Systemic stiffening of mouse tail tendon is related to dietary advanced glycation end products but not high-fat diet or cholesterol

C. Eriksen,1 R. B. Svensson,1 J. Scheijen,2 A. M. F. Hag,3 C. Schalkwijk,2 S. F. E. Praet,4 P. Schjerling,1 M. Kjær,1 S. P. Magnusson,1,5 and C. Couppé1,5

1Institute of Sports Medicine, Department of Orthopedic Surgery M, Bispebjerg Hospital and Center for Healthy Aging, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark; 2Department of Internal Medicine and Cardiovascular Research Institute Maastricht, Maastricht University Medical Center, The Netherlands; 3Cluster for Molecular Imaging, Faculty of Health and Medical Sciences and Department of Clinical Physiology, Nuclear Medicine and PET, Rigshospitalet, University of Copenhagen, Denmark; 4Department of Rehabilitation Medicine, MOVEFIT- Sports medicine, Erasmus University Medical Centre, Rotterdam, The Netherlands; and 5Department of Physical Therapy, Musculoskeletal Rehabilitation Research Unit, Bispebjerg Hospital, Denmark

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Eriksen C, Svensson RB, Scheijen J, Hag AM, Schalkwijk C, Praet SF, Schjerling P, Kjær M, Magnusson SP, Couppé C. Systemic stiffening of mouse tail tendon is related to dietary advanced glycation end product (AGE) cross-linking of non-weight-bearing mouse tail tendons. Twenty ApoE−/− male mice were used as a model for hypercholesterolemia along with 26 wild-type (WT) mice. One-half of the mice from each group was fed a normal diet (ND) and the other half was fed a high-fat diet (HFD) to induce obesity. All were killed at 40 wk, and tail tendon fascicles were mechanically tested to failure and analyzed for AGEs. Diets were also analyzed for AGEs. ApoE−/− mice displayed a 14% increase in plateau modulus compared with WT mice (P < 0.05), whereas HFD mice displayed a 13% decrease in plateau modulus (P < 0.05) and a 12% decrease in total modulus (P < 0.05) compared with ND mice. Tail tendons of HFD mice had significantly lower concentrations of AGEs [carboxymethyllysine (CML); 26%, P < 0.0001; methylglyoxal-derived hydroimidazolone (1MG-H1); 15%, P < 0.005; pentosidine; 13%, P < 0.0005]. The HFD had a 44-fold lower content of CML (P < 0.01), a 29-fold lower content of carboxyethyllysine (P < 0.005), and a 16-fold lower content of MG-H1 (P < 0.05) compared with ND. ApoE−/− increased, whereas HFD decreased mouse tail tendon stiffness. Dietary AGE content may be a crucial determinant for accumulation of AGE cross-links in tendons and for tissue compliance. The results demonstrate how systemic metabolic factors may influence tendon health, advanced glycation end products; tendon biomechanics; cholesterol or hypercholesterolemia are more prone to tendinopathy (1, 8, 22–25, 33, 49, 59), ruptures of the Achilles tendon (38, 43), and rotator cuff tendon tears (2, 58). However, very little is known about the mechanisms behind this, and conflicting results are provided with regard to the influence of metabolic disorders upon tendon mechanical properties.

The elastic modulus of different hypercholesterolemic animal tendons has been reported to decrease (6) or increase (7); and, furthermore, in mice fed with a high-fat diet (HFD), tendon modulus has been reported to decrease (11), while deformation as well as strain of collagen fibrils have been reported to increase in obese rats (9). Thus the results are not only sparse and conflicting, but most studies are performed on weight-bearing tissue, which makes it difficult to separate the potential influence of metabolic deterioration from that of mechanical loading, especially with increased body weight as in obesity.

The collagen fibril is the fundamental tensile-bearing structure in tendon tissue and is made up of collagen molecules linked together by cross-links. These bonds provide structural integrity to the fibrils and ensure optimal force transmission (3, 4, 19). The two general types of cross-links, one enzymatically controlled by lysyl oxidase during maturation and the other with a nonenzymatic glycation mechanism, may potentially be controlled differently by tendon loading and metabolic disorders (3, 20, 40). Whereas the enzymatic cross-link expression is demonstrated to be upregulated with increased mechanical loading (31) and downregulated with decreased loading (10), the nonenzymatic and irreversible formation of covalent advanced glycation end products (AGEs) via the Maillard reaction is influenced by age (3–5, 15, 40, 41) and dietary intake (55).

It is still unknown whether hypercholesterolemia and obesity affect mechanical properties of tendon directly systemically via a metabolic effect independent of mechanical loading. Furthermore, while obesity and hypercholesterolemia individually affect tendon mechanical properties, it is unknown whether there is any additive effect of the two and whether this effect is mediated by changes in AGEs as has been suggested (54). In this study, non-weight-bearing tail fascicles from apolipoprotein E-deficient (ApoE−/−) mice, on a normal diet (ND) and HFD, gave us the opportunity to observe the systemic effects
of hypercholesterolemia and diet-induced obesity on collagen mechanical properties and AGEs [methylglyoxal-derived hydroimidazolone 1 (MG-H1), carboxymethyllysine (CML), carboxyethyllysine (CEL), and pentosidine]. We hypothesized that ApoE deficiency and HFD would result in increased cholesterol levels and augment the accumulation of AGEs (54). Furthermore, we expected an association between higher AGE cross-link content and increased elastic modulus. The purpose of the present study was, therefore, to investigate the effect of ApoE deficiency and HFD on the mechanical properties and collagen cross-linking of mouse tail tendon fascicles.

MATERIALS AND METHODS

Mouse model. Homozygous ApoE<sup>−/−</sup> male mice (B6.129P2-<br>Apo<sup>em1Unc91</sup>N11, Taconic Europe, Lille Skensved, Denmark) were used as a model for hypercholesterolemia (n = 20) along with wild-type (WT) mice (C57BL/6Ntac) functioning as controls (n = 26). Mice were obtained at 8 wk of age, at which point one-half of the mice from each group was fed ad libitum HFD (protein, 17 kcal%; carbohydrate, 43 kcal%; fat, 41 kcal%; Western Diet, #D12079B, Research Diets, New Brunswick, NJ) to induce obesity, and the rest were fed ad libitum ND (protein, 27 kcal%; carbohydrate, 60 kcal%; fat, 13 kcal%; #1310, Altromin Spezialfutter & KG, Germany). Mice were killed at 40 wk of age by decapitation after having fasted overnight and being anesthetized for a period of 4 h. The blood was collected and centrifuged (3,200 rpm for 10 min), and plasma was transferred to a fresh tube and stored at −20°C. After removal of the organic solvent, the tendon samples were dried under a gentle stream of nitrogen at room temperature. To avoid AGE formation during hydrolysis, samples were reduced with 0.2 ml of 0.1 M NaBH<sub>4</sub> in 0.2 M borate buffer (pH 9.2) for 2 h at room temperature. The reduced samples were deproteinized in 1 ml of cold (4°C) 20% (vol/vol) trifluoroacetic acid and centrifuged at 4,300 × g, 4°C for 20 min, and the supernatant was discarded. The remaining pellets were hydrolyzed in 0.5 ml of 6 M HCl. From each hydrolysate, 40 µl were taken and mixed with 20 µl of internal standard before analysis. CML, CEL, and MG-H1 were analyzed by ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) while pentosidine was analyzed using HPLC fluorescence as described earlier (28, 47). Hydroxyproline (hyp) was analyzed by UPLC-MS/MS. In short, a dilution of the hydrolysate was mixed with internal standard trans-4-hydroxy-L-proline-2,5,5-d₃ (D3-hyp) and dried under a gentle stream of nitrogen at 70°C. The residue was dissolved in 1 ml of water-acetonitrile (1.9, vol/vol) and injected onto a hydrophilic interaction chromatography (HILIC) UPLC column (Acquity UPLC BEH HILIC, 1.7 µm, 2.1 × 50 mm). Solvent A was 10 mM ammonium formate-acetonitrile (1.9, vol/vol) and Solvent B was 10 mM ammonium formate-acetonitrile (5.5, vol/vol). A linear gradient was started at 95% Solvent A, which was changed to 60% Solvent A within 2 min. After the column with 100% Solvent B was cleaned for 1.5 min, the column was equilibrated for 4 min to the initial conditions. Injection volume was 0.2 µl (partial loop injection) at a column temperature of 45°C. Hyp and D3-hyp were detected in multiple-reaction monitoring electrospray positive mode (MRM-ESI) at a capillary voltage of 0.25 kV, a cone voltage of 25 V, and a desolvation temperature of 600°C (Acquity UPLC, Xevo TQ MS; Waters, Milford, MA). Quantification of hyp was performed by calculating the peak area ratio of hyp (MRM, 132.0 > 86.0) to the internal standard D3-hyp (MRM, 135.0 > 89.0). The levels of AGES were expressed as nanomoles of AGE per millimole hyp.

Except for the initial salt, acid, and chloroform extraction, diet samples were analyzed by the same protocol as the tissue samples, starting with NaBH<sub>4</sub> reduction. AGE levels in three ND and three HFD samples were analyzed as described earlier (expressed as nmol/g).

Plasma measurements. Cholesterol measurements were performed in duplicate with a standard kit (Cholesterol Chod-Pap; Roche Diagnostics, Germany). A more detailed description is available in a previous publication (27). Glucose measurements were done on 2 µl plasma with MediSense Precision PCX Plus (hand-held monitor and blood glucose test strips) (Abbott Laboratories, UK) using the venous mode of the monitor. A single fasted blood sample was taken from each mouse on the day of death (40 wk).

Data reduction and analysis. Fascicle diameter measurement was performed in Scion Image (v.3b; Scion, MD) at two locations in each
Fig. 1. Representative stress-strain curve obtained by stretching mouse tail fascicles to failure at a constant deformation rate of 2 mm/min (~10% strain/min). The curve had a characteristic appearance with a toe region and three linear phases separated by two yield points and a failure point. Maximal modulus occurred in the first linear phase, and in the third linear phase a plateau modulus was defined. Total modulus was defined as the average slope of the stress-strain curve, and failure energy density was determined as the area under the curve. See text for details.

Table 1. Biophysical and biochemical parameters

<table>
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<tr>
<th></th>
<th>WT</th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>WT</th>
<th>ApoE&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Effect of ApoE&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Effect of HFD</th>
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<tr>
<td>Fascicle CSA, µm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>ND (n = 14)&lt;sup&gt;a&lt;/sup&gt; 5.810 ± 590 7.240 ± 440 6.450 ± 450 7.190 ± 570 1.116 (96; 2.135)&lt;sup&gt;c&lt;/sup&gt; 277 (–782; 1345)</td>
<td>ND (n = 12)&lt;sup&gt;a&lt;/sup&gt; 6.450 ± 450 7.190 ± 570</td>
<td>HFD (n = 12)&lt;sup&gt;a&lt;/sup&gt; 13.0 (10.3; 15.8)&lt;sup&gt;*&lt;/sup&gt; 0.05</td>
<td>HFD (n = 8)&lt;sup&gt;a&lt;/sup&gt; 13.6 (10.6; 17.9)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.7 (0.1; 9.3)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.3 (8.8; 17.9)&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>Weight, g</td>
<td>ND (n = 14)&lt;sup&gt;a&lt;/sup&gt; 33.2 ± 0.9 35.6 ± 0.6 47.4 ± 1.3 47.2 ± 1.2 2.7 (–4.7; 51)</td>
<td>ND (n = 12)&lt;sup&gt;a&lt;/sup&gt; 47.2 ± 1.2</td>
<td>HFD (n = 12)&lt;sup&gt;a&lt;/sup&gt; 2.2 (–4.7; 51) 13.0 (10.3; 15.8)&lt;sup&gt;*&lt;/sup&gt; 0.05</td>
<td>HFD (n = 8)&lt;sup&gt;a&lt;/sup&gt; 4.6 (2.0; 7.2)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.7 (0.1; 9.3)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.3 (8.8; 17.9)&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>Plasma glucose, mM</td>
<td>ND (n = 14)&lt;sup&gt;a&lt;/sup&gt; 13.9 ± 1.4 10.9 ± 0.6 15.7 ± 1.9 10.0 ± 0.66 4.1 (–6.5; 1.6)&lt;sup&gt;‡&lt;/sup&gt; 0.36 (–2.6; 3.4)</td>
<td>ND (n = 12)&lt;sup&gt;a&lt;/sup&gt; 15.7 ± 1.9</td>
<td>HFD (n = 12)&lt;sup&gt;a&lt;/sup&gt; 4.1 (–6.5; 1.6)&lt;sup&gt;‡&lt;/sup&gt; 0.36 (–2.6; 3.4)</td>
<td>HFD (n = 8)&lt;sup&gt;a&lt;/sup&gt; 4.6 (2.0; 7.2)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.7 (0.1; 9.3)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.3 (8.8; 17.9)&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>Plasma Cholesterol, mM</td>
<td>ND (n = 14)&lt;sup&gt;a&lt;/sup&gt; 11.2 ± 1.0 15.8 ± 0.72 15.9 ± 1.9 33.8 ± 1.9 4.6 (2.0; 7.2)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>ND (n = 12)&lt;sup&gt;a&lt;/sup&gt; 15.9 ± 1.9</td>
<td>HFD (n = 12)&lt;sup&gt;a&lt;/sup&gt; 4.6 (2.0; 7.2)&lt;sup&gt;<em>&lt;/sup&gt; 13.0 (10.3; 15.8)&lt;sup&gt;</em>&lt;/sup&gt; 0.05</td>
<td>HFD (n = 8)&lt;sup&gt;a&lt;/sup&gt; 4.6 (2.0; 7.2)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.7 (0.1; 9.3)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.3 (8.8; 17.9)&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>CML, nmol/mmol hyp</td>
<td>ND (n = 14)&lt;sup&gt;a&lt;/sup&gt; 35.3 ± 1.0 36.9 ± 1.4 28.3 ± 1.2 24.4 ± 0.9 0.4 (–4.4; 3.6)</td>
<td>ND (n = 12)&lt;sup&gt;a&lt;/sup&gt; 24.4 ± 0.9</td>
<td>HFD (n = 12)&lt;sup&gt;a&lt;/sup&gt; 0.4 (–4.4; 3.6) 13.0 (10.3; 15.8)&lt;sup&gt;*&lt;/sup&gt; 0.05</td>
<td>HFD (n = 8)&lt;sup&gt;a&lt;/sup&gt; 4.6 (2.0; 7.2)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.7 (0.1; 9.3)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.3 (8.8; 17.9)&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>CEL, nmol/mmol hyp</td>
<td>ND (n = 14)&lt;sup&gt;a&lt;/sup&gt; 9.09 ± 0.40 9.90 ± 0.47 8.30 ± 0.92 8.76 ± 0.87 0.70 (–0.60; 1.99)</td>
<td>ND (n = 12)&lt;sup&gt;a&lt;/sup&gt; 8.76 ± 0.87</td>
<td>HFD (n = 12)&lt;sup&gt;a&lt;/sup&gt; 0.70 (–0.60; 1.99) 13.0 (10.3; 15.8)&lt;sup&gt;*&lt;/sup&gt; 0.05</td>
<td>HFD (n = 8)&lt;sup&gt;a&lt;/sup&gt; 4.6 (2.0; 7.2)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.7 (0.1; 9.3)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.3 (8.8; 17.9)&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>MG-H1, nmol/mmol hyp</td>
<td>ND (n = 14)&lt;sup&gt;a&lt;/sup&gt; 164.8 ± 9.1 174.2 ± 6.6 148.1 ± 5.1 136.4 ± 5.0 1.6 (–15.2; 18.5)</td>
<td>ND (n = 12)&lt;sup&gt;a&lt;/sup&gt; 136.4 ± 5.0</td>
<td>HFD (n = 12)&lt;sup&gt;a&lt;/sup&gt; 1.6 (–15.2; 18.5) 13.0 (10.3; 15.8)&lt;sup&gt;*&lt;/sup&gt; 0.05</td>
<td>HFD (n = 8)&lt;sup&gt;a&lt;/sup&gt; 4.6 (2.0; 7.2)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.7 (0.1; 9.3)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.3 (8.8; 17.9)&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>Pentosidine, pmol/mmol hyp&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND (n = 14)&lt;sup&gt;a&lt;/sup&gt; 90.2 ± 2.6 91.7 ± 1.5 80.3 ± 4.7 77.3 ± 3.3</td>
<td>ND (n = 12)&lt;sup&gt;a&lt;/sup&gt; 77.3 ± 3.3</td>
<td>HFD (n = 12)&lt;sup&gt;a&lt;/sup&gt; 0.1 (&lt;–0.7; 7.1) 13.0 (10.3; 15.8)&lt;sup&gt;*&lt;/sup&gt; 0.05</td>
<td>HFD (n = 8)&lt;sup&gt;a&lt;/sup&gt; 4.6 (2.0; 7.2)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>4.7 (0.1; 9.3)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.3 (8.8; 17.9)&lt;sup&gt;*&lt;/sup&gt;</td>
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Values are means ± SE for each group. ApoE<sup>−/−</sup>, apolipoprotein E deficiency; WT, wild type B6; HFD, high-fat diet; ND, normal diet; CEL, carboxymethyllysine; CML, carboxyethyllysine; MG-H1, methylglyoxal-derived hydromydialalone 1. Mean difference (95% CI) for effects of ApoE<sup>−/−</sup> and HFD. *Significant effect (P < 0.05). ‡The complete ANOVA model was not significant, although the 95% confidence interval (CI) indicates a difference. *N is lower for some parameters because of missing samples. Weight, n = 13:10:8:8; Cholesterol and glucose, n = 13:11:8:8; CEL, CML, MG-H1, and pentosidine, n = 14:12:11:8, respectively. *Note that pentosidine is reported as pmol rather than nmol.
Mechanical properties. ApoE\(^{-/-}\) mice displayed a significantly increased plateau modulus compared with WT mice (276 ± 12 MPa vs. 242 ± 10 MPa, \(P < 0.05\)), an increased yield strain at both yield points (yield 1: 1.89 ± 0.04% vs. 1.73 ± 0.03%, \(P < 0.005\); yield 2: 4.77 ± 0.06% vs. 4.48 ± 0.11%, \(P < 0.05\)), and a trend toward increased total modulus (614 ± 28 MPa vs. 553 ± 19 MPa, \(P = 0.07\)). Conversely, HFD mice displayed a significantly decreased plateau modulus (238 ± 12 MPa vs. 272 ± 10 MPa, \(P < 0.05\)) and total modulus (539 ± 20 MPa vs. 610 ± 23 MPa, \(P < 0.05\)) compared with ND mice (Table 2 and see Fig. 4). No interactions were observed in any mechanical parameters.

Fascicles broke near to the clamps (end breakage) in 50% (71/141) of the tests. No differences were observed between the groups, but end breakage was associated with decreased maximal strain (\(P < 0.05\)) and failure energy (\(P < 0.05\)).

Advanced glycation end products. Tendon samples of HFD mice had significantly decreased concentrations of CML (\(P < 0.0001\), MG-H1 (\(P < 0.005\)), and pentosidine (\(P < 0.0005\)). CEL was unaffected by diet. None of the AGEs were affected by ApoE deficiency (Table 1).

Correlations. There was a positive within-group correlation between weight and maximum stress (\(r^2 = 0.19\), \(P < 0.01\)), yield 1 stress (\(r^2 = 0.12\), \(P < 0.05\)), yield 2 stress (\(r^2 = 0.17\), \(P < 0.05\)), and failure energy (\(r^2 = 0.18\), \(P < 0.01\)) (Fig. 3). Plasma cholesterol did not correlate significantly with any mechanical parameters, or with weight, or with any of the AGEs. CML correlated negatively with yield 1 strain (\(r^2 = 0.18\), \(P < 0.005\)) and yield 2 strain (\(r^2 = 0.20\), \(P < 0.005\)). Finally, pentosidine also correlated negatively with total modulus within groups (\(r^2 = 0.11\), \(P < 0.05\)). No other correlations were found with any of the AGEs.

DISCUSSION

This study is, to the best of our knowledge, the first study to investigate the systemic effects of apolipoprotein E deficiency (ApoE\(^{-/-}\)) and HFD on the mechanical properties of tendon. It was found that both conditions significantly altered the mechanical behavior of mice tail tendon fascicles, although, surprisingly, in opposite directions. ApoE\(^{-/-}\) mice displayed an increased plateau modulus, total modulus (\(P = 0.07\)), and yield point strains compared with WT mice (Table 2 and Fig. 4A). The HFD yielded the reverse effect, with decreased plateau modulus and total modulus compared with mice eating the normal diet (ND) (Table 2 and Fig. 4C and D). Together, these results suggest a stiffening of the connective tissue with ApoE deficiency and a softening with HFD. We also found reduced AGE density (CML, pentosidine, and MG-H1) in tendons of HFD compared with ND mice (Table 1), which could be related to a higher AGE concentration in the ND (manufactured with heating) compared with the HFD (Fig. 2).

Mechanical changes were mainly observed in the region of plastic deformation, i.e., where tissue damage takes place, and these loading magnitudes are unlikely to be reached during normal loading. However, the occurrence of a tendinopathy is believed to involve microruptures where the tissue is locally loaded to failure. Therefore, changes that influence the properties in the plastic region may well be an important factor affecting the development of a tendinopathy (36).

Diet. Over the past decades there has been increasing focus on metabolic factors as contributors to tendon pathology.

Table 2. Mechanical parameters

<table>
<thead>
<tr>
<th></th>
<th>WT ND (n = 14)</th>
<th>ApoE(^{-/-}) ND (n = 12)</th>
<th>WT ND (n = 12)</th>
<th>ApoE(^{-/-}) HFD (n = 8)</th>
<th>Effect of ApoE(^{-/-})</th>
<th>Effect of HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max strain, %</td>
<td>12.1 ± 0.46</td>
<td>11.5 ± 0.55</td>
<td>12.7 ± 0.51</td>
<td>12.7 ± 0.64</td>
<td>-0.4 (-1.5; 0.70)</td>
<td>0.9 (-0.20; 1.9)</td>
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<tr>
<td>Max stress, MPa</td>
<td>68.4 ± 4.0</td>
<td>72.8 ± 2.8</td>
<td>66.8 ± 4.5</td>
<td>67.7 ± 3.9</td>
<td>3.1 (-4.4; 10.6)</td>
<td>-3.3 (-11.2; 4.6)</td>
</tr>
<tr>
<td>Modulus (max), MPa</td>
<td>1.48 ± 64</td>
<td>1.54 ± 67</td>
<td>1.52 ± 73</td>
<td>1.39 ± 67</td>
<td>-16 (-155; 124)</td>
<td>-46 (-186; 94)</td>
</tr>
<tr>
<td>Modulus (plateau), MPa</td>
<td>253 ± 13</td>
<td>294 ± 15</td>
<td>230 ± 17</td>
<td>250 ± 18</td>
<td>34 (1.7; 66)*</td>
<td>-34 (-66; -1.8)*</td>
</tr>
<tr>
<td>Modulus (total), MPa</td>
<td>567 ± 23</td>
<td>661 ± 39</td>
<td>536 ± 31</td>
<td>543 ± 21</td>
<td>61 (-5.9; 129)</td>
<td>-72 (-133; -10.0)*</td>
</tr>
<tr>
<td>Yield 1 strain, %</td>
<td>1.73 ± 0.04</td>
<td>1.87 ± 0.05</td>
<td>1.72 ± 0.04</td>
<td>1.93 ± 0.06</td>
<td>0.17 (0.07; 0.27)*</td>
<td>0.01 (-0.1; 0.1)</td>
</tr>
<tr>
<td>Yield 1 stress, MPa</td>
<td>22.2 ± 1.0</td>
<td>24.1 ± 0.8</td>
<td>22.3 ± 1.2</td>
<td>22.3 ± 0.7</td>
<td>1.1 (-0.8; 3.1)</td>
<td>-0.8 (-2.8; 1.3)</td>
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<tr>
<td>Yield 2 strain, %</td>
<td>4.61 ± 0.13</td>
<td>4.80 ± 0.07</td>
<td>4.33 ± 0.19</td>
<td>4.73 ± 0.10</td>
<td>0.29 (0.04; 0.54)*</td>
<td>-0.20 (-0.5; 0.1)</td>
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<tr>
<td>Yield 2 stress, MPa</td>
<td>46.6 ± 2.4</td>
<td>51.9 ± 2.0</td>
<td>44.8 ± 3.1</td>
<td>45.7 ± 1.9</td>
<td>3.7 (-1.3; 8.6)</td>
<td>-3.9 (-9.1; 1.3)</td>
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<td>Failure energy, J/m³</td>
<td>5.81 ± 0.48</td>
<td>5.79 ± 0.38</td>
<td>5.96 ± 0.53</td>
<td>5.95 ± 0.57</td>
<td>-0.03 (-0.98; 0.92)</td>
<td>0.16 (-0.8; 1.1)</td>
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</table>

Values are means ± SE for each group. Mechanical parameters were obtained by stretching the tail fascicles to failure. Mean difference (95% CI) for effects of ApoE\(^{-/-}\) and HFD. \(*P < 0.05\), significant effect.
Based on epidemiological and clinical case-control studies, obesity has been proposed as a risk factor for developing tendon pathology, independent of the increased mechanical loading associated with obesity (24, 25). Mechanical changes of weight-bearing tendons have been reported in obese animal models. Biancalana et al. (9) found increased maximal strain of the deep digital flexor tendon in obese leptin receptor deficient rats. This occurred primarily in the plateau region of the stress-strain curves. Total modulus was not reported in that study, but, when trying to calculate this parameter from the data provided (maximum stress divided by maximum strain), there was an 8% decrease in total modulus in obese rats (obese: 938 MPa vs. lean: 1,024 MPa) compared with a 13% decrease in the present study. Supporting these findings, another study also found decreased modulus in the Achilles tendon of mice fed with a HFD (11). In the present study, we found almost similar results, a softening of the tail tendons in HFD mice compared with ND mice. To elucidate a possible cause of the group differences, we looked at within-group correlations to the mechanical parameters. Positive correlations were found between weight and stress values (maximum, yield 1 and yield 2) within the groups. The positive correlations suggest that it is not the increased weight in the HFD group per se that causes the softening (if anything, it may counteract softening). Because the tendon stress related values all depend on the measured fascicle CSA, there was a risk that the correlation to weight was driven by changes in tendon CSA. However, tendon CSA did not correlate to any of the tendon stress parameters or to weight. It should be noted that correlations to modulus parameters were also positive, although not significant (P = 0.09, P = 0.1, P = 0.4 for maximum, plateau, and total modulus, respectively).

Interestingly, we found a reduced AGE density (CML, pentosidine, and MG-H1) in the tail tendons of HFD mice. The ability of AGEs to affect mechanical properties was somewhat supported by the negative within-group correlation between the AGE adduct CML and yield strains, suggesting a reduced compliance. Conversely, the AGE cross-link pentosidine correlated negatively with total modulus, suggesting an increased compliance, and the remaining AGEs did not display any significant correlation to mechanical properties. Therefore, the relation between tissue AGEs and mechanical properties remains inconclusive.

It is unclear why the HFD mice would display lower AGE concentrations in their tail tendons, since obesity is known to be associated with decreased glucose tolerance, hyperglycemia, and AGE accumulation (21). Analyzes of the AGE content in the diet showed that the HFD contained significantly fewer AGEs than the normal diet (Fig. 2). The reason for this difference is likely related to lower carbohydrate (sugar) content in the HFD and the processing of the diet. The normal diet used in the present study is heated during production, and heating is known to increase AGE formation in foods via the so-called Maillard reaction (37, 51). A fraction of dietary AGEs may enter the bloodstream and bind to matrix proteins (fibronectin) via glycation intermediates (34), thereby being incorporated into tissue, and in rats it has recently been demonstrated that a high dietary AGE content resulted in higher AGE concentrations in tail tendons and other organs (heart) in
rats (45). The accumulation of AGEs in tail tendons was, in a separate study, associated with increased tissue stiffness (26).

In addition to a direct effect of dietary AGEs, high diet AGE content has been shown to induce oxidative stress resembling that of metabolic disease such as insulin resistance and diabetes (14, 46). The oxidative stress induces an inflammatory cascade, which can again increase AGE formation and tissue cross-linking (44, 48, 55, 56).

Thus, it is possible that high diet fat content per se is less important in the aging process. In contrast, the low dietary AGE content in the HFD may have been protective in relation to collagen cross-linking (44, 48, 55, 56).

In a separate study, the accumulation of AGEs in tail tendons was associated with increased tissue stiffness (26).

Immobilization is also known to cause decreased stiffness of tendon tissue, but only in weight-bearing tendons (16, 39). If physical inactivity has a systemic effect on tendon tissue, the decreased modulus observed in the obese mice in our study may be explained by a decreased physical activity level. The spontaneous physical activity of the mice was, unfortunately, not monitored in the present study.

**ApoE deficiency.** Hypercholesterolemia affects millions of people worldwide. Besides having an elevated risk of cardiovascular disease, hypercholesterolemic individuals may also be more prone to tendon overuse disease (23, 38, 58). Clinical studies have shown associations between elevated serum cholesterol and Achilles tendinopathy (23), Achilles tendon rupture (38, 43), and rotator cuff tendon tears (2, 58). The mechanical properties of tendons in hypercholesterolemic individuals are, however, sparsely investigated.

Another research group has recently found decreased maximal modulus in the patellar tendon of ApoE−/− mice (6) and increased maximal modulus in the supraspinatus tendon of ApoE−/− mice, as well as in hypercholesterolemic rats and monkeys (7). However, in ApoE deficiency, it remains unknown whether weight-bearing tendons respond differently from non-weight-bearing tendons.

The present study, performed on non-weight-bearing tendon fascicles, showed no differences in maximal modulus but an increase in modulus in the plateau region of the stress-strain curve. These data suggest a stiffening of the collagen tissue in the ApoE−/− mice. The synergistic effect (statistical interaction) of ApoE−/− and HFD on cholesterol was not observed for the mechanical parameters, but, in contrast, ApoE−/− and HFD mice showed opposing trends. Furthermore, no correlation was seen between cholesterol and any of the mechanical parameters, suggesting that plasma cholesterol per se was not an important explanation of the mechanical behavior in the ApoE−/− mice. Lack of the ApoE gene in itself may, independent of the cholesterol level, affect the mechanical behavior because of abnormal lipid deposition in the tendons (53), as in the Achilles tendon of patients with familial hypercholesterolemia (LDL receptor deficiency) (52). Cholesterol-rich lipoproteins may be entrapped by the extracellular matrix, just like in atheromatous plaque formation, where they are oxidized and taken up by macrophages (foam cells) initiating an inflamma-

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### Fig. 4
Illustration of the stress-strain curves of the four groups. The curves are drawn on the basis of mean values (±SE) of stress and strain at the two yield points and at the failure point.
ory reaction (50). The process may eventually lead to fibrous degeneration and stiffening of collagen tissue in ApoE\textsuperscript{--/--} mice (57).

Although ApoE\textsuperscript{--/--} mice develop atherosclerosis, they do not accumulate fat in the liver and adipose tissue, and this may result in higher insulin sensitivity and reduced blood glucose levels (32). In accordance with this, the ApoE\textsuperscript{--/--} mice in our study demonstrated reduced blood glucose levels compared with WT mice, suggesting better glucose control. This is somewhat surprising in light of our mechanical data, because improved glucose control is assumed to protect against formation of tissue AGEs. However, there was no correlation between any of the measured AGEs in tail tendon and blood glucose, indicating that the difference in blood glucose between groups may have been insufficient to affect AGE accumulation.

Limitations. End breakage in the mechanical test is probably the result of stress concentration close to the clamps and cannot be completely avoided even with the best gripping techniques (42). In the present study, end breakage was observed in 50\% of the fascicles equally distributed between the groups, and this was associated with decreased maximal strain and failure energy density. Premature failure due to stress concentration would likely increase the variation in plateau and failure region mechanical properties, making it more difficult to detect any difference between the groups.

In conclusion, in the present study, ApoE\textsuperscript{--/--} deficiency resulted in higher stiffness of tail tendons, which did not correlate with cholesterol. We unexpectedly found lower AGE levels in tail tendons of animals on a HFD, which may be explained by significantly lower AGE content in the diet itself. The hypothesized relation between AGE accumulation and tendon mechanical properties was inconclusive, but the tendons of HFD mice were markedly more compliant than the tendons of ND mice. These alterations of non-weight-bearing tendon mechanical properties may explain how metabolic deterioration can serve as an independent cause of systemic tendon pathology.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


