

1 **The effect of exercise on the protein profile of rat knee joint intra- and**
2 **extra-articular ligaments**

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1 **Abstract**

2 Injuries to the intra-articular anterior cruciate ligament (ACL) and the extra-articular medial
3 collateral ligament (MCL) result in significant knee joint instability, pain and immobility.
4 Moderate endurance type exercise can increase ligament strength but little is known on the
5 effect of short-term regular bouts of high intensity exercise on the extracellular matrix (ECM)
6 structure of knee ligaments . Therefore, this study aimed to identify the effect of short-term
7 regular bouts high exercise on the proteome of the rat ACL and MCL using mass spectrometry.
8 Sprague Dawley male rats (n=6) were split into control and exercise groups, and subjected to
9 high intensity training for four 4 weeks followed by proteomic analyses of the ACL and MCL.
10 Knee joint health status was assessed using OARSI and a validated histological scoring system.
11 Histopathological analyses demonstrated no significant changes in either in cruciate, collateral
12 ligaments or cartilage between the control and exercised knee joints. However significant
13 proteins were found to be more abundant in the exercised ACL compared to ACL control group
14 but not between the exercised MCL and control MCL group. The significant abundant proteins
15 in ACL exercise groups were mostly cytoskeletal, ribosomal and enzymes with several
16 abundant matrisomal proteins such as collagen proteins and proteoglycans being found in this
17 group. In conclusion, our results indicate that short term regular bouts of high intensity exercise
18 has an impact on the intra-articular ACL but not extra-articular MCL ECM protein expression.

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1 INTRODUCTION

2
3 Ligaments are short bands of fibrous connective tissues and are responsible for providing a
4 mechanical connection from bone to bone across joints ¹. Their function is to guide and limit
5 normal joint motion assisted by joint surface geometry and musculotendinous forces ². The
6 anterior cruciate ligament (ACL) ³ is located intra-articularly in the mammalian knee joint and
7 is one of the most frequently injured ligaments within this joint. Injuries to the ACL together
8 with the extra-articular medial collateral ligament (MCL), account for over 95% of all multi-
9 ligament injuries in the knee joint ^{4,5}, resulting in significant joint instability and causing major
10 physical, social ⁶ and financial implications ⁷. ACL injuries can also lead to significant
11 functional impairment in athletes as a result of joint instability and muscle atrophy, and are
12 associated with the development of osteoarthritis ^{5,8} leading to a major clinical challenge in
13 orthopaedic medicine ⁹.

14 Exercise is known to exert beneficial effects on the musculoskeletal system by enhancing
15 muscle mass, increasing bone strength ¹⁰ and contributing to the mechanical strength of
16 ligaments and tendons ^{11,12}. In tendons, such as Achilles and patellar tendon, endurance-type
17 exercise has been shown to increase stiffness ¹³, tensile strength ^{12,14} and increases in the cross-
18 sectional area ¹⁵⁻¹⁷. In the mouse Achilles and patellar tendons, it has been demonstrated that
19 short-term treadmill running enhances levels of growth factors such as insulin-like growth
20 factor 1 (IGF-1) ¹⁸⁻²⁰ and transforming-growth factor beta (TGF β) ^{21,22}. Together these data,
21 along with increased collagen synthesis observed after both acute exercise and endurance
22 training, suggest that tendon is dynamic in its response to mechanical loading ²³⁻²⁶.

23 In ligaments, it has been shown that enforced treadmill running has a beneficial effect on the
24 strength of the MCL in canine knees²⁷.

25 In the ACL, increased intercellular activity of fibroblasts in and decreased average fibril
26 diameters have been observed in adult rats after acute treadmill running, which is indicative of

1 increased collagen metabolism²⁸. Endurance treadmill training in rats has been shown to be
2 beneficial for ACL strength and mechanical stiffness¹¹. Other studies have shown an increase
3 in extracellular matrix (ECM) components such as elastin microfibrils in ACLs from an
4 exercising dog breed (e.g. greyhound) compared with more sedentary dog breeds (e.g.
5 Labrador retriever)²⁹.

6 To date, the comparative response to mechanical loading between the MCL and ACL has not
7 been studied, and its response could be used in potential therapeutic strategies to aid these
8 ligaments with repair and to avoid degeneration. The MCL has been found to heal adequately,
9 whilst the ACL has been found to have poor capacity for healing, even following direct
10 apposition with suture repair³⁰. These differences may be due to factors which are mechanical
11 and biologic in origin³⁰, alterations in the cellular metabolism after injury³¹ and to intrinsic
12 cell deficiencies of the ACL³². There is a paucity of knowledge on the effect of mechanical
13 loading in terms of exercise between the ACL and MCL and whether it may be beneficial to
14 ECM protein structure of these ligaments. In this study, we hypothesised that short-term high
15 intensity exercise would result in alterations to the ACL and MCL protein profile which would
16 be structure specific and impact on knee joint health. Therefore, this study aimed to identify
17 the effect of short-term regular bouts of high intensity exercise using treadmill training on the
18 proteome of the rat ACL and MCL. The proteome was assessed using our previously
19 established proteomic workflow with label-free quantification³³. Knee joint health was
20 evaluated using histology to identify pathological changes to structures within the rat knee
21 joint, including the cruciate and collateral ligaments and articular cartilage.

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1 METHODS

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3 *Training exercise regime and tissue collection*

4 The study was conducted under United Kingdom (UK) Home Office project license number
5 PPL 70/7210. Male Sprague Dawley rats (n=12) were assigned to an exercise (n=6) or control
6 (n=6) group. The rats were eight weeks old at the beginning of the study. All rats were housed
7 in the animal facility for one week prior to the start of the study to acclimatise to their
8 surroundings. The rats were group housed and allowed free cage activity and provided with a
9 standard pelleted rat chow and water ad libitum. The rats in the exercise group were introduced
10 to the treadmill (Linton Instrumentation, UK) over a two-week period before commencing a
11 four-week treadmill-training programme. The training programme consisted of running five
12 days/week on the treadmill at 0° incline with increasing speed up to 17m/s for two 30-minute
13 sessions 30 minutes apart (Supplementary Figure 1). At the end of the four weeks rats were
14 euthanased and the left and right knee joints harvested. The right knee joints were removed and
15 prepared for whole joint histological analysis. The left knee joint was dissected and the MCL
16 and ACL removed for protein extraction and proteomic analysis. The left MCL and ACL
17 samples were snap frozen in liquid nitrogen and stored at -80°C until required.

18

19 *Proteomics*

20 ACL and MCL samples were homogenized and proteins were extracted, as previously
21 described,³³ using 4M guanidine-HCl followed by RapiGestTM extraction and the protein
22 concentration of each soluble fraction was measured using the PierceTM 660 nm protein assay.
23 30µg of the soluble fraction was subjected to an in-solution digestion on 10 µl StratacleanTM
24 resin (Agilent Genomics, UK) followed by reduction and alkylation in 3 mM dithiothreitol
25 (DTT) and 9 mM iodoacetamide with trypsin at a ratio of 50:1 protein: trypsin³³. Liquid
26 chromatography tandem-mass spectrometry (LC-MS/MS) analysis was performed using an

1 Ultimate 3000 nano system (Dionex/Thermo Fischer) coupled online to a Q-Exactive
2 Quadrupole-Orbitrap mass spectrometer. 2 μ L loading of digests (equivalent to 60 ng peptides)
3 were loaded on to the column on a one-hour gradient with an inter-sample 30 minutes blank as
4 described previously^{34,35}.

5 Proteins were identified using PEAKS studio 8.5 (Bioinformatics Solutions, Waterloo, ON,
6 Canada) using the Uniprot Rat database (UP000002494) as described previously^{33,36}. In brief,
7 instrument configuration was set up as Orbitrap (Orbi-Orbi) and high collisional dissociation
8 (HCD) fragmentation. Parameters used were; 10.0 ppm parent mass error tolerance and 0.01
9 Da fragment mass error tolerance; trypsin monoisotypic enzyme; one missed cleavage; one
10 nonspecific cleavage; fixed modification, carbamidomethylation; variable modification,
11 methionine oxidation and hydroxylation; and 3 variable PTMs per peptide. Searches were
12 adjusted to confidence score > 50%; protein-10lgP \geq 20, 1% false discovery rate (FDR) and
13 unique peptides \geq 2. Label free (LF) quantitative analysis was performed firstly by comparing
14 both the ACL and MCL control and exercise groups together. After that, pair-wise comparisons
15 were performed between the samples in the control and exercise groups on Progenesis^{QI}
16 software (Waters, Elstree Hertfordshire, UK)^{34,37}. In brief, the spectra for each feature was
17 searched against the UniProt Rat database on Mascot (v2.6 Matrix Science, London, UK) using
18 the same parameters. Identified peptides hits were re-imported and assigned to proteins and
19 filtered at a threshold score corresponding to a 1% FDR. Identification of protein with two or
20 more peptides were used for quantification, and greater than two-fold abundance with a FDR
21 adjusted p-values <0.05 were considered to be significant. The proteomics data set for this
22 study has been deposited in the ProteomeXchange Consortium via the PRIDE³⁸ partner
23 repository (identifier PXD016516).

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1 *Gene Ontology, Pathway Enrichment Analysis and Protein Network Analysis*

2 Further analysis of the proteomic data was only performed on ACL control and exercise groups
3 as no significant changes were found between MCL control and exercise group.

4 Significant abundant proteins quantified using proteomic analysis between ACL control and
5 exercise groups were used to assess the gene ontology (GO), pathway enrichment analysis and
6 protein network analysis. Interactions between proteins were visualised using bioinformatics
7 software to gain a broader understanding of functional changes derived from the ACL exercise
8 group. ToppGene software was used for functional enrichment analysis of the up-regulated
9 proteins in the ACL exercise group with Benjamini-Hochberg false discovery rate adjusted p -
10 value cut-off < 0.05 ³⁹. Biological process GO terms and associated FDR values generated
11 through ToppGene were then summarized and formed into a network of processes using
12 REViGO⁴⁰, which was then exported to Cytoscape software⁴¹ to generate interactive graphs.
13 In addition, Strings Bioinformatics tool⁴² was used for GO and to produce a further network
14 to visualise connections between up-regulated and down-regulated proteins between ACL
15 control versus exercise group, as described previously³⁶.

16 Using the same significant data sets, the canonical pathways, further networks and biologic
17 process pathways were produced using Ingenuity Pathway Analysis software (IPA,
18 Ingenuity Systems, Redwood City, CA, USA). The accession and p -values of those
19 significant proteins were used to form a core analysis as described previously³⁶.

20 *Western Blotting Validation of Beta-actin Abundance:*

21 Western blotting was performed to validate the up-regulation of beta-actin abundance between
22 ACL control and exercise groups using previously established methods⁴³. In brief, 10 μ g of
23 ACL control and exercise samples were electrophoresed and separated on a pre-cast 12 well
24 gel (Bio-Rad Criterion 10% TGX). Separated proteins were then transferred to a nitrocellulose
25 membrane and blocked with 10 ml LICOR Odyssey[®] blocking buffer (LI-COR, Cambridge,
26 UK) for 1 hour at room temperature. Subsequently primary antibody (β -actin, Abcam, ab8227,

1 Cambridge, UK) was added to the membrane at a 1:1000 dilution directly into the buffer and
2 allowed to incubate overnight at 4°C. The membrane was washed and incubated in a secondary
3 goat anti-rabbit (LI-COR, IRDye® 680RD Goat anti-Rabbit IgG, Cambridge, UK) at 1:20,000
4 dilution was incubated for one hour at room temperature. The membrane was imaged by
5 Odyssey® LI-COR CLx imaging system at a wavelength of 700 nm. As a normalising control,
6 GAPDH was also probed following the same steps as above using GAPDH primary antibody
7 (Sigma, Poole, UK) at a 1:1000 dilution with goat – anti rabbit secondary at 1:20,000 (LI-COR,
8 IRDye® 800CW Goat anti-Rabbit IgG, Cambridge, UK). ImageJ software
9 (<http://rsbweb.nih.gov/ij/>) was used to quantify bands using densitometry. Results were
10 normalized to GAPDH loading control as reported previously ⁴⁴.

11 *Histopathological examination of the knee joints*

12 Surrounding soft tissues were removed from the right rat knee joints leaving only the joint
13 capsule and its contents. The dissected joints were stored in 4% paraformaldehyde for 24 hours.
14 Samples were then decalcified for eight weeks in a solution of 25 g EDTA in 175 mls distilled
15 water (pH= 4-4.5) ⁴⁵ and embedded coronally in paraffin wax and cut at a thickness of 6 µm
16 using a microtome (HM355S, Thermofischer). Sections from the paraffin blocks that were cut
17 prior to the femoral condyles becoming visible were discarded and thereafter three cross-
18 sectional cuttings were mounted per slide. Cutting of the sample paraffin blocks continued
19 throughout the joint until the femur began to disappear visually from samples ⁴⁶. Collected
20 sections were stained with haematoxylin & eosin (H&E) ⁴⁷ and toluidine blue & fast green ⁴⁸
21 and histologically scored for the knee joint and knee collateral and cruciate ligament. Sections
22 were scored by two observers blind sighted to the samples using a validated OARSI grading
23 system for rat knee joints ⁴⁹. In brief, grade 0= normal, grade 1= minimal degeneration of
24 articular cartilage 5-10% affected, grade 2= mild degeneration, 11- 25% affect, grade 3=
25 moderate degeneration, 26-50%, grade 4= marked degeneration, 51- 75% affected, grade 5=

1 severe degeneration, 76-100% affected. Medial and lateral aspects of the tibia and femur were
2 scored individually across the whole joint compartment producing a maximum (most severe)
3 grade and overall “average” maximum grade in each group of rats. In addition, a mean score
4 was produced for each joint and these similarly were used to produce an overall ‘average’ mean
5 grade in each group of rats ⁵⁰. The scoring for ligaments was adapted from Kharaz, Canty-
6 Laird, Tew, Comerford ⁵¹ and scoring was performed based on strength of ECM staining, cell
7 hypertrophy, cell clustering, loss of alignment and ossification and were graded from 0-4 based
8 on the extent of changes ((0= 0% increase; normal, 1= 5-25% increase; mild abnormality, 2=
9 26-50% increase; moderate abnormality, 3= 51-75% increase; marked abnormality, 4= 76-
10 100% increase; severe abnormality). Inter and intra-observer variability was calculated using
11 Cohen’s Kappa statistics using an online software tool:
12 (http://www.statstodo.com/CohenFleissKappa_Pgm.php)

13

14 *Statistical analysis*

15 Statistical analysis for proteomic label free datasets was performed by Progenesis^{Qi} on all
16 detected features using transformed normalized abundances for one-way ANOVA as
17 previously described ^{33,36}. Identification of proteins with two or more peptides, greater than
18 two-fold abundance and with a *q* value (*p*-value adjusted to FDR) <0.05 were considered
19 significant. Quantitative analysis was initially performed by comparing the four groups of
20 samples together. After that, pair-wise comparison were performed between ACL control and
21 ACL exercise, MCL control and MCL exercise, ACL control and MCL control and ACL
22 exercise and MCL exercise as has been used with similar data previously ³⁶. Normal
23 distribution for the histological and Western blot data sets was assessed with GraphPad Prism
24 (Version 7, GraphPad Software, USA) using a Kolmogorov-Smirnov test. A one-way ANOVA
25 with Bonferroni *post-hoc* test was performed on histological scoring results between the

1 cruciate and collateral ligaments and t-test was performed on mean and maximum OARSI joint
2 scores and Western blot analysis.

3

4 RESULTS

5

6 Rat body weight at the start of the study did not differ significantly between groups (control –
7 432.8 ± 18.9 g; exercise - 413.7 ± 11.7 g). Both groups of rats gained weight during the study
8 (control – 85.0 ± 32.6 g; exercise - 72.7 ± 17.1 g) but this was not significantly different
9 between groups and the rats weights at the end if the study (control – 517.8 ± 34.8 g; exercise
10 – 486.3 ± 22.4 g) were not significantly different between groups. Inclination to run on the
11 treadmill varied between rats. The better runners reached a top speed of 17m/s while the poorest
12 runner reached a top speed of 10m/s. This difference resulted in a range of total distance
13 covered by individual rats in the exercise group from 10058.9 to 17506.3 metres.

14 *Histological findings*

15

16 In general, minor changes were observed histologically in knee joint health between the
17 control and exercise groups. Histological observation of staining of the cruciate ligaments from
18 control and exercise groups showed a normal alignment with variation in fibre orientation in
19 fibre orientation and a similar intensity of toluidine blue staining (Figure 1A, 1Aa, D and Da).
20 For the collateral ligaments, the level of ECM staining between the control and the exercise
21 group were similar with no obvious disorganisation of fibres alignment (Figure 1B, 1Ba, E and
22 Ea). Histological staining of the articular cartilage knee joints showed a smooth undisrupted
23 articular cartilage surface with none to minor degradation and lesions observed in both control
24 and exercise group (Figure 1C, Ca, F and Fa). However, in one joint from the exercise group
25 that were good runners, lesions were observed on the surface of the articular cartilage as
26 highlighted in Figure 1F and 1Fa. Histological analysis resulted in average scoring in the ACLs

1 of 3.53 ± 0.92 and 3.66 ± 1.55 for the control and exercise groups respectively. For the MCLs,
2 scores reached 1.69 ± 1.02 in the control group and 2.54 ± 1.03 in the exercise group (Figure
3 4G). No significant difference was found between control and exercise group in both cruciate
4 and collateral ligaments ($p=0.08$, $p=0.19$).

5 An average OARSI of 0.37 ± 0.3 and 0.92 ± 0.54 was achieved for the control and exercise
6 group, respectively. Overall, the mean OARSI scores for the control and exercise group were
7 calculated at 0.025 ± 0.024 and 0.063 ± 0.045 respectively. This difference between the two
8 groups was not statistically significant ($p=0.072$) (Figure 4H and 4I).

9

10 *Proteomics*

11 1) Protein concentration and identification

12

13 The average protein content ($\mu\text{g}/\text{mg}$ wet wt) of 24.6, 23.1, 30.1 and 23.1 measured for ACL
14 and MCL control and ACL and MCL exercise groups, respectively was not significantly
15 different (Supplementary Figure 2). A total number of peptides of 4065, 5135, 5517, 4546
16 assigned to 381, 473, 589 and 451 proteins were identified in ACL and MCL control and ACL
17 and MCL exercise, respectively (Figure 2A, Supplementary Table 1). A higher number of
18 unique and total proteins were identified in ACL exercise group in comparison to ACL control
19 group, however in the MCL a similar number of total and unique proteins were identified in
20 both control and exercise group (Figure 2A).

21 2) Quantitative label-free (LF) analysis

22 Quantitative LF analysis demonstrated a set of 332 proteins within the four groups with a fold
23 change >2 and unique peptides >2 (Supplementary Table 2). Principle component analysis
24 (PCA) was used to identify the major variance between the groups. This analysis revealed that
25 the control ACL and MCL samples were distinctly grouped, whereas ACL and MCL exercise
26 samples were clustered closer together (Figure 2B).

1 Quantitative differences between ACL control and exercise group samples resulted in 124
2 proteins that were significantly different. Of these proteins, 122 were abundant in the ACL
3 exercise group and two proteins were abundant in ACL control group (Figure 2C, and
4 Supplementary Table 3). The majority of significantly abundant proteins in ACL exercise
5 groups were cytoskeletal, ribosomal and enzymes (Table 1). Several abundant matrisomal
6 proteins such as collagen alpha-3 (IX) chain, collagen type XVIII alpha 1 chain, collagen alpha-
7 1(XIV) chain, asporin, lumican, thrombospondin-3, periostin and TGF β were found to be up-
8 regulated in ACL exercise group. A summary of the classification of these proteins is provided
9 in Table 1.

10 No statistically significant differences in proteins abundance were identified between MCL
11 control group when compared to the MCL exercise group (Figure 2D and Supplementary Table
12 3) as all proteins had a FDR adjusted p -values greater than 0.05.

13 When the ACL control was compared to MCL control group samples, 73 proteins were
14 abundant in ACL control and 217 in MCL control (Figure 2E and Supplementary Table 4). The
15 ACL control group samples were more abundant in fibrocartilaginous proteins such as cartilage
16 intermediate layer protein and hyaluronan and proteoglycan link protein 1, whilst the MCL
17 control group samples had more asporin and keratocan (Figure 1E). Between the ACL and
18 MCL exercise groups only HAPLN was found to be significantly upregulated in the ACL
19 exercise group (Figure 2F and Supplementary Table 5).

20

21 *Gene Ontology and Ingenuity Pathway Analysis*

22 Following Cytoscape software analysis of the significantly upregulated proteins in the ACL
23 exercise group, they were found to be proteins mostly associated with respiration and
24 metabolism (Supplementary Figure 3). In addition, gene response to stimuli, protein
25 localisation and cell migration were also significantly upregulated. String analysis

1 demonstrated some similarities with the most predominant linkage involving the ribosomal
2 proteins in ACL exercise group (Figure 3). Further linkage was also seen between the heat
3 shock proteins, actins and collagens. The most common biological processes highlighted by
4 the String analysis software included metabolic ($p= 7.08e-16$) and cellular ($p=2.05e-11$)
5 pathways (Figure 3).

6 The IPA of the differentially abundant proteins in ACL exercise group compared to ACL
7 control group generated several networks that were enriched (Figure 4A and 4B). According
8 to the top scoring networks, the differentially expressed proteins were associated with
9 metabolic and disease development, cell signalling and post-translational modifications
10 (Figure 4A). Proteins that were found to be enriched included metabolism of ATP and
11 nucleoside triphosphate, aerobic respiration, mitochondrial disorder, respiratory chain and
12 trifunction protein deficiency and organisation of cytoskeleton (Figure 4B). Significant IPA
13 canonical pathways that were upregulated eukaryotic initiation factor, integrin and actin
14 cytoskeletal and paxicillin signalling (Figure 4C).

15 Western blot analysis of beta-actin abundance was in agreement with the mass spectrometry
16 data and was significantly greater ($p = 0.017$, (Figure 4D) in the ACL exercise group than
17 ACL control group.

18

19 *Data availability*

20 The data that support the findings of this study are openly available in bioRxiv at
21 <https://doi.org/10.1101/2020.01.09.900142>⁵².

22

23 DISCUSSION

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25 This is the first study to measure the effect of an imposed and controlled exercise regime on
26 the proteome of the rat intra- articular ACL and extra-articular MCL. Our findings demonstrate

1 that short-term (4 weeks) of high intensity treadmill training influences intra-articular ACL
2 protein expression, but not that of the extra-articular MCL compared to control groups. These
3 changes in protein expression in the ACL as a response to exercise may contribute to a
4 protective or degenerative role in these ligaments. The health of the knee joint, as assessed by
5 histopathological examination, demonstrated no significant differences in the ACL, MCL and
6 cartilage in the exercise groups compared to rats undertaking only cage activity suggesting that
7 the added exercise regime was not detrimental to the soft tissues of the joint.

8 In this study 8-weeks old rats were chosen as they are sexually mature. We aimed to measure the
9 effects of exercise on mature tissue rather than the effect that exercise may have on the development
10 process in ligaments.

11 Histopathological changes observed in the current study demonstrated no significant changes
12 in neither in cruciate, collateral and cartilage of the knee joint, indicating that exercise regime
13 in the study did not have substantial impact on the tissue structure and the health of several
14 tissues with the rat knee joint. Additional further studies are required to measure the
15 biomechanical changes of the ligaments to assess the stress and functionality of the ligament
16 with exercise.

17 For our proteomic analysis, we used label-free quantification to identify differentially abundant
18 proteins between the control and exercise group of both ligaments and between ACL and MCL
19 tissues. Interestingly the ACL and MCL from non-exercised animals showed a significantly
20 different protein profile, which included ECM proteins. The ACL had more abundant levels of
21 proteins associated with fibrocartilagenous tissue suggesting that this ligament may be
22 subjected to other force types in addition to tensile force. With respect to exercise, this analysis
23 produced 124 significant proteins that were more abundant in ACL exercise than ACL control
24 group. However, no significant differential proteins were identified between MCL control and
25 MCL exercise group. The differences found in protein expression in this study between ACL

1 and MCL exercise groups may be due to altered mechanical loading between the intra- and
2 extra-articular ligaments. In humans, during athletic tasks such as jump landing, the ACL has
3 been found to exhibit greater loading and strain and greater contribution to knee restraint, in
4 comparison to the MCL ⁵³. In the current study, the rat MCL may be similar to the human
5 MCL and may be subject to less strain compared to the ACL. Our exercise regime was given
6 in a straight line, with no twisting or turning, which as result may not have had significant load
7 on the MCL and consequently resulted in different protein expression in ACL during exercise.
8 Furthermore the intra-articular rat ACL is exposed to cytokines and other mediators released
9 from other joint tissue into synovial fluid, which therefore may also have led to an altered
10 protein profile with in ACL with exercise ⁵⁴.

11 Further studies are required to understand the rat knee joint loading during *in vivo* tasks and
12 may provide insight that enhances the efficacy of injury prevention protocols.

13 Our proteomic analysis between ACL control and exercise group samples demonstrated an
14 increase in mainly cellular proteins such as tubulins, ribosomal and heat shock proteins. We
15 also found actins to be abundant in ACL exercise group which were then validated through
16 western blot analysis. Actin participates in many cellular processes such as muscle contraction,
17 cell motility, division, cytokinesis and signalling, where many of these processes are mediated
18 by extensive and intimate interactions of actin with cellular membranes ^{55,56}. In tendon, the
19 disruption of actin cytoskeleton has been found to decrease tissue elastic modulus during
20 development ⁵⁷. Therefore, the increased actin protein found in our study in ACLs following
21 exercise could contribute to the improved tissue mechanical properties. Whilst the majority of
22 abundant proteins were cellular associated, several matrisomal collagens, proteoglycan and
23 glycoprotein proteins such as collagen type IX, XIV and XVIII, lumican, asporin, periostin,
24 thombrospodin-3 and TGF β were also upregulated in ACL exercise group. The exact role and
25 mechanism of these matrisomal proteins is not known after exercise, but the presence of
26 collagen type IX may indicate a chondrocytic phenotype of ACL and corresponds with another

1 study in mouse Achilles tendon where intense exercise resulted in cartilaginous changes⁵⁸. The
2 upregulation of TGFβ found in this study could indicate local release in the ACL tissue and
3 agrees with previous tendon exercise studies where elevations of TGFβ have been
4 demonstrated in response to exercise^{25,59}. In tendon, mechanical loading following exercise
5 has been shown to release active TGFβ, which has been demonstrated to regulate ECM protein
6 expression such as collagen type I²⁵, proteoglycans⁶⁰, and also microRNA molecules with
7 known roles in cell proliferation and extracellular matrix synthesis⁶¹. The upregulation of
8 TGFβ in the ACL exercise group may be associated with regulation of ECM proteins and is
9 likely to stimulate many anabolic pathways that control exercise-mediate ACL adaption.

74 Gene ontology revealed that metabolic and cellular processes were overrepresented in ACL
75 exercise group in comparison to the control group. This was also evident using IPA, where the
76 analysis of differential networks identified significant pathways in relation to metabolic
77 development and cell signalling. Ingenuity pathway analysis (IPA) also showed upregulation
78 of several canonical pathways including eukaryotic initiation factor 2 (EIF2) and integrin
79 signalling. Eukaryotic initiation factor 2 signalling enhances the initiation of translational and
80 transcriptional activators⁶²) and integrins play a crucial role in linking the ECM to the
81 cytoskeleton playing a role in mechanotransduction of muscle and tendon^{59,63}. In the current
82 study, the exact role of the signalling factors in the ACL exercise group cannot yet be elucidated
83 and additional studies are required to understand whether induced activation of these pathway
84 aid in the organisation of ACL ECM.

85 In conclusion, we have shown for the first time the effect of short- term bouts of high impact
86 exercise on intra- and extra- articular knee joint ligaments. This study demonstrated that short-
87 term strenuous treadmill exercise impacts ACL protein expression, whilst MCL proteome is
88 not altered. These differences in response may be due to difference in mechanical loading and
89 previously identified structural and ECM compositional difference between the two tissue types

1 ⁵¹. Although increases in matrisomal associated proteins were observed between ACL control
2 and exercise group, the majority of differential abundant proteins were cellular, indicative of
3 an intracellular response and whether these changes are protective or degenerative in ACL is
4 yet to be elucidated.

5

6 PERSPECTIVE

7 Exercise is extremely important in the field of sports medicine; both in terms of inducing
8 injuries as well as being essential in their rehabilitation. To date, information on any alteration
9 in knee ligament tissue structure, in terms of a cellular and extracellular matrix proteins, to
10 controlled exercise such as treadmill running both in humans and animals is limited. Our study
11 examined the effect of controlled exercise on key knee ligaments such as the intraarticular
12 anterior cruciate ligament (ACL) and extraarticular medial collateral ligament (MCL). We
13 found that that short-term high intensity exercise had a significant impact on the expression of
14 cellular and extracellular matrix proteins in exercised ligaments compared to non-exercised
15 groups. Our work has major implications for future translation in sports medicine as it
16 demonstrates that exercise, even in the short term, can quantifiably cause cellular responses in
17 key ligaments of the knee joint organ which may lead to altered protein production. Identifying
18 and quantifying any alterations in knee ligament proteins in future work may be very useful in
19 terms of validating methods of non-invasive imaging of knee ligaments after exercise and
20 injury with the ultimate aim of modulating rehabilitative exercise aligned to changes in
21 ligament structure.

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4

5 DECLARATION

6 The authors have declared no conflict of interest.

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		Higher in ACL exercise group	Higher in ACL control group
ECM Proteins	Collagens	3	0
	Glycoproteins	6 (4.8%)	0
	Proteoglycans	2 (1.6%)	0
	ECM affiliated proteins	5 (4%)	0
	Total number of proteins	16 (12.9%)	0
	Non-ECM proteins	Cytoskeletal	17 (13.7%)
Histones		2 (1.6%)	
Cell membrane		7 (5.6%)	
Ribosomal		13 (10.5%)	
Enzymes		41 (33.1%)	
Transcription/translation		3 (2.4%)	
Other/unknown		23 (18.5%)	2
	Total number of proteins	106 (85.5%)	2 (1.6%)

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3 Table 1. Classification of differentially abundant proteins (identified using ≥ 2 peptides, >2 -
4 fold change, FDR adjusted $p < 0.05$) in the ACL exercise group compared ACL control group.

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- 1 Figure 1. Histological comparison between ACL (A, Aa), MCL (B, Ba), cartilage (C, Ca)
- 2 control groups and ACL (D, Da), MCL (E, Ea) and cartilage exercise (F, Fa) groups. No
- 3 significant difference was found in the ligament score (G) and OARSI mean (H) and maximum
- 4 (I) score between the control and exercise groups.

Figure 2. Qualitative and quantitative differences in proteins identified between ACL, MCL control and exercise groups. (A) Venn diagram demonstrating the total number of proteins identified following MS in both ACL and MCL control and exercise group as well as common proteins between the groups. (B) Principle component analysis between ACL and MCL control and exercise groups produced by Progenesis^{Q1} after ANOVA analysis with identified proteins at p-value < 0.05. (C-F) volcano plots (-10lgP of FDR adjusted p-value vs log2 fold change). (C) ACL control vs ACL exercise, (D) MCL control vs exercise, (E) ACL control vs MCL control and (F) MCL exercise vs ACL exercise. Volcano plots of quantified proteins in C, D, E indicated up-regulation and down-regulation of proteins with up-fold and down-fold change with significance. This was not the case in volcano plot D as quantified proteins had a p-value (adjusted to FDR) > 0.05.

Figure 3. String analysis of upregulated proteins in ACL exercise group versus ACL control group. The above figure shows the greatest linkage of proteins predominantly involves those associate with ribosomes, also there is further linkage of actins, heat shock proteins and collagens. The main principal gene ontology processes were identified as metabolic ($p= 7.08e-16$) and cellular ($p=2.05e-11$).

Figure 4. IPA analysis between differential abundant protein between ACL control and exercise group significant networks were related to metabolic, disease development (**A**), and cell signalling, posttranslational modification and protein synthesis (**B**). Red nodes, greater protein abundance in the ACL exercise group; white nodes, proteins not differentially abundant between the ACL exercise and control group. Intensity of colour is related to higher fold-change. Key to the main features in the networks is shown. Significant functions related to network 1 included metabolism of ATP, metabolism of nucleoside triphosphate, beta-oxidation and catabolism of fatty acid, mitochondrial disorder respiratory chain and trifunctional protein deficiencies (**A**, $p < 0.0001$). Diseases and functions related to network 2 included organisation of the cytoskeleton (**B**, $p < 0.0001$). (C) A number of canonical pathways shown to be upregulated in the ACL exercise compared to the ACL control group. (D) Western blot analysis between of Beta-actin in ACL control and ACL exercise. Statistical differences were assessed between the ACL control and exercise group using a T-tests.