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 ($\chi^2$) 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:
1. Introduction

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

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

The \textit{TNFRSF1A} gene encodes tumour necrosis factor receptor 1 (TNFR1), a cell receptor that can signal apoptosis in response to the pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-\(\alpha\))\(^11,12\). TNFR1 mRNA and protein have recently been identified in human Achilles tendon\(^12\) and in cultured tenocytes\(^13\). Importantly TNFR1 is known to be highly expressed in tenocytes isolated from Achilles tendinosis\(^12\) but the functional or pathologic significance of this is not clear. Single nucleotide polymorphisms (SNPs) within the \textit{TNFRSF1A} gene, especially the rs4149577 variant, have previously been associated with other musculoskeletal\(^14\) and inflammatory diseases\(^15\). However, the role of this variant as a risk factor for ATP has not been investigated.
Caspases form a family of proteases that are important in the regulation of apoptosis\(^\text{16}\). Caspase-3 can selectively cleave target proteins after aspartate residues in their primary sequence\(^\text{16,17}\). Although apoptosis can be activated through a number of complex pathways, caspase-3 appears to have a critical role in chromatin condensation and DNA fragmentation\(^\text{18}\). The \textit{CASP3} rs1049253 variant resides within the 3' untranslated region (UTR) of the gene and can influence the binding of miR-885-5p to \textit{CASP3} mRNA\(^\text{19}\). Guan et al. (2013) showed that the CC genotype of this variant influences levels of \textit{CASP3} mRNA expression\(^\text{19}\). Like the \textit{TNFRSF1A} gene, the role of the \textit{CASP3} rs1049253 variant in predisposing to ATP is unknown.

Another important regulator of apoptosis is caspase-8. Caspase-8, encoded by the \textit{CASP8} gene, can activate downstream effector caspases\(^\text{11}\). It is known to regulate apoptosis of tendon fibroblasts\(^9\). Regulating the balance between synthesis and degradation is essential in maintaining ECM homeostasis and the removal of damaged tendon fibroblasts during normal tendon turnover is important\(^9\). However, atypical tenocyte apoptosis has been shown in tendinopathy, with elevated expression levels of \textit{CASP8} observed\(^\text{10}\). Two SNPs within the \textit{CASP8} gene have been associated with ATP\(^\text{10}\) but the role of larger scale variation within this gene as a risk factor has not been considered.

Although our understanding of the role that SNPs play in ATP is growing\(^4,6,8,10\), there has yet to be an investigation into the influence of copy number variation (CNV) as a predisposing factor. Copy number variants (CNVs) are segments of DNA greater than 1kb in size, which show altered copy number (CN) when compared to a reference genome\(^\text{20}\). CNVs can influence phenotypes by altering gene dosage and disrupting coding sequences of DNA\(^\text{21}\). Indeed, predisposition to certain diseases appears to be associated with CNV\(^\text{21,22}\). The \textit{CASP8} gene is known to harbour a CN variant that spans intron 11 - intron 12 of the nucleotide sequence (as reported in the Database of Genomic Variants (DGV) (http://projects.tcag.ca/variation/)). With regard to the role of caspase 8 in apoptosis, and as
SNPs within this gene have been associated with ATP\textsuperscript{10}, we considered that CNV within 
*CASP8* might predispose to ATP. Additionally, for the reasons outlined in the preceding paragraphs, we decided to investigate whether the *TNFRSF1A* rs4149577 and *CASP3* rs1049253 variants were additional risk factors for this pathology.

### 2. Methods

One hundred and thirty one British Caucasian participants diagnosed with ATP and 131 asymptomatic British Caucasian controls (CON) were recruited for this genetic association study. ATP participants were recruited through The County Clinic in Northampton, UK. Participants within the CON group (physically active individuals without any history of ATP) were recruited from the East Midlands region of the UK. Cases of Achilles tendinopathy typically presented with gradual progressive pain with early-morning pain/stiffness in the Achilles tendon area. Affected individuals were diagnosed by the clinical author using the criteria published elsewhere\textsuperscript{5,23,29} Diagnosis of tendinopathy was objectively confirmed, in most cases, by MRI of the affected Achilles tendon. The ATP group consisted of participants with noninsertional (N=47) or insertional (N=29) tendinopathy along with those presenting with partial or complete Achilles tendon rupture (N=23). We further recruited an additional 29 cases with more than one type of tendinopathy (insertional and noninsertional). Three individuals originally recruited as controls later self-reported symptoms of ATP but the specific type of tendinopathy for these was not obtained. Subsequent to initial diagnosis, 15 cases with Achilles tendinopathy went on to develop ruptures. As each subpathology (insertional, non-insertional and rupture) was relatively small in number we pooled all cases as having the combined condition of Achilles tendon pathology and this was the main phenotype we chose to investigate. Additional association analysis on each subpathology was, nevertheless, investigated but this was statistically underpowered due to the small sample sizes. All participants completed a physical activity/medical history/injury questionnaire, and gave written, informed consent. The study was approved by the Research Ethics Committee of the University of Northampton, United Kingdom.
DNA was extracted from 2 mL of saliva collected using ORAGENE-DNA kits (OG-500) and DNA purification was carried out using the prepIT-L2P DNA extraction kit (DNA Genotek Inc., Ontario, Canada). Quantitative PCR (qPCR) reactions were run on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, California, USA) in 96-well plates, using fluorescence-based TaqMan assays. The genotyping assays for the TNFRSF1A rs4149577, CASP3 rs1049253 and CASP8 variants were selected from Applied Biosystems. PCR reactions contained probes and primers in a mastermix containing AmpliTaq DNA Polymerase Gold and 10 ng of DNA. The cycling conditions consisted of a holding phase at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Quality control for our SNP genotyping consisted of running both within and between plate replicates to assess for reproducibility in genotype call along with the running of 4 no DNA template controls (NTC) in each qPCR run. For the copy number assays, each sample was repeated in quadruplicate along with the inclusion of NTCs.

For the TNFRSF1A rs4149577 and CASP3 rs1049253 SNPs, the TaqMan Genotyping Assays (C_2645708_10 and C_11683739_10 respectively) contained both FAM and VIC reporter dye labelled probes to discriminate between genotypes, along with ROX dye as the passive reference. Genotypes were called using StepOne Software version 2.1 (Applied Biosystems, Foster City, California, USA). CNV spanning intron 11 - intron 12 of the CASP8 gene was determined using the TaqMan Copy Number Assay Hs02601709_cn along with the reference assay for RNase P. QPCR was performed as a duplex reaction and all CN data were exported into CopyCaller Software version 2.0 (Applied Biosystems, Foster City, California, USA) for calculation of discrete and continuous CN. CN of the target gene was normalised relative to the reference RNase P gene. We used CopyCaller Software to calculate confidence and z-score quality metrics. CN calls were inspected for reproducibility 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 \( E = 10^{(-1/m) - 1} \).

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 (χ²) test was used to determine any significant differences between the gender of the ATP and CON groups. A χ² 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). 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/ 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 ±
11.3, n = 122) and ATP (45.1 ± 14.2, n = 127) groups. There was a small but significant
difference in age between the CON and RUP groups (p=0.036). Full details of the study
participant characteristics (including the TEN and RUP subgroups) can be seen in Table 1.
The FAM labelled TaqMan Copy Number Assay and the VIC labelled Reference Assay
RNase P gave PCR efficiencies of 106% and 104% respectively. We found no interaction
between the variants investigated and participant characteristics for the entire cohort (data
not shown). We also found that none of the variants investigated associated with either the
insertional, noinsertional, rupture or mixed pathologies (P>0.05). However, investigating
potential associations between the various subpathologies and genotype was not the focus
of our study as the N values for each subpathology was small and analysis was statistically
underpowered.

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

For the CASP3 rs1049253 variant, there were no significant differences in genotype (p =
0.643) or allele (p = 0.635) frequencies between the ATP and CON groups. There was no
significant difference in genotype (p = 0.142) or allele (p = 0.104) frequencies between the
female ATP and CON group. There was also no significant difference in genotype (p =
0.072) or allele (p = 0.058) frequencies between the male ATP and CON group, although
there was a trend towards significance.
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\textsuperscript{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\textsuperscript{14,15,27}.

Nell \textit{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\textsuperscript{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 \textit{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 \textit{et al.} (2012). This might
be related to a number of factors. Firstly, SNPs like the \textit{CASP8} rs1045485 variant may have a more profound impact on the development of ATP, compared to larger-scale variants such as CNV. This assertion is supported by the fact that the \textit{CASP8} rs1045485 SNP is a non-synonymous G>C polymorphism that results in an amino acid change (Asp302His), which may affect interaction with other proteins\textsuperscript{9}. In contrast, the functional effect of altered CN at the \textit{CASP8} locus we investigated is presently unknown. Secondly, the association of this region of the \textit{CASP8} gene with ATP might be a population specific effect, as association with the \textit{CASP8} rs1045485 SNP was reported in South African and Australian cohorts\textsuperscript{10} and our data were obtained from British Caucasians. With this in mind, we would envisage further studies on this British cohort using the SNPs investigated by Nell et al (2012) to establish whether they specifically associate with ATP. This would confirm or refute any population difference with high resolution. An additional haplotype based association study using these SNPs would also be worthwhile.

Although we did not find an association between the \textit{CASP3} rs1049253 variant and the ATP group as a whole, we did observe that both genotype and allele frequency distributions approached significance when male CON and male ATP were compared. Furthermore, we did find a quantitative overrepresentation of the CC genotype in males with insertional tendinopathy compared to male controls (data not shown). However, a detailed statistical analysis of this was not possible due to the small number of males in our cohort who presented with this sub-pathology, nevertheless, it does warrant further investigation.

Our study seems to exclude the \textit{TNFRSF1A} rs4149577, \textit{CASP3} rs1049253 and \textit{CASP8} CN loci from a role in ATP. However, we must be cautious in interpreting these findings as the study has some limitations. Firstly, although our investigation was sufficiently powered to detect relatively large effect sizes given the total sample size of 262, it lacked power to detect associations with more modest effects. Secondly, while we found no association between discrete CN calls and ATP for the \textit{CASP8} locus, we were aware that it has been suggested
that rounding CN data into discrete calls might introduce a degree of error. However, to circumvent this possible issue, we also analysed our CN data as a continuous variable and found a concordant lack of association that agreed with the discrete CN findings (data not shown).

To our knowledge, no previous study has investigated whether the TNFRSF1A rs4149577 and CASP3 rs1049253 variants were associated with ATP. Additionally, this was the first study to investigate whether genomic CNV (in this case within the CASP8 locus) was a possible predisposing factor for ATP. To date, studies on the genetics of ATP have been conducted predominantly in South African and Australian cohorts10,29-31 Our study was the first to investigate the effect of these variants in British-based individuals.

In conclusion, the TNFRSF1A rs4149577, CASP3 rs1049253 and CASP8 variants do not associate with ATP. Although we recommend that this study be repeated in a larger cohort, and where possible using cases where only each subpathology is evident, these preliminary findings exclude three plausible candidate gene variants from a role in a common musculoskeletal pathology.

7. Acknowledgements
This research was supported by a grant to Dr Stuart M. Raleigh from the Rosetrees Trust and The University of Northampton. The authors would also like to thank Professor Malcolm Collins and Dr Mike Posthumus at the University of Cape Town’s UCT/MRC Research Unit for Exercise Science and Sport Medicine for discussions concerning the questionnaire design. Authors S.M.R. and W.J.R. have filed patents on the application of specific sequence variations (not included in this manuscript) related to risk assessment of Achilles tendinopathy and anterior cruciate ligament injuries.

References


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

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

Values are expressed as means ± SD for study participant characteristics. The total number of participants (n) is in parentheses. cm: centimetres; kg: kilograms; m: metres.

\(^a\) Age of CON is age of recruitment, while age of ATP, TEN and RUP is age of initial injury.

One-way ANOVA was used to analyse differences in the characteristics of CON vs. ATP, CON vs. TEN and CON vs. RUP groups. Significance was accepted when p < 0.05.

\(^b\) The RUP group includes those that initially presented with rupture (N=23) and those that later developed ruptures from an initial diagnosis of tendinopathy (N=15).
### Table 2. Genotype and allele frequency distributions of the *TNFRSF1A* rs4149577 and *CASP3* rs1049253 variants within cases (ATP) and controls (CON).

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>ATP</th>
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<th>Male</th>
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<tbody>
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<td>Female</td>
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<td>Female</td>
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<td><strong>TNFRSF1A</strong></td>
<td></td>
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</tr>
<tr>
<td>N</td>
<td>131 (82)</td>
<td>81 (49)</td>
<td>49 (50)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CC</td>
<td>23.7 (31)</td>
<td>25.6 (21)</td>
<td>23.5 (19)</td>
<td>20.4 (10)</td>
<td>18.0 (9)</td>
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<tr>
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<td>48.9 (64)</td>
<td>47.6 (39)</td>
<td>42.0 (34)</td>
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<tr>
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<td>26.8 (22)</td>
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<td>49.4 (81)</td>
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<td>0.742</td>
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Values are expressed as a frequency (%) with number of participants (n) in parenthesis.

HWE: Hardy-Weinberg equilibrium. MAF: minor allele frequency. Differences in genotype and allele frequencies between CON and ATP were analysed using a Pearson’s \( \chi^2 \) or Fisher’s exact test. Significance was accepted when \( p < 0.05 \).
Table 3. CN frequency distribution within the *CASP8* gene in cases (ATP) and controls (CON).

<table>
<thead>
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<th>&lt; 2 CN</th>
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<td>8</td>
<td>39</td>
<td>-</td>
</tr>
<tr>
<td>Male ATP</td>
<td>1</td>
<td>44</td>
<td>7</td>
<td>52</td>
<td>0.703</td>
</tr>
<tr>
<td>Female CON</td>
<td>1</td>
<td>16</td>
<td>6</td>
<td>23</td>
<td>-</td>
</tr>
<tr>
<td>Female ATP</td>
<td>3</td>
<td>26</td>
<td>3</td>
<td>32</td>
<td>0.277</td>
</tr>
</tbody>
</table>

Differences in discrete CN between CON and ATP were analysed as described in the Methods section. Significance was accepted when p < 0.05.