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1 **Human Achilles tendon glycation and function in diabetes**

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28  
29 Running Title: The Human Diabetic Tendon

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36  
37

38 **Abstract**

39 Diabetic patients have an increased risk of foot ulcers, and glycation of collagen may increase tissue  
40 stiffness. We hypothesized that the level of glycemic control (glycation) may affect Achilles tendon  
41 stiffness, which can influence gait pattern. We therefore investigated the relationship between  
42 collagen glycation, Achilles tendon stiffness parameters and plantar pressure in poorly ( $n = 22$ ) and  
43 well ( $n = 22$ ) controlled diabetic patients, including healthy age matched (45-70 yrs) controls ( $n =$   
44 11). There were no differences in any of outcome parameters (collagen cross-linking or tendon  
45 stiffness) between patients with well-controlled and poorly controlled diabetes. The overall effect of  
46 diabetes was explored by collapsing the diabetes groups (DB) compared to the controls. Skin  
47 collagen cross-linking lysylpyridinoline (LP), hydroxyllysylpyridinoline (HP), (136%, 80%,  $P <$   
48 0.01) and pentosidine concentrations (55%,  $P < 0.05$ ) were markedly greater in DB. Furthermore,  
49 Achilles tendon material stiffness was higher in DB (54%,  $P < 0.01$ ). Notably, DB also  
50 demonstrated higher forefoot/ rearfoot peak plantar pressure (PPP)-ratio (33%,  $P < 0.01$ ). Overall,  
51 Achilles tendon material stiffness and skin connective tissue cross-linking were greater in diabetic  
52 patients compared to controls. The higher foot pressure indicates that material stiffness of tendon  
53 and other tissue (e.g skin and joint capsule) may influence on foot gait. The difference in foot  
54 pressure distribution may contribute to the development of foot ulcers in diabetic patients.

55

56 Key words: Diabetes, Enzymatic and non-enzymatic collagen cross-linking, Achilles tendon  
57 mechanics, Foot ulcer

58

59  
60

61 **Introduction**

62 Pathological conditions of the feet remain an extensive clinical problem in persons with diabetes  
63 (6), and advanced diabetes ulcerations of the forefoot are the main reason for lower extremity  
64 amputations (20). In fact, approximately 25% of all hospital admissions of diabetic patients  
65 encompass pathological conditions of the feet, and about 15% of all diabetes patients will develop a  
66 foot ulcer (20). In addition to this, Achilles tendon problems are more pronounced in patients with  
67 diabetes (1), but it is unknown to what extent this is due to altered tendon tissue properties in  
68 diabetes, or rather is secondary to altered gait pattern or skin ulcers.

69 Patients with poorly controlled diabetes have elevated plasma glucose concentrations, and  
70 this is associated with the accumulation of AGE (Advanced Glycation Endproducts) derived cross-  
71 links in various collagenous tissues such as skin, via the Maillard reaction (37). There is evidence  
72 that compromised tissue function is a consequence of such increases in AGE cross-linking (4, 37-  
73 39, 46). In vitro experiments have shown that glycation increases tendon stiffness and strength (3,  
74 26, 27, 45). Increased collagen and tendon stiffness, due to the accumulation of intermolecular AGE  
75 cross-links, has been proposed as a concomitant factor in the development of pathological foot  
76 conditions in diabetes (23, 41), but reports on AGE accumulation in the human diabetic tendon is  
77 sparse (24, 52).

78 Evidence of mechanical changes in diabetic tendons is currently inconclusive, since the  
79 effect of diabetes on animal tendon has been reported to result in increased (2, 35, 42) or decreased  
80 (7, 12, 18) stiffness properties. In addition, it has not been investigated if the quality of glycemic  
81 control in diabetic patients affects AGE cross-linking and tendon stiffness. At the micro-structural  
82 level, the extent to which tendon collagen fibrils are affected by diabetes is also sparsely  
83 investigated (23). A few animal studies (3, 43) and a single human study (23) have demonstrated  
84 significant changes in tendon fibril morphology (increased fibril density and decreased mean fibril

85 area). The biomechanical consequences of these changes in terms of potential alterations in tendon  
86 tissue stiffness currently remain unknown.

87 The influence of Achilles tendon stiffness on gait patterns in diabetic patients is unknown,  
88 but elevated Achilles tendon stiffness may well decrease dorsiflexion capacity of the ankle joint,  
89 and reduced dorsiflexion has been reported to increase forefoot loading (17). Moreover, excessive  
90 plantar pressure has been shown to result in elevated tissue breakdown and delayed wound healing  
91 in the foot (41) and could be a risk factor for diabetes related pathological foot conditions (51).  
92 Therefore, the purpose of the present study was to investigate the hypothesis that poorly controlled  
93 diabetes is associated with greater accumulation of AGE cross-links, greater tendon stiffness and  
94 altered gait pattern compared to well-controlled diabetes, that may lead to development of foot  
95 ulcers. This hypothesis was tested by examining the concentration of enzymatic and non-enzymatic  
96 collagen cross-links in skin and tendon, Achilles tendon stiffness, and the modulation in plantar  
97 pressure during gait in poorly and well-controlled diabetic patients compared to healthy age-  
98 matched controls.

99

## 100 **Methods**

101 The present cross-sectional study was designed to compare the effect of glycemic control (based on  
102 2 year average HbA1c) in two groups of male diabetic patients (type I and type II) with either well  
103 ( $n = 22$ , HbA1c  $< 7.5\%$ ; WCD) or poorly- ( $n = 22$ , HbA1c  $> 9\%$ ; PCD) controlled diabetes. The  
104 number of type 1 diabetic patients was: 1 in WCD and 3 in PCD. Subject characteristics are shown  
105 in Table 1. A smaller healthy control group was also included to provide baseline healthy  
106 characteristics ( $n = 11$ , HbA1c  $< 6\%$ ; CON). Subjects were matched for age (45-70 years) and  
107 physical activity. Exclusion criteria in both WCD, PCD and CON included neuropathy of non-  
108 diabetic origin, severe neuropathy, foot ulcers, severe arterial insufficiency, arthritis of the ankle or

109 foot, previous foot surgery, previous Achilles tendon rupture, amputations, previous Charcot foot,  
110 body mass > 110 kg and use of anti-thrombotic medication. The presence of clinical neuropathy  
111 was assessed by use of Semmes-Weinstein 5.07 monofilament exam and biothesiometry. The Ethics  
112 Committee of the Capital Region of Denmark approved study (journal number 25543), and all  
113 procedures conformed to the Declaration of Helsinki. Written, informed consent was obtained from  
114 all subjects prior to study onset.

115

#### 116 *Physical Activity*

117 Physical activity was assessed using the International Physical Activity Questionnaire - (IPAQ,  
118 Swedish version translated into Danish) quantified as weekly metabolic equivalent of task-(MET)  
119 minutes.

120

#### 121 *Blood Sampling*

122 Blood samples of 10 mL were collected before the test day and sent for standard clinical blood tests  
123 for triglycerides, high and low-density lipoprotein cholesterol (HDL and LDL), total cholesterol and  
124 HbA1c as a measure of mean glucose load over the previous 2-3 months (14, 40). For the diabetic  
125 patients (WCD, PCD), the two-year average HbA1c was also determined based on data from their  
126 medical records (3-4 measurements).

127

#### 128 *Biopsy Sampling*

129 After biomechanical testing was performed (details given below) biopsy specimens of the Achilles  
130 tendon were obtained in the non-dominant leg at the distal end of the tendon 4 cm proximal to the  
131 calcaneus. Using ultrasound imaging, the biopsy site was marked on the skin and under local  
132 anesthetic (1% lidocaine) the biopsy was obtained with a 16 gauge Bard Monopty triggered biopsy

133 instrument (C. R. Bard Inc, Covington GA). Skin biopsies were performed using a 4 mm biopsy  
134 punch (Miltex, York PA) in the gluteal region under local anesthetic (1% lidocain). Both tendon  
135 and skin biopsies were immediately frozen in liquid nitrogen for cross-link analysis, and a small  
136 segment from the tendon was also placed in 0.05 M phosphate buffered 2% glutaraldehyde for  
137 electron microscopy.

138

### 139 *Collagen Cross-links*

140 The concentrations of enzymatic cross-links lysylpyridinoline (LP) and hydroxylysylpyridinoline  
141 (HP), and non-enzymatic AGE cross-link pentosidine in the biopsy samples were quantified as  
142 previously described (8, 32). In brief, the tendon biopsy was hydrolysed in 6 M HCl and run on a  
143 reversed-phase high performance liquid chromatography column with detection by  
144 autofluorescence. The cross-link content was normalized to total collagen content based on  
145 hydroxyproline measurement by 4-dimethylaminobenzaldehyde color reaction after oxidation, as  
146 previously described (8, 32). Three tendon and 5 skin biopsies were lost during processing for  
147 cross-link analysis.

148

### 149 *Electron Microscopy*

150 Transmission electron microscopy was performed as previously reported (30, 32). In brief,  
151 glutaraldehyde fixated samples were stained en-bloc with OsO<sub>4</sub> and embedded in epon. Ultrathin  
152 ( $\approx 100\text{nm}$ ) cross-sections were cut and stained with uranyl acetate and lead citrate. Ten  $10 \times 10 \mu\text{m}^2$   
153 images were obtained in a random pattern across each section to avoid selection bias. In each image  
154 36 unbiased counting frames and an unbiased point grid were used to determine collagen fibril  
155 density, volume fraction and size. Five biopsies were lost during processing for electron  
156 microscopy.



157

158

159 *Achilles Tendon Morphology*

160 Details of the tendon morphology measurements have previously been published (29). In brief, the  
161 subject was sitting with the hip, knee and ankle at 90° and using a 100 mm long ultrasound probe  
162 the full length of the free tendon from its insertion on the calcaneus to its fusion with the soleus  
163 muscle was imaged in B-mode. Using the ultrasound “shadow” of a long needle, the calcaneus and  
164 soleus insertions were marked on the skin with a permanent marker. Three evenly spaced marks  
165 were placed between the two ends (proximal, mid and distal), and axial ultrasound images were  
166 recorded at each point for determining tendon cross-sectional area (CSA) as previously described  
167 (29). The average tendon CSA was calculated and used for analysis. The paired student's t-test  
168 (systematic error), Pearson correlation coefficient (strength of relationship) and typical error percent  
169 for duplicate measures within day were 0.64, 0.93 and 3% for proximal, 0.70, 0.90 and 4% for mid  
170 tendon, and 0.57, 0.90 and 4% for distal tendon. The Achilles tendon moment arm was determined  
171 as the distance from the foot axis of rotation (mean of medial and lateral malleoli) to the tendon line  
172 of action (mid line between calcaneus and soleus insertion) as previously described (29).

173

174 *Achilles Tendon Mechanical Properties*

175 Mechanical properties of the Achilles tendon were assessed using a method that has previously been  
176 described and validated in detail (29). In brief, subjects were seated in a rigid chair with the hip,  
177 knee and ankle at 90°. The foot was resting on a footplate with the foot axis of rotation vertically  
178 above the plate axis of rotation (see Figure 1). The knee was immobilized by a steel cross-bar to  
179 prevent lower limb motion (29). A load cell fixed to the footplate was used to measure the plantar  
180 flexor moment. Electromyography (EMG) electrodes were attached to the tibialis anterior and

181 soleus muscles to monitor muscle activation and correct for antagonist co-activation as previously  
182 described (29). Achilles tendon deformation was monitored using B-mode ultrasound imaging  
183 (Hitachi EUB-6500) with a 100 mm long 10 MHz probe positioned along the tendon to visualize  
184 the insertion at the calcaneus and soleus.

185 Achilles tendon mechanics were assessed during slow (10s) isometric plantar flexion ramps  
186 to maximum voluntary contraction. Force and EMG were recorded synchronously with ultrasound  
187 video (29). To correct the Achilles tendon force for antagonist muscle co-activation, the relationship  
188 between tibialis anterior EMG amplitude and its resulting dorsiflexor moment was determined  
189 during a maximal isometric dorsiflexion lasting 5 seconds (29).

190 Tendon deformation was obtained from the ultrasound videos by feature tracking of the  
191 calcaneus and soleus insertions (29). The force-deformation data were fitted to a 3rd order  
192 polynomial and this fit was used for further analysis. Stiffness was measured as the slope over the  
193 last 20% of tendon deformation. Material properties - stress, strain and modulus - of the Achilles  
194 tendon were obtained by dividing force with the mean tendon CSA and dividing deformation with  
195 the initial free tendon length. In order to compare tendon properties at identical load, all parameters  
196 were also determined at the largest common tendon force observed across participants. To avoid the  
197 highly nonlinear toe region commonly observed in tendon at low load, 7 participants (all from the  
198 diabetic groups) with particularly low force production were omitted from this comparison. The  
199 decision to omit the data points in these 7 participants were made prior to running any between-  
200 group analyses. The selected common tendon force level was 1815 N. Five participants did not  
201 complete all morphology and mechanical tests due to logistical reasons.

202

203 *Gait Analysis*

204 Load distribution on the foot during walking was determined using a pressure plate (4 sensors/cm<sup>2</sup>,  
205 Emed, Novel, Germany) integrated into a wooden walking path. Subjects were instructed to walk  
206 normally along the path and the pressure plate was hit at the third step after start. The mean pressure  
207 distribution during 5 steps from each foot was calculated and pressure distribution was assessed by  
208 the forefoot/rearfoot peak plantar pressure ratio (PPP-Ratio). Two participants did not complete gait  
209 analyses due to logistical reasons.

210

### 211 *Data Reduction and Statistics*

212 The study was initially powered for the comparison of the WDC and PDC groups, with the healthy  
213 controls (CON) included only as a baseline. Tendon stiffness was considered the primary outcome  
214 and sample size was determined to be 21 for an effect size of 0.2 with 80% power and a  
215 significance level of 5%. Differences between WCD and PCD were determined by an unpaired two-  
216 tailed Student's t-test corrected for unequal variances. No differences were observed between the  
217 two diabetic groups for any of the outcome variables related to the hypothesis. For this reason it was  
218 decided to also report findings relative to the healthy group as a more exploratory approach, in spite  
219 of this group being underpowered. Acknowledging that the study is underpowered, we also report  
220 some near-significant trends as a basis for future investigation. Diabetic patients were combined  
221 into a merged diabetes (DB) group and subsequently compared to CON using unpaired two-tailed  
222 Students t-tests corrected for unequal variances. Pearson product-moment correlation analysis was  
223 used to analyze the strength of relationships between variables within the merged diabetes group  
224 (DB).  $P < 0.05$  was considered significant. Results are reported as mean  $\pm$  standard error (SE)  
225 unless otherwise reported. Student's t-tests were performed using Excel for Mac 2011 (Microsoft  
226 corporation) while all correlation analysis was performed using Prism 6 (Graphpad Software Inc.).  
227

228 **Results**

229 *Subject characteristics*

230 Diabetes duration was not different between the WCD and PCD groups. HbA1c concentration was  
231 higher in PCD compared to WCD, both at present ( $8.9 \pm 1.7\%$  vs.  $7.2 \pm 0.9\%$ ,  $P < 0.01$ ) and as 2-  
232 year average ( $9.4 \pm 1.4\%$  vs.  $6.9 \pm 0.5\%$ ,  $P < 0.01$ ). Subject characteristics are shown in Table 1.  
233 Body mass was greater in DB compared to CON ( $P < 0.01$ ). The difference in IPAQ score was not  
234 significant between the groups.

235

236 *Collagen cross-linking*

237 Tendon collagen cross-link data are shown in Table 2. None of the parameters collagen,  
238 pentosidine, HP and LP concentration, differed significantly between DB and CON. Tendon  
239 pentosidine was positively related to age ( $r = 0.42$ ,  $P < 0.01$ ). Skin collagen cross-link data are  
240 shown in Table 2. In contrast to tendon, skin pentosidine ( $P < 0.05$ ), LP ( $P < 0.01$ ) and HP ( $P <$   
241  $0.01$ ) concentrations were higher in DB than CON. Two year HbA1c correlated with skin HP ( $r =$   
242  $0.34$ ,  $p < 0.05$ ) and pentosidine ( $r = 0.31$ ,  $p < 0.05$ ).

243

244 *Collagen fibril characteristics*

245 Collagen fibril data are shown in Table 2. Tendon fibril density was greater in DB compared to  
246 CON ( $P < 0.05$ ).

247

248 *Achilles Tendon Morphology*

249 The Achilles tendon moment arm was greater in DB compared to CON ( $4.26 \pm 0.07$  vs.  $3.94 \pm 0.10$   
250 cm,  $P < 0.05$ ). However, no other differences were observed between DB and controls with respect

251 to average Achilles tendon CSA ( $0.73 \pm 0.02$  vs.  $0.79 \pm 0.03$  cm<sup>2</sup>,  $P = 0.23$ ) or free Achilles tendon  
252 length ( $6.5 \pm 0.2$  vs.  $6.1 \pm 0.4$  cm,  $P = 0.47$ ).

253

#### 254 *Mechanical Tendon Properties*

255 Mechanical properties of the Achilles tendon at maximum force are shown in Table 3. DB did not  
256 differ from CON although there was a trend toward reduced Achilles tendon strain in DB compared  
257 to controls (effect size 0.9%,  $P = 0.075$ ). Mechanical properties of the Achilles tendon at largest  
258 common force are shown in Table 3. DB had higher Achilles tendon modulus at common force than  
259 CON ( $P < 0.001$ ).

260

#### 261 *Gait Analysis*

262 Gait data are shown in Table 3. DB demonstrated greater forefoot/rearfoot PPP-Ratio than CON ( $P$   
263  $< 0.05$ ).

264

#### 265 **Discussion**

266 To the best of our knowledge the present study is the first to investigate if diabetes in humans is  
267 associated with greater Achilles tendon glycation and stiffness, and altered gait. In contrast to our  
268 initial hypothesis, we could not demonstrate any differences in collagen cross-linking or  
269 biomechanical Achilles tendon stiffness between patients with well-controlled and poorly-  
270 controlled diabetes. However, in skin collagen cross-linking (HP, LP and pentosidine  
271 concentrations) was markedly greater in diabetic patients compared to healthy age-matched  
272 controls. Furthermore, Achilles tendon modulus, which represents the material stiffness after  
273 accounting for tendon dimensions, was higher in diabetic patients compared to controls. Notably,  
274 diabetic patients also demonstrated higher forefoot/rearfoot peak plantar pressure ratio (PPP-ratio)

275 indicating a more forward distributed loading pattern on the foot. This difference in foot pressure  
276 distribution may contribute to the development of foot ulcers in diabetic patients. These findings  
277 lend some support to the hypothesis that diabetes leads to increased stiffness in the Achilles tendon  
278 and an elevated forefoot pressure.

279

#### 280 *Collagen cross-linking*

281 In diabetes there is an increased rate of non-enzymatic formation of AGE cross-links, which may  
282 also affect the protein structure and function of connective tissue such as tendon and skin. In  
283 collagen one such cross-link is pentosidine, and in the present study the concentration of  
284 pentosidine was greater in skin of diabetic patients, although somewhat surprisingly not elevated in  
285 the Achilles tendon. In agreement with the present skin data, previous work on experimental animal  
286 and human skin composition also show increased pentosidine concentration (16, 37) and other  
287 glycation products with diabetes (5, 13). In contrast, data on cross-links in the diabetic tendon are  
288 scarce. A greater glycation in the tendon of diabetic human digastric muscle and diaphragm has  
289 been shown, although pentosidine was not measured specifically (24, 52). In diabetic animals,  
290 increased glycation of tendon has also been reported (35, 42). The difference between tendon and  
291 skin data in the present study may relate to differences in tissue turnover. Tendons have very slow  
292 turnover, and may even be maintained throughout adult life (25), while skin has a much more rapid  
293 turnover rate (50), as also indicated by the lower pentosidine concentrations presently observed in  
294 skin biopsies compared to tendon biopsies. Consequently, pentosidine in tendon most likely  
295 represent an average over a longer time period than that of skin, and therefore the relative effect of  
296 the period with diabetes may be smaller in tendon tissue.

297 Another factor potentially affecting the pentosidine concentration in Achilles tendons is the  
298 level of physical activity of the subjects. It has recently been shown that the pentosidine

299 concentration of the patellar tendon is reduced in elderly life-long regular endurance runners  
300 (master athletes) compared to sedentary controls (9), and that resistance training can reduce  
301 pentosidine concentration in patellar tendons (28). If loading of tendons can ameliorate AGE  
302 accumulation, it may also explain why greater AGE accumulation was observed in the diabetic  
303 digastric tendon as previously mentioned, since this tendon is not weight bearing.

304         The present study also revealed markedly greater HP and LP concentrations in the skin of  
305 diabetic patients compared to healthy controls. The concomitant greater in glycation and enzymatic  
306 cross-links is in agreement with previous reports on skin collagen in diabetic conditions (5).  
307 Conversely, in the Achilles tendon we did not observe a similar greater cross-linking (HP and LP ( $P$   
308 = 0.10)) with diabetes, which to our knowledge has not previously been examined in human  
309 diabetic tendons. A simultaneous greater HP, LP and pentosidine with aging have been  
310 demonstrated in the human patellar tendon (8). Based on the 'synchronized' changes in non-  
311 enzymatic and enzymatic cross-links reported in both diabetes and aging, it is reasonable to  
312 speculate that some mechanistic link(s) may exist between the two cross-linking processes. The  
313 finding that serum two-year average HbA1c and skin pentosidine in the present study demonstrated  
314 a weak relationship ( $r = 0.31$ ,  $P < 0.05$ ) while this was not the case in the tendon ( $r = 0.03$ ,  $P =$   
315  $0.84$ ). This may indicate that the skin tissue is subjected to a systemic effect of AGEs with less  
316 protection by physical activity and mechanical loading, which thereby could lead to greater  
317 accumulation of non-enzymatic cross-links in skin compared to tendon. Despite superior glycemic  
318 control (Hb1Ac) in WCD compared to PCD there were no differences in any of the collagen cross-  
319 linking parameters examined, which is in agreement with observations by Lyons et al. who reported  
320 similar skin pentosidine content in type 1 diabetic patients with better glycemic control (34).  
321 Monnier et al (37) reported an approximately 20% lower skin pentosidine in diabetic patients with  
322 improved glycemic control and considering the absolute difference observed in the present study,

323 there is in fact a similar difference, so the lacking effect may reflect a sample size issue.

324

### 325 *Collagen fibril morphology*

326 Some studies have reported on tendon microstructural changes in diabetes. Both animal and human  
327 studies have reported greater collagen fibril density and decreased mean fibril area (3, 23, 43). The  
328 present study revealed a 25% higher fibril density in diabetic patients compared to controls.

329 Furthermore, mean fibril diameter and mean fibril area tended ( $P = 0.096$ ) to be reduced (11%) in  
330 diabetic patients compared controls, confirming previous findings (3, 23, 43). Why diabetic tendon  
331 collagen fibrils display higher fibril density is unknown. It has been speculated that closer packing  
332 density could be a result of AGEs binding together collagen fibrils (3, 33). Another mechanism  
333 could be that the higher density is a compensating mechanism for a lower mean fibril diameter  
334 thereby maintaining total collagen content and volume fraction in agreement with our findings.  
335 However these mechanisms need to be explored further.

336

### 337 *Achilles Tendon Mechanical Properties*

338 In the present study we observed no difference in Achilles tendon mechanics expressed in absolute  
339 terms between WCD and PCD, however a 54% greater Young modulus was observed in diabetic  
340 patients compared to healthy controls, indicating that qualitative differences exist between diabetic  
341 and healthy Achilles tendon tissue. Diabetes has previously been associated with mechanical  
342 changes in different tissues including tendon. In experimental diabetic animals greater stiffness has  
343 been extensively reported in non-weight bearing rat-tail tendon (2, 19, 21, 22, 35, 42, 53) and knee  
344 ligaments (15). Likewise, in various human non-weight bearing connective tissue such as blood  
345 vessels (49) and the lens of the eye (44), it has been reported that diabetes induces greater tissue  
346 stiffness. A modest increased stiffness has also been demonstrated in weight bearing diabetic canine



347 patellar tendon under long-term insulin therapy (31). In contrast, lower stiffness of the Achilles  
348 tendon has been reported in several experimental diabetic animal studies (7, 12, 18), and this may  
349 be attenuated by weight bearing physical activity (11). It was recently shown that Achilles tendon,  
350 strains are less during walking in human diabetic patients than in controls, which may indicate that  
351 greater tendon stiffness could be related to observed differences in the gait pattern of these patients  
352 (10). To our best knowledge the present study is the first to directly measure the mechanical  
353 properties of human diabetic Achilles tendons in vivo. Our data show a markedly (54%) higher  
354 Achilles tendon material stiffness (modulus) compared to controls, however, absolute tendon  
355 stiffness was not significantly different despite it was numerically 27% greater in diabetic patients.  
356 The difference between the modulus and stiffness lies in the tendon dimensions, with the diabetic  
357 tendon towards a greater tendon length and reduced cross-sectional area (neither significant), which  
358 counteracts the greater material stiffness. It is possible that the Achilles tendon dimensions of  
359 diabetic patients may have adapted to counteract increased material stiffness in order to maintain  
360 functional stiffness, but this hypothesis cannot be addressed by the data obtained in the present  
361 study.

362         Cross-linking by AGEs is the likely mechanism underpinning tissue stiffening with diabetes  
363 (38), and AGE cross-links have been shown to increase tendon stiffness in vitro, where tendon is  
364 incubated with a reducing sugar (26, 27, 45). In the present study the material stiffness of the  
365 Achilles tendon was greater with diabetic patients, however no differences were observed in  
366 pentosidine or HP, LP cross-link concentrations. In addition, collagen content also did not differ  
367 between diabetic patients and healthy controls. The diabetic patients had a higher fibril density, but  
368 due to their tendency ( $P = 0.096$ ) to toward a lower fibril size the total volume fraction, and thus the  
369 load bearing cross-sectional area was unaltered.

370

371 *Gait*

372 In the present study, diabetic patients demonstrated higher forefoot/rearfoot PPP-ratio indicating  
373 increased forefoot loading during walking. This finding is in agreement with our initial hypothesis.  
374 A forward shift in pressure could be caused by an increased ankle joint stiffness; however, the  
375 hypothesized relation to absolute Achilles tendon stiffness was not observed. As previously  
376 discussed, the weight bearing nature of the Achilles tendon may render it less susceptible to diabetic  
377 changes than other tissues crossing the joint. Since diabetes is a systemic disease these other tissues  
378 are likely also affected and may contribute to overall joint stiffness. One concern could be that the  
379 difference in tendon moment arm observed between the two subject groups would influence these  
380 findings, however, the moment arm was not correlated to either forefoot/rearfoot PPP-ratio or  
381 tendon modulus, respectively. However, the potential influence of the Achilles tendon should not  
382 be completely disregarded, since the modulus was greater and there were tendencies for both  
383 greater absolute stiffness and reduced strain, and as such, a lack of sensitivity may have prevented.  
384 Stiffening of the Achilles tendon material properties combined with the observed tendency for  
385 decreased tendon strain (potentially causing reduced dorsiflexor ROM during the late stance phase)  
386 could *per se* cause an increased magnitude of forefoot loading, and any systemic glycation effect  
387 would likely also stiffen other connective tissues surrounding the joint. Notably, reduced  
388 dorsiflexion ability has been shown to increase peak plantar pressure during walking (17) while  
389 excessive plantar pressure has been shown to result in accelerated tissue breakdown and delayed  
390 wound healing (41).

391

392 *Study Limitations*

393 The present investigation is a cross-sectional case-control study and, therefore, has inherent  
394 limitations. Furthermore, while a fairly large number of diabetic patients were recruited, a larger

395 number of control subjects would have improved the statistical strength. In the present study the  
396 only measured AGE marker was pentosidine, which constitutes a small fraction of AGE cross-links  
397 (47). Even though pentosidine is reported to correlate well with diabetic tissue complications (48),  
398 total AGE fluorescence (36, 48) and with more abundant AGEs such as carboxymethyllysine  
399 (CML)(4), it is possible that investigating other AGE targets (47) could have provided additional  
400 information to help explain the greater Achilles tendon mechanics in our diabetic patients.

401 In vivo mechanical measurements are also affected by several limitations. The tendon load  
402 is estimated from external moments, and while muscle activation was partly accounted for by EMG  
403 measurements, there are still uncertainty in such measures. In addition, the CSA used for  
404 determining tendon stress was measured by ultrasound, which is less precise than for example MRI.  
405 Finally, tendon deformation is also determined with ultrasound in 2D and some uncertainty may be  
406 present due to out of plane motion. These factors combine to increase the variance of the  
407 measurements, but should affect the groups equally.

408 There were differences in the baseline characteristics of the two groups, which could affect  
409 the outcome. The diabetic group had a higher body mass, and as would be expected peak plantar  
410 pressure did correlate with body mass ( $r = 0.23$ ,  $P = 0.1$ ), the forefoot/rearfoot PPP-ratio was not  
411 correlated to body mass ( $r = 0.06$ ,  $P = 0.66$ ). Furthermore, tendon stiffness correlated with body  
412 mass ( $r = 0.34$ ,  $P = 0.03$ ), but body mass was not linked to modulus ( $r = 0.22$ ,  $P = 0.14$ ).  
413 Moreover, the moment arm in diabetic group was higher than in controls. In the present study, the  
414 method used to determine moment arm may have some limitations that could have influenced our  
415 results. Using e.g. x-ray would have been more precise. However, we were not able demonstrate  
416 that the difference in moment arm correlated with the outcome parameters (Forefoot/rearfoot PPP-  
417 ratio:  $r = -0.10$ ,  $P = 0.48$ , Modulus:  $r = -0.16$ ,  $P = 0.30$ ). In addition, the higher moment arm in  
418 the diabetic group would have underestimated modulus and thereby cannot be the reason for the

419 observed increase in the diabetic group. To our knowledge there is no evidence that diabetes results  
420 in altered moment arm and so we would believe that the difference observed in the present study is  
421 spurious. Altogether, if we include mass and moment arm as confounding factors in an ANOVA,  
422 the main findings of increased forefoot/rearfoot PPP-ratio and modulus in the diabetic group remain  
423 significant.

424

#### 425 *Conclusions*

426 For the first time it was demonstrated that irrespective of hyperglycemia severity Achilles tendon  
427 material stiffness was greater in diabetic patients compared to age-matched healthy controls. The  
428 finding that well and poor glycemic controlled diabetic patients did not differ in terms of  
429 biomechanical Achilles tendon properties was in contrast to our initial hypothesis. Surprisingly,  
430 collagen cross-linking also did not differ in the Achilles tendon of the diabetic patients compared to  
431 that of controls. In contrast, when assessed in the skin HP, LP and pentosidine cross-link  
432 concentrations were markedly greater in diabetic patients compared to controls. Furthermore,  
433 diabetic patients showed higher forefoot/rearfoot PPP-ratio during walking, however, a direct  
434 relation to increased Achilles whole tendon stiffness was not found, indicating that altered Achilles  
435 tendon material stiffness and possibly also in other tissues (e.g skin and joint capsule) may  
436 influence plantar pressure distribution during gait habitual walking. Collectively, our data suggest  
437 that both the material stiffness of the Achilles tendon and foot pressure distribution are altered in  
438 diabetic patients. Such changes in tendon material properties and loading may have implications for  
439 the development of diabetic foot ulcers.

440

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594 **Tables**

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	<b>WCD</b>	<b>PCD</b>	<b>DB</b>	<b>CON</b>
Number of participants	22	22	44	11
Age (yrs)	60 ± 7	58 ± 7	59 ± 7	58 ± 5
Height (cm)	177 ± 5	180 ± 6	178 ± 6	177 ± 5
Mass (kg)	91 ± 13	96 ± 10	93 ± 12**	83 ± 8
BMI (kg·m <sup>-2</sup> )	29 ± 4	30 ± 4	29 ± 6	27 ± 3
Diabetes duration (yr)	12 ± 6	15 ± 8	13 ± 7	-
HbA <sub>1c</sub> 2yr average (%)	6.9 ± 0.5	9.4 ± 1.4###	8.1 ± 0.3	-
(mmol·mol <sup>-1</sup> )	51 ± 6	79 ± 16	65 ± 3	-
HbA <sub>1c</sub> present (%)	7.2 ± 0.9**	8.9 ± 1.7###, **	8.0 ± 0.3**	5.5 ± 0.3
(mmol·mol <sup>-1</sup> )	61 ± 9	73 ± 18	64 ± 3	36 ± 4
Triglyceride (mmol·l <sup>-1</sup> )	1.7 ± 0.3	1.7 ± 0.2	1.7 ± 0.1	1.7 ± 0.4
Total cholesterol (mmol·l <sup>-1</sup> )	4.6 ± 0.3	4.9 ± 0.2	4.7 ± 0.2	5.6 ± 0.4
HDL Cholesterol (mmol·l <sup>-1</sup> )	1.30 ± 0.09	1.21 ± 0.10	1.25 ± 0.07	1.37 ± 0.13
LDL Cholesterol (mmol·l <sup>-1</sup> )	2.4 ± 0.2	2.9 ± 0.1	2.7 ± 0.2*	3.6 ± 0.3
IPAQ (MET Score)	2300 ± 1800	1700 ± 1800	2000 ± 300	1400 ± 900

596 **Table 1 - Subject characteristics. WCD = well-controlled diabetic patients, PCD = poorly-controlled diabetic**  
597 **patients, DB = merged diabetic patients (WCD + PCD), CON = healthy, age -matched controls.** Data are given as  
598 mean ± SD). Different from WCD, #*P* < 0.05, ###*P* < 0.01\*Different from CON, \**P* < 0.05. \*\**P* < 0.01.  
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	WCD	PCD	DB	CON
<b>Tendon Composition</b>				
Number of participants	21	21	42	10
Collagen (mg·mg <sup>-1</sup> dry wt)	0.73 ± 0.03	0.70 ± 0.02	0.72 ± 0.03	0.75 ± 0.03
Hydroxylysyl pyridinoline (HP, mmol·mol <sup>-1</sup> collagen)	1230 ± 80	1340 ± 70	1250 ± 50	1220 ± 80
Lysyl pyridinoline (LP, mmol·mol <sup>-1</sup> collagen)	52 ± 3	53 ± 3	52 ± 2 (*) P = 0.101	43 ± 5
Pentosidine, (mmol·mol <sup>-1</sup> collagen)	33 ± 2	30 ± 3	31 ± 2	28 ± 2
<b>Skin Composition</b>				
Number of Participants	21	20	41	9
Collagen (mg·mg <sup>-1</sup> dry wt)	0.62 ± 0.01	0.64 ± 0.02	0.63 ± 0.01	0.65 ± 0.02
Hydroxylysyl pyridinoline (HP, mmol·mol <sup>-1</sup> collagen)	35 ± 10	54 ± 10	45 ± 6**	19 ± 4
Lysyl pyridinoline (LP, mmol·mol <sup>-1</sup> collagen)	8 ± 1	9 ± 2	9 ± 1**	5 ± 1
Pentosidine, (mmol·mol <sup>-1</sup> collagen)	13 ± 2	16 ± 2	14 ± 1*	9 ± 2
<b>Tendon Fibril Morphology</b>				
Number of Participants	18	22	40	10
Volume fraction (%)	53 ± 2	54 ± 1	53 ± 1	57 ± 2
Density (#fibril·μm <sup>-2</sup> )	132 ± 10	130 ± 11	131 ± 7*	105 ± 8
Mean fibril diameter (nm)	64 ± 4	65 ± 3	64 ± 2(*) P = 0.096	73 ± 14
Mean fibril area (nm <sup>2</sup> )	4300 ± 500	4400 ± 400	4400 ± 300	5500 ± 600

**Table 2. Tendon collagen cross-link and fibril composition.** Data are given as mean ± SE. Different from CON, \*P < 0.05, \*\* P < 0.01. Compared with CON (\*).

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	WCD	PCD	DB	CON
<b>Achilles Tendon Mechanics</b>				
<b>(At maximum force)</b>				
Number of participants	20	21	41	9
Deformation (mm)	1.80 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	2.3 ± 0.3
Max force (N)	2600 ± 200	2400 ± 200	2500 ± 200	2800 ± 200
Stiffness (kN·mm <sup>-1</sup> )	3.4 ± 0.3	3.4 ± 0.1	3.4 ± 0.3	3.1 ± 0.5
Stress (MPa)	41 ± 5	36 ± 3	39 ± 3	40 ± 3
Strain (%)	2.8 ± 0.3	2.7 ± 0.2	2.8 ± 0.2(*) <i>P</i> = 0.075	3.7 ± 0.4
Modulus (GPa)	3.1 ± 0.2	3.2 ± 0.4	3.1 ± 0.3	2.5 ± 0.3
<b>Achilles Tendon Mechanics</b>				
<b>(At common force)</b>				
Number of Participants	17	17	34	9
Deformation (mm)	1.6 ± 0.3	1.6 ± 0.2	1.6 ± 0.2	1.9 ± 0.3
Stiffness (kN·mm <sup>-1</sup> )	2.7 ± 0.3	2.7 ± 0.3	2.7 ± 0.2	2.0 ± 0.4
Stress (MPa)	28 ± 2	25 ± 1	27 ± 1	26 ± 1
Strain (%)	2.5 ± 0.4	2.5 ± 0.2	2.5 ± 0.2	3.2 ± 0.4
Modulus (GPa)	2.5 ± 0.2	2.5 ± 0.3	2.5 ± 0.2**	1.7 ± 0.1
<b>Foot pressure mapping</b>				
Number of Participants	21	22	43	10
Peak Plantar Pressure (PPP) (kPa)	650 ± 40	620 ± 40	640 ± 30	580 ± 50
Forefoot PPP (kPa)	630 ± 40	600 ± 40	620 ± 30	530 ± 60
Rearfoot PPP (kPa)	410 ± 20	440 ± 30	42 ± 20	450 ± 30
Forefoot/rearfoot PPP-Ratio	1.7 ± 0.2	1.5 ± 0.1	1.6 ± 0.1*	1.2 ± 0.1

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**Table 3. Achilles tendon mechanics and foot pressure mapping.** Data are given as mean  $\pm$  SE. Different from CON, \* $P < 0.05$ , \*\*  $P < 0.01$ .  
Achilles tendon mechanical properties determined at maximum and highest common force of 1815 N. Note: Modulus is based on average Achilles tendon CSA.

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617 **Figure legends**

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619 **Figure 1**

620 The Achilles tendon stress-strain relationship based on largest common tendon force observed for

621 merged Diabetic patients (DB) and age-matched healthy controls. Data are given as mean  $\pm$  SE. DB

622 showed higher Achilles tendon modulus than controls at highest common tendon force ( $P < 0.001$ ).

623 A = Ultrasound Transducer, B = Strain Gauge.

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