Symptom duration-dependent protein- and peptide-level abundance in symptomatic and asymptomatic tendon in early-stage unilateral patellar tendinopathy

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KEYWORDS Early-phase tendinopathy, Patellar tendon, Peptides and proteins, Overuse injury, Temporal proteomics, Extracellular matrix

ABSTRACT

Tendinopathy is a painful overuse disorder marked by progressive functional decline. Although chronic disease mechanisms have been described, molecular changes during the first three months of symptoms remain poorly defined. Here, we performed temporal proteomic profiling of human patellar tendon biopsies across one, two, and three months of symptom duration and evaluated proteome differences between symptomatic and non-symptomatic contralateral tendons. Biopsies were collected from participants with unilateral patellar tendinopathy and symptoms lasting three months or less for analysis by data-independent acquisition liquid chromatography-mass spectrometry. Peptide- and protein-level abundances were quantified from tryptic digestion products as part of a bottom-up proteomics workflow and assessed using two-way ANOVA with factors symptom status (symptomatic or non-symptomatic) and symptom duration (one, two, or three months). Symptom duration had a significant main effect: twelve proteins and fourteen peptides changed significantly over time, with most peptides corresponding to the altered proteins. However, there was no interaction between symptom status and symptom duration, no sex differences, and no detectable proteomic difference between symptomatic and non-symptomatic tendons. These findings indicate that protein-level changes are detectable within the first three months of tendinopathy and similar proteomic changes were also observed in contralateral tendons. Together, these observations suggest that early phase patellar tendinopathy is associated with time-dependent proteomic changes that are not confined to the symptomatic tendon.

INTRODUCTION

Tendinopathy is a clinical diagnosis characterized by tendon pain and impaired function that arises from mechanical overload[1]. It is a challenging injury to manage because symptoms vary between individuals, and athletes with tendinopathy often do not know how to manage the amount/type of exercise that should be performed during the early phase of the disease. People with tendinopathy are not as physically active as they were prior to injury and this can drastically affect their quality of life[2, 3]. In the United States, musculoskeletal disorders cost $380 billion annually and are the most expensive category of health care spending, amounting to more than diabetes or cardiovascular diseases[4]. Nearly 80% of the cost of tendinopathy is estimated to be attributed to physiotherapy visits[2], demonstrating that people with tendinopathy value professional advice to guide their rehabilitation, especially with respect to load management.

The molecular and structural alterations observed in chronic tendinopathy are comparatively well characterized, whereas those arising in early tendinopathy (defined here as <3 months of symptoms) remain less clearly understood. It has been reported for both early and chronic tendinopathy that there is tendon swelling, hypervascularization, and increased cellularity[5-10]. Markers of inflammation and resolution are already evident in early patellar tendinopathy, which could be accompanied by proteomic changes[10]. In chronic tendinopathy, one study reported proteome changes compared to healthy controls, but it was based on a small sample size[11]. Turnover of collagen and collagen gene expression are also increased in chronic tendinopathy[8, 12], but these changes may be restricted to the tendon interfascicular matrix (IFM), where type I collagen turnover has been shown to be higher than in the collagen fascicles of adult rat tendons[13]. Studies on protein alterations in chronic patellar tendinopathy have focused on elevated proteoglycan synthesis and increased levels of the large proteoglycans, versican and aggrecan[6, 14, 15]. However, the molecular changes which guide these structural changes and elicit symptoms in early-stage patellar tendinopathy are still unknown.

There is a paucity of human tendon proteomic studies because it is difficult to solubilize the collagen-rich extracellular matrix[16], resulting in exclusion from many atlas studies, including The Human Protein Atlas (proteinatlas.org)[17, 18]. There is only one study that has investigated the human patellar tendon proteome, which reported a larger difference in proteome composition between patellar tendon and anterior cruciate ligament than between individuals in patellar tendon proteome composition[19]. There were minimal sex differences within the non-symptomatic patellar tendon, with 7% of proteins (12/166) differentially expressed between males and females[19]. However, there is very limited knowledge of the human tendon proteome and protein networks, which make it challenging to discern whether changes in the proteome contribute to symptom progression or resolution in tendinopathy.

To address this knowledge gap, male and female patients with early phase unilateral patellar tendinopathy (<3 months symptom duration) were recruited and biopsies from the symptomatic and non-symptomatic patellar tendons were obtained. To study the molecular progression of early tendinopathy, we subdivided the samples by symptom duration of 0-1 months, 1-2 months, and 2-3 months, and analyzed using bottom-up proteomics to quantify peptide- and protein-level abundance. Our hypothesis is that tendinopathic tissue would have a distinct proteomic profile from the non-symptomatic contralateral tenon and that proteomic changes might emerge over the early course of symptom development.

EXPERIMENTAL METHODS

Study Design

Human patellar tendon biopsies were obtained from a previous cross-sectional study[9]. Informed and written consent from participants were obtained. The purpose of this study was to examine proteomic changes in people with early patellar tendinopathy. In a cross-sectional design, participants were allocated into a time-group based on symptom duration: 0-1 month (T1), 1-2 months (T2), 2-3 months (T3).

Ethics

The ethical considerations of the study were guided by the Declaration of Helsinki and approved by local Ethical Committee of the Capital Region Copenhagen (H-1-6019-857). The study was reported to the Danish register (Datatilsynet; approval number BFH-2016-019, I-Suite nr. 04519) and was performed in accordance with Danish law (Lov om behandling af personoplysninger). The study was registered on ClinicalTrials.gov (BBH-128, NCT02797925).

Participants

The study recruited sports-active patients with palpatory soreness and exercise-related pain present for less than three months in the patellar tendon. Tendinopathy was confirmed by standardized clinical examination, carried out by a medical doctor, as described previously[9]. This present study used a subset of patellar tendon biopsies collected from individuals with unilateral patellar tendinopathy. All participants had the risks, burdens, and benefits explained and gave informed consent to participate. Recruitment took place between August 2016 and October 2018.

Ultrasound Measurements

Ultrasound measurements of patellar tendon thickness were obtained bilaterally as previously described[9]. Ultrasound-recordings on tendon dimensions and power Doppler signals were manually quantified by the investigator in a blinded fashion using a plugin for ImageJ (FIJI, macOS 10.14.4).

Patellar Tendon Biopsy

Twenty-seven subjects were selected to have biopsies on their symptomatic and non-symptomatic patellar tendons. Patients were lying prone with knees bent at a 45° angle. The patellar tendon area was sterilized, a local anesthetic (1% lidocaine, 2-3 mL) was injected, and an incision of 5-10 mm was made distal to the patella with care to obtain tissue from the symptomatic area. The biopsy needle (14G) was placed in the biopsy instrument (Bard Magnum, Bard biopsy systems, USA) and the biopsy taken at a 45° angle relative to the long axis of the patellar tendon. Biopsies were dissected free of subcutaneous tissue and frozen in an isopentane liquid nitrogen isopentane bath. Tissues were stored at -80°C until homogenization. Four symptomatic samples were used for an initial pilot study and therefore not used in the present study. The digestion workflow from the pilot study differed. Therefore, all data from these samples were excluded. The final dataset was generated from 50 biopsies from 27 subjects (16 male, 11 female) (see Table 1).

Protein Extraction

Biopsy tissue (~ 10 mg) was cut into 0.5 mm slices. TRI Reagent (Molecular Research Center, cat no. TR 118) was originally used for RNA isolation and followed manufacturer's directions[9]. Briefly, the tissue samples were homogenized with ice-cold 1 mL TRI Reagent and shaken with stainless-steel beads for 15 sec. Phase separation was performed with 1-bromo-3-chloropropane.

The phenol phase was isolated and frozen, then shipped for protein isolation, digestion, and LC-MS/MS proteomics.

Liquid Chromatography-Tandem Mass Spectrometry

Phenol extracts were centrifuged for 15 min at 17,000 rcf and the supernatant transferred to a new tube. Ice cold acetone was added to 80% and the sample was vortexed, then centrifuged at 17,000 rcf for 5 min and the supernatant discarded. The pellet was then washed with 1 mL ice cold acetone and centrifuged. The wash step was repeated two more times. The pellet was resuspended in 250 µL 6 M urea/2 M thiourea in 100 mM TEAB (triethylammonium bicarbonate) pH 8.5 using three rounds of sonication followed by vortexing. The first round of protein digestion was performed using 4 µg endoproteinase Lys-C (Wako) at for 4 h at 37°C, then the solution was diluted 4-fold with 50 mM TEAB. The second round of digestion was performed using 20 µg trypsin (TPCK, Worthington) overnight at 37°C. Peptides were loaded onto Evotips (Evosep) and analysed on an Evosep One liquid chromatography system connected to a TimsTOF Pro (Bruker) mass spectrometer. The 60spd standard Evosep method was used for separation, and the MS data were acquired with 8 diaPASEF scans per TIMS-MS scan with an accumulation and ramp time of 100 ms, for a total cycle time of 0.95 seconds. The ion mobility range was set to 0.85-1.3 Vs/cm2. Each mass window isolated was 25 m/z wide, ranging from 475-1000 m/z with an ion mobility-dependent collision energy that increased linearly from 20 eV to 59 eV between 0.6-1.6 Vs/cm2. Raw MS data were searched in DIA-NN v1.8.1[20] in double pass mode against a predicted library generated from the UniProt human proteome database (reviewed-only UP000005640, downloaded 18th May 2023). Peptides were permitted one missed cleavage and a length of 7-30 amino acids over a range of 300-1800 m/z with charge state 1-4. Modifications were fixed cysteine carbamidomethylation (to exclude cysteine-containing peptides), N-terminal methionine excision, and variable methionine oxidation. Both MS1 and MS2 mass error were set to 10 ppm, and RT-dependent cross-run normalization was enabled. A precursor FDR of 1% was applied in DIAN-NN outputs. Identified peptides needed to be present in at least 50% of either tendinopathic or contralateral patellar tendon to qualify for further analysis and imputation of missing values (see Supplemental Data 1). Proteomic processing and data acquisition were conducted under blinded conditions. Laboratory analysts received tendon samples coded as left or right, without access to clinical information or symptom status, to reduce observer bias.

Bioinformatics

Different imputation methods were tested with NAGuideR[21] and the imputation method which best fit our data was the Glmnet Ride Regression method[21, 22] (Supplemental Figure 1A). Imputed peptide values were inspected against real values and imputed values were within range of measured values (Supplemental Figure 1B). It is possible for peptides to be found in more than one protein, thus posing a challenge for accurate protein identification and quantification. Peptides shared between multiple proteins were retained and assigned to a corresponding protein group, whereas peptides uniquely matching a single protein informed distinct protein groups, allowing both shared and unique peptides to contribute to quantitative analysis. Peptide measurements from the same protein should correlate in amount with other peptides from the same protein. To use actin isoforms as an example, the six isoforms share at least 93% amino acid sequences with each other. The cytoplasmic actins (β and γ) are nearly identical and only differ by four amino acids[23]. In our samples, we had peptides from skeletal muscle actin and cytoplasmic actin. We first correlated all peptides that belonged to cytoplasmic actin and noticed that some peptides were not correlated with each other (Supplemental Figure 1C). For the peptides that did not correlate, it was observed that these peptide sequences were shared between cytoplasmic and skeletal muscle actins (not shown). We next considered the correlation between peptides that were unique to cytoplasmic actin and observed a better correlation between peptides (Supplemental Figure 1D). Therefore, we decided to aggregate only peptides with identical matches to proteins, e.g. only cytoplasmic actin or both skeletal and cytoplasmic actin. This approach allowed the quantification of peptides that were shared between proteins but also allowed for a more accurate quantification of specific protein levels. In the raw data file, these are the "Protein IDs" and represent what is referred to throughout the manuscript (Supplemental Data 1). The "Protein Groups" are generated from the classical analysis where the razor peptides which dominate the shared peptides determine the identity of a single protein group (Supplemental Data 1). The peptides were aggregated using MSstats (v. 4.12.1, log2, TMP, highQuality and uninformative feature removal)[24].

Peptide and Protein Nomenclature

Proteins were identified using the UniProt IDs, e.g. P60709, but named using the gene that encodes them. Multiple protein IDs can be assigned to the same gene (for example due to alternative splicing of a single gene or proteolytic cleavage of a larger precursor peptide) and this is indicated with an underscore and number after the gene name (example: "P60709;P63261" =

“ACTB;ACTG1_1”, “P60709;P63261;Q6S8J3” = “ACTB;ACTG1_2” and “P60709;P62736;P63261;P63267;P68032;P68133” = “ACTB;ACTG1_3”). Multiple peptides are indicated with a decimal place after the gene names (i.e. COL6A3, COL6A3.1, COL6A3.2, etc).

Matrisome Identification and STRING Protein Network Analysis

Proteins were annotated as core matrisome (ECM glycoproteins, collagens, proteoglycans), matrisome-associated (ECM affiliated, ECM regulators, secreted factors), or non-matrisome using MatrisomeAnalyzeR v1.0.1[25]. Matrisome proteins (core and matrisome affiliated) were analyzed with a full STRING network (version 12.0; function and physical and protein associations) with a minimum required interaction score of high confidence (0.700)[26]. In the cases where multiple GeneIDs were associated with a protein, the first geneID was used. Interaction sources included: text-mining, experiments, databases, co-expression, neighborhood, gene fusion, and co-occurrence. Markov Cluster Algorithm (MCL) clustering was performed to find natural clusters with an inflation parameter of 2.

Statistics

Participant Characteristics: Fisher’s exact test was used to compare the proportion of males and females between symptomatic and non-symptomatic tendons. Chi-square tests were used to evaluate differences in the distribution of participants across time groups (T1, T2, T3) according to symptom status (symptomatic or non-symptomatic) and sex (male vs female).

Ultrasound measurements: To test for a difference in patellar tendon thickness, a paired t-test was used to test for differences in symptomatic versus non-symptomatic patellar tendon. A one-way ANOVA was used to test for a difference across time (T1, T2, T3) with data from the symptomatic and non-symptomatic tendon included in each time group. The separate healthy control group was tested for a left versus right patellar tendon thickness with a paired t-test.

Peptide- and Protein-Level Abundance: Statistical tests were performed for both peptide and protein-level abundances in R 4.4.1. All p-values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) procedure. To test the effect of sex on the peptide- and protein-level abundnaces, the symptomatic and non-symptomatic side were first considered separately, and a linear model (lm and anova in R) was used to test for sex differences. Subsequently, a two-way ANOVA with time group and gender as co-variates were used to test all data for sex differences in the peptide and protein levels. Fold change calculations for male vs female comparisons were performed such that a positive value indicates higher abundance in male patellar tendon and negative value is higher abundance in female patellar tendon. The changes in peptide- and protein-level abundance with symptom duration were first assessed with a linear model on only the tendinopathic samples. To test the interaction between symptom status (non-symptomatic vs symptomatic) and time (T1, T2, T3), we used a two-way repeated measures ANOVA. Lastly, the symptomatic samples and the non-symptomatic contralateral samples were also compared with a t-test (Supplemental Data 2 and 3).

Non-symptomatic vs. Symptomatic: Power analyses were performed using the R package pwr. SD for each peptide/protein was calculated as the mean SD for each individual group (symptom status * symptom duration). p-value cut-off was adjusted for FDR adjustment, assuming at least one real change, $p < 0.05$ / (total number of peptides/proteins). That is, $p < 0.00010$ for proteins and $p < 0.000034$ for peptides.

Data Availability

Raw proteomic data was deposited to the ProteomeXchange Consortium via the PRIDE partner repository (http://www.ebi.ac.uk/pride/archive/) with the data set identifier PXD071372. All data are available in the Supplemental Data files.

RESULTS

Participant Characteristics

Twenty-seven participants (16 males, 11 females; mean age 31 years ± 2, range: 18-58)) with unilateral patellar tendinopathy with less than 3 months of symptoms were included in the study (Table 1). A total of 54 tendon samples were collected from the symptomatic and non-symptomatic legs. Four symptomatic patellar tendon samples were allocated to a pilot study and therefore excluded from the analyses reported here. Symptom duration ranged from 3 to 88 days and subjects were subsequently divided into three groups based on symptom duration: T1 (0-1 month), T2 (1-2 month), T3 (2-3 month) (Table 1). There was no difference in sex distribution between symptomatic and non-symptomatic tendons (Fisher exact test two-tailed P value = 0.7759), and the proportion of participants across symptom-duration categories (T1, T2, T3) did not differ by leg or sex (Chi square P value for symptom status = 0.936675, Chi square P value for sex = 0.245843) (Table 1).

Group	**Subjects**	**Mean age (y) (range)**	**Number of symptomatic biopsies analyzed**	**Number of non-symptomatic biopsies analyzed**
T1 (0-1 mo.)	9 (5M / 4F)	30.1 ± 3.68 (18-50)	7 (2 of the male samples not available for this study)	All 9 analyzed
T2 (1-2 mo.)	11 (8M / 3F)	32.5 ± 3.34 (18-58)	9 (2 of the male samples not available for this study)	All 11 analyzed
T3 (2-3 mo.)	7 (3M / 4F)	29.2 ± 3.77 (19-29)	All 7 analyzed	All 7 analyzed
Total	27	30.9 ± 2.01 (18-58)	23 (4 used in a pilot study)	27

Table 1. Participant characteristics and number of samples analyzed. Total number of subjects (n = 27) and patellar tendon samples (n = 50), grouped by symptom status and duration.

The symptomatic patellar tendon was thicker than the non-symptomatic tendon (mean 4.58 mm vs. 3.89 mm) (paired t-test p = 0.02971) (Supplemental Figure 2, Supplemental Table 1).

However, there was no difference in thickness across the time groups (T1, T2, T3) when including the symptomatic and non-symptomatic data at each timepoint (one-way ANOVA p = 0.323). For context, a separate healthy control group demonstrated no difference in mean patellar tendon thickness of 4.36 mm (left) and 4.19 mm (right) patellar tendon (paired t-test p-value = 0.4986) (Supplemental Figure 2, Supplemental Table 1).

Peptide and Protein Dataset Overview

After imputation and peptide-to-protein assignment, 1457 peptides were quantified and grouped into 484 proteins, of which at least 50% were detected in either the symptomatic or non-symptomatic tendon (Supplemental Tables 2, 3). To provide an unsupervised overview of the dataset, we applied principal component analysis (PCA) to the data and observed no obvious groupings by sex or symptom status within the peptide or protein datasets (Figure 1). For the peptide data, PC1 accounted for 25% of the variance and PC2 for 13% of the variance within the data (Figure 1A). In the proteome, the first two principal components explained less of the variance, with PC1 accounting for 17% of the variance and PC2 15% of the variance (Figure 1B).

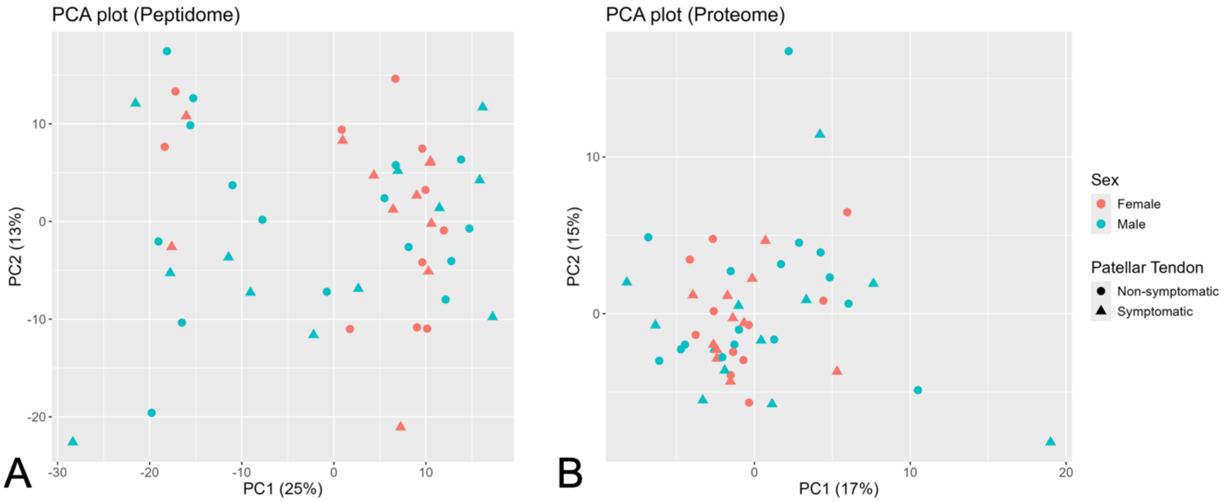

Figure 1. Principal component analysis (PCA) of the proteomics data. PCA was performed on the log2 transformed data of **(A)** 1457 peptides and **(B)** 484 proteins. Each data point represents a patellar tendon sample: 23 paired tendinopathic (symptomatic) and non-symptomatic samples and 4 unpaired non-symptomatic samples. Non-symptomatic: circles. Symptomatic: triangles. Male: blue. Female: red.

Protein-Protein Interactions in Patellar Tendon

We then profiled the patellar tendon proteome (using all proteins identified from non-symptomatic and symptomatic patellar tendon) and mapped protein-protein interactions to provide a baseline for later comparisons. Using Matrisome databases, 53 core matrisome and 37 matrisome-associated proteins were identified from 670 total interaction pairs between 484 unique proteins (Supplemental Figure 3, Supplemental Table 4). STRING network analysis of all matrisome-annotated proteins confirmed 335 significant interactions (average local clustering coefficient = 0.557, PPI enrichment p-value <1.0e-16), representing both physical and functional interactions (Supplemental Figure 4; Supplemental Table 4). The network of matrisome proteins revealed dense interactions among core matrix proteins, with collagens (COL1A1, COL1A2, COL10A1) and large proteoglycans (VCAN), and small leucine rich proteoglycans (DCN, BGN, FMOD, LUM) forming the central network (Figure 2). Matrisome-associated proteins were arranged in peripheral clusters, consistent with the known organization of extracellular matrix networks (Figure 2). There were 20 unique matrisome-associated proteins that had 56 interactions with 12 matrisome core proteins, comprising 8 ECM glycoproteins (FGB, FGA, FGG, FN1, VTN, NID1, THBS1, MGP), 3 proteoglycans (PRG4, DCN, LUM), and one collagen (COL18A1) (Figure 2).

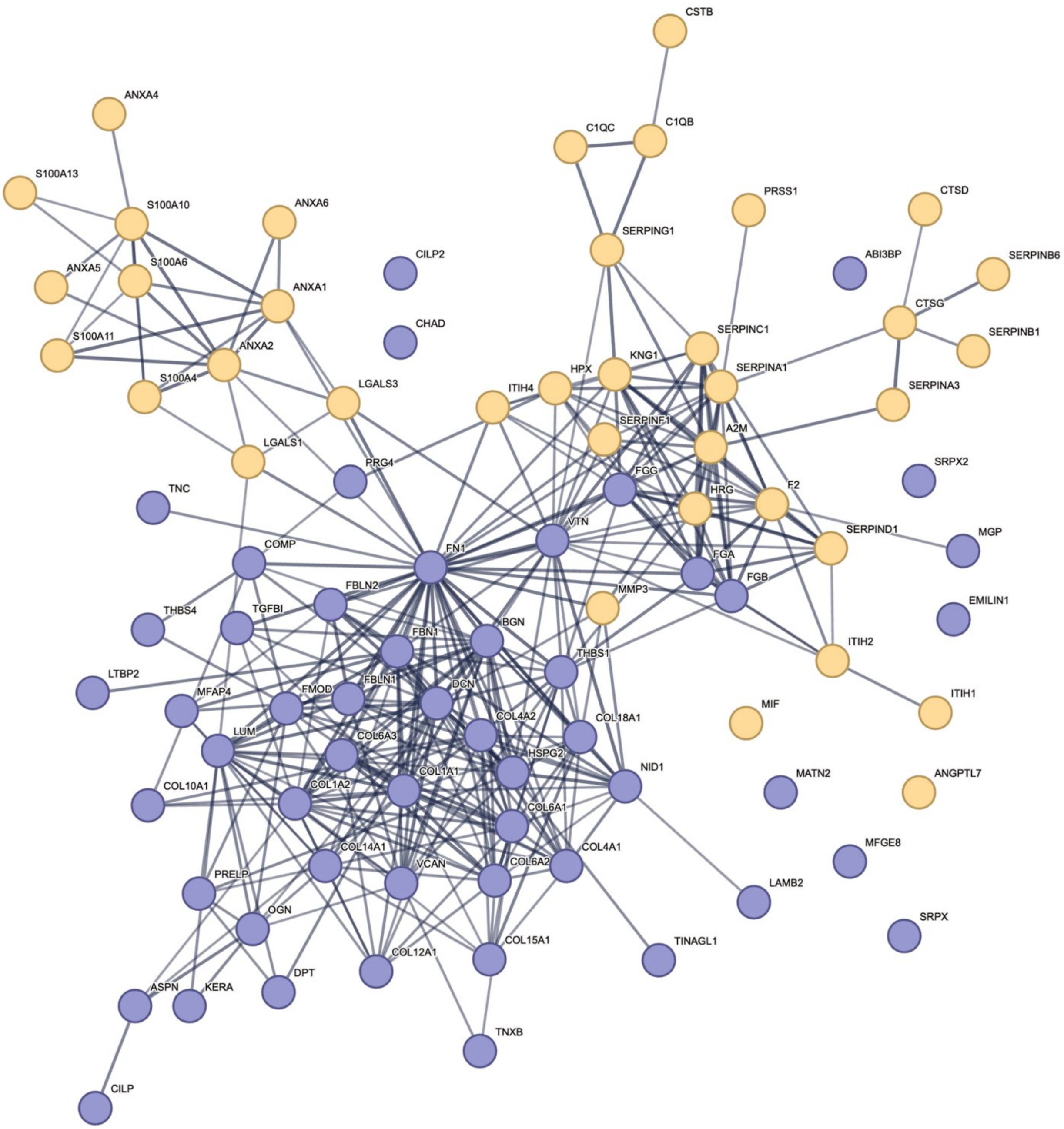

Figure 2. STRING analysis of matrisome-annotated proteins. Nodes represent 53 core matrisome proteins and 37 matrisome-associated proteins. Edges represent functional and physical protein associations at high confidence (score ≥ 0.700), with line thickness indicating the strength

of interaction evidence. The total number of observed edges (335) exceed the number of expected edges by chance (10).

To identify functional nodes within the matrisome proteins, Markov Cluster Algorithm (MCL) clustering was applied, which yielded five clusters (Supplemental Figure 4). Cluster 1 contained 57 proteins, and most were core matrisome proteins. Cluster 1 also had 14 matrisome-associated proteins, including several serine protease inhibitors. Cluster 2 contained 12 calcium-dependent binding proteins, which included the annexins and S100 alarmins (Supplemental Figure 4). Annexin A1 and annexin A2 had a predicted interaction combined score of 0.989 and the interactions stronger than that were: ANXA2-S100A4 (score = 0.999), ANXA2-S100A10 (score = 0.999), ANXA1-S100A11 (score = 0.998), ANXA2-S100A11 (score = 0.996) (Supplemental Table 4).

No Effect of Sex in the Patellar Tendon Peptide- or Protein-Level Abundances

To formally test the absence of sex-related differences suggested by the PCA, we next performed statistical analyses at both the peptide- and protein-levels. First, we used a linear model that considered only the non-symptomatic samples and statistically tested for sex differences in the peptide- and protein-level abundances using an ANVOA with sex (M or F) as variables and found no peptides or proteins with significant differences in abundance (FDR < 0.05) (Table 2). We next analyzed only the symptomatic samples in a two-way ANOVA with sex and symptom duration (T1, T2, T3) as co-variates, which also revealed no sex-dependent differences in peptides or protein abundance in the patellar tendon (FDR<0.05; Table 3).

Peptide dataset (top 15)			
Peptide ID	**Log2FC**	**p value**	**FDR**
MMP3_1.1	0.615414	0.001811	0.86711
TNC.7	0.764068	0.002738	0.86711
TNC.2	0.739446	0.004466	0.86711
TNC.3	0.970803	0.004574	0.86711
COL6A3.75	0.501897	0.004986	0.86711
ORM1_1	0.694693	0.005377	0.86711
RPL13	-0.328	0.005629	0.86711
MMP3_2	0.9526	0.007656	0.86711
NID1_1.1	0.328634	0.008249	0.86711
ASPN.8	-0.36934	0.008872	0.86711
COL6A1.2	0.590277	0.009531	0.86711
RAP1A_3	0.422497	0.013009	0.86711
H2AC18.H2AC20_2	-0.27227	0.013476	0.86711
TNC	0.618175	0.013591	0.86711
TNC.8	0.759994	0.014164	0.86711
Proteome (top15)			
Protein ID	**Log2FC**	**p value**	**FDR**
RPL13	-0.328	0.005629	0.74343
TNC	0.67047	0.007172	0.74343
MMP3_1	0.629846	0.007236	0.74343
MMP3_2	0.9526	0.007656	0.74343

SOD2	-0.39916	0.012723	0.74343
RAP1A_3	0.422497	0.013009	0.74343
H2AC18_H2AC20_2	-0.27227	0.013476	0.74343
SLC30A9	-0.59968	0.014349	0.74343
HSPA2	0.436776	0.018124	0.74343
AKR1C2_2	0.421931	0.018572	0.74343
IGHG2_2	0.553573	0.019738	0.74343
ATP5F1A	-0.21002	0.019857	0.74343
RPS27A_UBA52_UBB_UBC	-0.18535	0.019968	0.74343
H2B*	-0.37358	0.025298	0.87459
MYH11_2	0.458672	0.029681	0.90282

Table 2. ANOVA analysis of sex effects on peptide- and protein-level abundances in non-symptomatic patellar tendon. The top 15 peptides and proteins are shown (see Supplemental Table 2 and 3 for precursors). A positive log2FC value indicates a higher abundance in male patellar tendon. *H2B = H2BC12_H2BC12L_H2BC13_H2BC14_H2BC15_H2BC18_H2BC4_H2BC5_H2BC9_4.

Peptide dataset (top 15)			
Peptide ID	**L2FC**	**p value**	**FDR**
DPP4.1	-0.4777394	0.00027986	0.4077584
ATP5F1B.5	-0.3021371	0.00209305	0.9556763
H2BC(pep)*	-0.5856873	0.0021454	0.9556763

TNC	0.47430535	0.00542841	0.9556763
HSPA1A.HSPA1B_2.1	-0.4375278	0.00556981	0.9556763
A2M_2.2	-0.6347577	0.00659947	0.9556763
GLUD1_4.3	-0.264106	0.00744696	0.9556763
H1.4_3	-0.3958015	0.00766503	0.9556763
COL6A3.25	-0.5979725	0.00771157	0.9556763
FABP5	-0.3206039	0.00873908	0.9556763
TALDO1	-0.3403077	0.00976611	0.9556763
A2M_1.1	-0.5191107	0.0097855	0.9556763
COL6A3.28	-0.3880131	0.01128147	0.9556763
A2M_1.4	-0.7637455	0.01176099	0.9556763
RPS27A.UBA52.UBB.UBC	-0.1858318	0.01213422	0.9556763
Proteome (top15)			
Protein ID	**L2FC**	**p value**	**FDR**
DPP4	-0.3706074	0.00059714	0.2890152
H2BC(pr)*	-0.4952232	0.00196034	0.4744025
H4C1	-0.3375016	0.00473461	0.5308795
HSPA1A_HSPA1B_2	-0.3139819	0.00707399	0.5308795
CRYAB	-0.4722531	0.00731508	0.5308795
H1_4_3	-0.3958015	0.00766503	0.5308795
FABP5	-0.3206039	0.00873908	0.5308795
RPS27A_UBA52_UBB_UBC	-0.185965	0.00877487	0.5308795
FHL1	-0.4452031	0.01062284	0.5712729

TNC	0.44989331	0.01206792	0.5840871
TALDO1	-0.2239404	0.01338613	0.5889898
VAT1	-0.2486511	0.0198696	0.8014072
TMEM109	-0.4041042	0.02960104	0.8026326
COL6A2	-0.3566929	0.03379055	0.8026326
SOD2	-0.4349329	0.0371423	0.8026326

Table 3. Two-way ANOVA of sex and symptom duration on peptide- or protein-level abundances in in symptomatic patellar tendon samples. The top 15 peptides and proteins are shown (see Supplemental Table 2 and 3 for precursors). A positive log2FC indicates a higher abundance in male patellar tendon. *H2BC(pep) = H2BC12.H2BC12L.H2BC13.H2BC14.H2BC15.H2BC18.H2BC4.H2BC5.H2BC9_2.1. *H2BC(pr) = H2BC12_H2BC12L_H2BC13_H2BC14_H2BC15_H2BC18_H2BC4_H2BC5_H2BC9_2.

Changes in Peptide and Protein Abundance with Duration of Tendinopathy Symptoms

There was a significant effect of symptom duration (T1, T2, T3) on the abundance of 14 peptides and 12 proteins when tested as a two-way ANOVA that considered symptom status (non-symptomatic and symptomatic) and symptom duration (Figures 3, 4; Supplemental Table 5). The peptides that that showed significant changes with symptom duration in both non-symptomatic and symptomatic patellar tendons included those from the proteins, annexin A1 and A2 (ANXA1, ANXA2) and collagen alpha 3(VI) (Figure 3, Supplemental Table 5). All significantly changed peptides decreased with time in both the non-symptomatic and symptomatic tendon except COL6A3.58 (Figure 3). Two collagen VI peptides that changed with symptom duration are located at adjacent positions in the COL6A3 protein (P12111). COL6A3.58 (SQDDVSRFAQVIR) is at position 1552 – 1564. COL6A3.61 (SSGIVSLGVGDR) is at position 1565-1576. Both peptides are in a von Willebrand factor domain A containing 8 (VWFA8) within a nonhelical region. COL6A3.58 increased with symptom duration, while COL6A3.61 decreased with symptom duration.

Twelve proteins that significantly decreased with symptom duration were annexin A1 (ANXA1), vimentin (VIM), heat shock 70 kDa protein 8 (HSPA8), dermatopontin (DPT), immunoglobulin heavy constant gamma 1 (IGHG1), immunoglobulin heavy variable 3 (IGHV3), ribonuclease/angiogenin inhibitor 1 (RNH1), annexin A2 (ANXA2), catalase (CAT), potassium channel tetramerization domain containing 12 (KCTD12), complement factor B (CFB), and vimentin (VIM) (Figure 4, Supplemental Table 5 – Proteome). The proteins which had peptides and protein groups that decreased with symptom duration were: ANXA1, VIM, HSPA8, IHGV3, and ANXA2. To assess whether these changes followed a common trend, a pairwise correlation between the significant peptides were performed (Supplemental Figure 5). All peptides showed a strong positive correlation with each other except for COL6A3.58. Levels of COL6A3.58 had a strong negative correlation with the other peptides but was not correlated with COL6A3.61. In the same way, correlations between the proteins were significant except for the following pairs: CFB-DPT (r=0.25), KCTD12-IGHV3 (r=0.28), CFB-KCTD12 (r=0.09), and VIM3-CFB (r=0.27) (Supplemental Figure 6).

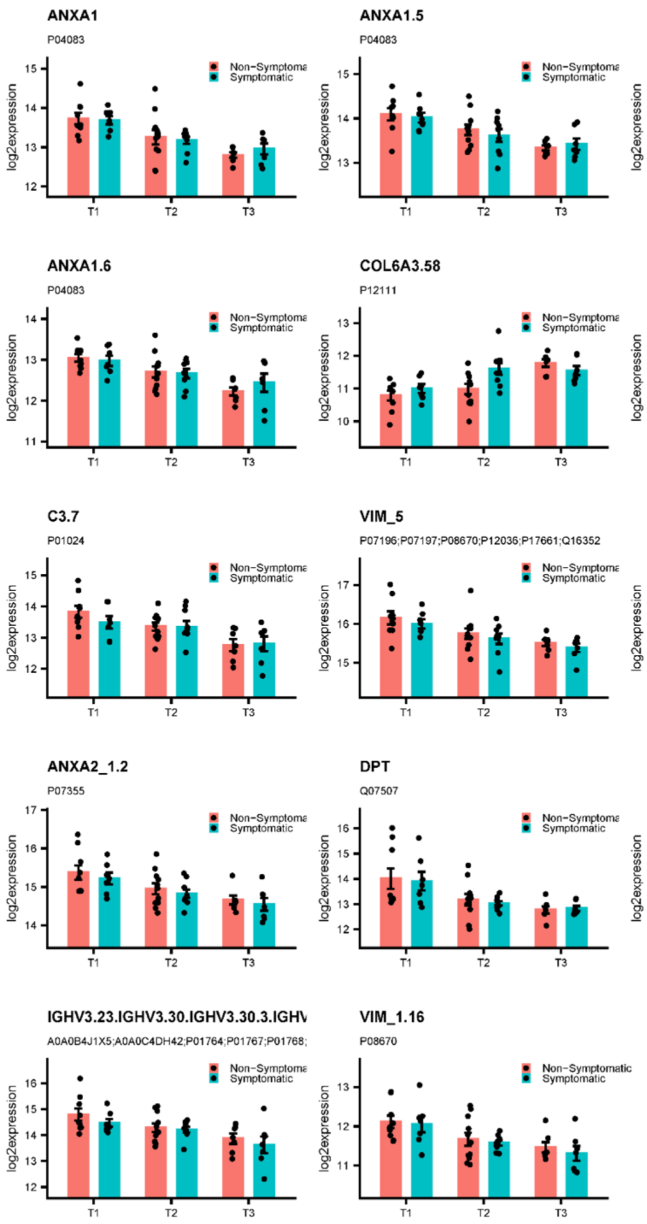

Figure 3. Early tendinopathy has peptidome alterations in symptomatic and non-symptomatic patellar tendon. The significant peptides were ANXA1, ANXA1.5, IGHG1_4, ANXA1.6, COL6A3.58, ANXA2_2.12, C3.7, VIM_5, HSPA8_4, ANXA2_1.2, DPT, COL6A3.61, IGHV3.23.IGHV3.30.IGHV3.30.3.IGHV3.30.5.IGHV3.33.IGHV3.53.IGHV3.66_1, and VIM_1.16. All peptides decreased with time except COL6A3.58.

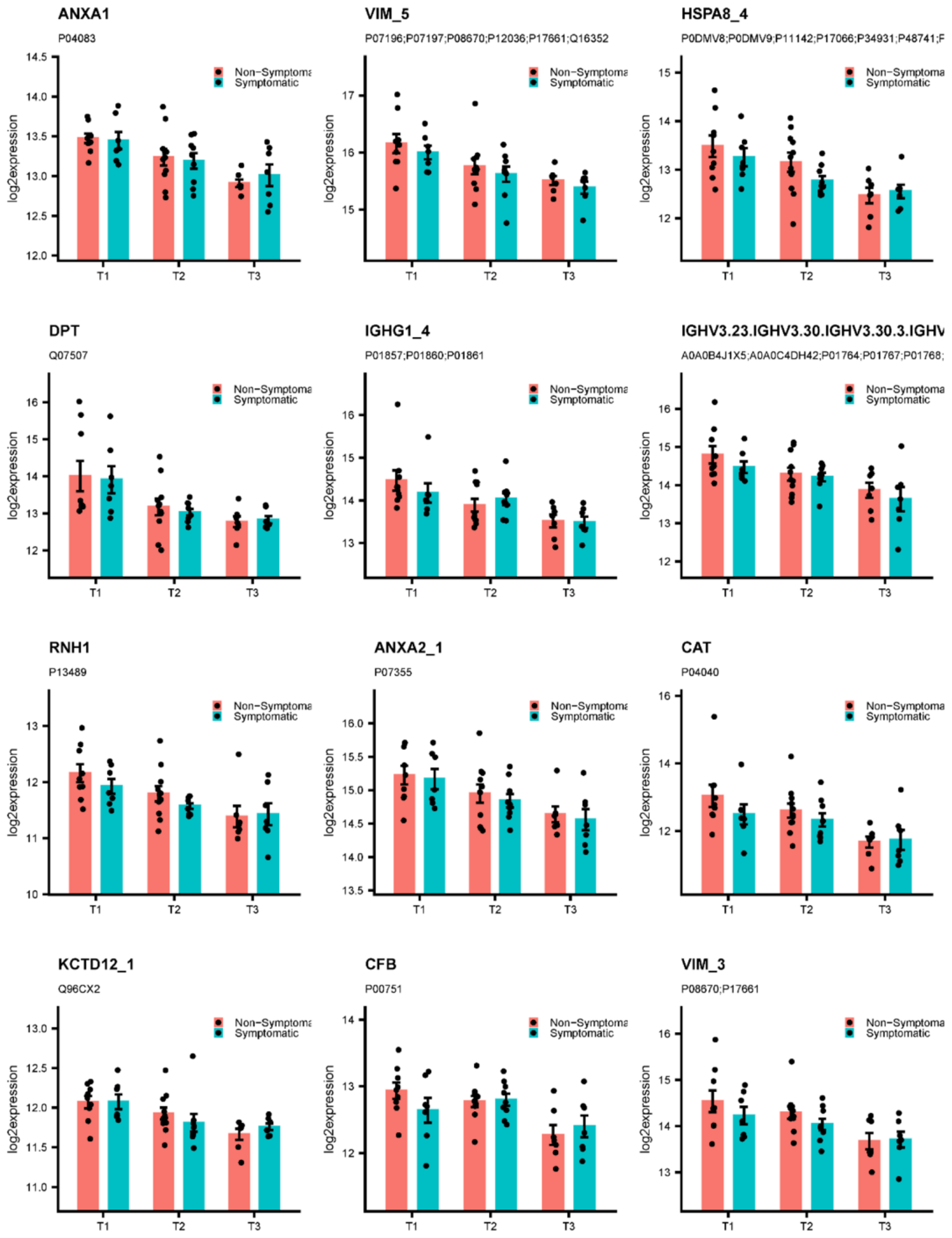

Figure 4. Proteome alterations in symptomatic and non-symptomatic patellar tendon. There was no effect of tendinopathy on the proteome. However, twelve proteins, ANXA1, VIM_5, HSPA8_4, DPT, IGHG1_4, IGHV3_23_IGHV3_30_IGHV3_30_3_IGHV3_30_5_IGHV3_33_IGHV3_53_IGHV3_66_1, RNH1, ANXA2_1, CAT, KCTD12_1, CFB, and VIM_3, decreased with time.

Changes in Peptide and Protein Abundance in Symptomatic and Non-Symptomatic Tendons

There was no effect of symptom status (non-symptomatic vs symptomatic) on the peptidome or proteome when tested as a two-way ANOVA with symptom status and symptom duration as factors (Supplemental Table 5). We also tested for differences in peptide or protein abundance between the non-symptomatic and symptomatic patellar tendons using a t-test and found no differences (FDR>0.05, t-tests; Supplemental Table 6). Post-power analyses showed a high power (2-fold change) for most of the peptides/proteins in these tests (453 of 484 and 1219 of 1457, respectively) and even a 1.5-fold change should be detectable for a large fraction of the proteins/peptides (Supplemental Figure 7).

DISCUSSION

By analyzing early-stage biopsies with the asymptomatic contralateral tendon, this study reveals previously uncharacterized molecular features of early patellar tendinopathy. While the absence of an independent healthy control group and the modest sample sizes within symptom-duration groups are important considerations that are addressed below, this design provides a unique within-individual comparison during the earliest phase of disease. The present proteomics dataset (1457 peptides and 484 proteins) is comparable to the previous proteomic study on human patellar tendon with a similar number of peptides (1586)[19]. The number of protein groups identified in the present study are not comparable to other studies because we made protein groups that contain only unique peptides, which allowed more specificity in the proteins identified. Proteomics studies typically take shared peptides that are assigned to multiple protein groups and assign all peptides to the protein with the most peptides. The present stricter grouping approach increases confidence in the specific proteins identified and provides a more precise dataset for understanding the molecular changes in early patellar tendinopathy.

In contrast to our hypothesis, no differences in any peptides or proteins between symptomatic and non-symptomatic patellar tendons were observed. It is unclear why symptoms develop in one tendon despite a similar proteomic profile in both patellar tendons. This may suggest that the symptoms associated with early tendinopathy do not relate to a detectable proteomic change. It is also possible that relevant pain mechanisms involve proteins below the detection threshold, components of the insoluble proteome, or changes outside the sampled tendon area. However, conclusions are limited by the absence of a true healthy control group. Previous studies have shown that while the overall levels of the pain neurotransmitter substance P may not differ in tendinopathic and healthy tendons, the arrangement of nerve fibers can change[27]. In chronic patellar tendinopathy, sprouting of substance P-positive nerve fibers into the tendon proper has been observed and may contribute pain[27]. Another possibility is that pain arises from tendon swelling, given the tendency toward the increased thickness observed even in asymptomatic tendons. Such swelling may activate pressure-sensitive nociceptors without corresponding alterations in the proteome[28, 29].

Despite the lack of changes between symptomatic and non-symptomatic tendons, we did observe changes in the peptidome and proteome that were associated with symptom duration. One interesting finding that emerged from the peptidome data was that the two COL6A3 peptides (COL6A3.58 and COL6A3.61) located adjacent to each other in the full amino acid sequence were differentially regulated in their abundance. COL6A3.61 levels decreased and COL6A3.58 increased with symptom duration in both tendons. While no biological function has yet been described for this specific region of COL6A3, post-translational modifications and region-specific regulation of COL6A3 have been observed in other contexts. For example, proteomic fingerprinting of COL6A3 in ultraviolet-irradiated skin microfibrils revealed domain-specific changes in peptide spectral matches within von Willebrand factor A domains, even though overall collagen VI ultrastructure appeared preserved[30]. Such findings suggest that stress conditions can differentially regulate peptide abundance of COL6A3, consistent with our observation that adjacent peptides show opposite regulation in early tendinopathy. Further studies will be needed to determine whether this reflects structural changes, isoform usage, or novel post-translational modifications of COL6A3 in tendon disease.

The annexins and immunoglobulins had corresponding peptides and proteins that decreased with symptom duration in both tendons. The annexins are calcium-dependent phospholipid binding proteins[31]. Annexin A1 is proposed to be a glucocorticoid regulated mediator of inflammation[32] and it is possible that there was an initial inflammatory response which coincided with symptom onset and subsequently dampened. Annexin proteins were also identified in early-stage equine superdigital flexor tendon injury, suggesting conserved mechanism of tissue response to injury across species[33]. Because the definition of early tendinopathy used in this study was pragmatic rather than rooted in set biological changes, it is possible that inflammatory changes were already present before the first month, meaning some processes may have preceded the defined early window. Data from a small pilot study supports an inflammatory response in people with early patellar tendinopathy, yet these changes seemed to persist in the chronic condition[10]. The time course of the inflammatory response in early tendinopathy remains under investigation.

Our data presents the first matrix protein network for human patellar tendon and suggests a possible alteration in actin dynamics, possibly within the IFM in tendinopathy. The organization of matrix proteins dictates the force transfer function of the patellar tendon, and it is without surprise that the core matrisome proteins interact with each other and have few connections to matrisome-associated proteins. Notably, most matrisome-associated proteins had functional and/or physical connection to ECM glycoproteins rather than the main fibrillar collagens in tendon. One cluster contained calcium-dependent binding proteins, including S100 proteins (S100A8 and S100A9), which have been implicated in inflammatory modulation in tendinopathy[34]. Here, we observed interactions of high confidence between annexin A1/2 and S100A4/10/1, which may be relevant to tendon IFM cells because IFM cells express S100A4, S100A10, and S100A11 at higher levels than fascicular matrix cells[35]. The S100A10/annexin A2 complex is well studied and association of S100A10 with annexin A2 increases the calcium sensitivity of annexin A2 for membrane binding[36]. The S100A10/annexin A2 complex is known to be enriched in dynamic actin structures, such as those necessary for cell protrusions and motility[37]. These data could support a hypothesis about altered IFM activity and actin dynamics with reduced annexin A2 levels during the early phase of tendinopathy[36]. Future work should focus on investigating the early changes in the IFM region.

In this early tendinopathy dataset, we observed no differences between the male and female patellar tendon peptide- or protein-level abundances, despite known differences in tendinopathy rates, where males are more likely to get patellar tendinopathy than females, even when participating in the same sport[38, 39]. The only study which has investigated the human patellar tendon proteome found 12 proteins differentially expressed between healthy tendons from males and females[19]. Interestingly, it was found that the male patellar tendon contained more type III collagen than female and suggested that this could predispose men to tendinopathy[19]. However, it was not possible to quantify collagen III levels in the present dataset because too few samples contained measurable peptides, suggesting that sex-specific proteomic differences may not be evident during early pathological stages, or that such differences are subtle and context-dependent.

The finding that there were peptide- and protein-level abundances varied with symptom duration, yet did not differ between symptomatic and non-symptomatic patellar tendons, raises the possibility that tendinopathy involves bilateral molecular adaptations. The lack of significant differences between the non-symptomatic and the symptomatic patellar tendon was strengthened by a post-power analysis indicating that this is a real lack of difference (not a type II error). It has been observed that unilateral electrical stimulation can induce a bilateral increase in cellularity and vascularity in rabbit Achilles tendon[40]. In humans, structural disruptions detected by ultrasound tissue characterization have been observed in both the symptomatic and non-symptomatic tendons of people with unilateral Achilles or patellar tendinopathy. While the structural changes in the asymptomatic tissue are distinct from a separate healthy control population, there is a greater degree of disorganization observed in the symptomatic tendon[41, 42]. In the patellar tendon, a greater amount of tissue disorganization is linked to patellar tendinopathy[43]. These data suggest that tendinopathy is a bilateral disease with structural changes occurring in both limbs, yet symptoms initially develop on one side.

Limitations

A key limitation of our study is the absence of a separate healthy control group. The non-symptomatic contralateral tendon was initially considered a suitable internal control, based on the assumption that proteomic changes would be localized to the symptomatic tendon. However, our findings suggest that the contralateral tendon may not serve as a healthy baseline. Including a truly healthy control group would have clarified whether the observed similarities between non-symptomatic and tendinopathic tendons reflect bilateral adaptations already present before symptom onset, or instead arise as a consequence of early tendinopathy or shared loading patterns.

Furthermore, as the study is cross-sectional in nature, it cannot be ruled out that the absence of between-group differences reflects substantial between-subject variability. In addition, subdivision of participants into symptom-duration groups resulted in relatively small sample sizes at individual time points, which may have limited statistical power to detect subtle temporal or symptom-related effects. However, previous data suggest that the variability is greater between anatomically distinct tendons or ligaments than between individuals[19].

The size of the biopsy tissue also provides a few limitations. First, the biopsy tissue collected might not be representative of changes happening in the whole tendon, although care was taken to target the affected region identified by ultrasound. Second, the tissue samples are small, so to allow for molecular analyses of RNA[9] as well as protein, a TRI reagent -based phase separation and acetone precipitation was used to efficiently recover the total protein from the tendon biopsies after RNA isolation. However, bottom-up proteomics remains inherently biased against highly cross-linked extracellular matrix (ECM) proteins such as mature fibrillar collagens, which are poorly digested and therefore underrepresented. Future studies aiming to capture mature, cross-linked ECM proteins may benefit from specialized chemical strategies, such as hydroxylamine or cyanogen bromide-based workflows, which have been shown to enable detection of collagen-derived peptides that are not accessible using conventional trypsin approaches[44].

Finally, this study did not include additional experimental validation of individual protein changes, such as immunohistochemistry. Such validation in human tendon biopsies is challenged by disrupted tissue architecture in tendinopathy and the limited size of the samples. Future studies using independent cohort including a healthy control group will be important to confirm the findings.

Conclusions

This study provides the first characterization of the proteome of early patellar tendinopathy in humans. While we found no differences between the symptomatic and non-symptomatic patellar tendon, we did find time-dependent proteomic changes between symptom onset (0-1 months symptoms) and 2-3 months of symptom duration. These results raise the possibility that proteomic alterations are not confined to the symptomatic tendon in early tendinopathy. Whether such bilateral changes have implications for rehabilitation approaches warrant further investigation.

SUPPORTING INFORMATION

Supplemental Figure 1. Supporting information for proteomics. (.PDF)

Supplemental Figure 2. Patellar tendon transverse thickness. (.PDF)

Supplemental Figure 3. Matrisome analysis. (.PDF)

Supplemental Figure 4. STRING analysis of matrisome-annotated proteins with MCL clustering. (.PDF)

Supplemental Figure 5. Correlations between significant peptides that change with symptom duration. (.PDF)

Supplemental Figure 6. Correlations between significant proteins that change with symptom duration. (.PDF)

Supplemental Figure 7. Power analysis for the difference between non-symptomatic and symptomatic patellar tendon. (.PDF)

Supplemental Table 1. All participant characteristics. (.XLS)

Supplemental Table 2. Peptide quantification results. (.XLS)

Supplemental Table 3. Proteome quantification results. (.XLS)

Supplemental Table 4. Matrisome analysis and STRING protein-protein networks made of 670 protein-protein interactions. (.XLS)

Supplemental Table 5. Significant peptides and proteins with symptom duration (ANOVA). (.XLS)

Supplemental Table 6. T-test of non-symptomatic vs. symptomatic peptides and proteins. (.XLS)

Supplemental Data 1. DataFile_Raw-Data_Peptides-Proteins.xlsx. (.XLS)

Supplemental Data 2. DataFile_Peptide_Result-Statistics.xlsx. (.XLS)

Supplemental Data 3. DataFile_Proteome_Result-Statistics.xlsx. (.XLS)

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Author Contributions

D.S., P.S. and P.H.T.T. analyzed and prepared the data. S.F. and R.F. performed the liquid chromatography-tandem mass spectrometry analysis. P.H.T.T. performed the ultrasound measurements. P.H.T.T., N.M.M.-C. and M.F.R.M. collected the biopsies. S.G.D. and M.K.

conceived the study. M.K. and C.-Y.C.Y. acquired the funding. D.S., P.S., and C.-Y.C.Y. drafted and revised the manuscript. All authors reviewed and have given approval to the final version of the manuscript.

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Notes

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ABBREVIATIONS

A2M, alpha-2-macroglobulin;AKR1C2, aldo-keto reductase family 1 member C2; ANOVA, analysis of variance; ANXA, annexin; ASPN, asporin; ATP5F1A, ATP synthase F1 subunit alpha, mitochondrial; BGN, biglycan; CAT, catalase; CFB, complement factor B; COL1A1, collagen α-1(I); COL1A2, collagen α-2(I); COL6A3, collagen α-3(VI); COL10A1, collagen α-1(X); COL18A1, collagen α-1(XVIII); CRYAB, alpha-crystallin B chain ; DCN, decorin; DIA-NN, data-independent acquisition - neural networks; DPP, decapentaplegic; DPT, dermatopontin; ECM, extracellular matrix; F, female; FAPB5, fatty acid binding protein 5; FBG, fibrinogen beta chain; FDR, false discovery rate; FGA, fibrinogen alpha chain; FGG, fibrinogen gamma chain; FHL1, four-and-a-half LIM domains 1; FMOD, fibromodulin; FN1, fibronectin; GLUD1, glutamate dehydrogenase 1; H1, histone 1; H2AC, H2A clustered histone; H2B, histone H2B; H4C1, H4 clustered histone 1; HSPA2, heat shock protein family A member 2; IFM, interfascicular matrix; IGHG1/2, immunoglobulin heavy constant gamma 1/2; IGHV3, immunoglobulin heavy variable 3; KCTD12, potassium channel tetramerization domain containing 12; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LUM, lumican; M, male; MCL, Markov Cluster Algorithm; MGP, matrix Gla protein; MMP, matrix metalloproteinase; MYH11, smooth muscle myosin heavy chain 11; NID1, nidogen 1; PC, principal component; ORM1, orosomucoid 1; PCA, principal component analysis; PRG4, lubricin; RAP1A, RAS-related protein 1A; RNH1, ribonuclease/angiogenin inhibitor 1; RPL, ribosomal protein L; RSP27A, ribosomal protein S27a; S100A, S100 calcium-binding protein A; SD, standard deviation; SOD2, superoxide dismutase 2; SLC30A9, solute carrier family 30 member 9; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; T1, symptom duration of 0-1 month; T2, symptom duration of 1-2 months; T3, symptom duration of 2-3 months; TALDO1, transaldolase 1; TEAB, triethylammonium bicarbonate; THBS1, thrombospondin 1; TMEM109, transmembrane protein 109; TNC, tenascin C; VAT1, vesicle amine transport 1; VCAN, versican; VIM, vimentin; VTN, vitronectin;

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