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Promoter methylation status of the *TIMP2* and *ADAMTS4* genes and patellar tendinopathy

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Abstract

Objectives Patellar tendinopathy (PT) is a debilitating and prevalent condition that tends to affect those who are physically active or engaged in jumping sports. Although tendinopathies are known to have a genetic basis, the role of DNA methylation as an epigenetic factor and risk determinant for human PT has never been described.

We sought to determine whether differences existed between the methylation profiles of both the *TIMP2* and *ADAMTS4* gene promoter sequences in a cohort of males having undergone surgery for patellar tendinopathy compared to controls.

Design Case-control epigenetic study using DNA from 10 males with PT and 10 males with healthy tendons.

Methods We used PCR and targeted pyrosequencing to interrogate the methylation profiles of CpG sites upstream of both the *TIMP2* (4 sites) and *ADAMTS4* (6 sites) genes. We compared methylation differences between the two groups using t-tests.

Results We report no significant (p>0.05) methylation differences within the *TIMP2* gene promoter between the PT group and controls across the 4 CpG sites investigated. In contrast, we detected a significant (p=0.016) difference in the methylation status of 1 CpG site, approximately 3 Kb upstream of the *ADAMTS4* gene between the PT group and controls.

Conclusions To our knowledge, this is the first study to investigate how DNA methylation impacts on the risk of human tendinopathy. Our data indicate that the methylation status of the *ADAMTS4* gene is altered in patellar tendinopathy and we speculate on how this change might modify the patellar tendon extra-cellular matrix environment.

KEY WORDS: Tendon, genetics, epigenetics, sports injury, tendinopathy

Introduction

Patellar tendinopathy (PT), also known as jumper's knee, is an activity-dependant chronic condition characterised by anterior pain to the knee[1]. Injuries to the patellar tendon are common[2,3]. They account for nearly 30% of knee injuries in US high school athletes[4] and are prevalent amongst volleyball (45%) and basketball (32%) players alike[1]. PT is also found in military cohorts and the injury can lead to prolonged periods of disability[5]. Both tendon neovascularisation and thickening are associated with PT injuries[6,7]. Furthermore, some authors report the injury to associate with an increase in collagen type III fibres along with elevated levels of glycosaminoglycans[8]. A number of risk factors for PT have been tentatively reported in the literature such as increased weight and BMI[9] and there is a growing body of evidence to suggest that genetics can play a role in the predisposition to human tendinopathy[9,10]. However, to date, and to our knowledge, no single study has

investigated the role of human DNA methylation (as an epigenetic factor) in sports injury phenotypes like PT.

DNA methylation occurs when a methyl group binds to a cytosine nucleotide base converting it to 5-methylcytosine[11]. This takes place predominantly in CpG rich regions (CpG islands) which are typically located in the promoter regions of genes. DNA methylation tends to down regulate gene expression by interfering with the accessibility of genes to transcription factors[12]. Hence, methylation can profoundly affect transcription levels and an epigenetic mode of inheritance might significantly modify the effect of genotype upon phenotype. Furthermore, methylation results from either directionally programmed changes, part of the ageing process, or from stochastic and spontaneous alterations attributed to environmental factors [13,14]. It is difficult to predict, as of yet, the reason why and at which rate some non-age-associated CpG sites undergo changes in methylation.

The *TIMP2* (tissue inhibitor of metalloproteinase 2) gene encodes a protein that has an important role in extra-cellular matrix (ECM) homeostasis as it degrades a number of metalloproteinases (MMPs)[15]. Recently, a single nucleotide polymorphism (SNP) within the *TIMP2* promoter (rs4789932) was found to associate with Achilles tendon pathology (ATP)[15] where *TIMP2* expression levels are altered. For example, Jones and colleagues (2006) reported a decrease in *TIMP2* expression in tendinopathic tissue compared to healthy tendons[16] while Karousou et al. (2010) reported the opposite effect[17]. Interestingly, *TIMP2* expression is under tight epigenetic control and hypermethylation has been shown to switch off transcription[18]. However, the effect of *TIMP2* methylation on the risk of PT has not been investigated.

The *ADAMTS4* (A disintegrin and metalloproteinase with thrombospondin motifs 4) gene encodes a protein that degrades aggrecan and a number of other components of the ECM[19]. *ADAMTS4* expression has been found to increase in knee osteoarthritis and this might

exacerbate the condition by contributing to the elevated degradation of proteogylcans[19]. *ADAMTS4* mRNA expression levels have been shown to differ in ruptured Achilles tendons compared with normal and tendinopathic tendons[20]. Furthermore, in an equine tendon model *ADAMTS4* expression level decreased in response to surgical transection. The decrease paralleled an increase in aggrecan, versican and lumican[21]. Hence, the expression of *ADAMTS4* likely plays an important role in the tendinopathic process but, as yet, our understanding of how *ADAMTS4* expression is controlled in tendinopathy is unclear. Furthermore, like *TIMP2, ADAMTS4* is under epigenetic control and loss of methylation has been shown to be responsible for the upregulation of ADAMTS4 protein in osteoarthritic cartilage[22].

It is known that an individual's methylation profile may change in response to physical activity[23]. However, somewhat surprisingly, the role that DNA methylation plays, as an epigenetic factor, has never been investigated in relation to human tendinopathy. With this in mind, the aims of the present study were to establish, for the first time, whether epigenetics, in particular DNA methylation, was changed in tissue isolated from patellar tendinopathy compared to healthy tissue. Our study specifically focused on the DNA methylation statuses of the *TIMP2* and *ADAMTS4* genes in a targeted approach as both, as discussed above, undergo changes in expression during tendinopathy but the mechanisms underlying these changes are unknown.

Material and methods

Patellar tendon tissue was obtained from 10 male participants with healthy patellar tendons (CON) undergoing ACL reconstruction surgeries using a patellar tendon graft. We also obtained 10 patellar tendinopathy (PT) samples from males selected for surgical debridement for recalcitrant overuse patellar tendinopathy. All tissue was immediately stored at -80^{o}C until processing. The DNA samples used in this study were collected from

participants recruited from 2003-2005 that were originally described elsewhere in studies focusing only on proteogylcan and RNA levels[24]. Participant information was obtained from surgery theatre lists and was limited to age, sex and ethnicity. Unfortunately, additional demographic information such as height, weight, BMI and other relevant medical history were not made available to us. Hence our cohort should be considered as a convenience sample. All participants were otherwise healthy Caucasians aged from 19 to 41 years and gave written informed consent. Approval was obtained from the University of Northampton's School of Health Research Ethics Committee and La Trobe University Human Ethics Committee (Application number 12-086).

DNA was obtained from all patellar samples using the PureLink® Genomic DNA Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. The DNA quantity was determined using a NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). The DNA samples (50 ng), in addition to a universal non-methylated control DNA sample from the EpiTect® PCR Control DNA Set (Qiagen, Hilden, Germany), were bisulfite treated using the EpiTect® fast DNA bisulphite Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. The Pyrosequencing assays for the *TIMP2* (Hs_TIMP2_01_PM) and *ADAMTS4 (*Hs_NDUFS_01_PM) genes used in this study were selected from the pre-designed PyroMark® CpG Assays (Qiagen, Hilden, Germany). The single assay designed for the CpG island within the *TIMP2* promoter region measured methylation at 4 CpG sites. (-488 bp, -478 bp, -467 bp, and -455 bp from the first exon). The assay chosen for the CpG sites within the promoter region of the *ADAMTS4* gene was selected to cover a wider area of the promoter region and spanned 6 CpG sites that were -2964 bp, -2973 bp, -2995 bp, -2998 bp, -3000 bp and -3002 bp from the first exon. We took a pragmatic approach to the choice of CpG sites that we investigated. In some cases it was not possible to study certain regions of the promoters as sequencing assays did not meet quality control standards. However, as *ADAMTS4* is regulated by a large promoter

sequence[25], we were especially interested to determine how methylation of CpG sites that are distant to the transition start site impacted on injury risk.

All bisulfite converted DNA samples in addition to hypermethylated and hypomethylated controls were PCR amplified using the pre-designed primer sets of the PyroMark® CpG Assays (Qiagen, Hilden, Germany). A Techne TC-512 thermocycler (Bibby Scientific Ltd, Staffordshire, UK) was used to amplify the target sequences according to the manufacturer protocol. To confirm successful PCR amplification the products were run on a 1x agarose gel alongside a GeneRuler™ 100 bp ladder (MBI Fermentas, UK). The biotinylated PCR products were then immobilised to Streptavidin-coated Sepharose high-performance beads (GE Healthcare, Buckinghamshire, UK) according to the PyroMark® Q24 manufacturer's recommendations (Qiagen, Hilden, Germany). The immobilised PCR products were captured using the PyroMark® Q24 vacuum (Qiagen, Hilden, Germany) and released into a 24 well PyroMark® Q24 plate containing 1x sequencing primer diluted in 25 µl PyroMark® Annealing Buffer. Before placing the Q24 plate into the pyrosequencer, the sequencing primer was allowed to anneal on a preheated hot-plate at 80 °C for 2 min followed by room temperature incubation for 10 min. Following pyrosequencing, pyrograms were visualised and percentage methylation of the CpG sites was determined using the PyroMark® Q24 v2.0.6 software. Data analysis was performed using SPSS Version 20 program (IBM Corp. Armonk, NY). A t-test was used to compare the mean percentage methylation of each CpG site between the CON and the PT samples for both the *TIMP2* and *ADAMTS4* CpG sites. Significant differences in methylation were called when $p<0.05$. Correction for multiple testing was not conducted for reasons previously described by Posthumus et al (2011) [26]

Results

PCR products for the *TIMP2* and *ADAMTS4* promoter sequences investigated were detected as single bands with the expected amplicon lengths (154 bp and 244 bp respectively) as shown in figure 1. Typical pyrograms obtained showing percent methylation

at the 4 CpG sites for *TIMP2* (panels A and B) and the 6 CpG sites for *ADAMTS4* (panels C and D) can be seen in figure 2. In our cohort the CON and PT groups were similarly matched for age (p=0.449) and gender. For *TIMP2* we found no significant difference (p=0.885) in the mean methylation percentages across all 4 sites between the CON and the PT groups. Furthermore, there was no significant difference in methylation for any of the 4 CpG sites when analysed independently between the CON and the PT groups as shown in figure 3. However, for the *ADAMTS4* gene, CpG site 3 was significantly (P=0.016) more methylated in the PT group compared to the CON group (figure 3). Further data analysis showed no significant difference between CON and PT in the remaining 5 sites when analysed independently (figure 3), or for the mean percentages of methylation across the 6 sites (p=0.294).

Discussion

To our knowledge, this is the first study to investigate DNA methylation differences in clinically resected human tendinopathy samples compared to healthy tendon tissue. Unlike the recent study of Trella and co-workers[27] who used a genome wide design in a mouse tendinopathy study, we employed a hypothesis-driven, targeted approach to study methylation profiles of two genes (*TIMP2* and *ADAMTS4*) that have been associated with the tendinopathic process in human[15,20].

TIMP2 is known to be under epigenetic control with a CpG island in the promoter region of the gene extending from the transcription initiation site upstream for approximately 900 bp[18]. The methylation of the CpG sites located between positions -295 and -145 bp was found to reduce the expression of *TIMP2* in prostate cancer samples[18]. Likewise, the overlapping region ranging from -350 to -240 bp was also found to be over-methylated in cervical cancer[28]. However, our work showed that there were no methylation changes within the CpG Island ranging from position -488 to -455 between the CON and PT tissue samples.

Moreover, all analysed samples showed very low methylation levels which might infer that this region of the *TIMP2* promoter is not involved in regulating *TIMP2* expression or deregulation during the process of patellar tendinopathy. Furthermore, the lack of change in *TIMP2* methylation status between PT tissue and controls shown here is consistent with the absence of *TIMP2* expression level changes between PT and control tissue previously shown by Parkinson et al (2010)[24]. The fact that we found no difference in *TIMP2* methylation status between PT tissue and controls it interesting. This might suggest that previously observed differences in *TIMP2* expression in Achilles tendinopathy[17] are not an active part of the tendinopathic process but possibly no more than an artefact of the injury. Of course, we only interrogated a small number of CpG sites for *TIMP2* in this study and investigating a larger area might well have revealed significant differences between the PT and CON samples.

Of the 6 CpG sites investigated 3 kb upstream of the *ADAMTS4* gene we found one site, located at position -2995, that was significantly ($p=0.016$) more methylated in the PT group compared to the CON group. Although only one of the 6 CpG sites was more methylated in the tendinopathic tissue compared to controls, the impact of this when considering the risk of patellar tendinopathy might be far reaching. To be specific, the *ADAMTS4* gene is known to be under epigenetic control[22] and regulated by a large promoter (-4109 to +406) that consists of two NFAT and several Runx binding elements[25]. One of the NFAT binding regions resides only 103 bp away from the CpG site that we investigated and it is plausible that a change in methylation status at that site, as measured by us, could affect the binding of NFAT transcription factors and alter the expression of *ADAMTS4*. Interestingly, an increase in the accumulation of proteoglycans including aggrecan was found previously in PT tissue taken from the participants in this study[24] and others[29]. However, somewhat surprisingly, *ADAMTS4* levels in this patient cohort did not differ compared to controls[24]. Recently, the expression of *ADAMTS4* was shown to decrease, in a region specific manner, in an ovine model of tendinopathy[30]. The reduction in *ADAMTS4* expression was found to be

commensurate with an increase in aggrecan[30]. Therefore, we speculate that in some situations the accumulation of proteoglycans such as aggrecan may be explained, at least in part, by reduced levels of ADAMTS4 caused by hypermethylation at CpG sites within the *ADAMTS4* gene promoter. It is also worth noting that methylation change at single CpG sites can have profound effects on phenotypes. For example, Fürst and colleagues[31] have observed this phenomenon with respect to the expression of estrogen receptor alpha (*ESR1*) and methylation changes at single sites upstream of the *SLC23A2* and *NCOR2* genes have been shown to influence the severity of spinal muscular atrophy[32].

Although our study was the first, to our knowledge, to report DNA methylation changes associated with human tendinopathy there were some limitations. Firstly, although our participants were all male with no significant age differences between the PT cases and controls we were unable to control for height, weight or level of physical activity in our dataset as this information was not available to us. The acquisition of such data in future experiments would improve confidence in the interpretation of the results. Secondly, our study was limited to a small number of clinical samples that were available from Caucasian men. Therefore, the changes we observed may not represent methylation changes that might occur in other populations. Hence, this work should be validated in larger, non-Caucasian cohorts and in female patients. Finally, this study has revealed that an over-methylation event in the promoter sequence of the *ADAMTS4* gene associates with patellar tendinopathy. The hypermethylated CG site sits close to an important DNA regulatory element and might influence risk of tendinopathy by modifying *ADAMTS4* levels leading to structural or regulatory changes within the ECM components of the tendon. Such changes might predispose an individual to develop the injury.

Conclusions

We provide the first evidence that DNA methylation (an epigenetic factor) within the promoter sequence of the human *ADAMTS4* gene might be important in patellar tendinopathy. Our data have clear implications as set out below:

Practical implications

- As DNA methylation changes are modified by the environment it might be possible, in the future, to alter methylation status, through adaptions to an athlete's training programme, to reduce the risk of tendinopathy.

- DNA methylation changes might well explain discrepant gene association findings that have been observed in some studies on human tendinopathy.

- DNA methylation, as an epigenetic factor, should be considered as a potential modifier of genotype and other biochemical factors when considering risk of tendinopathy.

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References

1 Lian Ø, Holen KJ, Engebretsen L, et al. Relationship between symptoms of jumper's knee and the ultrasound characteristics of the patellar tendon among high level male volleyball players. *Scand J Med Sci Sports* 1996; 6(5):291–296.

2 Kujala UM, Kvist M, Österman K. Knee injuries in athletes. *Sport Med* 1986; 3(6):447–460.

3 King J, Cook J, Khan K, et al. Patellar Tendinopathy. *Sport Med* 2000; 8(1):86–95.

4 Swenson DM, Collins CL, Best TM, et al. Epidemiology of knee injuries among U.S.

high school athletes, 2005/2006-2010/2011. *Med Sci Sports Exerc* 2013; 45(3):462–469. Doi: 10.1249/MSS.0b013e318277acca.

5 Rutland M, O'Connell D, Brismée J-M, et al. Evidence-supported rehabilitation of patellar tendinopathy. *N Am J Sports Phys Ther* 2010; 5(3):166–178.

6 Schwartz A, Watson JN, Hutchinson MR. Patellar Tendinopathy. *Sport Heal A Multidiscip Approach* 2015; 7(5):415–420. Doi: 10.1177/1941738114568775.

7 Zhang ZJ, Ng GYF, Lee WC, et al. Changes in morphological and elastic properties of patellar tendon in athletes with unilateral patellar tendinopathy and their relationships with pain and functional disability. *PLoS One* 2014; 9(10). Doi: 10.1371/journal.pone.0108337.

8 Rosso F, Bonasia DE, Cottino U, et al. Patellar tendon: From tendinopathy to rupture. *Asia-Pacific J Sport Med Arthrosc Rehabil Technol* 2015; 2(4):99–107. Doi: 10.1016/j.asmart.2015.07.001.

9 Collins M, Raleigh SM. Genetic Risk Factors for Musculoskeletal Soft Tissue Injuries. *Med Sport Sci* 2009; 54:136–149.

10 Collins M, September A V, Posthumus M. Biological variation in musculoskeletal injuries : current knowledge , future research and practical implications. *Br J Sports Med* 2015; 49:1–8. Doi: 10.1136/bjsports-2015-095180.

11 Robertson KD. DNA methylation and human disease. *Nat Rev Genet* 2005; 6(8):597–610.

12 Golbabapour S, Abdulla MA, Hajrezaei M. A concise review on epigenetic regulation: Insight into molecular mechanisms. *Int J Mol Sci* 2011; 12(12):8661–8694.

13 Ashapkin V V, Kutueva LI, Vanyushin BF. Aging Epigenetics : Accumulation of Errors or Realization of a Specific Program ? *Biochem* 2015; 80(11):1406–1417. Doi: 10.1134/S0006297915110024.

14 Zampieri M, Ciccarone F, Calabrese R, et al. Reconfiguration of DNA methylation in aging. *Mech Ageing Dev* 2015; 151:1–11. Doi: 10.1016/j.mad.2015.02.002.

15 El Khoury L, Posthumus M, Collins M, et al. Polymorphic variation within the ADAMTS2, ADAMTS14, ADAMTS5, ADAM12 and TIMP2 genes and the risk of Achilles tendon pathology: A genetic association study. *J Sci Med Sport* 2013; 16(6):493–498. Doi: 10.1016/j.jsams.2013.02.006; 10.1016/j.jsams.2013.02.006.

16 Jones GC, Corps AN, Pennington CJ, et al. Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human achilles tendon. *Arthritis Rheum* 2006; 54(3):832–842. Doi: 10.1002/art.21672.

17 Karousou E, Ronga M, Vigetti D, et al. Collagens, proteoglycans, MMP-2, MMP-9 and TIMPs in human achilles tendon rupture RID C-9861-2010. *Clin Orthop Relat Res* 2008; 466(7):1577–1582. Doi: 10.1007/s11999-008-0255-y.

18 Pulukuri SM, Patibandla S, Patel J, et al. Epigenetic inactivation of the tissue inhibitor of metalloproteinase-2 (TIMP-2) gene in human prostate tumors. *Oncogene* 2007; 26(36):5229–5237.

19 Shiraishi A, Mochizuki S, Miyakoshi A, et al. *Development of human neutralizing antibody to ADAMTS4 (aggrecanase-1) and ADAMTS5 (aggrecanase-2)*. vol. 469. 2016.

20 Corps AN, Jones GC, Harrall RL, et al. The regulation of aggrecanase ADAMTS-4 expression in human Achilles tendon and tendon-derived cells. *Matrix Biol* 2008; 27(5):393–401.

21 Jacobsen E, Dart AJ, Mondori T, et al. Focal Experimental Injury Leads to Widespread Gene Expression and Histologic Changes in Equine Flexor Tendons. *PLoS One* 2015; 10(4):e0122220. Doi: 10.1371/journal.pone.0122220.

22 Cheung KSC, Hashimoto K, Yamada N, et al. Expression of ADAMTS-4 by chondrocytes in the surface zone of human osteoarthritic cartilage is regulated by epigenetic DNA de-methylation. *Rheumatol Int* 2009; 29(5):525–534. Doi: 10.1007/s00296-008-0744-z.

23 Raleigh SM. Epigenetic regulation of the ACE gene might be more relevant to endurance physiology than the I/D polymorphism. *J Appl Physiol* 2012; 112(6):1082 LP-1083.

24 Parkinson J, Samiric T, Ilic MZ, et al. Change in proteoglycan metabolism is a characteristic of human patellar tendinopathy. *Arthritis Rheum* 2010; 62(10):3028–3035.

25 Thirunavukkarasu K, Pei Y, Moore TL, et al. Regulation of the human ADAMTS-4 promoter by transcription factors and cytokines. *Biochem Biophys Res Commun* 2006; 345(1):197–204. Doi: 10.1016/j.bbrc.2006.04.023.

26 Posthumus M, Collins M, van der Merwe L, et al. Matrix metalloproteinase genes on chromosome 11q22 and the risk of anterior cruciate ligament (ACL) rupture. *Scand J Med Sci Sports* 2011; 22(4):523–533. Doi: 10.1111/j.1600-0838.2010.01270.x.

27 Trella KJ, Li J, Stylianou E, et al. Genome-wide analysis identifies differential promoter methylation of Leprel2, Foxf1, Mmp25, Igfbp6, and Peg12 in murine tendinopathy. *J Orthop Res* 2016. Doi: 10.1002/jor.23393.

28 Ivanova T, Vinokurova S, Petrenko A, et al. Frequent hypermethylation of 5′ flanking region of TIMP-2 gene in cervical cancer. *Int J Cancer* 2004; 108(6):882–886.

29 Attia M, Scott A, Carpentier G, et al. Greater glycosaminoglycan content in human patellar tendon biopsies is associated with more pain and a lower VISA score. *Br J Sports Med* 2014; 48(6):469–475. Doi: 10.1136/bjsports-2013-092633.

30 Smith MM, Sakurai G, Smith SM, et al. Modulation of aggrecan and ADAMTS

expression in ovine tendinopathy induced by altered strain. *Arthritis Rheum* 2008; 58(4):1055–1066. Doi: 10.1002/art.23388.

31 Fürst RW, Kliem H, Meyer HHD, et al. A differentially methylated single CpG-site is correlated with estrogen receptor alpha transcription. *J Steroid Biochem Mol Biol* 2012; 130(1):96–104. Doi: 10.1016/j.jsbmb.2012.01.009.

32 Zheleznyakova GY, Nilsson EK, Kiselev A V., et al. Methylation Levels of SLC23A2 and NCOR2 Genes Correlate with Spinal Muscular Atrophy Severity. *PLoS One* 2015; 10(3):e0121964. Doi: 10.1371/journal.pone.0121964.

FIGURE 1. Agarose gel (1%) electrophoresis images of the *TIMP2* and *ADAMTS4* gene promoter sequences investigated. **Panel A** (*TIMP2*): Well 1, 100 bp ladder; well 2, no DNA (negative control); well 3 and 4, control samples; wells 5 and 6, DNA from PT; well 7, hypermethylated control; well 8, hypomethylated control; well 9, no DNA (negative control) and well 10 bisulphite converted hypomethylated control. **Panel B** (*ADAMTS4*): Well 1, 100 bp ladder; wells 2 and 4, control DNA samples; wells 5 to 7, DNA from PT; well 8, hypermethylated control; well 9, hypomethyated control; well 10, bisulfite converted hypomethylated control and well 11, no DNA (negative control). Gels were run as described in the Materials and Methods section.

FIGURE 2. Typical pyrograms showing the methylation status of the *TIMP2* and *ADAMTS4* gene promoter sequences investigated from both control and PT patients. **Panel A:** Percent methylation at each of the four CpG sites within the *TIMP2* gene promoter from healthy tendon. **Panel B:** Percent methylation at each of the four CpG sites within the *TIMP2* gene promoter from a patient with patellar tendinopathy. **Panel C:** Percent methylation at each of the six CpG sites within the *ADAMTS4* gene promoter from healthy tendon. **Panel D:** Percent methylation at each of the six CpG sites within the *ADAMTS4* gene promoter from a patient with patellar tendinopathy. In each figure the grey boxes indicate the percentage of each CpG site that is methylated in the sample that is run.

FIGURE 3. Bar graphs showing the mean (± SD) percent methylation status at each of the CpG sites investigated within the *TIMP2* (Panel A) and *ADAMTS4* (Panel B) gene promoter sequences. For both genes the CpG sites for healthy tendons are shown as white bars and those for tendinopathic tendons are shown as black bars.

*p=0.016.

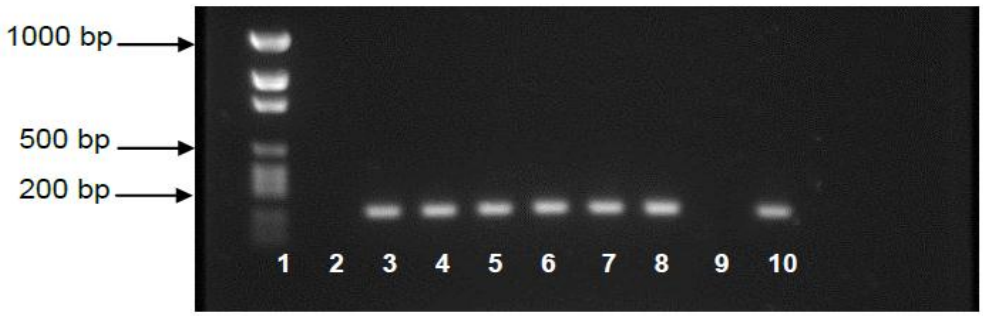

FIGURE 1 Panel A

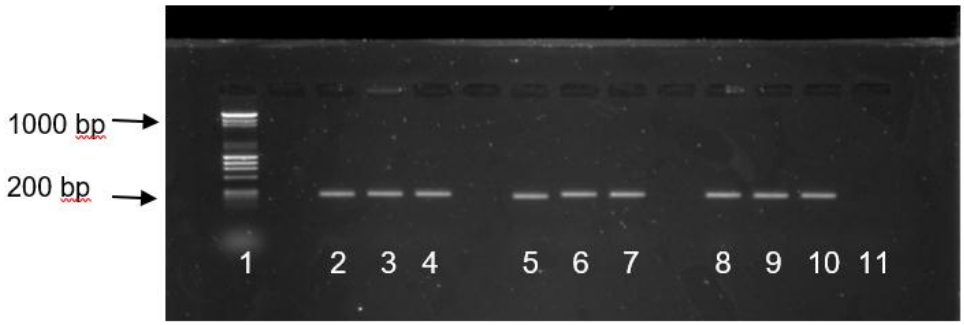

FIGURE 1 Panel B

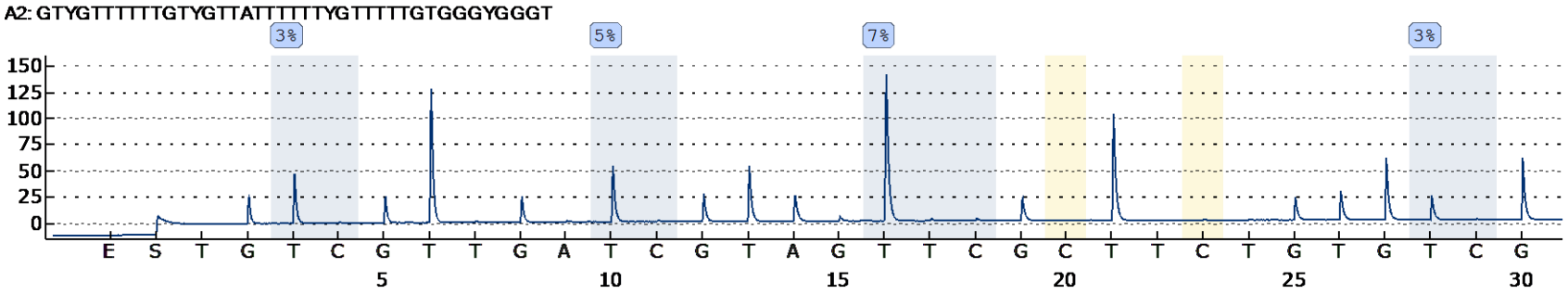

FIGURE 2 Panel A

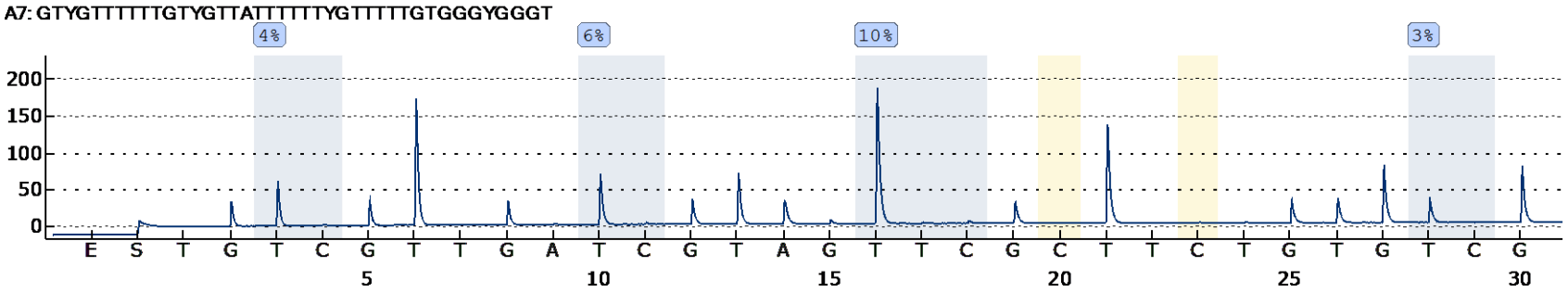

FIGURE 2 Panel B

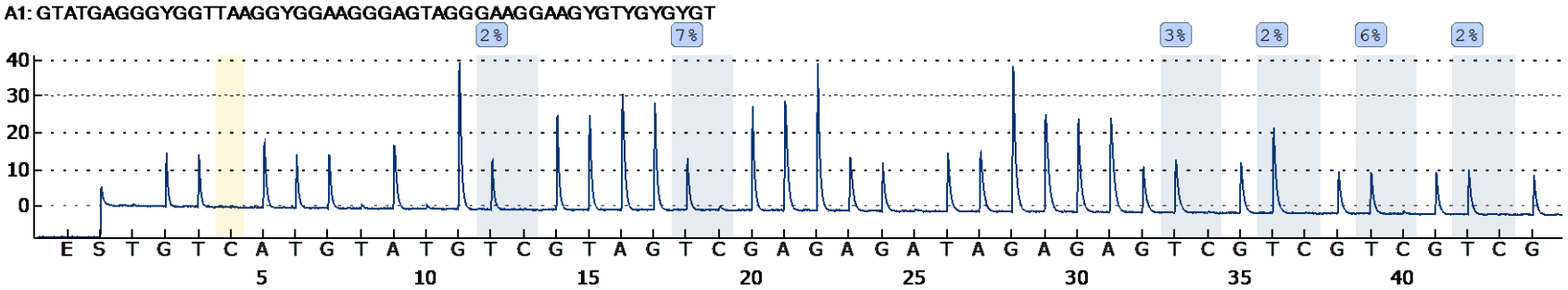

FIGURE 2 Panel C

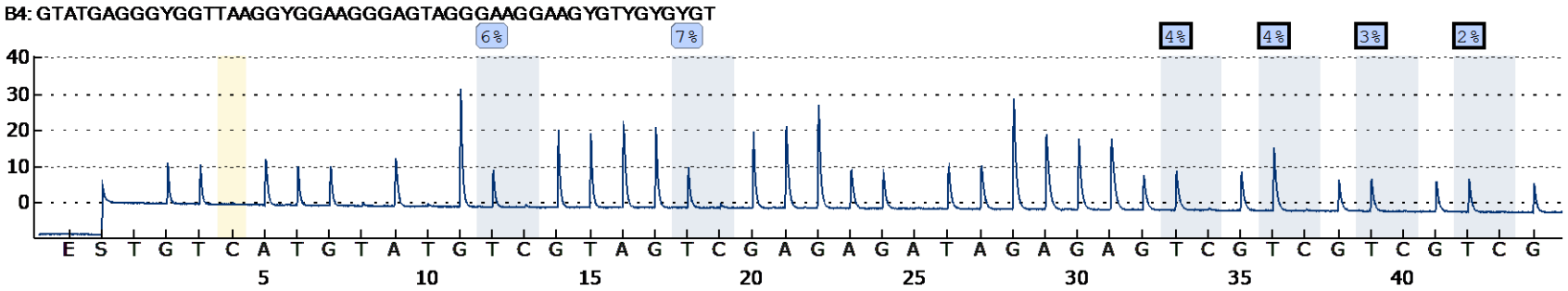

FIGURE 2 Panel D

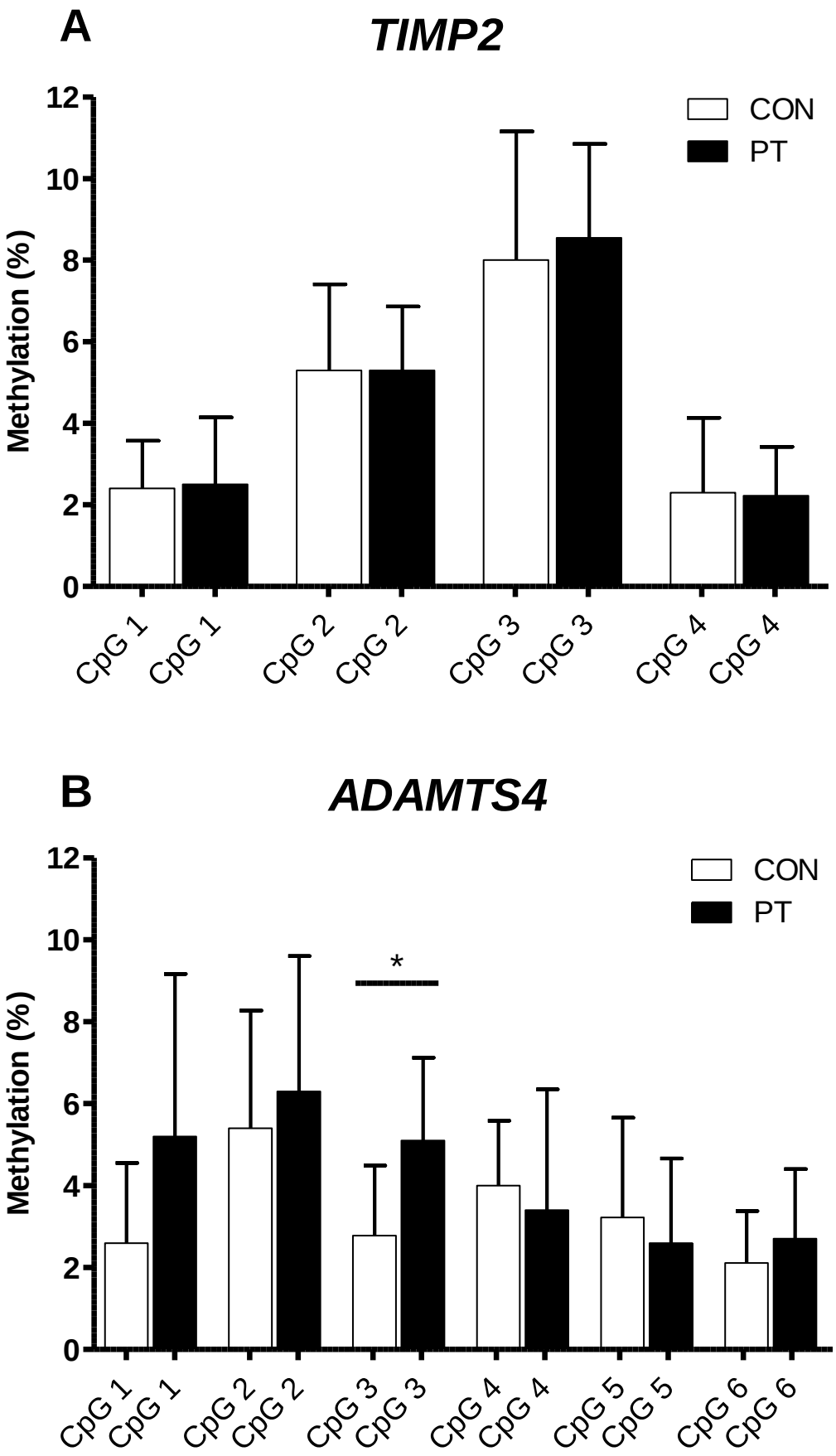

FIGURE 3