1 **Structure-function Specialisation of the Interfascicular Matrix in the Human Achilles**

2 **Tendon**

3 Dharmesh Patel[1]; Danae E. Zamboulis[2]; Ewa M Spiesz[3]; Deborah Simpson[4]; Helen L. Birch[5];

4 Peter D. Clegg[2]; Chavaunne T. Thorpe[6#]; Hazel R.C. Screen[1#*]

5 [#]Joint Last Authorship

6

7 [1]Institute of Bioengineering, School of Engineering and Materials Science, Queen Mary
8 University of London, Mile End Road, London, E1 4NS, UK.
9
10 [2] Department of Musculoskeletal Biology, Institute of Life Course and Medical Sciences,
11 University of Liverpool, West Derby Street, Liverpool, L7 8TX, UK.
12 [3]Department of Bionanoscience, Delft University of Technology, Building 58, Van der
13 Maasweg 9, 2629 HZ Delft, The Netherlands.
14
15 [4]Centre for Proteome Research, Biosciences Building, Institute of Systems, Molecular &
16 Integrative Biology, University of Liverpool, Crown Street, Liverpool, L69 7ZB, UK.
17 [5]Institute of Orthopaedics and Musculoskeletal Science, UCL, Stanmore Campus, Brockley
18 Hill, Stanmore, Middlesex, HA7 4LP, UK.
19
20 [6]Department of Comparative Biomedical Sciences, Royal Veterinary College, Royal College
21 Street, London, NW1 0TU, UK.
22

23 [*]Corresponding Author: h.r.c.screen@qmul.ac.uk

24

25 **ABSTRACT**

26 **Objective**

27 Tendon consists of highly aligned collagen-rich fascicles surrounded by interfascicular matrix

28 (IFM). Some tendons act as energy stores to improve locomotion efficiency; these tendons

29 are prone to debilitating injuries, the incidence of which increases with ageing. In equine

30 tendons, energy storage is achieved primarily through specialisation of the IFM. However, no

31 studies have investigated IFM structure-function specialisation in human tendons. Here, we

32 compare the positional anterior tibialis and energy storing Achilles tendons, testing the

33 hypothesis that the Achilles IFM has specialised composition and mechanical properties,

34 which are lost with ageing.

35 **Methods**

36 We used a multidisciplinary combination of mechanical testing, immunolocalisation and

37 proteomics to investigate structure-function specialisations in functionally distinct human

38 tendons and how these are altered with ageing.

39 **Results**

40 The IFM in the energy storing Achilles tendon is more elastic and fatigue resistant than the

41 IFM in the positional anterior tibialis tendon, with a trend towards decreased fatigue

42 resistance with age in the Achilles IFM. With ageing, alterations occur predominantly to the

43 proteome of the Achilles IFM.

44 **Conclusion**

45 The Achilles tendon IFM is specialised for energy storage, and changes to its proteome with

46 ageing are likely responsible for the observed trends towards decreased fatigue resistance.

47   Knowledge of key energy storing specialisations and their changes with ageing offers insight

48   towards developing effective treatments for tendinopathy.

49

50

51   **Keywords:** fascicles; interfascicular matrix; mechanical testing; mass spectrometry; ageing,

52

Key messages:

What is already known about this subject?

- Energy storing tendons in animals and humans are particularly prone to injury and the incidence increases with increasing age.

- Previous work in some animal models has shown that the specialisation of tendon properties for energy storage is achieved primarily through adaptation of the interfascicular matrix, with specialisation lost in ageing. However, the structural specialisations that provide the human Achilles tendon with its energy storing ability, and how these are affected by ageing, remain to be established.

What does this study add?

- We demonstrate that the interfascicular matrix in the human Achilles tendon is specialised for energy storing, with increased elastic recoil and fatigue resistance, and that these specialisations are partially lost with ageing, likely due to alterations to the proteome of the interfascicular matrix.

How might this impact on clinical practice or future developments?

- Short term, the specialist IFM mechanics we have demonstrated can be detected with new developments in ultrasound functional imaging, offering improved opportunities for contextual tendinopathy diagnostics. Personalised rehabilitation programmes can now be explored and designed specifically to target IFM mechanics.

- Longer term, the knowledge of key specialisations in injury prone energy storing tendons and how they are affected by ageing, offers crucial insight towards developing cell or tissue engineering treatments targeted at restoring tendon structure and function post-injury, specifically targeted at the IFM.

76   **INTRODUCTION**

77   The Achilles tendon has two main functions; transferring muscle-generated forces to move

78   the skeleton and storing energy during locomotion. Efficient energy storage requires the

79   ability to stretch and recoil with each stride, and the mechanical properties of energy storing

80   tendons are specialised to increase locomotory efficiency,[1, 2]. The Achilles tendon is

81   exposed to high stresses and strains during use, and has a much lower safety factor than most

82   primarily positional tendons, such as the anterior tibialis tendon,[3, 4], making it prone to

83   tendinopathy,[5-7]. While fundamental tendon structure is well defined, the structural

84   specialisations that provide the Achilles with the mechanical properties required for energy

85   storage remain unclear. This information is crucial to develop novel, targeted treatments that

86   recover Achilles function post-injury.

87   All tendons have the same general structure, comprised of aligned, collagen-rich fascicles

88   bound by the interfascicular matrix (IFM); a looser, less organised matrix also referred to as

89   the endotenon,[8, 9]. Our previous studies using the equine model have identified the

90   importance of the IFM for tendon function, with structural and compositional specialisations

91   within the IFM of the energy storing superficial digital flexor tendon (SDFT) resulting in a

92   highly extensible and fatigue resistant material which likely translates to greater extensibility

93   and fatigue resistance within the whole tendon,[10-14]. However, these specialisations

94   become compromised with ageing, likely contributing to the age-related increased risk of

95   injury to energy storing tendons in horses,[12, 15, 16].

96   While structure-function relationships in equine tendon are now well established, little work

97   has been undertaken to elucidate structure-function relationships in human tendon or how

98   these alter with ageing. Indeed, to the authors' knowledge, only one study has directly

99   compared functionally distinct human tendons, demonstrating that the Achilles has a lower

5

100   elastic modulus than the anterior tibialis tendon, which is accompanied by several

101   compositional differences,[17]. While studies have assessed the mechanical properties of the

102   Achilles tendon *in vivo*,[1, 18], or obtained healthy and/or diseased Achilles tissue via biopsy

103   or during surgery,[19-21], the limited amount of tissue available with these approaches

104   restricts the subsequent analysis that can be performed. Further, no studies have compared

105   subunits of functionally distinct human tendons to establish their role in tendon structure-

106   function relationships.

107   The aim of this study was therefore to compare the composition and mechanical properties of

108   the human Achilles and anterior tibialis tendons and their subunits, and identify any age-

109   related alterations. We hypothesise that the IFM in the energy storing Achilles tendon has

110   specialised composition and mechanical properties, and these specialisations are lost with

111   ageing.

112   **METHODS**

113   **Sample collection and processing**

114   Collection, storage and use of human tendon was approved by the Human Tissue Authority

115   (HTA; REC number: 14/NE/0154). Achilles and anterior tibialis tendons were collected,

116   either post-mortem from the Centre for Comparative and Clinical Anatomy, University of

117   Bristol (HTA licence: 12135), or the Newcastle Surgical Training Centre, Freeman Hospital,

118   Newcastle upon Tyne (HTA licence: 12148), or as surgical waste from limbs amputated for

119   tumour treatment at the Royal National Orthopaedic Hospital, Stanmore (UCL/UCLH

120   Biobank for Studying Health and Disease; HTA license: 12055; R&D approval from

121   UCL/UCLH/RF JRO (Ref: 11/0464)). Donors were divided into two age groups (n=9 per

122   group); middle-aged: 31–58 years, mean 47.6 years (3 female, 6 male); old aged: 72–94

123   years, mean 84.8 years (4 female, 5 male). Paired Achilles and anterior tibialis tendons were

6

124   processed <24 hours post-mortem by quartering longitudinally and either frozen for

125   mechanical analysis or prepared for proteomic and histological analysis as described in

126   supplementary methods,[12, 22].

127   All donor tendons underwent mechanical characterisation of IFM and fascicles. Owing to the

128   challenges associated with gripping short specimens for mechanical testing, and time required

129   to laser-capture tissue for proteomic analysis, only 5 paired tendons from each age group

130   underwent quarter tendon testing, histologic and proteomic analysis. See supplementary table

131   1 for donor and analysis details.

132   **Mechanical characterisation of tendon, fascicles and IFM**

133   *Tendon quasi-static mechanical properties* – Immediately before testing, one quarter from

134   each tendon was thawed and its cross-sectional area measured,[23]. Samples were

135   preconditioned then pulled to failure. See supplementary methods for details of testing

136   parameters.

137   *Fascicle and IFM quasi-static mechanical properties* – Samples for determination of fascicle

138   and IFM quasi-static properties were dissected and prepared from one of the tendon quarters

139   as previously described,[10, 24]. Samples were preconditioned then pulled to failure. See

140   supplementary methods for details of dissection and testing parameters.

141   *Calculation of viscoelastic and failure properties* - Force and extension data were recorded at

142   100Hz. Hysteresis and stress relaxation were calculated during preconditioning. For tendons

143   and fascicles, maximum modulus, failure strain and stress were calculated during the pull-to-

144   failure test, and for IFM samples, maximum stiffness, and force and extension at yield and

145   failure were calculated as previously described,[11].

146 *Fascicle and IFM fatigue properties* - Samples for determination of fascicle and IFM fatigue

147 properties were dissected from one of the tendon quarters and fatigue properties measured by

148 subjecting samples to cyclic loading until failure as described previously,[13, 25], with some

149 modifications. See supplementary methods for details of testing parameters. Force and

150 displacement data were collected at 100Hz, with cycles to failure and secondary creep rate

151 calculated for each sample.

152 *Statistical Analysis* –A general linear mixed model was applied to assess differences between

153 tendon type and with ageing, using tendon type and age as crossed factors and donor as a

154 nested random effect factor (R, version 3.6.1; www.r-project.org). Shapiro-Wilk tests

155 indicated non-normal distribution of the data, hence data transformations using box-cox

156 transformations were used.

157 **Protein Immunolocalisation**

158 Immunohistochemistry was used to localise decorin, fibromodulin, lubricin and versican, as

159 described previously,[22]. See Supplementary Table 2 for antibodies and blocking

160 conditions. Elastin distribution was also assessed by elastic van Gieson's staining.

161 **Quantification of elastin content**

162 The elastin content of Achilles and anterior tibialis tendons from middle-aged and old donors

163 (n=3 per group) was quantified using the FASTIN^{TM} Elastin Assay (Biocolor, UK) as

164 previously described,[14], and differences between tendon types assessed using paired t-

165 tests.

166 **Mass Spectrometry Analyses**

167 *Laser-Capture Microdissection* – Laser-capture microdissection of IFM and fascicles from

168 tendon cryosections was performed as previously described,[12], with a haematoxylin

169 staining step (1 min.) to visualise cell nuclei.

170 *Protein Extraction and mass spectrometry analyses* – Protein extraction and mass

171 spectrometry analysis of laser-captured samples was performed as described previously,[12],

172 using an Ultimate 3000 Nano system (Dionex/Thermo Fisher Scientific) coupled to a Q-

173 Exactive Quadrupole-Orbitrap mass spectrometer (Thermo-Scientific).

174 *Peptide Identification and Quantification* - Fascicle and IFM proteins were identified,

175 searching against the UniHuman reviewed database (http://www.uniprot.org/proteomes/)

176 using parameters and filters as described previously,[26] (Peaks® 8.5 PTM software;

177 Bioinformatics Solutions, Canada). Label-free quantification was performed separately for

178 IFM and fascicles from each tendon and age group (Peaks® 8.5 PTM software). Protein

179 abundances were normalised for collected laser-capture area and differentially abundant

180 proteins between groups identified using fold change ≥ 2 and $p < 0.05$ (PEAKS adjusted p-

181 values).The mass spectrometry data have been deposited to the ProteomeXchange

182 Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner

183 repository,[27] with the dataset identifier PXD018212.

184 *Gene Ontology and Network Analysis* – The dataset of differentially abundant proteins

185 between groups were classified for cell compartment association with Ingenuity Pathway

186 Analysis (IPA, Qiagen) and for matrisome categories using The Matrisome Project

187 database,[28]. Protein pathway analysis for differentially abundant proteins was performed in

188 IPA.

189 **RESULTS**

190 **Tendon, fascicle and IFM mechanical properties**

9

191   There were no differences in tendon mechanical properties between age groups or tendon

192   types, except for maximum modulus and hysteresis, which were significantly greater in the

193   anterior tibialis tendon (Fig. 1). However, notable variability between donors was evident. At

194   the fascicle level, ultimate tensile stress, hysteresis and stress relaxation were significantly

195   greater in fascicles from the anterior tibialis tendon compared to those from the Achilles,

196   however, no changes in fascicle mechanical properties were evident with ageing. Similarly,

197   significantly greater hysteresis and force relaxation were evident in the IFM of the anterior

198   tibialis compared to the Achilles, but no changes occurred with ageing. There were also no

199   differences in IFM extension or force at the yield point, defined as the point at which

200   maximum stiffness was reached and indicating the limit of elastic behaviour, between tendon

201   types or age groups (Supplementary Fig. 1).

202   **Fascicle and IFM fatigue properties**

203   Fascicle fatigue resistance did not differ between tendon types or with ageing. By contrast,

204   the IFM in the Achilles was more fatigue resistant than that in the anterior tibialis tendon,

205   characterised by a significantly greater number of cycles to failure ($p < 0.001$) and lower rate

206   of secondary creep ($p < 0.001$). These differences were lost with ageing, due to indications of

207   reduced fatigue resistance in the ageing Achilles IFM, seen in a trend towards a decrease in

208   number of cycles to failure ($p = 0.09$; Fig. 2).

209   **Protein Immunolocalisation**

210   Lubricin, versican and elastin localised to the IFM in both tendon types. Fibromodulin

211   staining was greater in the fascicles, with little or no staining in the IFM. Decorin was present

212   throughout the extracellular matrix (ECM) in both tendon types (Fig. 3).

213   **Tendon elastin content**

214 Elastin content in the Achilles from middle-aged and old donors was 2.4±1.6% and

215 1.05±0.4% respectively. Elastin content in the anterior tibialis was 1.7±0.9% in middle-aged,

216 and 2.1±0.6% in old tendon. See supplementary figure 2 for individual data. There was a

217 trend towards a lower elastin content in the old Achilles compared to the old anterior tibialis

218 tendon, but this did not reach statistical significance (p=0.07).

219 **Protein identification and ontology**

220 Overall, more proteins were identified in the IFM than in fascicles, and a greater proportion

221 of those identified in fascicles were classified as ECM or ECM-related proteins (Table 1).

	Fascicular Matrix				Interfascicular Matrix			
	Achilles		Anterior tibialis		Achilles		Anterior tibialis	
	Middle	Old	Middle	Old	Middle	Old	Middle	Old
Total Protein Number	152	152	148	153	265	259	243	211
Number of ECM Proteins	54 (35.5%)	69 (45.4%)	58 (39.2%)	53 (34.6%)	77 (29.1%)	81 (31.3%)	82 (33.7%)	74 (35.1%)
ECM Glycoproteins	16 (10.5%)	22 (14.5%)	17 (11.5%)	16 (10.5%)	28 (10.6%)	31 (12.0%)	31 (12.8%)	29 (13.7%)
Collagens	15 (9.9%)	16 (10.5%)	15 (10.1%)	15 (9.8%)	20 (7.5%)	18 (6.9%)	17 (7.0%)	17 (8.1%)
Proteoglycans	9 (5.9%)	10 (6.6%)	9 (6.1%)	8 (5.2%)	11 (4.2%)	11 (4.2%)	10 (4.1%)	10 (4.7%)
ECM Affiliated Proteins	4 (2.6%)	6 (3.9%)	6 (4.1%)	5 (3.3%)	9 (3.4%)	8 (3.1%)	9 (3.7%)	7 (3.3%)
ECM Regulators	8 (5.3%)	13 (8.6%)	10 (6.8%)	8 (5.2%)	6 (2.3%)	11 (4.2%)	13 (5.3%)	9 (4.3%)
Secreted Factors	2 (1.3%)	2 (1.3%)	1 (0.7%)	1 (0.7%)	3 (1.1%)	2 (0.8%)	2 (0.8%)	2 (0.9%)

222

223 **Table 1. Protein number in each of the tendon compartments as identified by LC-**

224 **MS/MS and categorisation of matrisome-associated proteins.** Numbers in brackets

225 indicate percentage of total protein number.

226 *Differences in protein abundance between tendon types and age groups* – More proteins were

227 identified as differentially abundant in the IFM than in fascicles, with the majority of changes

228 occurring in the old Achilles (Fig. 4&5). Many of these proteins were ECM or ECM-

11

229   associated, with a predominance of proteoglycans and glycoproteins. By contrast, fewer

230   alterations in protein abundance were observed in fascicles, and the majority of those that did

231   change were either collagens, or were not ECM-related. Radar plots indicate that in the IFM,

232   changes in collagen abundance were consistent for different collagen types, whereas

233   differences in proteoglycan/glycoprotein abundance differed with protein type (Fig. 4). A

234   similar pattern was seen for collagens in fascicles as in the IFM, with greater abundance of

235   several collagens in the old Achilles (Fig. 5).

236   *Pathway analysis* – IPA was used to identify potential upstream regulators in each group.

237   TGF-β1 was predicted to be activated in the old Achilles, and inhibited in the anterior tibialis

238   tendon, in both the IFM and fascicles (Fig. 6).

239   **DISCUSSION**

240   The results support our hypothesis, demonstrating specialisation of the IFM in the Achilles

241   tendon, which is more elastic and fatigue resistant than the IFM in the anterior tibialis tendon.

242   In contrast to our hypothesis, few changes in mechanical properties were observed with

243   ageing, although there was a trend towards decreased fatigue resistance with ageing in the

244   Achilles IFM only. Proteomic analysis revealed a more complex proteome in the IFM, with

245   age-related alterations in protein abundance predominantly occurring in the Achilles IFM.

246   Overall, the results we present here are similar to those reported previously in functionally

247   distinct equine tendons, with greater elasticity in energy storing compared to positional

248   tendons and their subunits,[10, 11]. However, we observed fewer differences between human

249   tendons than seen previously in equine tendon, in which whole tendons and fascicles also

250   show several differences in mechanical properties between tendon types,[10, 13]. This may

251   simply arise from the lower numbers of available human samples and high sample variability,

252   but may also be associated with the unmatched age ranges of human and equine samples, or

12

253   with differences in energy storing function, as the Achilles is a less extreme energy store than

254   the highly specialised equine SDFT,[29, 30].

255   We were not able to obtain tendons from donors under 30 years old, so we are comparing

256   middle-aged and old, rather than young and old, providing less age contrast than in our

257   previous studies of equine tendon. It is possible that specialisation of the Achilles was already

258   diminishing in the middle-aged group, as we observed previously in equine tendon,[15], so

259   fewer age-related alterations in mechanical properties were evident. Indeed, it is well

260   established that Achilles tendinopathy is most prevalent in the fourth decade of life,[7, 31],

261   which may well result from this diminishing specialisation coinciding with continual or

262   increasing usage as individuals take up new sporting activities.

263   However, it is also apparent that there are large variations in tendon mechanical properties

264   between individuals, which may mask differences between tendon types or with ageing. The

265   source of this variation is uncertain, but it is to be noted that we had a mixed-sex population,

266   and minimal information about the health, exercise or injury status of donors. Tendons with

267   any macroscopic signs of degeneration were excluded from all analyses. However, whilst

268   some donors did have documented diabetes, in others information of any systemic diseases

269   were lacking. While the sex of each donor was known, limited sample numbers means it was

270   not possible to establish if any variability arose from sex-related differences at baseline or

271   with ageing, as previously reported,[32]. Despite these limitations, it is notable that we still

272   identified significant compositional and mechanical differences between energy storing

273   Achilles and positional anterior tibialis tendons.

274   Few studies have investigated the mechanical or structural properties of functionally distinct

275   human tendons and their subunits. Those that have, typically analyse a single tendon type to

276   explore limited mechanical or compositional aspects. The IFM of the human Achilles tendon

277   has received little attention previously, with data supporting the results we present here,

278   demonstrating localisation of lubricin to the IFM,[33], and identifying capacity for

279   interfascicular sliding,[34]. Indeed, a recent modelling study indicated that sliding of tendon

280   subunits, enabled by IFM, was necessary to accurately predict tendon viscoelasticity and

281   failure,[35]. However, in contrast to our previous findings in functionally distinct equine

282   tendons,[10, 11, 15], we did not identify any differences in interfascicular sliding between

283   tendon types or age groups, as measured by IFM extension at the point of maximum stiffness.

284   While the capacity for interfascicular sliding does not appear to differ between tendon types,

285   our results demonstrate enhanced elasticity and fatigue resistance in the Achilles IFM,

286   specialisations that likely contribute to efficient energy storage in this tendon.

287   Histological analysis confirmed that the IFM is rich in proteoglycans, particularly lubricin

288   and decorin, and also elastin, as seen previously in tendons from other species,[22, 36, 37].

289   Mass spectrometry allowed a comprehensive characterisation of the IFM and fascicular

290   proteomes and comparison between tendon types and age groups, reveal a greater complexity

291   in the IFM proteome in both tendon types, with almost double the number of glycoproteins

292   identified in this region compared to the FM, supporting previous findings in equine

293   tendon,[12]. The protein profile identified is also similar to that detected previously in the

294   whole Achilles tendon, with many collagens and proteoglycans present,[38]. Elastin detection

295   by mass spectrometry requires the inclusion of an elastase digestion step,[38] which was not

296   possible with our samples due to the limited volumes collected by laser capture. However, by

297   combining quantitative assays to determine whole tendon elastin content, and

298   immunolocalisation to identify its spatial arrangement, we were able to establish that elastin

299   was localised to the IFM, with a trend towards lower elastin content in the old Achilles

300   compared to the old anterior tibialis tendon. Previous research demonstrates a decline in

301   elastin content in the energy storing equine SDFT with ageing,[14]. In our samples, a

14

302   particularly large individual variation in elastin content in the middle-aged Achilles tendon

303   was evident (see supplementary figure 1), which may mask a decline in elastin content in this

304   tendon with ageing. Previous studies have demonstrated highly variable energy storage

305   capacity between the Achilles tendons of middle-aged individuals,[39], which also indicates

306   significant individual variability in human tendons with ageing.

307   While no significant changes in tendon or subunit quasi-static mechanics were identified with

308   age, the superior fatigue resistance of the Achilles IFM was lost with ageing, suggesting some

309   effect of ageing on the Achilles IFM specifically. Indeed, proteomic analysis revealed that the

310   majority of changes in protein abundance were measured in the IFM from old Achilles

311   tendons relative to the other groups. Many of these changes were ECM-related, suggesting

312   dysregulation of homeostasis which may be responsible for the loss of superior fatigue

313   resistance observed. This is in contrast to previous transcriptomic analysis of the ageing

314   Achilles, which showed little changes in ECM proteins at the gene level,[40]. It may be that

315   the changes in ECM protein abundance we observed are post-transcriptionally regulated, or

316   that separate analysis of IFM and fascicles allows detection of differences that are not

317   apparent when the tendon is analysed as a whole. Indeed, very few proteins changed in

318   abundance in both fascicles and IFM with ageing, instead ageing changes were

319   predominantly in the IFM, suggesting differential age-related regulation of protein

320   homeostasis across fascicles and IFM. Of interest, we measured increased abundance of

321   collagen VI in the Achilles IFM with ageing. Collagen VI is overexpressed in several fibrotic

322   diseases, and collagen VI-null mice exhibit improved cardiac structure and function post-

323   myocardial infarction,[41, 42].

324   TGF-β signalling was predicted to be activated in the Achilles tendon from old donors. TGF-

325   β signalling is essential for tendon development, and is expressed predominantly within the

326   IFM of developing and adult tendons,[43-45]. Indeed, our recent work demonstrates

15

327   upregulation of TGF-β in the IFM of the energy storing equine SDFT upon commencement

328   of loading during development,[26]. However, TGF-β signalling has also been shown to be

329   dysregulated in several age-associated diseases, including atherosclerosis, neurodegenerative

330   diseases and arthritis, and is upregulated in tendon injury,[46, 47]. In cartilage, TGF-β

331   switches from a protective to a detrimental role with ageing, which is associated with

332   osteoarthritis development,[48]. It is possible that dysregulation of TGF-β signalling in the

333   old Achilles drives the changes in the IFM proteome that we report here.

Conclusions

335   In this study, we demonstrate that the IFM is specialised in the energy storing Achilles

336   tendon, with greater elasticity and fatigue resistance than in the positional anterior tibialis

337   tendon. Further, we identify age-related alterations in the IFM proteome of the Achilles

338   tendon which is likely related to the loss of fatigue resistance observed.

Author Contributions

340   DP: Investigation, formal analysis, writing – review & editing; DEZ: Investigation, formal

341   analysis, visualisation, writing – review & editing; EMS: Investigation, writing – review &

342   editing; DS: Methodology, investigation, writing – review & editing; HLB:

343   Conceptualisation, funding acquisition, writing – review & editing; PDC: Conceptualisation,

344   funding acquisition, supervision, writing – review & editing; CTT: Conceptualisation,

345   funding acquisition, writing – original draft; HRCS: Conceptualisation, supervision, funding

346   acquisition, writing – review & editing.

Acknowledgements

16

350   Research Council (MRC) and Versus Arthritis as part of the MRC-Arthritis Research UK

351   Centre for Integrated research into Musculoskeletal Ageing (CIMA).

352   **References:**

353   1.   Lichtwark GA, Wilson AM. In vivo mechanical properties of the human Achilles
354   tendon during one-legged hopping. J Exp Biol. 2005;208(24):4715-25.
355   2.   Lichtwark GA, Wilson AM. Is Achilles tendon compliance optimised for maximum
356   muscle efficiency during locomotion? J Biomech. 2007;40(8):1768-75.
357   3.   Magnusson SP, Aagaard P, Rosager S, Dyhre-Poulsen P, Kjaer M. Load-displacement
358   properties of the human triceps surae aponeurosis in vivo. The Journal of Physiology.
359   2001;531(1):277-88.
360   4.   Ker RF, Alexander RM, Bennett MB. Why are mammalian tendons so thick? J Zool.
361   1988;216(2):309-24.
362   5.   Kujala UM, Sarna S, Kaprio J. Cumulative incidence of achilles tendon rupture and
363   tendinopathy in male former elite athletes. Clinical journal of sport medicine : official journal
364   of the Canadian Academy of Sport Medicine. 2005;15(3):133-5.
365   6.   de Jonge S, van den Berg C, de Vos RJ, van der Heide HJL, Weir A, Verhaar JAN, et
366   al. Incidence of midportion Achilles tendinopathy in the general population. British Journal
367   of Sports Medicine. 2011;45(13):1026.
368   7.   Clayton RAE, Court-Brown CM. The epidemiology of musculoskeletal tendinous and
369   ligamentous injuries. Injury. 2008;39(12):1338-44.
370   8.   Kastelic J, Galeski A, Baer E. The multicomposite structure of tendon. Connective
371   tissue research. 1978;6(1):11-23.
372   9.   Kannus P. Structure of the tendon connective tissue. Scandinavian Journal of
373   Medicine and Science in Sports. 2000;10(6):312-20.
374   10.   Thorpe CT, Udeze CP, Birch HL, Clegg PD, Screen HR. Specialization of tendon
375   mechanical properties results from interfascicular differences. Journal of the Royal Society
376   Interface. 2012;9(76):3108-17.
377   11.   Thorpe CT, Godinho MS, Riley GP, Birch HL, Clegg PD, Screen HR. The
378   interfascicular matrix enables fascicle sliding and recovery in tendon, and behaves more
379   elastically in energy storing tendons. Journal of the Mechanical Behavior of Biomedical
380   Materials. 2015;52:85-94.
381   12.   Thorpe CT, Peffers MJ, Simpson D, Halliwell E, Screen HR, Clegg PD. Anatomical
382   heterogeneity of tendon: Fascicular and interfascicular tendon compartments have distinct
383   proteomic composition. Scientific Reports. 2016;6:20455.
384   13.   Thorpe CT, Riley GP, Birch HL, Clegg PD, Screen HR. Fascicles and the
385   interfascicular matrix show adaptation for fatigue resistance in energy storing tendons. Acta
386   Biomater. 2016;42:308-15.
387   14.   Godinho MSC, Thorpe CT, Greenwald SE, Screen HRC. Elastin is Localised to the
388   Interfascicular Matrix of Energy Storing Tendons and Becomes Increasingly Disorganised
389   With Ageing. Scientific Reports. 2017;7(1):9713.
390   15.   Thorpe CT, Udeze CP, Birch HL, Clegg PD, Screen HR. Capacity for sliding between
391   tendon fascicles decreases with ageing in injury prone equine tendons: a possible mechanism
392   for age-related tendinopathy? European cells & materials. 2013;25:48-60.
393   16.   Thorpe CT, Riley GP, Birch HL, Clegg PD, Screen HRC. Fascicles and the
394   interfascicular matrix show decreased fatigue life with ageing in energy storing tendons. Acta
395   Biomaterialia. 2017;56:58-64.

396 17.   Birch HL. Tendon matrix composition and turnover in relation to functional
397 requirements. Int J Exp Pathol. 2007;88(4):241-8.
398 18.   Fouré A. New Imaging Methods for Non-invasive Assessment of Mechanical,
399 Structural, and Biochemical Properties of Human Achilles Tendon: A Mini Review. Front
400 Physiol. 2016;7:324-.
401 19.   Dakin SG, Newton J, Martinez FO, Hedley R, Gwilym S, Jones N, et al. Chronic
402 inflammation is a feature of Achilles tendinopathy and rupture. British journal of sports
403 medicine. 2018;52(6):359-67.
404 20.   Pingel J, Lu Y, Starborg T, Fredberg U, Langberg H, Nedergaard A, et al. 3-D
405 ultrastructure and collagen composition of healthy and overloaded human tendon: evidence
406 of tenocyte and matrix buckling. J Anat. 2014;224(5):548-55.
407 21.   Magnusson SP, Qvortrup K, Larsen JO, Rosager S, Hanson P, Aagaard P, et al.
408 Collagen fibril size and crimp morphology in ruptured and intact Achilles tendons. Matrix
409 Biol. 2002;21(4):369-77.
410 22.   Thorpe CT, Karunaseelan KJ, Ng Chieng Hin J, Riley GP, Birch HL, Clegg PD, et al.
411 Distribution of proteins within different compartments of tendon varies according to tendon
412 type. Journal of Anatomy. 2016;229(3):450-8.
413 23.   Goodship AE, Birch HL. Cross sectional area measurement of tendon and ligament in
414 vitro: a simple, rapid, non-destructive technique. Journal of Biomechanics. 2005;38(3):605-8.
415 24.   Thorpe CT, Riley GP, Birch HL, Clegg PD, Screen HR. Fascicles from energy-
416 storing tendons show an age-specific response to cyclic fatigue loading. Journal of the Royal
417 Society Interface. 2014;11(92):20131058.
418 25.   Legerlotz K, Jones GC, Screen HRC, Riley GP. Cyclic loading of tendon fascicles
419 using a novel fatigue loading system increases interleukin-6 expression by tenocytes.
420 Scandinavian Journal of Medicine & Science in Sports. 2013;23(1):31-7.
421 26.   Zamboulis DE, Thorpe CT, Ashraf Kharaz Y, Birch HL, Screen HRC, Clegg PD.
422 Postnatal mechanical loading drives adaptation of tissues primarily through modulation of the
423 non-collagenous matrix. eLife. 2020;9:e58075.
424 27.   Vizcaíno JA, Côté RG, Csordas A, Dianes JA, Fabregat A, Foster JM, et al. The
425 Proteomics Identifications (PRIDE) database and associated tools: status in 2013. Nucleic
426 Acids Res. 2013;41(D1):D1063-D9.
427 28.   Hynes RO, Naba A. Overview of the matrisome--an inventory of extracellular matrix
428 constituents and functions. Cold Spring Harbor perspectives in biology. 2012;4(1):a004903.
429 29.   Patterson-Kane JC, Rich T. Achilles Tendon Injuries in Elite Athletes: Lessons in
430 Pathophysiology from Their Equine Counterparts. ILAR Journal. 2014;55(1):86-99.
431 30.   Thorpe CT, Screen HR. Tendon Structure and Composition. Advances in
432 experimental medicine and biology. 2016;920:3-10.
433 31.   Hess GW. Achilles Tendon Rupture:A Review of Etiology, Population, Anatomy,
434 Risk Factors, and Injury Prevention. Foot & Ankle Specialist. 2010;3(1):29-32.
435 32.   Pease LI, Clegg PD, Proctor CJ, Shanley DJ, Cockell SJ, Peffers MJ. Cross platform
436 analysis of transcriptomic data identifies ageing has distinct and opposite effects on tendon in
437 males and females. Sci Rep. 2017;7(1):14443.
438 33.   Sun Y-L, Wei Z, Zhao C, Jay GD, Schmid TM, Amadio PC, et al. Lubricin in human
439 achilles tendon: The evidence of intratendinous sliding motion and shear force in achilles
440 tendon. Journal of Orthopaedic Research. 2015;33(6):932-7.
441 34.   Haraldsson BT, Aagaard P, Qvortrup K, Bojsen-Moller J, Krogsgaard M, Koskinen S,
442 et al. Lateral force transmission between human tendon fascicles. Matrix Biol. 2008;27(2):86-
443 95.
444 35.   Obuchowicz R, Ekiert M, Kohut P, Holak K, Ambrozinski L, Tomaszewski KA, et al.
445 Interfascicular matrix-mediated transverse deformation and sliding of discontinuous tendon

446 subcomponents control the viscoelasticity and failure of tendons. Journal of the Mechanical
447 Behavior of Biomedical Materials. 2019;97:238-46.
448 36.   Funakoshi T, Schmid T, Hsu H, Spector M. Lubricin Distribution in the Goat
449 Infraspinatus Tendon: A Basis for Interfascicular Lubrication. J Bone Joint Surg Am.
450 2008;90(4):803-14.
451 37.   Kohrs RT, Zhao C, Sun Y-L, Jay GD, Zhang L, Warman ML, et al. Tendon fascicle
452 gliding in wild type, heterozygous, and lubricin knockout mice. J Orthop Res.
453 2011;29(3):384-9.
454 38.   Sato N, Taniguchi T, Goda Y, Kosaka H, Higashino K, Sakai T, et al. Proteomic
455 Analysis of Human Tendon and Ligament: Solubilization and Analysis of Insoluble
456 Extracellular Matrix in Connective Tissues. Journal of Proteome Research.
457 2016;15(12):4709-21.
458 39.   Arampatzis A, De Monte G, Karamanidis K, Morey-Klapsing G, Stafilidis S,
459 Brüggemann G-P. Influence of the muscle-tendon unit's mechanical and morphological
460 properties on running economy. Journal of Experimental Biology. 2006;209(17):3345-57.
461 40.   Peffers MJ, Fang Y, Cheung K, Wei TK, Clegg PD, Birch HL. Transcriptome
462 analysis of ageing in uninjured human Achilles tendon. Arthritis Res Ther. 2015;17:33.
463 41.   Cescon M, Gattazzo F, Chen P, Bonaldo P. Collagen VI at a glance. Journal of Cell
464 Science. 2015;128(19):3525-31.
465 42.   Luther DJ, Thodeti CK, Shamhart PE, Adapala RK, Hodnichak C, Weihrauch D, et al.
466 Absence of type VI collagen paradoxically improves cardiac function, structure, and
467 remodeling after myocardial infarction. Circ Res. 2012;110(6):851-6.
468 43.   Pryce BA, Watson SS, Murchison ND, Staverosky JA, Dünker N, Schweitzer R.
469 Recruitment and maintenance of tendon progenitors by TGFβ signaling are essential for
470 tendon formation. Development. 2009;136(8):1351-61.
471 44.   Kuo CK, Petersen BC, Tuan RS. Spatiotemporal protein distribution of TGF-βs, their
472 receptors, and extracellular matrix molecules during embryonic tendon development.
473 Developmental Dynamics. 2008;237(5):1477-89.
474 45.   Russo V, Mauro A, Martelli A, Di Giacinto O, Di Marcantonio L, Nardinocchi D, et
475 al. Cellular and molecular maturation in fetal and adult ovine calcaneal tendons. Journal of
476 Anatomy. 2015;226(2):126-42.
477 46.   Krieglstein K, Miyazono K, ten Dijke P, Unsicker K. TGF-β in aging and disease.
478 Cell and Tissue Research. 2012;347(1):5-9.
479 47.   Morita W, Snelling SJB, Dakin SG, Carr AJ. Profibrotic mediators in tendon disease:
480 a systematic review. Arthritis Research & Therapy. 2016;18(1):269.
481 48.   Hodgson D, Rowan AD, Falciani F, Proctor CJ. Systems biology reveals how altered
482 TGFβ signalling with age reduces protection against pro-inflammatory stimuli. PLOS
483 Computational Biology. 2019;15(1):e1006685.

484

485

Figure Legends

Figure 1. Failure and viscoelastic properties of Achilles and anterior tibialis tendons and their subunits from middle-aged and old donors. Due to limited sample numbers, individual data points are plotted for tendon tests (solid line denotes median). Distribution of fascicle and IFM data is shown by violin plots (solid line denotes median, dashed lines indicate the interquartile range and width corresponds to frequency of data points).

Figure 2. Fatigue properties of fascicles and IFM in Achilles and anterior tibialis tendons. Data are plotted on a $\log_{10}$ scale. Distribution of data is shown by violin plots (solid line denotes median, dashed lines indicate the interquartile range and width corresponds to frequency of data points).

Figure 3. Localisation of proteins in the Achilles and anterior tibialis tendons. Representative immunohistochemical images showing distribution of tendon proteoglycans (brown) and elastin (black) in the Achilles and anterior tibialis tendons from middle-aged donors. IFM is indicated by *. Scale bar = 100 µm.

Figure 4. Most changes in the IFM protein abundance are observed in the old Achilles tendon. (A) Heatmap of differentially abundant proteins in the IFM middle-aged anterior tibialis (ATT M) and Achilles tendon (AT M), and old anterior tibialis tendon (ATT O) and Achilles tendon (AT O) ($p<0.05$, fold change ≥ 2). Heatmap colour scale ranges from blue to white to red with blue representing lower abundance and red higher abundance. MatrisomeDB was used to assign protein classifications. (B) Radar plots of collagens, proteoglycans and glycoproteins that showed differential abundance with age or tendon type in the IFM ($p<0.05$, fold change ≥ 2).

Figure 5. Most changes in fascicle protein abundance are observed in the old Achilles tendon. (A) Heatmap of differentially abundant proteins in the fascicles of middle-aged

20

510   anterior tibialis (ATT M) and Achilles tendon (AT M), and old anterior tibialis tendon (ATT

511   O) and Achilles tendon (AT O) (p<0.05, fold change ≥2). Heatmap colour scale ranges from

512   blue to white to red with blue representing lower abundance and red higher abundance.

513   MatrisomeDB was used to assign protein classifications. (B) Radar plots of collagens,

514   proteoglycans and glycoproteins that showed differential abundance with age or tendon type

515   in fascicles (p<0.05, fold change ≥2).

516   **Figure 6. Pathway analysis identified TGF-β1 as an upstream regulator.** TGF-β1 is

517   predicted to be activated in the Achilles tendon, but inhibited in the anterior tibialis tendon

518   from aged donors. IPA networks for TGFB1 in the IFM (A) and fascicles (B) of the Achilles

519   tendon and anterior tibialis tendon of old donors.

	Tendons	Fascicles	IFM
Failure stress/force	Stress (MPa)	Stress (MPa), p=0.025, p<0.001	Force (N)
Failure strain/extension	Strain (%)	Strain (%)	Extension (mm)
Maximum modulus/stiffness	Modulus (MPa), p=0.044	Modulus (MPa), p=0.005	Stiffness (N)
Hysteresis	Hysteresis (%), p=0.02	Hysteresis (%), p<0.001, p<0.001	Hysteresis (%), p<0.001, p<0.001
Stress/force relaxation	Stress relaxation (%)	Stress relaxation (%), p=0.001	Force relaxation (%), p<0.001, p=0.001
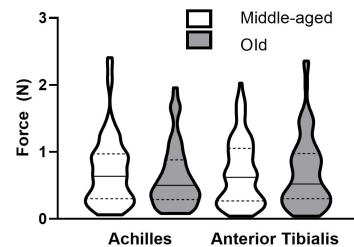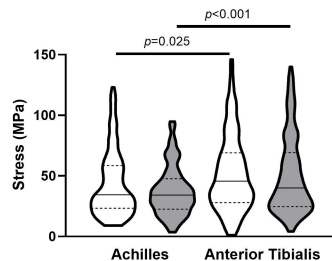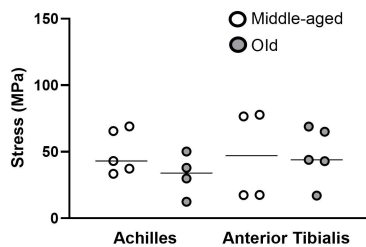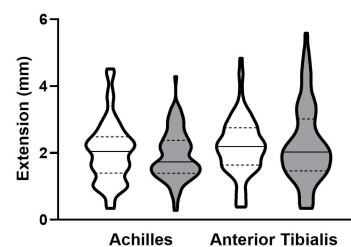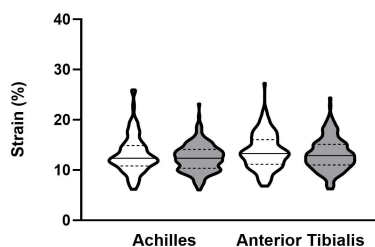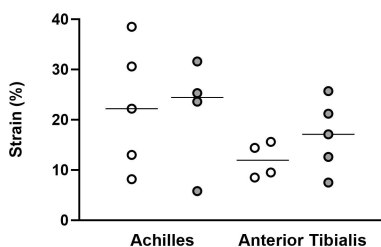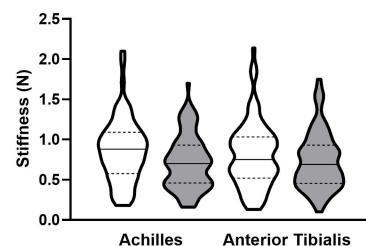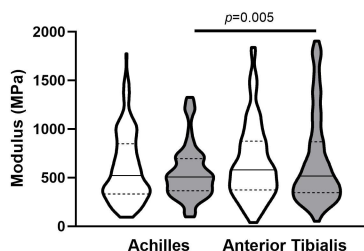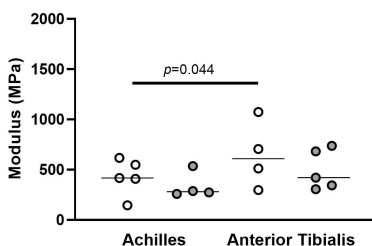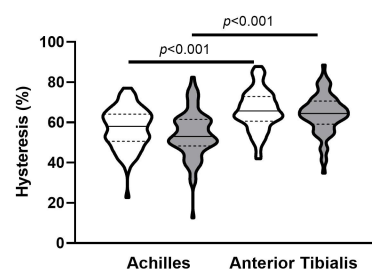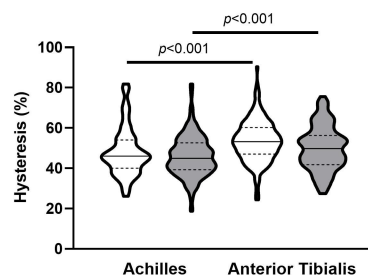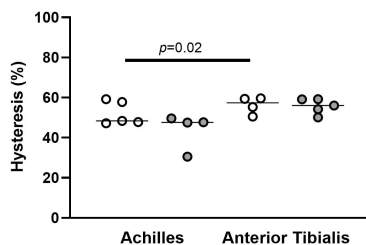
Legend: Middle-aged (open), Old (grey/filled). X-axis categories: Achilles, Anterior Tibialis.
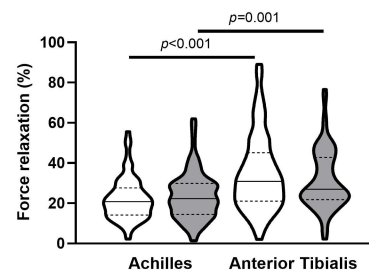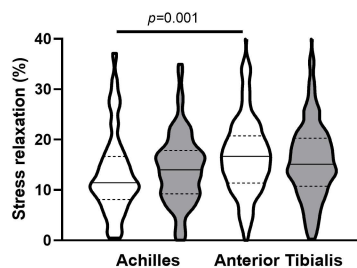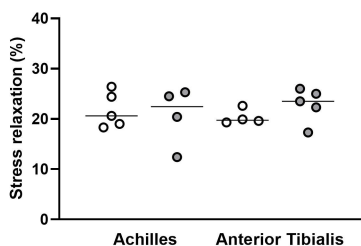
Fascicles
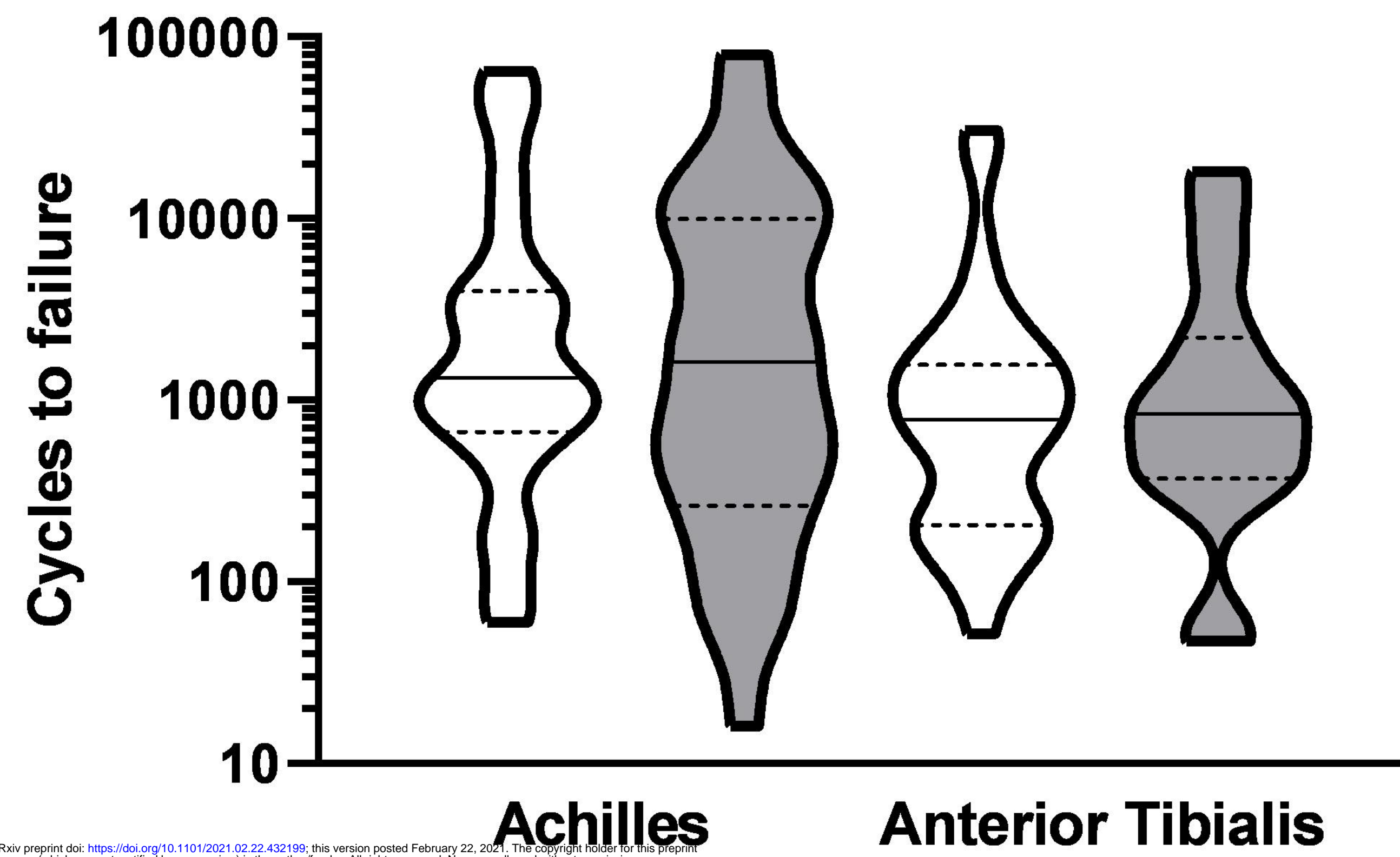
IFM
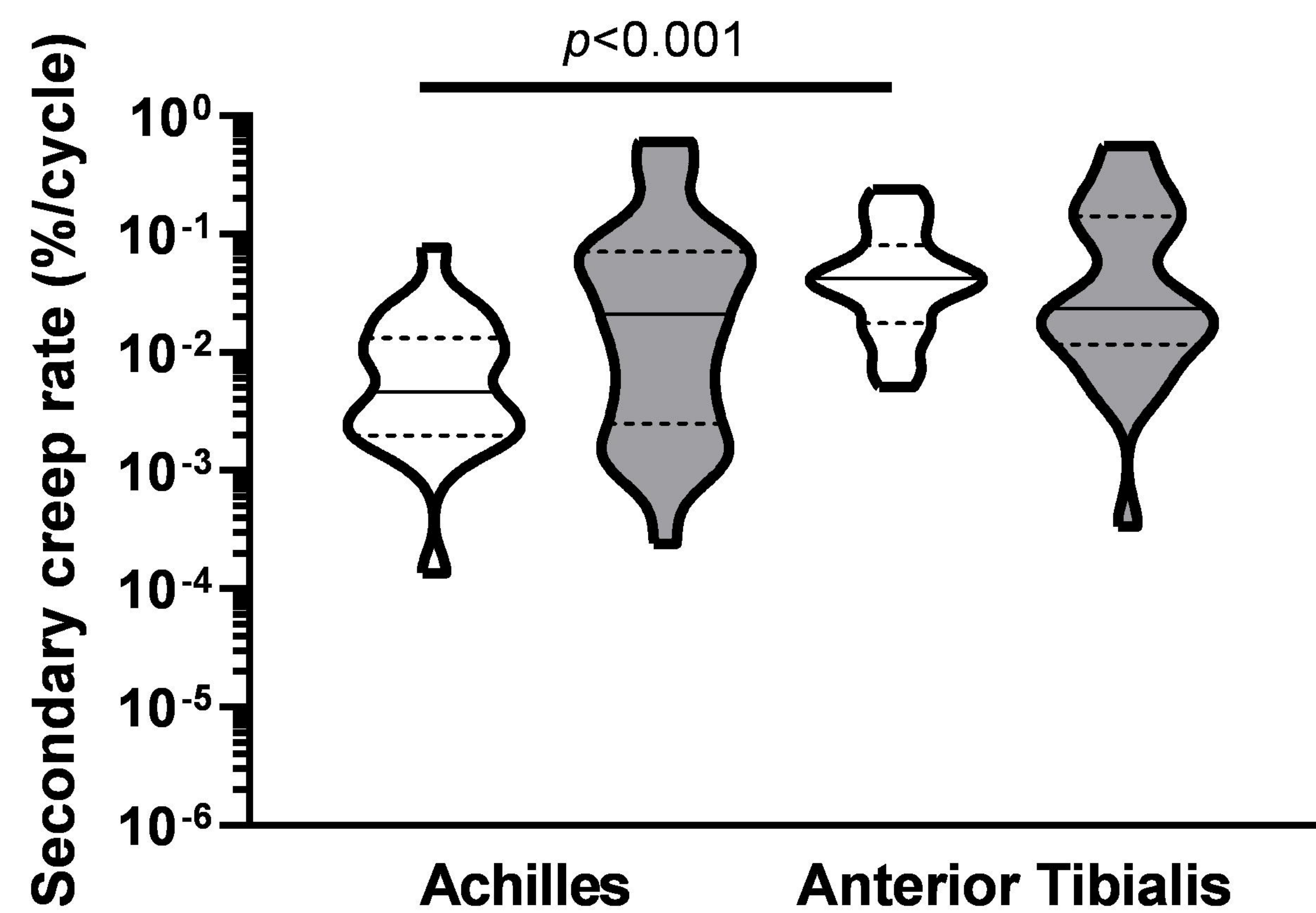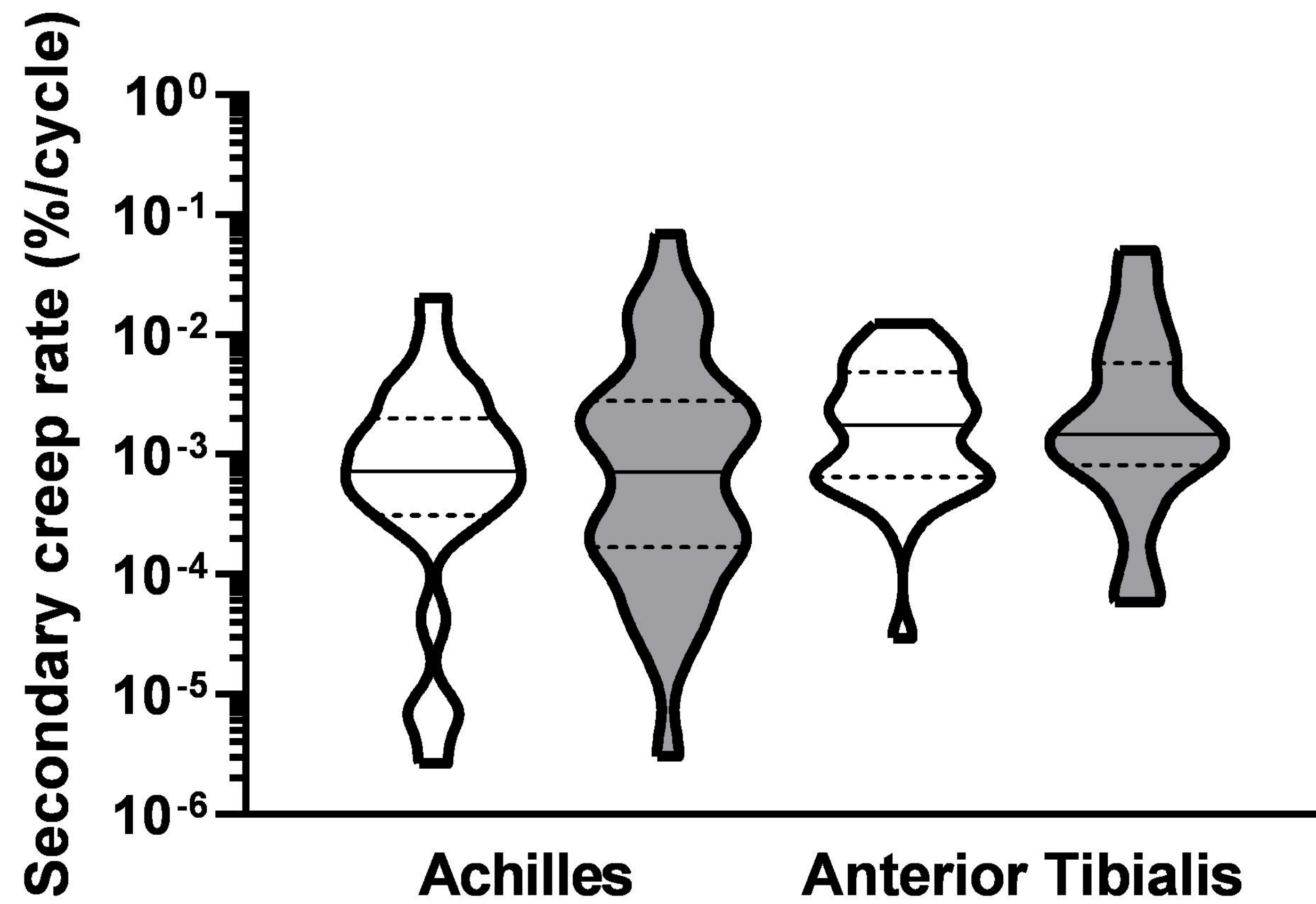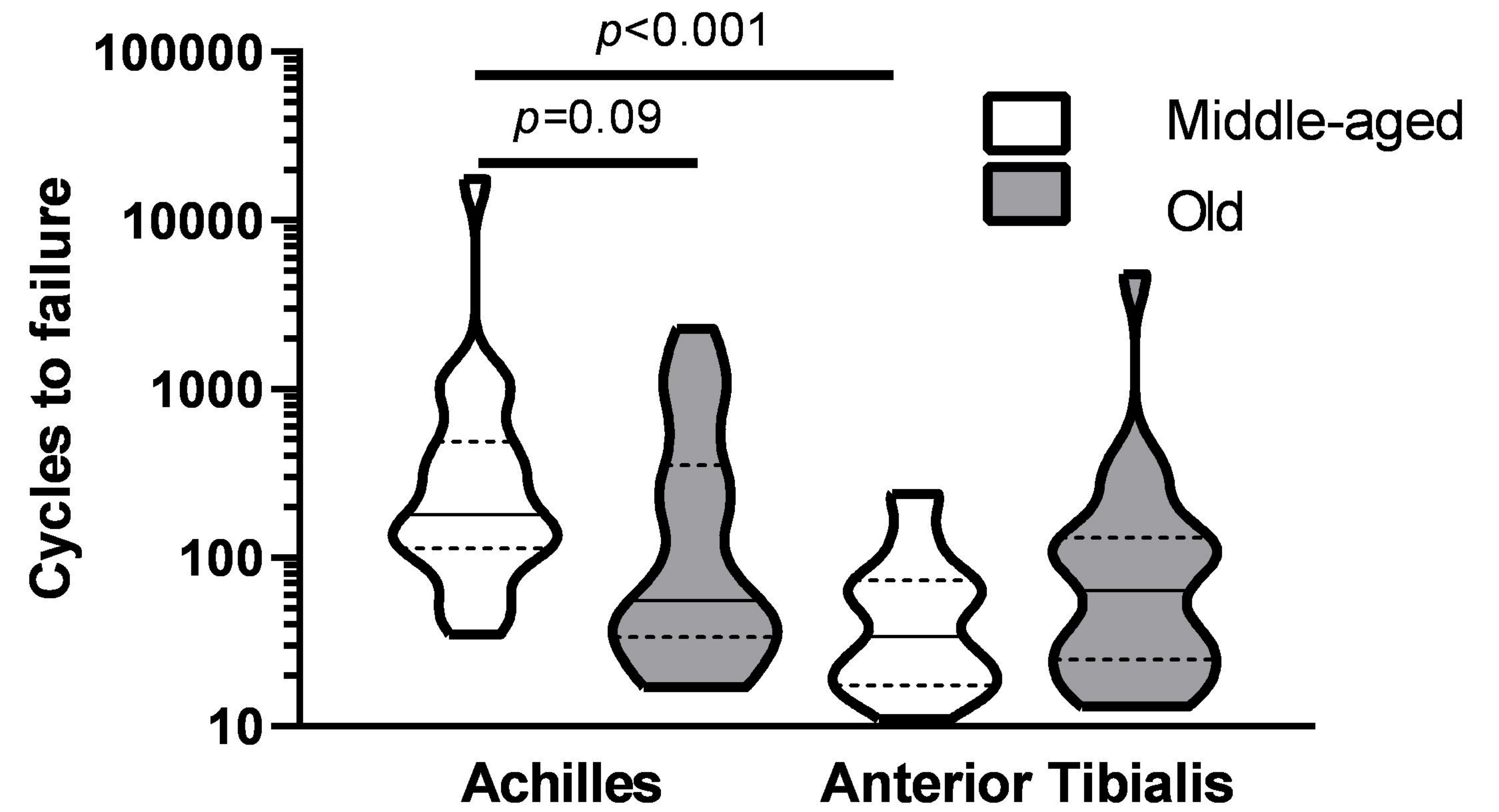
Scale bar = 100 micrometers

	Achilles Tendon	Anterior Tibialis Tendon
Decorin		
Fibromodulin		
Lubricin		
Versican		
Elastin		
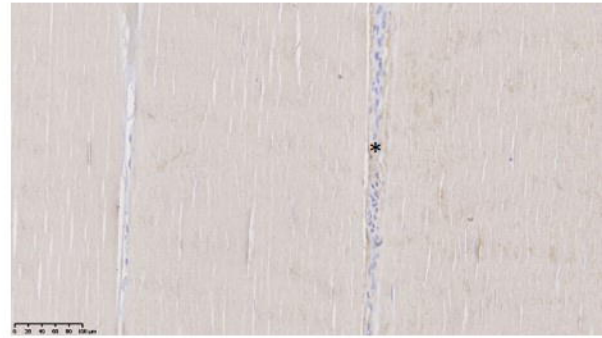
A

IFM

PRG4	Core matrisome	Proteoglycans
BGN	Core matrisome	Proteoglycans
PRELP	Core matrisome	Proteoglycans
COL5A3	Core matrisome	Collagens
COL6A1	Core matrisome	Collagens
COL6A2	Core matrisome	Collagens
COL6A3	Core matrisome	Collagens
COL14A1	Core matrisome	Collagens
AMBP	Matrisome-associated	ECM Regulators
MMP3	Matrisome-associated	ECM Regulators
LGALS1	Matrisome-associated	ECM-affiliated Proteins
C9	involved in immune response	
TAGLN	biomarker of ageing, may contribute to replicative senescence	
MAMDC2	overexpressed in replicative senescence	
CFB	involved in immune response	
IGHM	involved in immune response	
APCS	involved in apoptosis	
TNC	Core matrisome	ECM Glycoproteins
DPT	Core matrisome	ECM Glycoproteins
POSTN	Core matrisome	ECM Glycoproteins
FBN1	Core matrisome	ECM Glycoproteins
COMP	Core matrisome	ECM Glycoproteins
CILP	Core matrisome	ECM Glycoproteins
FGG	Core matrisome	ECM Glycoproteins
FGB	Core matrisome	ECM Glycoproteins
FGA	Core matrisome	ECM Glycoproteins
HP	heme iron recycling	
TF	iron transport, saturation associated with telomeric ageing	
ALB	main protein of plasma	

ATT M   AT M   ATT O   AT O

B

Collagens

Proteoglycans and glycoproteins

Fascicles

A

FMOD	Core matrisome	Proteoglycans
BGN	Core matrisome	Proteoglycans
MMP3	Matrisome-associated	ECM Regulators
TIMP3	Matrisome-associated	ECM Regulators
COL6A2	Core matrisome	Collagens
COL6A3	Core matrisome	Collagens
COL3A1	Core matrisome	Collagens
COL5A1	Core matrisome	Collagens
COL5A2	Core matrisome	Collagens
VTN	cell adhesion and spreading factor	
APCS	involved in apoptosis	
CLU	upregulated in ageing and in increased levels of organismal stress	
PLA2G2A	involved in cellular senescence, may contribute to ageing inflammatory disorders	
TF	iron transport, saturation associated with telomeric ageing	
KRT10	major epithelial keratin	
KRT2	major epithelial keratin	
KRT9	major epithelial keratin	

ATT M   AT M   ATT O   AT O

B

A

IFM

AT old

ATT old

B

Fascicles

AT old

ATT old

Growth factor

Peptidase

Transmembrane Receptor

Transporter
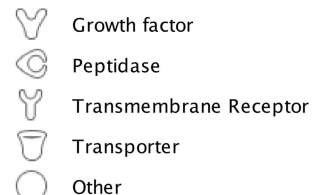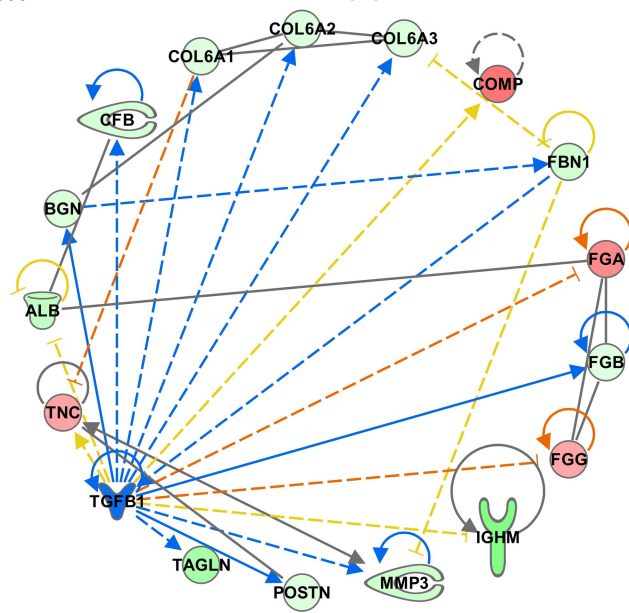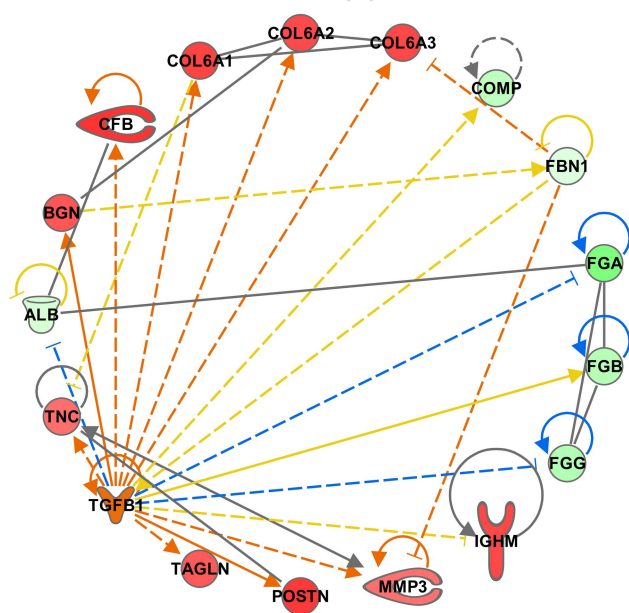
Other
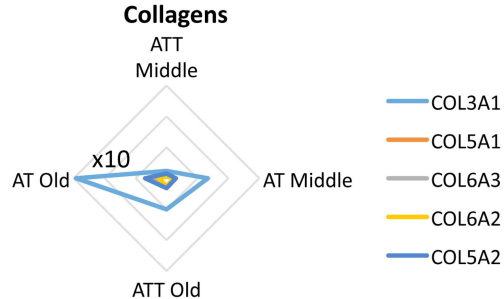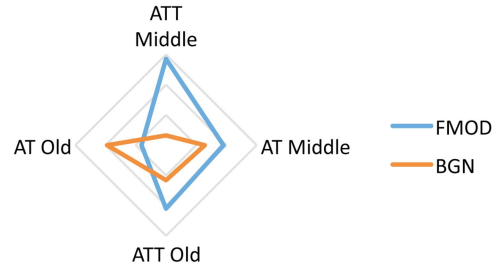
Direct Relationship

Indirect Relationship

Prediction Legend

more extreme in dataset   less

Increased measurement

Decreased measurement

more confidence   less

Predicted activation

Predicted inhibition

Glow Indicates activity
when opposite
of measurement

Predicted Relationships

Leads to activation

Leads to inhibition

Findings inconsistent
with state of downstream
molecule

Effect not predicted