, and RUP groups is at the age of initial injury, while the age of the CON groups is at the age of recruitment.

^b weight of the ATP, TEN, and RUP groups at the time of initial injury, while the weight of the CON groups is at the time of recruitment

cm, centimetres; kg, kilograms; m, meters

Table 2. The genotype and allele frequency distribution of *MMP3* rs679620 variant within the British control (CON) and Achilles tendon pathology (ATP) groups, as well as the chronic Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) sub-groups.

					Female				Male			
	CON	ATP	TEN	RUP	CON	ATP	TEN	RUP	CON	ATP	TEN	RUP
<i>MMP3</i> rs679620	n=131	n=118	n=93	n=25	n=49	n=47	n=39	n=8	n=82	n=71	n=54	n=17
AA	26.7 (35)	31.4 (37)	33.3 (31)	24.0 (6)	22.6 (12)	31.9 (15)	33.3 (13)	25.0 (2)	28.0 (23)	31.0 (22)	33.3 (18)	23.5 (4)
AG	54.2 (71)	45.8 (54)	49.5 (46)	32.0 (8)	61.2 (30)	46.8 (22)	48.7 (19)	37.5 (3)	50.0 (41)	45.1 (32)	50.0 (27)	29.4 (5)
GG	19.1 (25)	22.9 (27)	17.2 (16)	44.0 (11)	14.3 (7)	21.3 (10)	17.9 (7)	37.5 (3)	22.0 (18)	23.9 (17)	16.7 (9)	47.1 (8)
P Value	0.413 ^a		0.564 ^b	0.021^c	0.358 ^a		0.499 ^b	0.248 ^c	0.831 ^a		0.684 ^b	0.122 ^c
HWE	0.301	0.396	0.879	0.056	0.097	0.716	0.990	0.500	0.973	0.428	0.834	0.120
G allele	46.2 (121)	45.8 (108)	41.9 (78)	60.0 (30)	44.9 (44)	44.7 (42)	42.3 (33)	56.3 (9)	47.0 (77)	46.5 (66)	41.7 (45)	61.8 (21)
P Value	0.925 ^a		0.373 ^b	0.073 ^c	0.976 ^a		0.731 ^b	0.399 ^c	0.934 ^a		0.391 ^b	0.116 ^c

Values are expressed as a frequency with the number of participants (n) in parentheses.

^a CON vs ATP

^b CON vs TEN

^c CON vs RUP

Table 3 The genotype and allele frequency distribution of *TIMP2* rs4789932 variant within the British control (CON) and Achilles tendon pathology (ATP) groups, as well as the chronic Achilles tendinopathy (TEN) and Achilles tendon rupture (RUP) sub-groups

					Female				Male			
	CON	ATP	TEN	RUP	CON	ATP	TEN	RUP	CON	ATP	TEN	RUP
<i>TIMP2</i> rs4789932	n=131	n=118	n=93	n=25	n=49	n=47	n=39	n=8	n=82	n=71	n=54	n=17
CC	26.7 (35)	34.7 (41)	34.4 (32)	36.0 (9)	36.7 (18)	27.7 (13)	30.8 (12)	12.5 (1)	20.7 (17)	39.4 (28)	37.0 (20)	47.1 (8)
CT	54.2 (71)	46.6 (55)	48.4 (45)	40.0 (10)	44.9 (22)	51.1 (24)	48.7 (19)	62.5 (5)	59.8 (49)	43.7 (31)	48.1 (26)	29.4 (5)
TT	19.1 (25)	18.6 (22)	17.2 (16)	24.0 (6)	18.4 (9)	21.3 (10)	20.5 (8)	25.0 (2)	19.5 (16)	16.9 (12)	14.8 (8)	23.5 (4)
P Value	0.364 ^a		0.464 ^b	0.425 ^c	0.636 ^a		0.841 ^b	0.525 ^c	0.038^a		0.112 ^b	0.038^c
HWE	0.301	0.640	0.979	0.346	0.621	0.861	0.924	0.445	0.077	0.500	0.924	0.120
T allele	46.2 (121)	41.9 (99)	41.4 (77)	44.0 (22)	40.8 (40)	46.8 (44)	44.9 (35)	56.3 (9)	49.4 (81)	38.7 (55)	38.9 (42)	38.2 (13)
P Value	0.342 ^a		0.315 ^b	0.776 ^c	0.403 ^a		0.589 ^b	0.248 ^c	0.061 ^a		0.087 ^b	0.236 ^c

Values are expressed as a frequency with the number of participants (n) in parentheses.

^a CON vs ATP

^b CON vs TEN

^c CON vs RUP

Abbreviations

ATP:	Achilles tendon pathology
ECM:	Extra cellular Matrix
MMP:	Matrix metalloproteinase
RUP:	Rupture
TEN:	Tendinopathy
TIMP2:	Tissue inhibitors of metalloproteinases

Highlights

- *MMP3* and *TIMP2* gene variants associate with Achilles tendon pathology phenotypes.
- Males carrying a *TIMP2* genetic variant are more at risk than females.
- Variability within *MMP3* and *TIMP2* genes might interact with geographic location to modify risk.