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The Association between Mechanical and Biochemical/Histological Characteristics in Diabetic and Non-Diabetic Plantar Soft Tissue

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Abstract

Diabetes, and the subsequent complication of lower limb ulcers leading to potential amputation, remains an important health care problem in United States, even with declining amputation rates. It has been well documented that diabetes can alter the mechanical properties (i.e., increased stiffness) of the plantar soft tissue, although this finding is not universal. Similarly, biochemical and histological changes have been found in the plantar soft tissue, but, as with the mechanical changes, these findings are not consistent across all studies. Our group's work has demonstrated that diabetes increases plantar soft tissue modulus and increases elastic septal thickness. The purpose of the current study was to explore the association between mechanical, biochemical and histological properties. Using previously collected data, a linear mixed effects regression was conducted. The correlations were weak; of the 32 that were tested, only 3 (modulus to septal thickness when location was accounted for, energy loss to total collagen, and energy loss to collagen/elastin ratio) were statistically significant, none with an R^2 greater than 0.10. The main differences in the means were increase tissue stiffness and increase septal wall thickness, both trends were supported in the literature. However, as the correlations were weak, it is likely that another unexamined biochemical factor (perhaps collagen crosslinking) is associated with the mechanical tissue changes.

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Conflict of interest statement

The authors have no conflicts to report.

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Keywords

foot; diabetes; subcutaneous; ulceration; plantar soft tissue; adipose

INTRODUCTION

Plantar ulceration and subsequent lower limb amputation are complications of diabetes mellitus that are important clinical problems in the United States. Over 8% of the population has diabetes, disproportionally leading to nearly two thirds of all non-traumatic amputations (nearly 66,000 in 2006) (CDCP, 2011). Recently, there has been a trend of reduced non-traumatic amputation rates, both in veterans (Tseng et al., 2011) and the general population (Belatti and Phisitkul, 2013; Li et al., 2012), likely a result of improved preventative care, increase revascular interventions, and evolving orthopaedic management (Belatti and Phisitkul, 2013). However, in terms of the sheer number of amputations per year (Belatti and Phisitkul, 2013; Tseng et al., 2011), and by the fact diabetic subjects undergo a disproportionate percentage of all amputations (CDCP, 2011; Li et al., 2012), diabetic foot ulceration remains an issue that requires further study.

Ulcer development is a complex and multi-factorial process, with aspects related to autonomic and peripheral neuropathy, poor circulation and aberrant mechanical tissue loading (Sumpio, 2000). Many groups have studied the mechanical properties of diabetic plantar soft tissue, often with ultrasound devices on living subjects. The findings have not always been repeatable and are sometimes contradictory. Studies have found that diabetic tissue is thicker than normal tissue (Chao et al., 2011; Gooding et al., 1986) and that diabetic plantar skin is harder (Piaggesi et al., 1999). Diabetic plantar soft tissue was shown to have increased energy loss (Hsu et al., 2007; Hsu et al., 2002; Hsu et al., 2000) but no change in elastic modulus (Hsu et al., 2002; Hsu et al., 2000). Conversely, it has been found that diabetic, elderly tissue is stiffer and thinner than non-diabetic, younger tissue, but age may have confounded these findings (Zheng et al., 2000). Others have found that diabetic plantar soft tissue is stiffer at the metatarsal heads, but not at the heel pad (Klaesner et al., 2002). One recent study contradicted earlier work and found no change in thickness in diabetic tissue, but did confirm that diabetic tissue was stiffer (Sun et al., 2011). More recently, it has been shown that diabetic tissue has increased Young's and relaxation moduli (Jan et al., 2013). Changing modalities, one group has used indentors and an MRI scanner to determine that older diabetic subjects had increase shear and compressive modulus compared to younger, healthy non-diabetic subjects (Gefen et al., 2001). Magnetic resonance elastography has also been used to demonstrate increased stiffness in diabetic heel pads (Cheung et al., 2006). Our own research group mechanically tested diabetic plantar tissue isolated from cadaveric specimens and demonstrated increased modulus in compression and shear, but no difference in energy loss and very little change in the relaxation properties in either loading mode (Pai and Ledoux, 2010; 2011; 2012).

In addition to the mechanical changes in the diabetic plantar soft tissue, there is also some evidence that the histomorphometric characteristics are altered, although most work has been associated with the heel pad (Buschmann et al., 1995; Jahss et al., 1992; Waldecker and

Lehr, 2009). Some of the original studies indicated thicker, frayed septal walls and decreased adipocyte size (Buschmann et al., 1995; Jahss et al., 1992), but a more recent study (Waldecker and Lehr, 2009) found no change in adipocyte size between healthy and diabetic tissue, findings that agreed with our own group's research (Wang et al., 2011). This contradiction might be explained by the fact that the studies from the 1990's used limbs that were amputated due to vascular disease. However, our work has confirmed the finding that thick, damaged elastic septa are found in diabetic plantar soft tissue (Wang et al., 2016; Wang et al., 2011).

Finally, concerning the effect of diabetes on the biochemistry of the plantar soft tissue, it is known that diabetes can induce alterations in the metabolism of the macromolecules present in the body. These biochemical changes are complex and have been found to be dependent on the tissue type and the macromolecule being evaluated (Sternberg et al., 1985), but the evaluation of the biochemistry of diabetic plantar soft tissue is not well understood. Our group has shown that there is no difference in the amount of collagen nor collagen I to III ratios, and minimal differences in the amount of elastin between diabetic and non-diabetic plantar soft tissue (Wang et al., 2016).

In summary, there have been many studies that have explored quantitative differences between diabetic and non-diabetic plantar soft tissue, but the relationship between microstructural characteristics (biochemical and histomorphological properties) and macrostructural characteristics (mechanical properties) is not clear. The purpose of the current study was to explore the direct association between the biochemical/histological characteristics of the plantar soft tissue and the mechanical properties of the plantar soft tissue at six locations beneath the foot. It is our hypothesis that the mechanical characteristics (e.g., increased stiffness or increased energy loss) will be directly associated with biochemical/histological characteristics (e.g., increased elastic septal thickness or increased amounts of collagen).

METHODS

All cadaveric foot specimens were obtained from the National Disease Research Interchange (NDRI; Philadelphia, PA) and our protocols were all approved by the University of Washington Institutional Review Board.

Mechanical testing

Two subsets of the mechanical testing data of the plantar soft tissue presented here have been partially published elsewhere with detailed methodologies (Pai and Ledoux, 2010; 2011). Whereas previously we reported on data collected from 4 diabetic and 4 non-diabetic specimens, here we have data from a larger sample including 5 additional non-diabetic donors. Briefly, specimens were collected from six locations, including the: hallux (big toe), first, third and fifth metatarsal heads, lateral midfoot and the calcaneus (heel) (Figure 1). Cylindrical specimens (1.905cm diameter) from these locations were excised and separated from the skin and bone to maintain approximate *in vivo* thickness (3 to 11 mm). Specimens were kept cool on ice until immediately prior to testing.

Testing was conducted in an environmental chamber (near 100% humidity and 35 degrees Celsius) with each specimen placed between two platens covered in 220 grit sand paper. The apparatus was integrated with an ElectroForce 3200 (Bose Corporation; Minnetonka, MN). A nominal load of 0.1N was applied and the initial thickness was determined. The target load was based on the donor weight and specimen cross-sectional area, and the required displacement needed to achieve the target load was determined (Pai and Ledoux, 2010). The specimens were tested in compression using triangle waves of varying frequencies (1, 2, 3, 5, and 10 Hz). Each test consisted of 30 triangle waves; after allowing each specimen to precondition, trials 27–29 were sampled at 1000 Hz for the 1, 2, 3 and 5 Hz tests, and 5000 Hz for the 10 Hz tests. Peak stress, peak strain, toe modulus (i.e., slope of the stress-strain curve before the inflection point), modulus (i.e., slope of the stress-strain curve after the inflection point), and energy loss (the area between the loading and unloading curve) were determined for each specimen and frequency. Stress relaxation tests were also conducted (Pai and Ledoux, 2011). Using the same target load and displacement, the specimens were preconditioned with ten 1 Hz sine waves, before undergoing a ramp (0.1s) and hold (300s) test. The data were sampled at 1000 Hz, down sampled to 200 Hz, and linear slopes from $t = t_0$ to $t = 0.5$, $t = 10$ to $t = 15$, and $t = 290$ to $t = 300$ (all in seconds) were used to approximate the initial, middle and final relaxation rates.

Biochemical Quantification of Collagen and Elastin

The biochemical properties of the plantar soft tissue, and the methodologies employed to determine them, have been previously reported elsewhere (Wang et al., 2016) and are described briefly below. Before biochemical quantification of the extracellular proteins, adipose tissue was carefully removed from the skin and defatted.

Collagen content was quantified using an established hydroxyproline assay (Bergman and Loxley, 1963). Defatted samples were lyophilized and subjected to acid hydrolysis with 6M hydrochloric acid (HCl) for 15 hours. The acid was neutralized before collagen quantification. After incubation with Chloramine-T solution and Ehrlich's reagent, absorbance was read at 550 nm (Model 680 microplate reader, Biorad, Hercules, CA). The hydroxyproline content was determined from a standard curve for trans-4-hydroxy-L-proline. Total collagen per sample was calculated using a conversion factor of 6.94, based on the fact that hydroxyproline represents 14.4% of the amino acid composition of collagen in most mammalian tissues (Samuel, 2009).

The Fastin Elastin assay (Biocolor Ltd, Carrickfergus, U.K.) was used to quantify total soluble and insoluble elastin content. Defatted samples were lyophilized, weighed and subjected to sequential acid digestion with oxalic acid and ethanolic potassium hydroxide. The extracted elastin was quantified as per the manufacturers instructions. Absorbance was read at 515 nm and elastin content was determined from a standard curve for α -elastin.

Collagen I:III ratio was determined by interrupted SDS gel electrophoresis based on methods of Sykes et al. (1976) and the modifications made by Samuel (2009). Briefly, collagen was extracted from the tissue using 0.5M acetic acid followed by a pepsin acetic acid solution. The extracts were lyophilized and weighed. The lyophilized samples were reconstituted using loading buffer containing urea and heated before loading onto gels. 30%

β -mercaptoethanol was added to each sample well for 60 minutes to separate the type I collagen chains from the type III collagen chains. Gels were stained with Coomassie blue and the collagen I and III band ratio was calculated using measurements made with a densitometer with quantitative software (GelDoc XR, Biorad, Hercules, CA).

Histomorphometric evaluation

As with the mechanical and biochemical properties, and the methodologies employed to determine them, the histomorphometric properties have been reported elsewhere (Wang et al., 2016; Wang et al., 2011). Soft tissue samples (1 x 1cm) of the plantar foot containing epidermis, dermis and subcutaneous fat (hypodermis), were fixed in 10% neutral buffered formalin. Vertical uniform random (VUR) sampling of the specimens (Baddeley et al., 1986) was used to obtain unbiased, isotropic sections when combined with stereological sampling probes as described previously (Wang et al., 2011). Sequential 5 μ m sections were stained with hematoxylin and eosin (H&E) and a modified Hart's procedure for elastin, all according to standard protocols (Wang et al., 2011). Image analysis of the histological sections was performed using a Nikon microscope (Eclipse 80i, Nikon, Inc; Melville, NY) and digitized with a 12.6 megapixel digital camera (DXM-1200C, Nikon, Inc; Melville, NY). Images were analyzed in a blind fashion.

The thickness of the septal walls was measured in sections stained with modified Hart's protocol and imaged at 4x. A stereological approach was used to make random measurements of the septal wall thickness as described previously (Wang et al., 2011). An approximation of the true thickness was calculated using the harmonic and arithmetic mean, which overcomes the overestimation of the measured values (Ferrando et al., 2003; Mayhew, 1991).

An optical-dissector probe (25,000 μ m² area) was placed randomly over H&E stained 10x images using systematic random sampling rules (Cruz-Orive and Weibel, 1990; Nyengaard and Gundersen, 2006). The size was measured for each cell adipocytes lying within the dissector and touching the top and right planes (Wang et al., 2011). Adipocytes that were damaged or overly distorted owing to processing were not included in the measurements.

Statistical methods

Student's t-tests were used to assess differences in specimen demographics. Linear mixed effects regression was used to test for mean differences in mechanical, biochemical and histological measure (the dependent variable) by diabetes status (the independent fixed effect) with foot ID as a random effect. Linear mixed effects regression was used to test the association of selected biomechanical measures with biochemical and histological measures (Table 1). Biomechanical measure was the dependent variable, and biochemical measure or histological measure (averaged over location within specimen) was modeled as the independent fixed effect. Correlations were summarized using slope of change in the mechanical variable per increase in the biochemical or histological variable, 95% confidence intervals and approximate R^2 (Edwards et al., 2008). To assess if the correlations were confounded by tissue location on the foot models were rerun with the addition of location as a fixed effect covariate. Interaction terms between location and the independent variable

were tested for significance when graphical evidence suggested associations may be influenced by location. Random effects were modeled for foot, and location within foot. For relaxation measures, models were adjusted for strain rate. For biochemical variables that were ratios, associations were assessed using the numerator as the fixed effect of interest and the denominator as a model covariate, i.e. slopes were estimated for the numerator adjusting for the denominator. Due to skewness, hypothesis testing for the modulus was conducted using log modulus as the dependent variable. Significance was set as $p < 0.05$ and analyses were carried out using R 3.1.0 (Team, 2014), using the lme4 (Bates et al., 2014) and pbkrtest (Halekoh and Højsgaard, 2013) packages to carry out the linear mixed effects regression and the R^2 estimates respectively.

Results

Demographically, the diabetic and non-diabetic specimens were similar in age, sex and height, but the diabetic specimens were statistically significantly heavier ($p=0.02$) and had a greater body mass index (BMI, $p=0.002$, Table 2). The diabetic specimens had a duration of diabetes of 20.3 ± 8.1 years.

The mechanical, biochemical and histological properties indicated mean differences between diabetic and non-diabetic tissue (Table 3). In particular, the diabetic tissue had a 94% increased modulus ($p=0.0008$) and an 11%, but non-significant ($p=0.055$), increase in energy loss, as well as a 66% increase in the middle relaxation modulus ($p=0.0040$). Biochemical properties were similar between the two groups, except for a 25% increase in total elastin ($p=0.045$). Histologically, the septal thickness was 79% greater in the diabetic specimens ($p<0.0001$), but the adipose area was not statistically different.

The correlations between mechanical properties (modulus, toe modulus, energy loss, and relaxation rates) and biochemical properties (total elastin, total collagen, collagen/elastin ratio, collagen I/III ratio) or histological properties (septal thickness, adipocyte size) were weak (Table 4). Of the 32 relationships we tested, only three were significantly correlated: modulus to septal thickness (when location was accounted for), energy loss to total collagen and energy loss to collagen/elastin ratio. While significant, the biomechanical or histological measures explained relatively little of the variability in the mechanical measures (R^2 of 0.10, 0.07 and 0.06, respectively; Table 4, Figures 2 and 3). Of note, the relationship between modulus and septal thickness was masked by tissue location due to tissue from the calcaneus region having lower mean modulus and higher mean septal thickness than tissue from other locations. Consequently, adjustment for location yielded a significant association between modulus and septal thickness. Further analysis showed that the association between modulus and septal thickness differed by location (location by septal thickness interaction, $p=0.019$). Mean modulus increased with septal thickness at all locations except for the calcaneus (Figure 2). Conversely, the two energy loss relationships were no longer significant when location was considered, indicating that differences in energy loss and total collagen (unadjusted or adjusted for total elastin) by location accounted for the significant associations, with tissue from the metatarsal locations having higher mean energy loss and higher total collagen than tissue from other regions. In Figure 3, the black line shows the significant slope of change when location is not accounted for. However, the red and blue

lines show that for metatarsals and other locations, separately, the slope is relatively flat. A similar pattern resulted when total collagen was adjusted for total elastin (data not shown). There was no evidence that the association between total energy loss and total collagen differed by location ($p>0.66$).

Discussion

Although amputation rates due to diabetic ulceration have decreased, there are still a significant number of amputations each year. The processes that lead to an ulcer and then to a subsequent amputation are complex and multi-factorial. One aspect is related to the alteration in the mechanical, histological and biochemical properties of diabetic tissue. Previous work has shown that the diabetic tissue is stiffer and has frayed septal walls, but there are minimal differences in the biochemistry. In this study, we aimed to explore the relationships between the various properties, hypothesizing that changes in the mechanical characteristics would be associated with histological and/or biochemical alterations, i.e., in effect, the histology and/or biochemistry would explain the mechanical findings.

There were several limitations to this study. First, the BMI of the diabetic subjects was significantly greater than the non-diabetic group, potentially confounding our data. Second, we only had 4 unique diabetic specimens, so it is possible our small number of subjects might have skewed our results. It is also possible that false negative (Type II) errors could occur, meaning that there were significant findings that were not found due to low study power. Finally, while the mechanical and histological parameters chosen were comprehensive (including modulus and energy loss, and septal thickness and adipocyte size, respectively), it is possible that the biochemical parameters chosen (all related to the amount of collagen and elastin) failed to account for an important aspect of how diabetic tissues are altered by diabetes. Namely, the amount of collagen crosslinking was not quantified – the might explain the lack of strong correlations in our study.

Our findings indicated that there was a weak correlation between septal wall thickness and modulus; the relationship was seen at all locations, except for the calcaneus (Figure 2). The association between energy loss and total collagen (both unadjusted and adjusted for total elastin) was confounded by location (Figure 3). However, none of the correlations were strong; had they been, it would have been suggestive of causation, but the lack of a strong correlation is suggestive that the wrong biochemical/histological parameters were studied. Although differences in mechanical, histological and biochemical properties between normal and diabetic soft tissue have been noted in the literature, little work has been done correlating these parameters on the same specimens. Recently, it has been shown in living diabetic subjects that the blood biochemistry (amount of triglycerides and fast blood sugar) does correlate with mechanical properties (stiffness and/or energy absorbed) (Chatzistergos et al., 2014). Others have used computational models of the foot and associated soft tissues to explore the relationship between tissue microstructure (i.e., essentially, histological properties) and the mechanical behavior (Natali et al., 2012).

We also examined the mechanical, biochemical and histological data for differences between diabetic and non-diabetic tissue. The diabetic tissue was found to have an increased modulus

(mean $\pm$ standard error, 1147 ± 93 kPa vs. 594 ± 62 kPa), which has been shown previously both by our research group on a subset of these data (Pai and Ledoux, 2010) and by others (Jan et al., 2013; Klaesner et al., 2002; Sun et al., 2011; Zheng et al., 2000). The increase in relaxation modulus (-0.233 ± 0.023 N/s vs. -0.148 ± 0.015 N/s) in the diabetic tissue has been found in our early analysis of a subset of data (Pai and Ledoux, 2011), however this finding was not significant once peak force was accounted for. The diabetic tissue was found to have a non-significant trend of increased energy loss ($68.5 \pm 2.9\%$ vs. $61.8 \pm 2.0\%$), which was in contradiction to our group's analysis of a subset of these data (Pai and Ledoux, 2010), but was supported by the work of other laboratories (Hsu et al., 2007; Hsu et al., 2002; Hsu et al., 2000). It is possible that our initial analysis suffered from a lower number of specimens and that the subsequent, more inclusive data set was more representative. Biochemical properties between diabetic and non-diabetic tissues were similar, with, as noted in our previous work (Wang et al., 2016), only the total elastin being different in diabetic tissue (0.141 ± 0.012 mg/mg vs. 0.113 ± 0.008 mg/mg). Increased elastin in diabetic tissue has been found previously in the lung (Sahebjami and Denholm, 1987) and kidneys (Thongboonkerd et al., 2004). Finally, histologically, the septal thickness was greater in diabetic tissues (184 ± 12 μ m vs. 103 ± 8 μ m), supporting our work (Wang et al., 2016; Wang et al., 2011) and several other groups (Buschmann et al., 1995; Jahss et al., 1992) and while the adipocyte size differed by 15% (2052 ± 290 μ m² vs. 1767 ± 193 μ m²), there was significant variability, which has been shown to lead to non-significance by our group (Wang et al., 2016; Wang et al., 2011) and others (Waldecker and Lehr, 2009).

In summary, we have determined the mechanical, histological and biochemical properties of the both normal and diabetic plantar soft tissue. Mean differences included, among others, increased diabetic tissue stiffness, total elastin, and septal thickness, all generally supported by the literature. However, there were not strong correlations between the mechanical properties and histological/biochemical properties. There are likely other biochemical properties, (e.g., collagen crosslinking) that would better correlate to the mechanical properties.

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Figure 1.

Specimen locations a) at the hallux (ha), first, third, and fifth metatarsal heads (m1, m3, and m5), lateral midfoot (la), and calcaneus (ca) as well as b) a typical plantar tissue specimen before skin removal. Reprinted from Journal of Biomechanics, Vol 43, Pai, S. & Ledoux, W. R., The compressive mechanical properties of diabetic and non-diabetic plantar soft tissue, 1754–60, 2010, with permission from Elsevier.

Figure 2.
Log modulus by septal thickness for the calcaneus and all other locations. Regression lines are shown for each group (blue and red).

Figure 3.

Energy loss by amount of collagen for the metatarsals and all other locations. Regression lines are shown for each group (blue and red) and for all data together (black).

Table 1

The specific associations that were tested using linear mixed effects regression.

Number	Hypothesis
1	Modulus will positively correlate with the septal thickness
2	Modulus will positively correlate with the amount of collagen
3	Modulus will positively correlate with the collagen/elastin ratio
4	Modulus will negatively correlate with the collagen I:III ratio
5	Modulus will positively correlate with the adipocyte size
6	Toe modulus will positively correlate with the amount of elastin
7	Energy loss will positively correlate with the septal thickness
8	Energy loss will positively correlate with the amount of collagen
9	Energy loss will positively correlate with the collagen/elastin ratio
10	Energy loss will positively correlate with the collagen I:III ratio
11	Energy loss will positively correlate with the adipocyte size
12	Energy loss will positively correlate with the amount of elastin
13	Initial relaxation rate will negatively correlate with the amount of elastin
14	Middle relaxation rate will negatively correlate with the amount of elastin
15	Final relaxation rate will negatively correlate with the amount of elastin
16	Initial relaxation rate will positively correlate with the adipocyte size
17	Middle relaxation rate will positively correlate with the adipocyte size
18	Final relaxation rate will positively correlate with the adipocyte size

Demographic information for tissue specimens, mean (standard deviation) or, in the case of gender, male to female ratio.

Table 2

	Age (years)	Gender (M:F)	Height (m)	Weight (N)	BMI
Diabetic (n=4)	70.5 (6.8)	2:2	1.68 (0.11)	923 (246)	33.0 (5.1)
Non-diabetic (n=9)	70.9 (6.6)	5:4	1.71 (0.10)	637 (150)	22.1 (4.2)
p-value *	0.92	1.0 **	0.62	0.02	0.002

BMI = body mass index,

* = student's t-test,

** Fisher's exact test, bold = statically significant (< 0.05)

Table 3

Mechanical variables means [standard error] and mean differences (95% confidence interval) by diabetes status from linear mixed effects regression of outcome variable on diabetes status with foot as a random effect

	Diabetic	n [§]	Non-diabetic	n [§]	D - N difference	p-value
Mechanical						
Modulus (kPa) *	1147 [93]	810	594 [62]	360	553 (335, 770)	0.0008
Toe modulus (kPa)	17.0 [1.7]	810	14.0 [1.1]	360	3.0 (-1.0, 7.1)	0.13
Energy loss (%)	68.5 [2.9]	810	61.8 [2.0]	360	6.7 (-0.2, 13.6)	0.055
Initial relax. rate (s ⁻¹) [†]	-45.4 [4.3]	54	-39.3 [2.8]	24	-6.1 (-16.1, 3.9)	0.21
Middle relax. rate (s ⁻¹) [†]	-0.233 [0.023]	54	-0.148 [0.015]	24	-0.085 (-0.139, -0.032)	0.0040
Final relax. rate (s ⁻¹) [†]	-0.0048 [0.0008]	54	-0.0035 [0.0005]	24	-0.0013 (-0.0032, 0.0007)	0.19
Biochemical						
Total collagen (mg/mg)	0.488 [0.028]	50	0.449 [0.019]	24	0.039 (-0.035, 0.115)	0.25
Total elastin (mg/mg)	0.141 [0.012]	50	0.113 [0.008]	24	0.028 (0.001, 0.055)	0.045
Collagen:elastin ratio	3.8 [0.5]	19	4.2 [0.3]	18	-0.4 (-1.5, 0.7)	0.30 **
Collagen I:III ratio	1.11 [0.03]	50	1.02 [0.02]	24	0.08 (0.00, 0.15)	0.061 **
Histological						
Septal thickness (µm)	234 [15]	50	134 [10]	22	100 (64, 136)	<0.0001
Adipocyte area (µm ²)	2052 [290]	50	1767 [193]	22	285 (-397, 968)	0.38

* Hypothesis testing carried out on log modulus

[†] Adjusted for strain rate

** Significance based on models of the numerator with the denominator as a covariate

[§] The full component for the mechanical properties was 810 for the non-diabetic specimens (9 feet, 6 locations, 5 frequencies and 3 trials) and 360 for the diabetic specimens (4 feet, 6 locations, 5 frequencies and 3 trials). The full component of specimens for the relaxation rates, biochemical properties and histological properties (all of which were measure once per specimen) was 54 for the non-diabetic (9 feet and 6 locations) and 24 for the diabetic (4 feet and 6 locations). However, for the biochemistry and histology, there were numerous instances where the sample was accidentally destroyed during processing, resulting in less than complete data sets. relax. = relaxation, **bold** = statistically significant (p< 0.05)

Table 4

Slopes (95% confidence interval), R^2 and p-value for the significance of the association between mechanical variable and biomechanical or histological variable from linear mixed effects models. Data were reanalyzed adjusting for tissue location.

	Slope (95 % CI)	R^2 , p	Slope (95 % CI) adjusted for location	R^2 , p
Correlations with log Modulus				
Septal thickness	7.8e-4 (-4.0e-4, 20.1e-4)	0.02, 0.19	19.0e-4 (5.5e-4, 32.6e-4)	0.10, 0.0062
Total collagen	0.17 (-0.32, 0.67)	<0.01, 0.48	0.09 (-0.38, 0.56)	<0.01, 0.71
Collagen adjusted for elastin	0.17 (-0.32, 0.67)	<0.01, 0.49	0.09 (-0.38, 0.56)	<0.01, 0.71
Collagen I adjusted for collagen III	3.2e-4 (-2.5e-4, 9.3e-4)	0.03, 0.26	4.6e-4 (-1.0e-4, 10.4e-4)	0.06, 0.11
Adipose area	5.2e-5 (-11.9e-5, 23.0e-5)	<0.01, 0.53	6.3e-5 (-10.5e-5, 23.0e-5)	<0.01, 0.46
Correlations with toe modulus				
Total elastin	-27.1 (-61.8, 10.2)	0.03, 0.16	-16.9 (-51.6, 18.0)	0.01, 0.34
Correlations with energy loss				
Septal thickness	0.018 (-0.010, 0.048)	0.02, 0.19	0.011 (-0.018, 0.040)	<0.01, 0.45
Total elastin	37.8 (-21.3, 97.8)	0.02, 0.20	-15.8 (-67.2, 35.8)	<0.01, 0.54
Total collagen	13.0 (0.9, 24.7)	0.07, 0.036	6.0 (-3.6, 15.5)	0.02, 0.21
Collagen adjusted for elastin	12.7 (0.5, 24.3)	0.06, 0.041	6.0 (-3.5, 15.5)	0.02, 0.21
Collagen I adjusted for III	-8.0e-3 (-22.8e-3, 7.6e-3)	0.02, 0.32	-6.1e-3 (-18.1e-3, 6.0e-3)	0.02, 0.31
Adipose area	1.3e-3 (-2.5e-3, 5.2e-3)	<0.01, 0.47	1.7e-3 (-1.6e-3, 5.0e-3)	0.01, 0.30
Correlations with relaxation rate (initial) *				
Total elastin	58 (-39, 148)	0.02, 0.24	26 (-69, 121)	<0.01, 0.58
Adipose area	-0.0021 (-0.0073, 0.0031)	<0.01, 0.42	-0.0020 (-0.0071, 0.0033)	<0.01, 0.45
Correlations with relaxation rate (middle) *				
Total elastin	0.15 (-0.22, 0.51)	<0.01, 0.44	0.14 (-0.26, 0.53)	<0.01, 0.48
Adipose area	-1.3e-5 (-3.6e-5, 1.1e-5)	0.02, 0.28	-1.4e-5 (-3.9e-5, 1.1e-5)	0.02, 0.26
Correlations with relaxation rate (end) *				
Total elastin	-0.008 (-0.032, 0.010)	<0.01, 0.44	-0.011 (-0.034, 0.010)	0.01, 0.34
Adipose area	2.3e-7 (-7.2e-7, 13.4e-7)	<0.01, 0.70	4.2e-7 (-10.0e-7, 18.3e-7)	0.01, 0.53

* adjusted for strain rate

bold = statistically significant (p< 0.05)