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Human Achilles tendon glycation and function in diabetes

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Running Title: The Human Diabetic Tendon

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Abstract

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

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

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Introduction

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

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

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

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

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

Methods

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

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

Physical Activity

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

Blood Sampling

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

Biopsy Sampling

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

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

Collagen Cross-links

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

Electron Microscopy

Transmission electron microscopy was performed as previously reported (30, 32). In brief, glutaraldehyde fixated samples were stained en-bloc with OsO_4 and embedded in epon. Ultrathin (≈100nm) cross-sections were cut and stained with uranyl acetate and lead citrate. Ten 10x10 μm^2 images were obtained in a random pattern across each section to avoid selection bias. In each image 36 unbiased counting frames and an unbiased point grid were used to determine collagen fibril density, volume fraction and size. Five biopsies were lost during processing for electron microscopy.

Achilles Tendon Morphology

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

Achilles Tendon Mechanical Properties

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

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

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

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

Gait Analysis

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

Data Reduction and Statistics

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

Results

Subject characteristics

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

Collagen cross-linking

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

Collagen fibril characteristics

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

Achilles Tendon Morphology

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

to average Achilles tendon CSA (0.73 ± 0.02 vs. 0.79 ± 0.03 cm^2, P = 0.23) or free Achilles tendon length (6.5 ± 0.2 vs. 6.1 ± 0.4 cm, P = 0.47).

Mechanical Tendon Properties

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

Gait Analysis

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

Discussion

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

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

Collagen cross-linking

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

Another factor potentially affecting the pentosidine concentration in Achilles tendons is the 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,

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

Collagen fibril morphology

Some studies have reported on tendon microstructural changes in diabetes. Both animal and human studies have reported greater collagen fibril density and decreased mean fibril area (3, 23, 43). The present study revealed a 25% higher fibril density in diabetic patients compared to controls. Furthermore, mean fibril diameter and mean fibril area tended ($P = 0.096$) to be reduced (11%) in diabetic patients compared controls, confirming previous findings (3, 23, 43). Why diabetic tendon collagen fibrils display higher fibril density is unknown. It has been speculated that closer packing density could be a result of AGEs binding together collagen fibrils (3, 33). Another mechanism could be that the higher density is a compensating mechanism for a lower mean fibril diameter thereby maintaining total collagen content and volume fraction in agreement with our findings. However these mechanisms need to be explored further.

Achilles Tendon Mechanical Properties

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

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

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

Gait

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

Study Limitations

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

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

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

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

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

Conclusions

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

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Tables

	WCD	PCD	DB	CON
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 ($kg \cdot m^{-2}$)	29 ± 4	30 ± 4	29 ± 6	27 ± 3
Diabetes duration (yr)	12 ± 6	15 ± 8	13 ± 7	-
HbA_1c 2yr average (%)	6.9 ± 0.5	9.4 ± 1.4##	8.1 ± 0.3	-
($mmol \cdot mol^{-1}$)	51 ± 6	79 ± 16	65 ± 3	-
HbA_1c present (%)	7.2 ± 0.9**	8.9 ± 1.7##,**	8.0 ± 0.3**	5.5 ± 0.3
($mmol \cdot mol^{-1}$)	61 ± 9	73 ± 18	64 ± 3	36 ± 4
Triglyceride ($mmol \cdot l^{-1}$)	1.7 ± 0.3	1.7 ± 0.2	1.7 ± 0.1	1.7 ± 0.4
Total cholesterol ($mmol \cdot l^{-1}$)	4.6 ± 0.3	4.9 ± 0.2	4.7 ± 0.2	5.6 ± 0.4
HDL Cholesterol ($mmol \cdot l^{-1}$)	1.30 ± 0.09	1.21 ± 0.10	1.25 ± 0.07	1.37 ± 0.13
LDL Cholesterol ($mmol \cdot l^{-1}$)	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

Table 1 - Subject characteristics. WCD = well-controlled diabetic patients, PCD = poorly-controlled diabetic patients, DB = merged diabetic patients (WCD + PCD), CON = healthy, age -matched controls. Data are given as 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
Tendon Composition				
Number of participants	21	21	42	10
Collagen (mg·mg^{-1} dry wt)	0.73 ± 0.03	0.70 ± 0.02	0.72 ± 0.03	0.75 ± 0.03
Hydroxylysyl pyridinoline (HP, mmol·mol^{-1} collagen)	1230 ± 80	1340 ± 70	1250 ± 50	1220 ± 80
Lysyl pyridinoline (LP, mmol·mol^{-1} collagen)	52 ± 3	53 ± 3	52 ± 2 (*) P = 0.101	43 ± 5
Pentosidine, (mmol·mol^{-1} collagen)	33 ± 2	30 ± 3	31 ± 2	28 ± 2
Skin Composition				
Number of Participants	21	20	41	9
Collagen (mg·mg^{-1} dry wt)	0.62 ± 0.01	0.64 ± 0.02	0.63 ± 0.01	0.65 ± 0.02
Hydroxylysyl pyridinoline (HP, mmol·mol^{-1} collagen)	35 ± 10	54 ± 10	45 ± 6**	19 ± 4
Lysyl pyridinoline (LP, mmol·mol^{-1} collagen)	8 ± 1	9 ± 2	9 ± 1**	5 ± 1
Pentosidine, (mmol·mol^{-1} collagen)	13 ± 2	16 ± 2	14 ± 1*	9 ± 2
Tendon Fibril Morphology				
Number of Participants	18	22	40	10
Volume fraction (%)	53 ± 2	54 ± 1	53 ± 1	57 ± 2
Density (#fibril·μm^{-2})	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^2)	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
Achilles Tendon Mechanics (At maximum force				
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 ($kN \cdot mm^{-1}$)	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(*) $P = 0.075$	3.7 ± 0.4
Modulus (GPa)	3.1 ± 0.2	3.2 ± 0.4	3.1 ± 0.3	2.5 ± 0.3
Achilles Tendon Mechanics (At common force)				
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 ($kN \cdot mm^{-1}$)	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
Foot pressure mapping				
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

Table 3. Achilles tendon mechanics and foot pressure mapping. Data are given as mean ± 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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Figure legends

Figure 1

The Achilles tendon stress-strain relationship based on largest common tendon force observed for merged Diabetic patients (DB) and age-matched healthy controls. Data are given as mean ± SE. DB showed higher Achilles tendon modulus than controls at highest common tendon force ($P < 0.001$). A = Ultrasound Transducer, B = Strain Gauge.

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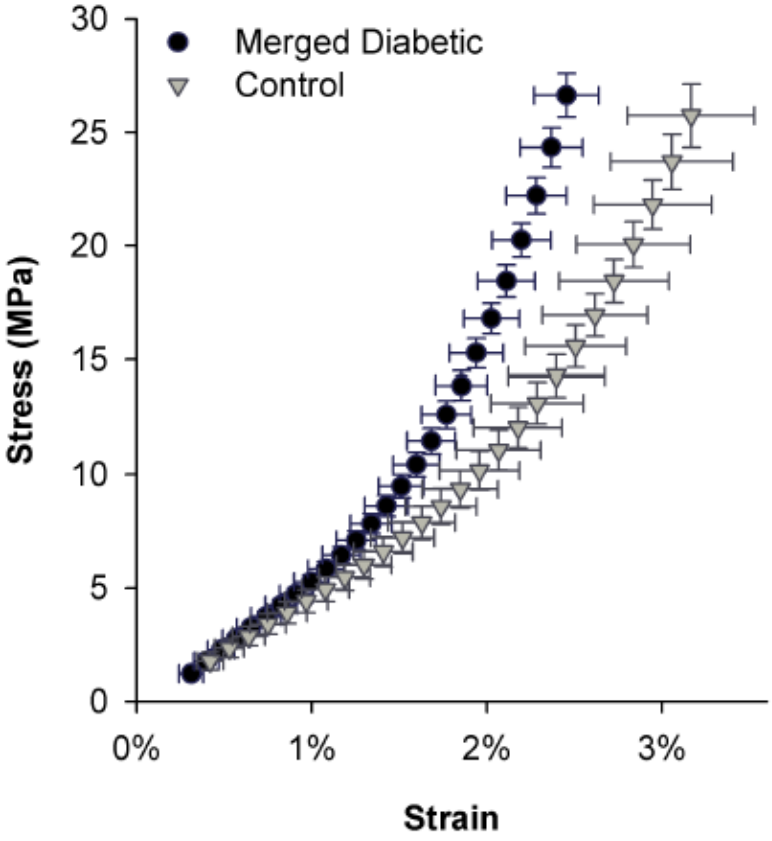
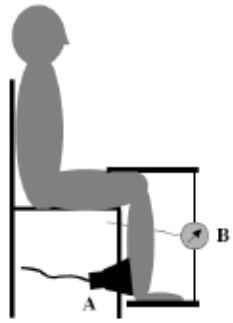
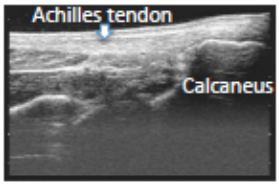

Merged Diabetic
Control
Stress (MPa)
30
25
20
15
10
5
0
0%
1%
2%
3%
Strain


B
A


Achilles tendon
Calcaneus