THE MOLECULAR AND CELLULAR DIFFERENCES BETWEEN TENDONS AND LIGAMENTS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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March 2015

ABSTRACT

Tendons and ligaments play key roles in the musculoskeletal system in both man and animals. Both tissues can often be injured as result of contact based accidents or ageing and disease, causing discomfort, pain and increased susceptibility to degenerative joint disease. To date, tendon and ligament biology is relatively under-studied in healthy, non-diseased tissues. This information is essential to understand the pathology of these tissues and vital for future development of tendon and ligament tissue-engineered structures.

This thesis aims to investigate the molecular and cellular differences between tendons and ligaments around the canine stifle joint. The biochemical composition, structural, and morphological characteristics were identified between the different regions of the intra-articular cranial cruciate ligament (CCL) and extra-articular medial collateral ligament (MCL), and the positional long digital extensor tendon (LDET) and energy storing superficial digital flexor tendons (SDFT). Differences in proteome composition were also assessed between CCL and LDET. Cells isolated from canine CCL and LDET were cultured in a 3D *in vitro* fibrin culture model and measured for differences in structural, biochemical and proteome composition.

Statistical significant differences in extracellular matrix (ECM) composition in terms of glycosaminoglycan (GAG) and elastin content were primarily detected in CCL in comparison to the other three tissues. The CCL was also found to have morphological differences including less compact collagen architecture, differences in cell nuclei phenotype, and increased (GAG) and elastin content. Proteomic comparison between CCL and LDET resulted in significantly abundant fibrocartilage proteins such as collagen type II, aggrecan, versican and chondroadherin in CCL, while the LDET was more abundant in asporin and thrombospondin-4. 3D tendon and ligament constructs were able to recapitulate tendon and ligamentous tissue characteristics particularly with regards to ECM proteins present, however both construct were less abundant in ECM protein and contained a greater proportion of cellular proteins, corresponding with low collagen and high level of DNA content measured in both constructs. 3D tendon and ligament constructs derived from tendon and ligament cells had similar ECM, proteomic and structural composition, indicating that cell source may not be an important factor for tendon or ligament tissue engineering.

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LIST OF ABBREVIATIONS

AB-PAS	Alcian blue- periodic acid Schiff
ACL	Anterior cruciate ligament
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CCL	Cranial cruciate ligament
Da	Dalton
DAB	3,3'-Diaminobenzidine
dH20	Distilled water
DMBA	Dimethylaminobenzaldehyde
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
DMMB	Dimethyl-methylene blue
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FDR	False discovery rate
Glycosaminoglycan	GAG
GnHCl	Guanidine hydrochloride
GO	Gene ontology
H & E	Hematoxylin and Eosin
HCL	Hydrochloride
HPLC	High-performance liquid chromatography
HRP	Horse-radish peroxidase
IAA	Iodoacetamide

kN	Kilonewtons
LDET	Long digital extensor tendon
M	Molar
mM	Millimolar
MCL	Medical collateral ligament
mg	Miligram,
ml	Mililitre
MS	Mass spectrometry
Nm	Nanomolar
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLRPs	Small leucine rich proteoglycans
sGAG	Sulphated glycosaminoglycans
SEM	Standard error of the mean
SDFT	Superficial digital flexor tendon
TBS	Tris-buffered saline
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
OD	Optical density
PBS	Phosphate buffer saline
PCA	Principle component analysis
PG	Proteoglycan
µl	Microlitre

ACKNOWLEDGEMENT

I would like to take this opportunity to acknowledge and express my gratitude to everyone in the Comparative Musculoskeletal Science Research Group, Department of Musculoskeletal Biology who has supported me throughout my PhD.

First and foremost I would like to express my sincerest gratitude to my primary supervisor, Dr Eithne Comerford, for her profound expertise, continued guidance, invaluable support and for giving me opportunity to advance my research career. I would also like to thank my secondary supervisors, Dr Elizabeth Canty-Laird and Simon Tew for their help, profound knowledge and assistance.

I would like to acknowledge Medical Research Council and University of Liverpool for the generous funding. I would also like to acknowledge the University of Liverpool Technology Directorate for co-funding the proteomic study

I am also very grateful to Deborah Simpson at Proteomic Facility Centre and Marion Pope for processing my samples for proteomic analysis and transmission electron microscopy, respectively. I also want to express my gratitude to Mandy Peffers for helping and assisting me with the proteomic data analysis.

I must also thank my laboratory colleagues Ben, Sumaya, Alan, Rhianon, Kate, Louise, Eleri and Othman for their positive energy and encouragement throughout my PhD.

My final thanks goes to my wonderful family; my parents for motivating and always believing in me; my loving husband, Ali, for his unwavering support, patience, encouragement, and devotion to extra childcare duties, none of this would have been possible without his help; my beautiful daughter, Elika, whose always keeps me smiling even during hard times of PhD pursuit.

CHAPTER 1

GENERAL INTRODUCTION

1.1 CONNECTIVE TISSUE

There are four basic types of tissues in the human or animal body, namely epithelial, muscle, nervous and connective tissue. Of these, connective tissues are one of the most abundant, being extensively distributed and encompassing a variety tissues with differing functional properties (Van de Graaff et al. 2010, Tortora and Derrickson 2013).

In general, the key functions of connective tissues are to bind, support and strengthen other body tissues, protect and insulate internal organs, and serve as major transport with the body (Van de Graaff et al. 2010, Tortora and Derrickson 2013). Connective tissues consist of cells and an extracellular matrix (ECM) of fibres, fluid and ground substance (Ross and Pawlina 2006, Tortora and Derrickson 2013). The role and function of the various connective tissues is reflected in the types of cells, fibres and the character of ground substance in the ECM (Ross and Pawlina 2006). The ground substance is a unstructured material that fills the space between cells and fibres of connective tissue and is primarly composed of water and proteoglycans and cell adhesion proteins (Marieb and Hoehn 2007).

Connective tissue classification is not always clear due to some similarity between characteristics of the many tissues and is therefore somewhat subjective and open to dispute. Based on differences of composition and organisation of the cellular and ECM component and functional properties, connective tissues could be classified into embryonic connective tissue, connective tissue proper and specialised connective tissue subgroups (Aughey and Frye 2001, Ross and Pawlina 2006) (Figure 1.1).

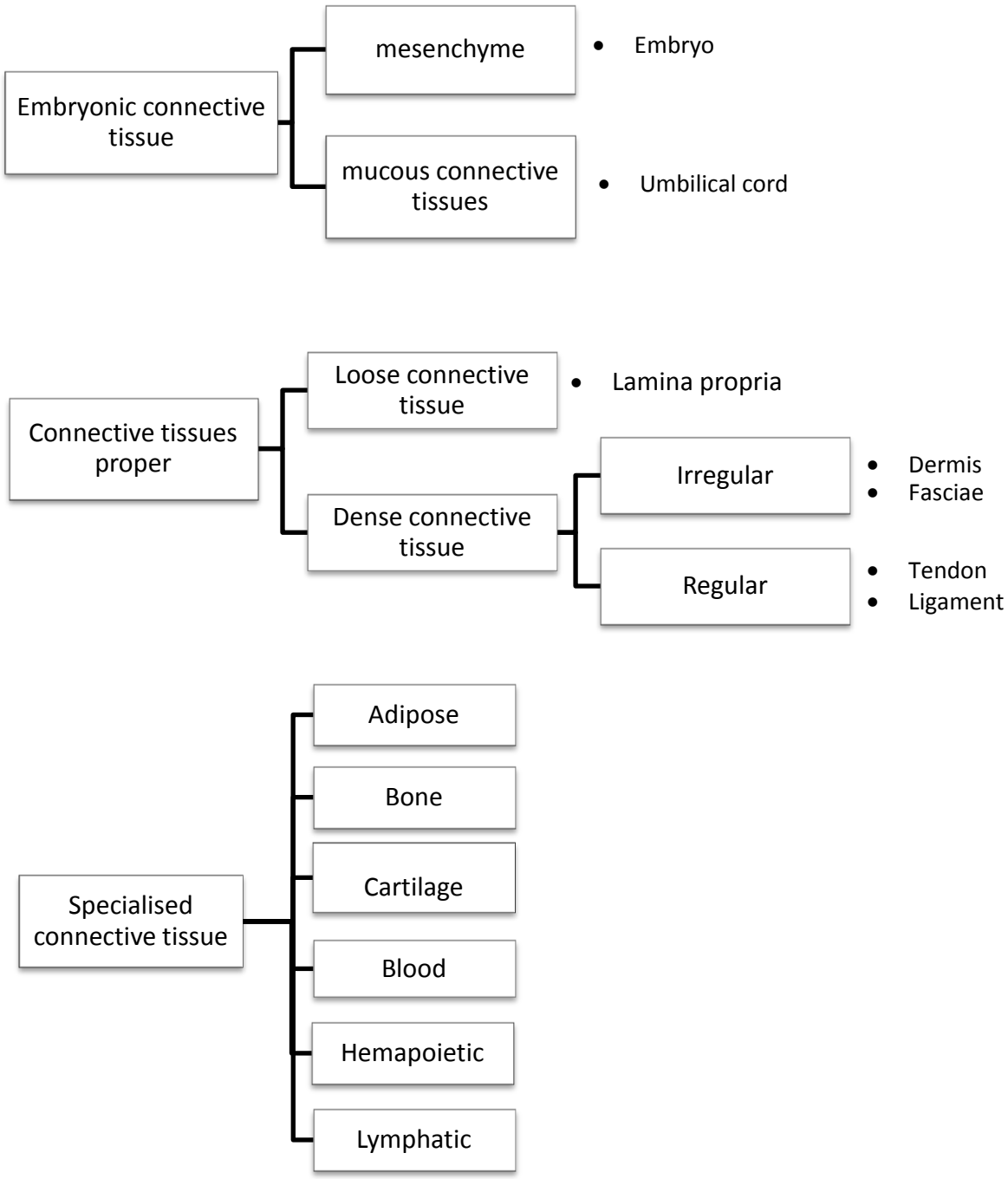


Figure 1.1. Classification of connective tissues. Connective tissues are categorised into subgroups based on differences in cellular and ECM composition, organisation and functional properties adapted from Ross and Pawlina (2006) and Aughey and Frye (2001)

1.1.1 Embryonic connective tissue

Almost all connective tissues develop from the mesoderm, which is the middle embryonic germ layer that forms through proliferation and migration of the mesodermal and specific neural crest cells into primitive connective tissue referred to as mesenchyme (Ross and Pawlina 2006, Marieb and Hoehn 2007). The mesenchyme is established in the early embryo and leads to the formation of several connective tissues such as cartilage, tendon or bone (Lorda-Diez et al. 2014). It also develops into other tissues e.g. muscle, vascular and urogenital systems (Ross and Pawlina 2006). Mesenchymal tissue consist of unspecialised cells, that have a homogenous and spindle shaped appearance, and ECM with a sparse arrangement of fine reticular fibres and ground substance (Ross and Pawlina 2006, Tortora and Derrickson 2013).

Another subtype of embryonic connective tissue is mucous connective tissue, which is located in the umbilical cord. The mucous connective tissue cells are widely scattered and appear much like fibroblasts and are embedded in a jelly-like extracellular matrix, known as Wharton's jelly (Aughey and Frye 2001, Ross and Pawlina 2006, Tortora and Derrickson 2013).

1.1.2 Connective tissue proper

Within this category connective tissues is further categorised into loose connective tissue and dense connective tissue.

1.1.2.1 Loose connective tissue

Loose or areolar connective tissue is a cellular connective tissue with thin, sparse and loosely arranged collagen fibres, however it has an amorphous ground substance (Ross and Pawlina 2006). It contains many scattered cells of various types and it is widespread throughout the body surrounding vessels and nerves (Aughey and Frye 2001). Loose connective tissue has a space-filling function between organs and structures; for instance

between skin and musculature and between muscle fibres. It is found underneath epithelial tissue and is also a component of lamina propria (Krstić 1985).

1.1.2.2 Dense connective tissue

Dense connective tissues have substantially less cells, but contain more fibres that are thicker and more tightly packed together compared to loose connective tissue. This subtype can further be divided into dense irregular and regular subcategories (Aughey and Frye 2001, Tortora and Derrickson 2013).

Dense Irregular Connective Tissue: This type of tissue contains sparse cells and little ground substance, but a high proportion of collagen fibres that are usually irregularly arranged. The fibres are arranged in bundles and run in more than one plane. This irregular arrangement allows tissues of this type to resist extensive stretching and extension (Ross and Pawlina 2006, Marieb and Hoehn 2007). Examples of dense irregular connective tissue include the fascia, dermis of skin and the fibrous covering that surround some organs such as the heart and kidney (Marieb and Hoehn 2007, Tortora and Derrickson 2013).

Dense Regular Connective Tissue: This type of tissue provides strong attachment between various structures, which are able to withstand tension along long axis of fibres (Tortora and Derrickson 2013). Unlike in dense irregular connective tissue, collagen fibres are arranged in parallel array and are tightly packed to provide maximum strength. Ligaments and tendons are examples of dense regular connective tissue, where ligament joins bone to bone and tendon joins muscle to bone (Aughey and Frye 2001, Ross and Pawlina 2006). The structure of tendon and ligaments are being examined in this thesis and will be discussed in further detail later on in this Chapter.

1.1.3 Specialised connective tissue

Specialised connective tissues include cartilage, bone, adipose tissue, blood, hemapoietic tissue and lymphatic tissue. Similar to the other connective tissues, specialised connective tissue consists of cells and ECM, however the cell characteristics and the components of the

ECM differ in quantity, type and organisation (Ross and Pawlina 2006). For instance one feature that distinguishes bone from other connective tissues is the addition of collagen fibres and mineralisation of the bone matrix, giving it substantial strength and rigidity (Weatherholt et al. 2012). Conversely blood is a connective tissue that consists of different types of cells and a liquid ECM, allowing it to travel and transport nutrients, wastes, enzymes, plasma proteins and hormones (Marieb and Hoehn 2007, Tortora and Derrickson 2013). Another example of specialised connective tissue is cartilage, which contains specialised cells, chondrocytes, that maintain the ECM. Unlike other connective tissues cartilage does not contain nerves or blood vessels in its ECM. Cartilage resists both tension and compression, as it is composed of large amounts of glycosaminoglycans, firmly bound collagen fibres and has a high water content (Marieb and Hoehn 2007).

1.2 TENDON

1.2.1 Tendon function and gross structure

Tendons are dense connective tissues and are dominated by regularly arranged collagen fibres. They serve primarily to transfer the pull of muscles to bone (Benjamin and Ralphs 1998). They also play a fundamental role in locomotion, transferring the forces generated by our muscles to the skeleton, and thus facilitating movement (Screen 2009). In addition, they stabilise the joint, and act as shock absorber to limit muscle damage (Clegg et al. 2007). Some tendons have an additional energy storing function which when stretched under load they then recoil. This lessens the energetic cost of locomotion, as a reduced muscular effort is required to return the limb to the starting position (Birch et al. 2013). Examples of energy storing tendons are the human Achilles tendon and equine superficial digital flexor tendon (SDFT) (Thorpe et al. 2012, Birch et al. 2013). However, tendons such as the human anterior tibialis tendon and the equine common digital extensor tendon (CDET) act purely to position the limb and are relatively inextensible to allow efficient transfer of force from muscle to bone and precise placement of the limb (Thorpe et al. 2012).

Macroscopically healthy tendons are white in colour and have a fibro-elastic texture (Kannus 2000). They can vary remarkably in shape and size and in the way they are attached

to the bone; some tendon can be rounded or cord-like, straplike bands appear like, or flattened ribbons (Benjamin and Ralphs 1997, Kannus 2000, Benjamin et al. 2008). The point of unification of tendon to the muscle is referred to as the myotendinous junction (MTJ) and the point of unification of tendon with bone as the osteotendinous junction (OTJ). The connection of the proximal tendon of a muscle to bone is called the muscle origin, and the distal tendon connection is known as an insertion (Kannus 2000).The MTJ is important for force transmission of contracted muscle to tendon (Benjamin et al. 2002, Kostrominova et al. 2009). At this region collagen fibres of tendon are inserted into deep recesses formed by myocyte processes, which allows the tension that has been generated by contractile proteins of muscle fibres to be transferred to the collagen fibrils (Sharma and Maffulli 2005). The OTJ or enthesis is the interface between tendon and bone and is classified in to four zones: fibrous tissue, fibrocartilage, mineralised fibrocartilage and bone (Benjamin et al. 1986, Benjamin et al. 1995, Doschak and Zernicke 2005). The presence of fibrocartilage in tendon is an adaptation to resist compression and/or shear forces (Benjamin and Ralphs 1998).

1.3 LIGAMENT

1.3.1 Ligament function and gross structure

Ligament is another dense regular connective, that joins bone to bone (Ross and Pawlina 2006). Similar to tendons, ligaments vary in size, form, orientation and location (Frank 2004). Grossly, ligament appears white, firm, homogenous and fibrous (Frank et al. 1985). Ligament can be categorised into at least two major subgroups; those found in the musculoskeletal system (usually crossing joints) and those connecting other soft tissues, for instance the suspensory ligament in the abdomen. They are named based on the point of bony attachment, shape, function, their relation to joints or surfaces and their relationship to each other (Frank et al. 1985).

Skeletal ligaments play essential roles for accurate joint function. They passively stabilise the joint and are responsible for guiding movement of the joint through a normal range of motion. They are responsible for preventing or blocking abnormal joint movement within

set limits. Another function of articular ligament role in joint is in proprioception, which provides feedback relating position space of the joint and contributes to the coordinated movement of the limbs (Frank 2004, Birch et al. 2013).

Ligament contains different regions similar to what has been previously described in tendon. For instance the anterior cruciate ligament has a proximal origin from the femur and a distal insertion on the tibia (Zantop et al. 2006). The direct insertion or enthesis of ligament to bone also consists of four gradual transition zones as mentioned earlier (Doschak and Zernicke 2005), while indirect insertion of the ligament passes along the surface of the bone rather than bony or gradient transition zone. The cruciate ligaments of the knee joint are examples of ligaments with direct entheses at both the femural and tibial insertions, whilst medial collateral ligament (MCL) has an indirect enthesis at the tibial insertion (Woo et al. 2006).

The formation of fibrocartilaginous matrix in both tendon and ligament is thought to occur at sites that are under compression. Cells in both tendon and ligament are capable of detecting changes in mechanical load and co-ordinate their response to alter the composition of ECM (Benjamin and Ralphs 1998)

1.4 STRUCTURE OF TENDON AND LIGAMENT

1.4.1 Hierarchical structure

The collagen- rich composition of ligaments and tendons are organised in a complex hierarchical structure. This was first reported in tendon by Kastelic et al. (1978), who summarised that tropocollagen molecules are synthesised by tenocytes then self-assembled and grouped together in a highly ordered fashion to form fibrils, fibres and fascicle (fibre bundles). Each fascicle is separated by a loose connective tissue of interfascicular matrix termed endotendon. Grouped fascicles form the entire tendon, which is enclosed by another connective tissue referred to as epitenon (Figure 2.1) (Kastelic et al. 1978). In the ligament the endotendon is referred to endoligament, and the epitenon is called epiligament (Chowdhury et al. 1991, Lo et al. 2002).

The orientation of collagen fibrils tends to be in the direction of applied force (Kastelic et al. 1978) relative to the axis of tendon or ligament. In tendon forces are applied in a uniaxial direction, resulting in parallel alignment of collagen fibrils. However, in ligament collagen fibrils are not as uniformly orientated as forces are applied in more than one direction (Amis 1998, Rumian et al. 2007).

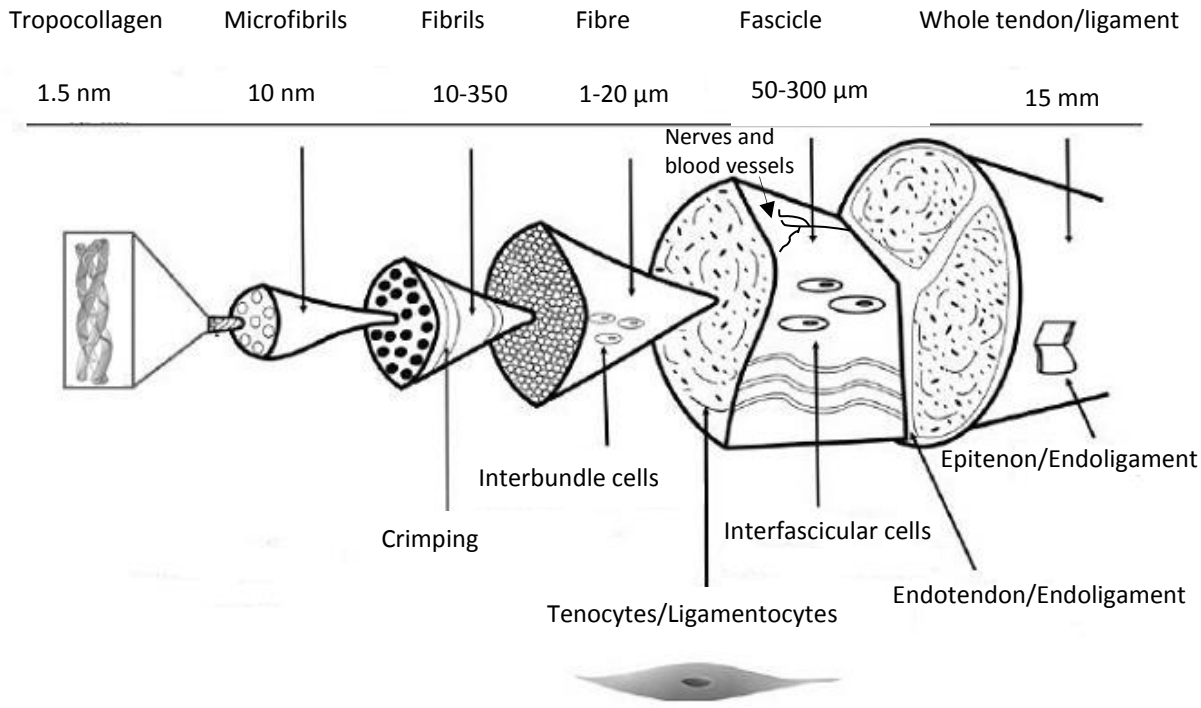


Figure 1.2. Hierarchical structure of tendon and ligament. Image from Kastelic et al. (1978) and Thorpe et al. (2012).

1.5 TENDON AND LIGAMENT COMPOSITION

1.5.1 Cells

Both tendon and ligament cells reside between collagen fibres within the fascicle and at the interfascicular regions (Lo et al. 2002, Clegg et al. 2007) (Figure 1.2). In both tendon and ligament the major cell type is the fibroblast which is responsible for the synthesis and assembly of ECM molecules (Tozer and Duprez 2005). Fibroblasts are referred to as tenoblasts/tenocytes in tendon and ligamentoblast/ligamentocytes in ligament, and comprises about 90-95% of the cellular element of both tissues (Kannus 2000, Hoffmann and Gross 2007). In tendon and ligament different morphological appearances of fibroblasts have been reported (Murray and Spector 1999, Kannus 2000, Clegg et al. 2007, Smith et al. 2012) Other populations of cells are also present but in lower number (5-10%)(Riley 2005, Hoffmann and Gross 2007) and include chondrocyte-like cells (fibrochondrocytes) at the bone origin and insertion sites, synovial cells, and vascular cells (Kannus 2000, Riley 2005, Hoffmann and Gross 2007). The existence of stem cells has also been documented in both tendon (Bi et al. 2007) and ligament tissue (Zhang et al. 2011).

Cells in tendon communicate by intercellular communication sites known as gap junctions, which are also necessary for strain-induced collagen synthesis (McNeilly et al. 1996, Clegg et al. 2007). Connections occur through cell cytoplasmic extensions that extend for long distances and connects to cytoplasmic extension of adjacent cells, forming an elaborate 3-dimensional structure (McNeilly et al. 1996). A similar intercellular communication system has also been described in ligament (Lo et al. 2002). The detection of gap junctions in association with these cell connections has also raised the possibility that cells coordinate cellular and metabolic responses throughout ligament and tendon tissue via cell- to-cell communication (McNeilly et al. 1996, Ralphs et al. 1998, Lo et al. 2002).

1.5.2 Extracellular matrix

The ECM is the non-cellular structural network that is secreted by cells, and provides not only essential physical scaffolding for the cellular constituents but also initiates essential biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis (Benjamin and Ralphs 1997, Kjaer 2004, Frantz et al. 2010). The general ECM composition of tendon and ligament consists of water and collagens, proteoglycans, elastin and glycoproteins, which are further described below (Benjamin and Ralphs 1997, Frank 2004, Thorpe et al. 2013).

1.5.2.1 Water

Tendon and ligament are composed of two–thirds water (Frank 2004, Kjaer 2004). A significant amount of this is associated with proteoglycans (PGs). The water and proteoglycans are thought to have spacing and lubricating roles necessary for the gliding of the fibres in the ECM of ligament and tendon (Amiel et al. 1995, Kjaer 2004).

1.5.2.2 Collagen

Collagen is the most abundant protein in the human body and makes up to 30% of its total protein content (Liu et al. 1995). Collagens are important for a wide range of functions including tissue scaffolding, maintaining the tissue structure, cell adhesion, chemotaxis, cell migration and the regulation of tissue remodelling during growth, morphogenesis and wound healing (Myllyharju and Kivirikko 2004, Kadler et al. 2007). Tendon and ligament contain approximately 70-80% dry weight collagen, of which ~85-95% is type I collagen (Frank 2004, Kjaer 2004, Riley 2005) depending on the type of tendon or ligament.

Collagen Classification- At least twenty eight collagens have been identified (Canty and Kadler 2005, Kadler et al. 2007), they are grouped based on their structure and supramolecular organisation into fibril forming collagens, fibril-associated collagens with interrupted triple helices (FACITs), network-forming collagens, transmembrane collagens, anchoring fibrils, beaded filament forming, endostatin producing collagens and others with unique functions (Kadler et al. 2007) (Table 1.1).

Collagen type	Classification	Distribution
I	*Fibril –forming*	Fibrils in tendon, bone, skin, cornea and blood vessels walls
II	*Fibril-forming*	Fibrils in cartilage
III	*Fibril-forming*	Forms heterotypic fibrils with type I collagen, especially in embryonic skin and hollow organs
IV	*Network-forming*	Network in basement membrane
V	*Fibril-forming*	Forms heterotypic fibrils with type I, especially in embryonic skin and in cornea
VI	*Beaded filament-forming*	Ubiquitous distribution, especially in muscle
VII	*Anchoring fibrils*	Fibrils in skin at the dermal/epidermal junction
VIII	*Network-forming*	Descement's membrane
IX	*FACIT*	Associated with type II collagen fibril, especially in cartilage
X	*Network-forming*	Hypertrophic cartilage
XI	*Fibril-forming*	Heterotypic fibrils with type II
XII	*FACIT*	Associated with type I fibrils
XII	*Transmembrane*	Neuromuscular junction, skin
XIV	*FACIT*	Associated with type I fibrils
XV	*Endostatin*	Specialised basement membranes, close structural homology to XVIII
XVI	*FACIT*	Specialised components of fibrillin 1 microfibrils and collagen fibrils
XVII	*Transmembrane*	Transmembrane component of hemidesmosomes, which attach epidermis to basement membrane skin
XVIII	*Endostatin*	Associated with basement membranes
XIX	*FACIT*	Infrequent, localised to basement membrane zones; contributes to muscle physiology and differentiation
XX	*FACIT*	Ubiquitous, most dominant in corneal epithelium
XXI	*FACIT*	Wide spread expression pattern
XXII	*FACIT*	Localised in specific tissue junctions such as myotendinous junction, cartilage and synovial fluid, hair follicle dermis
XXIII	*Transmembrane*	Limited tissue distribution
XXIV	*Fibril-forming*	Expressed in tissues containing type I collagen
XXV	*Transmembrane*	Precursor protein for CLAC (collagenous Alzheimer amyloid plaque component)
XXVI	*Beaded filament forming*	Expressed in testis and ovary of adult tissues
XXVII	*Fibril-forming*	Widespread expression especially in cartilage
Ectodysplasin	*Transmembrane*	Ectoderm
Gliomedlin	*Transmembrane*	Myelinating Schwan cells

Table 1.1. Collagen types, classification and distribution. Table adapted from Canty and Kadler (2005) and Kadler et al. (2007).

Collagen Structure- Collagen molecules consist of three polypeptide chains called α chains, in which each chain comprises a repeating Gly-X-Y triplet, where X and Y can be any residue but are usually proline and hydroxyproline respectively (van der Rest and Garrone 1991, Myllyharju and Kivirikko 2004). This triplet motif results in a left-handed helix that intertwines with two other helices to form a right hand triple-helical structure (van der Rest and Garrone 1991).

Collagen fibrils are the principal source of tensile strength in mammalian tissue. Fibrils can be identified by a characteristic banding pattern with 67nm axial periodicity. Fibrils can be up to millimetres in length and range in diameter from 12 nm to approximately 500nm, depending on the tissue and stage of development (Canty and Kadler 2005, Kadler et al. 2007). The three polypeptide α1 or α2 chains combine together to form a heterotrimer (two or three different chains) or a homotrimer (three identical chains) (Riley 2005). Fibrillar collagen type I is a heterotrimeric collagen, as it consists of two α1 chains and one α2 chain (Kadler et al. 2007). Heterotrimeric type I collagen is the main constituent of tendon and ligament and contributes to the high tensile strength of both tissues. Collagen type III is another fibrillar collagen present in both ligaments and tendon (Frank 2004, Kjaer 2004, Riley 2005). Collagen type III is believed to regulate the size of collagen type I fibrils and has been demonstrated to be important for normal fibrillogenesis (Kadler et al. 2007). Type III collagen has been found to be predominately localised at endotendon (Duance et al. 1977, Sodersten et al. 2013). Other types of collagen are also present but are present in much lower amounts. These include the fibrillar collagens type II and V, basement membrane collagen type IV and beaded filament forming collagens VI, XII and XIV (Frank 2004, Riley 2005).

Collagen Assembly- Collagen biosynthesis is characterised by the presence of an extensive number of co- and post-translational modifications of the polypeptide chains (Kjaer 2004). The formation of collagen fibrils starts with the synthesis of polypeptide chains on membrane bound ribosomes. The collagen polypeptides are secreted into lumen of the endoplasmic reticulum (ER) in which the following steps occur (Myllyharju and Kivirikko 2004):

a) Cleavage of the signal peptides
b) Hydroxylation of certain proline and lysine residues:

   Hydroxylation to 4-hydroxyproline, 3-hydroxyproline and hydroxylysine is due to action of the three hydroxylases; prolyl-4-hydroxylase (P4H), proline 3-hydroxylase (P3H) and lysine hydroxylase.
c) Glycosylation of some of the hydroxylysine residues to galactosylhydroxylysine and glucosylgalactosylhydroxylysine
d) Glycosylation of certain aspargine residues in the C or both N and C propeptides
e) Association of three C propeptides directed by specific recognition sequences
f) Creation of intramolecular and intermolecular disulfide bonds

After association of C propeptides and hydroxylation of around 100 proline residues, a nucleus for the assembly of the triple helix is formed in the C-terminal region and the triple helix is propagated towards the N-terminus (Myllyharju and Kivirikko 2004). The procollagen molecules are transported from the ER through the Golgi stacks and are aggregated during transport to form secretory vesicles. Subsequently, the N and C propeptides are cleaved by procollagen propeptides (Myllyharju and Kivirikko 2004). This process of N and C propeptides cleavage in tendon has been described by Canty and others to occur intercellulary, where N propeptides have been found to be removed earlier in the secretory pathway than the C propeptides (Canty and Kadler 2005, Canty-Laird et al. 2012). The ECM secreted collagen molecules are assembled in fibrils in a quarter stagger pattern to give D-periodic characteristics to the fibril (Kadler et al. 1996, Canty and Kadler 2005). This process is also referred to as collagen fibrillogenesis. Several molecules are involved in the regulation of tendon collagen fibrillogenesis such as collagen type III, small leucine proteoglycans and cartilage oligomeric matrix protein (COMP) (Banos et al. 2008). Formation of covalent crosslinks occurs within and between the formed triple-helical collagen molecules in fibrils (Figure 1.3) (Myllyharju and Kivirikko 2004, Canty and Kadler 2005).

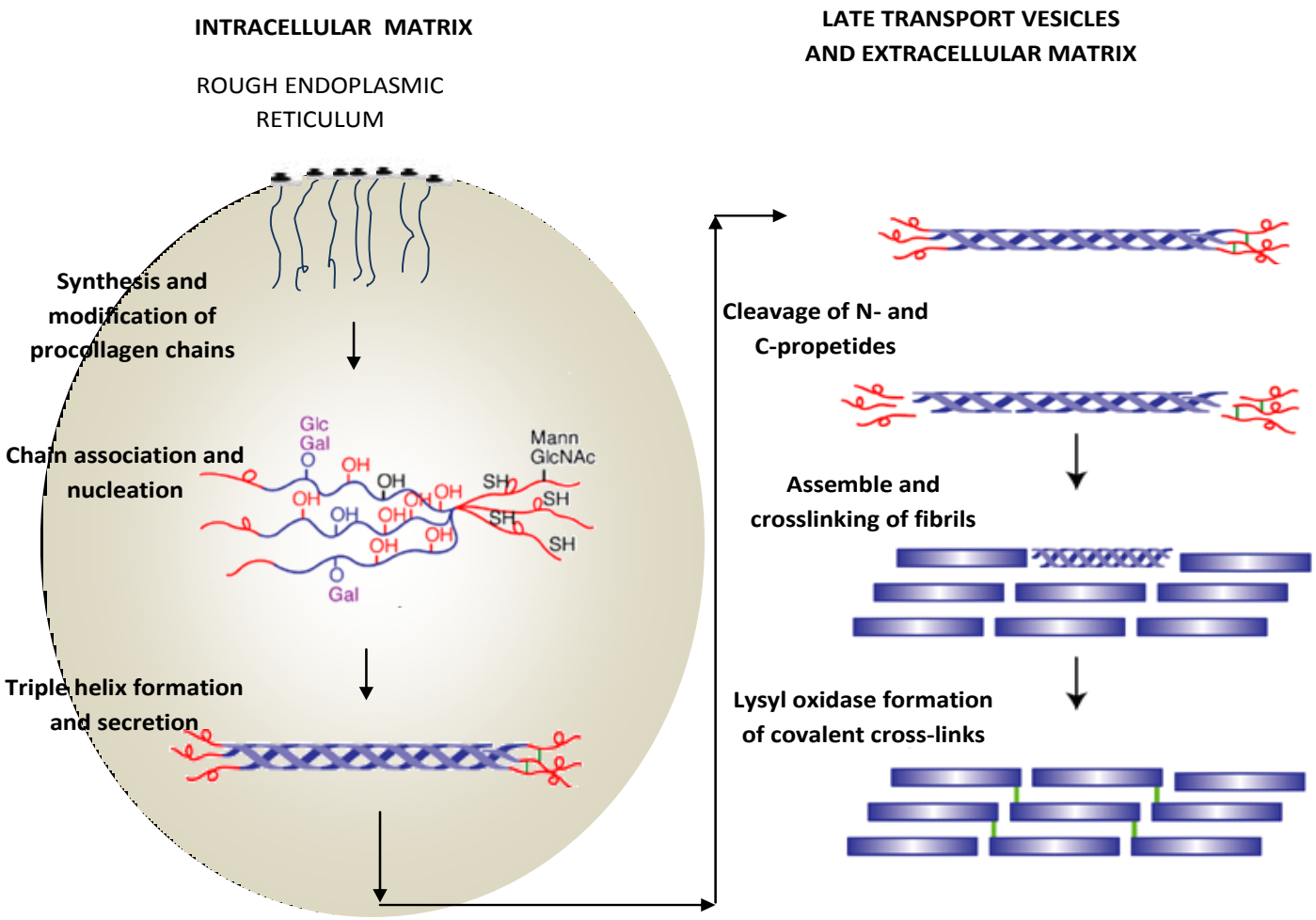


Figure 1.3 Intracellular and extracellular synthesis and processing of the collagen triple helix. Image adapted from Myllyharju and Kivirikko (2004).

Collagen Crosslinks- After extracellular fibril formation, covalent crosslinks are formed between collagen molecules (Kielty and Grant 1993). The type of cross-link can vary according to type of tissue, but is generally divided into three types; immature, mature and non-enzymatic (Knott and Bailey 1998).

Tendon and ligament contain both of the immature hydroxylysine aldehyde derived cross-links and the lysine aldehyde derived cross-links. Ligament mainly contains hydroxylysine aldehyde derived cross-links; whilst tendon has a higher content of lysine aldehyde derived cross-links (Amiel et al. 1984). Ligament also poses significant amount of type III collagen and a higher level of lysyl hydroxylation. This phenomenon might be due to the differences in turnover rate between these two tissues (Amiel et al. 1984).

1.5.2.3 Elastic Fibers

Although collagen fibrils are the principal source of tensile strength in ligament and tendon, (Canty and Kadler 2005), other non-collagenous components may contribute to the overall mechanical function of the tendon and ligament complex (Benjamin and Ralphs 1997, Frank 2004, Kjaer 2004, Thorpe et al. 2013)

Elastic fibres are major ECM assemblies that provide elasticity and resilience to many vertebrate tissues including arteries, lung, ligament, tendon, skin and elastin cartilage (Mithieux and Weiss 2005). Elastic fibres have at least three critical functions: 1) important mechanical properties in tissue elastic recoil and resilience (Butler et al. 1978, Eriksen et al. 2001), 2) regulation of cell functions such cell migration, and differentiation (Ito et al. 1997, Wendel et al. 2000) 3) regulation of the activity of the growth factor TGFβ family (Charbonneau et al. 2004, Feng and Derynck 2005). The arrangement, structure and organisation of elastic fibres are reflected in the function of the tissue. For instance, in arteries, elastic fibres are organised in concentric rings, while in the lung elastic fibres form fine branched network throughout the organ. In ligaments and tendons fibres are oriented longitudinally, parallel to collagen fibrils (Mithieux and Weiss 2005).

Elastic fibres are composed of a central cross-linked to core of elastin, surrounded by a sheath of fibrillin rich microfibrils. The fibrillin microfibrils are formed first and act as a

scaffold where secreted elastin molecules are deposited on the surface (Kielty 2006). The absence of fibrillin microfibrils during elastogenesis results in elastin sheets rather than formation of elastic fibres (Ross and Pawlina 2006).

Molecular Composition Of Elastic Fibres- Elastin is the most abundant component of elastin fibres and is extremely durable with little turnover in healthy tissues (Mithieux and Weiss 2005, Kielty 2006). Like collagen, elastin is rich in proline and glycine, but is not glycosylated and contains some hydroxylysine but no hydroxyproline (Alberts et al. 2002). Elastin is secreted as a 65-70 kDa protein, whose length depends on alternate splicing and is formed through lysine mediated crosslinking. Tropoelastin has a multidomain structure with repeating hydrophobic and lysine-rich crosslinking domains, each encoded by separate exons. Crosslinked elastin is formed through the action of the enzyme lysyl oxidase (LOX) and other members of this family (Mithieux and Weiss 2005, Kielty 2006). Proteoglycans, including biglycan have been detected within the elastin core (Baccarani-Contri et al. 1990). Moreover, it has been demonstrated that the elastin assembly may be influenced by the presence of sulphated proteoglycans (Kozel et al. 2004).

Microfibrils are considered to have a structural role in ligament and tendon and may have key roles in cellular matrix interactions (Ito et al. 1997, Wendel et al. 2000) as well as in the extracellular regulation of transforming growth factor (TGF) β (Charbonneau et al. 2004). Microfibrils (MFs) are chiefly composed of the structural glycoproteins fibrillin-1, fibrillin-2, and microfibril-associated glycoprotein-1 (MAGP1). Bundles of microfibrils are known as oxytalan fibres. Collectively, oxytalan and elastin fibres are referred to as elastic fibres (Kielty 2006, Smith et al. 2011). The fibrillins are large proteins (350 kDa) composed of multiple calcium-binding growth factor (cbEGF)-like domains and several eight-cysteine-containing (TB) motifs (Kielty et al. 2002, Kielty 2006). Fibrillin-1 and fibrillin-2 have overlapping expression patterns where fibrillin-2 is strongly expressed in developing tissues, whereas fibrillin-1 is expressed throughout life (Zhang et al. 1994, Charbonneau et al. 2003). Fibrillin 1 and 2 have been shown to co-localise within MFs and may overlap in function (Carta et al. 2006). The microfibril- associated molecule MAGP-1, a 31kDA glycoprotein, is most likely to be a structural component and widely co-localises with microfibrils (Cain et al. 2006). Other proteins associated with elastin microfibrils include MAGP-2 and latent TGF-β

binding protein (LTBP)-1, which also co-localise in certain tissues such as nuchal ligament and dermis (Gibson et al. 1998, Kielty 2006).

Elastic fibres are thought to account for about 1-2% of the dry weight of tendon (Kannus 2000) and ligament (Frank 2004), however this can vary between tendon and ligament type. For instance nuchal ligament has been found to contain 7.8% elastin (Uitto 1979), while the ACL was found to contain 5% elastin (Dodds and Arnoczky 1994). The distribution of elastic fibres in bovine tendon (Grant et al. 2013) and canine cruciate ligament complex (Smith et al. 2011) has been described, with elastic fibres being sparse and microfibrils abundant in both tissue types. In bovine tendon both fibrillins 1 and 2 were found to co-localise with elastin (Grant et al. 2013), while in ligament the majority of fibrillin 2 fibres did not stain for elastin (Smith et al. 2011). This different localisation of fibrillin 2 between ligament and tendon may demonstrate a fundamental difference between these two tissues.

1.5.2.4 Proteoglycans

Proteoglycans have numerous biological functions such as assembly and maintenance of ECM, participation in cell proliferation through interaction with growth factors, tumor cell growth and invasion (Halper 2014). In tendon and ligament proteoglycans play a role in collagen fibrillogenesis and organisation of collagen fibrils (Zhang et al. 2005, Franchi et al. 2007), and they interact with collagen fibres to yield viscoelastic properties (Rees et al. 2000, Woo et al. 2006, Franchi et al. 2007). They also contribute to the fibril and fibre structural integrity performing an important role alongside the fibrous elements of the ECM, by forming interfibrillar linkages (Franchi et al. 2010). Proteoglycans are a special class of glycoproteins that are heavily glycosylated, consisting of a core protein with one or more covalently attached glycosaminoglycans (GAGs). The function of different proteoglycans is determined by structure of the protein core and GAG chains (Parkinson et al. 2011). Most GAGs consist of repeating units containing uronic acid (glucuronic acid or iduronic acid) and an N-acetylated sugar (N-acetyl glucosamine or N-acetyl galactosamine). At least one of the repeating units has a negatively charged carboxylate group, apart from hyaluronic acid, which is not sulphated (Styrer 1996). The negatively charged carboxyl group of GAGs results in the hydrophilic nature of proteoglycans, which provides lubrication and water retention

(about 70% water) within tissues (Franchi et al. 2010). GAGs are distinguished according to their sugar, the type of linkage between sugars, and the number and location of sulphate groups (Alberts et al. 2002). These are:

1. Chondroitin sulphate (CS) and dermatan sulphate (DS)
2. Heparan sulfate (HS)
3. Keratan sulphate (KS)
4. Hyaluronan

Chondroitin sulphate plays a role in articular cartilage and bone metabolism by controlling cartilaginous matrix integrity and bone mineralisation. It contains disaccharide repeats of glucuronic acid and N-acetylgalactosamine (Bali et al. 2001). Dermatan sulphate proteoglycans are widely distributed in the ECM of skin, sclera, tendon and a wide variety of connective tissues. Its chains are a variant of chondroitin sulphate and are also O-linked to a protein core via a serine xylose-galactose-galactose structure (Rosenberg et al. 1986). Keratan sulphate is primarily found in cornea and skeletal tissues and brain. It is the only GAG without uronic acid residues. The hexosamine residue is commonly sulphated at its 6-position, but sulphation may also occur at the 6-position of the galactose residues (Yoon and Halper 2005). Heparan sulphate is present on the cell surface of all human and animal cells. It has a similar tetrasaccharides linkage as CS, and an analogous consensus sequence for attachment to serine residues (Yoon and Halper 2005). Hyaluronan serves as a lubricant and is believed to have a role in resisting compressive forces in tissues and joints as well as being an important component of joint fluid (Alberts et al. 2002). Within the GAGs hyaluronan is unique, as it does not contain a sulphate chain. In addition unlike the other GAGs, hyaluronan is not covalently attached to the proteoglycan protein core and binds indirectly to proteoglycans (Ross and Pawlina 2006).

The concentration of GAGs in ligaments and tendon can differ, and is thought to be related to their viscoelastic properties. Rabbit cruciate ligaments have been found to contain 2-4 times more GAG then any tendons studied (Vogel et al. 1993). Amiel et al. (1984) also found similar differences between rabbit cruciate ligaments and tendons (Achilles and patellar tendons). In addition, a comparative study in an ovine model showed that the ligaments

(ACL and PCL) have a higher GAG content than tendons (LDET, SDFT, and PT) (Rumian et al. 2007).

Proteoglycans are usually divided into two classes of small and large proteoglycans (Halper 2014). Their overall structure with their associated GAG chain is shown in Figure 1.4.

Small Leucine Rich Proteoglycans (SLRPs)- These proteoglycans are tissue organisers by orientating and ordering collagen fibrils during ontogeny, wound healing, tissue repair and interact with a number of surface receptors and growth factors (Iozzo et al. 2011). The central part of SLRPs contains 10 leucine-rich repeats region forming a parallel beta sheet which comprises a site for collagen binding (Halper 2014). The SLRPs are dividided into five classes (Halper 2014), however members of the first two classes are only described in this Chapter.

In tendon, SLRPs act to modulate formation and diameter of collagen fibrils during fibrillogenesis (Parkinson et al. 2011). The four principal SLRPs in ligament and tendon are decorin, biglycan, fibromodulin and lumican (Ilic et al. 2005, Yoon and Halper 2005). Decorin is a class I member SLRP with either single dermatan or chondroitin sulphate at its N-terminal region. At the leucine rich repeats region it contains binding sites for collagen type I, II, III and VI (Halper 2014). Decorin also binds to other proteins such as transforming growth factor β (TGFβ) (Hildebrand et al. 1994) and epidermal growth factor (EGF) (Santra et al. 2002). Apart from collagen fibrillogenesis, decorin hinders TGFβ activity (Hildebrand et al. 1994), normalises cell proliferation, stimulates the immune response (Yoon and Halper 2005) and can substitute for absent biglycan (Ameye and Young 2002).

Biglycan is another member of class I SLRPs, has similar homology to decorin and also plays important roles in collagen fibrillogenesis (Halper 2014). It has either two chondroitin or dermatan sulphate chains (Yoon and Halper 2005, Halper 2014) and can interact with collagen type I (Schonherr et al. 1995) and VI (Wiberg et al. 2002). Together, biglycan and aggrecan have been found to be increased at the fibrocartilaginous regions of tendon (Rees et al. 2009).

Asporin is another member of class I SLRPs, but has no glycosaminoglycan chains attached to its core protein (Ikegawa 2008). Asporin competes with decorin for collagen binding with

the high affinity site located in the C-terminal part of the protein (Kalamajski and Oldberg 2010). Asporin also plays a role in biomineralisation as it has the ability to bind to calcium in the polyaspartic N-terminal domain of the protein (Kalamajski et al. 2009).

Fibromodulin and lumican are members of class II SLRPs that consist of four keratan chains that are bound to the core protein. Both SLRPS have a binding sites at the leucine rich region for collagen I and II, though these are different to the decorin binding site (Yoon and Halper 2005, Halper 2014). Together decorin, biglycan, fibromodulin and lumican SLRPs play an important roles in the organisation of collagen fibrils, as mice deficent in these SLRPs demonstrated irregular collagens fibril diameters and reduced tensile strenghth of tendon (Danielson et al. 1997, Svensson et al. 1999, Ameye and Young 2002, Chakravarti 2002). Keratocan is another member of class II SLRPS. Keratocan and lumican together are the major keratan sulfate containing SLRPs that are primarly found in the cornea (Carlson et al. 2005). In the cornea the keratocan has been found to highly glycolysated, while in the tendon it is poorly sulphated. This SLRP is also likely to play an important role in regulating the collagenous matrix of tendon (Rees et al. 2009).

Large Aggregating Proteoglycans- Proteoglycans that indirectly bind to hyaluranon form giant macromolecules and are there referred to large aggregating proteoglycans (Ross and Pawlina 2006). Aggrecan is one of large aggregating that contains numerous chondroitin and keratan sulphate GAGs and three globular domains (G1,G2,G3) (Hardingham and Fosang 1992). The CS and KS GAG chains are attached to specific sites between the G2 and G3 domain, whilst the G1 domain interacts with hyaluronan (Hardingham and Fosang 1992, Riley 2005). Aggrecan is most abundant in cartilage. A major function of aggrecan is its capacity to retain water in this tissue due to the presence of many negatively charged GAG chains, which creates a strongly hydrophilic environment (Kiani et al. 2002, Heinegard 2009).

Versican is another large proteoglycan, present in a variety of soft tissues. It plays a role in cell adhesion, proliferation, migration and ECM assembly, regulation of cell phenotype (Wight 2002) and may be involved in chondrogenic changes (Zhang et al. 2001). Versican lacks a globular domain 2 and is divided into sections known as GAGα and GAGβ for its GAG

binding domain (Wight 2002). Versican has been found in bovine ligaments (Campbell et al. 1996) and in tendon, where it forms an integral part of a pericellular matrix that organises the tendon cells in linear arrays between collagen fascicles (Ritty et al. 2003). Versican has been found to interact with other ECM proteins and plays a important role in ECM assembly. Versican interacts occurs with proteins such as hyaluronan, tenascin R, fibrillin-1, fibrillin-2 and elastic fibers (Wight 2002). It has been noted that Versican interacts with the elastic fibre associated protein fibrillin-1 and co-localises with microfibrils (Isogai, Aspberg et al. 2002).

In general proteoglycans comprise less than 3% of the dry weight of ligaments and tendons (Ilic et al. 2005, Franchi et al. 2007, Parkinson et al. 2011). The major proteoglycan in tendons and ligament is decorin, which accounts for 80% and 90% of total proteoglycan in respectively (Samiric et al. 2004, Ilic et al. 2005). The other SLRPs including biglycan fibromodulin and lumican have been detected in both tendon and ligament (Rees et al. 2009, Yang et al. 2012). These SLRPs are primarily located at the interfibrillar matrix, which are able to bind to collagen fibrils at specific sites in tendon (Thorpe et al. 2013). Other SLRPS that such as keratocan and asporin have been less characterised in both tendon and ligament. Keratocan has been identified in both the compresive and tensile regions of tendon (Rees et al. 2009) and asporin in tensile region of both tendon and ligament (Little et al. 2014). The large aggregating proteoglycans, versican and aggrecan have been found to be present at similar levels in the tendon tensile region, though the versican might be predominant in the tensile region of ligament (Ilic et al. 2005).

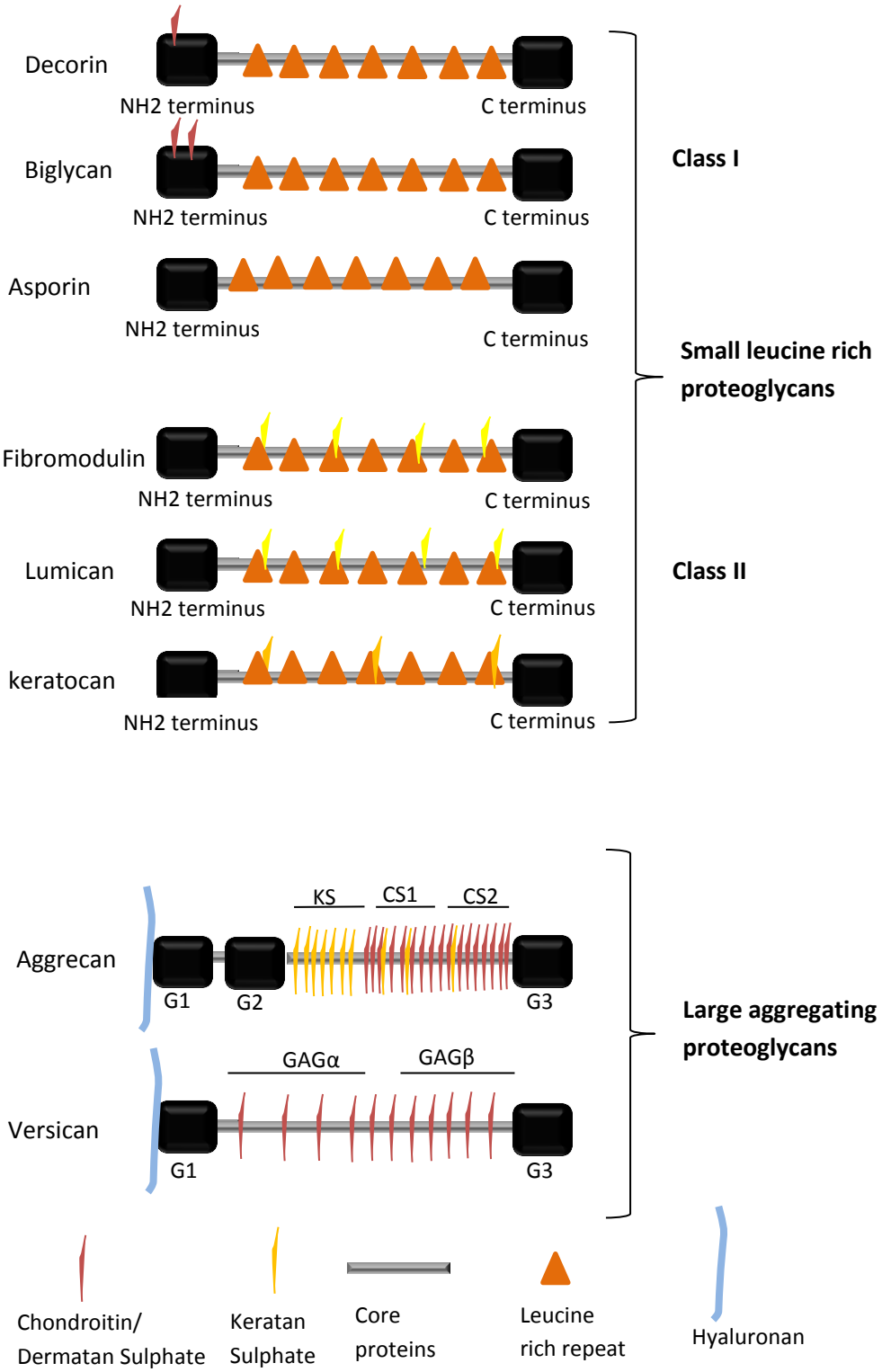


Figure 1.4. Proteoglycans present in tendon and ligament. The structure of each proteoglycan with its associated GAGs is represented. This image is modified fromRiley (2005).

1.6 LIGAMENTS AND TENDONS OF THE KNEE JOINT

1.6.1 Functional anatomy

The knee joint (tibiofemoral joint) is the largest and most complex joint of the body (Tortora and Derrickson 2013). The knee joint is a synovial joint formed between the three bones, tibia, femur and patella. It is a hinge joint allowing for wide range of flexion and extension. The articulations consist of the patellofemoral and tibiofemoral joint. The joint capsule within the knee is thin and attaches on the margin of the femur and tibia and encloses the joint (Gosling et al. 2008). The patellar tendon, medial collateral ligament (MCL) and lateral collateral ligament (LCL), oblique popliteal and arcuate popliteal ligaments are extracapsular. The patellar tendon is located below the patella. It inserts at the top of the tibia and spreads over the top of the patella where it connects to the quadriceps tendon (Figure 1.5). The MCL and LCL extend from the medial and lateral condyles of the femur to the medial condyle of the tibia and lateral side of the head of the fibula and provide stability of the inner and outer part of the knee, respectively (Tortora and Derrickson 2013).

Knee intracapsular ligaments include the anterior cruciate ligament (ACL) and the posterior cruciate ligament (PCL). The cruciate ligaments (CLs) are the primary stabilisers of the kee joint which are attached to the tibia, located within the joint capsule and are surrounded by a layer of synovium. The ACL and PCL cross each other and are in intimate contact, forming the CL complex (Arnoczky and Marshall 1977, Woo et al. 2006, Smith et al. 2011). The PCL is twice the strength of ACL, and infrequently injured (Kannus et al. 1991). The increased strength of the PCL is thought to be with the large cross-sectional area of the PCL, and the large broad femoral attachment (Amis et al. 2006).

The ACL plays a fundamental role in joint stability being the main restraint to anterior translation of the tibia with regard to the femur. It also functions as a key secondary restraint to internal rotation when the joint is near full extension and restraint to valgus angulation (Duthon et al. 2006). The ACL is enveloped by the synovial membrane from the knee joint capsule originating at the medial side of the lateral femoral condyle and runing an oblique course through the intercondylar fossa distoanteromedial to the insertion at the medial tibial eminence (Zantop et al. 2006, Petersen and Zantop 2007). The ACL has distinct functional bands, which vary the tension among the fibers in the ligament at different

ranges of motion. The ACL is differentiated into two distinct fibre bundles, namely the anteromedial (AM) and posterolateral (PL) bundle (Zantop et al. 2006, Petersen and Zantop 2007). The AM bundle is thought to be important as a restraint to anterior–posterior translation of the knee, while the PL bundle is thought to be an important restraint to rotational moments about the knee (Woo et al. 2006).

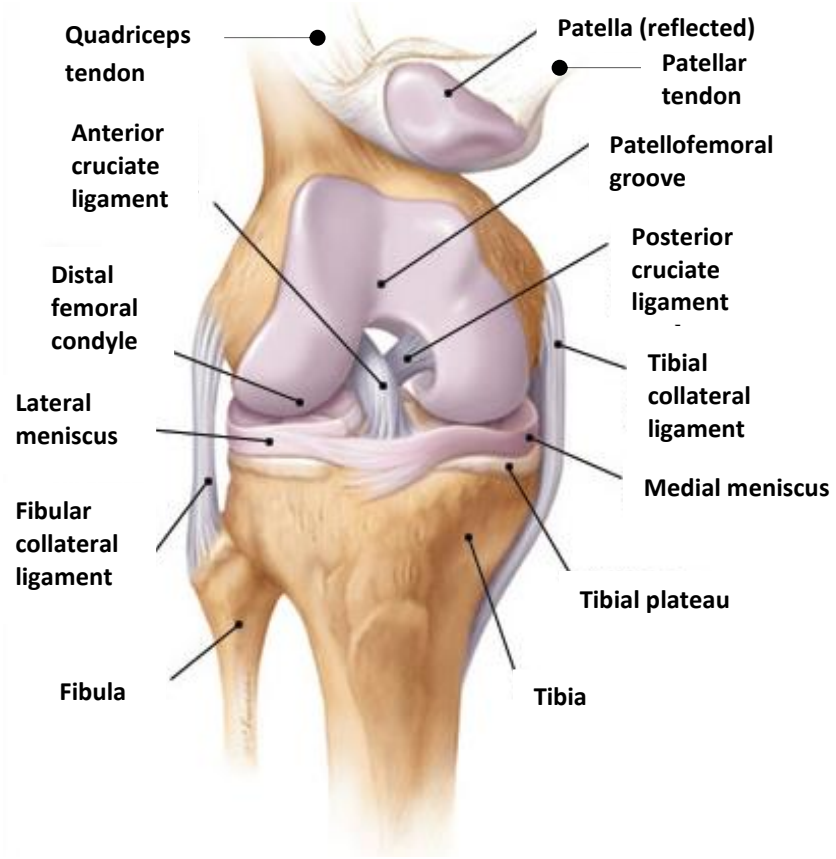


Figure 1.5. Anatomy of the human knee joint. Image adapted from Calmbach and Hutchens (2003). The patellar tendon has been cut to allow observation of cruciate ligaments.

Translational animal models including mouse, rabbit, goat and sheep, pig, dog and horse are used to study the human knee structures. From this group, the dog model is thought to be the closest to a gold-standard animal model for knee osteoarthritis currently available (Gregory et al. 2012). The canine stifle (knee) joint is remarkably similar to the human knee.

Apart from size, the macroscopic and microscopic anatomical structures are very similar (Cook et al. 2010). As in the human knee joint, the canine stifle joint consists of medial and lateral femorotibial compartments and a patellofemoral compartment. The canine stifle joint also contains similar structures such as the ACL, PCL, menisci, meniscal ligament, fat pad and patellar tendon to the human knee joint (Cook et al. 2010). The major anatomical difference is that the canine stifle joint consists of sesamoid bones in the popliteus and lateral and medial heads of the gastrocnemius muscles and a long digital extensor tendon (LDET) that is intra-articulary located. In the canine stifle joint the ACL is referred as the cranial cruciate ligament (CCL) and is comparable to the human ACL (Arnoczky 1983).

In this thesis, the canine stifle joint was the model chosen to examine knee joint tendon and ligaments including, the intra-articular CCL, LDET and extra- articular MCL and SDFT are studied. The CCL originates at lateral femoral condyle runs diagonally across and is inserted at the tibial eminence. The MCL runs its course from femur to tibia by fusing with the join capsule and medial meniscus. (Evans et al. 1979). The LDET originates from the extensor fossa on the lateral aspect of the femoral condyle and runs lateral to the stifle joint and becomes the muscle located along the lateral side of the tibia. The SDF muscle originates from the lateral supracondylar tuberosity of the femur, and continues distally into SDF tendon. The SDFT twists across the medial surface of the gastrocnemius muscle, travels distally over the tuber calcanei and attaches on each side. It then runs further distally before bifurcating at the tarsus and each of these tendons bifurcates to insert on the middle phalanges of digits II, III, IV and V. (Evans et al. 1979).

1.6.2 Blood supply

Tendons and ligaments have a low blood supply in comparison with other associated tissues such as the muscle, synovium or bone (Benjamin and Ralphs 1997). Blood supply and source of vasculature are different in specific tendons such as Achilles tendon (Ahmed et al. 1998) and posterior tibial tendon (Petersen et al. 2002). Ligaments such as the ACL and MCL have been reported to have a difference in vascular anatomy (Bray et al. 1990)

In this chapter we will first describe the general blood supply in tendon, ligament and the focus on ACL, MCL blood supply.

General Tendon Blood Supply- The vascularisation of tendon and the presence of vessels is important for the normal function of tendon cells and the ability of tendons to repair. The vessels generally run longitudinally, parallel to the fascicle and within the endotenon (O'Brien 2005). The blood supply in tendons could occur from three main sources 1) the musculotendinous junction; 2) the osteotendinous juction; 3) the extrinsic system through the paratenon or the synovial sheath (Carr and Norris 1989, Fenwick et al. 2002, Sharma and Maffulli 2005). At the musculotendinous junction the blood supply is from the superficial vessels in surrounding tissues, in which perimysial vessels continue between the fascicles of the tendon (Carr and Norris 1989, O'Brien 2005). Blood vessels originating from the muscle are not likely to extend past the proximal third of tendons (Sharma and Maffulli 2005). In the middle portion of tendon the main blood supply is via the paratenon, in which small blood vessels run transversely towards the tendon, and branch several times before running parallel to the long axis of tendon. The vessels enter tendon along the endotenon and the arterioles run longitudinally flanked by two venules (O'Brien 2005). The blood supply from the osteotendinous region is limited, as vascular supply is at the lower one third of the and there is no direct communication between the vessels because of the avascular fibrocartilaginous layer between the tendon and bone (Benjamin et al. 1986, Carr and Norris 1989, O'Brien 1992, O'Brien 2005).

Blood Supply In ACL and MCL- The anterior cruciate ligament is supplied by branches of the lateral and medial inferior geniculate artery. Proximal and distal vessels support a synovial plexus from which small vessels run into the ACL and align longitudinally parallel to the collagen bundles (Arnoczky 1985, Zantop et al. 2005). Both ACL and PCL are covered by a synovial membrane consisting supplied with vessels. The synovial vessels divide into more branches and form a web-like network of periligamentous vessels ensheathing the entire ligament. These periligamentous vessels then enter the ligament transversely and anastomose with the network of endoligamentous vessels. These vessels then reach the attachment of the ligament to the femur and tibia (Arnoczky 1983).

The distribution of blood supply in the ACL is not homogenous, as the mid part of the cruciate ligaments is less vascularised than the proximal and distal parts. The PCL may have a more substantial blood supply as they appear to have more epiligamentous vessels (Arnoczky et al. 1979). In the ACL avascular zones are located within the fibrocartilaginous regions at the femoral and tibial insertion site and at the anterior part where the ligament faces the anterior rim of the intercondylar fossa (Petersen and Tillmann 1999, Petersen and Tillmann 2002).

The ACL may gain nutrition from the synovial fluid, as even though the ACL has been considered extra-articular due to the enveloping synovial epiligament, free passage of macromolecules from intra-articular synovial fluid to the substance of the ACL has been demonstrated (Kobayashi et al. 2006).

The MCL is supplied from the inferior medial geniculate artery, which travels longitudinally, transversely within and next to the substance of the ligament (Wallace and Amiel 1991). The MCL is more vascularised than ACL, as it has a relatively vascularised epiligament, with vessels penetrating the mid substance region (Bray et al. 2003).

In the canine the LDET and SDFT blood supply is not well described. However in the horse the blood supply of SDFT is described to be afforded proximally and distally through connection with arterial supply of the SDFT muscle and by vessels carried through the digital sheath. The tendon is least well vascularised within its middle third and is dependent on the paratendinous covering with this region, which supplied by many branches arising from the medial palmar artery (Kraus-Hansen et al. 1992).

1.7 TENDON AND LIGAMENT INJURY AND DISEASE

1.7.1 Fatigue and stress

Tendons and ligament encounter loads as parts of everyday activities. These loads can either be due to tension, compression or shearing and can result in different tissue strain characteristics (Doschak and Zernicke 2005). A typical strain-stress curve of tendon and ligament consists of a toe region (Region I, Figure 1.6), which is the result of the waviness of

the collagen fibres (crimp) straightening out. The strain of the tissue at the end of this region has been reported to be between 1.5% and 4% (Butler et al. 1978). In the elastic region (Region II, Figure 1.6) the tendon or ligament shows a relatively linear response to stress, as the collagen fibres take up force, become more parallel and lose their wavy or crimped appearance. The slope of the curve in this region is often referred to as the elastic modulus or Young's modulus of elasticity. Beyond the elastic limits of the tissue (Region III, Figure 1.6), fibre failures occur strain limit the strain of this region (linear region). Higher levels of strain beyond the linear region result in tensile failure and shear failure occurring between the fibres progressing to complete rupture (Region IV, Figure 1.6) (Butler et al. 1978, Goh et al. 2003, Doschak and Zernicke 2005).

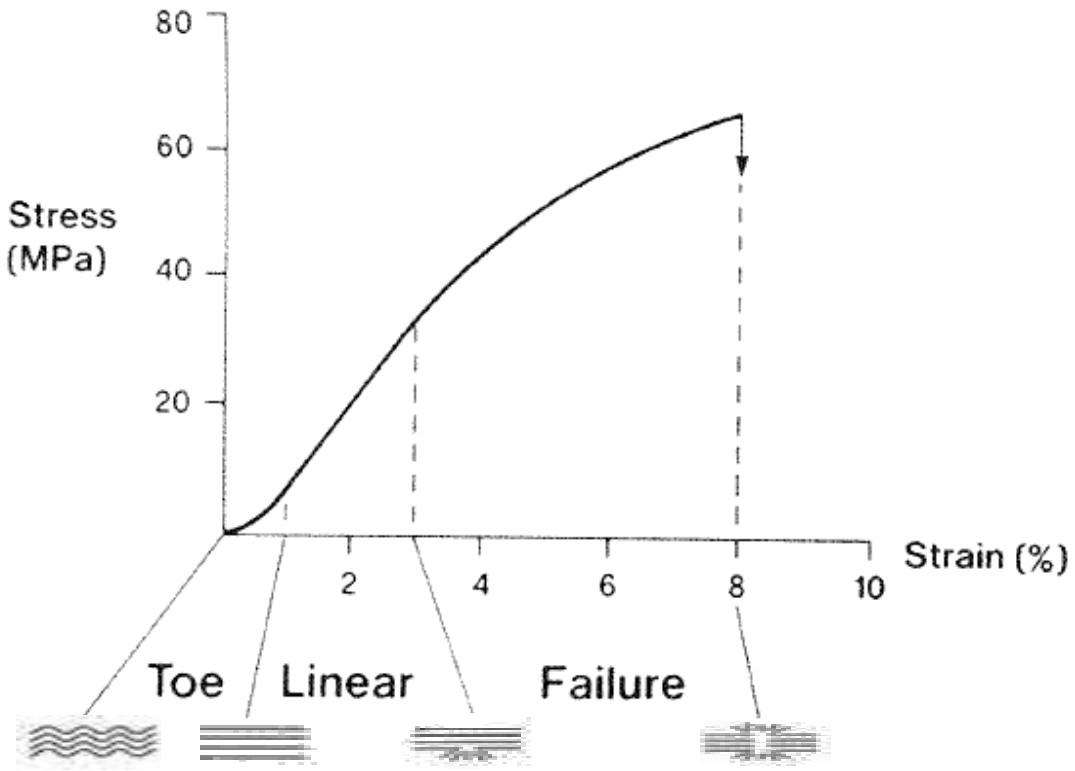


Figure 1.6 A typical tensile stress-strain curve for tendon and ligament. Image from Butler and others. (Butler et al. 1978).

Tendons and ligaments have precise mechanical properties to support their specific function (Birch et al. 2013). This difference in mechanical behaviour compared to other connective

tissues has been shown between tendons such as, the human Achilles tendon and the anterior tibialis, with the Achilles tendon possessing ultimate tensile strength (force to failure) of 4 kN and anterior tibialis 1.5 kN. Differences in mechanical properties have also been shown in material strength (ultimate stress) and stiffness between tendon and ligament, such as the equine suspensory ligament (SL), SDFT and CDET, where the SL was found to contain lower ultimate stress and elastic modulus (Birch et al. 2013). Similar findings have also been reported between human Achilles tendon, patellar tendon, meniscofemoral ligament and PCL, with both ligaments containing a lower elastic modulus and tensile strength than tendons (An et al. 2004, Harner et al. Martin et al. 1998). These diverse mechanical properties of tendons and ligaments are as result of a distinct matrix composition and structural alignment of these tissues and contribute to the understanding of how these two connective tissues function.

1.7.2 Tendon and ligament injuries

Tendon and ligament injuries are increasingly common in human and in comparative species such as the dog and horse (Burt and Overpeck 2001, Adirim and Cheng 2003, Comerford et al. 2011, Clegg 2012). More than 30 million tendon injuries are reported per year worldwide (Maffulli et al. 2003), with 30-50% of these being sports-related (Rees et al. 2009). In humans, Achilles, rotator cuff and patellar tendon injuries are the most common and can be as a result of trauma or overuse (Moshiri and Oryan 2013). Whilst in the horse, SDFT lacerations are among the most common tendon injuries (Thorpe et al. 2010). Tendon injuries are divided into acute and chronic injuries and are caused by intrinsic or extrinsic factors either alone or in combination (Sharma and Maffulli 2005). Acute injuries are caused by trauma or overload, whilst chronic injuries are not associated with one traumatic event, but are caused by repeated microtrauma or overuse of tendon, which is thought to be the consequence of repeated exposure to low-magnitude force (Riley 2004). The terminology of chronic tendon pathologies is unclear. Unless the presence of inflammation or degeneration has been clearly demonstrated, most tendon pain and dysfunction is best described as a ‘tendinopathy’. In chronic tendinopathy both intrinsic and extrinsic factors play a role. Intrinsic factors include age, bodyweight, disease, gender and extrinsic factors include occupation, physical load, environmental conditions and fatigue (Maffulli et al. 2003,

Riley 2004). Histological observation of tendinopathies shows different features such as collagen fibre disorganisation, cell rounding, cell density change, increased glycosaminoglycans deposition, lipid accumulation and calcification (Astrom and Rausing 1995, Movin et al. 1997, Khan and Maffulli 1998, Riley et al. 2001, Riley 2005).

The ACL is one of the most frequently injured ligaments (Woo et al. 2000), with ~37 tears per 100,000 people (Gianotti et al. 2009). The ACL together with the MCL accounts for 95% of all multi-ligament injuries in the knee joint (Funakoshi et al. 2007), resulting in significant joint instability causing pain and immobility in the affected individual (Woo et al. 1999). Injury also leads to significant functional impairment in athletes, and is associated with induction of degenerative joint disease such as osteoarthritis (OA) (Daniel et al. 1994, Maffulli et al. 2003). ACL injury caused by trauma or contact sport only accounts for about 30 percent of ACL injuries. The remaining 70 percent of ACL tears are from non-contact injuries (Cimino et al. 2010). Risk factors such as age (Hasegawa et al. 2012), gender (Harmon and Ireland 2000, Toth and Cordasco 2000), bodyweight (Uhorchak et al. 2003) and genetics (Posthumus et al. 2009) are involved in the pathogenesis of non-contact ligament ruptures. Histological examination of degenerative ACL has been shown to be associated with an increase in cell number, disorientation of collagen fibres, inflammation and GAG accumulation and chondroid metaplasia (Hasegawa et al. 2012, Hasegawa et al. 2013). Chondroid metaplasia is characterised by cells becoming more rounded with chondroid transformation that are being arranged in chains (Vasseur et al. 1985, Narama et al. 1996, Comerford et al. 2006).

1.8 HEALING OF TENDINOUS AND LIGAMENTOUS TISSUE

Tendon and ligament have slower healing rates than other connective tissues, most likely due to the hypocellular and hypovascular nature of these tissues (Tozer and Duprez 2005). The process of ligament and tendon healing is complex, with the responses of these specific tissues being dramatically different and ranging from spontaneous healing to little or no healing (Woo, Hildebrand et al. 1999; Jung, Fisher et al. 2009).

For instance, some ligaments such as the MCL have good healing potential whereas others such as the ACL demonstrate poor healing, especially in the case of complete ligament

rupture (Murray 2009). Conventional ligament and tendon healing occurs in three phases: inflammation, proliferation, and matrix remodeling (Figure 1.7) (Woo et al. 1999, Sharma and Maffulli 2005, James et al. 2008).

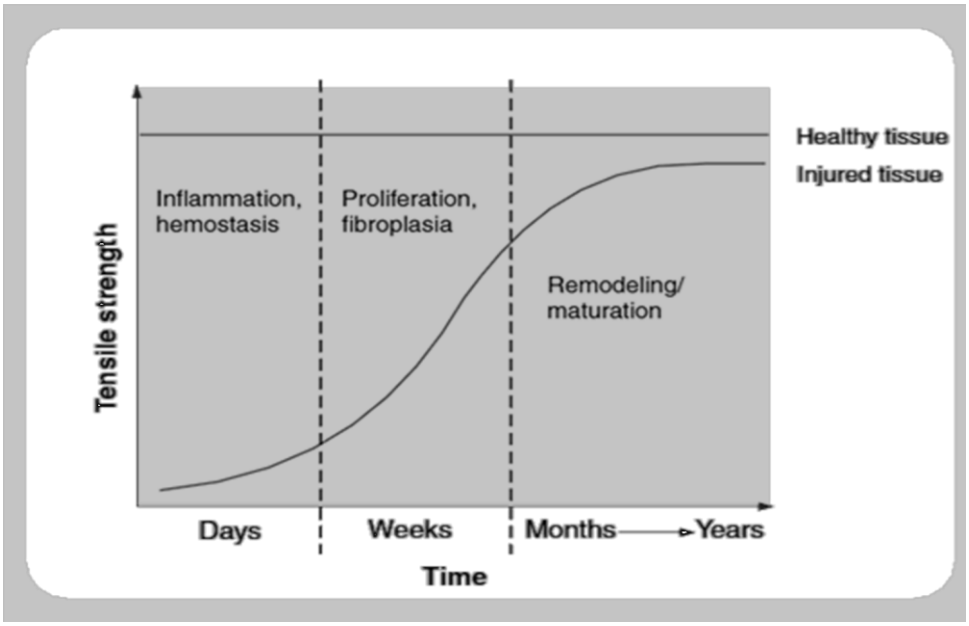


Figure 1.7. Typical tendon and ligament healing process. Image adapted from Lin et al. (2004).

In tendon the healing starts with an inflammatory/haemostasis stage, where erythrocytes, platelets, neutrophils, monocytes, and macrophages migrate to the site of injury. Theinflammatory cells engage in the phagocytosis of necrotic tissue and debris breaking down the blood clot (Lin et al. 2004, James et al. 2008). During this phase, there is an increase in DNA, fibronectin, GAGs, water and collagen type III content (Lin et al. 2004). In the proliferative phase, type III collagen and DNA reach their peak amounts in the entire reparative process. Within this stage the wound has a scar-like appearance surrounded by an extensive blood vessel network. Water and GAGs content remain high during this stage (Sharma and Maffulli 2006, James et al. 2008). In the remodelling stage, which begins 6-8 weeks after injury, there is a decrease in cellularity, reduced ECM synthesis, and collagen fibers begin to orient themselves longitudinally along the long axis of the tendon. As the scar enters the maturation phase there is a notable reduction of the type III to type I

collagen ratio, water, GAGs and DNA concentration (Lin et al. 2004, James et al. 2008). The repaired tendon tissue never achieves the mechanical properties it had prior to injury and the biochemical and ultrastructural characteristics remain abnormal even at 12 months post injury (Miyashita et al. 1997, Yang et al. 2013). Histologic studies of healing tissue have shown higher cell numbers and a less parallel collagen fibre organisation (Proctor et al. 1997, Provenzano et al. 2001). These altered structural properties may contribute to degenerative changes, inferior function, and an increased risk of re-rupture (Proctor et al. 1997, Rokito et al. 1999, Lin et al. 2004). Matrix metalloproteinases (MMPs) are important regulators of ECM network remodelling and are also altered during tendon and ligament healing (Foos et al. 2001, Ireland et al. 2001, Riley et al. 2002, Jones et al. 2006).

The extra- articular MCL tends to heal successfully, whilst the intra-articular ACL has poor healing capacity (Murray 2009). This could be due to several factors such as inadequate blood supply of the ACL (Arnoczky et al. 1979, Vasseur et al. 1985, Bray et al. 1996), presence of synovial fluid (Kobayashi et al. 2006), deficiencies in stimulation or intrinsic deficiencies of cell migration or proliferation (Nagineni et al. 1992, Geiger et al. 1994, Amiel et al. 1995, Spindler et al. 1996). Murray et al. (2000) illustrated that following rupture repair of the human ACL undergoes four histological phases, inflammation, epiligamentous regeneration, proliferation, and remodelling, however the ligament ends retract by forming layers of synovial tissue over the ruptured surface, which may impede ligament repair. Studies on ACL and MCL healing have demonstrated differences in terms of cellular properties and proliferation (Yoshida and Fujii 1999, Chun et al. 2003), stem cells properties (Zhang et al. 2011), MMP expression (Zhang et al. 2009) and lysyl oxidase expression (Xie et al. 2013).

As with tendons, healing of the MCL been shown to heal with a scar that is histologically, biochemically and biomechanically different to normal ligament. This scar is weaker and larger and creeps more than normal ligament (Thornton et al. 2000). Following healing of the MCL differences in structure and function such as altered collagen types (Amiel et al. 1987), failure of collagen crosslinks to mature (Frank et al. 1995), altered proteoglycans (Plaas et al. 2000), presistence of small diameter collagen fibrils (Frank et al. 1992) and increased vascularity (Bray et al. 1996).

1.9 CURRENT METHOD OF TENDON AND LIGAMENT REPAIR AND TISSUE ENGINEERING

The current methods of tendon and ligament repair depend on the grade of injury. In tendon, injuries with mild tissue defects have been found to respond to a gradual return to exercise (Soroceanu et al. 2012, Moshiri and Oryan 2013). About 80 percent of patients with mild tendon injury recover within 12 months with conservative therapy, however surgery may remain an option if the conservative therapy doesn't work (Moshiri and Oryan 2013). Large tendon and ligament injuries require surgical treatment and/or surgical reconstruction (Ronel et al. 2004, Maffulli et al. 2012, Moshiri and Oryan 2013). Surgical reconstruction involves the use autografts or allografts as the current biological substitutes in large tendon and ligament injuries (Rodrigues et al. 2013), however both grafts have associated limitations. For example ACL replacement with allogeneic tissue which is from donor recipients, is associated with an increased risk of infection, disease transmission and graft rejection as well as limited tissue availability (Strickland et al. 2003, Robertson et al. 2006). Whilst ACL autogenous graft sources for ACL injury are from the patient's own central third patellar or hamstring tendon, which can avoid complications associated with the use of allografts. Limitations with autografts include donor site morbidity, anterior knee pain, patella fracture, and residual hamstring weakness (Kartus et al. 2001, Tashiro et al. 2003).

Tissue engineering of tendons and ligaments offers an attractive approach to treat tendon and ligament rupture and could have the potential to provide an alternative graft source that avoids the donor site morbidity and is readily available (Rodrigues et al. 2013). A variety of methods are being currently investigated for enhancing repair and regeneration of tendon such as cell therapy, growth factors and gene therapy (Sharma and Maffulli 2005, Moshiri and Oryan 2013, Hirzinger et al. 2014), however this thesis will only focus on currently described scaffold based tendon and ligament tissue engineering strategies

The tissue engineering approaches commonly involve an artificial ECM (scaffold) on which cells can proliferate and differentiate with subsequent new tissue generation (Ahmed et al. 2008). The ideal scaffold should provide a high surface area for cell-polymer interaction, sufficient space for ECM regeneration, and minimal constraint during *in vitro* culture. The scaffold should be biodegradable and resorb once it has served its purpose with its

degradation products not provoking inflammation or toxicity when implanted *in vivo* (Freed et al. 1994).

Current tissue engineering strategies for tendon and ligament involve the use of natural or synthetic scaffolds that are biocompatible and biodegradable. Commonly used synthetic scaffolds for tendon and ligament tissue engineering include chemical substances such as poly-a-hydroxyesters, polyglycolic acid (PLGA) or polylactic acid (PLA) (Kuo et al. 2010, Rodrigues et al. 2013). Advantages of synthetic scaffolds include reproducibility of mechanical and chemical properties and degradation through hydrolysis (Kuo et al. 2010, Moshiri and Oryan 2013). However the lack of functional groups for signalling molecules, the release of acidic by-products and unnatural polyesters into the bloodstream during degradation may cause potential complications (Kuo et al. 2010). Collagen and fibrin are amongst the natural scaffolds. Both collagen and fibrin scaffolds allow cells to create three-dimensional (3D) structures *in vitro* (Kapacee et al. 2008, Abraham et al. 2010). An important trait of fibrin gels that may benefit tendon/ligament tissue engineering is that they allow cells to freely migrate, proliferate, degrade fibrin and produce their own ECM (Huang et al. 2005). In comparison to collagen scaffolds, the fibrin scaffolds do not suppresses ECM synthesis and can promote greater collagen synthesis (Grassl et al. 2002). A recent comparison of fibrin and collagen scaffolds using tendon progenitor cells demonstrated increased tenogenic expression, improved ECM organisation and mechanical properties with fibrin tissue-engineered constructs (Breidenbach et al. 2014). This study indicates that fibrin-based constructs are more suitable for tissue-engineered tendon/ligament repair (Breidenbach et al. 2014). To date engineered tendon and ligament constructs with fibrin scaffolds have been created with cells from different species such rat tendon (Calve et al. 2004), chick embryonic tendon (Kapacee et al. 2008, Kalson et al. 2010, Paxton et al. 2010, Herchenhan et al. 2012), human bone marrow stem cells (Kapacee et al. 2010), human adult ACL (Hagerty et al. 2012) and human adult tendon cells (Bayer et al. 2010), whilst in the dog tendon and ligament cells have not previously been cultured in 3D fibrin culture.

1.10 SUMMARY OF TENDON AND LIGAMENT COMPARISON STUDIES

Studies have demonstrated that while tendons and ligaments are composed of similar proteins, they contain different proportions of ECM macromolecules. This has been demonstrated in both rabbit (Amiel et al. 1984) and ovine (Rumian et al. 2007) tendons and ligaments. While the above studies used biochemical analysis, a recent proteomic study between human patellar tendon and ACL demonstrated differential protein expression of several ECM proteins (Little et al. 2014). These findings might relate to different functions between these tissue types. However function can also be related to the dissimilar structure of tendon and ligament tissue types. Structural differences between tendon and ligament have been demonstrated to some extent by Amiel et al. (1984), Rumian et al. (2007) and Zhu et al. (2012). Amiel et al. (1984) showed a different cell morphology between rabbit intra- and extra-articular ligaments and tendons, while Rumian et al. (2007) demonstrated different collagen fibril diameters between ovine tendons and ligaments, with ligaments (ACL, MCL and LCL) containing a greater proportion of smaller diameter fibrils. Zhu et al. (2012) compared structural differences between human ACL and hamstring tendon and found a more complex arrangement of collagen fibres and a different proteoglycan and distribution in ACL. Neither of the above mentioned studies fully examined the distinct morphological and structural characteristics and the arrangement of ECM components between tendons and ligaments at the different regions.

As highlighted in Section 1.9, tendon and ligament fibroblasts from different species have been used for the creation of tissue engineered tendon and ligament constructs (Bayer et al. 2010, Hagerty et al. 2012, Herchenhan et al. 2013). However it is unknown whether tendon or ligament cells retain the specific identifiable characteristics of the original tissue. It is yet to be determined whether the analogous tendon and ligament constructs reproduce specific phenotypic, biochemical and proteomic characteristics of the tendinous and ligamentous tissue when cultured *in vitro* in 3D fibrin culture.

1.11 HYPOTHESIS AND AIMS

This study will address the hypothesis that canine tendons and ligaments around the stifle joint will be different in terms of their cellular and ECM composition. This difference will be reflected in a distinct cellular morphology, structural arrangement, localisation of ECM molecules and proteome characteristics between tendons and ligaments. It is further hypothesised that canine tendon and ligament cells retain similar morphological, ECM composition and proteomic characteristics to the original tendon and ligament tissue when cultured in 3D fibrin culture.

To answer our hypothesis the aims and objectives of this study are to:

1. Determine measurable differences in cellular and ECM macromolecular composition between canine tendons and ligaments with regard to the location and function of the tissue
2. Identify structural and morphological tissue characteristics using a semi-objective histological scoring system and identify the distribution and localisation of the ECM components in both tissue types.
3. Determine whether canine tendon/ligament cells are able to recapitulate the formation of tendinous or ligamentous tissue using 3D cultures and characterise whether 3D tendon and ligament constructs retain biochemical and morphological characteristic of the original tissue.
4. Identify the differences between the proteomes of native tendon and ligament as well as engineered tendon and ligament 3D constructs.

CHAPTER 2

GENERAL MATERIAL AND METHODS

2.1 TISSUE COLLECTION

The canine cranial cruciate ligament (CCL), medial collateral ligament (MCL), long digital extensor tendon (LDET) and superficial flexor tendon (SDFT) (Figure 2.1) were harvested from disease-free cadaveric canine hindlimbs from skeletally mature Staffordshire bull terrier crossbreeds on the same day of euthanasia. The dogs were euthanased for purposes not related to this study and were clinical waste material donated to the University of Liverpool. Ethical approval was granted by the Veterinary Research Ethical committee (VREC64) to use material in this research project.

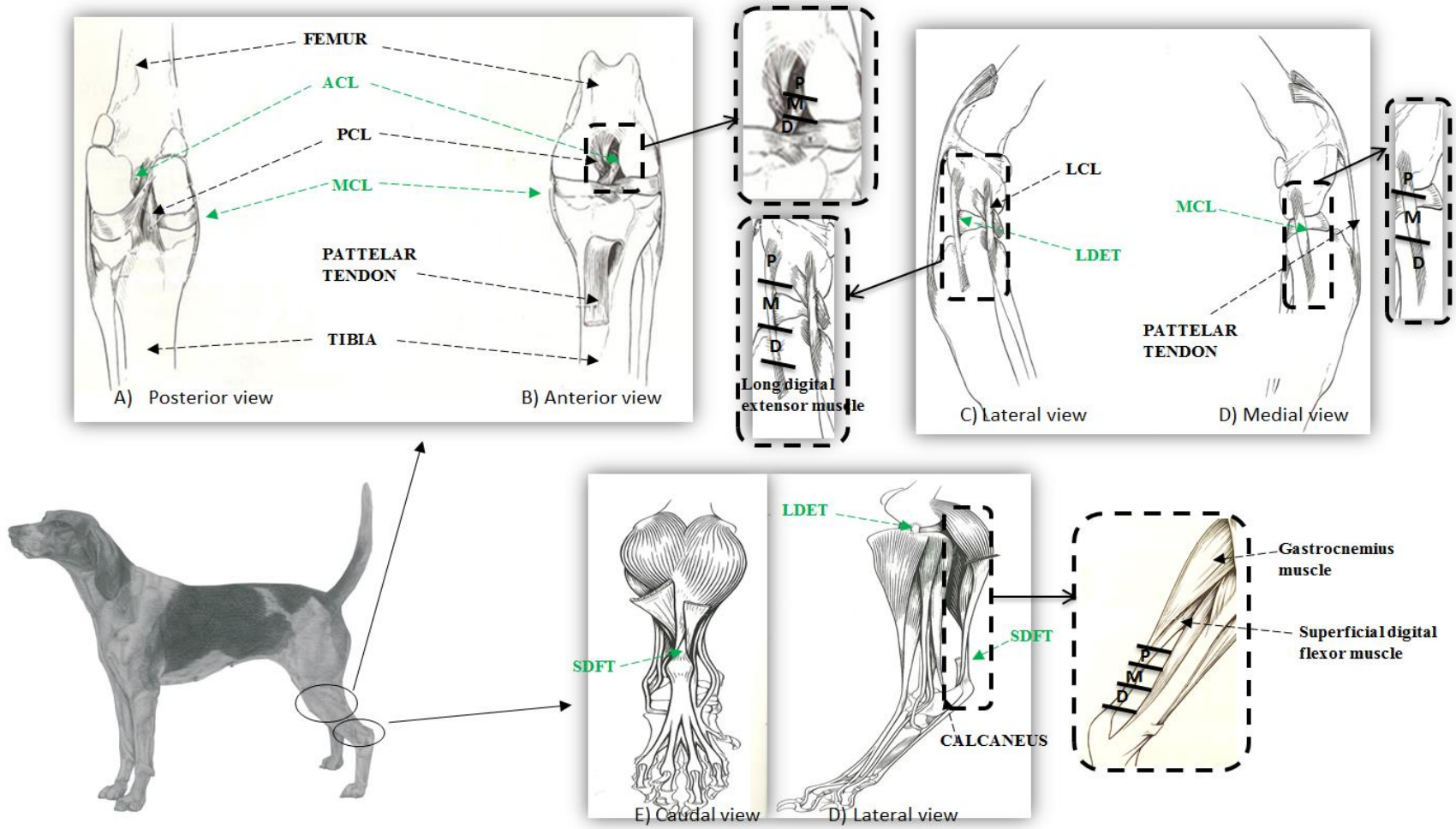


Figure 2.1. Anatomy of canine stifle or knee joint which is adapted from Evans et al. (1979). Locations of tissues collected are indicated by the green arrow. Figure A, B, C and D indicate the left and right stifle with the position of CCL, MCL and LDET. Figure D indicates lateral view of pelvic limb, showing the position of LDET and SDFT. Dashed images represent the location of division into proximal, middle and distal parts of ligaments and tendons.

Sample ID	Tissues obtained		Experimental testing protocol
	Left stifle	Right stifle	
A	CCL, MCL, LDET and SDFT	CCL, MCL, LDET and SDFT	Both left and right knee tissues were used biochemical and histological analysis
B	CCL, MCL, LDET and SDFT	CCL, MCL, LDET and SDFT	Samples A, B and C were used for tissue immunohistochemistry/ immunofluorescence
C	CCL, MCL, LDET and SDFT	CCL, MCL, LDET and SDFT	
D	CCL, MCL, LDET and SDFT	CCL, MCL, LDET and SDFT	
E	CCL, MCL, LDET and SDFT	CCL, MCL, LDET and SDFT	
F	CCL and LDET	CCL and LDET	Left stifle joint samples were used for cell isolation and creation tendon and ligament constructs for biochemical analysis, histology and mass spectrometry
G	CCL and LDET	CCL and LDET	
H	CCL and LDET	CCL and LDET	
I	CCL and LDET	CCL and LDET	Right stifle joint tissues were only used for mass spectrometry
J	CCL and LDET	CCL and LDET	TEM analysis was also performed with constructs created from isolated cells from samples F,G and H of left stifle joint

Table 2.1. Tissue collection and associated experimental testing purposes. All tissues were obtained from skeletally mature (<5 years) Staffordshire bull terrier crossbreeds with disease free stifle joints.

2.2 TISSUE PREPARATION

2.2.1 Samples for biochemistry, histology and immunohistochemistry/immunofluorescence analysis (Chapters 3 and 4)

CCL, MCL, LDET and SDFT were divided into proximal, middle and distal regions (Figure 2.1). In both LDET and SDFT any residual muscle was removed. The AM and PL bundles in CCL were not dissociated in this study. Further subdivision into thirds through each longitudinal section allowed one third to be snap frozen in liquid nitrogen and stored at -80°C for biochemical analysis and one third to be fixed in 4% paraformaldehyde for histological analysis. The remaining third was embedded on a cork disc in Tissue-TEK OCT (Sakura Finetek; Torrance, CA, USA), snap frozen in isopentane and stored at -80°C until required for analysis.

2.2.2 Samples for cell culture, construct development and proteomics (Chapter 5 and 6)

CCL and LDET were obtained for creation of tendon and ligament constructs and for proteomic analysis. The left stifle joint tissues were used for cell culture and development of tendon and ligament constructs, while the right stifle joint tissues were used for proteomic analysis (Table 2.1).

For proteomics analysis native LDET and CCL tissues of the right stifle joint were prepared by firstly removing the LDET muscle and washing three times in PBS. Tissues were then transferred into clean eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C until further analysis (Section 2.8).

2.3 DEVELOPMENT OF TISSUE ENGINEERED LIGAMENT AND TENDON CONSTRUCTS

2.3.1 Cell isolation and processing

CCLs and LDETs were sectioned into small pieces of around 1 mm^3 and placed in Dulbecco's modified Eagle's medium (DMEM, life technologies, MD, USA) containing 10% foetal bovine serum (Gibco, Paisley, UK), 100 units/ml penicillin/streptomycin (Penstrep, Life technologies, Paisley, UK), 500 ng/ml Amphotericin (Life technologies, Paisley, UK), 20mM L-glutamine (Life Techonologies, Paisley, UK) and 0.1% collagenase (Worthington, Lakewood, NJ). The solution was incubated at 37°C overnight and then filtered through a 70 µm cell strainer (SLS, Yorkshire, UK). Following centrifugation at 1100 rpm for 4 minutes, cells were resuspended with 12 mls of DMEM, 10% FBS and antibiotics. Both ligamentocytes and tenocytes were grown to ~95-100% confluence at 37°C in 5% carbon dioxide. Tenocytes and ligamentocytes were split no further than the 3rd passage prior to being used in construct set up.

2.3.2 Preparation of culture plates

Each well of a six well plate was coated with a ~2 mm- thick layer (1.5 ml) of SYLGARD (SYLG184, WPI, Hertfordshire, UK) and incubated overnight at 55ºC to induce polymerisation. Next, two short silk sutures (0.8 cm) (W328H, sutures Ethicon, Bunzl Healthcare, London, UK) were pinned onto the coated plates with minutien insect pins (0.1 mm diameter) (Fine Science Tools, Interfocus Ltd, Cambridge, UK). Sutures were pinned at a distance of 1 cm. Prior to cell seeding each six well plate was sterilised by immersion in 100% ethanol for 1 hour under UV light.

2.3.3 Tendon and ligament construct development

Both tenocytes and ligamentocytes were removed from tissue culture flasks using trypsin-EDTA (Lonza, UK), centrifuged at 300 g for 10 min and re-suspended inculture medium containing DMEM supplemented with penicillin (100U/ml), streptomycin (100 µg/ml), L-

glutamine (2 mM) and FCS (10%). The cell density was measured, where 10 µl of cell suspension was pipetted into a haemocytometer (Hawskley, Sussex, UK) and manually counted. For three technical replicates of fibrin constructs, 1.2 mls of $1.5x10^6$ cells/ml were resuspended in 250 µl of 20 mg/ml fibrinogen (F8630, Sigma-Aldrich, UK) and 25 µl of 200 U/ml thrombin (T4648, Sigma-Aldrich, UK), 480 µl of the mixture was immediately deposited in each well and vigorously shaken to ensure even mixing and formation of a flat layer of the fibrin gel. After the deposition of the mixture the plates were incubated for 30 minutes at 37°C, 5% CO_2 to allow the gels to set. Cell-fibrin layers were scored with a fine pipette tip to prevent adhesion to the side of the well, then incubated with 2 mL culture medium (as above) supplemented with L-ascorbic acid 2-phosphate (200 µM; Sigma-Aldrich, UK), non-essential amino acids at 10 µl/ml (Sigma-Aldrich, UK) and aprotinin at 10 µl/ml. The gel was scored and media changed every two days until it had contracted to form a linear construct between the pinned sutures. All constructs were fully contracted between the fixed anchors at fourteen days.

2.3.4 Harvesting of constructs

Constructs formed were excised from the plates using a scalpel blade. Both tendon and ligament constructs were washed for three times in PBS, snap frozen in liquid nitrogen and stored at -80ºC until ready for biochemical and proteomic analysis. Constructs were also fixed in 4% paraformaldehyde and 2.5% glutaraldehyde for histology staining and transmission electron microscopy (Section 2.7) respectively.

2.4 BIOCHEMICAL ANALYSIS

Double stranded DNA (dsDNA), total sulphated glycosaminoglycan (sGAG), total collagen and elastin content were measured. The details of each assay are provided below.

2.4.1 Water content

Samples were thawed at room temperature and wet weight measured. To obtain water content and dry weight samples were freeze dried overnight at -60°C and reweighed.

2.4.2 Papain digestion

A papain digest was performed to determine the dsDNA, total collagen and sulphated glycosaminoglycans (sGAG) content of ligament and tendon samples. Papain buffer (500 µl; 10 unit/ml papain (P4762, Sigma-Aldrich, UK) in sterile phosphate buffered saline (PBS) with 100mM sodium acetate, 2.4mM EDTA and 5mM cysteine HCL, pH 5.8) was added to the samples (5-20 mg dry weight) which were then incubated for 24hrs at 60°C (Farndale et al. 1986). The samples were vortex mixed several times during digestion to ensure all tissue was solubilised. Once completely solubilised samples were stored at -20°C until used for analysis.

2.4.3 Oxalic acid digestion

Oxalic acid digestion was performed to extract the insoluble elastin from the tissue in the form of soluble cross-linked polypeptide elastin fragments; α-elastin. This was done by adding 750 µl of 0.25 M oxalic acid (35295, Sigma-Aldrich, UK) and by heating samples to 95°C. They were centrifuged at 3000g for 10 minutes and the supernatant extracted. This process was repeated 5 times for all tissues to extract all elastin.

2.4.4 Cellular content

2.4.4.1 Double stranded DNA content

Total DNA was measured using Quant-iT^{TM} Picogreen ® dsDNA reagent and kits (P7589, Invitrogen, UK). Quant-iT^{TM} PicoGreen® dsDNA reagent is an ultrasensitive fluorescent nucleic acid stain for quantitating double stranded DNA in solution (Singer et al. 1997). Triplicate aliquots of 100 µl of pre-diluted papain-digested ligament and tendon samples were incubated with 100 µl Quant-iT^{TM} Picogreen® dsDNA reagent for 5 minutes at room temperature, protected from the light. Fluorescence was read on fluorescence microplate reader (Bio-Tek FLX800) at wavelength 480 nm excitation and 520 nm emission. DNA concentration was calculated by comparing to a standard curve constructed using bacteriophage lambda DNA standards from 0-1000 ng/ml. DNA content was expressed as µg per mg dry weight tissue.

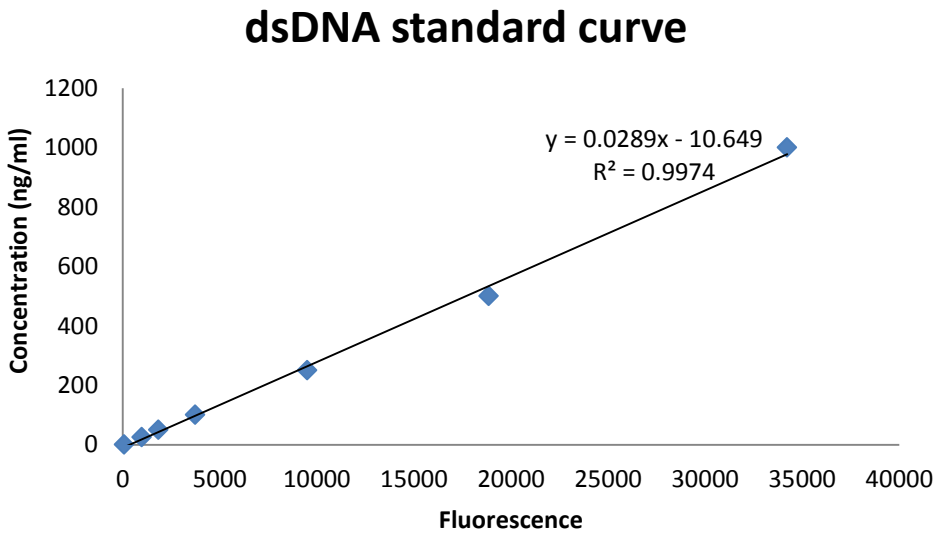


Figure 2.2. Typical standard curve for dsDNA quantification. Final concentrations of λDNA (100 µl) ranging from 1-1000 ng/ml were entered into wells of 96-well plate in triplicate and 100 µl Quant-iT^{TM} Reagent PicoGreen® reagent dye was added into each well. After 5 minutes incubation the fluorescence value were read at 480 nm/520 nm excitation and emission.

2.4.5 Extracellular matrix composition

2.4.5.1 Sulphated glycosaminoglycan content

Total sulphated GAG was measured using the 1, 9-dimethlylmethylene blue (DMMB) dye binding assay, which forms a complex that absorbs light and is measured spectrophotometrically (Farndale et al. 1986). In brief, 40 µl aliquots of papain-digested ligaments and tendons samples were placed into a transparent flat bottomed 96-well plates. From the stock solution of DMMB dye (16 mg 1-9 dimethyl methylene blue, 2 g sodium formate, 2 ml formic acid; made up to 1 litre dH_20, pH 3.5), 250 µl was pipetted into each sample and standard. Absorbance was immediately read on the spectrophotometer at a wavelength of 570 nm. The assay was calibrated by the use of standards of shark chondroitin sulphate (C4384, Sigma-Aldrich, UK) up to 70 µg/ml, and sGAG concentration obtained by comparison with the standard curve. sGAG concentration was expressed as µg per mg dry weight tissue.

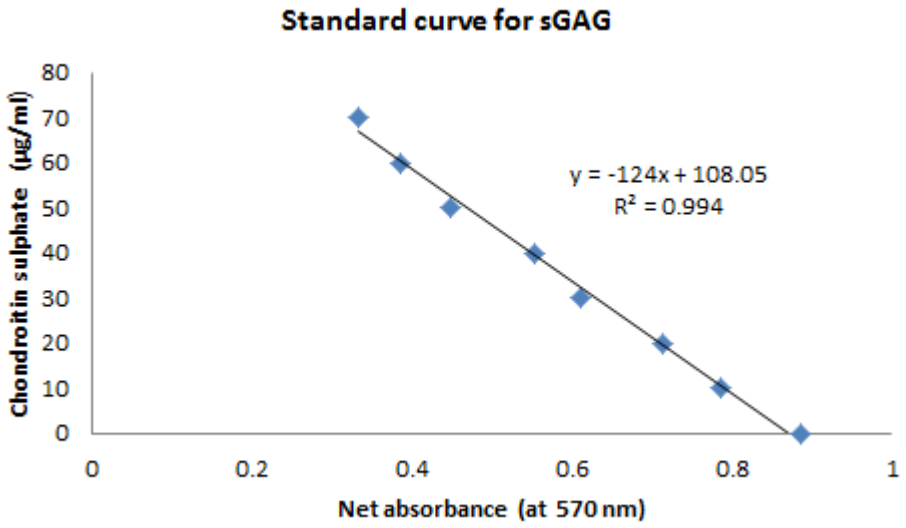


Figure 2.3.Typical standard curve for sGAG quantification. Final concentrations of chondroitin sulphate (40 µl) ranging from 0-70 µg/ml were entered into wells of 96 well plate in triplicate and 250 µl DMMB assay dye added into each well. Absorbance was immediately read on a spectrophotometer at an absorbance of 570 nm.

2.4.5.2 Collagen content

Collagen content was calculated by measuring the concentration of the imino acid hydroxyproline (Bergman and Loxley 1963). This assay involves the oxidation by chloramine T, coupled with dimethylaminobenzaldehyde (DMBA) resulting in a coloured product which can be visualised at 550 nm using a spectrophotometer. Aliquots of papain digested tissue (100 µl) were hydrolysed with 100 µl of concentrated (12 M) hydrochloric acid (HCL), dried and re-dissolved in 10 ml deionised water. Samples (1 ml) were then mixed with 1 ml diluent (2 parts propan-2-ol to 1 part deionised water), 1 ml of oxidant was added (0.42 g chloroamine T, 5 ml deionised water. water, 25 ml stock buffer) and the samples left to stand for 20 minutes. Colour reagent (1 ml; 3 g DMBA, 4.5 ml 70% percholoric acid, 25 ml propan-2-ol) was then added and the samples left for 14 minutes before heating (70°C, 20 minutes). Absorbance was read on a spectrophotometer (CECIL CE2040, CECIL instrument limited) after allowing the samples to cool for 10 minutes. Concentration was calculated by comparison to a standard curve prepared using L-hydroxyproline standards (56250, Sigma-Aldrich, UK) from 0-10 µg/ml (final concentration). Collagen content was calculated assuming hydroxyproline to be present at 14%. The total collagen content was then expressed as a percentage of dry tissue weight (Rumian et al. 2007).

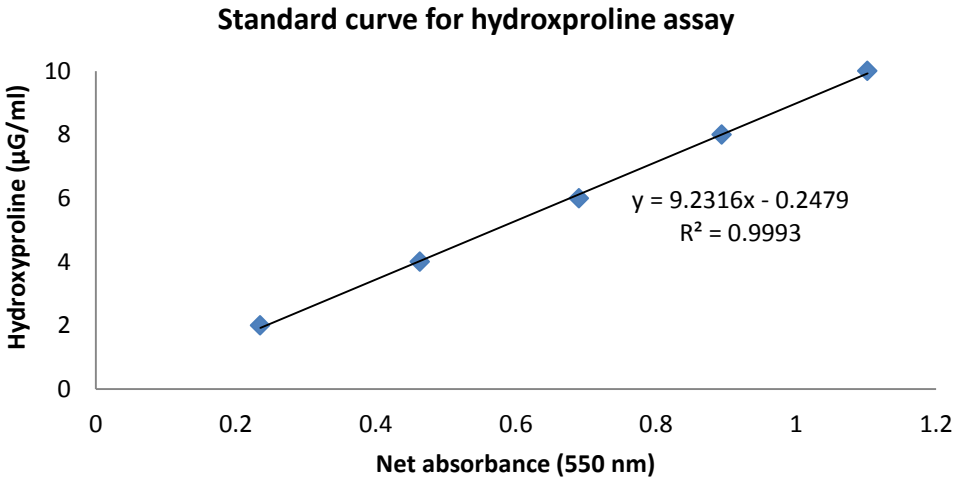


Figure 2.4. Typical standard curve for hydroxproline quantification. Final concentrations of hydroxyproline (1 ml) ranging from (0-10 µg/ml) were mixed with 1 ml diluent and 1 ml of oxidant. Colour reagent was added and samples incubated at 70°C for 20 minutes. Absorbance was read on a spectrophotometer at an absorbance of 550 nm.

2.4.5.3 Elastin content

Elastin was measured using the Fastin™ dye-binding assay (Biocolor Ltd, N. Ireland) previously described by Smith et al. (2013). The average dry weight of samples used for the elastin assay was 14.3mg. Oxalic acid extracts were pooled together and were precipitated with elastin precipitation reagent (trichloroacetic acid and hydrochloric acid). Following centrifugation (10000g) for 10 minutes, the precipitate was incubated on a mechanical shaker with 1ml of elastin fastin dye reagent (5,10,15,20-tetraphenyl-21,23-porphine tetrasulphonate) for 90 minutes to allow reaction between α-elastin and the dye. Following centrifugation (1000g) for 10 minutes, the bound dye was suspended in a 250 µl of dissociation agent (guanidine HCl and propan-1-ol) and transferred to a 96 well plate after 10 minutes to ensure that all bound dye had passed into solution. Absorbance was measured on the plate reader (Mulltiskan EX, Therma) at 550nm and compared to a standard curve generated from known concentrations of alpha-elastin. Elastin concentration was expressed as a percentage of dry weight.

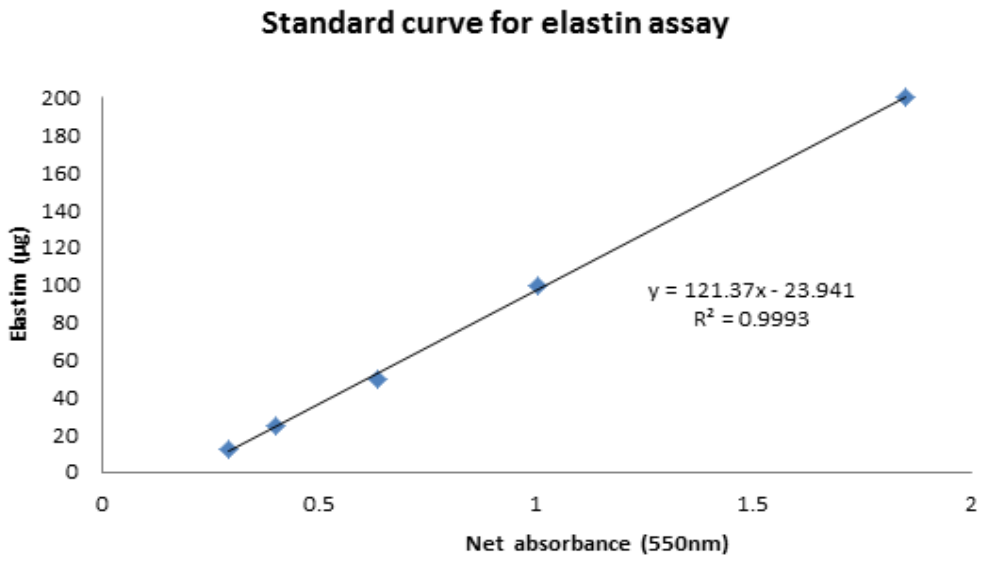


Figure 2.5. Typical standard curve for elastin quantification. Final concentrations of α-elastin (0-100 µl) ranging from (0-100 µg) were entered into 1.5 ml eppendorf tubes, precipitated with an equal volume of elastin precipitation reagent and incubated with elastin bind dye complex. The bound dye was suspended with 250µl of dissociation reagent and transferred into duplicate wells of a 96 well plate. Absorbance was read on a spectrophotometer at an absorbance of 550 nm.

2.5 HISTOLOGY STAINING AND SCORING

2.5.1 Sample preparation

Tendon and ligament native samples (Table 2.1: Samples A-E) and constructs created from isolated cell samples (Table 2.1: Samples F-J) were fixed in 4% neutral buffered paraformaldehyde (PFA) and further processed by the Division of Veterinary Pathology, School of Veterinary Science, where samples were longitudinally embedded in paraffin, cut in 4 μm sections and mounted on polylysine slides

2.5.2 Histology staining

Histological sections were stained with Hematoxilin and Eosin (H&E), Alcian blue periodic acid Schiff (PAS) and Miller's stain. In addition to the above mentioned stains, tendon and ligament constructs were also stained with Masson's trichrome. All histological sections were visualised using a Nikon eclipse 80i microscope and pictures were acquired with Nikon DS-L2 standalone control unit.

2.5.2.1 Alcian blue and periodic acid Schiff (AB-PAS) stain

The AB-PAS was used for the detection of the areas rich in GAGs (John and Bancroft 1996). Sections were deparaffinised and rehydrated in two washes of xylene, 100% and 95% ethanol for 10 minutes and two washes of ultrapure water for 5 minutes. Once rehydrated, the slides were stained in 1x Alcian blue (1 g Alcian blue, 3ml glacial acetic acid, 97 mls of distilled water) for 3 minutes. The slides were then washed in running water for 2 minutes, rinsed in distilled water and treated with 0.5% periodic acid (HC6405, TCS Biosciences Ltd, Buckingham, UK) for 10 minutes. The slides were then further washed in running tap water for 5 minutes, rinsed in distilled water, treated with Schiff's reagent (3952016, Sigma-Aldrich, UK) for 15 minutes and washed in running tap water for 5 minutes. The nuclei were stained with Mayer Haemalum for 2 minutes and washed in tap water for 5 minutes. The slides were then dehydrated in two washes of 95% ethanol, 100% ethanol, and xylene for 10

seconds in the first wash and 5 minutes in the second. Once dehydrated, the slides were coverslipped with D.P.X. (Di-n-butyl phthalate in xylene, Sigma-Aldrich, UK) mounting media.

2.5.2.2 Miller's stain

Miller's stain was used to stain for elastic fibres (Miller 1971). In brief, sections were deparaffinised and rehydrated, treated with 1% potassium permanganate for 5 minutes and rinsed in distilled water. The slides were then decolourised in 1% oxalic acid for 10 minutes followed by a brief wash in running water and a rinse in 95% ethanol untill the colour disappeared. The slides were stained with Miller's stain (HS235, TCS Biosciences Ltd, Buckingham, UK) for 3 hours, rinsed in 95% alcohol to remove the excess stain and washed in running tap water for 3 minutes. Slides were counter stained with Van Gieson (HS780, TCS, Biosciences LtD, Buckingham, UK) for 4 minutes followed by dehydration and mounted with D.P.X.

2.5.3 Histology scoring methods

Histological sections of the CCL, MCL, LDET and SDFT were scored by a three part scoring system to assess the cells and extracellular matrix of the tissues. All sections were read by two observers blinded to section location and tissue type on two separate occasions at least two weeks apart.

H&E- H&E sections were assessed to determine differences in terms of tissue architecture, cell morphology, cell distribution, vascularization and inflammation. The scoring system was modified from Stoll, John et al (2011), whereby each parameter was numerically graded from 0-2 based on changes seen for each parameter listed (Table 2.2). For each parameter the average score between inter and intra-observers was converted into percentage distribution by counting the occurrence of the graded numerical scores. This was multiplied by 100% and then divided by the number of biological replicates. For instance for ECM organisation parameter, the presence of grade 2 and 1 and 0 score was counted, the

number of occurrence of each grade was then converted to percentage score as explained above.

Miller's stain- A modified scoring system from Smith (2010) was used to quantify the differences in term of elastin and microfibril staining. In brief, the distribution and increased staining in interfascicular, interbundle and ligament substance regions, as well as the extent and degree of pericellular staining was scored. For the general distribution and presence of pericellular staining a score between 0-1 was given. For the interfascicular, interbundle, intrabundle and pericellular staining a score from 0-3 was awarded based on the extent of staining 0%= 0, absent, 0-25%= 1, staining percent in up to 25% of tissue, 25-50%=2, staining present in 25-50 percent of tissue, >50%= 3, marked staining in above 50% of the tissue. The overall score was added up for each sample, giving a range of 0-14. The average scores within and between observers was calculated (Table 2.3).

AB-PAS stain- a similar scoring system like the Miller's score was self-developed based on Smith (2010) to quantify differences in GAGs staining. For general GAG distribution and chondrocytic cell change shape a score between 0-1 was given. For more detailed location of staining i.e. interfascicular, interbundle, substance and cellular staining, a score from 0-3 was awarded based on the extent of positive staining, The overall score was added up for each sample, giving a range of 0-14. The average scores between inter- and intra-observers were calculated (Table 2.4).

Each parameter of H&E scoring, Miller's scoring and AB-PAS scoring were assessed for agreement between inter and intra-observer scorings results using a Kendall's coefficient concordance.

1) Extracellular matrix organization of the whole ligament or tendon

Score

- Wavy, compact and parallel arranged collagen fibre (Normal collagen architecture and density) — 2
- In part compact, in part loose or not orderly (Reduced density) — 1
- Loosely composed, not orderly (Absent, minimal density) — 0

2) Cellular shape, distribution and alignment

a) Shape

- Spindle shape (normal) — 2
- Mixed — 1
- Oval to round shape — 0

b) Distribution

- Normal distribution of cells — 1
- focal areas of elevated cell density (Cell clustering or formation of cell chains) — 0

c) *Alignment*

- Uniaxial — 2
- Areas of irregular arranged cells — 1
- More than 50% of cell with no uniaxial alignment — 0

3) Vascularity of the whole ligament and/or tendon

- hypo-vascularised — 1
- hyper-vascularised (increased number of smaller or larger capillaries) — 0

Table 2.2. H&E scoring sheet adapted from Stoll, John et al (2011)

1) Distribution and location of Microfibril staining **Score**

a) *Distribution*

- Normal (sparse fibres throughout) 0
- Increased 1

b) *Location*

	>50%	25-50%	0-25%	0%
• Interfascicular				
	>50%	25-50%	0-25%	0%
• Interbundle				
	>50%	25-50%	0-25%	0%
• Substance				

2) Pericellular staining

a) Pericellular deposition

- not present 0
- present 1

b) Degree of pericellular deposition

>50%	25-50%	0-25%	0%

Table 2.3. Miller's scoring sheet adapted from Smith, Clegg et al. (2013).

1) Glycosaminoglycans distribution and location

a) Overall

- normal (absent) 0
- Increased (focal or generalised) 1

b) location

	>50%	25-50%	0-25%	0%
• Interfascicular				
	>50%	25-50%	0-25%	0%
• Interbundle				
	>50%	25-50%	0-25%	0%
• Substance				

2) Cell with 'chondroid' change

a) Cell shape

- No change (well-spaced, spindle shaped) 0
- Oval to rounding of nuclei (halos around them) 1

b) Location of stain with cells

	>50%	25-50%	0-25%	0%
• Around the cells				

Table 2.4. Alcian-blue-PAS scoring sheet.

2.6 TISSUE IMMUNOSTAINING

2.6.1 Immunohistochemistry for collagens and proteoglycans staining

For collagen type I, III. VI, asporin, decorin, biglycan, keratocan, agreccan, and versican immunostaining (Table 2.5) sequential sections of 4 µm from paraffin-embedded samples (Table 2.1: Samples A-C) were deparaffinised and rehydrated in two washes of xylene, 100% and 95% ethanol solution for 10 minutes and in two washes of ultrapure water for 5 minutes.

Endogenous peroxidase activity was blocked by incubating the tissue sections with 3% H_2O_2 for 10 minutes. After endogenous peroxidase block, the slides were washed two times for 5 minutes in water and then in Tris buffered saline (TBS) for an additional 5 minutes. Sections for decorin, biglycan, asporin, keratocan and versican immunostaining were pre-digested with chondroitinase ABC (Sigma) (0.5 U/ml) in 100 mM Tris-HCL pH 7.2-7.4 for 30 minutes at room temperature followed by two washes of 5 minutes in TBS.

Each section was subsequently blocked with 200 µl of 10% normal goat serum (PCN500, Invitrogen, CA, USA) for 1 hour at room temperature to prevent non-specific antibody binding. The solution was replaced with 100µl of primary antibody diluted in TBS (Table 2.5). The slides were incubated overnight at 4°C. After incubation, the sections were washed for three times for 5 minutes in TBS and incubated with appropriate of secondary antibody for 1 hour at room temperature. For the primary antibodies that were raised in rabbit, 100µl of Zytochem Plus HRP polymer goat antirabbit was used as secondary antibody (ZUC032), Zytomed system, DE). For the primary antibodies that were raised in mouse, 100µl goat antimouse was used (A0545, Sigma-Aldrich, UK) as secondary antibody.

Following incubation with secondary antibody, the slides were washed three times in TBS before adding 400µl of 3,3- diaminonemzidine (Sigmafast DAB, Sigma Aldrich, UK) for 1 minute. The slides were immersed in ultrapure water for 5 minutes, counterstained with Mayer's Haemalum for 1 minute and washed in tap water. The sections were then dehydrated and mounted with D.P.X.

As control, non-immune polyclonal rabbit or mouse IgG (isotope control) (Abcam, Cambridge, UK) and buffer were used instead of the primary antibodies.

Every collagen and proteoglycan antibody has been validated using western blot analysis. All immunohistological sections were examined using a Nikon eclipse 80i microscope and images were acquired with a Nikon DS-L2 standalone control unit.

2.6.2 Immunofluorescence for elastic fibre staining

Elastic fibre immunostaining were assessed using sections stained with elastin, fibrillin 1 and fibrillin 2 antibodies (Table 2.5), previously described by Smith et al. (2011). (Table 2.1, Samples A-C). Elastin was dual stained with fibrillin 1 or fibrillin 2 together (Table 2.5). Longitudinal sections (5 µm) of OCT-embedded samples were cut on a cryostat (Leica CM 1900), adhered to poly-L-lysine slide (polysine, VWR, UK), allowed to dry and stored at -20°C until required.

Tissue sections were fixed for 20 minutes in 100% methanol at -20°C and then rinsed three times for 5 minutes in PBS. This was followed by an overnight incubation at room temperature with hyaluronidase (4800 IU/ml in PBS, H3884) solution containing a protease inhibitor cocktail (complete mini, Roche). Sections were rinsed three times for 5 minutes with PBS, and then blocked with 200 µl of 10% normal goat serum (PCN5000, Invitrogen, CA, USA) in PBS for 1 hour at room temperature. After blocking the goat serum was drained off and the tissue sections were incubated overnight at 4°C with 100 µl of primary elastin antibody diluted in PBS. The sections were washed three times for 5 minutes in PBS and followed by a second overnight incubation at 4°C with 100µl of fibrillin 1 or 2 antibody. Slides were rinsed in three times in PBS for 5 minutes, followed by secondary antibody incubation (100ul) (antimouse IgG or antirabbit IgG) for 1 hour at room temperature and rinsed in PBS.

After washing, all section were incubated with DAPI (1:1000 diluted in PBS) (Invitrogen, USA) for 5 minutes at room temperature, rinsed with PBS and mounted with mounting media (H-1400, Vector Laboratories, UK).

Negative controls were included with rabbit and mouse Isotope IgG and normal serum in place of primary antibody. Elastin and fibrillin antibodies have previously been validated in canine connective tissue (Smith et al. 2011).

Sections were examined with confocal microscope (Nikon Ti Eclipse) and images were captured with Nikon EZ-C1 free viewer (version 3.90, Nikon corporation).

Primary antibody	Manufacturer	Secondary antibody
Collagen I (1:100)	Abcam (ab292)	ZytoChemPlus (HRP) Polymer anti-Rabbit (ZUC032)
Collagen III (1:100)	Abcam (ab7778)	ZytoChemPlus (HRP) Polymer anti-Rabbit (ZUC032)
Collagen VI (1:100)	Abcam (ab6588)	ZytoChemPlus (HRP) Polymer anti-Rabbit (ZUC032)
Asporin (1:100)	Abcam (ab58741)	ZytoChemPlus (HRP) Polymer anti-Rabbit (ZUC032)
Decorin (70.6) (1:50)	Donated by B.Caterson/ C Hughes (Cardif university)	Anti-Mouse IgG (1:50) (A4416, Sigma, UK)
Biglycan (PR8A4) (1:50)	Donated by B.Caterson/C.Hughes	Anti-Mouse IgG (1:50) (A4416, Sigma, UK)
Keratocan (KER-1) (1:50)	Donated by B.Caterson/ C. Hughes	Anti-Mouse IgG (1:50) (A4416, Sigma, UK)
Agreccan (7D1) (1:50)	Donated by B.Caterson/C. Hughes	Anti-Mouse IgG (1:50) (A4416, Sigma, UK)
Versican (1:100)	Hybridoma (12C5)	Anti-Mouse IgG (1:50) (A4416, Sigma, UK)
Elastin (1:100)	Abcam (ab9519)	Anti-Mouse IgG (1:500) (A11001, Invitrogen, USA)
Fibrillin 1 (1:50)	Donated by B. Mecham (Washington University)	Anti-Rabbit IgG (1:500) (A11011, Invitrogen, USA)
Fibrillin 2 (1:50)	Donated by Tim Ritty (Penn State University)	Anti-Rabbit IgG (1:500) (A11011, Invitrogen, USA)

Table 2.5. Primary and secondary antibodies used in tissue immunostaining for detection ECM macromolecules.

2.7 TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy (TEM) of LDET and CCL constructs (Table 2.1: Samples F-H) was performed by Marion Pope in the School of Veterinary Science, Division of Veterinary Pathology. In brief, constructs were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for about 8 hours. After three times washing, this was followed by a secondary fixation and contrast stain with 1% osmium tetroxide for 90 minutes. Samples were stained with 8% uranyl acetate in 0.69% maleic acid for 90 minutes, dehydrated in ascending ethanol concentrations (50,70, 90,95%) and embedded in epoxy resin. Ultrathin cross-sections (60-90 nm) were cut with a Reichert- Jung Ultracut on an ultramicrotome using a diamond knife. Cut cross sections were then mounted on 200 mesh copper grids and stained with 'Reynold's Lead citrate' stain for 4 minutes. Images were viewed in Philips EM208S Transmission Electron Microscope at 80kV.

2.8 PROTEOMIC ANALYSIS

2.8.1 Sample preparation

Both native LDET, CCL and harvested tendon and ligament constructs samples (Table 2.1, Samples F-J) were first washed with PBS, snap frozen in liquid nitrogen and stored at -80°C until required. Prior to protein extraction all samples were freeze dried overnight and dry weight was measured.

2.8.2 Protein extraction

All lyophilised samples were disrupted using a micro-Dismembrator U (B.Braun Biotech. International, Germany). 200 µl of chondroitinase buffer (100 mM Trisacetate, mini protease inhibitor cocktail with EDA (Roche, UK) containing 1 unit/ml chondroitinase ABC (C2905-10 UN , Sigma-Aldrich, St Louise, MO) was added to approximately 3 mg of dismembranated lyophilised tissue. Samples were then placed in hybridizer roller tube at 37°C and incubated for 6 hours. After chondroitinase treatment, samples were centrifuged at 15000 rpm for 15 minutes and media was removed. 500 µl of GnHCL extraction buffer (4 M guanidine HCL, 50 mM sodium acetate, 65 mM DTT with mini protease inhibitor cocktail tablet) was added to the media removed from the samples and incubated for 48 hours at 4°C on a shaker. The samples were then centrifuged at 15000 rpm at 4°C for 15 minutes and the soluble fraction removed. Both the soluble and insoluble fractions were stored at -20°C. The soluble fraction was used for in-solution trypsin digestion and analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) (see Section 2.8.5), but prior to that protein concentration was measured and an aliquot of each sample run on a 4-12% Tris-Bris SDS-PAGE gel (section 2.8.4).

2.8.3 Protein concentration

The protein concentration of the samples was measured with the Pierce™ 660nm protein assay (Thermo scientific, Hertfordshire, UK.). Samples were diluted with water and 50µl of each sample was added to a 96-well plate with 150 µl of the protein assay reagent. The plate was shaken for 1 minutes and left to incubate at room temperature for 5 minutes. Absorbance was measured on a plate reader at 650 nm (Mulltiskan EX, Therma). Concentration are calculated by comparison to a standard curve (Figure 2.6) prepared using BSA (0-2 mg/ml).

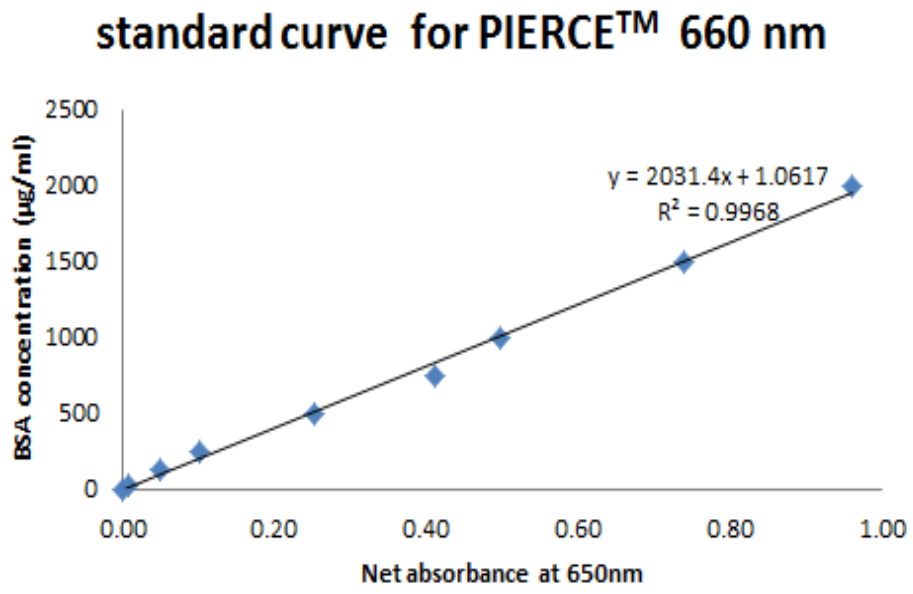


Figure 2.6. Typical standard curve for PIERCE™ protein assay. Final concentrations of BSA (50 µl) ranging from (0-2 µg/ml) were measured in a 96-well-plate with 150 µl of protein assay reagent. Absorbance was read on a spectrophotometer at an absorbance of 650nm.

2.8.4 SDS-PAGE and Silver stain

A 50µl (~50 µg) aliquot of the GnHCL soluble fraction was precipitated overnight with 100% cold ethanol, centrifuged for 15 minutes at 1200 rpm speed at 4°C and washed with 90% cold ethanol. Samples were re-centrifuged for 15 minutes at maximum speed at 4°C. The ethanol was discarded and the pellet air dried for 3 hours. The air dried pellet was then resuspended in 20 µl of 7 M Urea, 2 M Thiourea, 4% Chap and 3 mM Tris at pH 8. Once mixed, 10 µl of 2x SDS loading buffer containing 8% mercaptoethanol was added to the samples.

2.8.5 In-solution tryptic digestion and LC-MS/MS

The soluble 4M GnHCL extracted proteins were send to the University of Liverpool Centre for Proteome Research facility. In-solution tryptic digestion was performed by one of the technical team, Dr Deborah Simpson, who also ran the samples on liquid chromatography tandem mass spectrometry.

For tryptic digestion, the samples were 8-fold diluted to 0.5 M with 100 mM ammonium bicarbonate (AMBIC) and normalised to the sample that had lowest protein concentration. This gave about 18.5 µg of protein from each sample which were diluted to a volume of 160uL with AMBIC. Each protein sample was supplemented with digestion enhancer; 10 µl Rapigest for 10 minutes at 80°C (Waters, Manchester, UK). Protein samples were reduced and alkylated with DTT (10 µl of 9.2 mg/ml at 60°C for 10minutes) and then IAA added (10 µl of 33mg/ml for 30min in the dark room temperature). Trypsin (10 µl) was added and incubated overnight at 37°C and inactivated with 0.5% trifluoroacetic acid for 30 minutes. A desalting procedure (Zip-tip) on trypsin digested samples was performed. About ~93 ng protein per each sample was loaded on liquid chromatography and mass spectrometry.

2.8.6 Liquid chromatography and tandem mass spectrometry

Liquid chromatography separation All peptide separations were carried out using an Ultimate 3000 nano system (Dionex/Thermo Fisher Scientific). For each analysis the sample was loaded onto a trap column (Acclaim PepMap 100, 2 cm x 75 µm inner diameter, C_{18}, 3 µm, 100Å) at 5 µL/min with an aqueous solution containing 0.1% (v/v) TFA and 2%(v/v) acetonitrile. After 3 min, the trap column was set in-line with an analytical column (Easy-Spray PepMap® RSLC 15cm x 75 µm inner diameter, C_{18}, 2 µm, 100Å) (Dionex). Peptide elution was performed by applying a mixture of solvents A and B. Solvent A was HPLC grade water with 0.1%(v/v) formic acid, and solvent B was HPLC grade acetonitrile 80%(v/v) with 0.1%(v/v) formic acid. Separations were performed by applying a linear gradient of 3.8% to 50% solvent B over 30 minutes at 300 nL/min followed by a washing step (5 minutes at 99% solvent B) and an equilibration step (10 minutes at 3.8% solvent B).

Q Exactive set-up- The Q Exactive instrument was operated in data dependent positive (ESI+) mode to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (*m/z* 300-2000) were acquired in the Orbitrap with 70,000 resolution (*m/*z 200) after accumulation of ions to $1x10^6$ target value based on predictive automatic gain control (AGC) values from the previous full scan. Dynamic exclusion was set to 20s. The 10 most intense multiply charged ions ($z \geq 2$) were sequentially isolated and fragmented in the octopole collision cell by higher energy collisional dissociation (HCD) with a fixed injection time of 100ms and 35,000 resolution. Typical mass spectrometric conditions were as follows: spray voltage, 1.9kV, no sheath or auxillary gas flow; heated capillary temperature, 250°C; normalised HCD collision energy 30%. The MS/MS ion selection threshold was set to 1×10^4 counts and a 2 *m/z* isolation width was set.

2.8.7 Proteomic data analysis

Proteomic data was analysed for protein identification, label-free quantification, and post translation modifications. PEAKS® software (Version 6, Bioinformatics Solutions, Waterloo, Canada) and Progenesis™ LC-MS software (Nonlinear dynamics) were used for data analysis. Raw MS/MS data were imported into PEAKS® where the peptides and proteins were identified. PEAKS® searches were performed against the canine taxonomy in which protein sequence database was imported from ensemble (http://www.ensembl.org/info/data/ftp/index.html). Details of PEAKS® search parameters, filter parameter and classification of protein categories is described in Chapter 6 (Section 6.3.5a).

For label free quantitative analysis PEAKS® data search results were imported into Progenesis™ LC-MS software, where statistical significant differentially abundant proteins were identified between group comparisons (Chapter 6, section 6.3.5c).

Post-translational modifications were assessed using the PEAKS® PTM finder algorithm (Chapter 6, Section 6.3.5c).

2.9 GENERAL STATISTICAL ANALYSIS

Statistical analysis was performed on biochemical assays data and histology scoring results. A one-way of analysis of variance (ANOVA) with Bonferroni post-hoc test was performed using Graphpad Prism (version 6, GraphPad Software, La Jolla California, USA). A Univariate analysis was also performed using SPSS (IBM SPSS Statistics, Version 20.0, and Chicago). The significance level was set at $p<0.05$ and exact p-values are presented for all data sets as appropriate.

For proteomic label free quantification data sets, one-way ANOVA was performed by Progenesis™ LC-MS software. Identified proteins were regarded as significant, with adjusted p-value to false discovery (FDR) rate to be <0.05, at greater than 2 fold change and when two or more peptides were identified.

CHAPTER 3

A BIOCHEMICAL COMPARISON OF THE EXTRACELLULAR MATRIX COMPOSITION OF TENDONS AND LIGAMENTS AROUND THE CANINE STIFLE JOINT

3.1 INTRODUCTION

The macromolecular extracellular matrix composition of tendons and ligaments are similar, however the precise matrix components, cellular morphology and arrangement and mechanical properties are specialised for the efficient functioning of each tissue type and for tissues within each type e.g. SDFT vs CDET tissue (Rumian et al. 2007, Mienaltowski and Birk 2014). In general both tissues consist of cells and extracellular matrix (ECM), where ECM has been reported to contain collagens (70-80% of dry weight) and non-collagenous proteins including glycoproteins, proteoglycans and elastin. The water content of tendons and ligaments ranges from 55 to 70% and a substantial part of this is associated with proteoglycans in the ECM (Benjamin and Ralphs 1997, Frank 2004, Kjaer 2004, Rumian et al. 2007).

The composition of tendon and ligaments is related to their specific function and mechanical properties (Mienaltowski and Birk 2014). Composition and cellular arrangements alters with different tendon and ligament types, locations and regions (Mienaltowski and Birk 2014). This has been shown between different tendons and ligaments in rabbit (Amiel et al. 1984) and sheep (Rumian et al. 2007), where altered proportions of molecular components and a different collagen organisational structure was found between tendons and ligaments (Rumian et al. 2007). Specialised tendon types such as the energy storing SDFT and positional CDET have also been shown to have structural and compositional differences, which relate to differing function of these tendons (Birch 2007, Birch et al. 2008, Franchi et al. 2009, Thorpe et al. 2010, Thorpe et al. 2012). While ligaments at different locations around the knee joint such as the inter-articular anterior cruciate ligament (ACL) and extra-articular medial collateral ligament (MCL) have been reported to have different collagen content (Fujii et al. 1994), ultrastructural morphometry (Hart et al. 1992) and cellular morphology (Newton et al. 1990). Other ligaments with discrete biomechanical properties including the ACL, the ligamentum teres of the hip (LT), and the iliofemoral ligament (IL) have been found to have differences in collagen type I, elastin, fibromodulin and biglycan expression indicating molecular heterogeneity between anatomically distinct ligaments with differing biomechanical demands (Lorda-Diez et al. 2013). Regional variation of tendons and ligaments can occur as a result of changes in mechanical loading, where regions under mechanical compression can exhibit increased

fibrocartilaginous matrix composition (Benjamin and Ralphs 1998). Alterations in hydroxyproline and collagen crosslinks, at different locations (femoral and tibial insertions and the midsubstance) within the ligaments and a tendon of the human knee (Hanada et al. 2014) have been reported. Vogel et al. (1993) demonstrated different cell morphology and increased glycosaminoglycan (GAG) content in human posterior tibialis tendon at the compressed regions where it is subjected to more compressive forces. This phenomenon has also been identified in tendons of other species such as the dog (Okuda et al. 1987), cow (Koob and Vogel 1987) and rabbit (Daniel and Mills 1988). Heterogeneity in cell morphology at different regions in canine cranial cruciate ligament (CCL) has also been reported, indicating the adaptation of ligament to mechanical or physiological environment and reflects variation in stresses and strains (Smith et al. 2012).

The CCL is comparable to human ACL (Arnoczky 1983) and is prone to cranial cruciate ligament disease (CCLD), where degenerative changes occur and result in altered extracellular matrix (ECM) and cellular metabolism, eventually leading to a non-contact injury (Comerford et al. 2011). This situation is closely analogous to that in man where non-contact ACL rupture has been reported (Serpell et al. 2012). Risk factors such as age, hormonal influence and exercise have been reported for both human and dogs with non-contact ACL injury (Comerford et al. 2011, Serpell et al. 2012). An additional risk factor in dogs for non-contact injury is the genetic or breed predisposition, where non-diseased CCLs from dogs at high risk dogs to CCLD (Labradors retriever) have an increased ECM degeneration and collagen turnover compared to low risk dog breeds (Greyhounds) (Comerford et al. 2005). Ultrastructural differences with increased collagen fibril diameters and a fibrocartilaginous appearance of the ligaments were identified in low risk dogs, reflecting the higher maturation levels of collagen at the slower turnover rate and greater adaptability to mechanical loading (Comerford et al. 2006).

The distribution of ECM macromolecules have previously been identified in CCL (Smith et al. 2013), however it remains unknown whether canine ligaments and tendons with different functions around the canine stifle joint have similar or different compositions. Increased knowledge of the normal composition of these structures will aid in our understanding of the pathology of these tissues with regard to function related injury and in the future development of tendon and ligament tissue-engineered structures.

3.2 HYPOTHESIS & AIM

The hypothesis in this study was that canine inter-and extra- articular ligaments and flexor and extensor tendons have a different cellular and biochemical composition. The aim was to use the dog as the animal model for investigation and comparison of different tendons and ligaments, due to its comparable nature to man and because of the species predisposition to ligament disease and rupture. Normal (non-diseased) tendons and ligaments tissues were used from a breed with moderate risk (Staffordshire bull terrier cross) to CCLD (Whitehair et al. 1993). The objective was to measure the biochemical ECM composition of ligaments and tendons with regard to location (extra- and intra-articular), function (extensor and flexor) and region (origin, middle, insertion).

Our aim was to use the dog as our animal model for investigation and comparison of different tendons and ligaments, due to its comparable nature to man and because of the species predisposition to ligament disease and rupture. Normal (non-diseased) tendons and ligaments tissues were used from a breed with moderate risk (Staffordshire bull terrier cross) to CCLD (Whitehair et al. 1993). The objective was to measure the biochemical ECM composition of ligaments and tendons with regard to location (extra- and intra-articular), function (extensor and flexor) and region (origin, middle, insertion).

3.3 EXPERIMENTAL PROCEDURE

The methods described below are those pertinent to this chapter. More general detail is described in Chapter 2; General Material and Methods.

3.3.1 Tissue extraction and preparation

Cranial cruciate ligament (CCL), medial collateral ligament (MCL), long digital extensor tendon (LDET) and superficial extensor tendon (SDFT) were harvested from five paired disease free cadaveric canine hindlimbs from Staffordshire bull terrier cross dogs on the day of euthanasia. All harvested tissues (Chapter 2, table 2.2) were sectioned into proximal, middle and distal sections. Sections that were previously snap frozen and stored -80°C were used for biochemical analysis. Two of the animals were female and three were male. All animals were skeletally mature,age ranging between 2-5 years old and body score between 2 and 3.

3.3.2 Water content

The water content was calculated as described in Chapter 2, Section 2.4.1. The water content was calculated and expressed as % of wet weight.

3.3.3 Biochemical analysis

Biochemical analysis was performed on lyophilised tendon and ligament tissues from the different regions, relevant to Chapter 2, Section 2.4.

3.3.4 Cellular content

3.3.4.1 dsDNA content

Total dsDNA content was measured using the Quant-iT^{TM} Picogreen ® dsDNA reagent and kit (Singer et al. 1997), relevant to Chapter 2, section 2.4.4.1). DNA concentrations were determined by comparing to a standard curve using bacteriophage lambda DNA standards and expressed as μg per mg dry weight tissue.

3.3.5 Extracellular matrix composition

3.3.5.1 Collagen content

Total collagen content was indirectly determined by measuring the imino acid, hydroxyproline (Bergman and Loxley 1963), as described in Chapter 2, Section 2.4.5.2. The hydroxyproline concentrations were calculated by comparison against hydroxyproline standard curve and collagen content was calculated assuming hydroxyproline to be present at 14% (Rumian et al. 2007).

3.3.5.2 Elastin content

and elastin content was measured on pooled oxalic acid digested extracts using Fastin dye-binding assay (Biocolor) (Chapter 2, Section, 2.5.3). Elastin concentrations were determined by comparing against the alpha-elastin generated standard curve were expressed as a percentage of dry weight (Smith et al. 2013).

3.3.5.3 sGAG content

Total sulphated glycosaminoglycan (sGAG) concentrations were measured using the dimethylene blue (DMMB) dye binding assay (Farndale et al. 1986), relevant to Chapter 2,

Section 2.4.5.1. sGAG concentrations were measured against shark chondroitin sulphate and were expressed as µg per mg dry weight tissue.

3.3.6 Statistical analysis

Data are presented as means $\pm$ standard error of the mean. For comparison between the different locations statistical analysis using One-way ANOVA with Bonferroni *post-hoc* test was performed using Graphpad Prism (version 6). The significant differences between tissues was statistically analysed with SPSS (version 20) using a Univariate analysis with Bonferroni *post-hoc* test. For both analyses the significance level was set at $p<0.05$.

3.4 RESULTS

3.4.1 Water content

The average water content was 60.7% ± 0.7 in CCL, 57% ± 1.4 in MCL, 56.8% ± 0.9 LDET and 58.9% ± 0.6 in SDFT (Table 3.1). No statistically significant differences were found in water content between proximal, middle and distal regions or between different tissues (Figure 3.1).

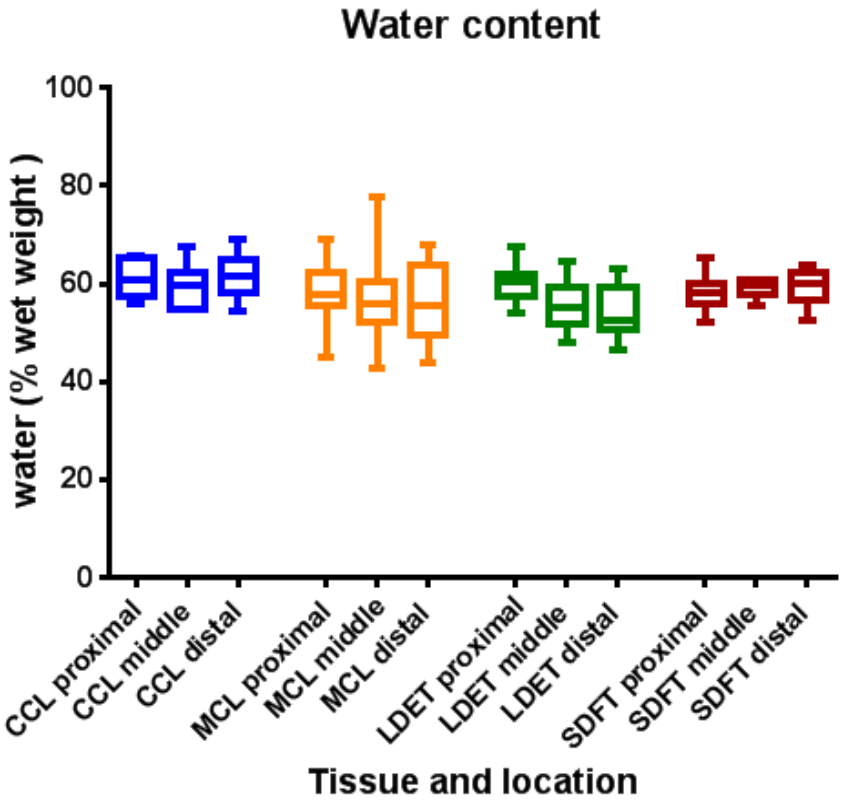


Figure 3.1. Box plot summaries of CCL, MCL, LDET and SDFT (n=5 paired) water content (%). No statistically significant differences were between the different locations or between tissues.

3.4.2 DNA content

The mean dsDNA content was 2.9 ± 0.3 µg per mg dry weight tissue in CCL, 5.2 ± 0.8 µg/ mg in MCL, 3.8 ± 0.3 µg/ mg in LDET and 3 ± 0.3 µg/ mg in SDFT (Table 3.1). Between the proximal, middle and distal regions of tissues no statistically significant difference was found. Significantly greater DNA content was found in the MCL compared to CCL (p=0.012) and SDFT (p=0.017) (Figure 3.2).

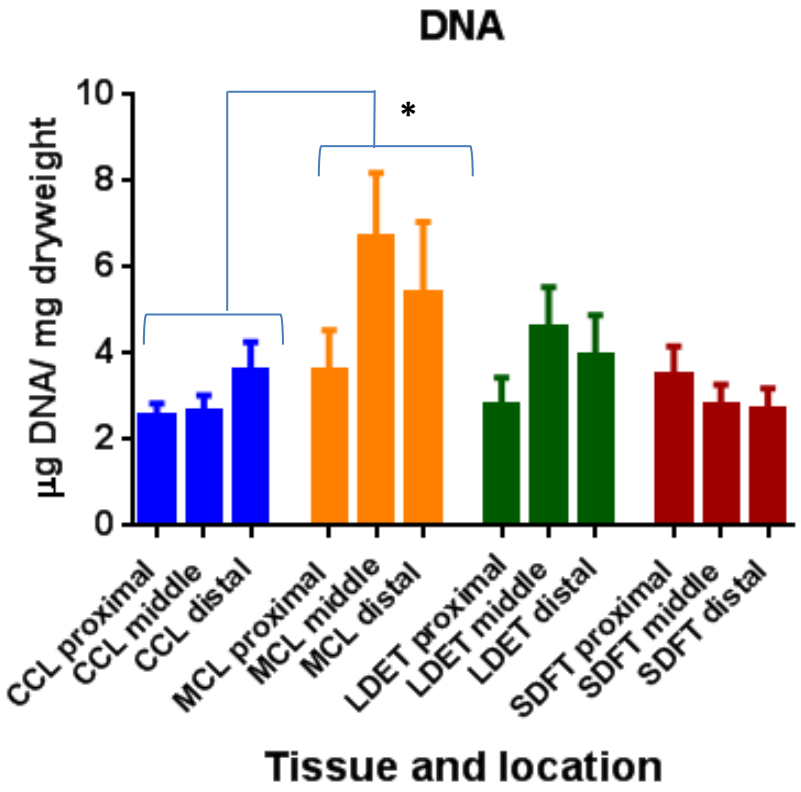


Figure 3.2. DNA content (µg DNA/ mg dry weight) of the proximal, middle and distal regions of CCL, MCL, LDET and SDFT. n = 5 paired. Values are mean and error bars represent SEM. DNA content was significantly higher in MCL in comparison to CCL (p=0.012) and SDFT (p=0.017). No statistically significant differences were found between different locations. * indicates p=<0.05.

3.4.3 Collagen content

The average collagen as a percentage of dry weight was 65.6% ± 1.8 in CCL, 70.4% ± 2.6 in MCL, 71.2% ± 1.4 in LDET and 50.8% ± 1.2 in SDFT (Table 3.1). There were no statistically significant differences in collagen content between the proximal, middle and distal location in each tissue. However, the SDFT had statistically significantly less collagen compared to CCL (p=0.0001), MCL (p=0.0001) and LDET (p=0.0001) (Figure 3.3).

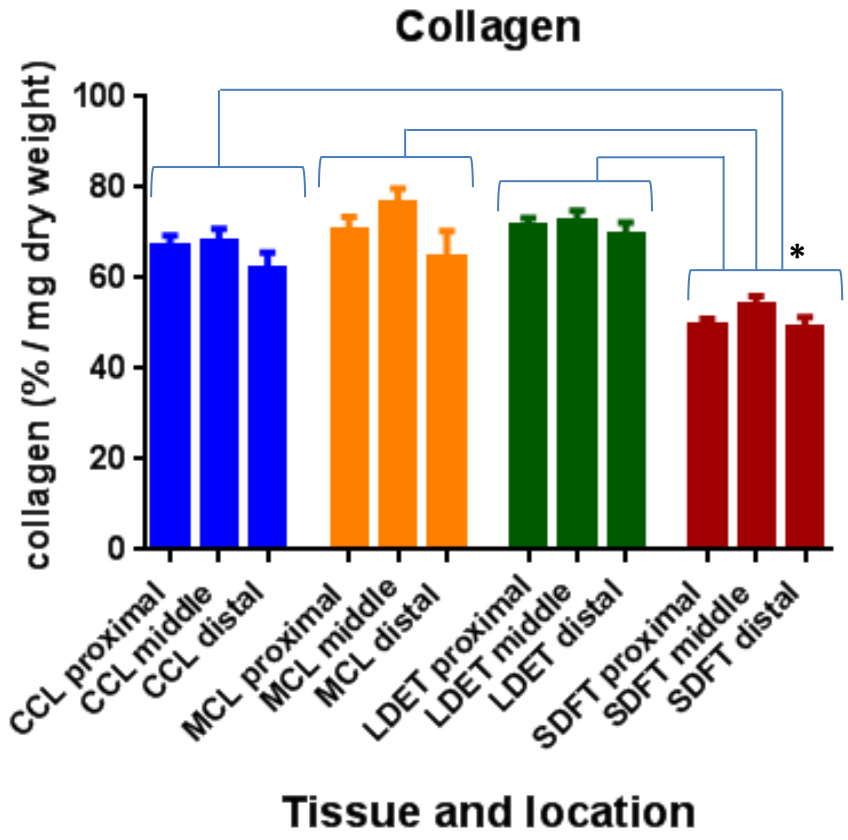


Figure 3.3. Total collagen content (% /mg dry weight) of proximal, middle and distal regions of CCL, MCL, LDET and SDFT. n = 5 paired. Values are mean and error bars represent SEM. No variation was found between locations, but SDFT was significantly lower (p=0.0001) than ACL, MCL and LDET. * indicates p=<0.05.

3.4.4 Elastin content

Elastin content (percentage of dry weight) was 4.6 % ± 0.3 in CCL, 1.9 ± 0.2 in MCL, 2.4 ± 0.2 in LDET and 2.9 ± 0.2 in SDFT (Table 3.1). There was no statistically significant variation between proximal, middle and distal regions with the tissues. CCL contained significantly higher elastin content when compared to MCL (p=0.0001), LDET (p=0.0001) and SDFT (p=0.0001) (Figure 3.4).

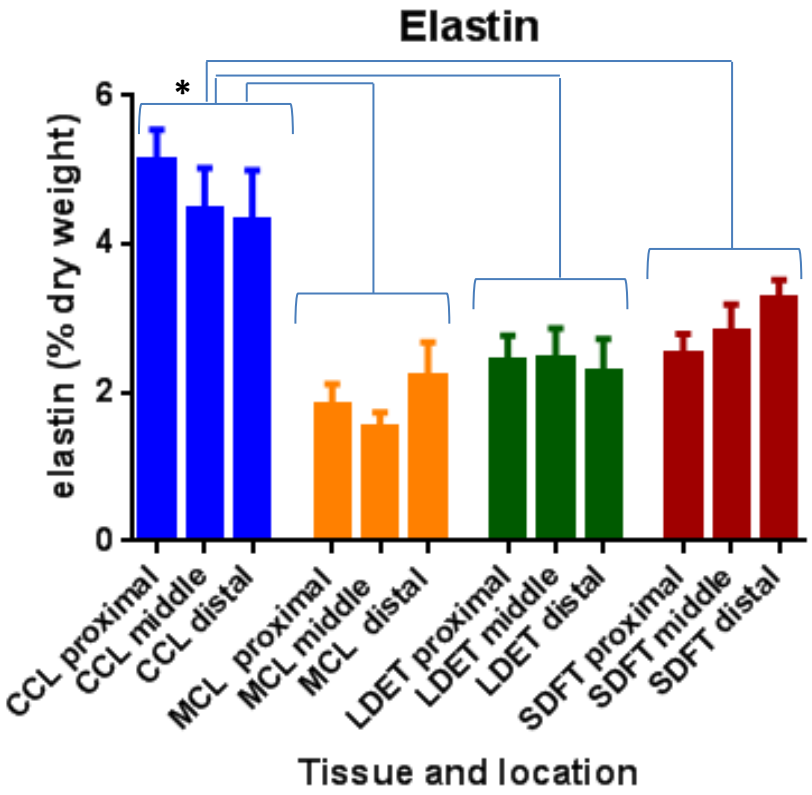


Figure 3.4. Elastin content (% /mg dry weight) of the proximal middle, distal regions of CCL, MCL, LDET and SDFT. n= 5 paired. Values are mean and error bars represent SEM. No variation was found between the different locations within tissues, but CCL was significantly higher than MCL (p=0.0001), LDET (p=0.0001) and SDFT (p=0.0001). * indicates p=<0.05.

3.4.5 sGAG content

The median sGAG content as µg/mg dry weight was 15.5 ± 1.1 in CCL, 9.9 ± 0.7 in MCL, 8.3 ± 0.7 in LDET and 11.1 ± 0.8 in SDFT. There was no statistically significant difference found between proximal, middle and distal locations. CCL had statistically greater sGAG content compared to MCL (p=0.0001), LDET (p=0.0001) and SDFT (p=0.01) (Figure 3.5).

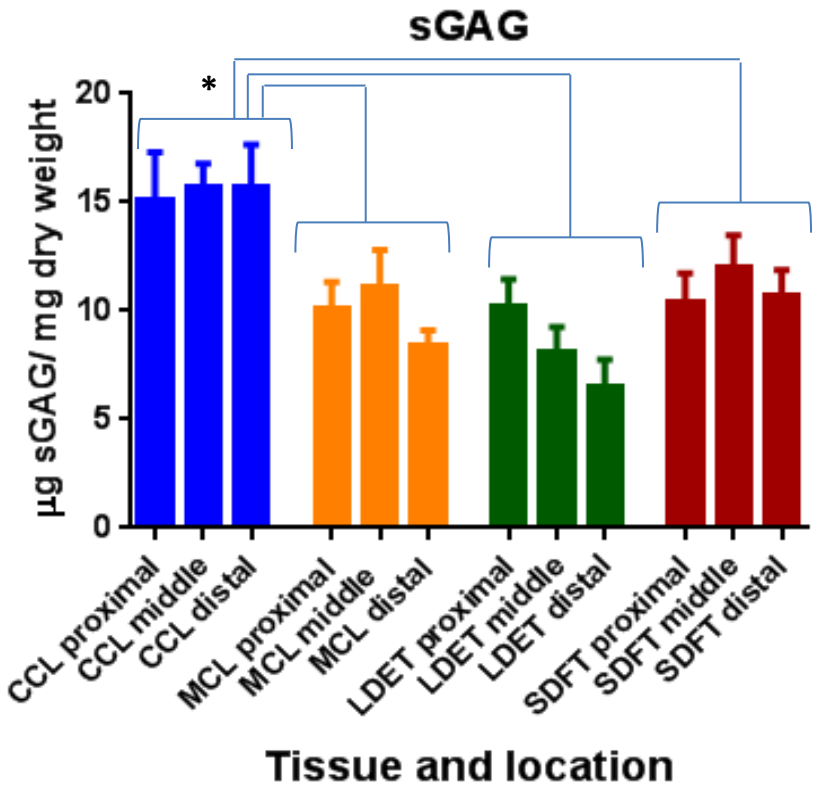


Figure 3.5. sGAG content (µg sGAG/ mg dry weight) of the proximal, middle and distal regions of CCL, MCL, LDET and SDFT. n = 5 paired. Values are mean and error bars represent SEM. No significant variation was found between proximal, middle and distal locations within tissues, but CCL was significantly higher than MCL, LDET and SDFT. * indicates p=<0.05.

	Water (% per wet weight)	dsDNA (µg/ mg dry weight)	Collagen (%/mg dry weight)	Elastin (%/mg dry weight)	sGAG (µg/ mg dry weight)
ACL proximal	61.1 ± 1.2	2.6 ± 0.3	67.1 + 2.5	5.1 ± 0.4	15.1 ± 2.2
ACL middle	59.5 ± 1.4	2.6 ± 0.4	68.1 + 2.9	4.5 ± 0.6	15.7 ± 1.1
ACL distal	61.7 ± 1.4	3.6 + 0.7	61.9 + 3.8	4.3 ± 0.7	15.7 ± 1.9
MCL proximal	58.1 ± 2.1	3.6 ± 0.9	70.3 + 3.3	1.8 ± 0.3	10.1 ± 1.2
MCL middle	57.1 ± 2.9	6.7 ± 1.5	76.6 + 3.2	1.5 ± 0.2	11.1 ± 1.7
MCL distal	55.9 ± 2.6	5.4 ± 1.6	64.4 + 5.9	2.2 ± 0.6	8.4 ± 0.7
LDET proximal	60.3 ± 1.2	2.8 ± 0.6	71.5 + 1.8	2.4 ± 0.4	10.2 ± 1.3
LDET middle	55.9 ± 1.6	4.6 ± 0.3	72.5 + 2.4	2.5 ± 0.4	8.1 ± 1.1
LDET distal	54.3 ± 1.6	3.9 ± 0.9	69.5 + 2.8	2.3 ± 0.5	6.6 ± 1.2
SDFT proximal	58.1 ±1.2	3.5 ± 0. 7	49.5 + 1.6	2.5 ± 0.3	10.4 ± 1.3
SDFT middle	59.2 ± 0.5	2.8 ± 0.5	54.1 + 1.9	2.8 ± 0.4	11.9 ± 1.5
SDFT distal	59.3 ± 1.2	2.7 ± 0.5	48.8 + 2.5	3.3 ± 0.3	10.7 ± 1.2

Table 3.1. Water content and tissue concentration of DNA, collagen, elastin and sGAG of CCL, MCL, LDET and SDFT at proximal, middle and distal regions. Values are mean (n = 5 paired) ± SEM.

3.5 DISCUSSION

Determination of the content of ECM macromolecules between different functional tendons and ligaments will aid in finding tissue specific characteristics, which is vital for understanding the pathology of these tissues and for future development of regeneration and reparative strategies. In this study we have demonstrated a different ECM composition between inter- and extra-articular ligament and tendon, but no variation was identified at different regions in each tissue. CCL had significantly greater GAG and elastin content compared to the MCL, LDET and SDFT. Significantly higher DNA content was measured in MCL, while SDFT had the lowest collagen content. The higher sGAG content in CCL in our study corresponds with previous comparison of the knee joint ligaments and tendons in sheep (Rumian et al. 2007) and rabbit (Amiel et al. 1984), where intra-articular ligaments had greater sGAG measurement than tendons and the extra-articular ligaments. Together the increased sGAG and elastin content in CCL may be due to a greater requirement for viscoelastic properties and elastic recoil mechanisms in CCL.

In this study, DNA concentration was measured to assess the tissue cellularity. Generally tendons and ligaments have relatively few cells and have been documented as hypo-cellular structures (Lo et al. 2002, Yin et al. 2010) with the vast majority of tendon and ligament cells being tenocytes and ligamentocytes, respectively (Hoffmann and Gross 2007). Cells in tendons communicate by intercellular communication sites known as gap junctions, necessary for strain-induced collagen synthesis (McNeilly et al. 1996). This finding has also been reported in meniscus, intervertebral disc (Bruehlmann et al. 2002) and in the ovine ACL and MCL (Lo et al. 2002). In this study a greater DNA content in MCL indicated a higher cellularity in MCL in comparison to CCL and SDFT. Although no correlation has been found between high cell content and intercellular communication, this result might indicate a better cell-cell communication in the MCL. This result supports a previous study, which demonstrated that human MCL contained higher cell numbers compared to ACL (Yoshida and Fujii 1999). The same group identified different cellular properties including slower growth rates and a lower response to growth factors in the ACL compared to the MCL (Yoshida and Fujii 1999). A comparison study of the human ACL and MCL stem cells demonstrated differential properties, with ACL stem cells exhibiting a lower capacity of

colony formation, slower proliferation and shorter period of self-renewal capability (Zhang et al. 2011). Together, these cellular differences between MCL and ACL may be indicative of a different healing capacity between the two tissue types.

Collagens provide major tensile strength and are the abundant proteins in tendons and ligaments by contributing to 70-80% of their dry weight (Provenzano and Vanderby 2006, Mienaltowski and Birk 2014). In general both tendons and ligaments have been reported to contain collagen types I, II, III, V, VI and XI, XIV (Frank 2004, Franchi et al. 2007, Mienaltowski and Birk 2014). By measuring the hydroxyproline content, of which 14% is present in collagen (Bergman and Loxley 1963), total collagen can be quantified. The collagen content in canine CCL, MCL, LDET were not significantly different and ranged between 65-71% of dry weight tissues. Canine SDFT on the other had significantly less collagen ($p=0.0001$) compared to the other three tissues, representing 50.8% of the dry weight of the tissue. This finding does not correspond with a study comparing ovine tendons (patellar, LDET and SDFT) and ligaments (cruciate ligaments, MCL and LCL) (Rumian et al. 2007), and may be explained by the species differences. The ovine SDFT had a collagen content of 78.8% of the dry weight and cruciate ligament contained 55-58% collagen per dry weight (Rumian et al. 2007). A similar concentration of collagen in equine SDFT (75.8% of dry weight) has been reported, while the positional CDET has been documented to contain a significantly higher collagen content (80.4%) than the energy storing SDFT (Thorpe 2010). The different collagen content between the energy storing SDFT and positional LDET in this study is similar to that found in the horse energy storing SDFT and positional CDET (Thorpe 2010). The finding might indicate that the canine LDET is stiffer than SDFT, however the ultimate tensile strength of each of these tendons has yet to be determined.

Elastic fibres are present in elastic connective tissues such as aorta and large arteries, skin, lung, and ligaments (Kielty 2006). A network of elastic fibres in the ECM of these tissues gives a required resilience that is needed for recoil after transient stretch (Alberts et al. 2002). Other reported functions of elastic fibres include imparting mechanical properties (Butler et al. 1978, Eriksen et al. 2001) and cell-regulatory functions (Ito et al. 1997, Wendel et al. 2000). The presence of elastic fibres has been demonstrated in canine cruciate ligament (Smith et al. 2011) and in bovine tendon (Grant et al. 2013). A general estimate of elastin in human tendon is 1-2% of the total dry weight tissue (Kannus 2000), but in the

human ligament it can range from 5%, in the ACL (Dodds and Arnoczky 1994), 7.3% in the posterior longitudinal ligament (Nakagawa et al. 1994) and 1.7% in the intervertebral disc (Mikawa et al. 1986). The measurement of elastin content in the present study was similar to that (Fastin assay) previously used in canine cruciate ligament (Smith et al. 2013), but also has been measured in a number of other tissues and species (Isayama et al. 2014, Latimer et al. 2014). This method measures all of the elastin including elastin precursor and degraded elastin peptides in the tissue. The previous study in canine cruciate ligament indicated an average elastin content of 9.87% (Smith et al. 2013), which is higher than the measured elastin content of 4.64% in this study. This may be as result of the different used dog breed or exercise background of the animals as compared to the greyhounds dogs from a racing background used by Smith et al. (2013). Although we did not have any information about dogs' exercise history, the elastin content in the CCL was found to be similar to the estimated reported human ACL elastin content. Further work is required to identify whether exercise plays an important factor role and is associated with the increase of elastin content. The higher elastin content in CCL compared to the other three tissues may be due to greater requirement for a stretch and recoil mechanism of this tissue(Rumian et al. 2007).

Proteoglycans consist of a core protein with one or more covalently attached GAGs, where the function of different proteoglycans is determined by structure of the protein core and GAG chains (Parkinson et al. 2011). In tendon and ligament, proteoglycans are reported to comprise about 1% of dry weight tissue, were they are likely to contribute to the biomechanical and structural properties of the tissues (Frank 2004, Yoon and Halper 2005, Halper 2014). Proteoglycans play a role in collagen fibrillogenesis and the organisation of collagen fibrils (Zhang et al. 2005, Franchi et al. 2007). They also interact with collagen fibres to yield viscoelastic properties (Rees et al. 2000, Woo et al. 2006, Franchi et al. 2007) in tendon and/or ligament. In the present study, the total sGAG content was measured to estimate the proteoglycan content of each tissue. The CCL resulted in 15.48 µg /mg (0.15%) dry weight of sGAG content, which was significantly more than the MCL, LDET and SDFT. This value was slightly higher than the sGAG content previously measured in greyhound CCL (0.081%/dry weight) (Smith et al. 2013), which may be again as a result of breed differences. These results support previous findings in a ligament and tendons comparison study in sheep (Rumian et al. 2007) and rabbit (Amiel et al. 1984), where higher GAG content was

found in cruciate ligaments compared to extra-articular collateral ligaments and several tendons. The greater proteoglycans in CCL may allow for more slippage between collagen fibrils and fibres, allowing a greater degree of deformation (Rumian et al. 2007). This altered viscoelastic property of CCL may signify an additional "shock absorbing" feature of this tissue. Together, the greater elastin and proteoglycan content in CCL may be important contributors to mechanical specialisation of the CCL.

Studies have shown variances of morphology and composition at different regions of ligaments and tendons (Vogel et al. 1993, Benjamin and Ralphs 1998, Waggett et al. 1998, Vogel and Peters 2005). The most proximal region of the human ACL contains round and ovoid cells, whereas the middle region contains more spindle shaped cells with higher collagen density and distal part is mainly rich in chondroblasts and ovoid fibroblasts (Duthon et al. 2006). The human ACL interface at the femoral origin and tibial insertion sites has been found to differ in collagen fibre orientation and has more sulphated GAGs and different collagen types such type II and X (Wang et al. 2006). A similar variation in cell morphology, collagen fibre arrangement and GAG content also been reported in at the different regions of human posterior tibialis tendon (Vogel et al. 1993). In human Achilles mid-tendon and insertion, Waggett et al. (1998) found more mRNA expression of collagen type II and aggrecan in the fibrocartilaginous regions and more versican in the midsubstance (Waggett et al. 1998). This was supported by a more recent study, where it was demonstrated that the distal region of rabbit Achilles tendon, which was closer to the bone had a more fibrocartiliginous tissue phenotype, with increased aggrecan expression (Huisman et al. 2014). In the present study, no statistically significant variation was found in ECM macromolecules at different regions of ligaments and tendons. This might indicate that there is no difference between compressed and tensile regions in canine ligaments and tendons or it could be that these differences cannot be detected when measuring the total content of main ECM macromolecules.

3.6 CONCLUSION

The results of this study demonstrated no significant differences between proximal, middle and distal regions of inter- and extra-articular ligament, and flexor and extensor tendons around the canine stifle joint. However, a significantly higher sGAG and elastin content in canine CCL was measured in comparison to MCL, LDET and SDFT, which may indicate that there is more of a stretch and recoil mechanism and a greater degree of deformation required in the inter-articular CCL (Rumian et al. 2007). These findings may be as result of specific tissue physiology, location and adaptation to mechanical loading.

CHAPTER 4

THE MORPHOLOGICAL AND STRUCTURAL DIFFERENCES AND EXTRACELLULAR MACROMOLECULES DISTRIBUTION BETWEEN TENDONS AND LIGAMENTS AROUND THE CANINE STIFLE JOINT

4.1 INTRODUCTION

The collagen-rich composition of ligament and tendon is arranged in a hierarchical structure, where collagen molecules are grouped together in a highly ordered fashion, forming fibrils, fibres and fascicles (Kastelic et al. 1978, Clark and Sidles 1990, Thorpe et al. 2012). A group of collagen fibres are referred to as bundles, divided by interbundle (IB) regions, and bundles are grouped together as fascicles, separated by a surrounding loose connective tissue septa referred to as endotenon in tendon and endoligament in ligament or known as the interfascicular matrix (IFM) (Clark and Sidles 1990, Smith et al. 2012). The IFM has been suggested to have a specialised structure that plays a role in facilitating sliding between fascicles within tendon (Thorpe et al. 2012).

Collagens fibrils confer the principal tensile strength to mammalian connective tissues and define the shape and form of tissues in which they occur (Canty and Kadler 2005). Tendons and ligaments mainly consists of type I collagen, but other collagens types including II, III, V,VI, IX and XI are also present (Frank 2004, Benjamin et al. 2008).

Besides collagens, tendon and ligaments contain other non-collagenous extracellular matrix (ECM) components such as proteoglycans and elastic fibres (Benjamin and Ralphs 1997, Frank 2004, Kjaer 2004, Thorpe et al. 2013). Proteoglycans (PGs) in tendons and ligament are primarily responsible for their viscoelastic behaviour (Benjamin and Ralphs 1997, Benjamin et al. 2008). They are composed of a protein core with one or more covalently attached glycosaminoglycans (GAG) side chains (Yoon and Halper 2005). In the tendon the majority of PGs are small leucine proteoglycans (SLRPS) with smaller amount of large aggregating PGs such as aggrecan and versican (Yoon and Halper 2005, Parkinson et al. 2011). Decorin is the most common SLRP in tendon, where it constitutes 80% of the overall proteoglycan content (Samiric et al. 2004). Other SLRPS found in tendon include biglycan, lumican, fibromodulin and keratocan (Rees et al. 2009). In the ligament, decorin is also the major constituent of proteoglycans, with the remainder also including biglycan, aggrecan and versican (Ilic et al. 2005). Furthermore, in the canine cruciate ligament the presence of other SLRPS including lumican, fibromodulin, prolargin and osteoglycin has been demonstrated (Yang et al. 2012).

Elastic fibres are composed of an elastin core covered with sheath of fibrillin-rich microfibrils, which acts as a scaffold on which the secreted elastin molecules are deposited (Kielty et al. 2002, Kielty 2006). Elastic fibres are considered to have a structural role in tendon and ligament, where the distribution in canine cruciate ligament (Smith et al. 2011) and bovine flexor tendon (Grant et al. 2013) has been demonstrated.

As mentioned previously in Chapter 1 (Section 1.10), a different ECM composition between tendon and ligament has been demonstrated in rabbit (Amiel et al. 1984) and in sheep (Rumian et al. 2007). In this study, similar findings have already been observed between canine tendons and ligaments around the stifle (knee) joint, where the canine cruciate ligament (CCL) contained elevated proteoglycans (based on sGAG measurement) and elastin content (Chapter 3).

To date there are little data regarding the distinct structural and morphological characteristics of different ligaments and tendons around the knee stifle joint in human/ dog and how they are related to the ligament and tendon functional role. Furthermore, the distribution and localisation of ECM macromolecules in the ligament and tendon in man and human has been not fully explored. These data will provide essential information on the fundamental structure of tendon and ligament tissues leading to increased understanding of the function relationship between these tissues types and will underpin future tissue engineering and regeneration strategies.

4.2 HYPOTHESIS AND AIMS

In this study, the canine intra-articular cranial cruciate ligament (CCL) has already been shown to have a different ECM composition when compared to the extra- articular medial collateral ligament (MCL), the positional long digital extensor tendon (LDET) and the energy storing superficial digital flexor tendon (SDFT) (Chapter 3). This finding might indicate how each tissue type functions in a distinct manner. However, the morphological differences and arrangement of ECM macromolecules between tendons and ligaments around the knee stifle joint has yet to be fully investigated.

In this study it was hypothesised that:

1. Canine CCL has a diverse structural property, with a greater abundance of proteoglycan and elastic fibres in comparison to MCL, LDET and SDFT.
2. Canine tendon and ligament have a different distribution in ECM collagens, proteoglycans and elastic fibres molecules and that there will be more proteoglycans and elastic fibres present at the IFM in ligament than tendon.

The aim was to use tendons and ligaments around the canine stifle joint, due to the comparable nature of the dog's stifle joint to the human knee joint (Cook et al. 2010). The canine stifle is an interesting model as it is predisposed to non-contact CCL injury (Comerford et al. 2011), which is analogous to the reported human ACL non-contact injuries (Serpell et al. 2012). Furthermore, there is a breed variation in ligament disease and rupture (Whitehair et al. 1993). In this study normal non- diseased tendons and ligaments were obtained from a breed with moderate risk (Staffordshire bull terrier cross) to cranial cruciate ligament disease (see Chapter 3).

The objectives in this study were

1. To identify the differential structural characteristics of ligaments (CCL and MCL) and tendons (flexor and extensor) at the different regions (proximal, middle, and distal) through semi-objective histological scoring analysis.
2. To determine tendon-ligament differences in the distribution and organisation of collagens, proteoglycans and elastic fibre proteins using immunostaining techniques.

4.3 EXPERIMENTAL PROCEDURES

The procedures written below are an outline of the performed experiments. More detail of the materials and methods of this section are described in Chapter 2.

4.3.1 Sample collection, tissue extraction and preparation

Harvested cranial cruciate ligament (CCL), medial collateral ligament (MCL), long digital extensor tendon (LDET), and superficial digital flexor tendon (SDFT) tissues (n=5 paired) (Table 2.1) were sectioned into proximal, middle and distal sections. Divided sections were fixed in 4% paraformaldehyde or embedded in OCT. Two of the animals were female and three were male. All animals were skeletally mature, age ranging between 2-5 years old and body score between 2-3.

4.3.2 Determination of morphological differences based on histology staining

4.3.2.1 Tissue preparation and histology staining

Fixed tissues in 4% paraformaldehyde were paraffin embedded and cut into 4µm sections. Sections were stained with haematoxylin and eosin (H&E) for general observation of tissue, Alcian blue/PAS (AB-PAS) for detection of GAGs (Bancroft et al. 1996) and Miller's stain for elastic fibres (Miller 1971).

4.3.2.2 Objective measurements of histological parameters

A three part scoring system was used to assess the cells and extracellular matrix of the tissues (Chapter 2, Table 2.1, 2.2 and 2.3). The scoring system used for H&E staining was modified from Stoll et al. (2011) and for Miller's elastic stain from Smith (2010). Scoring of AB-PAS staining was developed in house, based on that of Smith (2010). All sections were read by two observers blinded to the section location and tissue type on two separate occasions at least two weeks apart.

4.3.3 Distribution of ECM macromolecules using immunostaining

Distributions of the main ECM components were assessed on the mid-substance of CCL and LDET (n=3) using immunohistochemistry and immunofluorescence staining for different collagen types, proteoglycans and elastic fibres. The antibodies used were reactive against collagen type I, III, VI, aggrecan, versican, decorin, biglycan, keratocan and asporin, elastin, fibrillin 1 and fibrillin 2 (Chapter 2, Table 2.5 for manufacturer, concentration and secondary antibodies used). All antibodies (apart from elastin, fibrillin 1 and fibrillin 2) were assessed using immunohistochemistry methodology as described previously (Chapter 2, section 2.6.1), using 4µm paraffin embedded sections. Frozen sections of 5 µm were used for immunostaining of elastin, fibrillin 1 and fibrillin 2 (Chapter 2, section 2.6.2). The distribution and arrangement of the selected collagens and proteoglycans were visualised with a Nikon DS-L2 standalone control unit, while elastic fibres were assessed with the confocal microscope ((Nikon Ti Eclipse).

4.3.4 Statistical analysis

Hematoxillin and eosin histological scoring were individually assessed for each parameter and presented as an average % score. For AB-PAS and Miller's analysis the scores were accumulated together and the total score was presented with means ± standard error of the mean. Each histological parameter was assessed for statistical significance and integrity of agreement. Statistical analysis included comparison between the different locations (proximal, middle, distal) and between different tissue types (intra- and extra-articular tendon and ligament). Normal distribution for each data set was assessed with Graphpad Prism (Version 6) using Kolmogorov-Smirnov test.

For comparison between the different locations statistical analysis using one-way ANOVA with Bonferroni *post-hoc* test was performed using Graphpad Prism. The significant differences between tissues was statistically analysed with SPSS (Version 20) using a univariate analysis with a Bonferroni *post-hoc* test. For both analyses the significance level was set at $p<0.05$. The integrity of agreement was calculated for intra- and inter-observer

concordance between and within both observers respectively using the Kendall's coefficient of concordance for each histological parameter.

4.4 RESULTS

4.4.1 Morphological characteristics of intra- and extra-articular tendons and ligaments

4.4.1.1 Histology H&E staining

ECM organisation **-** The general architecture of collagen fibres bundles of both ACL and MCL ligament was less tightly packed than both LDET and SDFT tissue (Figure 4.1A and 4.1B). In both the LDET and SDFT tendon the collagen fibres were more dense and compact with parallel collagen fibres (Figures 4.2A and 4.2B,). The IF region in CCL appeared to be thicker than in the LDET and SDFT, where the IF region appeared narrower and the cells in this region were closer to each other (Figure 4.2B). Histological scoring of ECM architecture resulted in no statistically significant differences between different regions of either ligaments or tendons. However, CCL was significantly different in its architecture compared to LDET ($p=0.0001$) and SDFT ($p=0.036$), where a more wavy, compact and parallel aligned collagen architecture was observed on both the tendons (Figure 4.3A). This difference was also observed when MCL, was compared with LDET ($p=0.0001$)(Figure 4.3A).

Cell shape **-** In general, heterogeneous cell nuclei phenotype was seen in all tissue samples with a mixed population of rounded and spindle cell nuclei morphologies. It was notable however that there were more rounded and elliptical cell nuclei in the CCL in comparison to the other three tissues (Figure 4.1A). The MCL, LDET and SDFT substance cell nuclei were more spindle-shaped and also more elongated in the LDET and SDFT (Figure 4.1B, 4.2A and B). This observation was found to be statistically significant between LDET and ACL ($p=0.003$), with the ACL having a more mixed cell shape population (Figure 4.3B). No statistically significant difference was found between different locations within both tendons and ligaments.

Cellular distribution **-** Assessment of cellular distribution in the different tendons and ligaments were considered normal if cells were not focally increased. This increased cell

density was often seen at the IF regions in both tendons and ligaments, with cells showing a variation in length and orientation located at this region (4.1B and 4.2A and B). Statistical analysis of cellular distribution showed no significant differences either between different regions or between the tissues ($p>0.05$) (Figure 4.3C).

Cell alignment **-** Alignment of cells was assessed based on orientation of cells along the collagen fibre bundles. In tendons, in particular the LDET, the cells were mostly uniaxial and parallel to collagen fibres bundles (Figure 4.2A). This finding was seen less frequently in ligaments; the MCL and ACL cells were at times found to be more irregularly aligned (Figure 4.1A and 4.1B). Histological scoring of cell alignment demonstrated statistically significant differences in LDET than MCL ($p=0.034$), indicating a more uniaxial alignment of cells in LDET (Figure 4.3D). No statistical significant differences in cell alignment were found between different regions in both tendons and ligaments tissues.

Vascularisation **-** Blood vessels were observed in both intra- and extra-articular tendons and ligaments, however they were mainly found in the tendons, localised in the IF regions (Figure 4.2B). Histological scoring results demonstrated significantly more blood vessels in SDFT compared to ACL ($p=0.0001$), MCL ($p=0.001$) and LDET ($p=0.0001$) (Figure 4.3E). No statistically significant differences were found at the different regions in tendons and ligaments with regard to their vascularisation.

Inflammation **-** The presence of Inflammation in the tissues was assessed based on the presence of infiltrates of cells, such as neutrophils, lymphocytes and macrophages. These cells were occasionally present in the epitenon of tendons and surrounding blood vessels in the interfasicular regions in tendon or ligament. Statistical analysis of histological inflammation score resulted in significantly more infiltrate cells in SDFT compared to ACL ($p=0.0001$), MCL ($p=0.0001$) and LDET ($p=0.032$). No statistically significant differences were found between the different locations in both intra- and extra-articular tendon and ligament tissues (Figure 4.3F).

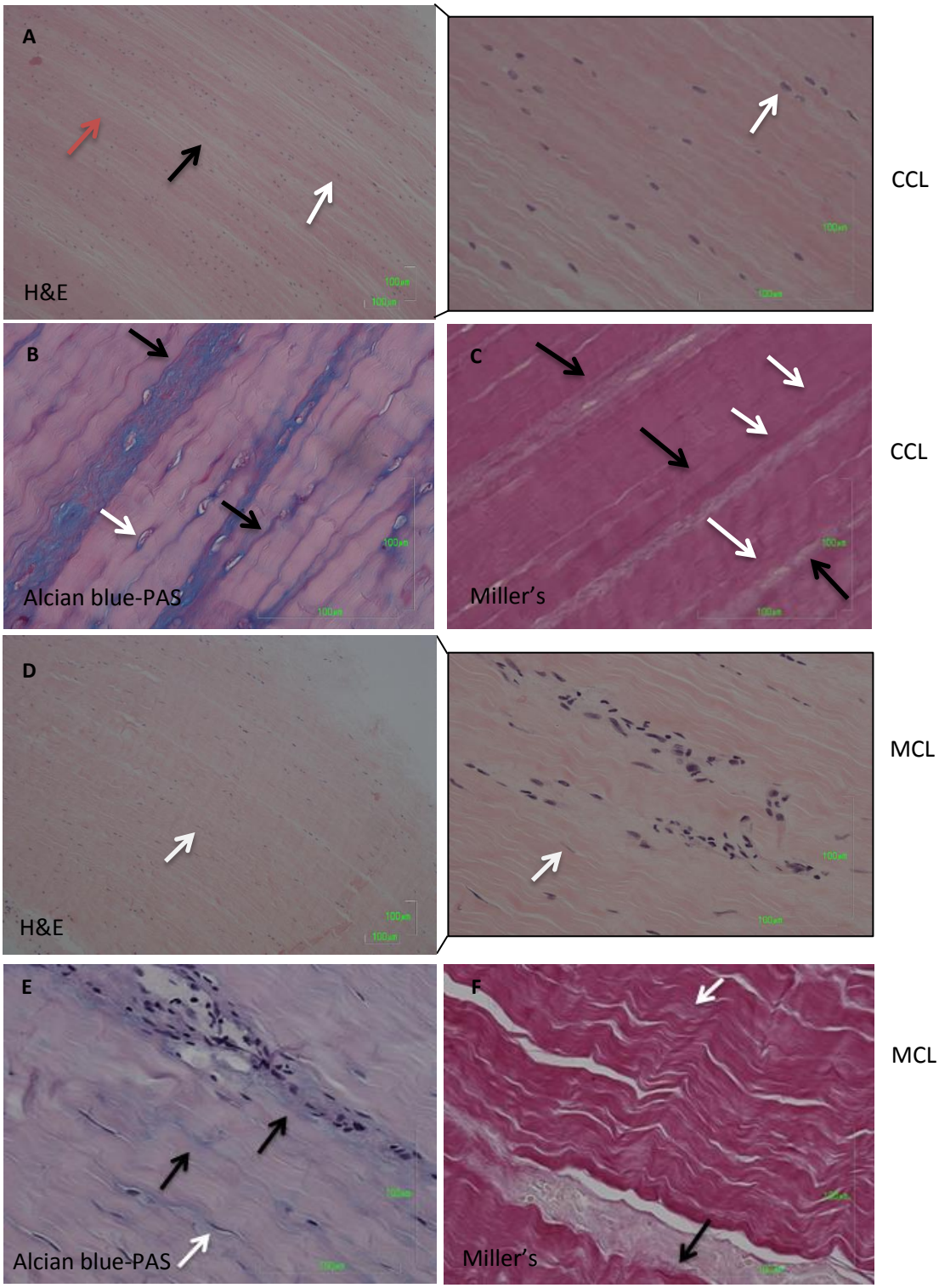


Figure 4.1. Representative images of histological staining of the mid-region of CCL and MCL. H&E staining of CCL (A) and MCL (D). (Bar 100µm, 10X and 40X magnifications). Ligaments were found to have less tightly packed collagen fibre bundles. This was observed more in CCL, where IF regions seemed more spacious (black arrow in A) and fascicles had less compact collagen fibres (orange arrow in A). Heterogeneous cell morphology was found in both CCL and MCL, but cell nuclei morphology was primarily more rounded and more elliptical shaped in CCL (white arrow in A), while MCL cell nuclei were more spindle shaped (white arrows in D and E). Alcian blue-PAS staining of CCL (B) and MCL (E). (Bar 100µm, 40x magnification). Glycosaminoglycans were mainly present at IF and at interbundle regions (black arrows in B and E). Increased staining of GAGs in CCL was observed with pericellular staining (white arrow B). Miller's staining of CCL (C) and MCL (F). (Bar 100µm, 40X magnifications). Elastic fibres were located mainly in IF and interbundle regions (black arrows in C and F) and orientated parallel aligned on collagen fibres (white arrows in C and F).

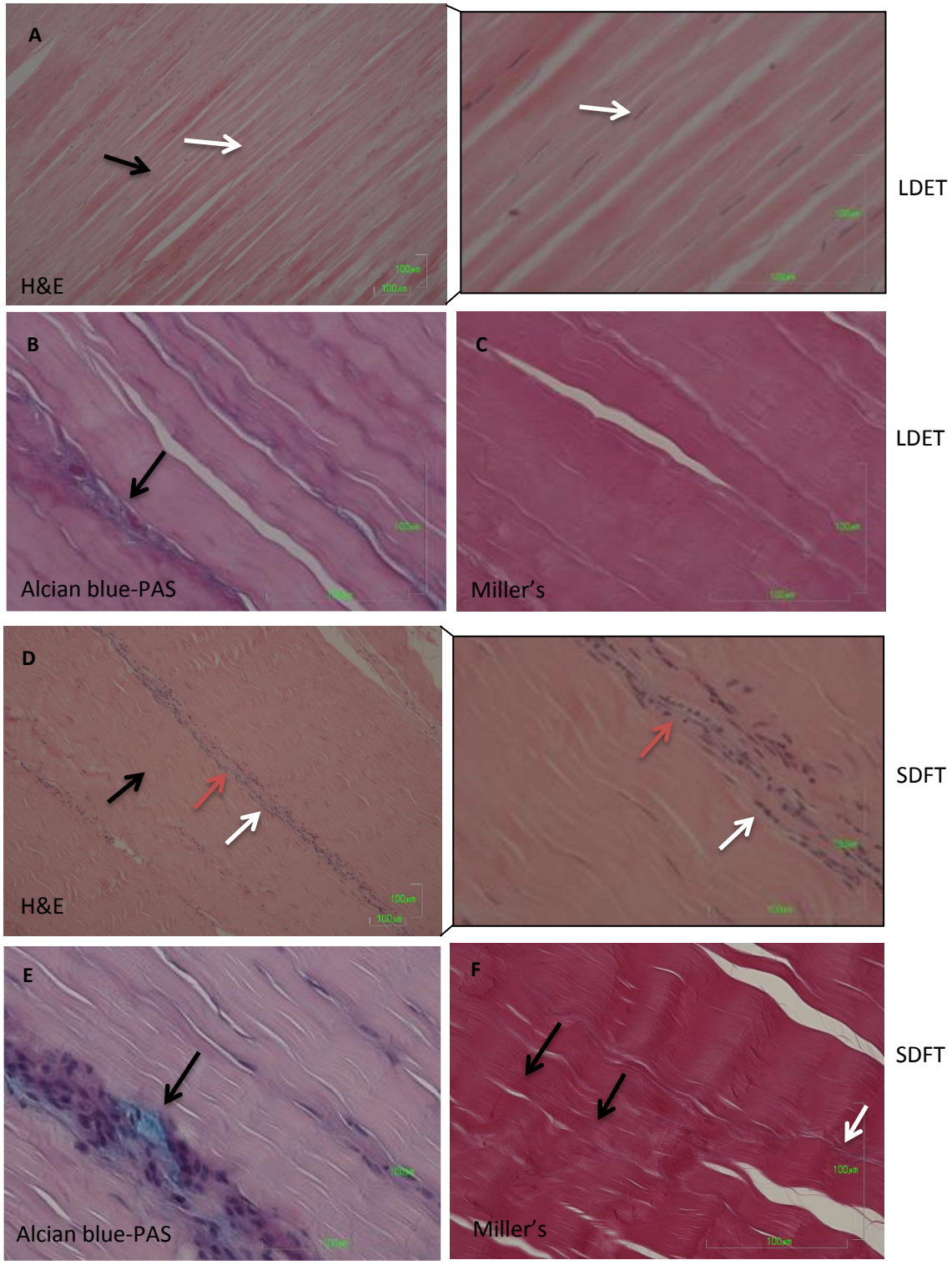


Figure 4.2. Representative images of histological staining of the mid-region of LDET and SDFT. H&E staining of LDET (A) and SDFT (D). (Bar 100µm, 10X and 40X magnifications). Both tendons appeared wavy and with generally compact collagen fibres architecture (black arrow in A and D). Heterogeneous cell nuclei morphology were present in LDET and SDFT, where cells in the IF regions were more rounded and seemed to be increased in number (white arrow in D). Cell nuclei morphology in the IF region was mainly spindle shaped in both tendons, but in LDET it also seemed more elongated (white arrow in A). Blood vessels were primarily seen in SDFT at IF regions (orange arrow in D). Alcian blue-PAS staining of LDET (B) and SDFT (E). (Bar 100 µm, 40x magnification). GAGs were mainly present in IF and at IB regions (black arrows in B and E). No pericellular GAG staining was identified in tendons. Miller's staining of LDET (C) and SDFT (F). (Bar 100µm, 40X magnifications). The presence of elastic fibres was identified in LDET and SDFT located in IF and IB regions (white arrow in F) and parallel aligned on collagen fibres (black arrows in F).

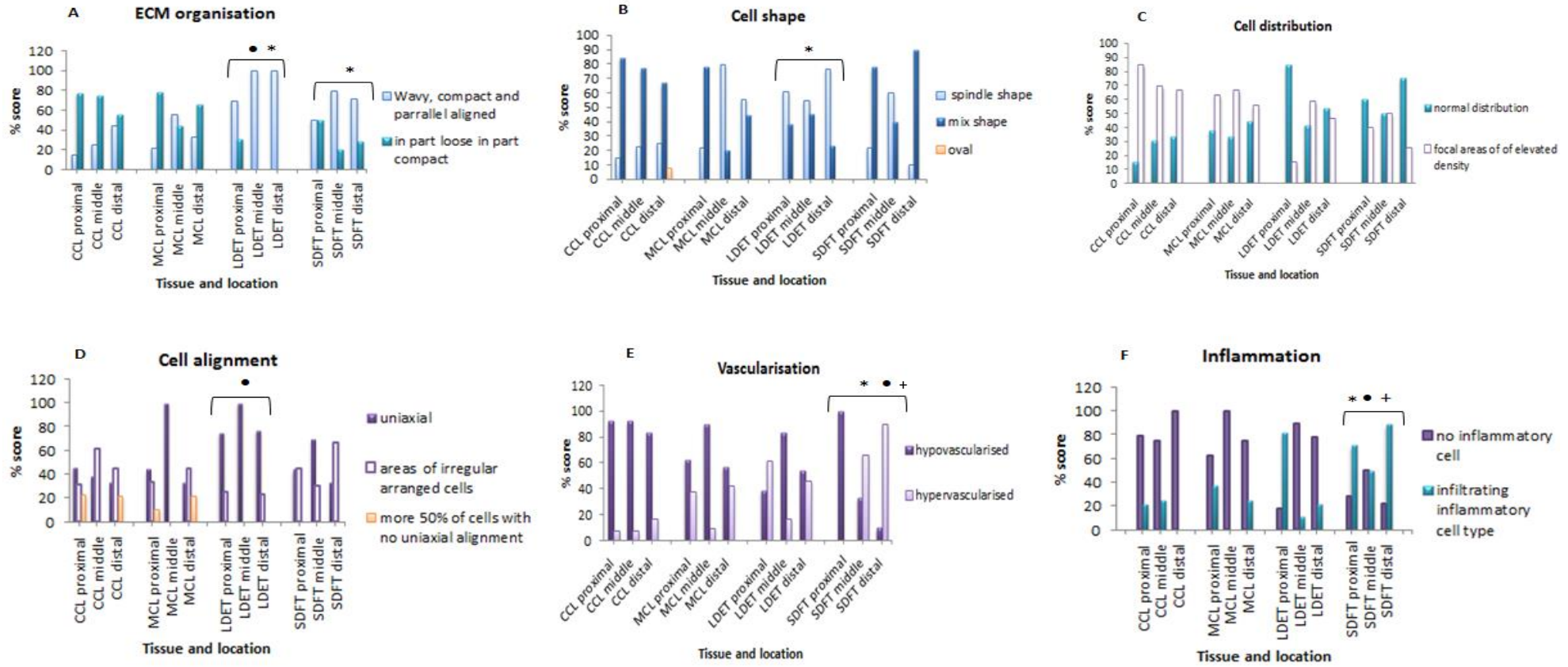


Figure 4.3. Histology scoring results of ECM organisation, cell shape, cell distribution, cell alignment, vascularisation and inflammation. (A) ECM organisation: Both ACL and MCL collagen fibre architecture was less dense compared to tendons. *p<0.05 vs ACL, • p<0.05 vs MCL. (B) Cell shape: A more heterogeneous mix cell shape population was seen in ACL compared to LDET, which had more spindle shaped cell nuclei. *p<0.05 vs ACL. (C) Cell distribution was not significantly different. (D) Cell alignment: LDET cells were significantly more uniaxial aligned along the collagen fibres compared to MCL, • p<0.05 vs MCL (E) Vascularisation: SDFT was statistically more vascularised compared to the other three tissues. *p<0.05 vs ACL, • p<0.05 vs MCL, +p<0.05 vs LDET. (F) Inflammation: SDFT had statistically more infiltrating inflammatory cells compared to the other three tissues. *p<0.05 vs ACL, • p<0.05 vs MCL, +p<0.05 vs LDET.

4.4.1.2 Histological staining for glycosaminoglycans

Glycosaminoglycans (GAGs) were distributed mainly in IF and IB regions in both tendon and ligaments. In the CCL, GAGs seemed to have a mesh-like structure with increased staining subjectively noted at IF/IB regions and surrounding the cells (Figure 4.1C). Compared with the CCL, in the MCL (Figure 4.1D) the LDET (Figure 4.2C) and SDFT (Figure 4.2D) less staining for GAGs were was seen in the IF and occasionally in IB region, with no staining for GAGs surroundings the cells. Statistical analysis of the histological scoring for differential GAG staining between ligaments and tendons, resulted in statistically higher AB-PAS score in the CCL than MCL (p=0.0001), LDET (p=0.0001) and SDFT (p=0.001)(Figure 4.4).

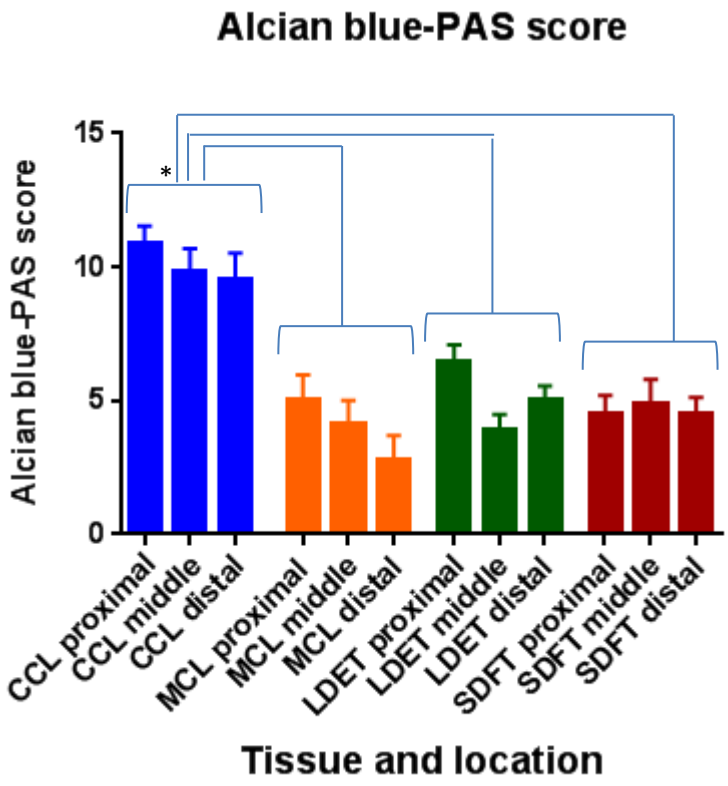


Figure 4.4. Alcian blue-PAS score for CCL, MCL, LDET and SDFT. Increased staining was noted in CCL which was statistically significant compared to MCL (p=0.0001), LDET (p=0.0001) and SDFT (p=0.001). No statistically significant differences were found between the different regions. Values are average of accumulated score, error bars represent SEM. *p<0.05

4.4.1.3 Histological staining for elastic fibres

Subjectively histological staining showed more elastic fibres in CCL compared to the other three tissues. Elastic fibres were mainly located between fascicles and fibre bundles, but were also found aligned parallel to the collagen fibres and pericellularly (Figure 4.2F). Further description of the arrangement of the elastin and microfibrillar glycoproteins fibrillin-1 and fibrillin 2 within the tendon and ligament tissues is described in Section 4.5.2. Histological scoring demonstrated statistically more elastic fibres in CCL compared to MCL (p=0.0001), LDET (p=0.0001) and SDFT (p=0.0001) (Figure 4.5).

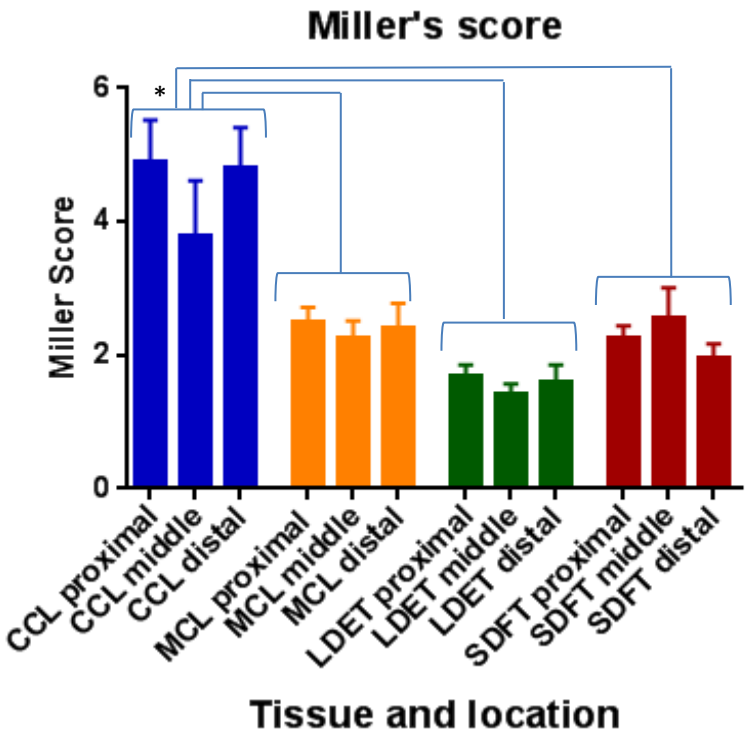


Figure 4.5. Miller's stain score for CCL, MCL, LDET and SDFT. More elastic fibres were identified in CCL compared to the other three tissues. Statistical analysis showed significantly more elastin fibres in CCL than MCL (p=0.0001), LDET (p=0.0001) and SDFT (p=0.0001). No statistically significant differences were found between the different regions. Values average of the accumulated score and error bars represents SEM. *p<0.05

4.4.1.4 Inter- and intra-observer agreement Histology scoring system

The integrity of agreement for both intra and inter-observer for each histology scoring parameter was assessed using Kendal coefficient of concordance analysis (Table 4.1). A Kendal coefficient values ranges from 0 to 1 with values closer to 1 suggestion a high degree of agreement.

		Intra-observer variation		**Inter-observer variation**
Observers		Ob1	Ob2	Ob1 and Ob2
ECM organisation	ACL	0.72	0.57	0.79
	MCL	0.7	0.83	0.83
	LDET	0.89	0.65	0.85
	SDFT	0.68	0.59	0.64
Cell morphology	ACL	0.65	0.66	0.77
	MCL	0.81	0.86	0.88
	LDET	0.71	0.54	0.72
	SDFT	0.62	0.74	0.79
Cell distribution	ACL	0.82	0.66	0.8
	MCL	0.67	0.62	0.78
	LDET	0.78	0.56	0.79
	SDFT	0.58	0.45	0.54
Cell alignment	ACL	0.71	0.43	0.74
	MCL	0.78	0.73	0.89
	LDET	0.81	0.61	0.74
	SDFT	0.65	0.63	0.8
Vascularity	ACL	0.92	0.57	0.79
	MCL	0.65	0.49	0.77
	LDET	0.91	0.73	0.9
	SDFT	0.76	0.72	0.67
Inflammation	ACL	0.63	0.61	0.61
	MCL	0.64	0.58	0.77
	LDET	0.79	0.87	0.79
	SDFT	0.67	0.53	0.7

Table 4.1. H&E scoring Kendall's coefficient of concordance results.

		Intra-observer variation		Inter-observer variation
Observers		Ob1	Ob2	Ob1 and Ob2
Alcian blue-PAS score	ACL	0.55	0.59	0.69
	MCL	0.58	0.63	0.66
	LDET	0.64	0.61	0.65
	SDFT	0.62	0.69	0.84
Millers score	ACL	0.68	0.61	0.69
	MCL	0.62	0.69	0.58
	LDET	0.71	0.73	0.83
	SDFT	0.76	0.65	0.82

Table 4.2. Kendall's coefficients of concordance for Alcian blue-PAS and Miller's histology score.

Kendall's coefficient concordance gave an average 0.71 and 0.64 for ob1 and ob2 intra-observer variation, while inter-observer variation an average value of 0.75 was measured. These indicated a good strength agreement between intra- and inter- observers.

4.4.2 Distribution of ECM macromolecules in CCL and LDET

4.4.2.1 Negative controls

Negative controls were included for every antibody immunostaining. Primary antibodies were raised either in rabbit or mouse. No immunostaining were detected with negative controls of non-specific rabbit and mouse IgG or in the absence of primary antibodies (Figure 4.6)

4.4.2.2 Collagens

- Collagen type I immunostaining was intense in both CCL and LDET and had a similar pattern distribution in both CCL and LDET. Although the most marked immunostaining of collagen type I was present in fascicular regions and was aligned along the fibres, the presence of collagen type I was also found between collagen fibre bundles in both CCL and LDET (Figure 4.7A and 4.7B).

- Collagen type III was mainly present in IF and IB regions of LDET, whilst in CCL collagen type III was more widespread and was found to be present in CCL substance, between collagen fascicles and fibre bundles (Figure 4.7C and 4.7D).

- Collagen type VI was mainly presented at the IF regions in both CCL and LDET (Figure 4.8A and 4.8B). Collagen type VI was found in close association with both CCL and LDET cells being localised at the pericellular regions of CCL and LDET, where they formed a dense mesh around groups of cells (Figure 4.8B and 4.8D).

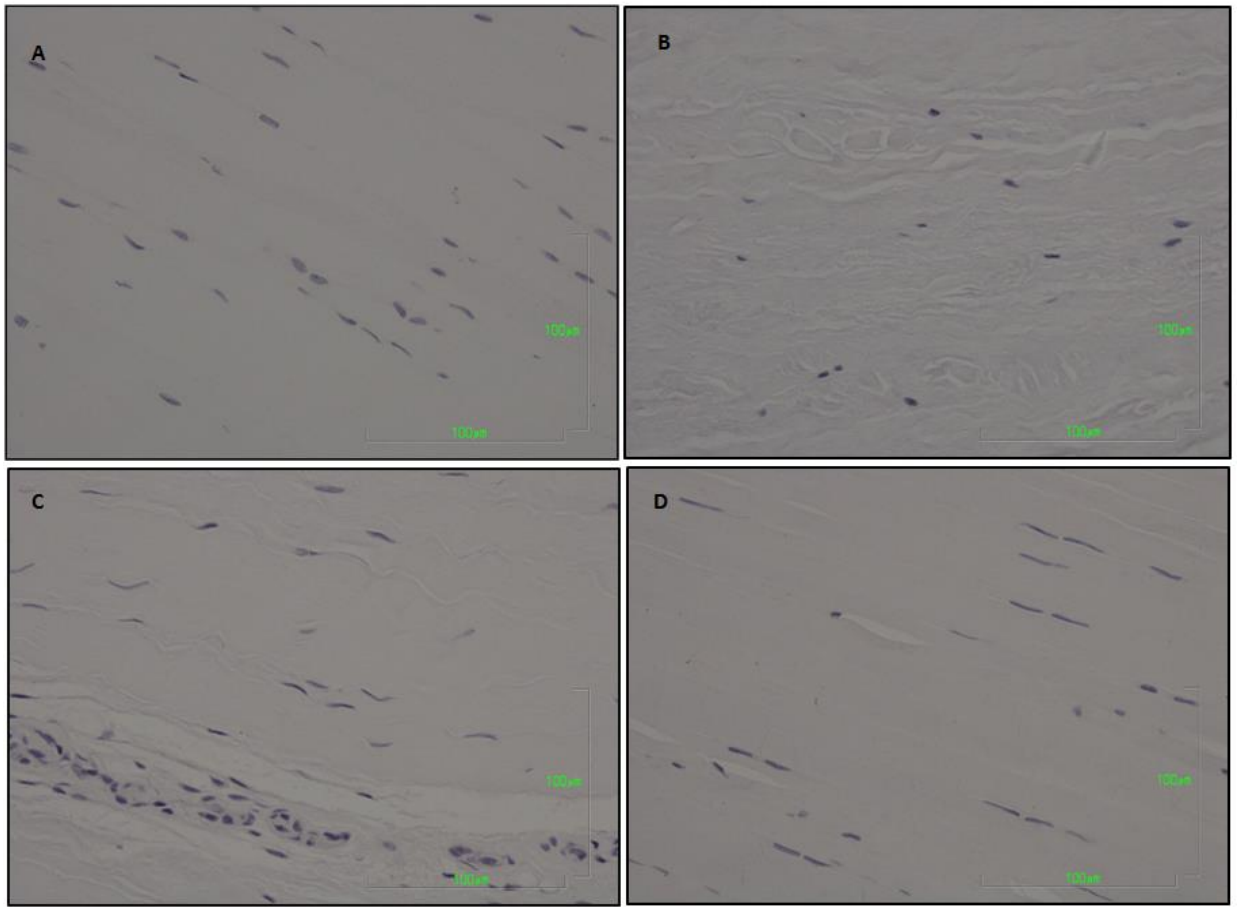


Figure 4.6. Representative immunostaining pictures of negative controls. Bar 100µm and 40X magnifications. No immunostaining was detected in the absence of primary antibody with addition of secondary goat antirabbit (A), secondary goat antimouse (B), rabbit IgG (C) and mouse IgG (D).

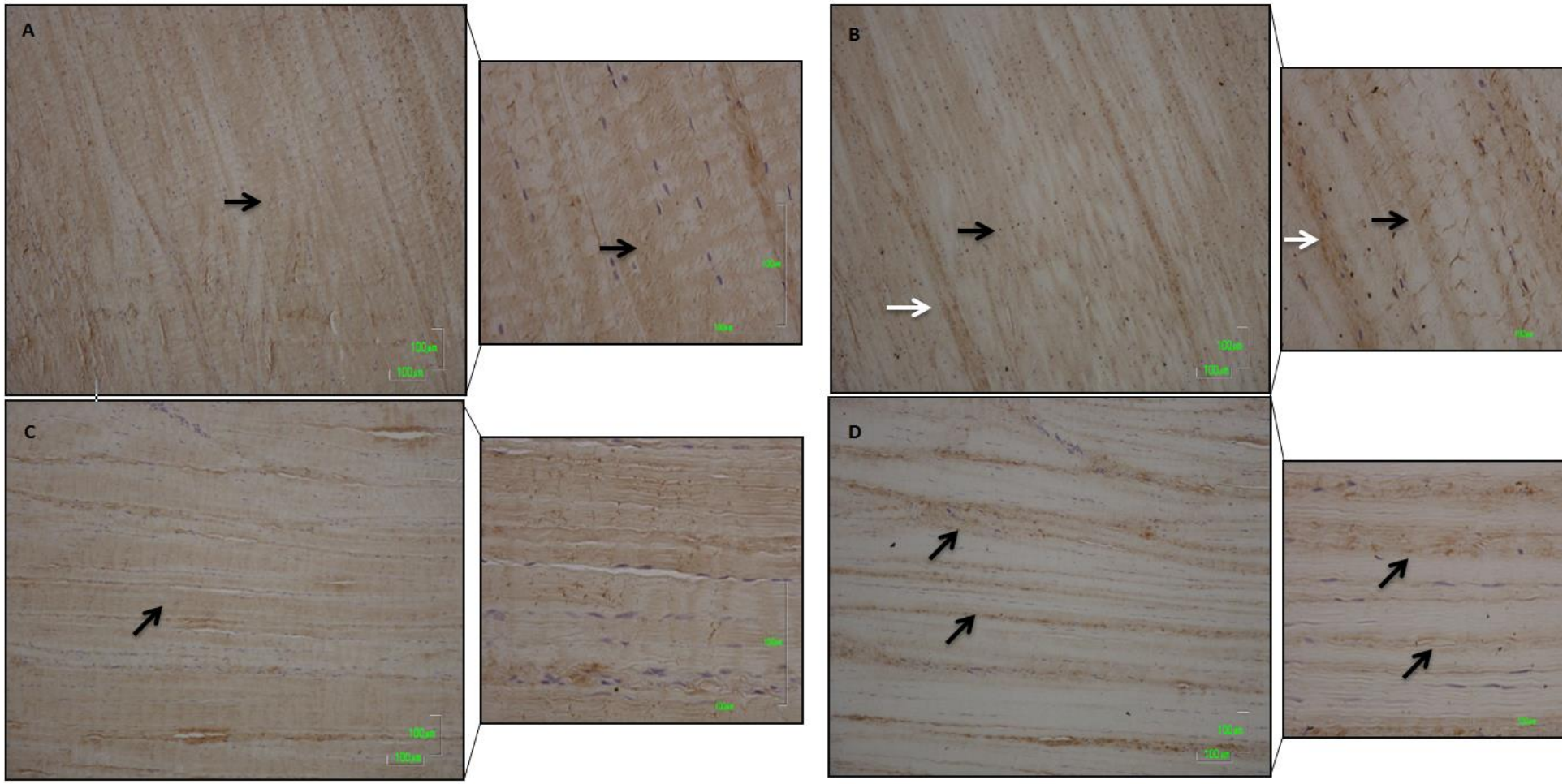


Figure 4.7. Immunostaining of collagen I and III. Microscopic longitudinal sections of canine CCL (A and B) and LDET (C and D). (Bar 100µm and 40X magnifications). Collagen type I immunostaining was mainly present in the aligned fibres (black arrows in A and C). Collagen type III immunostaining of CCL was present at both fascicular regions (black arrow in B) and between IF regions (white arrow in B), while in LDET collagen type III was primarily found at IF regions (black arrow in D).

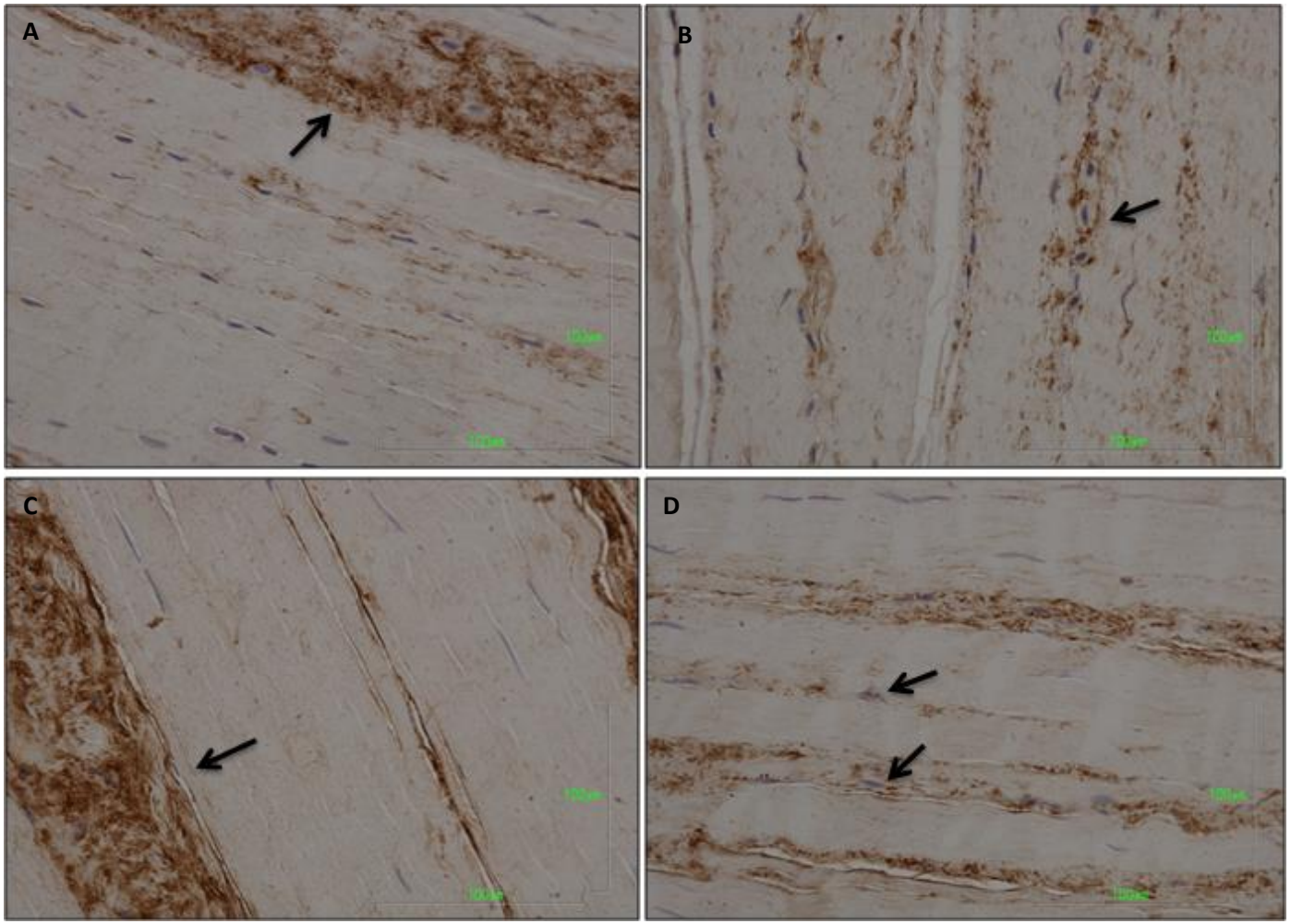


Figure 4.8. Immunostaining of collagen type VI. Microscopic longitudinal sections of canine CCL (A, B) and LDET (C,D). (Bar 100µm and 40X magnifications). Immunohistochemical localisation of type VI collagen. A marked immunostaining of type collagen VI was present at the IF regions (black arrows in A and B). Collagen VI was also localised in close proximity to tendon and ligament cells (B and D).

4.4.2.3 Proteoglycans

Large aggregating proteoglycans

- Aggrecan was mostly found at the interfascicular and interbundle regions of CCL and LDET. Marked immunostaining of aggreccan was observed in at IF regions of CCL compared to LDET. Aggrecan was highly localised around ligamentocytes (Figure 4.9A and 4.9B). This pericellular immunostaining of aggrecan was not observed in tendon and could be a key feature characteristic of ligament cells.

- Versican was found to be distributed between collagen fascicles and collagen fibre bundles in both CCL and LDET (Figure 4.8C and 4.8D). A noticeable immunostaining of versican was noted in CCL in comparison to LDET. Versican was not present on the aligned fibres throughout tendon and ligament tissue and was not found to be localised around ligament and tendon cells (Figure 4.9C and 4.9D).

Small leucine rich proteoglycans

- Decorin demonstrated marked immunostaining in both CCL and LDET and was present on the aligned collagen fibres at both fascicular and IF/IB regions (Figure 4.10A and 4.10B).

- Biglycan was present at the IF region of LDET with a minor immunoreactivity and no pericellular staining. However in the CCL biglycan was only found occasionally surrounding rounded cells (Figure 4.10C and 4.10D).

- Keratocan was only observed in the IF regions of both CCL and LDET although there was noticeable more immunoreactivity in the LDET (4.11A and 4.11B).

- Asporin immunostaining in LDET was in the IF region and surrounding tenocytes. In the CCL, whilst asporin immunostaining was localised to the pericellular region around most of ligament cells, no IF staining of asporin was found (Figure 4.11C and 4.11D).

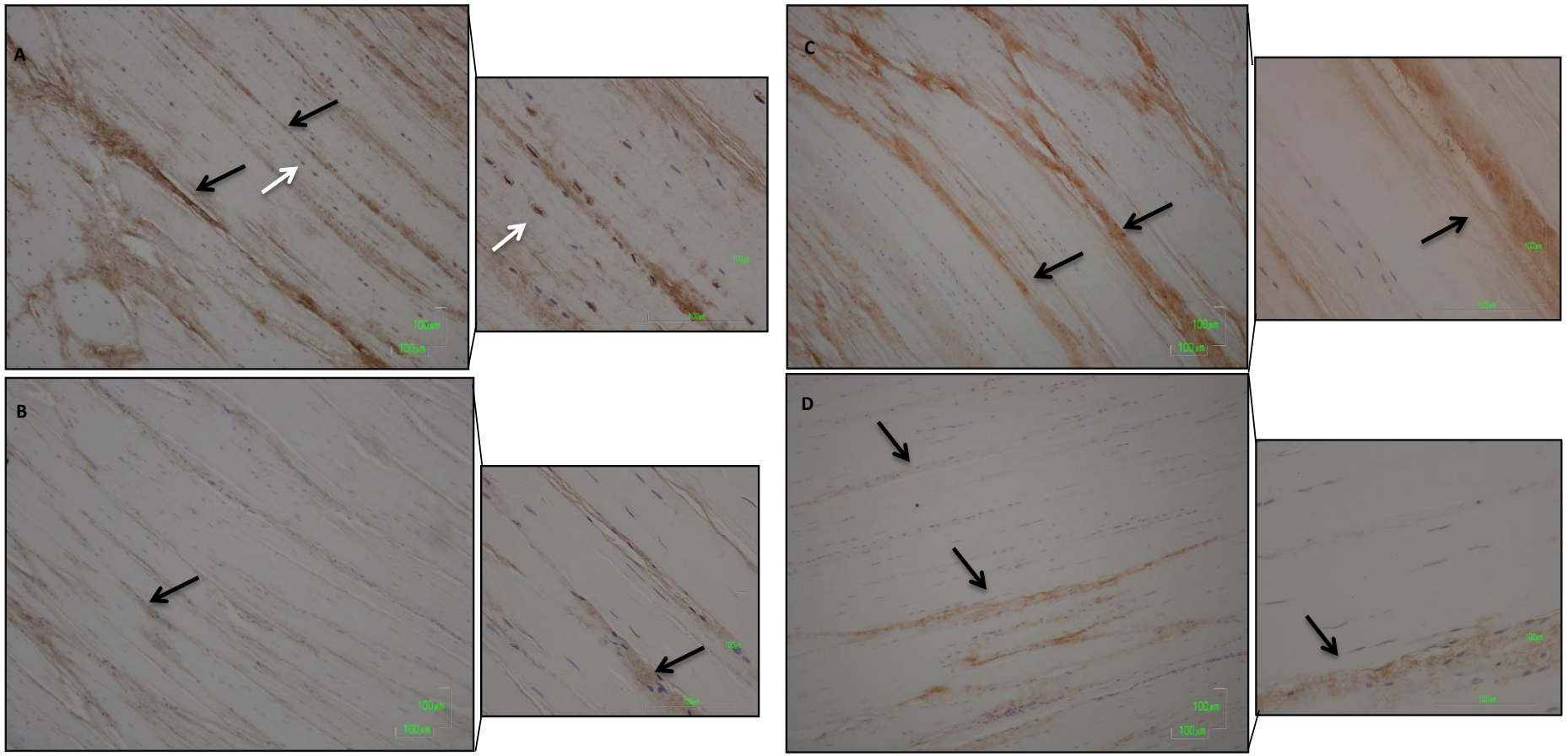


Figure 4.9. Immunostaining of aggrecan and versican. Microscopic longitudinal sections of canine CCL (A, C) and LDET (B,D). (Bar 100µm. 10X and 40X magnifications). Aggrecan was mainly present between collagen fascicles and fibre bundles in both CCL and LDET (black arrow in A and B), though this was noticeably higher in CCL. Immunostaining of aggrecan was also noted surrounding ligament cells (white arrows in A). Similar to aggrecan, a marked immunostaining of versican was found at IF and IB regions of CCL and LDET (black arrow in C and D), though greater immunostaining was observed in CCL in comparison LDET. No pericellular staining of versican was observed in either CCL or LDET.

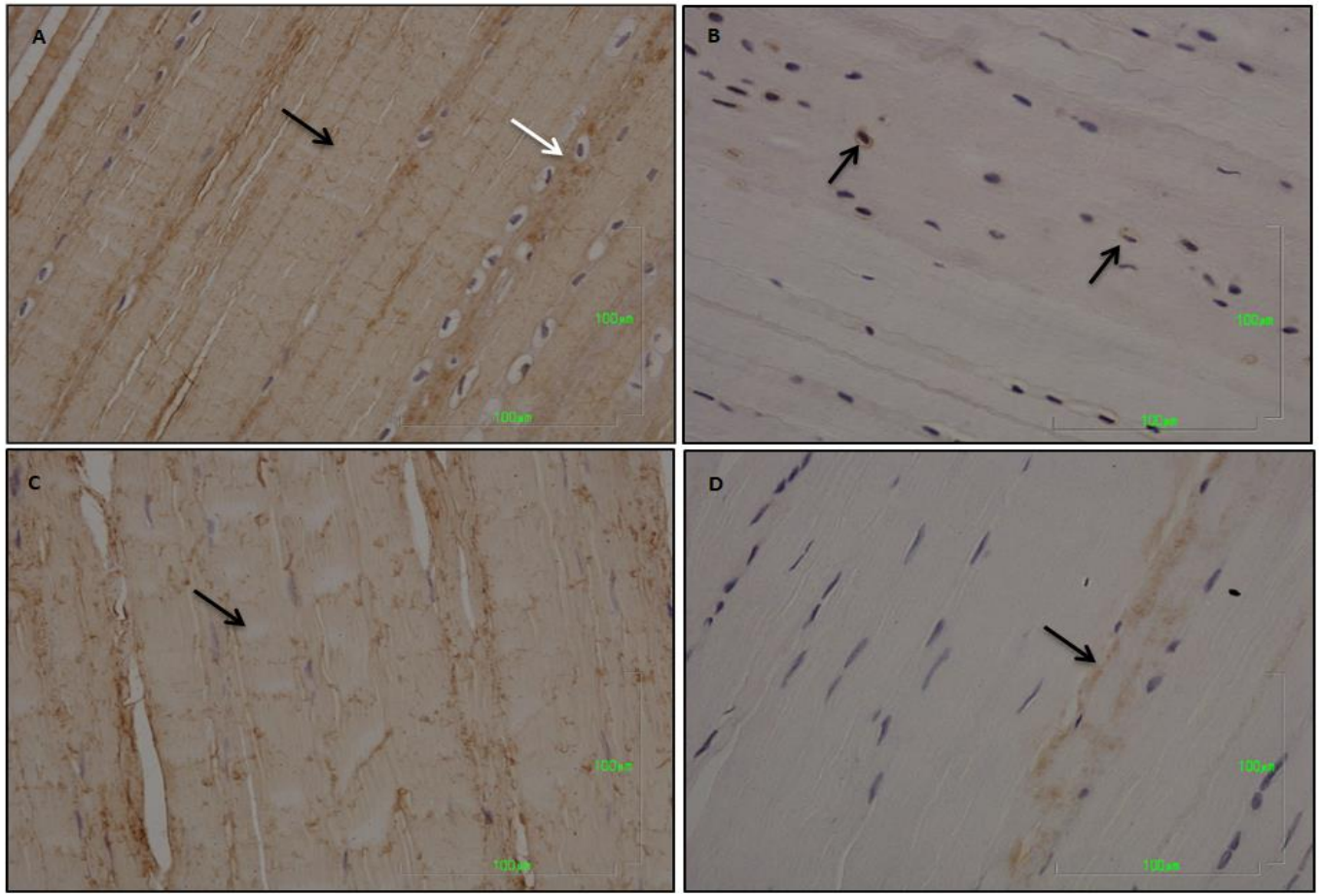


Figure 4.10. Immunostaining of decorin and biglycan. Microscopic longitudinal sections of canine CCL (A, B) and LDET (C, D). (Bar 100µm. 40X magnifications). Decorin immunostaining was present on the aligned collagen fibres (black arrows in A and C) and at the IF regions in both CCL and LDET (white arrow in A). Minor immunostaining of biglycan was detected in both tissues, where biglycan was only occasionally present pericellularly in CCL (black arrow B) and at the IF region in LDET (black arrow D).

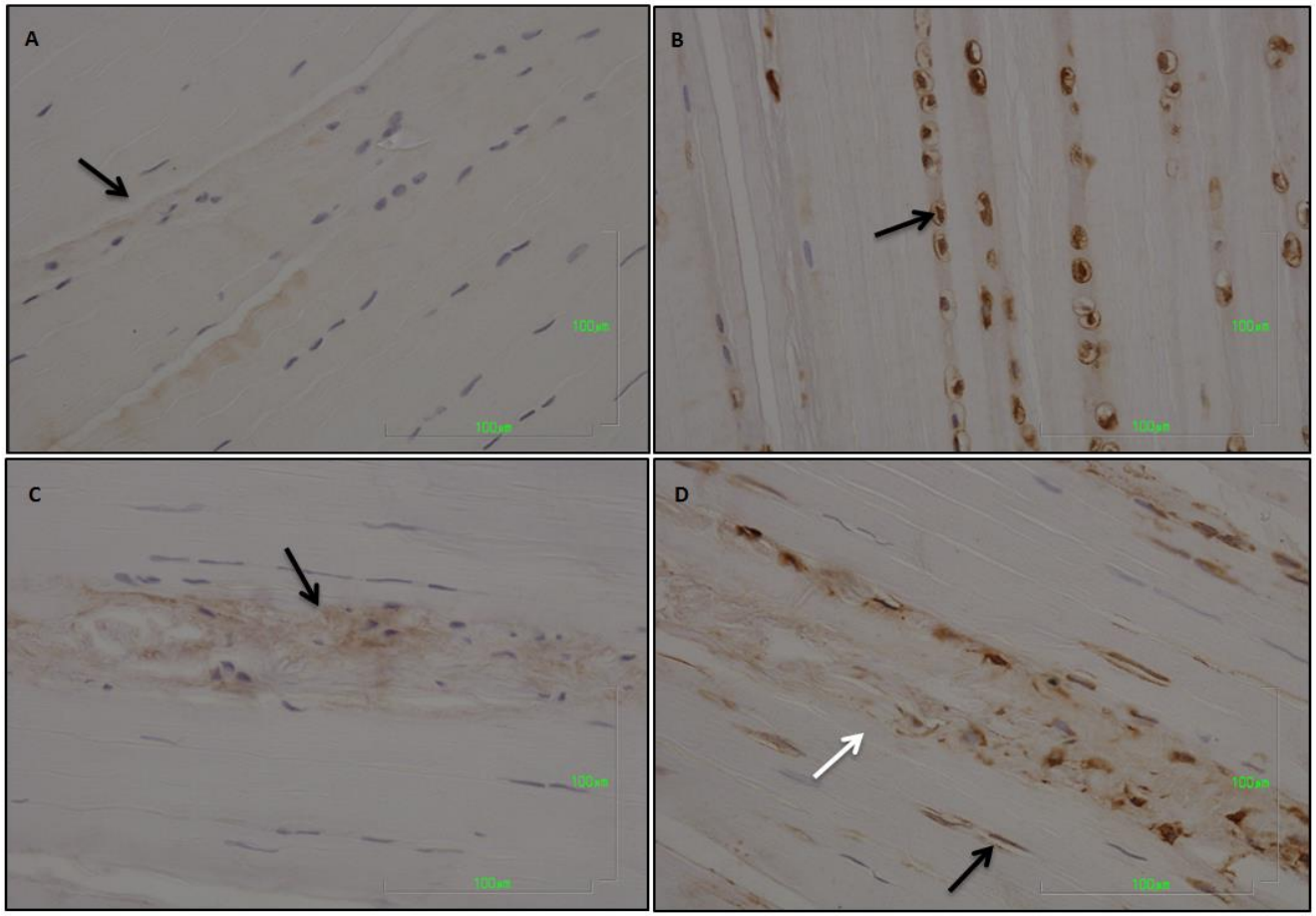


Figure 4.11. Immunostaining of keratocan and asporin. Microscopic longitudinal sections of canine CCL (A, B) and LDET (C, D). (Bar 100µm. 40X magnifications). Keratocan was present at the IF regions in both CCL and LDET (black arrows in A and C). Asporin immunostaining was found at the IF region (white arrow in D) in LDET and surrounding tenocytes (black arrow in D). In CCL, asporin was only present pericellularly (black arrow in B

4.4.2.4 Elastic fibres

- Fibrillin 1 and fibrillin 2

Fibrillin 1 localised to the interfascicular region, running longitudinally along the CCL and LDET in parallel alignment to collagen fibres and was highly localised around ligament and tendon cells. Stronger immunofluorescence was observed at interfascicular regions in both CCL and LDET, where the fibres formed a fine irregular meshwork (Figure 4.12A and C). The pattern of fibrillin 2 distribution was similar to fibrillin 1. Fibrillin 2 was broadly orientated parallel to collagen bundles with pericellular staining in both CCL and LDET. Fibrillin 2 immunostaining was also found to be more marked between fibres bundles and fascicles (Figure 4.12 B and D). No difference in the distribution of fibrillin 1 and fibrillin 2 was observed between CCL and LDET. Fibrillin 1 and 2 were unaffected following hyaluronidase enzymatic treatment.

- Elastin fibres and co-localisation with fibrillin 1 and fibrillin 2

Elastin fibres were sparse in comparison to the fibrillin 1 and fibrillin 2. Immuno-detection of elastin fibres was enhanced following treatment with hyaluronidase. In both CCL and LDET elastin fibres were found to be predominantly present at the interfascicular or interbundle regions and arranged in a fine, twisting meshwork either parallel or perpendicular to the long-axis of the tissue (Figure 4.13, white arrows in elastin images). All elastin fibres at this region were co-localised with either fibrillin 1 and 2 in both CCL and LDET (Figure 4.13 white arrows in elastin + fibrillin 1 images). Elastin fibres were also found at CCL and LDET substance where they were mostly oriented parallel to collagen bundles (Figure 4.13 orange arrows in elastin images). At this region elastin fibres were commonly co-localised with both fibrillin 1 and fibrillin 2, where they were in close conjunction to the cells (Figure 4.13 orange arrows elastin + fibrillin 1 and 2 images). In LDET it was occasionally noted that fibrillin 1 and 2 were independent, as elastin was occasionally not found to co-localise with fibrillin 1 and 2 (Figure 4.13 blue arrows in elastin+ fibrillin 1 and 2 images).

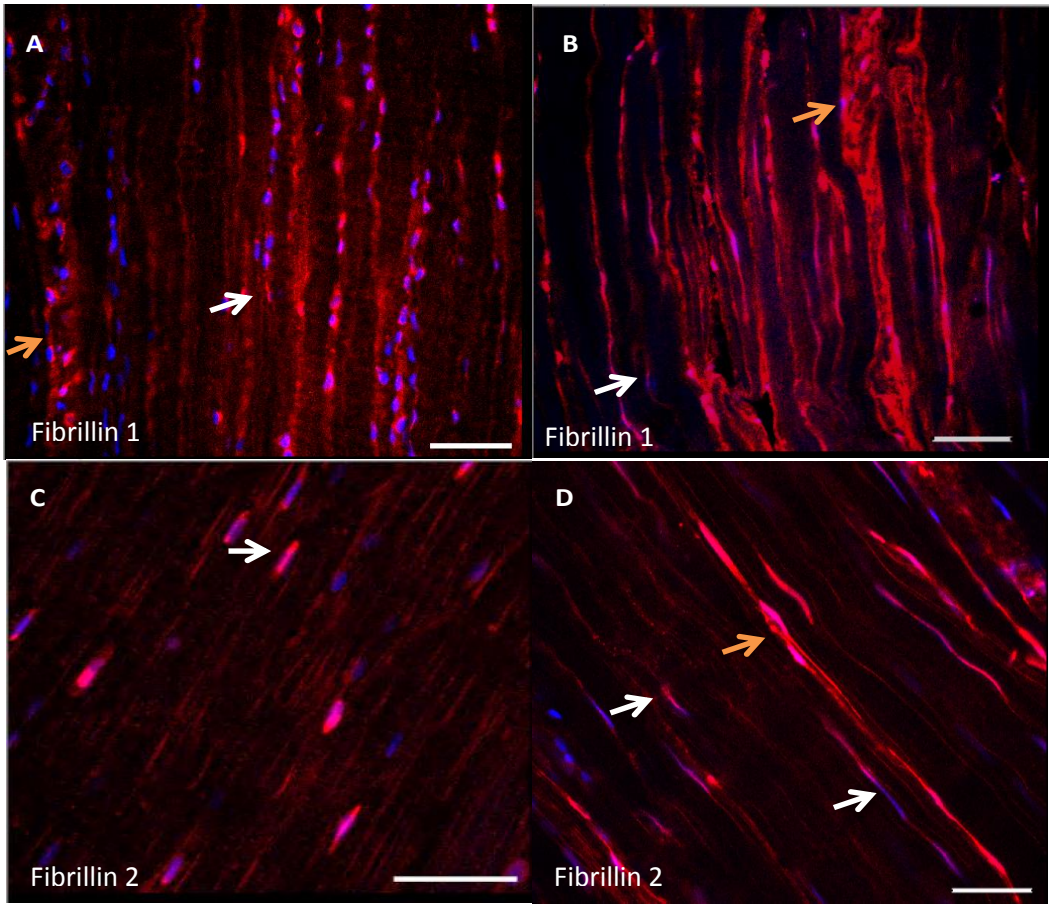


Figure 4.12. Immunostaining of fibrillin 1 (A, B) and fibrillin 2 (C, D). Microscopic longitudinal sections of canine CCL (A, C) and LDET (B,D). (Bar 50µm. 40X and 60X magnifications). Fibrillin 1 and fibrillin 2 (red) were found to be localised between collagen fascicles and bundles (orange arrows in A, B and D) with a parallel alignment to the long-axis of the tissue mainly surrounding the ligament and tendon cells (nuclei in blue) (white arrows in A, B, C and D).

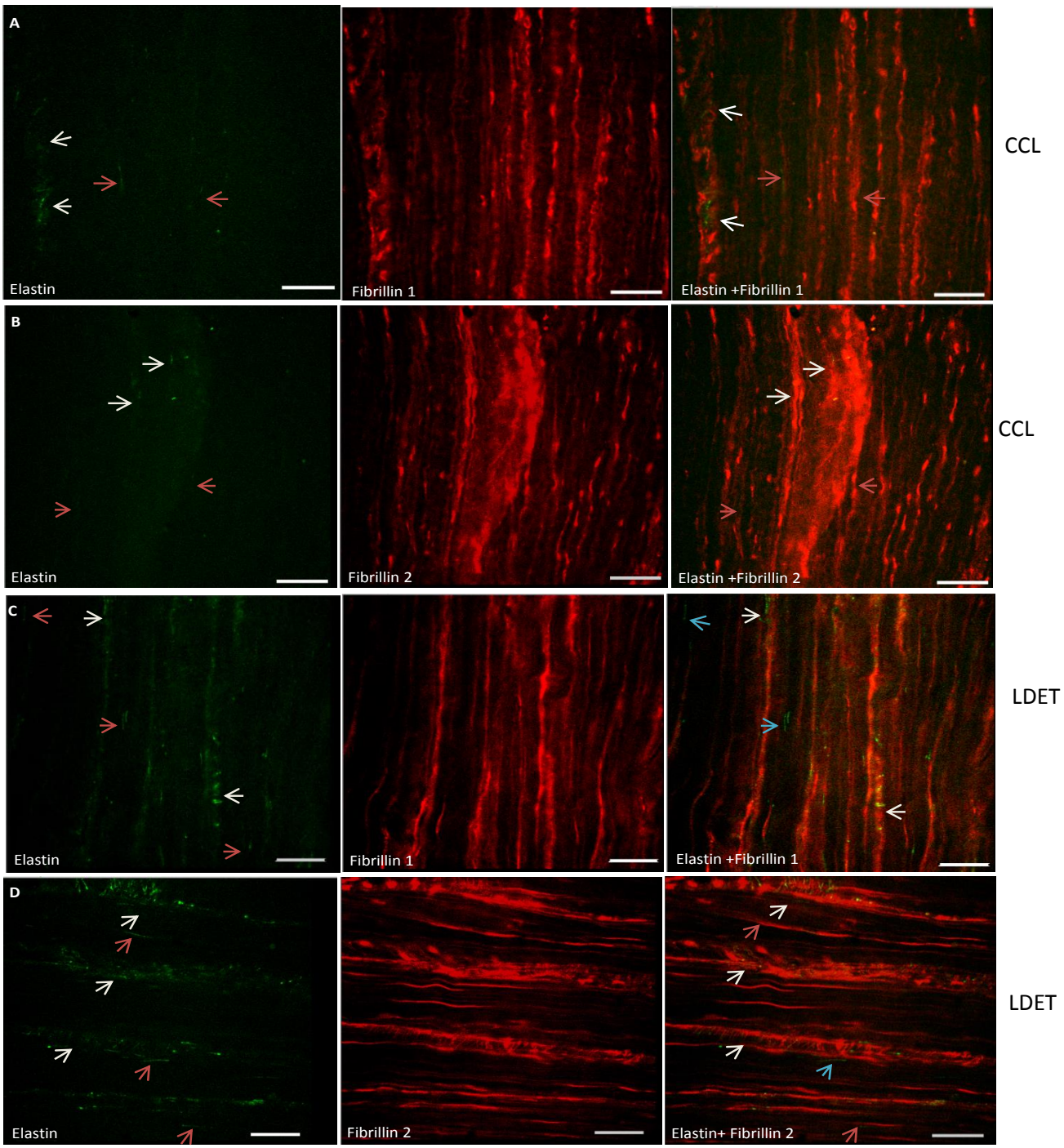


Figure 4.13. Immunostaining of elastic fibres with fibrillin 1 and 2. Microscopic longitudinal sections of canine CCL (A, B) and LDET (C, D). (Bar 50µm. 40X magnifications). Elastin fibre (green) distribution was sparse in comparison to fibrillin 1 and fibrillin 2 (red). In both CCL and LDET, elastin fibres were mainly distributed in IF regions and co-localised with fibrillin 1 and 2 at this region (white arrows A, B, C and D). Elastin fibres were also found within the CCL and LDET fascicles mostly parrallel aligned and co-localised to fibrillin 1 and 2 (orange arrows and A, B, C, D). Occasionally in LDET not all elastin fibres were found to co-localise with fibrillin 1 and 2 (blue arrows in C and D).

4.5 DISCUSSION

This study has demonstrated different morphological characteristics between the ligament and tendons that are in and around the canine stifle joint. However no significant regional differences within each tissue were found. A differential distribution of several ECM proteins between tendon and ligament was observed, which supports the second hypothesis, that canine tendon and ligament have distinct distributions of ECM macromolecules. Analysis of histological sections of ligaments (MCL and CCL) showed that they have a more loosely aligned collagen fibre bundle architecture and different cellular alignment compared to LDET. Ligaments such as the CCL were also found to have a more mixed population of cell morphologies than LDET. Vascularity was significantly more abundant in SDFT than both CCL and MCL. Similarly for inflammation, SDFT was found to have the highest number of infiltrative cells present compared to all three tissues. Immunostaining of the ECM macromolecules in CCL and LDET demonstrated a diverse distribution in collagen type III, aggrecan and asporin between the two tissue types. A more marked intensity of aggrecan and versican staining was observed in CCL. An altered distribution of collagens, proteoglycan and elastic fibres was observed at the fascicular regions, interfasicular and pericellular regions between and within tendons and ligaments, which may indicates the important role of ECM macromolecules for cellular function, regulation of the collagenous components viscoelasticity and elastic recoil mechanism.

4.5.1 Morphological and structural comparison of the different tendons and ligaments

4.5.2 Histological findings

ECM organisation- The overall architecture of intra- and extra-articular ligaments and extensor and flexor tendons was assessed based on the alignment of collagen fibres and spacing between fibre bundles and fascicles. The CCL and MCL had loosely arranged and less compact collagen fibres in comparison to LDET. This was more prominent in particular in the CCL where more spacing between collagen fibre bundles was observed and was statistically significant when compared to both LDET and SDFT. This finding might indicate a more complex structure of collagen fibre arrangement with regards to the IFM in CCL. Zhu et al.

(2012) demonstrated with scanning electron microscopy an undulating wave pattern known as crimp, in the human ACL, semitendinous and gracilis tendons. In addition, an helical wave pattern was also found in the ACL, indicating a more complex arrangement of collagen fibres in ACL, where tissue must resist multidirectional or unpredictable loading (Zhu et al. 2012). In the dog, both patellar tendon and CCL have been found to have crimp and a helical wave pattern of collagen fibres (Yahia and Drouin 1989). Crimp is involved in both transferring/absorbing forces and recoiling of tendon and ligaments (Franchi et al. 2009), and different crimp patterns may reflect different mechanical role of ligaments (Franchi et al. 2010). Franchi et al. (2009) demonstrated that the energy storing human vastus intermedius tendon required greater elastic recoil and therefore has larger crimp angles than that of both the positional rectus femoris and patellar tendon. Similar results have been found comparing the energy storing equine SDFT and positional CDET (Birch et al. 2013). In this study the different collagen structural architecture between tendon and ligament may be as result of the different axial forces applied in these structures. Forces are generally applied in a uniaxial direction in tendon resulting in parallel alignment collagen fibrils, whereas in the ligament collagen fibrils are not as uniformly aligned due to a multiaxial loading pattern (Amis 1998, Rumian et al. 2007). Further investigation with regard to crimp pattern and collagen fibril diameter between canine inter- and extra-articular ligament and extensor and flexor tendons in relation to functional and mechanical properties of these tissues is required.

Cellular morphology- Histological analysis showed different cellular morphologies in all ligament and tendon tissues throughout the different regions, indicating a heterogeneous cellularity among these tissues which is likely to be an adaptation to tissue mechanics or physiology (Benjamin and Ralphs 1998). This finding supports the variation in cell morphology previously reported in canine cruciate ligament (Smith et al. 2012). The majority of tendon and ligament cells are fibroblasts (Benjamin and Ralphs 1997), which are referred to as tenocytes in tendon and ligamentocytes in ligament (Hoffmann and Gross 2007). Apart from tenocytes, progenitor stem cells have been identified in human hamstring tendon (Bi et al. 2007) and in human ACL and MCL (Zhang et al. 2011). Furthermore more recently it has been shown that in mice Achilles tendon the epitenon and tendon substance have different populations of progenitors cells (Mienaltowski et al. 2013), with a unique

tenogenic differentiation (Mienaltowski et al. 2014). The presence of heterogeneous cell morphology in the ligament and tendons in this study may also be due to the occurrence of stem cell or progenitors cells. Though whether these stem cells reside in canine tendons and ligaments yet needs to be identified.

The scoring analysis result for cellular morphology demonstrated more spindle shaped cell nuclei in LDET in comparison to CCL which had more rounded and epileptically shaped cell nuclei ($p=0.003$). The spindle shaped cell nuclei morphology of tenoctyes in the canine tendons from the current study corresponds with other tendons from different species such as the horse (Clegg et al. 2007) and rabbit (Amiel et al. 1984). Similar to the present findings, Amiel et al. (1984) also found rabbit ACL contained more round and ovoid cells when compared to MCL, patellar and Achilles tendon. Murray et al. (2004) reported a majority of fusiform and rounded cell nuclei in normal human ACL which were also found in the canine CCL using histological analysis, indicating similar intrinsic properties of the fibroblasts between two species. The rounding and ovoid cell phenotype (chondrocytic) in tendon and ligament becomes more prominent where it approaches close to the bone (origin and insertion regions) in ligament (Duthon et al. 2006) and at the osteotendinous junction in tendon (Docking et al. 2013), and is likely to be as a result of compressive forces. In the human ACL a chondrocytic cell phenotype along with GAG accumulation, disorientation of collagen fibres and inflammation has been reported to be associated with degeneration and ageing (Hasegawa et al. 2012). The human ACL has also been shown to have different cellular changes with degeneration and ageing, where a decrease in the total cell number, MMP-1, 3 and 13 expression was found in normal ACL ageing but was increased in degenerated ACL (Hasegawa et al. 2013). In the canine CCL, a chondrocytic cell phenotype has been found in breeds with both a low (greyhound) and high risk (Labradors) of ligament rupture (Comerford et al. 2006). The low risk CCLs also had a fibrocartilaginous appearance with increased collagen fibril diameters, whereas the high risk breed had marked chondroid changes similar to what has been reported in human ACL with ageing and degeneration (Hasegawa et al. 2012). In this study, the CCLs were normal (disease free) and from young skeletally mature dogs (less than 5 years of age) at a moderate risk (Staffordshire bull terrier cross), to ligament rupture. It was found that the CCL ligamentocytes throughout the different regions to have a more epiliptical and round shaped cell nuclei being mostly

surrounded by GAGs as stained with Alcian blue-PAS. The “chondrocytic” appearance of ligamentocyte nuclei in cell chains and lacunae were also noted at the proximal, middle and distal regions of CCL. These findings particularly in the low and moderate risk dog breeds to ligament rupture may indicate that chondroid change may be a normal finding and a physical adaptation of CCL, rather than pathological degeneration. Nonetheless, further studies are required to identify cells type ontogeny and the effect of ageing and degeneratiion on canine CCL structure and whether this matches to the results reported in human ACL ageing and degeneration (Hasegawa et al. 2013).

Cellular distribution and alignment- In all tissues we found an increased cellular density in the interfasicular regions, with cells displaying rounded nuclei morphology with no particular orientation at this region. Although there was not any statistically significant differences for cellular distribution between different tissues, the CCL cells were more clustered and aligned in chains corresponding to the chondrocytic appearance as discussed earlier. Alignment of cells was assessed based on orientation of cells along the collagen fibre bundles. The tendon cells were more uniaxial and parallel to collagen fibres, while not all ligament cells were aligned in the same direction. This finding was statistically significant between MCL and LDET, which may be as result of different axial forces applied between these tissues.

Vascularity- Tendons and ligaments generally have a poor blood supply (Benjamin et al. 2008). Fibrocartilaginous regions of tendon and ligament are avascular, but there is some indirect anastomosis between the vessels (Petersen and Tillmann 1999, Fenwick et al. 2002, O’Brien 2005, Benjamin et al. 2008). Several tendons including the Achilles tendon, patella tendon and supraspinatus tendon appear to have regions of reduced vascularity, where avascular zones can be associated with degeneration and rupture (Fenwick et al. 2002). In human ACL non-homogenous vascularity and areas of hypovascularity have also been reported, which are within the core region of the ACL (Petersen and Tillmann 1999, Duthon et al. 2006). This has also been reported in the dog, with the proximal region containing a greater number of vessels, although a normal canine CCL is considered to be relatively hypovascular (Vasseur et al. 1985, Hayashi et al. 2011). This corresponds to our findings of low blood vascularity in non-diseased CCL. Blood vessels, when present, were found to be mainly located in the interfascicular regions of the different tendons and ligaments.

Histological scoring analysis demonstrated SDFT to be statistically more vascular in comparison to LDET, MCL and CCL, indicative of a better blood supply in canine SDFT.

Inflammation- In this study few inflammatory cells were found to be present in the tendon and ligaments examined. Occasionally infiltrating cell, which were primarily mononuclear-type cells where found at the epitenon and close to blood vessels. This finding was most evident in SDFT, which also had the highest number of blood vessels being statistically higher in comparison to LDET, MCL and CCL. The role of vascularity and inflammation in canine SDFT needs to be further explored with a focus on whether is it associated with disease or normal physiology.

Pathological tendons do not generally show signs of inflammation, however it may play a role in early initiation of the disease (Legerlotz et al. 2012). Studies have suggested that inflammatory cytokines and mediators may play a role contributing to disease as they have also been detected in painful posterior tibialis tendon and Achilles and rotator cuff tendons (Nakama et al. 2006, Legerlotz et al. 2012).

GAGs histological analysis- The accumulation of GAGs have been found to be associated with human ACL degeneration (Hasegawa et al. 2012), Achilles tendinopathy (Riley 2008), and supraspinatus tendinosis (Riley et al. 1994), although it has been shown to decrease in supraspinatus tendon with age (Riley et al. 1994). This has also been found in dog, where ruptured CCLs contained significantly higher GAG content when compared to intact CCLs from the same breed (Comerford et al. 2004). In the present study, the GAGs were mainly found at the IF and IB regions in all tissue, but also pericellularly in CCL. LDET, SDFT and MCL displayed relatively little GAG staining in comparison to CCL, which had significantly higher scores than the other three tissues ($p=0.001$). This finding corresponds with the increased GAG content found in CCL in comparison to MCL, LDET and SDFT (Chapter 3). Hasegawa et al. (2012) found an increased deposition of GAGs in the human ACL with the development of cartilage lesions, though no correlation was found between ACL ageing and mucoid degeneration, as they indicated that a relatively high percentage of young donors already had mucoid degeneration. This might also explain the high GAG content in our CCLs that were obtained from young skeletally mature donors. The high GAG content in CCL is most likely not as result of pathological degeneration, but is possibly related due to the physical

adaption of the tissue to different mechanical loading, which subsequently can lead to altered viscoelastic properties.

Elastic fibres- Histological staining of elastic fibres has previously been reported in canine CCL, demonstrating an increased quantity of staining and elastin content reported in a breed (greyhound) which has a low-risk of ligament rupture (Smith et al. 2013). These greyhounds were all exercised animals being ex-racing dogs (Smith et al. 2013). Although the exact effect of exercise on elastin content in canine cranial cruciate ligament has not been determined. A further study of the comparison between dog breeds demonstrated much less elastic staining in similarly aged moderate (beagle) and high risk breeds (Labrador retriever) (Smith 2010). This finding may be suggestive of a fundamental difference between breeds and may be due to the reduced production or increased destruction of elastic fibres (Smith 2010). In this study the elastic fibre intensity staining in the CCL was found to be similar to the previously described moderate risk breed (beagle) (Smith 2010). In both tendons and ligaments tissues elastic fibres were mainly present at the interfascicular and interbundle regions, but also found parallel aligned to collagen fibres. Comparison of the total Miller's score between the different tissues found a significantly higher score in the CCL than MCL, LDET and SDFT ($p=0.001$). This finding supports previous biochemical results showing a higher elastin content in CCL (Chapter 3) compared to other tendons and ligaments. Overall the higher elastin content in the CCL may be due to greater requirement for a stretch and recoil mechanism in this tissue.

Differences between regions- Regional variation of tendons and ligaments can alter as a result of changes in mechanical loading, where regions under mechanical stress can exhibit an increased fibrocartilaginous matrix composition (Benjamin and Ralphs 1998). The human ACL has been reported to have a different cellular morphology and collagen content at the proximal, middle and distal regions (Duthon et al. 2006). Ralphs et al. (1998) demonstrated altered cellular morphology in rat Achilles tendon at different regions, while Waggett et al. (1998) found more mRNA expression of aggrecan and versican in fibrocartilaginous regions compare to the mid-substance of the human Achilles tendon. However, no differences were found between the regional variances of the levels of matrix constituents in tendons and ligaments using either histological analysis or biochemical assays (Chapter 3). One possible

explanation could be that during dissection only a fragment of each proximal and distal region was analysed which may mask localised differences between the regions.

4.5.3 Distribution and organisation of collagens, proteoglycans and elastic fibres in CCL and LDET

In this study the distribution of the ECM macromolecules were assessed between CCL and LDET as representative ligaments and tendons respectively. Both CCL and LDET were primarily chosen based on the findings of a different ECM composition in these tissues (Chapter 3) and morphological and/or structural differences (as discussed above) in CCL. In comparison to CCL, LDET has been found to differ in terms of ECM content, structure and cellular morphology. As this positional tendon is also located in intra-articular in canine stifle joint it was therefore considered to be more comparable to the CCL. The distribution and organisation of ECM macromolecules were assessed on the middle region of the tissues as no regional difference were found (Chapter 3 and as discussed above), but also to avoid any of the fibrocartilage regions in both tissues.

Collagens

Distribution of collagen types I, III and VI- in both tendon and ligament- Collagens type I and III have been documented to be the major collagens in both tendon and ligament (Mienaltowski and Birk 2014). In the current study type I collagen immunostaining was found to be intense and mainly present in the fascicular regions in both LDET and CCL. Collagen type III was primarily located along the interfascicular and interbundle regions in LDET, similar to that previously reported for normal equine SDFT (Sodersten et al. 2013) and human extensor carpi radialis brevis tendon (Duance et al. 1977). In CCL, collagen type III displayed increased immunostaining and it was found to be located not only between collagen fibre bundles but also aligned on the fibre bundles. This finding agrees with previous reports comparing rabbit cruciate ligament with patellar tendon (Amiel et al. 1984) and human ACL with patellar tendon (Little et al. 2014). A quantitative study Wan et al. (2014) which used picrosirius red staining and tensile testing demonstrated that altered collagen type I and III ratio during ligament ageing is correlated with the mechanical

properties of ligaments, as the different type I and III ratios were found to be strongly related to the Young's modulus in the linear region of the stress-strain curve. This finding may indicate that different distribution of collagen type I and III in CCL and LDET in the current study may result in different mechanical behaviour in the two tissue types, yet this needs to be further elucidated.

Collagen type VI has a non-fibrillar organisation and is widely expressed in most tissues, including skeletal muscle, tendons and ligaments (Keene et al. 1988). It has been shown that collagen type VI interacts with many ECM molecules, including collagen I, II, and XIV, microfibril associated glycoprotein (MAGP-1), perlecan, decorin, biglycan, chondroitin sulphate, heparin and hyaluronan (Mienaltowski and Birk 2014). In this study collagen type VI immunostaining was mainly found between fascicles and bundles, but also localised around the CCL and LDET fascicular fibroblasts. No differences in distribution were noted between CCL and LDET. Pericellular localisation of collagen type VI in both canine CCL and LDET were also found, which supports previous findings in human ACL (Sardone et al. 2014) and human supraspinatus tendon (Thakkar et al. 2014). The absence of collagen type VI in knockout mice has been shown to result in dysfunctional tendon collagen fibrillogenesis (Izu et al. 2011) and points to a critical role of collagen VI in both tendons and ligaments.

Proteoglycans

Distribution of large aggregating and small leucine rich proteoglycans- The major proteoglycan in tendons and ligament are the small leucine proteoglycan (SLRPS) decorin, biglycan, fibromodulin and lumican (Rees et al. 2009, Parkinson et al. 2011, Yang et al. 2012). To date, other SLRPS are less well characterised, in these tissues. For instance the distribution of keratocan has only been determined in bovine digital flexor tendon (Rees et al. 2009), while the distribution of asporin has not been previously elucidated in either tendon or ligament. Large aggregating proteoglycans aggrecan and versican have also been found at the tensional region of bovine deep digital flexor tendon (Rees et al. 2000, Rees et al. 2009) and human ACL (Little et al. 2014). In this study the presence and distribution of aggrecan, versican, decorin, biglycan, keratocan and asporin was demonstrated with immunostaining in both CCL and LDET. An increased immunostaining of both aggrecan and versican was found in the midsubstance region of CCL compared to the midsubstance region

in LDET. Both aggrecan and versican were localised mainly between fascicular and bundle regions, however aggrecan was also found to be pericellularly located only in CCL. Both aggrecan and versican are large proteoglycans that bind to hyaluron (Halper 2014). Aggrecan is highly glycolysated with numerous chondroitin and keratan sulphate GAG chains, which attracts counter-ions and functions to hold water within the tissue, resulting in high tissue hydration that renders the tissue resistant to compressive loading (Riley 2005, Halper 2014). The structure of versican is similar to aggrecan, but lacks in one of the globular domain and contains much less GAGs (CS) (Riley 2005). An increase in versican content leads to expansion of ECM and a greater viscoelasticity of pericellular matrix, supporting cell-shape changes required for cell proliferation and migration (Halper 2014). In the human Achilles tendon, a higher expression of aggrecan has been found at the fibrocartilaginous regions where it is more associated with compressive forces, while versican mRNA was more expressed in mid-substance regions when compared to the insertion region (Waggett et al. 1998). Together the increase of aggrecan and versican immunostaining in this study may indicate higher tissue hydration and viscoelastic properties of CCL. In addition the increased pericellular location of aggrecan may also indicate that the CCL is subjected to more compressive forces throughout the tissue, which could be due to its functional adaption to protect the tissue from damage (Riley 2005).

SLRPs have regulatory roles in collagen fibrillogenesis during tendon development (Zhang et al. 2005, Chen and Birk 2013). In the current work a panel of SLRPS that are well described (decorin and biglycan) and less described (keratocan and asporin) were analysed. Asporin was also examined based on our proteomic finding between CCL and LDET (Chapter 6). Decorin acts to modulate collagen fibrillogenesis by binding to collagen fibres (Hedbom and Heinegard 1993, Parkinson et al. 2011). It can bind to several or several collagen types including type I, II, III, and VI (Halper 2014). In the present study the distribution of decorin in both CCL and LDET was found to be similarly intense, as it was found in both fascicular and between interfascicular regions, indicative of binding to collagen type I, III and VI. In contrast to decorin, biglycan immunostaining was present in LDET interfasicular regions and occasionally pericellulary in CCL. This finding supports studies where low mRNA expression and immunostaining of biglycan was observed in canine CCL (Yang et al. 2012). Asporin is the third member of class I SLRPs that lacks GAGs chains. It competes for type I collagen

binding with decorin via its leucine rich repeat domain with high affinity (Kalamajski and Oldberg 2010). To date asporin has only been identified thorough proteomic analysis in human ACL and patellar tendon (Little et al. 2014). Here, asporin was found in the LDET at interfascicular regions and pericellularly, whereas in CCL, asporin was only found pericellularly. Asporin has been found to bind to calcium and induces collagen mineralization (Kalamajski et al. 2009), but is a negative regulator of BMP-2 or TGF-β activity and could therefor play a factor in OA progression as well as susceptibility to the disease (Ikegawa 2008). In tendon and ligament the mechanism and role of asporin still needs to be explored. Keratocan is a member of the class II family of SLRPS and has been identified at both tensional and fibrocartilagenous regions in bovine deep digital flexor tendon (Rees et al. 2009). The tendon keratocan is poorly sulphated, in contrast to corneal keratocan, which is highly glycosylated possibly reflecting differences in function between these two tissues (Rees et al. 2009). Here, low intensity immunostaining of keratocan in both CCL and LDET was found, present only between fascicles and fibre bundles. This finding corresponds with a similar distribution of keratocan in bovine tendon endotenon (Rees et al. 2009). In addition Rees et al. (2009) also demonstrated co-localisation of keratocan with collagen type I at the endotenon indicating a role in the regulation of the collagenous components within this loose matrix in both tendon and ligament.

Elastin/Elastic fibres

Distribution of Elastic fibres- Elastic fibres are composed of an elastin core and microfibrils (fibrillin 1 and 2) (Mithieux and Weiss 2005). Previous studies have demonstrated the distribution of elastin, fibrillin 1, and fibrillin 2 in canine cruciate ligament (Smith et al. 2011) and bovine flexor tendon (Grant et al. 2013). Through the use of our histological analysis an increase in elastic fibres in CCL using the Miller's stain was demonstrated (section 4.4.1.2). The next aim was to identify the differences in distribution of elastic fibres between CCL and LDET using specific antibody staining. In this part of the study, the objective was to determine whether tendon and ligament from the same species and breed have a different or similar distribution of elastic fibres. Fibrillin 1 and 2 was found to be aligned along the long axis of the tissue and surrounding ligament and tendon cells. An increased intensity of

staining was also noted at the interfascicular regions. The similar distribution of both fibrillins may indicate co-localisation of both fibrillin-1 and fibrillin-2, which has been previously shown in bovine tendon (Grant et al. 2013). In comparison to fibrillin-1 and fibrillin-2, elastin fibres were sparse and were more located at the interfascicular and interbundle regions, but also found on CCL and LDET within fascicles. When elastin was present either in between collagen fibre bundles or orientated along the fibres, it was found to co-localise with both fibrillin-1 and fibrillin-2, where it was also in close conjunction to cells. These findings supports the previously demonstrated elastic fibre distribution in bovine tendon (Grant et al. 2013), but was slightly different to that previously reported for canine CCL, where elastin was found to co-localise with fibrillin-2, but not fibrillin-1 (Smith 2010). As discussed earlier, elastin content was also found be different between in this study and Smith et al. (2013) and these findings may be due to breed differences. One limitation of this part of the study was that the overall distribution of elastic fibres throughout the depth of the tissue was not presented due to software limitations in creating 3D images of different stack, as a result only representative 2D images were presented.

Studies have shown that type VI collagen and elastic fibres are in close conjunction with tendon cells (Thakkar et al. 2014) and ligament cells (Sardone et al. 2014) and are localised at the pericellular matrix. Finding in the current work are consistent with these studies, and may indicate a link between elastic fibres and collagen type VI. Apart from collagen type VI and elastic fibres, asporin (in both CCL and LDET) and aggrecan (only in CCL) were also found to be distributed pericellularly. Future work may address whether these protein are co-localised around the same cell. Together, these proteins may play an important role in the microenvironment of both LDET and CCL cells. The increased distribution of elastic fibres at interfascicular and interbundle regions may provide elastic recoil and offer stress protection of blood vessels and nerves at this region (Grant et al. 2013).

4.6 CONCLUSION

This study has demonstrated statistically significant structural and morphological differences between inter- and extra-articular ligaments and flexor and extensor tendons around the canine stifle joint. By using established and newly adapted scoring systems we have for the first time demonstrated semi-quantitative morphological and structural differences between the CCL and the other tendon and ligament tissues examined. These differences include a less compact collagen architecture, differences in cell nuclei phenotype, and increased staining for GAGs and elastic fibres in CCL. These findings may be a normal and a physical adaptation of CCL to mechanical loading, rather than pathological degeneration as suggested by the author Hasegawa et al. (2013) .

A different distribution of was also found for collagen type III, aggrecan, versican, asporin of CCL in comparison to LDET. While different collagen ratios may lead to different mechanical properties, the increase distribution of aggrecan and versican may result to increase the hydration and viscoelastic properties of CCL. Together, these finding may relate to different functioning of CCL and LDET and indicate that CCL is subjected to more compressive forces, resulting in different ECM composition and arrangement to protect the tissue from damage.

In both LDET and CCL collagens, proteoglycans and elastic fibres were either fascicular, interfascicular and pericellularly located. In particular ECM proteins including collagen type VI, elastic fibres, asporin and aggrecan (only in CCL) may play an important role in the cellular function and microenvironment of both LDET and CCL as these proteins were closely located adjacent to cells. Differences in the distribution and arrangement of ECM collagen, proteoglycans and elastic fibres between fascicular and interfascicular and/or interbundle regions in both LDET and CCL is suggestive of different shear forces between regions during deformation. Proteoglycans and elastic fibres in the interfascicle or interbundle matrix may be involved in the regulation collagenous matrix and could enhance the lubrication of collagen bundles and elastic recoil mechanism at this site.

CHAPTER 5

A COMPARISON OF THE EXTRACELLULAR MATRIX COMPOSITION OF NATIVE TENDON/LIGAMENT AND 3D TENDON/LIGAMENT CONSTRUCTS

5.1 INTRODUCTION

Tendon and ligament injuries can cause significant joint instability, which may lead to injury of other tissues and the development of degenerative joint disease (Woo et al. 1999). Depending on whether tendon and ligament injury has resulted in partial or complete tear a nonsurgical or surgical intervention is required for treatment. For instance, in nonsurgical treatment of human Achilles tendon progressive physical therapy and rehabilitation is often required (Soroceanu et al. 2012) and is usually also performed for pre- and post- operative ACL reconstruction (Adams et al. 2012). Strategies such as delivery of growth factors (Molloy et al. 2003, Thomopoulos et al. 2005) or cell therapy (Alves et al. 2011) have been considered to alter tendon and ligament repair and accelerate the healing process. With a complete tendon or ligament tear a surgical intervention is often necessary. At present both autologous and allogenic surgical treatments are used to treat tendon and ligament injuries, but are associated with complications such as infection (Robertson et al. 2006), chronic pain (Spindler et al. 2004), decreased muscle strength (Beynnon et al. 2002) and, donor site morbidity (Mastrokalos et al. 2005).

Tissue engineering can offer great potential in the treatment of tendon and ligament injury by seeking a biological replacement with a fully regenerated autologenous tissue that mimics the natural structure and function and has long term availability (Rodrigues et al. 2013). The engineering approach involves the acquisition and cultivation of adequate cells, growth-inducing stimuli and an artificial extracellular matrix (scaffold) in which cells can proliferate and differentiate with subsequent new tissue generation (Ahmed et al. 2008).

Fibrin is a natural scaffold with no toxic degradation or inflammatory reaction and can be used as an autologous scaffold for fibroblasts from connective tissue to create a three-dimensional (3D) structure (Ye et al. 2000). Use of this biological material in tissue engineering is advantageous compared to synthetic polymers and collagen gels where cost, inflammation, immune response and toxicity may be significant drawback (Ahmed et al. 2008). The generation of 3D tendon constructs using a fibrin gel from embryonic tendon cells has been described by Kapacee et al. (2008). This method was adapted from those used previously by Hecker et al. (2005) and Huang et al. (2005) to successfully culture smooth muscle and skeletal muscle cells *in vitro*. Kalson et al. (2010) demonstrated that

tendon constructs produced with embryonic chicken tendon cells are able to form after 10 days under static conditions and produce mechanical properties similar to 14- day- old chick embryonic tendon. 3D constructs made with human adult tendon fibroblasts revealed the cells to have the potential to produce a tendon-like structure *in vitro* with aligned collagen fibrils along the axis of tension (Bayer et al. 2010). 3D tendon constructs from adult cells have also been shown to develop larger collagen fibrils with increasing diameter resulting in an increasing resistance toward tensile mechanical forces (Herchenhan et al. 2013). Human adult ACL fibroblasts have also been used *in vitro* for creation of a ligament construct which demonstrated an increasing collagen content for maximal tensile load following the addition of transforming growth factor 1 (TGFβ1) and insulin-like growth factor (IGF) (Hagerty et al. 2012). Overall, it appears that these constructs have the potential to be used in studies of ligament and tendon regeneration and replacement. However it remains unknown to what extent 3D tendon and ligament constructs formed from tendon and ligament fibroblasts in 3D culture are able to recapitulate native tendon/ligamentous tissue characteristics.

5.2 HYPOTHESIS & AIMS

Previously we have identified biochemical and structural differences between canine intra- and extra- articular ligaments and tendons and identified tissue specific characteristics (Chapter 3 and 4). 3D constructs could provide an important role in tendon and ligament repair and replacement, but can also provide insight on whether cells derived from tendinous and ligamentous tissues have similar characteristics to original tissue when grown in a 3D culture system. Cells grown in 3D fibrin cultures have been reported to produce their own ECM system (Hecker et al. 2005). Therefore the biochemical characteristics of 3D tendon and ligament constructs can be measured and compared to native tendon and ligament tissue. We hypothesised that there are that there are differences in ECM macromolecules between native tendon and ligament tissue and 3D tendon and ligament constructs. The aims of this study were to identify whether canine isolated tendon/ligament cells are able to form *in vitro* 3D cultures and characterise the extent to which isolated tendon/ligament cells are able to recapitulate the formation of tendon/ligamentous tissue.

5.3 EXPERIMENTAL PROCEDURES

An overview of the performed experiments is highlighted below. More detail on the methods is described in the general material and methods Chapter 2.

5.3.1 Tissue extraction

Cranial cruciate ligament (CCL) and long digital extensor tendons (LDET) were harvested from five disease free skeletally mature Staffordshire bull terrier cross dogs cadaveric canine hindlimbs on the day of euthanasia (Table 2.1). Tissues were enzymatically digested overnight. Cells were isolated, cultured and split at no further than the 3rd passage.

5.3.2 Creation of 3D tendon and ligament constructs

Tendon and ligament 3D constructs were created as described in Chapter 2, section 2.3 3. All constructs were fully contracted between the anchored points and were harvested at 14 days post-seeding.

5.3.3 Histology staining

Tissue engineered constructs were fixed in 4% paraformaldehyde, paraffin embedded and 4 µm section cut. Sections were stained for hematoxilin and eosin (H&E) for general observation of tissue structure, alcian blue/PAS (AB-PAS) for detection of GAGs, Masson's trichrome for collagen staining (Bancroft et al. 1996) and Miller's stain for elastic fibres (Miller 1971).The methodology of these stains are explained in detail in Chapter 2, Section 2.5.2.

5.3.4 Transmission electron microscopy (TEM)

Transmission electron microscopy was performed by Marion Pope, technician, Division of Veterinary Pathology, School of the Veterinary Science (Chapter two, section 2.7). Ultra-thin transverse-sections were cut with a Reichert- Jung Ultracut on ultramicrotome with a diamond knife. Images were acquired in a Philips EM208S Transmission Electron Microscope at 80kV.

5.3.5 Biochemical analysis

Prior to biochemical analysis, tissue samples were freeze dried overnight and their dry weight was determined. For the measurement of sulphated glycosaminoglycan (sGAG), collagen and DNA content, samples were papain digested, whilst for elastin content measurement tissue samples were digested with 0.25M oxalic acid (Chapter 2, Section 2.4).

5.3.5.1 dsDNA

Quant-iT^{TM} Picogreen ® dsDNA reagent was used to measured DNA content in the papain digested samples (Singer et al. 1997)(Chapter 2, Section 2.4.4.1). DNA concentrations were determined by comparing to a standard curve constructed using bacteriophage lambda DNA standards and expressed as µg per mg dry weight tissue.

5.3.5.2 Collagen

Total collagen content was determined by measuring the amino acid of hydroxyproline (Bergman and Loxley 1963)(Chapter 2, Section 2.4.5.2). The hydroxyproline concentrations were calculated by comparison against a hydroxyproline standard curve and collagen content calculated assuming hydroxyproline to be present at 14% (Rumian et al. 2007).

5.3.5.3 sGAG

The dimethylmethylene blue (DMMB) dye binding assay (Farndale et al. 1986) was used to assess (Chapter 2, Section 2.4.5.1) the concentration of sulphated glycosaminoglycan (sGAG) in the samples. Concentrations were determined using sGAG calibration curves and chondroitin sulphate as a standard. sGAG concentrations were expressed as µg per mg dry weight tissue.

5.3.5.4 Elastin

Elastin content was measured using the Fastin^{TM} dye-binding assay on the pooled oxalic acid extracts (Chapter 2, Section 2.4.5.3). Unknown elastin concentration in the samples were compared to a standard curve generated from known concentrations of alpha-elastin. Elastin concentration was expressed as a percentage of dry weight(Smith et al. 2013).

5.3.5.5 Statistical analysis

Data are presented as means $\pm$ standard error of the mean (SEM) and statistically analysed using One-way ANOVA with a Bonferroni post-hoc correction using Graphpad Prism (Version 6). The significance level was set at $p<0.05$.

5.4 RESULTS

5.4.1 Formation of 3D tendon and ligament constructs

CCL and LDET fibroblasts within 3D ligament and tendon constructs displayed a rounded morphology following seeding (Figure 5.1A and 5.1B), but began to form cellular extensions 1 day after being embedded into the fibrin matrix. Cellular arrangements inside the constructs were more ordered and displayed elongated cell body extension after three days of construct formation (Figure 5.1C and 5.1D). In both 3D constructs fibroblasts became more organised forming a parallel arrangement during ECM formation (Figure 5.1E and 5.1F). Cells were initially seeded in a circular fibrin matrix which became fully contracted being at least partially replaced with collagen fibrils. A continuous tendon or ligament-like tissue was formed between the sutures after 14 days post seeding (Figure 5.1G and 5.1H).

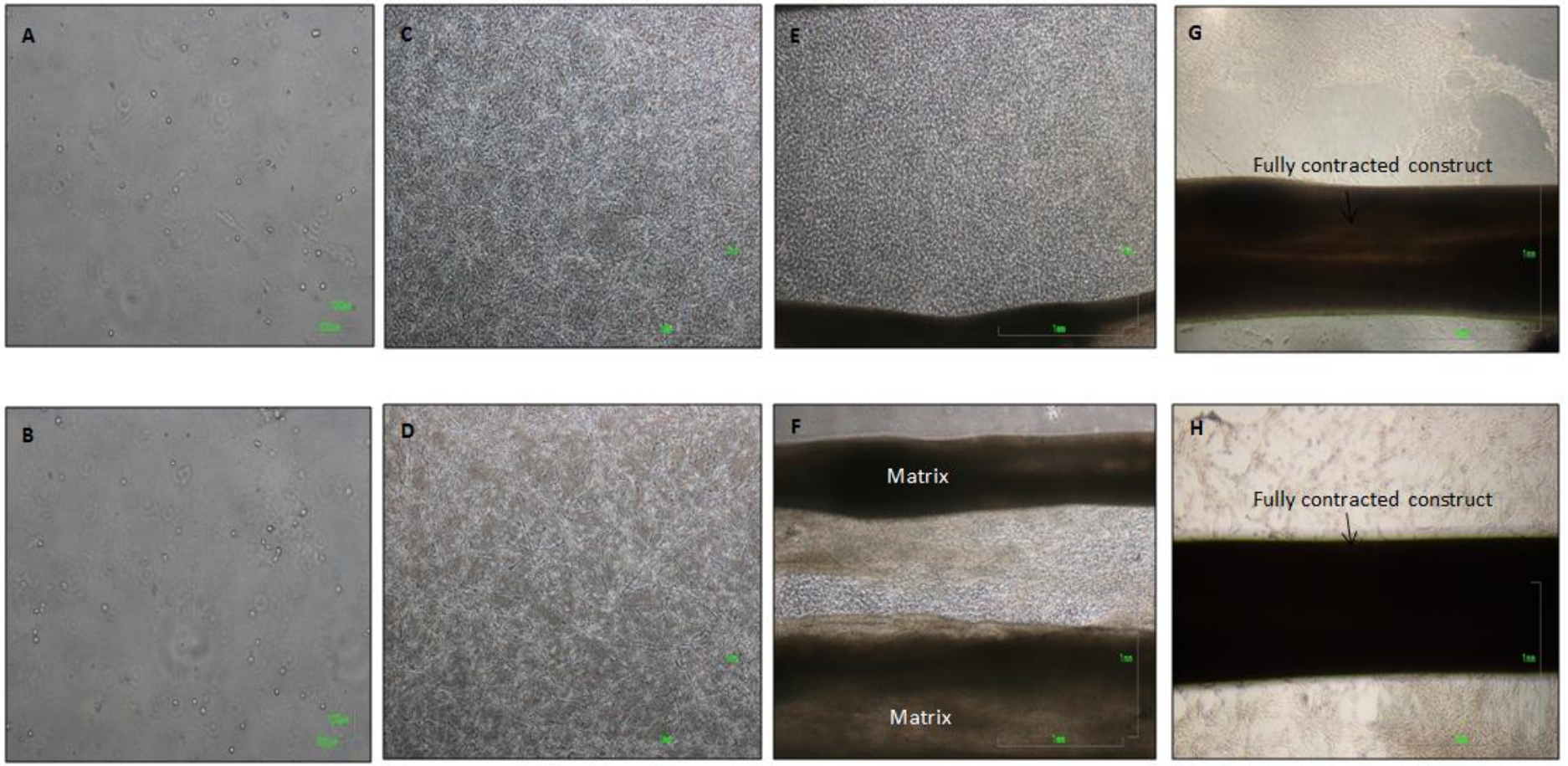


Figure 5.1. Canine CCL and LDET cells embedded in a 3D fibrin gel. Shortly after embedding in fibrin CCL (A) and LDET (B) fibroblasts displayed a rounded cell morphology. (10x magnification. Bar; 100µm). After 3 day of construct formation both CCL (C) and LDET (D) fibroblast displayed elongated cell bodies and long cellular extensions. (4x magnification Bar: 1mm). After 10 days of incubation of CCL (E) and LDET (F) fibroblast were located between ordered aligned matrix. (4x magnification. Bar; 1mm). At day 14 constructs were fully contracted and engineered tissues were formed (G and H). (4X magnification Bar; 1mm).

5.4.2 Ultrastructure of 3D tendon and ligament constructs

5.4.2.1 Histology

H&E staining displayed a general loose and not orderly architecture of both 3D ligament and tendon constructs with a high degree of cellularity that had no particular alignment. Towards the edge of the constructs, cell nuclei had more fibroblastic morphology, whereas in the centre of the constructs the cell nuclei had more rounded morphology. (Figure 5.2A and 5.3A).

AB-PAS staining displayed areas of blue staining indicating the presence of GAGs, which were mainly found next to collagen fibres. No pericellular staining was observed (Figure 5.2B and 5.3B).

Miller's staining- Both 3D tendon and ligament constructs demonstrated no elastic fibres, though intriguingly some structures which strongly resembled blood vessels were found in several tendons and ligaments constructs (Figure 5.2C and 5.3C).

Masson's trichrome indicated the presence of collagen (stained blue) in both 3D tendon and ligament constructs. Collagen fibres had a generally loose architecture and did not appear to be compact or dense with no particular orientation. Some areas of the fibrin matrix was stained red in several 3D tendon and ligament constructs, indicating that the fibrin matrix was not fully degraded (Figure 5.2D and 5.2E).

5.4.2.2 TEM

The presences of collagen fibrils were further confirmed with TEM, whereby close-packed narrow diameter collagen fibrils were visible in the extracellular space. Collagen fibrils were also found to be located in collagen fibripositors (Figure 5.4 and 5.5), which are actin rich plasma membrane protrusions that mediate collagen fibril organisation in embryonic tendon (Canty et al. 2004).

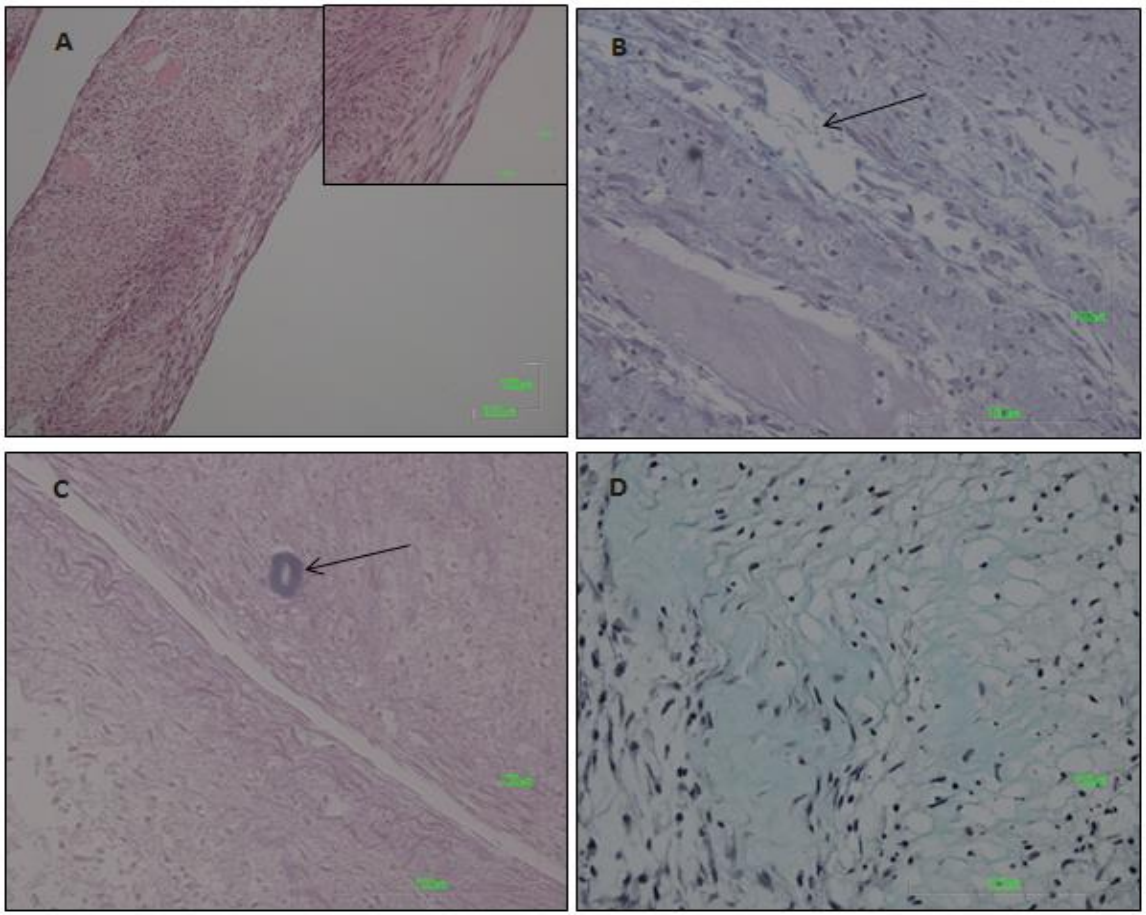


Figure 5.2. Representative histology staining pictures of 3D LDET construct. (A) – (D) longitudinally sectioned micrographs. (A) H&E staining indicated highly cellular structure of LDET construct. (10X magnification. Bar: 100µm). (B) AB-PAS staining. Areas of with GAGs were observed (black arrow). (40X magnification. Bar: 100µm). (C) Millers staining. No Elastic fibres were found, though a possible blood vessel was found within the construct (black arrow). (40X magnification. Bar: 100µm). (D) Masson's trichome staining. Collagen fibres were stained blue and cells were stained dark blue. Collagen fibres appeared loosely composed with minimal density. (40X magnification. Bar: 100µm).

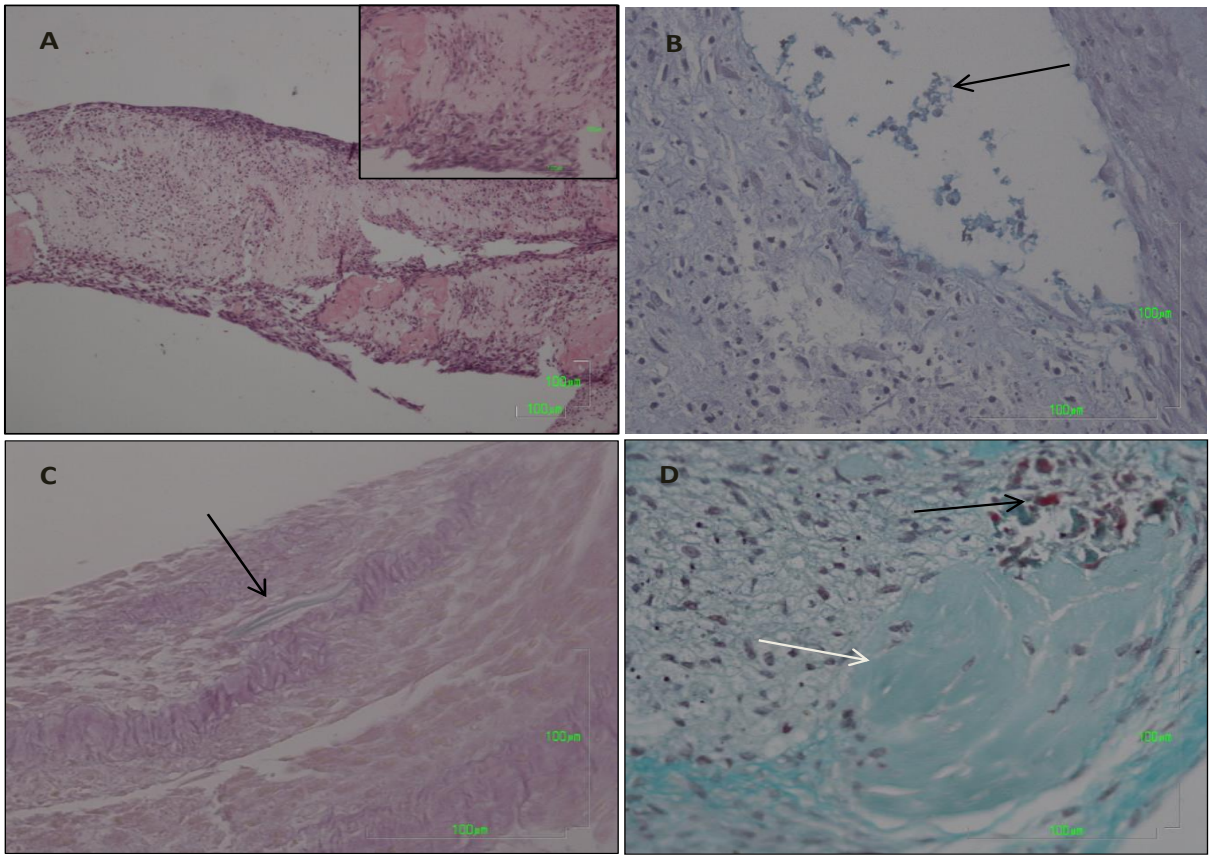


Figure 5.3. Representative histology staining pictures of 3D CCL construct. (A)–(D) longitudinally sectioned micrographs. (A) H&E staining demonstrated highly cellular structure of CCL construct. (10x magnification. Bar: 100µM). (B) AB-PAS staining. GAGs were identified (black arrow) magnification (40x Bar: 100µM) (C) Miller's staining. No Elastic fibres were found. Blood vessel-like structure was found (black arrow) magnification (40x Bar: 100µM) (D) Masson's trichome staining. Collagen fibres were stained blue and were general loose, but at some areas collagen fibres seemed more dense (white arrow). Areas of red staining indicated the presence of fibrin matrix (black arrow). (40x magnification. Bar: 100µM).

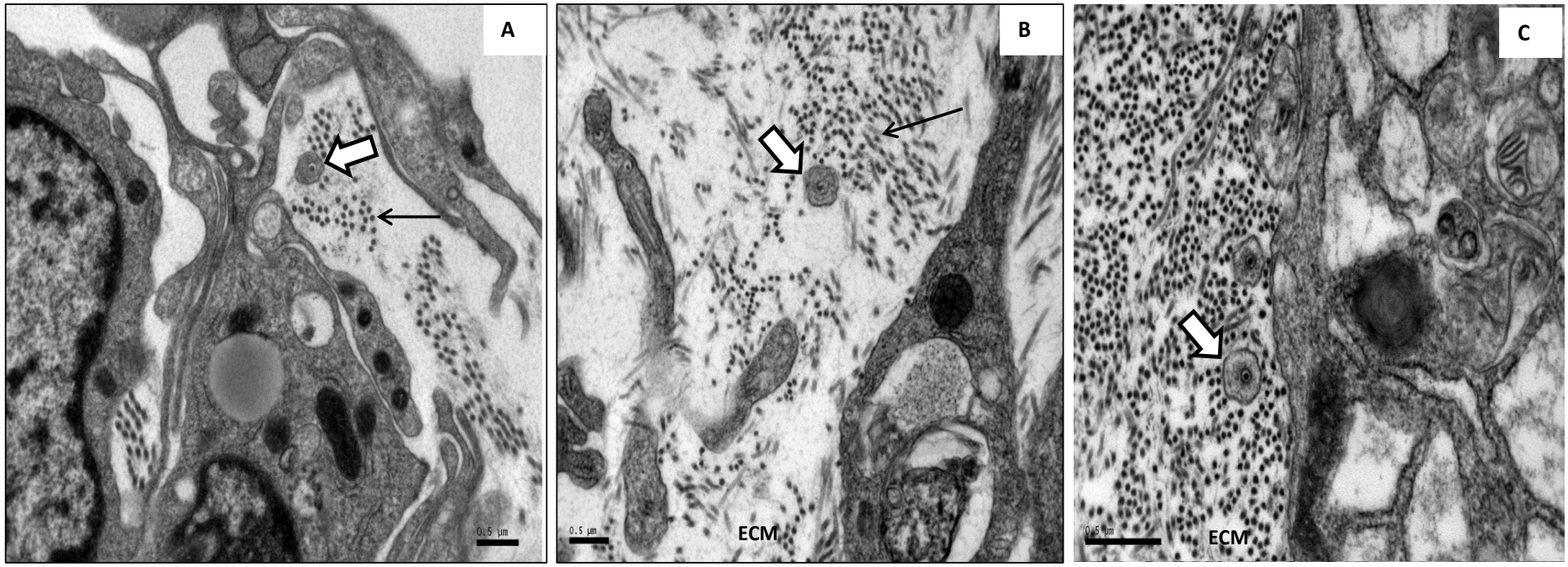


Figure 5.4. Representative pictures of transmission electron analysis of 3D CCL construct. (A) 2200x, (B) 24000x and (C) 28000x magnification. (A)-(C) transverse sectioned micrographs. (Bar: 0.5µm). Collagen fibrils were identified and appeared to be of a narrow diameter (~46nm). Collagen fibrils were located in extracellular space (black arrow). Collagen fibrils were also found with plasma membrane protrusions known as collagen fibripositors (white arrows).

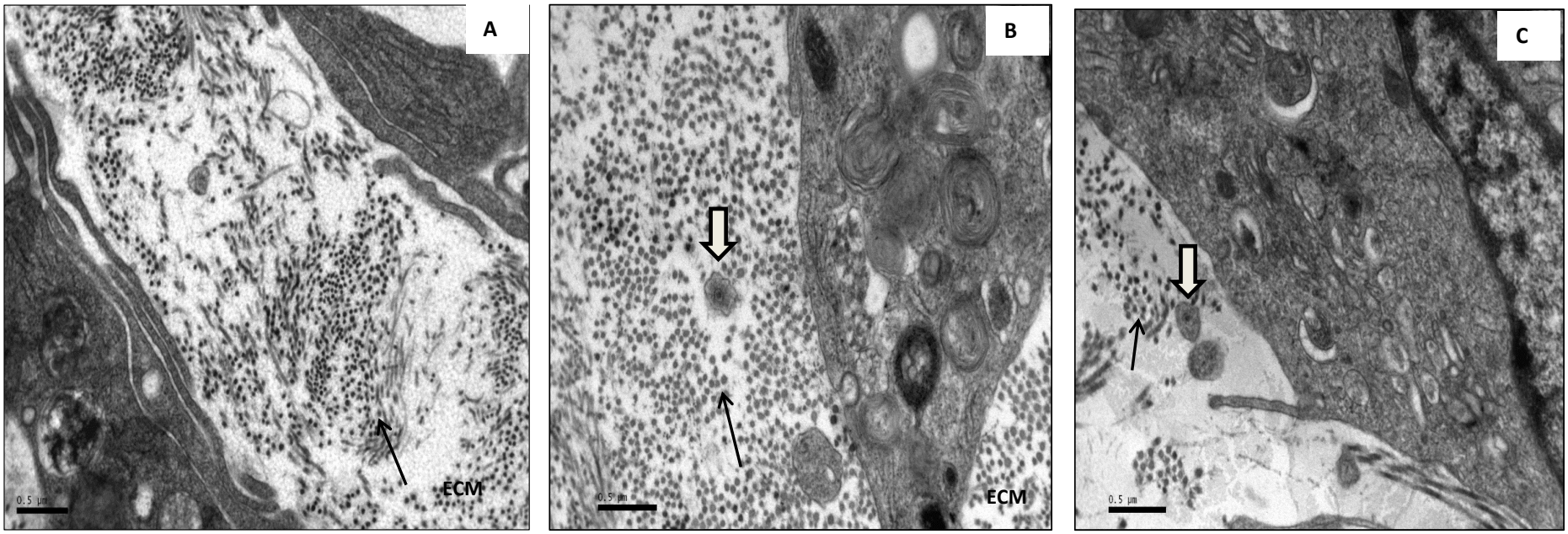


Figure 5.5. Representative transmission electron pictures of 3D LDET constructs. (A) 18000x , (B) 22000x, (C) 36000x transverse sectioned micrographs. (Bar 0.5µm). Collagen fibrils with uniformity can be seen being mainly identified in extracellular space (black arrow), but also in fibripositors (white arrow).

5.4.3 Biochemical comparison between native tissues and 3D tendon and ligament constructs

5.4.3.1 DNA content

3D tendon and ligament constructs were found to have significantly higher DNA content than both native tendon and ligament tissues (p=0.0157 and p=0.015) (Figure 5.6). DNA averaged 2.92 ± 0.32 mg/dry weight in native CCL and 3.94 ± 1.1 mg/dry weight in native tendon. 3D CCL and LDET constructs had an average of 10.6 ± 1.57 and 9.82 ± 1.46 DNA mg/dry weight, respectively. No significant differences were found between the native tendon and ligament or and between 3D tendon constructs compared to ligament construct.

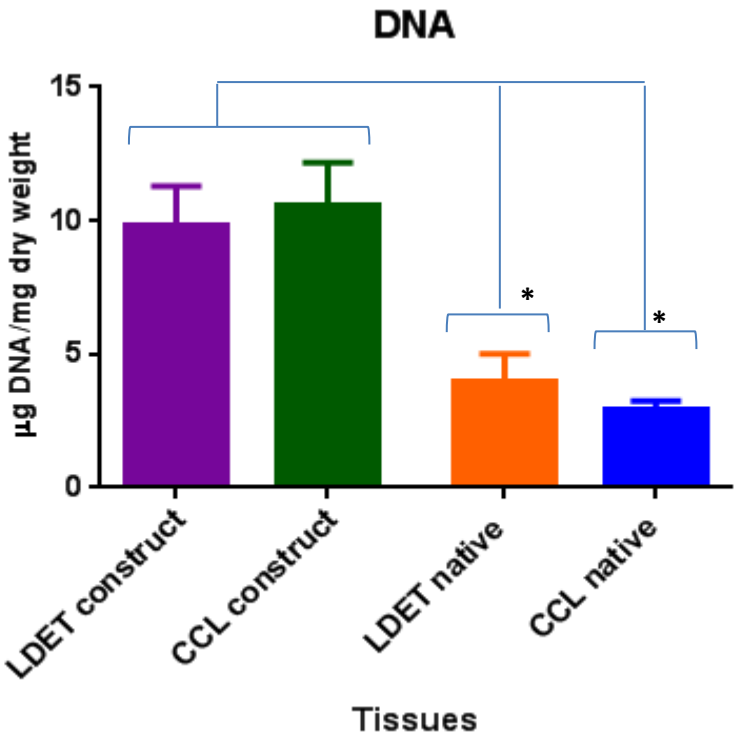


Figure 5.6. DNA content (µg/ mg dry weight). Values are mean and error bars represent SEM. Ligament construct had significantly higher DNA content than both native tendon and ligament tissues (p=0.0046, p=0.015). The DNA content in tendon constructs was also statistically significantly higher than native tendon (p=0.0157) and native ligament (p=0.012) tissues. There were not any statistically significant differences between native tissues types and 3D tendon and ligament constructs. * indicates p<0.05.

5.4.3.2 Collagen content

The total collagen content showed statistically significant differences between native tendon/ligament tissue and 3D tendon and ligament constructs (Figure 5.7). Both LDET and CCL constructs had significantly lower collagen content than native tissues (p=0.0001). The CCL and LDET had average values of 65.1% ± 1.02 and 70.4% ± 2.8 of dry weight tissue, while 3D CCL and 3D LDET had average values of 3.61 ± 0.9 and 2.89 ± 0.71 of dry weight. No significant variations were found between the two native tissues and between engineered tissue types.

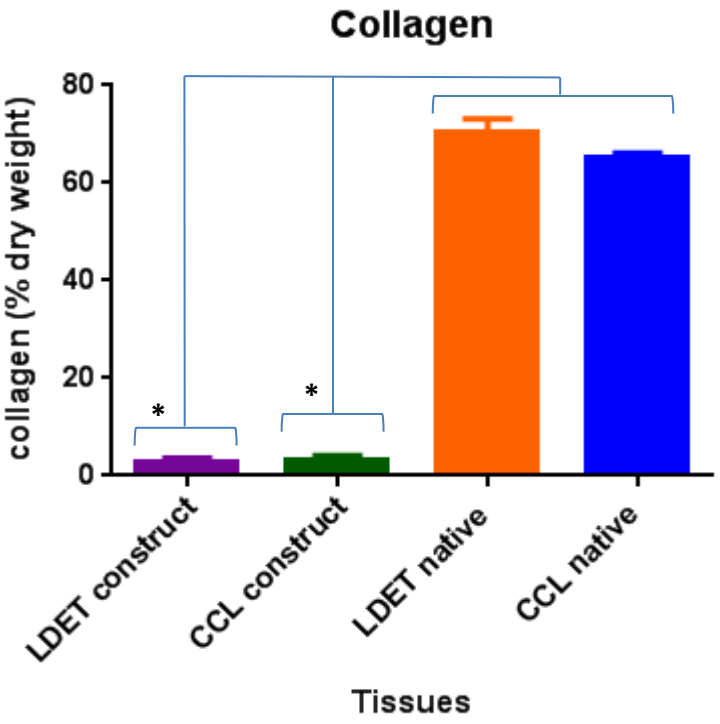


Figure 5.7. Collagen content/ mg % dry weight. Values are mean and error bars represent SEM. Collagen content in ligament and tendon constructs were both significantly lower than native tendon (p=0.0001) and ligament (p=0.0001). No significant differences were observed between native tendon and ligament and between 3D tendon and ligament construct.
* indicates p<0.05.

5.4.3.3 Total sulphated GAG content

sGAG content (as µg/mg dry weight) was an average of 15.1 ± 0.74 in native CCL, 8.29 ± 1.03 in native LDET, 14.4 ± 1.97 in 3D CCL and 11.1 ± 0.76 in 3D LDET. Native CCL contained significantly more sGAG than LDET (p=0.008). Only the 3D CCL construct had statistically higher sGAG than native LDET (p=0.028) (Figure 5.8).

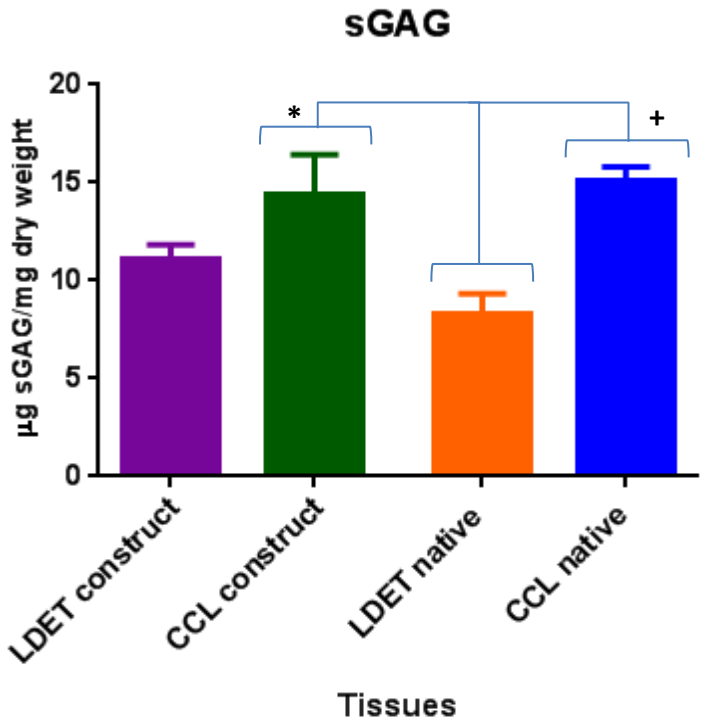


Figure 5.8. sGAG content (µg/ mg dry weight). Values are mean and error bars represent SEM. Ligament constructs had significantly higher than native tendon (p=0.028). Significant differences were found between native ligament and tendon tissues types (p=0.008), but not between engineered tissues. * and + indicates p<0.05.

5.4.3.4 Elastin content

Elastin content was 5.52 % ± 0.58 in native CCL, 2.42 % ± 0.18 in native LDET, 9.47% ± 1.39 in 3D CCL and 14.26% ± 0.49 in 3D LDET. Both 3D tendon and ligament constructs had higher elastin content than both native tendon and ligament tissue (p=0.0001). Statistically significant differences between native CCL and LDET were found (p=0.0038), with CCL containing significantly higher elastin. Between tissue engineered CCL and LDET constructs, the LDET constructs contained significantly more elastin (p=0.0026) (Figure 5.9).

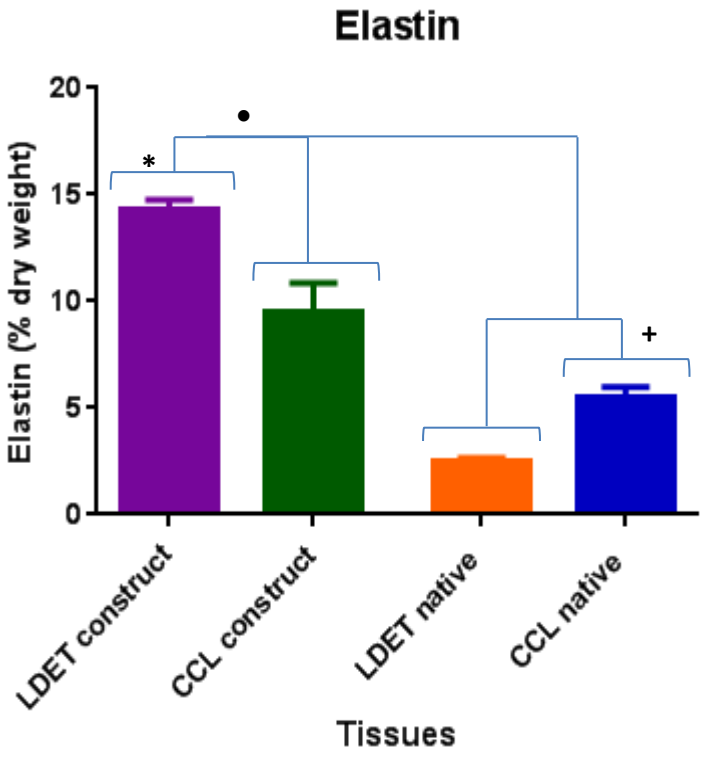


Figure 5.9. Elastin content (% mg dry weight). Values are mean and error bars represent SEM. Native tendon ligament tissues were significantly lower in elastin content than both native LDET (p=0.0001) and CCL (p=0.0001) 3D constructs. Statistically significant differences were found between native tendon and ligament (p=0.0038) and 3D tendon and ligament constructs (p=0.0026). *, + and • indicates p<0.05.

5.5 DISCUSSION

This study has demonstrated that 3D tendon and ligament fibrin constructs are able to produce ECM macromolecules. The constructs had a resemblance of an embryonic tendon or ligament, containing high cellular content and collagen fibripositors. Collagen content in both 3D tendon and ligament constructs was significantly lower compared to what was measured in native tendon and ligament tissue. A high content of non-collagenous ECM macromolecules including sGAGs and elastin were measured in both types of 3D constructs, indicating that these components mature faster than collagen and might play an important role in the development of 3D tendon and ligament constructs.

5.5.1 Tendon and ligament construct ultrastructure

Linear 3D tendon and ligament construct were created from mature canine long digital extensor tendon (LDET) and canine cranial cruciate ligament (CCL) fibroblasts using *in vitro* 3D cell fibrin cultures. Constructs from both tissues displayed a high degree of cellularity with collagen fibrils present and glycosaminoglycans identified in histological sections. The high degree of cellularity also corresponds to higher DNA content in both 3D tendon and ligament constructs compared to mature native tendon and ligament tissue, indicative of a high cell-to-matrix ratio in both 3D constructs. A high cell number could stimulate embryonic fibrillogenesis which have been identified in neonatal rat tendon (Calve et al. 2004), but also in human embryonic tendon (Chaplin and Greenlee 1975) using electron microscopy, Chaplin and Greenlee (1975) previously demonstrated that human digital embryonic tendon being as a highly cellular structure with few small diameter collagen fibrils. After 6 days, collagen fibrils developed in close proximity to the cells, whilst after 95 days, the extracellular space was filled with longitudinal aligned collagen fibrils containing a relatively high cell volume fraction and loose collagen packing density (Chaplin and Greenlee 1975). Comparing these electron microscopy pictures with our developed tendon and ligament construct at 14 days post-seeding, a resemblance is seen with the human embryonic digital tendon until about 60 days of gestation. The shorter period time for our constructs may be explained by the fact that cells are derived from mature adult tissue and ready to form ECM, whereas in the embryonic tendon the cells need to differentiate.

Transmission electron microscopy results in this study showed that collagen fibrils in the tendon and ligament constructs were mainly located in extracellular space, but also occasionally found in carriers, known as collagen fibripositors. Collagen fibripositors are plasma membrane protrusions that contain narrow diameter fibrils and have only been found in embryonic tendon (Canty et al. 2004). Despite active procollagen synthesis, fibripositors are absent in postnatal stages when fibrils increase in diameter by accretion of extracellular collagen (Canty et al. 2004). Fibripositors have been found to be associated with actin bundles suggesting cytoskeletal involvement in fibril assembly and coordination of collagen fibril alignment in tendon (Canty et al. 2006). Previous studies have identified collagen fibripositors in embryonic tendon fibroblasts, which are actin rich plasma membrane protrusions containing narrow collagen fibrils and occurring in tendon only during embryonic development when seeding of the ECM occurs (Canty et al. 2004, Canty et al. 2006). Together, the high cellularity and presence of collagen fibripositors reflect the immature state of the constructs in this study. This finding correlates with observations in rat tendon constructs obtained from postnatal tissue (Calve et al. 2004), chick embryo tendon constructs (Kapacee et al. 2008), and adult human tendon constructs (Bayer et al. 2010, Herchenhan et al. 2013), where the structural characteristics were similar to developing embryonic tendon.

5.5.2 Tendon and ligament construct ECM composition

Collagen content- The function of collagen content of the tissue is to withstand tensile forces (Canty and Kadler 2005), and it may be assumed that *in vitro* generated tissues with greater collagen concentration would be superior by providing more resistance to mechanical forces. Here, a low collagen content in 3D tendon and ligament constructs was found with only an average of 3.2% collagen in 3D tendon and ligament, whereas native tissues had an average 67.7% collagen. This may be explained by the immature state of constructs whereby collagen fibrillogenesis may still be in progress. The maturation of human Achilles tendon is associated with an increase of collagen fibril concentration and diameter as well as the presence of fewer cells that display altered cell morphology (Strocchi et al. 1991). An increase in ECM, decrease in cellularity and different morphological cell structure were also found with sheep tendon and ligament maturation, although

ligament cell morphology remained the same during postnatal development (Meller et al. 2009). To what extent 3D tissue engineered ligament and tendon are able to mature is not fully known. However, Paxton et al. (2012) demonstrated a more dense structure and an improved collagen content with chick embryonic construct when cultured over period of 5-weeks. This improvement was also observed in adult human constructs in terms of mechanical strength, stiffness, increase of collagen fibril diameter and density when subjected to static tension for 5 weeks (Herchenhan et al. 2013). Other studies have identified that factors such as loading (Paxton et al. 2012) and addition of growth factors such as TGFβ1 or insulin growth factor 1(IGF 1) (Paxton et al. 2010, Hagerty et al. 2012) can increase collagen content in 3D fibrin constructs. For instance, TGFβ is known to play role in tendon development (Pryce et al. 2009) and is thought to activate early growth response (EGR) 1 and 2 (Chen et al. 2006). TGFβ has been demonstrated to increase the collagen expression in invertebrate tendon by binding to *Col1a1* promoter (Lejard et al. 2011). A significant decrease in collagen content of tendon and ligament has been detected in the absence of EGR 1/2 (Lejard et al. 2011). In addition to TGFβ1, Hagerty et al. (2012) found an additive effect with IGF1 on the collagen content and maximal tensile load in human adult ACL fibrin constructs. This was supported by the work of Herchenhan et al. (2014) in human adult tendon constructs, where IGF1 increased collagen fibril diameter, overall collagen content and mRNA gene expression of collagen type I and III, tenomodulin and scleraxis (Herchenhan et al. 2014). Paxton et al. (2012) measured the effect of loading on tendon-like constructs derived from chick embryonic tendon and demonstrated that short cyclical loading with diverse strain rates and amplitudes resulted in an increase in collagen content within the engineered graft. Together, these variations and factors can be considered in future studies to get a better understanding of the development tendon and ligaments *in vivo*.

GAG and elastin content- In this study other non-collagenous ECM matrix components including sulphated glycosaminoglycan (sGAG) and elastin in 3D tendon and ligament constructs were measured. Significantly more sGAG and elastin were found, in native CCL and as compared to native LDET, indicative of greater viscoelastic properties and a stretch and recoil mechanism in CCL (also described in Chapter 3 and 4). Similar sGAG content was identified in both 3D tendon constructs compared to native tissues. Significantly higher

elastin content was found in both 3D constructs compared to native tissues. The high sGAG and elastin content in both 3D constructs suggest that these ECM components are rapidly acquired and mature much faster than the collagen matrix. Proteoglycans have been known to play role in the regulation of tendon collagen fibrillogenesis (Zhang et al. 2005). During tendon development the absence of several small leucine-rich proteoglycans causes distinct abnormalities, where different fibril diameter distributions with irregular profiles were observed in mice tendons with deficient lumican and fibromodulin in postnatal period (Ezura et al. 2000). Small leucine-rich proteoglycans have also been shown to prevent later fusion of collagen fibrils (Danielson et al. 1997, Raspanti et al. 2007). The data in the present study might indicate that the high GAG content might play an important role in the organisation of the collagen fibrils and the development of 3D tendon and 3D ligament constructs.

Elastic fibres have previously been identified in canine cruciate ligaments (Smith et al. 2011) and bovine flexor tendon (Grant et al. 2013). Elastic fibres have important mechanical (Butler et al. 1978, Eriksen et al. 2001), cell-regulatory (Ito et al. 1997, Wendel et al. 2000) and, recoil functions in tissue (Ritty et al. 2002). Elastic fibres are composed of an elastin core covered with a sheath of fibrillin- rich microfibrils, which acts as a scaffold onto which the secreted elastin molecules are deposited (Kielty et al. 2002, Kielty 2006). Although higher elastin content was identified in both 3D tendon and ligament constructs, no elastic fibres were detected in histological and TEM sections. Again this may be explained by the embryonic state of the constructs and agrees with the work of Brown et al. (2012), where elastic fibers were only detected at postnatal stage, while fibrillin-1 and tropoelastin were found to be present during embryonic development in the spinal ligament. Moreover, the elastin assay does not distinguish between tropoelastin precursor, insoluble elastin and degraded peptides, which could mean that higher elastin content in 3D tendon and ligament constructs may be the tropoelastin precursor.

Elastic fibres are also major component of blood vessels (Davis 1993) and are therefore stained with the Miller's stain. In this study the presence of blood vessel-like structures were also shown in both 3D tendon and ligament constructs with the Miller's stain. This might indicate that the endothelial cells from the native tendon and ligament tissue are able

actively remodel once placed in 3D culture. However, further validations through antibody immunostaining are required to verify the presence of blood vessels.

5.6 CONCLUSION

The results of this study shows that 3D tendon and ligament constructs have similar ECM matrix composition and are composed of different proportions of ECM proteins in comparison to the native tendon and ligament tissue. The low concentration of collagen and high cellularity of the construct might be explained by the embryonic nature of the tendon or ligament constructs. The increased sGAG and elastin content in the engineered constructs might indicate that these components mature faster than collagen matrix, as in particular sGAGs are important for collagen fibrillogenesis and might play an important role in the development of engineered connective tissue constructs.

CHAPTER 6

PROTEOMIC COMPARISON OF TENDON, LIGAMENT AND 3D TENDON/LIGAMENT CONSTRUCTS

6.1 INTRODUCTION

Proteomics is relatively a new technology that has been used widely in cancer, cartilage biology (Polacek et al. 2010) and osteoarthritis research (Ruiz-Romero and Blanco 2010), but also in several tendon and ligament studies (Johnson 2009, Little et al. 2014, Peffers et al. 2014) .

The concept of the proteome was first described by Wilkins et al. (1996) as "the entire set of proteins produced by the genome, or by a cell or tissue type". The functional proteome is the physiological state of a tissue represented by the protein complement of a given cell at a given time, including the set of all proteins isoforms and modifications (de Hoog 2004). Proteomics seeks to determine protein structure, modifications, localisation, and protein-protein interactions as well as protein expression levels (de Hoog 2004). Mass spectrometry (MS) is currently the most versatile technology in achieving these objectives (Aebersold and Mann 2003). MS allows identification and characterisation of thousands of proteins in a complex mixture by performing chromatographic separation prior to MS detection (Aebersold and Mann 2003, Domon and Aebersold 2006).

6.1.1 Mass spectrometry

Separation of proteins and peptides- Prior to MS separation of protein/peptides is required and achieved by gel electrophoresis or gel free techniques which normally involve liquid chromatography (LC).

Two dimensional gel electrophoresis (2DE) is an example of gel- based separation, where proteins are separated by isoelectric point and by molecular weight (Gorg et al. 2000, Gygi et al. 2000). Subsequently proteins can be visualised by staining techniques, excised from the gel and proteins can be identified by MS techniques.

Gel free separation involves the use of high performance liquid chromatography (HPLC) with reverse phase C18 column, where peptides are separated according to hydrophobicity (Wang et al. 2003). This is coupled to a mass spectrometer for online MS analysis and is therefore high throughput method.

The mass spectrometer- it consists of three main parts; the ion source, mass analyser, and detector. After LC, peptides enter the mass spectrometer and are ionised. The most commonly used ionisation is electrospray ionisation (ESI) and matrix-assisted laser desorption ionization (MALDI). The produced ions are transferred to a mass analyser where the mass-to-charge ratio (m/z) ratio is detected (Aebersold and Mann 2003, Matthiesen and Jensen 2008). In tandem mass spectrometry (MS/MS), the ions of particular interest m/z ratio are first selected in the mass analyser and are then fragmented in the second dimension of MS by collision induced dissociation (CID). The peptide CID spectra are more specific by mass mapping only as, in addition to the peptide mass, the peak pattern in the CID spectrum also delivers information about peptide sequence (Aebersold and Mann 2003). Examples of commonly used mass analysers are quadrupole, ion traps (IT), time of flight (TOF) and Fourier transform ion cyclotron (FT-MS)(Aebersold and Mann 2003). The detector is final part of the mass spectrometer, where the m/z vs intensity plot or a mass spectrum output is visualised (Matthiesen and Jensen 2008)

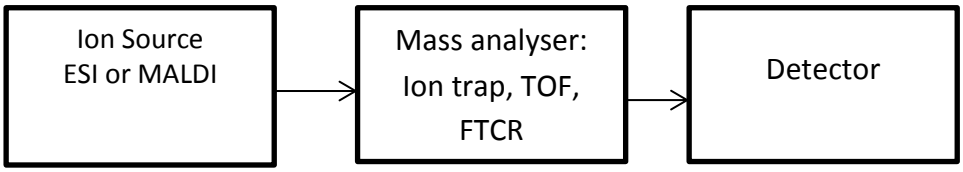


Figure 6.1. Outline diagram of mass spectrometer adapted from Matthiesen and Jensen (2008)

6.1.2 Protein quantification

Protein identification can be achieved by comparing the peptide profile of the unknown protein in comparison to theoretical peptide libraries generated from the sequences in the different databases (Barrett et al. 2005). Proteins can be quantified through absolute or relative quantification using either label-based or label-free methods. Absolute quantification is a targeted approach, where MS peptide peaks are compared alongside known internal standard concentrations (Kirkpatrick et al. 2005, Bantscheff et al. 2007). This quantification is used to calculate copy number or concentration. On the other hand relative quantification is used to define relative changes of protein between samples. This quantification is based on spectral counting and by using a relative peak intensity approach

(Bantscheff et al. 2007, Zhu et al. 2010, Bantscheff et al. 2012). Spectral counting is only used with label-free quantification and involves data-dependent analysis such as 'exponentially modified protein abundance index' (emPAI) (Ishihama et al. 2005, Zhu et al. 2010). The relative intensity peak approach is also used to quantify protein or peptide abundance by calculating MS peak intensity (Zhu et al. 2010). The abundance of each peptide or protein is associated with the relative peak intensity (peak area) (Zhu et al. 2009).

There is a growing interest in applying label-free LC-MS approaches. In comparison to the isotope label method, label-free methods have several advantages. These include higher dynamic range of quantification, reduced protein loading, fractionation, sample handling, and the ability to compare more conditions within one experiment (Bantscheff et al. 2007, Patel et al. 2009). However it must also be noted that with label-free quantification it is difficult to detect low-abundance peptides as there is less sensitivity (Zhu et al. 2010).

Tendon and ligament 3D fibrin constructs have been created using cells from several species such as chick embryonic tendon (Kapacee et al. 2008, Kalson et al. 2010, Paxton et al. 2010, Herchenhan et al. 2012), human adult ACL (Hagerty et al. 2012) and human adult tendon cells (Bayer et al. 2010) and canine tendon and ligament cells in this thesis. With the collagen fibrils having the ability to grow in size and with strength approaching that of adult human tendon (Herchenhan et al. 2013), these fibrin constructs have the potential for tendon and ligament replacement. However, to date an understanding of the proteome composition of these tendon and ligament-like structures is unknown. The ability of these structures to retain similar proteome characteristics to the original tissue and whether ligament and tendon fibroblasts have the ability to produce a distinct ligament and tendon proteome phenotype has yet to be identified. In this study a gel free separation technique with label free quantification was used to identify differences in protein abundances between samples.

6.2 HYPHOTHESIS & AIMS

In the previous chapters (Chapter 3 and 4) a comparison between canine intra- and extra articular tendons and ligament was performed. Through biochemical analysis and semi-objective histological scoring, the canine cranial cruciate ligament (CCL) had significantly more elastin and sulphated glycosaminoglycan content in comparison to long digital extensor tendon (LDET), medial collateral ligament (MCL) and superficial digital flexor tendon (SDFT) (Chapter 3 and 4). We have also shown that isolated cells from tendon (LDET) and ligament (CCL) grown in 3D culture have been able to form 3D tissue engineered tendon and ligament constructs with specific extracellular matrix (ECM) macromolecules being present (Chapter 5). The hypothesis in this part of the study was that native tendon and ligament have differential structural protein expression and that 3D constructs formed from tendon and ligament cells retain the proteome characteristics of the original tissues. Therefore, the study aimed to identify the differences between the proteomes of native tendon and ligament as well as engineered tendon and ligament 3D constructs. These findings would represent a level of comparison between native and tissue engineered ligaments and tendons that has not been previously reported.

6.3 EXPERIMENTAL PROCEDURE

An overview of experimental procedures is described below. For more detail of the procedures see general materials and methods (Chapter 2).

6.3.1 Tissue collection and preparation

The cranial cruciate ligament (CCL) and long digital extensor tendon (LDET) were harvested from five paired disease-free cadaveric canine hindlimbs from skeletally mature Staffordshire bull terrier dogs immediately after euthanasia. Tissues were used from both the right and left stifles joints (Chapter 2, table 2.1). The right stifle joint tissues were used for protein isolation and proteomic analysis of the native tissues. The left stifle joint tissues were used for cell isolation and creation of engineered tissues, which were subsequently used for protein isolation and proteomic analysis. All samples were snap frozen in liquid nitrogen and stored at-80°C until required for protein extraction.

6.3.2 Tendon and ligament construct formation

The isolation of cells and the generation of 3D constructs is described in detail in the general material and methods (Chapter 2, Section 2.3). All constructs were fully contracted between the anchored points and harvested at 14 days post-seeding.

6.3.3 Protein extraction

Prior to protein extraction all samples were lyophilised and then disrupted using a dismembrator. Approximately 3 mg of each dismembrenated sample were treated with 1 unit/ml chondroitinase ABC (1 unit/ml) and protein was extracted with 4 M GnHCL extraction buffer. The soluble fraction was used for in-solution trypsin digestion and analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Protein concentration was measured with the Pierce^{TM} 660 nm protein assay. For verification of isolated proteins an aliquot of each samples was run on a 4-12% Bis-Tris SDS-PAGE gel.

6.3.4 In-solution tryptic digestion and LC-MS/MS

In-solution tryptic digestion and LC-MS/MS was performed with technical assistance by Deborah Simpson at the University of Liverpool Centre for Proteome Research facility (Chapter, section 2.8.5). In brief, samples were reduced, alkylated and in-solution tryptic digested. Following desalting using procedure the digested peptide mixture was assessed using label-free identification and quantification on LC-MS/MS using Ultimate 3000 nano system for peptide separation which was coupled to Q Exactive instrument for tandem mass spectrometry.

6.3.5 Proteomic data analysis

a) Protein identification

Data was analysed with a PEAKS® de novo and database search against the canine protein sequence database to identify the protein composition of each tissue. A separate run was performed for each group (native ligament, native tendon, 3D ligament and 3D tendon) to identify protein composition. Instrument configuration was set up as Orbitrap (Orbi-Orbi) and high collisional dissociation (HCD) fragmentation. The following parameters were used for the PEAKS® search; parent mass error tolerance, 10.0 ppm; fragment mass error tolerance, 0.1 Da, precursor mass search type, monoisotypic; enzyme, trypsin; max missed cleavage, 1; non specific cleavage, 1; fixed modification; carbamidomethylation, 57.02Da; variable modification; oxidation, methionine, 15.99 Da, Hydroxylation, 15.99 Da; and variable PTMs per peptide, 3. The results were filtered on the basis of the following parameters; de novo average local confidence score (ALC) %) threshold, 50; protein -10lgP> 20; False discovery rate (FDR) at peptide spectrum matches (PSMs), 1%; and unique peptides $\geq$ 2. The ensemble protein accessions were put into Ingenuity Pathway Analysis (IPA, Inguinity Systems, Redwood City, CA, USA) and gene symbol with protein description and protein subcellular locations were then mapped. Tendon and ligaments contain a wide variety of 'matrisomal proteins' (Hynes and Naba 2012, Little et al. 2014). The matrisome is a part of the proteome that exhibits the complete complement of ECM proteins (Hynes and Naba 2012). Matrisomal proteins include collagens, proteoglycans, glycoprotein and other

proteins associated with ECM. Therefore the 'Matrisome Project' has been used to classify proteins into ECM categories (Hynes and Naba 2012, Naba et al. 2012). The remaining proteins were categorised according to UNIPROT function description. Gene ontology (GO) and protein network analysis was carried out using the String bioinformatics tool.

b) Quantification of differential expressed proteins

Differentially expressed proteins were analysed using Progenesis™ LC-MS software (Nonlinear dynamics) with data searched for protein identification in PEAKS® with de novo and database search against the canine protein sequence database. After adjusting to 1% false discovery rate (FDR) and unique peptides $\geq$ 2, the search hits were imported into Progenesis™ for label free quantification. Statistical analysis was performed on all detected features using transformed normalized abundances for one-way analysis of variance (ANOVA). Identification of proteins with two or more peptides, greater than 2 fold abundance and with a q value (p-value adjusted to FDR) <0.05 were considered significant. Quantitative analysis was initially performed by comparing the four groups of tissue samples together. After that pair-wise comparisons were performed between native ligament and tendon, native ligament and 3D ligament, native tendon and 3D tendon, and 3D ligament and 3D tendon.

c) Comparison of post-translational modifications (PTMs) between native tendon and ligament

Post-translational modification of protein (PTMs) can modulate the protein function as it can regulate numerous facets of a protein, including protein turnover and localisation, complex assembly, enzyme activity, protein-protein interactions and modulation of various signalling cascades (Karve and Cheema 2011). The PEAKS® software includes the advanced PEAKS® PTM algorithm for identifying peptides with unspecified modifications (Han et al. 2011). Using the parameters as detailed in Section 6.3.5a, a PEAKS PTM file for native tendon and ligament were used to compare PTMs of collagens, large proteoglycans and small leucine rich proteoglycans between the two tissue types.

6.4 RESULTS

6.4.1.1 Qualitative proteomes of native and tissue engineered tissues

A total of 3569, 3743, 4481 and 5790 peptides assigned to 167, 215, 442 and 561 proteins each with the presence of more than 2 unique peptides were identified in native ligament, native tendon, 3D ligament and 3D tendon respectively (Table 6.1). Between both native tissues and engineered tissues 93 proteins were common, which included both ECM and cellular proteins (Figure 6.2). The common matrisomal proteins between tendon and ligament native tissues and 3D constructs are highlighted in Table 6.2.

Identified proteins for each tissue were classified into matrisomal proteins (Matrisome Project) and into the other categories based on their function (Supplementary data, Chapter 8, Table S1).

N*ative tissues (CCL and LDET) protein composition*

The native ligament proteome consisted of 50% matrisomal proteins, 10.3% nucleosome related protein, 8.5% cytoskeleton and related proteins, 8.5% metabolic enzymes, 7.9% proteins associated with translation and signalling, 4.8% proteins associated with immune and inflammatory response, 3.6% proteins associated with transport and membrane activity, 2.4% proteins associated with chaperone, stress, and protein folding, 2.4% proteins associated with detoxification, 1.2% proteins associated with development and 0.6% associated with proteolytic activity (Figure 6.3A). About 53% of native ligament proteins locations were annotated to extracellular space and the rest were distributed between the cytoplasm, nucleus and plasma membrane (Figure 6.3B). Ligament matrisomal proteins were annotated as collagens (11), glycoprotein (27), proteoglycans (13), ECM-regulators (10), ECM-affiliated (11), and ECM-secreted factors (9). Analysis for String protein network and gene ontology (GO) resulted in connected clusters around ECM proteins and matrisome associated proteins with the principal gene ontology processes identified as ECM organisation, wound healing and collagen fibril organisation (FDR adjusted p-values of $3.75e^{-27}$ and $1.85e^{-14}$, $1.19e^{-13}$ respectively) (Figure 6.4).

The native tendon proteome contained 39.7% matrisomal proteins, 13.1 % nucleosome related protein, 10.7% cytoskeleton and related protein, 9.3% metabolic enzymes, 8.4% protein associated with translation and signalling, 4.7% proteins associated immune and inflammatory response, 4.2% proteins associated with transport and membrane activity, 4.2% proteins associated with chaperone, stress, and protein folding, 2.8% proteins associated with detoxification, 2.3% proteins associated with development and 0.5% associated with proteolytic activity (Figure 6.3C). About 45% of proteins locations were annotated to the extracellular space and the remainder to the cytoplasm, nucleus or plasma membrane (Figure 6.3D). Similar to ligament, the protein-protein interaction in tendon was mainly between ECM and other matrisome associated proteins, with similar ontology process identified such as extracellular matrix organisation, wound healing, collagen fibril organisation (FDR adjusted p-values $1.53e^{-26,}$ $1.94e^{-14}$, $2.01e^{-21}$ respectively) (Figure 6.5). Based on matrisome annotations, in tendon there were collagens (11), proteoglycans (13), glycoproteins (30), ECM regulators (11), ECM-secreted factors (8) identified.

Tissue engineered tendon and ligament 3D constructs protein compositions

The ligament 3D construct proteome contained 23.1% proteins associated with translation and signalling, 15.2% as proteins associated with nucleosome, 14.3% matrisomal proteins, 12.4% cytoskeletal proteins and related proteins, 12.2% metabolic enzymes, 7.5% proteins associated with transport and membrane activity, 6.6% chaperones, stress and protein folding, 3.6% proteins associated with detoxification, 2.0% proteins associated with immune and inflammatory response, 1.1% proteins associated with biodegradative metabolism and 1.1% associated proteolytic activity (Figure 6.6A). The tendon construct consisted of 20.5% proteins associated with translation and signalling, 16.6% metabolic enzymes, 14.6% proteins associated with nucleosome, 11.9% matrisomal proteins, 10.7% cytoskeletal proteins and related proteins, 8.9% proteins associated with transport and membrane activity, 6.1% chaperones, stress and folding domains, 3.2% proteins associated with detoxification, 2.7% proteins associated with biodegradative metabolism, 2.0% proteins associated with immune and inflammatory response, 1.6% as proteins associated with development and 1.2% as proteins associated proteolytic activity (Figure 6.6C). In both 3D

constructs an average of 66.3% protein were associated with cytoplasmic location, indicating high cellularity of these tissues (Figure 6.6B and 6.6D). This was further confirmed with String analysis whereby the main connected cluster was between ribosomal proteins (Figure 6.7 and 6.8). There was also association between cytoskeletal proteins, metabolic enzymes and heatshock proteins. Principle ontology for 3D ligament and 3D tendon involved translational elongation (FDR adjusted p-values $3.71e^{-65}$, $2.3e^{-63}$), protein targeting to ER (FDR adjusted p-values $9.98e^{-64}$, $1.75e^{-65}$) (Figure 6.6 and 6.7). Matrisomal protein in 3D ligament and 3D tendon consisted of 10 collagens, 18 glycoproteins and 11 ECM-regulators in both, 8 and 9 proteoglycans, 11 and 13 ECM-affiliated, and 5 and 6 ECM-secreted factors respectively.

Tissues	Identified PSM	Peptide sequences	Proteins	Unique peptides
Native ligament	14956	3569	167	129 ≥2 38= 2
Native tendon	14587	3743	215	156≥2 59= 2
3D ligament	13805	4481	442	339≥2 103=2
3D tendon	17009	5790	561	431>2 130=2

Table 6.1. Overview of PEAKS® database search results for native CCL and LDET tissue and 3D CCL and LDET construct.

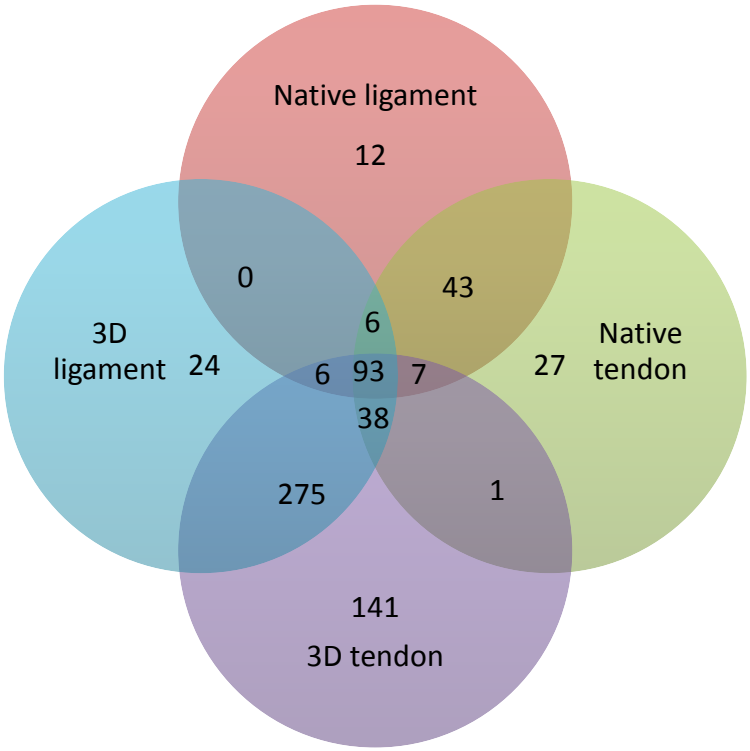


Figure 6.2. Venn diagram of native (CCL/LDET) and 3D tissues with the total number of proteins identified in each tissue as well as common proteins between the tissues.

ECM collagens	ECM proteoglycanS	ECM glycoproteins	ECM affiliated proteins	Secreted factors
Collagen type III, alpha 1	Asporin	Dermatopontin	Annexin A1	S100 calcium binding protein A10
Collagen type V, alpha 1	Biglycan	Dlastin microfibril interfacer 1	Annexin A2	S100 calcium binding protein A11
Collagen type VI, alpha 2	Decorin	Fibrillin 1	Annexin A4	S100 calcium binding protein A4
Collagen, type I, alpha 1	Heparan sulfate	Fibrinogen alpha chain	Annexin A5	S100 calcium binding protein A6
Collagen, type I, alpha 2	Proteoglycan 2	Fibrinogen beta chain	Lectin, galactoside-binding, soluble, 1	
Collagen, type I, alpha 2	Lumican	Fibrinogen gamma chain	Lectin, galactoside-binding, soluble, 3	
Collagen, type V, alpha 2	Osteoglycin	Fibulin 1		
Collagen, type VI, alpha 1	Proline/arginine-rich end leucine-rich repeat protein	Microfibrillar-associated protein 2		
Collagen, type VI, alpha 3		Milk fat globule-EGF factor 8 protein		
Collagen, type XII, alpha 1		Tenascin C		
		Thrombospondin 1		
		Transforming growth factor, beta-induced		
		Vitronectin		

Table 6.2.Matrisomal proteins in common between tendon and ligament native tissues and 3D constructs. Identified proteins had a -10lgP $\geq$20 ($p \leq 0.01$) and unique proteins $\geq$ 2.

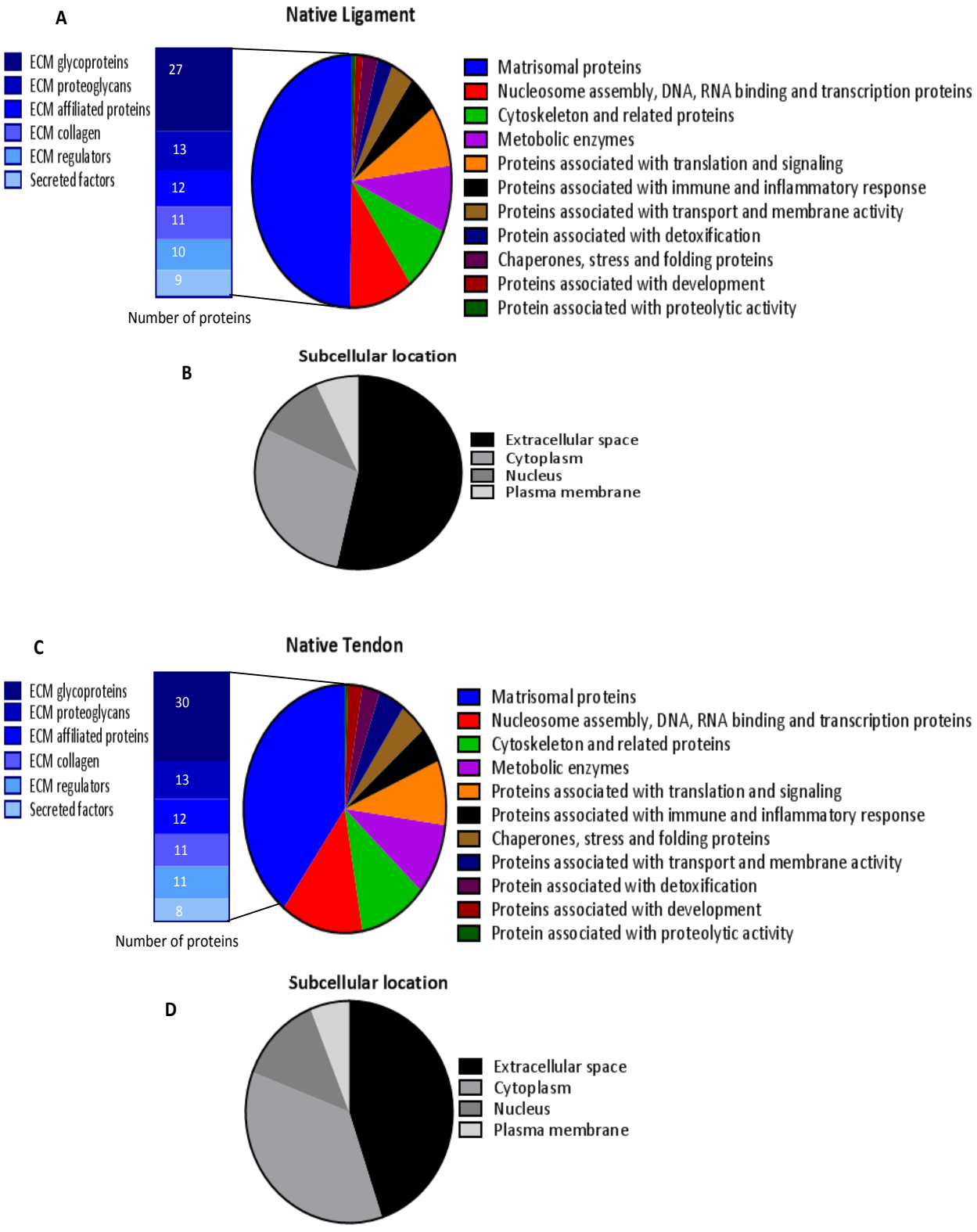


Figure 6.3. Overview of native ligament and tendon proteomes which were subdivided based on matrisomal proteins and on function (**A, C**) with the associated subcellular locations of the proteins in both tissues (**B,D**).

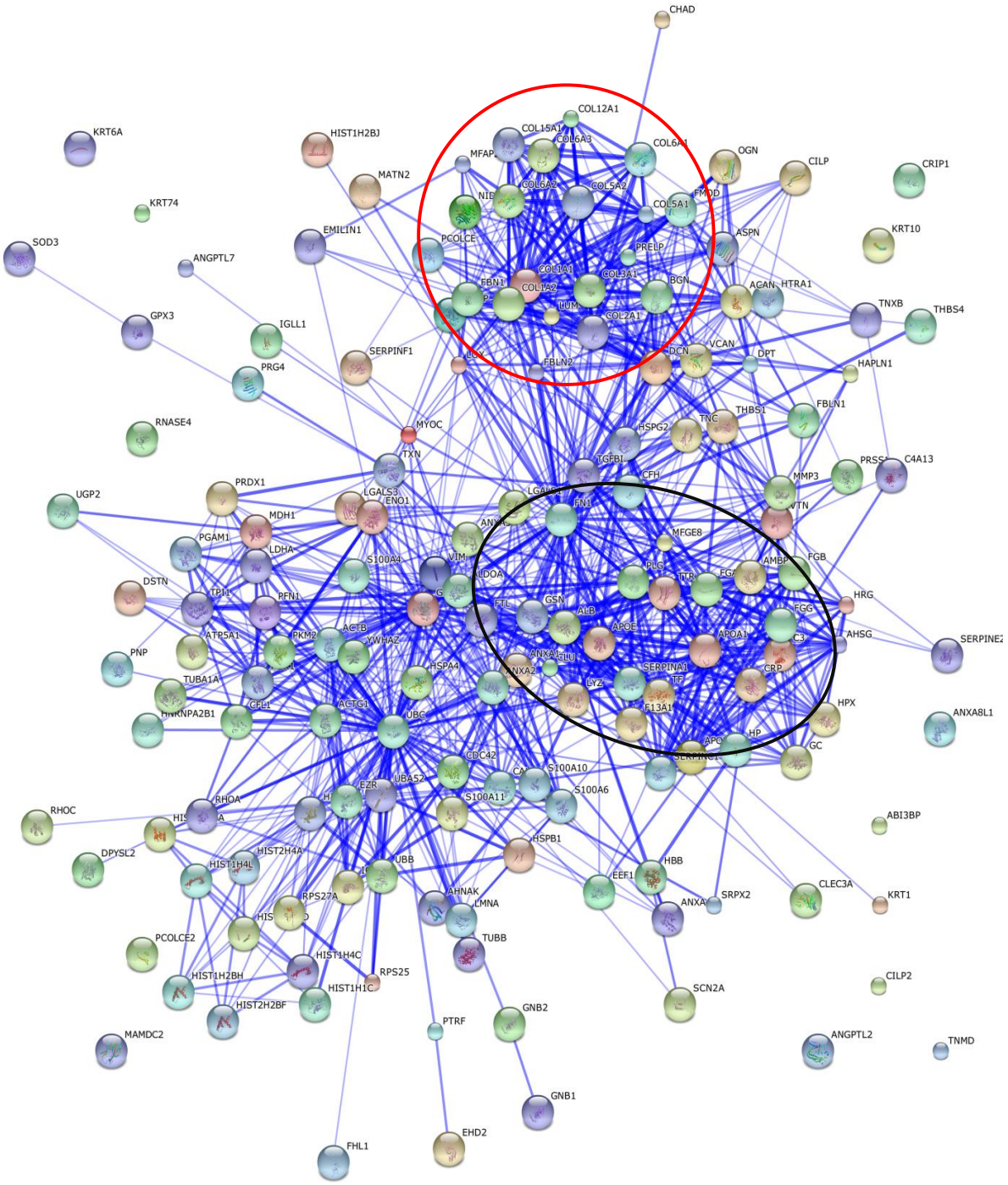


Figure 6.4. String analysis of native canine CCL. Stronger associations are represented by thicker lines. Two highly connected clusters were evident, with the first cluster between collagens, proteoglycans and elastin associated proteins (red circle) and second cluster between matrisome associated proteins (black circle). The main principal gene ontology processes were identified as ECM organisation ($p=3.75e^{-27}$), wound healing ($p=1.85e^{-14}$) and collagen fibril organisation ($p=1.19e^{-13}$).

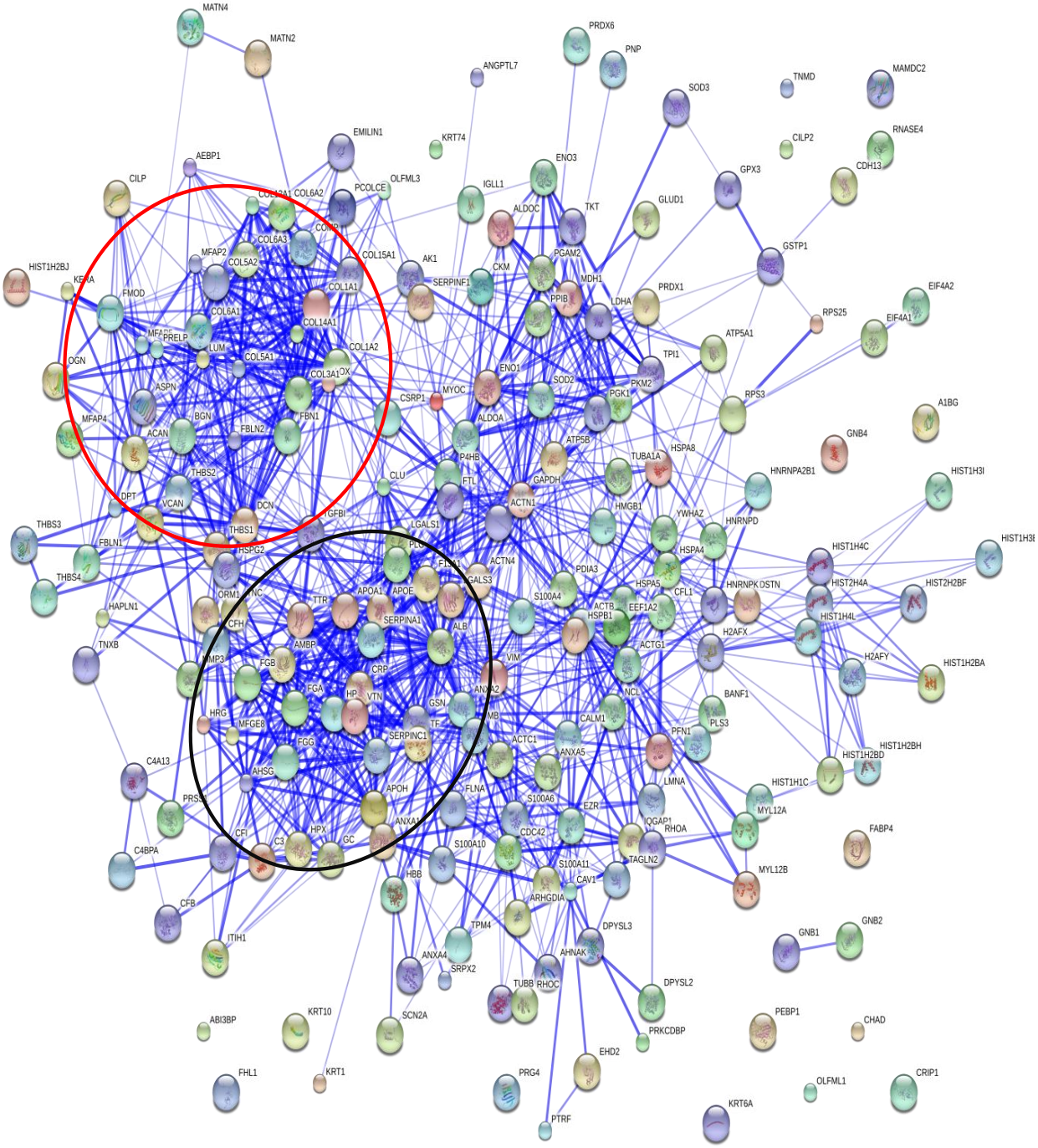


Figure 6.5. String analysis of native canine LDET. ECM proteins (red circle) and matrisome associated proteins (black circle) have the most evident protein-protein interaction. The main principal gene ontology processes were identified as ECM organisation ($p=2.1e^{-28}$), wound healing ($p=8.7e^{-15}$) and collagen fibril organisation ($p=1.5e^{-13}$).

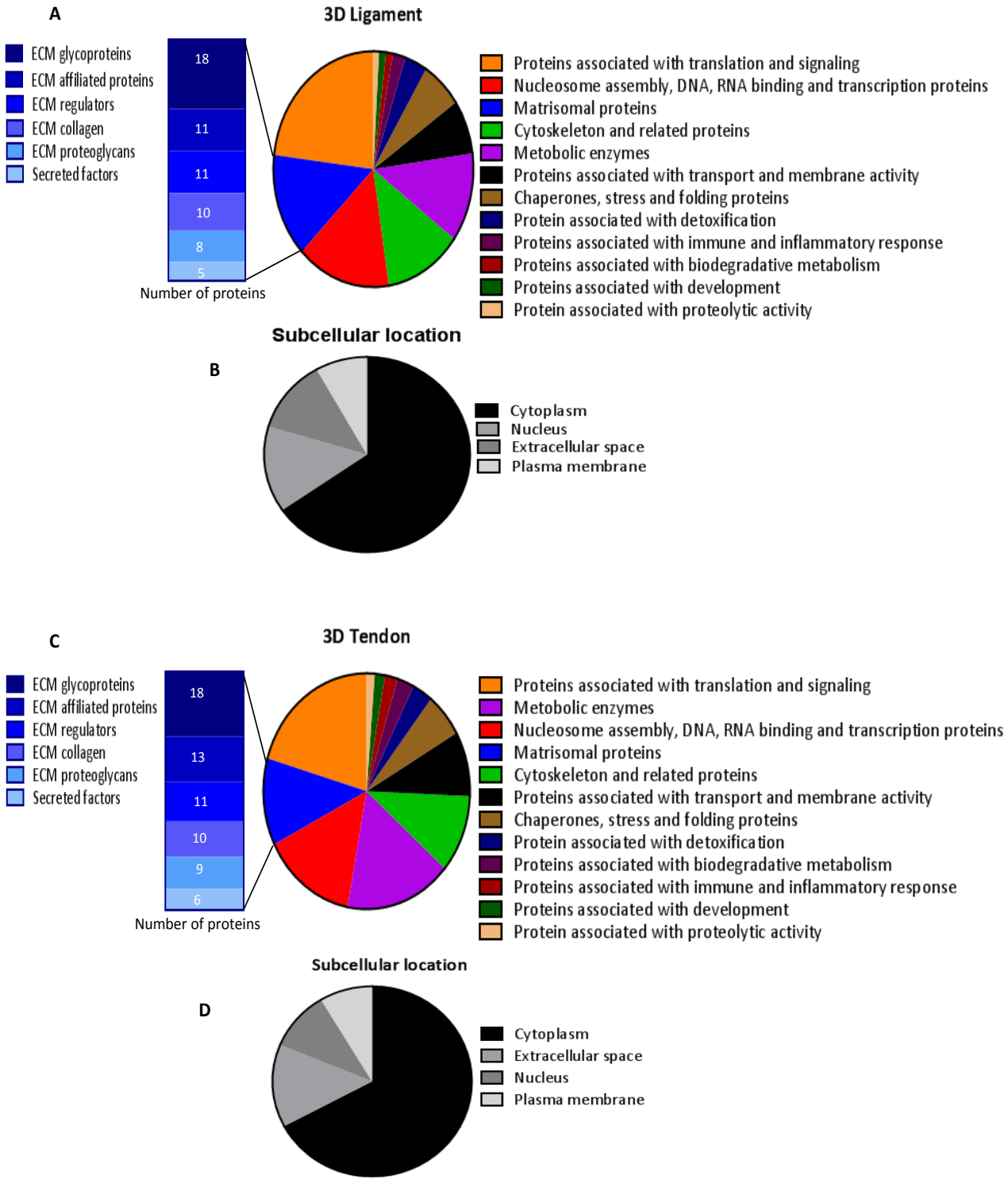


Figure 6.6. Overview of the proteomes of 3D ligament and 3D tendon constructs which were subdivided based on matrisomal proteins and on function (**A,B**) with the associated subcellular locations of the proteins in both tissues (**C, D**).

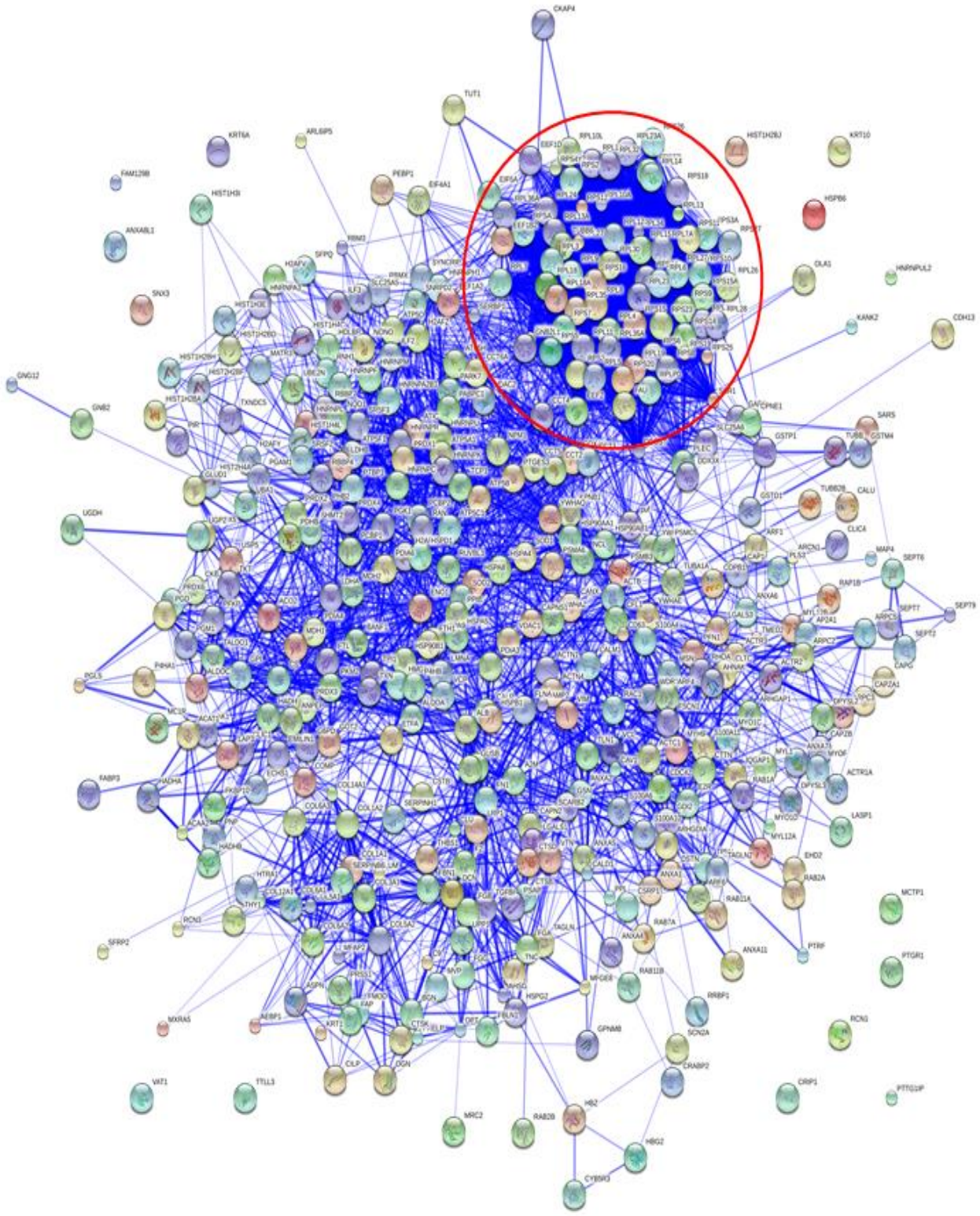


Figure 6.7. String analysis of 3D ligament construct. The strongest predicted protein-protein interaction is between the ribosomal proteins, which are mainly clustered together (red circle). The main principal gene ontology processes were identified as translational elongation ($p=3.71e^{-65}$) and protein targeting to ER ($p=9.98e^{-64}$).

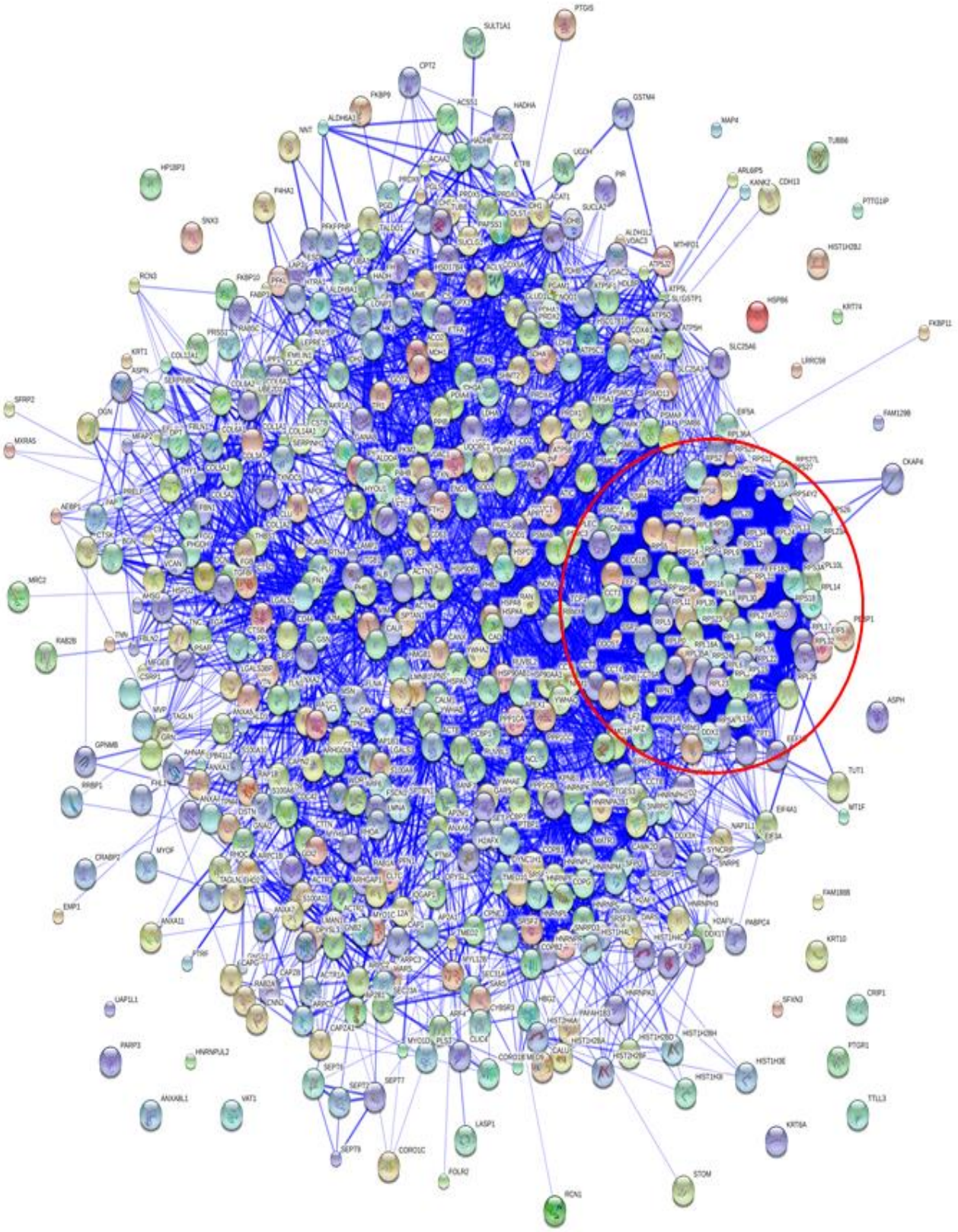


Figure 6.8. String analysis of 3D tendon construct. The most connected cluster was between ribosomal proteins (red circle). The main principal gene ontology processes were identified as translational elongation ($p=3.71e^{-65}$) and protein targeting to ER ($p=9.98e^{-64}$).

6.4.2 Label free quantification

Quantitative analysis was performed using Progenesis™ LC-MS software for group comparison between both native tissues and 3D constructs and pairwise comparisons as described earlier.

6.4.2.1 Quantitative differences of all group comparisons

Overall we identified 387 proteins that were different between the four tissue types, with 38 proteins being abundant in native ligament, 26 in native tendon, 4 in 3D ligament construct and 361 in 3D tendon construct. (Supplementary data, Chapter 8, Table S2). Principle component analysis (PCA) of either the peptide or protein PCA plots revealed that native ligament and tendon are grouped together and are therefore distinct, whereas 3D ligament and tendon did not split into discrete groups (Figure 6.9A and 6.9B).

6.4.2.2 Quantitative differences between native ligament and tendon

There were a total of 45 proteins identified with 2 or more peptides and more than 2-fold change that were different between native tendon and ligament (Figure 6.10A). Of the 45 proteins, 32 proteins had $p < 0.05$ (adjusted to FDR) (Table 6.3). Proteins more abundant in ligament included those also present in cartilaginous tissues, such as collagen, type II, alpha 1, agreccan, chondroadherin, and superoxide dismutase 3. While tendon abundant proteins were thrombospondin 4, asporin and collagen, type XII. The abundance of collagen type III, agrreccan, and versican, asporin corresponds with the previously reported immunohistochemistry results, where increased staining was observed. (Chapter 4),

6.4.2.3 Quantitative differences between native tissues and 3D constructs

Comparison of native ligament and 3D ligament construct

Between native ligament and 3D ligament there were 363 identified proteins there were different. All proteins identified had more than 2 peptides (q-value ≤0.05 and more than 2-fold change) (Figure 6.10C). 301 proteins were down-regulated in 3D ligament and 62 proteins were up- regulated for native ligament. The top ten proteins with highest fold changes in each tissue are depicted in Figure 6.11A. Both Figure 6.10C and 6.11A were curated from Supplementary data Table S3 (Chapter 8).

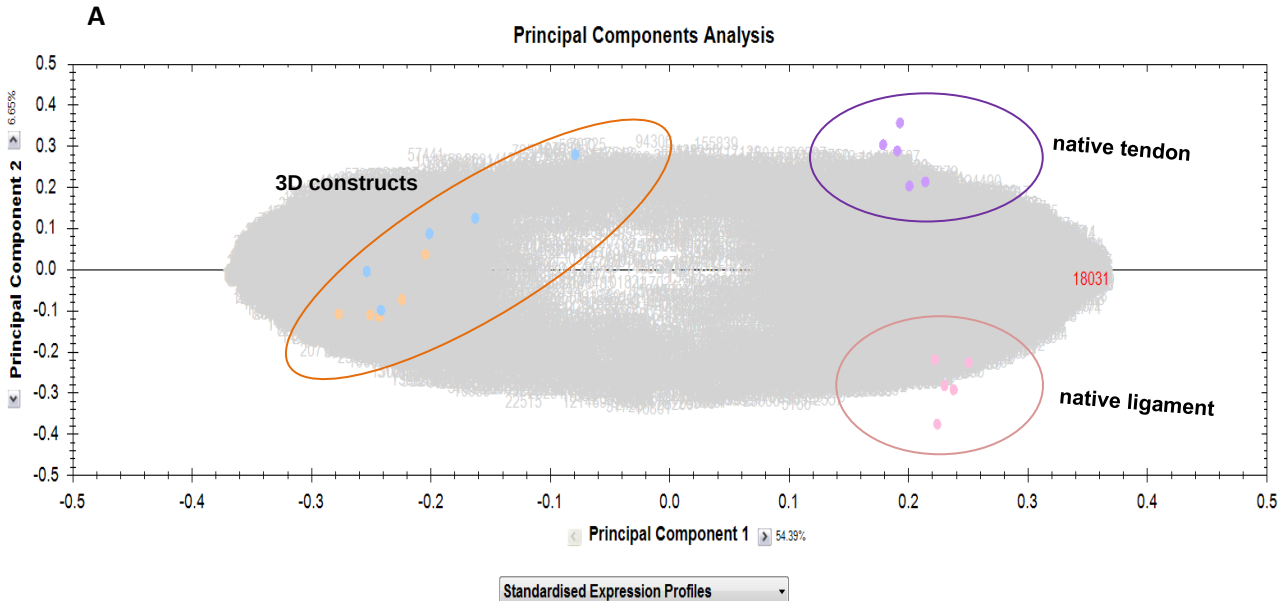
Comparison between native tendon and 3D tendon construct

Figure 6.10D shows a subset of 383 identified proteins that were different between native tendon and 3D tendon. All proteins identified had more than 2 peptides with p-value ≤0.05 (adjusted to FDR) and more than 2-fold change. 321 proteins were down-regulated in 3D tendon and 62 up-regulated native tendons. Figure 6.11B shows a subset of top twenty proteins with highest fold change. Both Figure 6.10D and 6.11B were curated from Table S4 (Supplementary data, Chapter 8).

Observation of abundant proteins in both native ligament and tendon against 3D ligament and tendon construct demonstrated not only the presence of significantly more ECM proteins, enzyme enhancer but also blood/plasma protein in both native tissues. On the other hand both 3D constructs had significantly more cellular protein, which has also been highlighted with qualitative proteomes of the both 3D tendon and ligament constructs.

Quantitative differences between 3D ligament and 3D tendon constructs

There were 4 proteins identified with 2 or more peptides and more than 2-fold change that were different between 3D ligament and 3D constructs (Figure 6.10B). Even though the ANOVA p-value was less than 0.05, when looking at the FDR adjust p-value (q-value), all values were more than 0.05. This may also be explained by looking expression plots, whereby an overlap between the two groups can be seen (Figure 6.12).



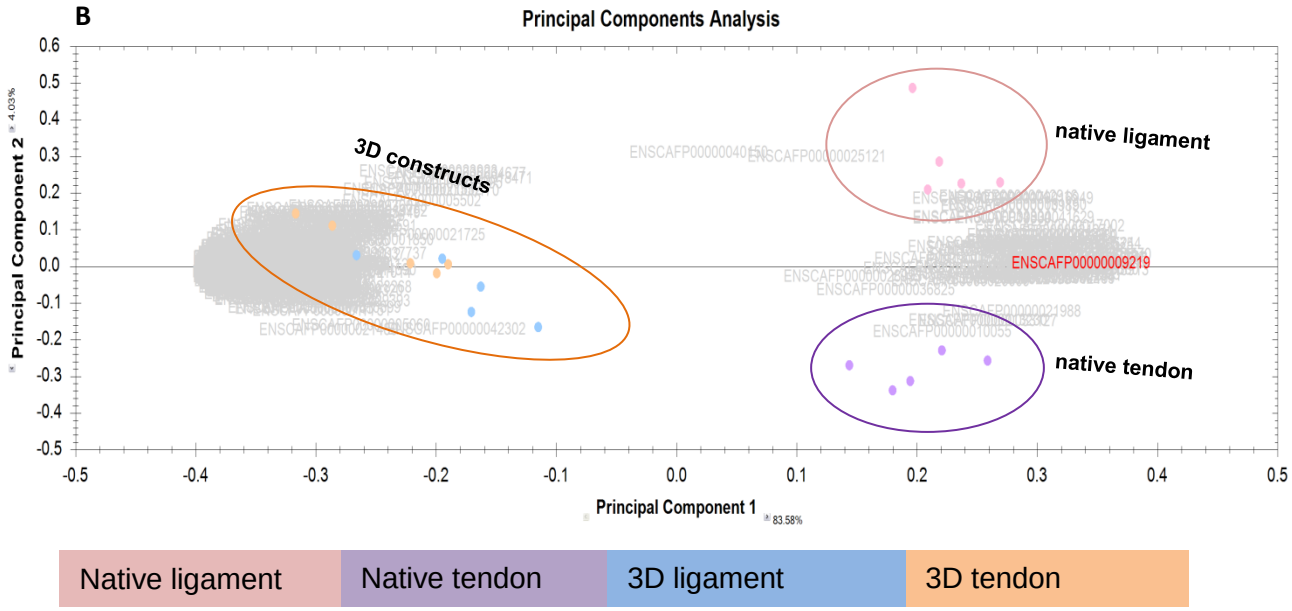


Figure 6.9. Principal component analysis (PCA) between native tendon and ligament and 3D tendon and ligament constructs at peptide (A) and protein level (B). This PCA plot was produced by Progenesis™ after ANOVA analysis with identified peptide and proteins at p-value $\leq$ 0.05. PCA was used to identify the major variance between the groups. Native tendon (purple dots) and ligament (pink dots) are grouped together and are therefore more distinct, whereas both 3D tendon and ligament constructs do no split into discrete groups (blue and orange dots).

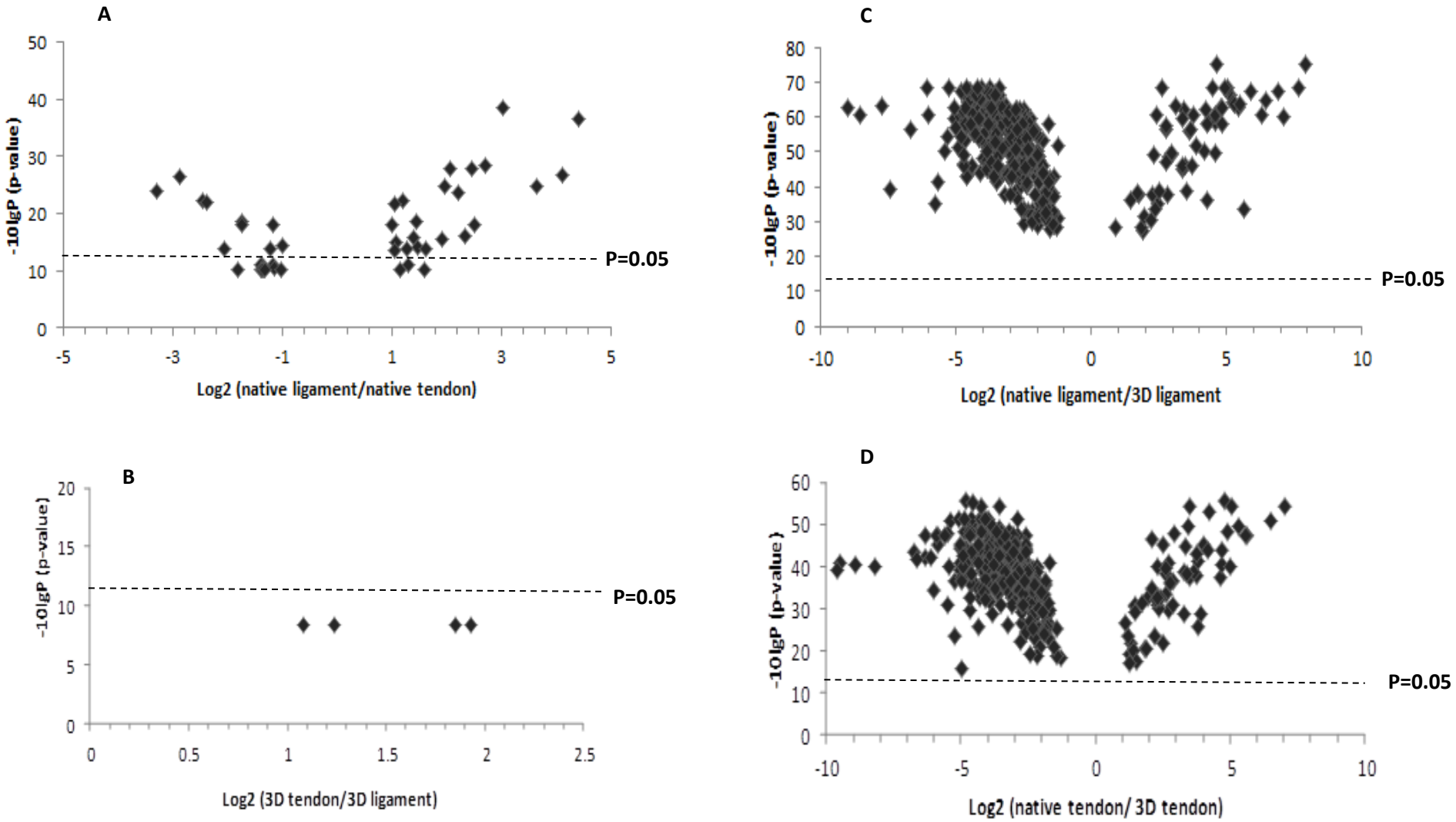


Figure 6.10. Volcano plots (-10lgP of FDR adjusted p-value vs log2 fold change) for: (A) Native ligament vs Native tendon, (B) 3D ligament vs 3D tendon, (C) Native ligament tendon vs 3D ligament, and (D) Native tendon vs 3D tendon. Volcano plots of quantified proteins in A, C and D indicated up-regulated and down-regulated proteins with up-fold and down-fold change with large significance. This was not the case in volcano plot D, as quantified proteins had a p-value (adjusted to FDR) >0.05.

Table 6.3. Differences in abundant proteins between native ligament and tendon*.

Accession	ID	Protein description	Peptide count	Anova (p)	q value	Max fold change	Highest mean condition	Log2 (L/T)	Function
ENSCAFP00000016949	ACAN	Aggrecan core protein	12	2.8E-06	0.0002	23.38	Ligament	4.42	Large aggregating proteoglycan that provides a hydrated gel structure via interaction with hyaluronan and link protein (Kiani et al. 2002)
ENSCAFP00000025121	CHAD	Chondroadherin	6	5.9E-05	0.0021	17.53	Ligament	4.13	Plays an important role in bone and cartilage homeostasis (Hessle et al. 2013)
ENSCAFP00000042916	HAPLN1	Hyaluronan and proteoglycan link protein 1	7	0.00014	0.0032	13.58	Ligament	3.64	Stabilizes the aggregates of proteoglycan monomers
ENSCAFP00000013230	THBS4	Thrombospondin 4	21	0.0002	0.004	9.84	Tendon	-3.28	Regulates the composition of the ECM at major sites of its deposition (Frolova et al. 2014)
ENSCAFP00000041629	PCOLCE2	Procollagen C-endopeptidase enhancer 2	2	1.2E-06	0.0001	8.04	Ligament	3.02	Binds to the C-terminal propeptide of types I and II procollagens and may enhance the cleavage of that propeptide by BMP1 (Steiglitz et al. 2002)
ENSCAFP00000021988	MYOC	Myocilin,	23	7.5E-05	0.0023	7.42	Tendon	-2.88	Modulates Wnt signalling and regulates the actin cytoskeleton (Kwon et al. 2009)
ENSCAFP00000018471	HTRA1	Serine protease HTRA1	9	2.3E-05	0.0014	6.65	Ligament	2.71	Serine protease with a variety of targets, including extracellular matrix proteins
ENSCAFP00000013330	COL2A1	Collagen, type II, alpha 1	26	0.00166	0.0152	6.00	Ligament	2.52	Specific for cartilaginous tissues. It is essential for the normal embryonic development of the skeleton
ENSCAFP00000010055	OLFML1	Olfactomedin-like 1	3	0.00036	0.006	5.56	Tendon	-2.44	Abundant expression in brain suggests that it may have an essential role in nerve tissue
ENSCAFP00000013989	Abi3bp	ABI gene family, member 3 binding protein	9	4.1E-05	0.0016	5.41	Ligament	2.46	Exact role unclear but has been found to play an important role in MSC biology(Hodgkinson et al. 2013)
ENSCAFP00000021176	FN1	Fibronectin 1	16	0.00338	0.0242	5.33	Ligament	2.35	Glycoprotein of the extracellular matrix that binds to membrane-spanning receptor proteins called integrins (Pankov and Yamada 2002).
ENSCAFP00000033127	ASPN	Asporin	17	0.00047	0.0063	5.18	Tendon	-2.38	Competes with collagen binding (Kalamajski et al. 2009)
ENSCAFP00000012600	VCAN	Versican	13	0.00032	0.0043	4.77	Ligament	2.21	Large proteoglycan that plays role in cell adhesion, proliferation, migration and extracellular matrix assembly (Wight 2002)
ENSCAFP00000023923	SERPINE2	Serpin peptidase inhibitor, clade E, member 2	2	4E-05	0.0016	4.34	Ligament	2.08	Antiprotease activity (Bouton et al. 2012)
ENSCAFP00000003954	COL12A1	Collagen, type XII, alpha 1	2	0.00914	0.0419	4.28	Tendon	-2.05	Interacts with type I collagen-containing fibrils
ENSCAFP00000001150	FBLN1	Fibulin 1	7	0.00014	0.0032	4.02	Ligament	1.96	Incorporated into fibronectin-containing matrix fibers and may play a role in cell adhesion and migration along protein fibers within the ECM

Accession	ID	Protein description	Peptide count	Anova (p)	q value	Max fold change	Highest mean condition	Log2 (L/T)	Function
ENSCAFP00000005060	CAV1	Caveolin 1, caveolae protein	3	0.00171	0.0152	3.44	Tendon	-1.74	May act as a scaffolding protein within caveolar membranes
ENSCAFP00000042302	AHNAK	AHNAK nucleoprotein	20	0.00116	0.0134	3.36	Tendon	-1.72	Interacts with dysferlin (Huang et al. 2007)
ENSCAFP00000017002	MFGE8	Milk fat globule-EGF factor 8 protein	9	0.00762	0.0402	3.12	Ligament	1.62	Important role in the maintenance of intestinal epithelial homeostasis and the promotion of mucosal healing (Raymond et al. 2009)
ENSCAFP00000004677	UGP2	UDP-glucose pyrophosphorylase 2	3	0.00124	0.0137	2.84	Ligament	1.46	Plays a central role as a glucosyl donor in cellular metabolic pathways
ENSCAFP00000024271	SOD3	Superoxide dismutase 3, extracellular	9	0.00703	0.0397	2.76	Ligament	1.48	Protect the extracellular space from toxic effect of reactive oxygen
ENSCAFP00000021570	COMP	Cartilage oligomeric matrix protein	2	0.00424	0.027	2.66	Ligament	1.39	role in the structural integrity of cartilage
ENSCAFP00000003680	COL15A1	Collagen, type XV, alpha 1	6	0.00861	0.041	2.45	Ligament	1.27	Stabilizes microvessels and muscle cells, both in heart and in skeletal muscle.
ENSCAFP00000023709	GLUD1	Glutamate dehydrogenase 1	6	0.00751	0.0402	2.38	Tendon	-1.21	Mitochondrial glutamate dehydrogenase that converts L-glutamate into alpha-ketoglutarate.
ENSCAFP00000025819	SRPX2	Sushi-repeat containing protein, X-linked 2	2	0.00042	0.006	2.32	Ligament	1.19	Acts as a ligand for the urokinase plasminogen activator surface receptor. (Royer-Zemmour et al. 2008)
ENSCAFP00000029164	ENO1	Enolase 1	13	0.00418	0.028	2.27	Ligament	1.93	Multifunctional enzyme. Plays role in glycolysis and other process such growth control and hypoxia tolerance
ENSCAFP00000027321	MYL12A	Myosin, light chain 12A, regulatory, non-sarcomeric	6	0.00171	0.0152	2.23	Tendon	-1.18	Responsible for many types of cell movements
ENSCAFP00000023831	ANXA8L1	Annexin A8-like 1	3	0.00148	0.0152	2.17	Ligament	1.00	Vascular anticoagulant (Hauptmann et al. 1989)
ENSCAFP00000034804	CLU	Clusterin	7	0.00776	0.0446	2.16	Ligament	1.06	Associated with cell apoptosis(Jones and Jomary 2002)
ENSCAFP00000025244	CILP	Cartilage intermediate layer protein	17	0.00536	0.0317	2.14	Ligament	1.08	Is upregulated with ageing and early OA and may be involved in the ectopic calcifications in the arthritic mice (Yao et al. 2004)
ENSCAFP00000039083	COL3A1	Collagen type 3, alpha 1	3	0.00052	0.0066	2.08	Ligament	1.06	Occurs in most soft connective tissues along with type I collagen.
ENSCAFP00000023240	TPM4	Tropomyosin 4	10	0.0063	0.0364	2.02	Tendon	-1	Binds to actin filaments in muscle and non-muscle cells. Binds to calcium (Crabos et al. 1991)

*Tables 6.3 includes 32 identified proteins that were different between native ligament and tendon with statistically significance. The following criteria were applied: 1) The proteins were identified and quantified with ≥ 2 unique proteins in all samples, 2) > 2-fold change and FDR adjusted p-value ANOVE $\leq$ 0.05 as determined by Progenesis^{TM}. Log2 T/C indicates Log 2 of native ligament / native tendon. Ligament was more abundant in cartilaginous protein such as agreccan, chondroadherin, collagen type II, alpha 1, while tendon had more thrombospondin 4 and asporin

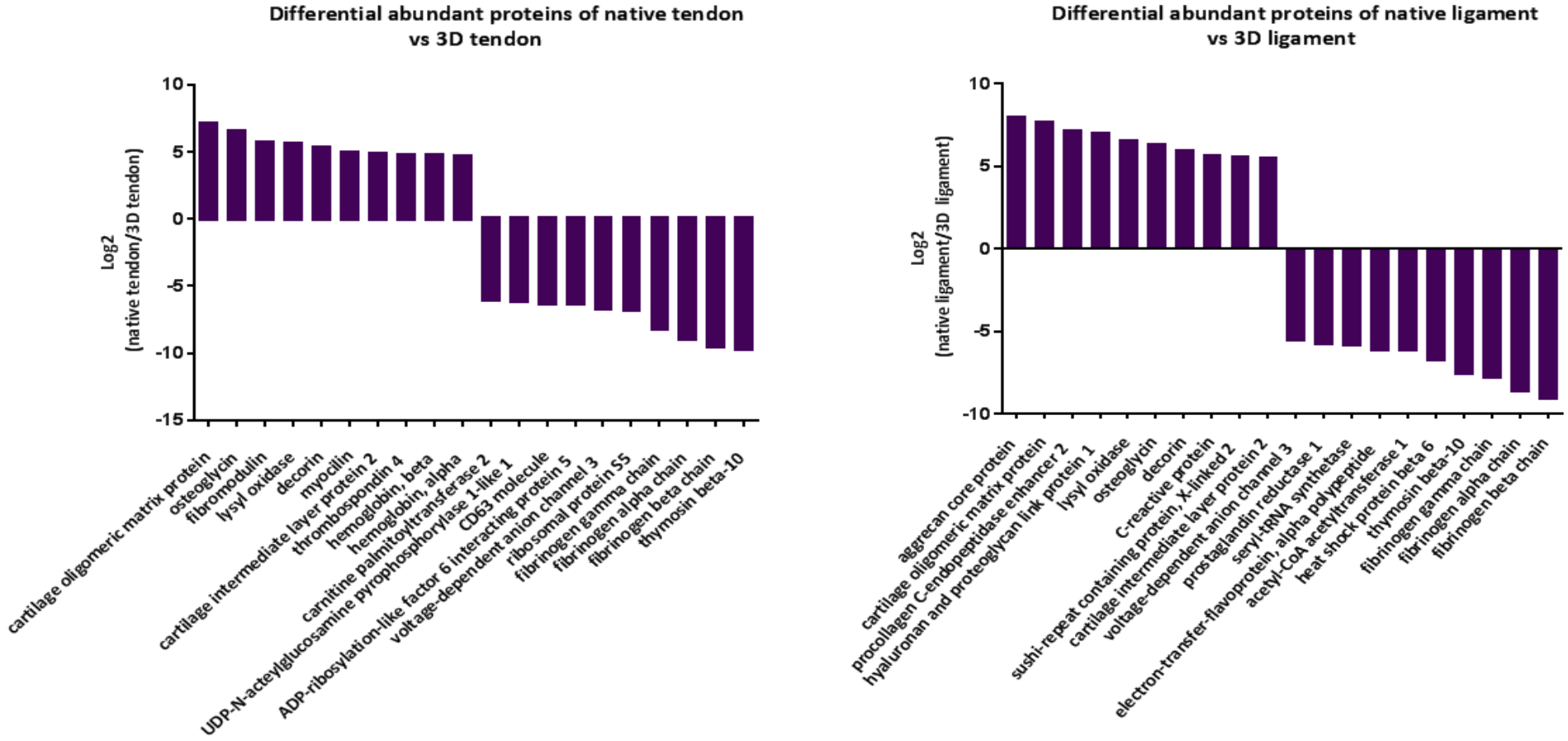


Figure 6.11. Different abundant proteins between native tendon and ligament tissues and 3D tendon and ligament constructs. The histograms depicts results of label-free relative protein quantification using Progenesis™ software for the top ten abundant proteins in each tissue type. Changes in protein expression of log2 native ligament/3D ligament (A) and log 2native tendon/3D tendon (B) for proteins identified with ≥2 peptides and FDR adjusted p-value ≤0.05 and more than 2-fold regulated are depicted.

Type	Proteins description	ID	Accession	Mean Log2 Fold change
Collagen	Collagen, type XV, alpha 1	COL15A1	ENSCAFP00000003680	3.9
Collagen	Collagen, type VI, alpha 1	COL6A1	ENSCAFP00000017530	2.7
Collagen	Collagen type VI, alpha 2	COL6A2	ENSCAFP00000041957	2.7
Collagen	Collagen, type VI, alpha 3	COL6A3	ENSCAFP00000018017	2.1
Collagen	Collagen type III, alpha 1	COL3A1	ENSCAFP00000039083	2.5
Collagen	Collagen, type II, alpha 1	COL2A1	ENSCAFP00000013330	2.7
Collagen	Collagen, type I, alpha 2	COL1A2	ENSCAFP00000029400	1.9
Collagen	Collagen, type I, alpha 1	COL1A1	ENSCAFP00000025056	1.9
ECM protein	ABI gene family, member 3 binding protein	Abi3bp	ENSCAFP00000013989	3.0
ECM protein	Aggrecan core protein	ACAN	ENSCAFP00000016949	5.4
ECM protein	Asporin	ASPN	ENSCAFP00000033127	2.6
ECM protein	Biglycan	BGN	ENSCAFP00000028297	4.2
ECM protein	Cartilage intermediate layer protein	CILP	ENSCAFP00000025244	4.0
ECM protein	Cartilage intermediate layer protein 2	CILP2	ENSCAFP00000042401	5.1
ECM protein	Cartilage oligomeric matrix protein	COMP	ENSCAFP00000021570	7.4
ECM protein	Decorin	DCN	ENSCAFP00000009230	5.6
ECM protein	Dermatopontin	DPT	ENSCAFP00000022484	2.6
ECM protein	Fibulin 1	FBLN1	ENSCAFP00000001150	2.6
ECM protein	Fibromodulin	FMOD	ENSCAFP00000013913	5.4
ECM protein	Fibronectin 1	FN1	ENSCAFP00000021176	2.3
ECM protein	Keratocan	KERA	ENSCAFP00000009210	4.9
ECM protein	Lumican	LUM	ENSCAFP00000009219	4.4
ECM protein	Matrilin 2	MATN2	ENSCAFP00000000680	1.7
ECM protein	Osteoglycin	OGN	ENSCAFP00000003355	6.4
ECM protein	Proline/arginine-rich end leucine-rich repeat protein	PRELP	ENSCAFP00000013916	5.2 3.7
ECM protein	Proteoglycan 4	PRG4	ENSCAFP00000020163	
ECM protein	Transforming growth factor, beta-induced	TGFBI	ENSCAFP00000001531	3.6
ECM protein	Thrombospondin 1	THBS1	ENSCAFP00000012843	4.0
ECM protein	Thrombospondin 4	THBS4	ENSCAFP00000013230	3.2
ECM protein	Tenascin C	TNC	ENSCAFP00000036170	2.4
ECM protein	Tenascin XB	TNXB	ENSCAFP00000043117	4.0
ECM protein	Vitronectin	VTN	ENSCAFP00000027547	3.8
Enzyme/enhancer	Procollagen C-endopeptidase enhancer	PCOLCE	ENSCAFP00000021146	2.61
Enzyme/enhancer	Procollagen C-endopeptidase enhancer 2	PCOLCE2	ENSCAFP00000041629	4.69
Enzyme/enhancer	Superoxide dismutase 3	SOD3	ENSCAFP00000024271	3.7
Enzyme/enhancer	Lysyl oxidase	LOX	ENSCAFP00000038725	6.0
Enzyme/enhancer	Matrix metallopeptidase 3	MMP3	ENSCAFP00000022195	2.4
Blood/plasma protein	Hemoglobin subunit alpha	HBA	ENSCAFP00000042411	4.4
Blood/plasma protein	Hemoglobin, beta	HBB	ENSCAFP00000021235	4.8
Blood/plasma protein	Serpin peptidase inhibitor, clade A member 1	SERPINA1	ENSCAFP00000031948	4.4
Blood/plasma protein	Serpin peptidase inhibitor, clade C member 1	SERPINC1	ENSCAFP00000021382	2.8
Blood/plasma protein	Serpin peptidase inhibitor, clade F member 1	SERPINF1	ENSCAFP00000028380	3.8
Blood/plasma protein	Transferrin	TF	ENSCAFP00000040309	2.7
Blood/plasma protein	Transthyretin	TTR	ENSCAFP00000026675	4.2
Blood/plasma protein	Histidine-rich glycoprotein	HRG	ENSCAFP00000020009	2.1
Blood/plasma protein	Hemopexin	HPX	ENSCAFP00000009637	2.4
Blood/plasma protein	Haptoglobin	HP	ENSCAFP00000029992	3.5
Blood/plasma protein	Albumin	ALB	ENSCAFP00000004489	3.1
Blood/plasma protein	Apolipoprotein A-I	APOA1	ENSCAFP00000019630	2.8
Blood/plasma protein	C-reactive protein, pentraxin-related	CRP	ENSCAFP00000017329	4.8
Blood/plasma protein	Glutathione peroxidase 3 (plasma)	GPX3	ENSCAFP00000038899	2.6
Blood/plasma protein	Milk fat globule-EGF factor 8 protein	MFGE8	ENSCAFP00000017002	3.6
Blood/plasma protein	Plasminogen	PLG	ENSCAFP00000001078	2.3
Other proteins	Angiopoietin-like 7	ANGPTL7	ENSCAFP00000024545	3.5
Other proteins	Clusterin	CLU	ENSCAFP00000034804	1.8
Other proteins	Glutamyl-prolyl-tRNA synthetase	EPRS	ENSCAFP00000016021	2.0
Other proteins	Olfactomedin-like 1	OLFML1	ENSCAFP00000010055	1.9
Other proteins	Sushi-repeat containing protein, X-linked 2	SRPX2	ENSCAFP00000025819	4.4

Table 6.4. Abundant proteins in both native tendon and ligament tissues in comparison 3D tendon and ligament constructs. Both native tissues had more abundant ECM, enzyme and blood/plasma proteins.

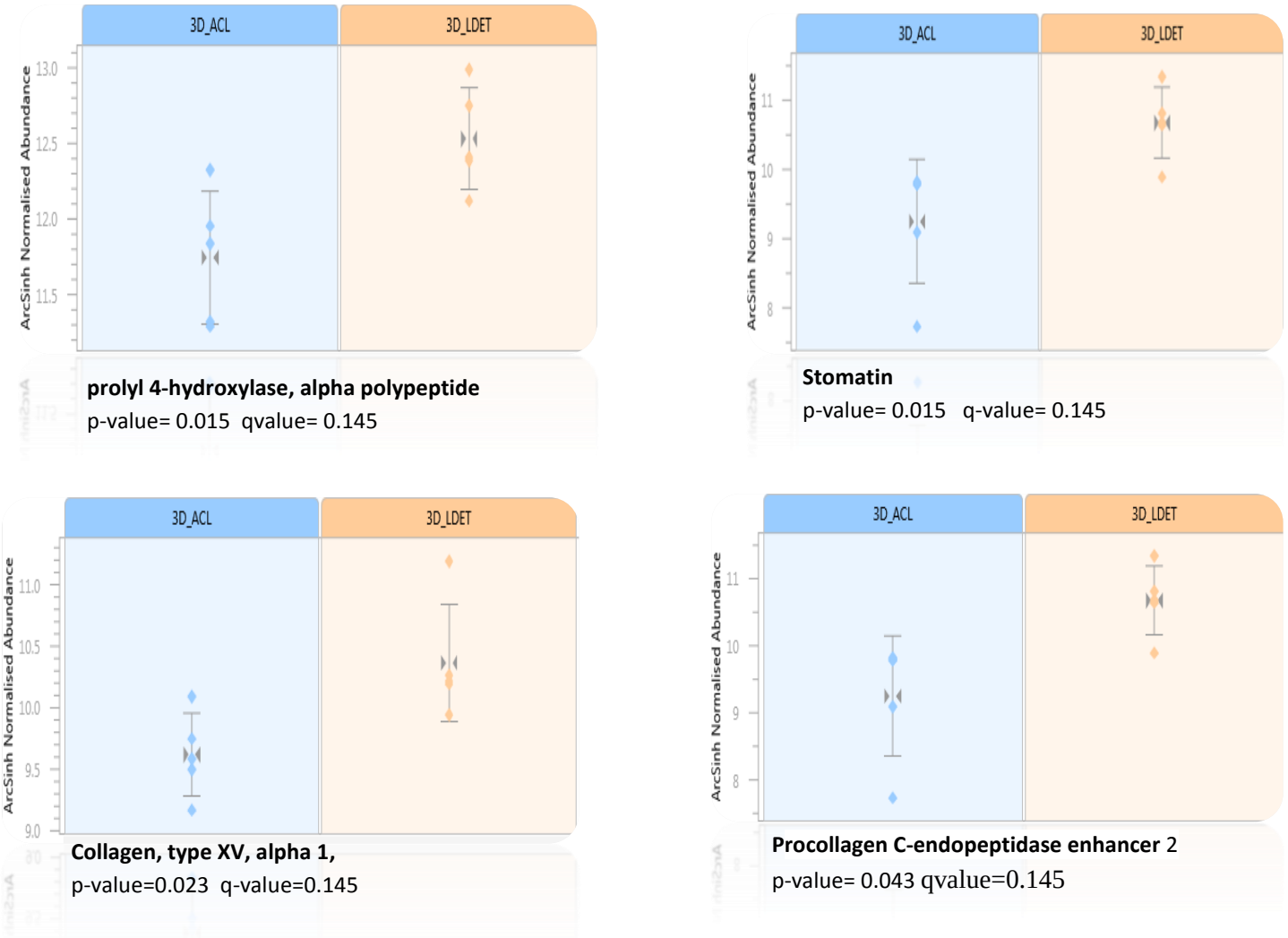


Figure 6.12. Expression plots of 3D ligament construct versus 3D tendon construct of selected proteins produced by Progenesis™ LC-MS. All proteins were identified by 2 or more peptides with greater than 2 fold abundance change and p values <0.05. 3D ligament constructs are blue dots on a blue background and 3D tendon constructs are orange dots and on a orange background. Plots display the mean Arcsinh transformed normalised volume for each group. Error bars demonstrate standard deviation within groups.

6.4.3 Post-translational modification differences between native tendon and ligament

PTMs comparisons of collagens, proteoglycans between native tendon (LDET) and ligament (CCL) tissue showed differences in the post-translational modification of collagen alpha-1(I), collagen alpha-1(I) and collagen alpha-1(III) with hydroxylation occasionally being present on different proline and lysine residues (Figure 6.13 and Figure 6.14).

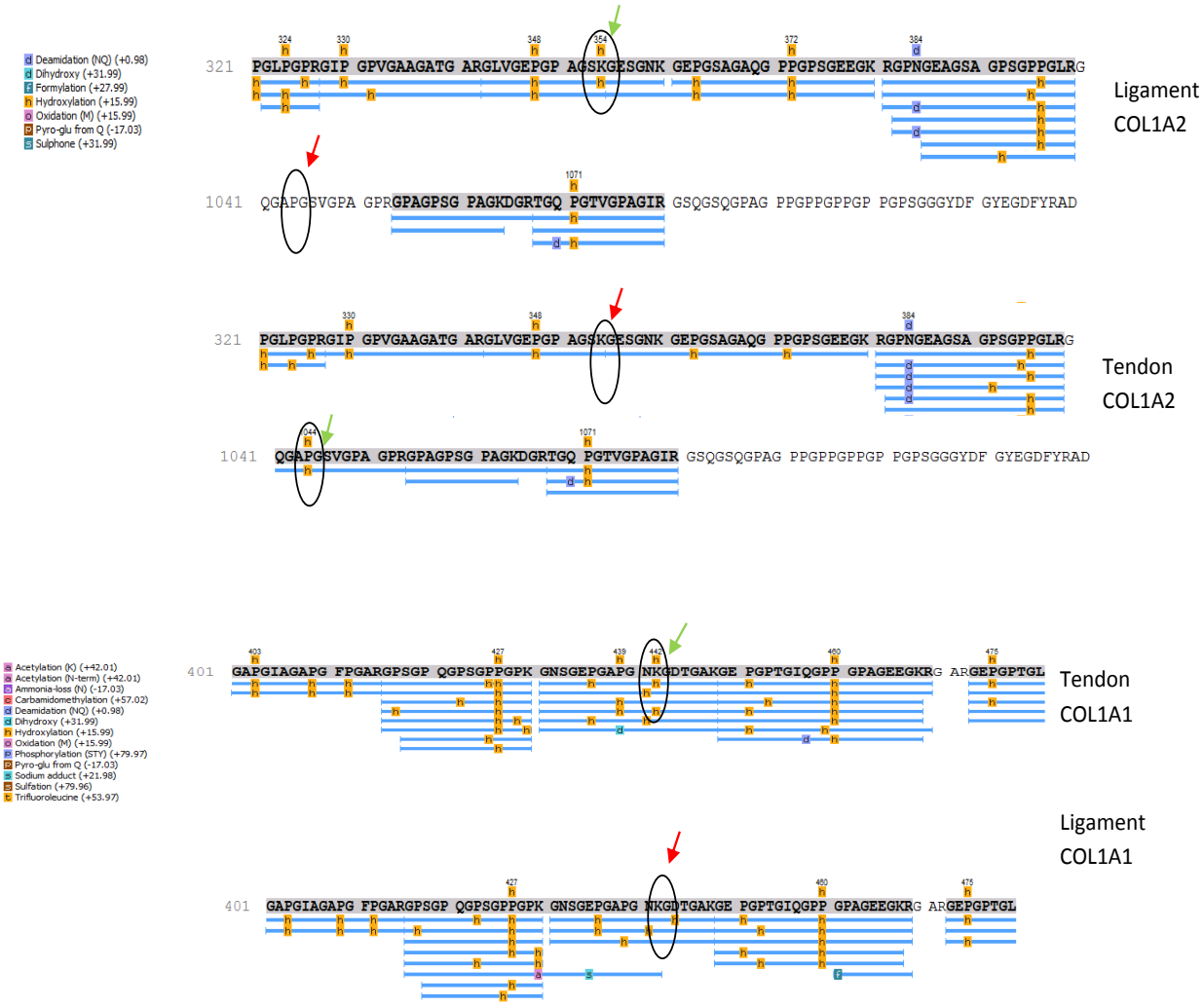


Figure 6.13. Post-translational modification of collagen alpha-1(I) and collagen alpha-2 (I) between native tendon and ligament. Differences were seen in post-translational hydroxylation, occasionally occurring on different proline and lysine sites. The green arrows represent the presence of post-translational hydroxylation and the red arrows represent the missed of post-translational hydroxylation located on the same amino acid. The partial sequence collagen alpha-1 (I) and collagen alpha-1 (II) is represented.

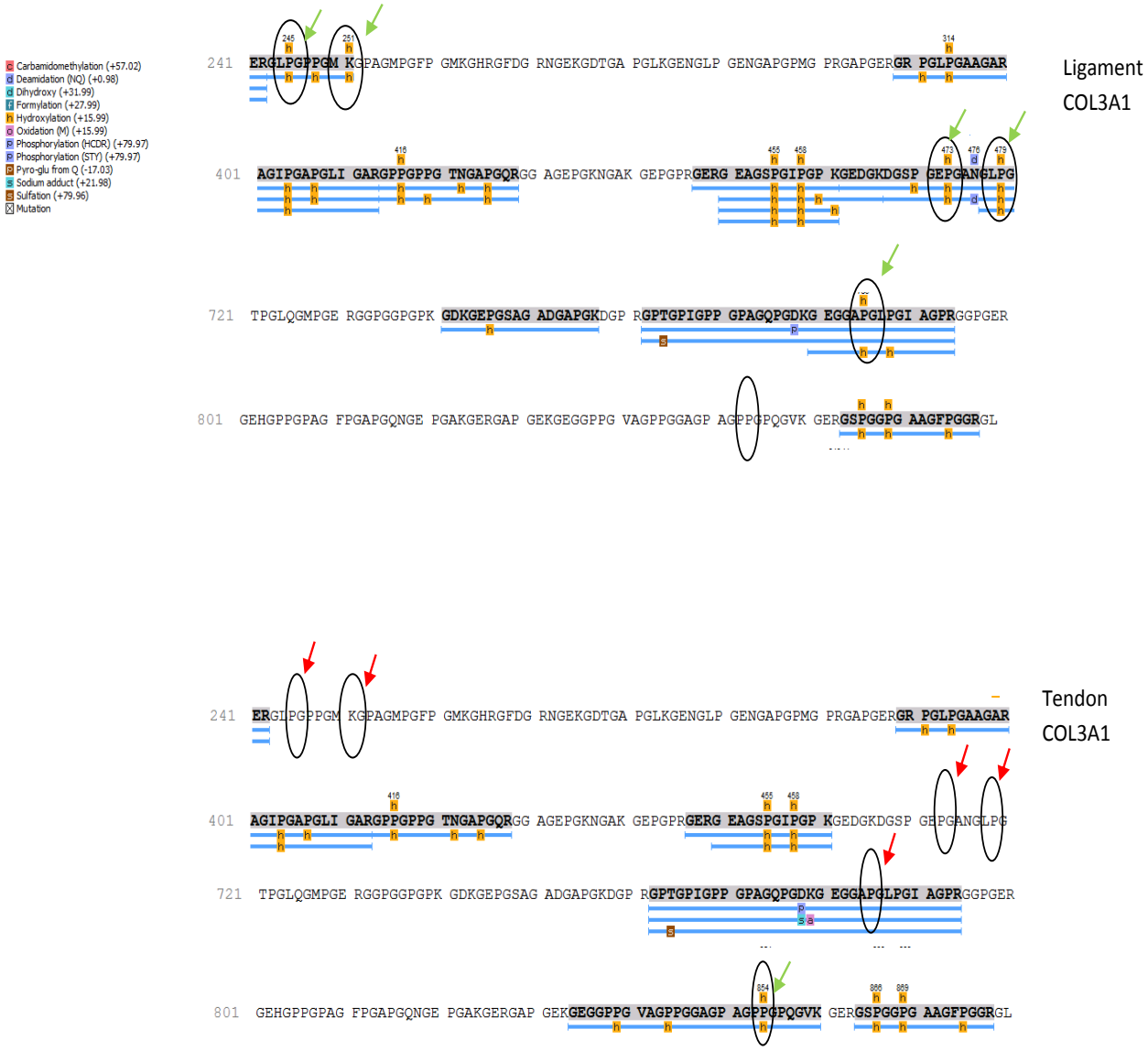


Figure 6.14. Post-translational modification of collagen alpha-1(III) between native tendon and ligament. Differences were seen in post-translational hydroxylation, occasionally occurring on different proline and lysine sites. The green arrow represent the presence of post-translational hydroxylation and the red arrows represent the absent of post-translational hydroxylation located on the same amino acid. The partial sequence of collagen alpha-1(III) is represented with PTMs variations.

6.5 DISCUSSION

In this study the proteomic comparison between native canine LDET and the CCL was performed. 3D fibrin tendon and ligament constructs were created from isolated canine LDET and CCL tissue adults cells. The proteome of 3D tendon and ligament constructs were characterised and compared to native tendon and ligament tissues. Our findings indicate specific protein differences between native tendon and ligament and demonstrated that 3D *in vitro* culture of tendon and ligament cells are able to recapitulate tendon and ligamentous tissue characteristics particularly with regards to ECM proteins.

6.5.1 Proteomic comparison between native tendon and ligament

Qualitative proteome comparison- the CCL and LDET proteomes had similar matrisomal and cellular proteins. More proteins were identified in tendon than ligament that were associated with cytoplasmic localisation. Protein network analysis resulted in a highly connected cluster between ECM proteins and other matrisomal proteins and indicated a complex relationship between these proteins.

Quantitative proteome comparison- Statistically significant quantitative proteome differences between (in different collagens, proteoglycans, glycoprotein and several cellular proteins) the ligament (CCL) and tendon (LDET) were identified

Collagen types II and III were found to be more abundant in ligament than tendon. The higher proportion of collagen type III agrees with previous reports, which compared rabbit cruciate ligament to patellar tendon (Amiel et al. 1984) and human ACL to patellar tendon (Little et al. 2014). It also agrees with the increase immunostaining of collagen type III in CCL, where it was found to have a more widespread distribution located on the CCL substance and between collagen fascicles and fibre bundles (Chapter 4). This might indicate that in the ligament collagen type III may play more of an essential role in bridging collagens with adjacent matrix, which could also be important for the pliability of the ligament.

Collagen type II has mainly been found at fibrocartilaginous regions in porcine ACL and posterior cruciate ligament (PCL) (Young et al. 2002) and in human ACL where it was found in pericellular matrix of the chondrocytes (Petersen and Tillmann 1999). In this study the

variance between different regions of the CCL was not discriminated, hence the higher collagen type II levels in the ligament may be reflected due to the localisation of collagen type II at the at the origin or insertion fibrocartilagenous regions. However, the presence of collagen type II has also been identified in mid-region of porcine ACL (Young et al. 2002), indicating the formation of fibrocartilage in central region of the ligament. This might alter the mechanical properties of the ligament, increasing its stiffness and changing the translation of loading across the femoro-tibial articulation (Young et al. 2002). In the canine CCL the localisation and organisation of collagen type II is yet to be identified. The higher collagen type II mRNA gene expression in porcine patellar tendon compared than porcine ACL (Pearse et al. 2009), does not agree with findings in this study and may be dependent on the presence of post-translational regulation pathways or differences in proteins deposited during development as compared to gene expression at a particular time point.

Higher levels of proteoglycan core proteins were found in ligament than in tendon, which corresponds with the data presented in Chapter 3, showing greater amounts of sGAG in the CCL. Previous studies have identified the presence of aggrecan in canine CL (Valiyaveettil et al. 2005) and versican in rabbit ACL (Tischer et al. 2007). Aggrecan and versican were also identified in human ACL but not in patellar tendon using proteomic analysis in a recent study (Little et al. 2014). These findings are supported by the current study, as both aggrecan and versican were elevated in ligament compared to tendon. Furthermore the elevated aggrecan and versican corresponds to the increased staining of these proteins located between collagen fibres (interfascicular area) of the CCL (Chapter 4, Section 4.4.2.3). Hyaluronic acid and link protein (HAPLN) was also found to be higher in ligament than in tendon, which could be due to the elevated level of aggrecan in ligament, as aggrecan has been demonstrated to interact with HAPLN at its globular 1 domain (Kiani et al. 2002). Together, these data might indicate that the increased large aggregating proteoglycans in the ligament may be as result to a functional response to compressive forces and might act as a lubricant layer that could modulate the sliding capacity between fascicles and fibre bundles.

The small leucine rich proteoglycan (SLRP) chondroadherin was up-regulated in ligament whereas another SLRP, asporin was more abundant in tendon. The predominance of asporin in tendon in this study was in contrast to findings in human ACL and patellar tendon (Little

et al. 2014). Asporin is known to compete for type I collagen binding with decorin with a high affinity site located in the C-terminal part of the protein. It binds to collagen and induces mineralisation through its variable domain that contains a polyaspartic tail, indicating that asporin has a role in osteoblast-driven collagen biomineralisation activity (Kalamajski et al. 2009). In periodontal ligament asporin has been found to prevent mineralisation by regulation BMP-2 activity. This data suggests that elevated levels of asporin in tendon might function to prevent mineralisation in this tissue. Chondroadherin is particularly highly expressed in cartilaginous tissues and has been suggested to have role in in the regulation of chondrocyte growth and proliferation (Shen et al. 1998). The absence of chondroadherin leads to a distinct skeletal phenotype, demonstrating that it has an important role in cartilage and bone turnover (Hessle et al. 2013). Identification of chondroadherin in this study may indicate an important role in ligament homeostasis and function.

A number of glycoproteins including cartilage oligomeric matrix protein, cartilage intermediate layer protein, fibronectin and fibulin were identified and were more highly expressed in ligament. Conversely, thrombospondin 4 was more abundant in tendon. These findings support previous reports on the proteomic comparison between ACL and patellar tendon, apart from the thrombospondin-4, which was up-regulated in ACL (Little et al. 2014).

ECM regulators and affiliated proteins such as annexin-8-like, clusterin, serpine protease inhibitor 2 were up-regulated in ligament. Principle gene ontology (GO) of ligament identified these proteins under the GO term ‘wound healing’. The role of annexin-8-like has been identified as a vascular anticoagulant (VAC-β) (Hauptmann et al. 1989), but it has also been associated with cancer (Gerke and Moss 2002). Clusterin is associated with cell apoptosis (Jones and Jomary 2002) and has previously been identified in tendinopathy (Millar et al. 2012). Serine protease inhibitor 2 is part of serine protease inhibitors which are reported have an antiprotease activity and play a predominant role in the homeostasis of the cardiovascular system. They are key modulators of cell adhesion, proliferation, and death (Bouton et al. 2012). Together, these ECM regulators and affiliated proteins may play an important role in the pathophysiology of the ligament.

Significantly more cellular proteins were identified in tendon than ligament, which correlates with qualitative proteome results of tendon, whereby more cellular associated proteins were found in the tendon proteome. This indicates higher cell content in tendon, which would result in more cellular proteins. However it was previously demonstrated that there are no differences in DNA content between CCL and LDET (Chapter 3), therefore the higher cellular proteins of LDET proteome could be due to the tissue specific metabolic and/or functional needs. Cellular proteins such as myosin, tropomyosin, glutamate dehydrogenase 1 and myocillin were higher in tendon. The predominance of myocillin may be an important finding in this study, as myocillin modulates Wnt signalling, regulates the actin cytoskeleton and interacts with fibronectin (Kwon et al. 2009). In the intervertebral disc the expression of myocilin appears to be up-regulated by transforming growth factor-beta (TGFβ) and by mechanical stimuli (Gruber et al. 2006). Therefore, increased expression of myocillin in tendon may suggest differential ability of tendon to respond to changes in growth factor signalling and mechanical loading compared to ligament.

Comparison of post-translational modifications (PTMs)- PTMs in tendon in ligament have not been studied before, yet they can reveal important differences that may be associated with tissue specific function. Here, the differences in PTMs between tendon and ligament collagens, SLRPS and large proteoglycan were analysed. Although no PTM variations were found in the SLRPS and large proteoglycans, differences in the post-translational modification of collagen alpha-1(I), collagen alpha-2(I) and collagen alpha-1 (III) between tendon and ligament were observed with hydroxylation occasionally present on different proline and lysine residues on each tissue. The process of hydroxylation plays an important role in stabilisation of collagen triple helix conformation and in the collagen synthesis process. An increase proline or lysine hydroxylation may indicate that the hydroxylases enzymes are more active, which as result could increase the collagen production. Further validations are required to confirm the different PTM hydroxylation pattern between tendon and ligament. This could be achieved through the use of high resolution mass spectrometry (Yang et al. 2012) or through the sequence algorithm that has been developed by Hu et al. (2010) for identifying the hydroxyproline and hydroxylysine sites.

In summary proteomic comparison between native tendon and ligament demonstrated differences in specific ECM proteins, in particular agreccan, chondroadherin, collagen type

II, alpha 1, asporin and thrombospondin-4, which is suggestive of fundamental differences between these tissue types. The abundance of fibrocartilage proteins in the CCL suggests the deposition of cartilage-like matrix in the ligament, which is most likely a physical adaptation of the tissue in response to intermittent compressive and shear forces.

6.5.2 Proteomic comparison between native tendon and ligament and tissue engineered tendon and ligament

Qualitative proteome comparison- The protein hits in the 3D tissue constructs were double those of the native samples of tendon and ligament (Table 6.1). 3D tendon and ligament constructs contained a greater proportion of cellular proteins than native tissues, as an average of 66.3% proteins in tendon and ligament constructs and 32.5% proteins in native tendon and ligament were associated with the cytoplasmic localisation. However both 3D engineered constructs also contained similar core matrisomal and matrisomal-associated proteins that were also found in the native tissues, including collagen I, III,V,VI and XII XIV, decorin, bigylcan, asporin, osteoglycin, fibronectin 1 and fibrillin 1. These findings indicate that 3D constructs are able to recapitulate specific tendon and ligament ECM proteins. String analysis revealed a highly connected cluster of ribosomal proteins in both 3D tendon and ligament constructs, while most protein-protein interactions in the native tissues were associated between extracellular proteins and matrisomal proteins. The principal gene ontology process in native tendon and ligament tissue were identified as ECM and collagen fibril organisation. Principal ontologies associated with the 3D tendon and ligament constructs involved protein translation and targeting.

Quantitative proteome comparison- when 3D tendon construct and native tendon were compared, there were 383 differentially expressed proteins: 321 proteins down-regulated in 3D tendon and 62 up-regulated in native tendon (Figure 6.10D). 3D ligament constructs and native ligament had 363 differentially expressed proteins: 301 proteins down-regulated in 3D ligament and 62 proteins were up-regulated in native ligament (Figure 6.10C). Both native tissues in comparison to 3D constructs were significantly abundant in not only ECM and enzyme/enhancers proteins but also in blood/plasma proteins, which correlates with our histological finding of blood vessels and reflects the vascularisation of the native tissues

(Chapter 4, Section 4.4.1). In contrast both 3D constructs had significantly more cellular proteins, correlating with qualitative proteome comparisons. The higher cellularity in 3D ligament and tendon construct is possibly due to higher cell content in these tissues compared to the native tissues (Chapter 5, Section) and reflects the immature state of the constructs.

Principle component analysis between native tendon, ligament and 3D tendon and ligament constructs demonstrated that 3D ligament and tendon groups do not split into discrete groups, whereas native tissues were more distinctly clustered together, indicating definite differences between their protein content. The results of the PCA plot might indicate why there were no significantly differentially expressed proteins between 3D tendon and ligament constructs. Although these findings suggest that there are specific protein characteristics between native tendon and ligament, fibroblasts of tendon and ligament do no result in distinct 3D constructs, thus cell source may not be an important factor for tissue engineering. This also indicates that tissue complexity rather than fibroblast phenotype or ECM composition may be a more significant challenge for tendon and ligament engineering. Tendon and ligament fibroblasts may become phenotypically distinct due to different response to the *in vivo* loading environment or extrinsic factors such as growth factors. Further optimisation and development of 3D tendon and ligaments constructs should provide insight into the tissue-specific modelling and remodelling mechanism of these tissues.

6.6 CONCLUSION

Significant proteomic quantitative differences were identified between native tendon, native ligament and 3D tendon and ligament constructs. Specific differences in the composition of native tissues were identified with prominent fibrocartilaginous proteins such as collagen alpha-1 (II), aggrecan, chondroadherin being more abundant in ligament and asporin and thrombospondin 4 more abundant in tendon. Differences in the post-translational modification of collagen alpha-1(I), collagen alpha-1 (I) and collagen alpha-1 (III) were observed between native tendon and ligament with hydroxylation occasionally present on different proline and lysine residues.

3D tendon and ligament constructs contained a greater proportion of cellular proteins than native tissues, reflecting their immature state. However both tissue constructs contained numerous ECM proteins that were also found in the native tissues, including collagen I, III, VI and XII, decorin, bigylcan, fibronectin 1 and fibrillin 1.

Tendon and ligament 3D constructs did not split into discrete groups in the PCA plot, whereas native tendon and ligament separated into more distinct groups. This matches similar to our proteomic finding between ligament and tendon constructs, as no significantly differential expressed proteins were observed between two tissue types.

These findings support the hypothesis that there are key structural protein differences between native tendon and ligament, but that tissue engineered tendon and ligament share similar characteristics with the native tissues particularly with regard to prominent ECM.

CHAPTER SEVEN

GENERAL DISCUSSION AND FUTURE DIRECTION

7.1 GENERAL DISCUSSION

Tendons and ligaments are important biological structures in both humans and animals. They are part of dense connective tissue and are crucial to the function of the musculoskeletal system. However, they are commonly damaged due to age-related wear and tear, trauma or sports related incidents (Riley 2004, Rees et al. 2009, Cimino et al. 2010). The healing process of ligament and tendon healing is complex and varies from tissue to tissue (Woo, Hildebrand et al. 1999; Jung, Fisher et al. 2009). In general, these tissues heal slowly following injury, resulting in pain and immobility in affected individuals, which can lead to injury of other tissues and the development of degenerative joint disease such osteoarthritis (Woo et al. 1999). This has resulted in a major clinical challenge in orthopaedic medicine (Hoffmann and Gross 2006). Tissue engineering can offer an attractive therapeutic approach to treat tendon and ligament injuries, however this is hampered by our poor understanding of the defining characteristics between the two tissue types.

The aim of this project was to understand the fundamental differences between tendon and ligaments at a molecular and cellular level. Information in this area is currently lacking and is vital for the future development of techniques to assist with tendon and ligament repair, as well as the preparation of tissue–engineered structures.

In this project the canine was chosen as model, as the canine stifle anatomy is similar to the human knee and is prone to non-contact injury, which reflects the human joint pathology. It was hypothesised that tendons and ligaments around the canine stifle joint have different extracellular matrix (ECM) and cellular composition, morphological characteristics and structural protein expression. It was further hypothesised that canine tendon and ligament cells would retain similar biochemical, morphological and proteomic characteristics as the original tendon and ligament tissue.

The first objective of this study was to determine specific measurable differences in composition (collagen, elastin and proteoglycans) between canine stifle joint tendons and ligaments with regard to the location, function and regions, as described in Chapter 3. The data demonstrated no significant differences between the different regions of intra-articular cranial cruciate ligament (CCL), extra-articular medial collateral ligament (MCL), superficial

digital flexor tendon (SDFT) and long digital extensor tendons (LDET). Nevertheless, significantly higher sulphated glycosaminoglycan (sGAG) and elastin content in canine CCL was measured in comparison to MCL, LDET and SDFT. The higher sGAG and elastin content in CCL may be due to the complex functional requirement of this tissue, where its role it to withstand anterior tibial translation of the tibia, rotational load and valgus angulation of the knee (Duthon et al. 2006, Kweon et al. 2013). The GAGs and water content are assumed to play a role in determining tendon and ligament viscoelastic properties (Frank et al. 1999). This highlights tendon or ligament ability to structurally adapt to constant or cyclic load to reach biomechanical equilibrium (Connizo et al. 2013). Therefore, the higher sGAG content measured in CCL could alter its viscoelastic properties, allowing greater degree of deformation in order to prevent damage or injury from activities such as running or jumping. This finding supports previous tendon and ligament comparison studies in the ovine (Rumian et al. 2007) and rabbit (Amiel et al. 1984), where higher sGAG content was found in cruciate ligaments compared to extra-articular collateral ligaments and several tendons. The greater degree of deformation in CCL may also be reflected on the increased elastin content that was measured against the other three tissues examined. Elastin has been reported to contribute to the mechanics of ligaments, primarily in the toe regions of the stress-strain curve of porcine MCL, thus contributing to its viscoelastic properties (Henninger et al. 2013). In addition, elastin has also been documented to provide significant resistance to ligament transverse and shear deformation (Henninger, 2015). These data indicate that the higher elastin content measured in CCL in this study may play a greater role in the mechanical integrity of this ligament tissue. Together, the increase in elastin and sGAG content indicate that a there is more of a stretch and recoil mechanism and a greater degree of deformation required in the inter-articular CCL.

Following the work of Amiel et al. (1984), Rumian et al. (2007) and Zhu et al. (2012), the distinct structural and morphological characteristics of the different ligaments and tendons were demonstrated in Chapter 4. Amiel et al. (1984) revealed altered ECM between rabbit knee joint ligaments and tendons, but also indicated different cell nuclei morphology of the tendons in comparison to MCL and cruciate ligaments, with both the cruciate ligaments containing heterogeneous cell nuclei morphology. Similarly, Rumian et al. (2007) also demonstrated differential ECM composition in ovine intra- and extra-articular ligaments and

tendons, but found ligaments to have different proportions of collagen fibre diameter. Zhu et al. (2012) performed an structural comparison study between human anterior cruciate ligament (ACL) and hamstring tendons, describing a more complex arrangement of collagen fibres and different proteoglycan content and distribution in the ACL. While the above studies describe the morphology differences between tendons and ligaments to some extent, neither of the above mentioned studies fully investigated the complete characterisation of tendon and ligament tissue properties at the different anatomical regions. In Chapter 4, a semi-objective histology scoring system was used and for the first time demonstrated quantitative morphological and structural differences between CCL, MCL, LDET and SDFT. This study revealed no significant differences between different regions of the tendons and ligaments. However, there was significantly less compact collagen architecture, more mixed population of cell morphology and increased staining for GAGs and elastic fibres specifically found in the CCL. The ligaments, in particular the CCL, contained less compact collagen fibres and larger interfascicular regions, while both LDET and SDFT consisted of more parallel and compact collagen fibres. This may be as result of different loading pattern in ligaments, but also indicates a more complex compositional architecture with regard to the matrix in CCL. The fibre bundles in the CCL could be exposed to different extent of loading according to the flexional or rotational location of the knee, resulting in structural alterations of fibre bundle orientation. One limitation of this analysis was that the overall ECM architecture was measured, rather than individual aspects of collagen structure. It would be interesting to further develop the histological analysis with regard to collagen architecture by measuring the exact thickness of the interfascicular regions, collagen fibre alignment, crimp pattern and collagen fibril diameter. Together these analyses will give better understanding regarding the functional and mechanical properties between the tissue types.

The CCL was found to contain a more hetergenous cell population in comparison to LDET in the current study. Whilst the LDET cell nuclei were mainly spindle shaped, the CCL cell nuclei were more epileptical or round in shape, surrounded by GAGs, suggesting a “chondrocytic” appearance. Our finding of the chondrocytic cell phenotype in the CCL agrees with previously reported findings in dogs with low and high risk of CCL rupture (Comerford et al. 2006). This may be a normal finding in the canine CCL and may be as result of physical

adaptation of the CCL, rather than pathological degeneration as reported in human ACL (Hasegawa et al. 2012). Further work is required to identify different cells types in CCL and the effect of aging and degeneration on the CCL structure. It may also be important to improve our understanding to the role of cell communication in ligament physiology and disease, which may be significant in the treatment of ligament failure and development of reparative constructs treatment.

Another important finding of the histology scoring in this study was the significantly increased GAG and elastin content in CCL against the other three tissues examined, which were primarily located between fibre bundles and fascicles. This finding supports the increased sGAG and elastin content found in CCL in comparison to MCL, LDET and SDFT (Chapter 3). Together, these data might implicate that the higher GAGs proteoglycans sidechains and elastin in CCL could increase sliding and recoil capacity between adjacent collagen fibres and fascicles, resulting in a greater extension of the CCL. The capacity of fascicle sliding has been demonstrated to be different between equine SDFT and CDET, which is as result of interfascicular differences. In the SDFT the interfascicular matrix has been reported to withstand more cyclic loading and is more elastic than the CDET (Thorpe et al. 2015). These data imply that the increased proteoglycan and elastin content at the interfascicular matrix of the CCL may lead to an increase in elastic properties of this tissue. Nevertheless, the role and function of the interfascicular matrix in ligaments is yet to be estabilished.

In this study, another aim was to define the key structural protein expression differences between ligament (CCL) and tendon (LDET), described in Chapter 6. This revealed significantly abundant fibrocartilage proteins in CCL such as aggrecan, chondroadherin, versican and collagen type II, while LDET contained more asporin, and thrombospondin 4. The increase of fibrocartilage proteins in CCL suggests the presence of a cartilage-like matrix in this ligament. This interpretation is supported by the presence of rounded cell morphology and the increase of proteoglycan content in the CCL. This could be as response to compressive and shear forces generated during twisting of the CCL as the stifle moves through flexion to extension and during rotational movements (Young et al. 2002). The presence of the fibrocartilage proteins is expected to alter the mechanical properties of CCL by increasing its tensile strength and reducing laxity, which may be physiologically beneficial

for the canine stifle joint during running and could protect the CCL against mechanical fragmentation due to repetitive compression (Comerford et al. 2006).

The differential abundance of several proteins such as collagen type III, aggrecan, versican, fibronectin and cartilage oligomeric matrix proteins in CCL supported a proteomic study between human ACL and pattelar tendon (Little et al. 2014). However, the same study found that thrombospondin 4 and asporin protein levels were higher in the ACL, which is contradictionary with our data and may be due species differences. To validate the proteomic data, several significant abundant proteins were validated using immunohistochemistry staining, described in Chapter 4. Chapter 4 also describes the distribution and localisation of several ECM proteins, which were assessed between CCL and LDET mid-region through immunohistochemical staining techniques. Extracellular matrix proteins including collagen I, decorin and biglycan, elastin, fibrillin 1 and 2 were analysed using immunohistochemistry staining. The distribution of aggrecan, versican, asporin and collagen type III was also analysed, as these proteins were found to be differentially abundant through proteomic data analysis (Chapter 6). Immunostaining of keratocan was chosen to assess its distribution in ligament, as this protein was observed in in our proteomic results, but had only previously been reported in tendon (Rees et al. 2009). Collagen type VI was also identified through proteomic data analysis in both CCL and LDET. This non-fibrillar collagen has been demonstrated in pericellular matrix of human rotator cuff tendons (Thakkar et al. 2014). The immunostaining of collagen type VI in this present study was also found to be pericellular, but also located at the interfascicular matrix in both CCL and LDET. Collagen type VI has been found to interact with decorin and biglycan (Wiberg et al. 2001), suggesting that the presence of collagen type VI in this study may provide an anchoring network with other non-collagenous proteins at the interfascicular and pericellular matrix. Immunostaining results also revealed a different distribution and intensity of staining for collagen type III, aspirin, aggrecan and versican between CCL and LDET, which supports the findings from the proteomic analysis in Chapter 6. Together, the increased aggrecan and versican content, as well as the presence of other fibrocartilage proteins in CCL and the different distribution of ECM protein between the two tissue types, may relate to the different functioning of CCL and LDET. The increased immunostaining of aggrecan and versican at the CCL mid-region indicates that CCL might also undergo

compression at the central region where it is twisted around the caudal cruciate ligament under tensile strength (Comerford et al. 2006). Therefore, the CCL results in a different ECM composition and arrangement to protect the tissue from damage and to better withstand compression. One limitation of this part of the study was the lack of quantifiable data, which made the observations subjective. Further studies, should include quantitative software analysis (Tissue Gnostics) to assess the different distribution and intensity of staining. In addition, further validation of immunostaining between CCL and LDET are required for the differentially abundant proteins such as thrombospondin-4, collagen type II or chondroadherin.

To answer the second hypothesis in this project, this study also aimed to ascertain whether canine tendon/ligament cells were able to grow in 3D fibrin cultures and to assess whether 3D tendon and ligament constructs retained the biochemical, morphological and proteome characteristics of the original tissues. Chapter 5 described the formation of 3D tendon and ligament constructs using a 3D *in vitro* fibrin culture model. Constructs were found to have a resemblance of embryonic tendon or ligament, as they contained high cellular content and plasma membrane protrusions called “collagen fibripositors”, which were occasionally found in the extracellular space of both constructs. Biochemical analysis of 3D tendon and ligament constructs demonstrated that both constructs had a high DNA content and significantly lower collagen content compared to native tendon and ligament tissue. Interestingly, the constructs contained similar levels of sGAG and elastin as the native tissues, indicating that these components mature faster than collagen matrix. It would also be interesting to perform immunoshistochemical staining on the constructs to identify whether high sGAG content is due to the presence of small leucine rich proteoglycans or large aggregating proteins. In addition, it would also be interesting to verify to presence of blood-vessel like structures in both tendon and ligament constructs through immunoshistochemical staining.

Chapter 6 also involved the proteomic analysis of 3D tendon and ligament constructs in comparison to native tendon and ligament. This revealed the presence of several ECM proteins such as collagen I, III, V, VI and XII and XIV, decorin, bigylcan, asporin, osteoglycin, fibronectin 1 and fibrillin 1 in both 3D tendon and ligament constructs, which are also found in both native tendon and ligament. This indicated that 3D constructs are able to

recapitulate specific tendon and ligament ECM proteins. Quantitative comparison between 3D constructs and native tissues revealed that both native tissues were significantly abundant primarily in ECM proteins. In contrast, both 3D constructs had significantly more cellular associated proteins, which is most likely explained by their higher cellular content and indicate the immature state of the 3D constructs.

Further optimisation and maturation of 3D tendon and ligament constructs could promote tendon or ligament tissue engineering approaches or treatments for tendon and ligament injuries. The typical treatment of tendon or ligament injuries involves the use of tissue allografts or autografts to allow patients to rapidly return to ordinary activities of daily living. However, evidence suggests that these procedures do not substantially convalesce long-term patient outcomes (Breidenbach et al. 2014, Lohmander et al. 2004, Streich et al. 2011). The use of autografts in ACL reconstruction is known as the current golden standard, where the patellar tendon is the common source of the autograft (Ma et al., 2012). However, the patellar tendon has been shown to differ in viscoelastic properties, as the initial stiffness and strain to failure rate are dissimilar, leading to an increasing failure incidences (Danto et al. 2008, Chandrashekar et al. 2008, Ma et al. 2012). The use of fibrin scaffold could provide treatment for tendon and ligament injures, as fibrin gel can be produced of the patients blood and can be used as an autologous scaffold for the seeding fibroblasts to create a three-dimensional structure (Ye et al. 2000). However, studies have demonstrated that the 3D tendon and ligament fibrin constructs have not fully reached the mechanical and structural integrity of the native tendon or ligament tissue (Bayer et al. 2010, Herchenhan et al. 2013, Hagerty et al. 2012, Kalson et al. 2010) and would require further development to create the tendon or ligament-like structure possessing sufficient mechanical and structural properties. Herchenhan et al. (2013) demonstrated that 3D fibrin tendon constructs, which were derived from adult human tendon cells and cultured *in vitro* for 5 weeks had an increase in mechanical strength and stiffness and collagen fibril diameter. In this study both 3D tendon and ligament constructs were cultured for 14 days, which may have led to low collagen content as both were not allowed to mature completely. Therefore, future studies will also aim to identify the optimum culture period with regard to the collagen content and biomechanical properties. Another future aim will be to measure the effect of growth factors on maturation of both canine 3D tendon and

ligament constructs. Transforming growth factor beta 1 and insulin growth factor 1 have previously been shown to have an additive effect on 3D tendons collagen synthesis from human adult tenocytes (Herchenhan et al. 2014) and 3D ligament construct from adult ligamentocytes (Hagerty et al. 2012). By measuring ECM composition, proteomic analysis, biomechanical properties and ultrastructural analysis, together the optimum culture period time and the effect of growth factor could be measured on both 3D tendon and ligament constructs.

In the current study, proteomic comparison of 3D tendon and ligament constructs revealed no significant protein differences, which might indicate that the cell source may not be an important factor for tissue engineering. However, it is yet to be confirmed and could be part of future work to discover whether tendon and ligament fibroblasts may become phenotypically distinct due to different *in vivo* loading environments, or exposure to extrinsic factors such as growth factors. It would be interesting to measure the effect of compressive and tensile loading on both 3D tendon and ligament constructs. It would be important to identify whether tenocytes or ligamentocytes produce fibrocartilage proteins that were also found within native ligaments when subjected to compressive loading. Finally, It would be compelling to identify whether other cell sources such mesenchymal stem cells or skin dermal fibroblasts differentiate into ligament or tendon when situated in a ligament or tendon *in-vivo* environment. This could be important in clinical aspect of future tendon and ligament tissue engineering strategies.

7.2 CONCLUSION

The work detailed in this thesis demonstrates differences in ECM composition between intra-articular CCL, extra-articular MCL, LDET and SDFT, where significant differences in elastin and sGAG content were primarily detected in CCL. This study is the first to report a histological scoring system demonstrating semi-quantitative morphological and structural differences between CCL in comparison to the other tendon and ligament tissues examined. These morphological differences include les compact collagen architecture, difference in cell nuclei phenotype, and increased GAG and elastin content. The work also reports differences between tendon and ligament using proteomic profiling resulting in significantly more

fibrocartilaginous proteins in the ligament. The localisation of key structural protein differences were found to differ between the two tissue types. Furthermore, this thesis has also demonstrated the development of 3D tendon and ligament constructs and novel proteomic methodologies that quantified distinct differences between constructs and native tissues.

7.3 FUTURE WORK

Further studies of native tendon and ligament comparison:

- Identification of different cell population in CCL
- Measure effect of ageing and degeneration in CCL
- Semiquantitative measurements of immunohistochemical staining
- Further validation of key structural protein difference between CCL and LDET, such as collagen type II, thrombospondin 4

Further development of 3D tendon and ligament constructs:

- Immunostaining of large proteoglycans and small leucine rich proteoglycans
- Measurement of biomechanical properties of 3D tendon and ligament constructs
- Longer culture period of 3D tendon and ligament construct to allow maturation to take place
- Measure of the growth factor including transforming growth factor β1 and insulin-like growth factor
- Effect of compressive and tensile loading and how do the tendon and ligament cells respond the different mechanical loading

CHAPTER 8

SUPPLEMENTARY DATA

Table S1 Proteins in all tissues

Table S2 Differential expressed proteins between both native tissues and 3D tissues

Table S3 Differential expressed protein between native ligament and 3D ligament

Table S4 Differential expressed proteins between native tendon and 3D tendon

REFERENCES

Abraham, T., J. Carthy and B. McManus (2010). "Collagen matrix remodeling in 3-dimensional cellular space resolved using second harmonic generation and multiphoton excitation fluorescence." J Struct Biol **169**(1): 36-44.

Adams, D., D. Logerstedt, A. Hunter-Giordano, M. J. Axe and L. Snyder-Mackler (2012). "Current concepts for anterior cruciate ligament reconstruction: a criterion-based rehabilitation progression." journal of orthopaedic & sports physical therapy **42**(7): 601-614.

Adirim, T. A. and T. L. Cheng (2003). "Overview of injuries in the young athlete." Sports Medicine **33**(1): 75-81.

Aebersold, R. and M. Mann (2003). "Mass spectrometry-based proteomics." Nature **422**(6928): 198-207.

Ahmed, I., M. Lagopoulos, P. McConnell, R. Soames and G. Sefton (1998). "Blood supply of the Achilles tendon." Journal of orthopaedic research **16**(5): 591-596.

Ahmed, T. A., E. V. Dare and M. Hincke (2008). "Fibrin: a versatile scaffold for tissue engineering applications." Tissue Engineering Part B: Reviews **14**(2): 199-215.

Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts and P. Walter (2002). "The extracellular matrix of animals."

Alves, A., A. A. Stewart, J. Dudhia, Y. Kasashima, A. E. Goodship and R. K. Smith (2011). "Cell-based therapies for tendon and ligament injuries." Veterinary Clinics of North America: Equine Practice **27**(2): 315-333.

Ameye, L. and M. F. Young (2002). "Mice deficient in small leucine-rich proteoglycans: novel in vivo models for osteoporosis, osteoarthritis, Ehlers-Danlos syndrome, muscular dystrophy, and corneal diseases." Glycobiology **12**(9): 107R-116R.

Amiel, D., C. R. Chu and J. Lee (1995). Chapter 15. "Effect of loading on metabolism and repair of tendons and ligaments." Repetitive Motion Disorders of the Upper Extremity: 217-230. American Academy of Orthopaedic Surgeons: USA

Amiel, D., C. Frank, F. Harwood, J. Fronek and W. Akeson (1984). "Tendons and ligaments: a morphological and biochemical comparison." J Orthop Res **1**(3): 257-265.

Amiel, D., C. B. Frank, F. L. Harwood, W. H. Akeson and J. B. Kleiner (1987). "Collagen alteration in medial collateral ligament healing in a rabbit model." Connect Tissue Res **16**(4): 357-366.

Amis, A. A. (1998). Chapter 15. "Biomechanics of bone, tendon and ligament". Sciences Basic to Orthopaedics: 222-239. Philadelphia: W.B. Saunders Company Ltd, In: Hughes SPF, McCarthy ID.

Amis, A. A., C. M. Gupte, A. M. Bull and A. Edwards (2006). "Anatomy of the posterior cruciate ligament and the meniscofemoral ligaments." Knee Surg Sports Traumatol Arthrosc **14**(3): 257-263.

An KN, Sun YL, Luo ZP (2004). "Flexibility of type I collagen and mechanical property of connective tissue". Biorheology **41**:239–246

Arnoczky, S. P. (1983). "Anatomy of the anterior cruciate ligament." Clin Orthop Relat Res 172 (1): 19-25.

Arnoczky, S. P. (1985). "Blood supply to the anterior cruciate ligament and supporting structures." Orthop Clin North Am **16**(1): 15-28.

Arnoczky, S. P. and J. L. Marshall (1977). "The cruciate ligaments of the canine stifle: an anatomical and functional analysis." Am J Vet Res **38**(11): 1807-1814.

Arnoczky, S. P., R. M. Rubin and J. L. Marshall (1979). "Microvasculature of the cruciate ligaments and its response to injury. An experimental study in dogs." J Bone Joint Surg Am **61**(8): 1221-1229.

Astrom, M. and A. Rausing (1995). "Chronic Achilles tendinopathy. A survey of surgical and histopathologic findings." Clin Orthop Relat Res **316**: 151-164.

Aughey, E. and F. L. Frye (2001). Chapter three. "Connective tissues" Comparative veterinary histology with clinical correlates: 31-49. Mansons Publishing: UK.

Baccarani-Contri, M., D. Vincenzi, F. Cicchetti, G. Mori and I. Pasquali-Ronchetti (1990). "Immunocytochemical localization of proteoglycans within normal elastin fibers." European journal of cell biology **53**(2): 305-312.

Bali, J. P., H. Cousse and E. Neuzil (2001). "Biochemical basis of the pharmacologic action of chondroitin sulfates on the osteoarticular system." Semin Arthritis Rheum **31**(1): 58-68.

Bancroft, J., A. Stevens and D. Turner (1996). "Theory and practice of histological techniques, Churchill Livingstone, New York." the text: 766.

Banos, C. C., A. H. Thomas and C. K. Kuo (2008). "Collagen fibrillogenesis in tendon development: current models and regulation of fibril assembly." Birth Defects Res C Embryo Today **84**(3): 228-244.

Bantscheff, M., S. Lemeer, M. M. Savitski and B. Kuster (2012). "Quantitative mass spectrometry in proteomics: critical review update from 2007 to the present." Anal Bioanal Chem **404**(4): 939-965.

Bantscheff, M., M. Schirle, G. Sweetman, J. Rick and B. Kuster (2007). "Quantitative mass spectrometry in proteomics: a critical review." Analytical and bioanalytical chemistry **389**(4): 1017-1031.

Barrett, J., P. M. Brophy and J. V. Hamilton (2005). "Analysing proteomic data." Int J Parasitol **35**(5): 543-553.

Bayer, M. L., C. Y. Yeung, K. E. Kadler, K. Qvortrup, K. Baar, R. B. Svensson, S. P. Magnusson, M. Krogsgaard, M. Koch and M. Kjaer (2010). "The initiation of embryonic-like collagen fibrillogenesis by adult human tendon fibroblasts when cultured under tension." Biomaterials **31**(18): 4889-4897.

Benjamin, M., E. J. Evans and L. Copp (1986). "The histology of tendon attachments to bone in man." J Anat **149**: 89-100.

Benjamin, M., E. Kaiser and S. Milz (2008). "Structure-function relationships in tendons: a review." J Anat **212**(3): 211-228.

Benjamin, M., T. Kumai, S. Milz, B. M. Boszczyk, A. A. Boszczyk and J. R. Ralphs (2002). "The skeletal attachment of tendons--tendon "entheses"." Comp Biochem Physiol A Mol Integr Physiol **133**(4): 931-945.

Benjamin, M., S. Qin and J. R. Ralphs (1995). "Fibrocartilage associated with human tendons and their pulleys." J Anat **187 (Pt 3)**: 625-633.

Benjamin, M. and J. R. Ralphs (1997). "Tendons and ligaments--an overview." Histol Histopathol **12**(4): 1135-1144.

Benjamin, M. and J. R. Ralphs (1998). "Fibrocartilage in tendons and ligaments—an adaptation to compressive load." J Anat **193**(4): 481-494.

Bergman, I. and R. Loxley (1963). "Two improved and simplified methods for the spectrophotometric determination of hydroxyproline." Analytical Chemistry **35**(12): 1961-1965.

Beynnon, B. D., R. J. Johnson, B. C. Fleming, P. Kannus, M. Kaplan, J. Samani and P. Renstrï (2002). "Anterior cruciate ligament replacement: Comparison of bone-patellar tendon-bone grafts with two-strand hamstring grafts a prospective, randomized study." The Journal of Bone & Joint Surgery **84**(9): 1503-1513.

Bi, Y., D. Ehirchiou, T. M. Kilts, C. A. Inkson, M. C. Embree, W. Sonoyama, L. Li, A. I. Leet, B. M. Seo, L. Zhang, S. Shi and M. F. Young (2007). "Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche." Nat Med **13**(10): 1219-1227.

Birch, H. L. (2007). "Tendon matrix composition and turnover in relation to functional requirements." Int J Exp Pathol **88**(4): 241-248.

Birch, H. L., C. T. Thorpe and A. P. Rumian (2013). "Specialisation of extracellular matrix for function in tendons and ligaments." Muscles Ligaments Tendons J **3**(1): 12-22.

Birch, H. L., S. Worboys, S. Eissa, B. Jackson, S. Strassburg and P. D. Clegg (2008). "Matrix metabolism rate differs in functionally distinct tendons." Matrix Biol **27**(3): 182-189.

Bouton, M.-C., Y. Boulaftali, B. Richard, V. Arocas, J.-B. Michel and M. Jandrot-Perrus (2012). "Emerging role of serpinE2/protease nexin-1 in hemostasis and vascular biology." Blood **119**(11): 2452-2457.

Bray, R., A. Fisher and C. Frank (1990). "Fine vascular anatomy of adult rabbit knee ligaments." Journal of anatomy **172**: 69-79.

Bray, R. C., C. A. Leonard and P. T. Salo (2003). "Correlation of healing capacity with vascular response in the anterior cruciate and medial collateral ligaments of the rabbit." Journal of orthopaedic research **21**(6): 1118-1123.

Bray, R. C., R. M. Rangayyan and C. B. Frank (1996). "Normal and healing ligament vascularity: a quantitative histological assessment in the adult rabbit medial collateral ligament." J Anat **188 (Pt 1)**: 87-95.

Breidenbach, A. P., N. A. Dyment, Y. Lu, M. Rao, J. T. Shearn, D. W. Rowe, K. E. Kadler and D. L. Butler (2014). "Fibrin Gels Exhibit Improved Biological, Structural, and Mechanical Properties Compared with Collagen Gels in Cell-Based Tendon Tissue-Engineered Constructs." Tissue Eng Part A. **21** (3-4): 438-450

Brown, J. P., R. M. Lind, A. F. Burzesi and C. K. Kuo (2012). "Elastogenic protein expression of a highly elastic murine spinal ligament: the ligamentum flavum." PloS one **7**(6): e38475.

Bruehlmann, S. B., J. B Rattner, J. R Matyas and N. A Duncan (2002). "Regional variations in the cellular matrix of the annulus fibrosus of the intervertebral disc." J Anat **201**(2): 159-171.

Burt, C. W. and M. D. Overpeck (2001). "Emergency visits for sports-related injuries." Annals of emergency medicine **37**(3): 301-308.

Butler, D. L., E. S. Grood, F. R. Noyes and R. F. Zernicke (1978). "Biomechanics of ligaments and tendons." Exerc Sport Sci Rev **6**: 125-181.

Cain, S. A., A. Morgan, M. J. Sherratt, S. G. Ball, C. A. Shuttleworth and C. M. Kielty (2006). "Proteomic analysis of fibrillin-rich microfibrils." Proteomics **6**(1): 111-122.

Calmbach, W. L. and M. Hutchens (2003). "Evaluation of patients presenting with knee pain: Part I. History, physical examination, radiographs, and laboratory tests." Am Fam Physician **68**(5): 907-912.

Calve, S., R. G. Dennis, P. E. Kosnik, K. Baar, K. Grosh and E. M. Arruda (2004). "Engineering of functional tendon." Tissue engineering **10**(5-6): 755-761.

Campbell, M. A., A. M. Tester, C. J. Handley, G. J. Checkley, G. L. Chow, A. E. Cant, A. D. Winter and W. E. Cain (1996). "Characterization of a large chondroitin sulfate proteoglycan present in bovine collateral ligament." Arch Biochem Biophys **329**(2): 181-190.

Chandrashekar, N., Hashemi, J., Slauterbeck, J., and Beynnon, B.D. (2008). "Low-load behavior of the patellar tendon graft and its relevance to the biomechanics of the reconstructed knee". Clin Biomech **23**: 918- 925.

Canty-Laird, E., G, Y. Lu and K. E. Kadler (2012). "Stepwise proteolytic activation of type I procollagen to collagen within the secretory pathway of tendon fibroblasts in situ." Biochemical Journal **441**(2): 707-717.

Canty, E. G. and K. E. Kadler (2005). "Procollagen trafficking, processing and fibrillogenesis." J Cell Sci **118**(Pt 7): 1341-1353.

Canty, E. G., Y. Lu, R. S. Meadows, M. K. Shaw, D. F. Holmes and K. E. Kadler (2004). "Coalignment of plasma membrane channels and protrusions (fibripositors) specifies the parallelism of tendon." J Cell Biol **165**(4): 553-563.

Canty, E. G., T. Starborg, Y. Lu, S. M. Humphries, D. F. Holmes, R. S. Meadows, A. Huffman, E. T. O'Toole and K. E. Kadler (2006). "Actin filaments are required for fibripositor-mediated collagen fibril alignment in tendon." Journal of Biological Chemistry **281**(50): 38592-38598.

Carlson, E. C., C.-Y. Liu, T.-i. Chikama, Y. Hayashi, C. W.-C. Kao, D. E. Birk, J. L. Funderburgh, J. V. Jester and W. W.-Y. Kao (2005). "Keratocan, a cornea-specific keratan sulfate proteoglycan, is regulated by lumican." Journal of Biological Chemistry **280**(27): 25541-25547.

Carr, A. J. and S. H. Norris (1989). "The blood supply of the calcaneal tendon." J Bone Joint Surg Br **71**(1): 100-101.

Carta, L., L. Pereira, E. Arteaga-Solis, S. Y. Lee-Arteaga, B. Lenart, B. Starcher, C. A. Merkel, M. Sukoyan, A. Kerkis and N. Hazeki (2006). "Fibrillins 1 and 2 perform partially overlapping functions during aortic development." Journal of Biological Chemistry **281**(12): 8016-8023.

Chakravarti, S. (2002). "Functions of lumican and fibromodulin: lessons from knockout mice." Glycoconj J **19**(4-5): 287-293.

Chaplin, D. M. and T. K. Greenlee (1975). "The development of human digital tendons." Journal of anatomy **120**(Pt 2): 253-274.

Charbonneau, N. L., B. J. Dzamba, R. N. Ono, D. R. Keene, G. M. Corson, D. P. Reinhardt and L. Y. Sakai (2003). "Fibrillins can co-assemble in fibrils, but fibrillin fibril composition displays cell-specific differences." Journal of Biological Chemistry **278**(4): 2740-2749.

Charbonneau, N. L., R. N. Ono, G. M. Corson, D. R. Keene and L. Y. Sakai (2004). "Fine tuning of growth factor signals depends on fibrillin microfibril networks." Birth Defects Research Part C: Embryo Today: Reviews **72**(1): 37-50.

Chen, S. and D. E. Birk (2013). "The regulatory roles of small leucine-rich proteoglycans in extracellular matrix assembly." FEBS J **280**(10): 2120-2137.

Chen, S. J., H. Ning, W. Ishida, S. Sodin-Semrl, S. Takagawa, Y. Mori and J. Varga (2006). "The early-immediate gene EGR-1 is induced by transforming growth factor-beta and mediates stimulation of collagen gene expression." J Biol Chem **281**(30): 21183-21197.

Chowdhury, P., J. R. Matyas and C. B. Frank (1991). "The "epiligament" of the rabbit medial collateral ligament: a quantitative morphological study." Connect Tissue Res **27**(1): 33-50.

Chun, J., T. L. Tuan, B. Han, C. T. Vangsness and M. E. Nimni (2003). "Cultures of ligament fibroblasts in fibrin matrix gel." Connect Tissue Res **44**(2): 81-87.

Cimino, F., B. S. Volk and D. Setter (2010). "Anterior cruciate ligament injury: diagnosis, management, and prevention." Am Fam Physician **82**(8): 917-922.

Clark, J. M. and J. A. Sidles (1990). "The interrelation of fiber bundles in the anterior cruciate ligament." J Orthop Res **8**(2): 180-188.

Clegg, P. (2012). "Musculoskeletal disease and injury, now and in the future. Part 2: Tendon and ligament injuries." Equine veterinary journal **44**(3): 371-375.

Clegg, P. D., S. Strassburg and R. K. Smith (2007). "Cell phenotypic variation in normal and damaged tendons." Int J Exp Pathol **88**(4): 227-235.

Comerford, E. J., J. F. Innes, J. F. Tarlton and A. J. Bailey (2004). "Investigation of the composition, turnover, and thermal properties of ruptured cranial cruciate ligaments of dogs." Am J Vet Res **65**(8): 1136-1141.

Comerford, E. J., K. Smith and K. Hayashi (2011). "Update on the aetiopathogenesis of canine cranial cruciate ligament disease." Vet Comp Orthop Traumatol **24**(2): 91-98.

Comerford, E. J., J. F. Tarlton, J. F. Innes, K. A. Johnson, A. A. Amis and A. J. Bailey (2005). "Metabolism and composition of the canine anterior cruciate ligament relate to differences in knee joint mechanics and predisposition to ligament rupture." J Orthop Res **23**(1): 61-66.

Comerford, E. J., J. F. Tarlton, A. Wales, A. J. Bailey and J. F. Innes (2006). "Ultrastructural differences in cranial cruciate ligaments from dogs of two breeds with a differing predisposition to ligament degeneration and rupture." J Comp Pathol **134**(1): 8-16.

Cook, J. L., K. Kuroki, D. Visco, J. P. Pelletier, L. Schulz and F. P. Lafeber (2010). "The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the dog." Osteoarthritis Cartilage **18 Suppl 3**: S66-79.

Connizzo, B.K., Yannascoli, S.M., Soslowsky. L.J. (2013). "Structure–function relationships of postnatal tendon development: A parallel to healing". Matrix Biology **32** (2):106-116

Crabos, M., T. Yamakado, C. Heizmann, N. Cerletti, F. Bühler and P. Erne (1991). "The calcium binding protein tropomyosin in human platelets and cardiac tissue: elevation in hypertensive cardiac hypertrophy." European journal of clinical investigation **21**(5): 472-478.

Daniel, D. M., M. L. Stone, B. E. Dobson, D. C. Fithian, D. J. Rossman and K. R. Kaufman (1994). "Fate of the ACL-injured patient a prospective outcome study." The American journal of sports medicine **22**(5): 632-644.

Daniel, J. C. and D. K. Mills (1988). "Proteoglycan synthesis by cells cultured from regions of the rabbit flexor tendon." Connect Tissue Res **17**(3): 215-230.

Danielson, K. G., H. Baribault, D. F. Holmes, H. Graham, K. E. Kadler and R. V. Iozzo (1997). "Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility." The Journal of cell biology **136**(3): 729-743.

Danto, M.I. and Woo, S.L.-Y. (1993). "The mechanical properties of skeletally mature rabbit anterior cruciate ligament and pa- tellar tendon over a range of strain rates". J Orthop Surg **11**: 58-67.

Davis, E. C. (1993). "Stability of elastin in the developing mouse aorta: a quantitative radioautographic study." Histochemistry **100**(1): 17-26.

de Hoog, C. L., and M.Man (2004). "Proteomics." Annual Review of Genomics and Human Genetics **5**: 267-293.

Docking, S., T. Samiric, E. Scase, C. Purdam and J. Cook (2013). "Relationship between compressive loading and ECM changes in tendons." Muscles Ligaments Tendons J **3**(1): 7-11.

Dodds, J. A. and S. P. Arnoczky (1994). "Anatomy of the anterior cruciate ligament: a blueprint for repair and reconstruction." Arthroscopy **10**(2): 132-139.

Domon, B. and R. Aebersold (2006). "Mass spectrometry and protein analysis." science **312**(5771): 212-217.

Doschak, M. R. and R. F. Zernicke (2005). "Structure, function and adaptation of bone-tendon and bone-ligament complexes." J Musculoskelet Neuronal Interact **5**(1): 35-40.

Duance, V. C., D. J. Restall, H. Beard, F. J. Bourne and A. J. Bailey (1977). "The location of three collagen types in skeletal muscle." FEBS Lett **79**(2): 248-252.

Duthon, V. B., C. Barea, S. Abrassart, J. H. Fasel, D. Fritschy and J. Menetrey (2006). "Anatomy of the anterior cruciate ligament." Knee Surg Sports Traumatol Arthrosc **14**(3): 204-213.

Eriksen, T. A., D. M. Wright, P. P. Purslow and V. C. Duance (2001). "Role of Ca2+ for the mechanical properties of fibrillin." Proteins: Structure, Function, and Bioinformatics **45**(1): 90-95.

Evans, H. E., G. C. Christensen and A. De La Hunta (1979). "The Skeletal and Muscular Systems". MILLER'S Guide to the Disection of the Dog. 2nd-Ed: 59-89. WB-Saunders: Philadelphia

Ezura, Y., S. Chakravarti, Å. Oldberg, I. Chervoneva and D. E. Birk (2000). "Differential expression of lumican and fibromodulin regulate collagen fibrillogenesis in developing mouse tendons." The Journal of cell biology **151**(4): 779-788.

Farndale, R. W., D. J. Buttle and A. J. Barrett (1986). "Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue." Biochim Biophys Acta **883**(2): 173-177.

Feng, X. H. and R. Derynck (2005). "Specificity and versatility in tgf-beta signaling through Smads." Annu Rev Cell Dev Biol **21**: 659-693.

Fenwick, S. A., B. L. Hazleman and G. P. Riley (2002). "The vasculature and its role in the damaged and healing tendon." Arthritis Res **4**(4): 252-260.

Foos, M. J., J. R. Hickox, P. G. Mansour, J. R. Slauterbeck and D. M. Hardy (2001). "Expression of matrix metalloprotease and tissue inhibitor of metalloprotease genes in human anterior cruciate ligament." J Orthop Res **19**(4): 642-649.

Franchi, M., V. De Pasquale, D. Martini, M. Quaranta, M. Macciocca, A. Dionisi and V. Ottani (2010). "Contribution of glycosaminoglycans to the microstructural integrity of fibrillar and fiber crimps in tendons and ligaments." ScientificWorldJournal **10**: 1932-1940.

Franchi, M., M. Quaranta, M. Macciocca, V. De Pasquale, V. Ottani and A. Ruggeri (2009). "Structure relates to elastic recoil and functional role in quadriceps tendon and patellar ligament." Micron **40**(3): 370-377.

Franchi, M., M. Quaranta, M. Macciocca, L. Leonardi, V. Ottani, P. Bianchini, A. Diaspro and A. Ruggeri (2010). "Collagen fibre arrangement and functional crimping pattern of the medial collateral ligament in the rat knee." Knee Surg Sports Traumatol Arthrosc **18**(12): 1671-1678.

Franchi, M., A. Trirè, M. Quaranta, E. Orsini and V. Ottani (2007). "Collagen structure of tendon relates to function." ScientificWorldJournal **7**: 404-420.

Frank, C., D. Amiel, S. Woo and W. Akeson (1985). "Normal ligament properties and ligament healing." Clin Orthop Relat Res **196**: 15-25.

Frank, C., D. McDonald, D. Bray, R. Bray, R. Rangayyan, D. Chimich and N. Shrive (1992). "Collagen fibril diameters in the healing adult rabbit medial collateral ligament." Connect Tissue Res **27**(4): 251-263.

Frank, C., D. McDonald, J. Wilson, D. Eyre and N. Shrive (1995). "Rabbit medial collateral ligament scar weakness is associated with decreased collagen pyridinoline crosslink density." J Orthop Res **13**(2): 157-165.

Frank, C. B. (2004). "Ligament structure, physiology and function." J Musculoskelet Neuronal Interact **4**(2): 199-201.

Frantz, C., K. M. Stewart and V. M. Weaver (2010). "The extracellular matrix at a glance." J Cell Sci **123**(Pt 24): 4195-4200.

Freed, L. E., G. Vunjak-Novakovic, R. J. Biron, D. B. Eagles, D. C. Lesnoy, S. K. Barlow and R. Langer (1994). "Biodegradable polymer scaffolds for tissue engineering." Nature Biotechnology **12**(7): 689-693.

Frolova, E. G., J. Drazba, I. Krukovets, V. Kostenko, L. Blech, C. Harry, A. Vasanji, C. Drumm, P. Sul and G. J. Jenniskens (2014). "Control of organization and function of muscle and tendon by thrombospondin-4." Matrix Biology **37**:35-48.

Fujii, K., T. Yamagishi, T. Nagafuchi, M. Tsuji and Y. Kuboki (1994). "Biochemical properties of collagen from ligaments and periarticular tendons of the human knee." Knee Surg Sports Traumatol Arthrosc **2**(4): 229-233.

Funakoshi, Y., M. Hariu, J. E. Tapper, L. L. Marchuk, N. G. Shrive, F. Kanaya, J. B. Rattner, D. A. Hart and C. B. Frank (2007). "Periarticular ligament changes following ACL/MCL transection in an ovine stifle joint model of osteoarthritis." Journal of orthopaedic research **25**(8): 997-1006.

Geiger, M. H., M. H. Green, A. Monosov, W. H. Akeson and D. Amiel (1994). "An in vitro assay of anterior cruciate ligament (ACL) and medial collateral ligament (MCL) cell migration." Connect Tissue Res **30**(3): 215-224.

Gerke, V. and S. E. Moss (2002). "Annexins: from structure to function." Physiological reviews **82**(2): 331-371.

Gianotti, S. M., S. W. Marshall, P. A. Hume and L. Bunt (2009). "Incidence of anterior cruciate ligament injury and other knee ligament injuries: a national population-based study." J Sci Med Sport **12**(6): 622-627.

Gibson, M. A., M. L. Finnis, J. S. Kumaratilake and E. G. Cleary (1998). "Microfibril-associated glycoprotein-2 (MAGP-2) is specifically associated with fibrillin-containing microfibrils but exhibits more restricted patterns of tissue localization and developmental expression than its structural relative MAGP-1." Journal of Histochemistry & Cytochemistry **46**(8): 871-885.

Goh, J. C., H. W. Ouyang, S. H. Teoh, C. K. Chan and E. H. Lee (2003). "Tissue-engineering approach to the repair and regeneration of tendons and ligaments." Tissue Eng **9 Suppl 1**: S31-44.

Gorg, A., C. Obermaier, G. Boguth, A. Harder, B. Scheibe, R. Wildgruber and W. Weiss (2000). "The current state of two-dimensional electrophoresis with immobilized pH gradients." Electrophoresis **21**(6): 1037-1053.

Gosling, J. A., P. F. Harris, J. R. Humpherson, I. Whitmore and P. L. Willan (2008). Chapter Six:"Lower Limb". Human Anatomy, Color Atlas and Textbook: Color Atlas and Textbook, Elsevier Health Sciences : 254-313

Grant, T. M., M. S. Thompson, J. Urban and J. Yu (2013). "Elastic fibres are broadly distributed in tendon and highly localized around tenocytes." Journal of anatomy **222**(6): 573-579.

Grassl, E., T. Oegema and R. Tranquillo (2002). "Fibrin as an alternative biopolymer to type-I collagen for the fabrication of a media equivalent." Journal of biomedical materials research **60**(4): 607-612.

Gregory, M. H., N. Capito, K. Kuroki, A. M. Stoker, J. L. Cook and S. L. Sherman (2012). "A review of translational animal models for knee osteoarthritis." Arthritis **2012**: 1-14.

Gruber, H., J. Ingram and E. Hanley Jr (2006). "Cellular immunohistochemical localization of the matricellular protein myocilin in the intervertebral disc." Biotechnic & Histochemistry **81**(4-6): 119-124.

Gygi, S. P., G. L. Corthals, Y. Zhang, Y. Rochon and R. Aebersold (2000). "Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology." Proc Natl Acad Sci U S A **97**(17): 9390-9395.

Hagerty, P., A. Lee, S. Calve, C. A. Lee, M. Vidal and K. Baar (2012). "The effect of growth factors on both collagen synthesis and tensile strength of engineered human ligaments." Biomaterials **33**(27): 6355-6361.

Halper, J. (2014). "Proteoglycans and diseases of soft tissues." Adv Exp Med Biol **802**: 49-58.

Han, X., L. He, L. Xin, B. Shan and B. Ma (2011). "PeaksPTM: Mass spectrometry-based identification of peptides with unspecified modifications." Journal of proteome research **10**(7): 2930-2936.

Hanada, M., M. Takahashi, D. Suzuki, M. Abe and Y. Matsuyama (2014). "A biochemical study of the distribution of collagen and its crosslinks in knee ligaments and the patellar tendon." Connect Tissue Res **55**(5-6): 378-383.

Hardingham, T. and A. Fosang (1992). "Proteoglycans: many forms and many functions." The FASEB Journal **6**(3): 861-870.

Harmon, K. G. and M. L. Ireland (2000). "Gender differences in noncontact anterior cruciate ligament injuries." Clin Sports Med **19**(2): 287-302.

Harner CD, Xerogeanes JW, Livesay GA, Carlin GJ, Smith BA, Kusayama T, Kashiwaguchi S, Woo SL (1995). "The human posterior cruciate ligament complex: an interdisciplinary study. Ligament morphology and biomechanical evaluation". Am J Sports Med **23**(6):736–745

Hart, R. A., S. L. Woo and P. O. Newton (1992). "Ultrastructural morphometry of anterior cruciate and medial collateral ligaments: an experimental study in rabbits." J Orthop Res **10**(1): 96-103.

Hasegawa, A., H. Nakahara, M. Kinoshita, H. Asahara, J. Koziol and M. K. Lotz (2013). "Cellular and extracellular matrix changes in anterior cruciate ligaments during human knee aging and osteoarthritis." Arthritis Res Ther **15**(1): R29.

Hasegawa, A., S. Otsuki, C. Pauli, S. Miyaki, S. Patil, N. Steklov, M. Kinoshita, J. Koziol, D. D. D'Lima and M. K. Lotz (2012). "Anterior cruciate ligament changes in the human knee joint in aging and osteoarthritis." Arthritis Rheum **64**(3): 696-704.

Hasegawa, A., S. Otsuki, C. Pauli, S. Miyaki, S. Patil, N. Steklov, M. Kinoshita, J. Koziol, D. D. D'Lima and M. K. Lotz (2012). "Anterior cruciate ligament changes in the human knee joint in aging and osteoarthritis." Arthritis & Rheumatism **64**(3): 696-704.

Hauptmann, R., I. Maurer-Fogy, E. Krystek, G. Bodo, H. Andree and C. P. Reutelingsperger (1989). "Vascular anticoagulant beta: a novel human Ca2+/phospholipid binding protein that inhibits coagulation and phospholipase A2 activity. Its molecular cloning, expression and comparison with VAC-alpha." Eur J Biochem **185**(1): 63-71.

Hayashi, K., J. Bhandal, S. Y. Kim, C. O. Rodriguez, Jr., R. Entwistle, D. Naydan, A. Kapatkin and S. M. Stover (2011). "Immunohistochemical and histomorphometric evaluation of vascular distribution in intact canine cranial cruciate ligament." Vet Surg **40**(2): 192-197.

Hecker, L., K. Baar, R. G. Dennis and K. N. Bitar (2005). "Development of a three-dimensional physiological model of the internal anal sphincter bioengineered in vitro from isolated smooth muscle cells." American Journal of Physiology-Gastrointestinal and Liver Physiology **289**(2): G188-G196.

Hedbom, E. and D. Heinegard (1993). "Binding of fibromodulin and decorin to separate sites on fibrillar collagens." J Biol Chem **268**(36): 27307-27312.

Heinegard, D. (2009). "Proteoglycans and more--from molecules to biology." Int J Exp Pathol **90**(6): 575-586.

Henninger, H.B., Underwood, C.J., Romney, S.J., Davis, G.L., Weiss, J.A., (2013). "Effect of elastin digestion on the quasi-static tensile response of medial collateral ligament". Journal of Orthopaedic Research **31**: 1226–1233.

Henninger, H.B., Valdez, W.R., Scott, S.A., Weiss, J.A. (2015). "Elastin governs the mechanical response of medial collateral ligament under shear and transverse tensile loading". Acta Biomaterialia. **25**: 304-312

Herchenhan, A., M. L. Bayer, P. Eliasson, S. P. Magnusson and M. Kjaer (2014). "Insulin-like growth factor I enhances collagen synthesis in engineered human tendon tissue." Growth Horm IGF Res. **25**(1): 13-19

Herchenhan, A., M. L. Bayer, R. B. Svensson, S. P. Magnusson and M. Kjær (2013). "In vitro tendon tissue development from human fibroblasts demonstrates collagen fibril diameter growth associated with a rise in mechanical strength." Developmental Dynamics **242**(1): 2-8.

Herchenhan, A., N. S. Kalson, D. F. Holmes, P. Hill, K. E. Kadler and L. Margetts (2012). "Tenocyte contraction induces crimp formation in tendon-like tissue." Biomechanics and modeling in mechanobiology **11**(3-4): 449-459.

Hessle, L., G. A. Stordalen, C. Wenglén, C. Petzold, E. K. Tanner, S.-H. Brorson, E. S. Baekkevold, P. Önnerfjord, F. P. Reinholt and D. Heinegård (2013). "The skeletal phenotype of chondroadherin deficient mice." PloS one **8**(6): e63080.

Hildebrand, A., M. Romaris, L. M. Rasmussen, D. Heinegard, D. R. Twardzik, W. A. Border and E. Ruoslahti (1994). "Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta." Biochem J **302 (Pt 2)**: 527-534.

Hirzinger, C., M. Tauber, S. Korntner, M. Quirchmayr, H. C. Bauer, A. Traweger and H. Tempfer (2014). "ACL injuries and stem cell therapy." Arch Orthop Trauma Surg **134**(11): 1573-1578.

Hodgkinson, C. P., V. Naidoo, K. G. Patti, J. A. Gomez, J. Schmeckpeper, Z. Zhang, B. Davis, R. E. Pratt, M. Mirotsou and V. J. Dzau (2013). "Abi3bp Is a Multifunctional Autocrine/Paracrine Factor that Regulates Mesenchymal Stem Cell Biology." Stem Cells **31**(8): 1669-1682.

Hoffmann, A. and G. Gross (2006). "Tendon and ligament engineering: from cell biology to in vivo application." Regen Med **1**(4): 563-574.

Hoffmann, A. and G. Gross (2007). "Tendon and ligament engineering in the adult organism: mesenchymal stem cells and gene-therapeutic approaches." Int Orthop **31**(6): 791-797.

Hu, L.-L., S. Niu, T. Huang, K. Wang, X.-H. Shi and Y.-D. Cai (2010). "Prediction and analysis of protein hydroxyproline and hydroxylysine." PloS one **5**(12): e15917.

Huang, Y.-C., R. G. Dennis, L. Larkin and K. Baar (2005). "Rapid formation of functional muscle in vitro using fibrin gels." Journal of Applied Physiology **98**(2): 706-713.

Huang, Y., S. H. Laval, A. van Remoortere, J. Baudier, C. Benaud, L. V. Anderson, V. Straub, A. Deelder, R. R. Frants and J. T. den Dunnen (2007). "AHNAK, a novel component of the dysferlin protein complex, redistributes to the cytoplasm with dysferlin during skeletal muscle regeneration." The FASEB Journal **21**(3): 732-742.

Huisman, E. S., G. Andersson, A. Scott, C. R. Reno, D. A. Hart and G. M. Thornton (2014). "Regional molecular and cellular differences in the female rabbit Achilles tendon complex: potential implications for understanding responses to loading." J Anat **224**(5): 538-547.

Hynes, R. O. and A. Naba (2012). "Overview of the matrisome—an inventory of extracellular matrix constituents and functions." Cold Spring Harbor perspectives in biology **4**(1): a004903.

Ikegawa, S. (2008). "Expression, regulation and function of asporin, a susceptibility gene in common bone and joint diseases." Curr Med Chem **15**(7): 724-728.

Ilic, M. Z., P. Carter, A. Tyndall, J. Dudhia and C. J. Handley (2005). "Proteoglycans and catabolic products of proteoglycans present in ligament." Biochem J **385**(Pt 2): 381-388.

Iozzo, R. V., S. Goldoni, A. D. Berendsen and M. F. Young (2011). Small leucine-rich proteoglycans. The Extracellular Matrix: an Overview, Springer**:** 197-231.

Ireland, D., R. Harrall, V. Curry, G. Holloway, R. Hackney, B. Hazleman and G. Riley (2001). "Multiple changes in gene expression in chronic human Achilles tendinopathy." Matrix Biology **20**(3): 159-169.

Isayama, N., G. Matsumura, H. Sato, S. Matsuda and K. Yamazaki (2014). "Histological maturation of vascular smooth muscle cells in in situ tissue-engineered vasculature." Biomaterials **35**(11): 3589-3595.

Ishihama, Y., Y. Oda, T. Tabata, T. Sato, T. Nagasu, J. Rappsilber and M. Mann (2005). "Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein." Mol Cell Proteomics **4**(9): 1265-1272.

Ito, S., S. Ishimaru and S. E. Wilson (1997). "Inhibitory effect of type 1 collagen gel containing α-elastin on proliferation and migration of vascular smooth muscle and endothelial cells." Cardiovascular surgery **5**(2): 176-183.

Izu, Y., H. L. Ansorge, G. Zhang, L. J. Soslowsky, P. Bonaldo, M. L. Chu and D. E. Birk (2011). "Dysfunctional tendon collagen fibrillogenesis in collagen VI null mice." Matrix Biol **30**(1): 53-61.

James, R., G. Kesturu, G. Balian and A. B. Chhabra (2008). "Tendon: biology, biomechanics, repair, growth factors, and evolving treatment options." J Hand Surg Am **33**(1): 102-112.

John, D. and A. S. Bancroft (1996). Chapter 10. "Carbohydrates". Theory and practice of histological techniques: 192-194. Churchill Livingstone: Uk.

Johnson, M. T. (2009). "Proteomics of tendinopathy." Front Biosci (Landmark Ed) **14**: 1505-1515.

Jones, G. C., A. N. Corps, C. J. Pennington, I. M. Clark, D. R. Edwards, M. M. Bradley, B. L. Hazleman and G. P. Riley (2006). "Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerate human achilles tendon." Arthritis Rheum **54**(3): 832-842.

Jones, S. E. and C. Jomary (2002). "Clusterin." The international journal of biochemistry & cell biology **34**(5): 427-431.

Kadler, K. E., C. Baldock, J. Bella and R. P. Boot-Handford (2007). "Collagens at a glance." J Cell Sci **120**(Pt 12): 1955-1958.

Kadler, K. E., D. F. Holmes, J. A. Trotter and J. A. Chapman (1996). "Collagen fibril formation." Biochem J **316 (Pt 1)**: 1-11.

Kalamajski, S., A. Aspberg, K. Lindblom and D. Heinegard (2009). "Asporin competes with decorin for collagen binding, binds calcium and promotes osteoblast collagen mineralization." Biochem. J **423**: 53-59.

Kalamajski, S. and A. Oldberg (2010). "The role of small leucine-rich proteoglycans in collagen fibrillogenesis." Matrix Biol **29**(4): 248-253.

Kalson, N. S., D. F. Holmes, Z. Kapacee, I. Otermin, Y. Lu, R. A. Ennos, E. G. Canty-Laird and K. E. Kadler (2010). "An experimental model for studying the biomechanics of embryonic tendon: Evidence that the development of mechanical properties depends on the actinomyosin machinery." Matrix Biol **29**(8): 678-689.

Kannus, P. (2000). "Structure of the tendon connective tissue." Scand J Med Sci Sports **10**(6): 312-320.

Kannus, P., J. Bergfeld, M. Jarvinen, R. J. Johnson, M. Pope, P. Renstrom and K. Yasuda (1991). "Injuries to the posterior cruciate ligament of the knee." Sports Med **12**(2): 110-131.

Kapacee, Z., S. H. Richardson, Y. Lu, T. Starborg, D. F. Holmes, K. Baar and K. E. Kadler (2008). "Tension is required for fibripositor formation." Matrix Biology **27**(4): 371-375.

Kapacee, Z., C.-Y. C. Yeung, Y. Lu, D. Crabtree, D. F. Holmes and K. E. Kadler (2010). "Synthesis of embryonic tendon-like tissue by human marrow stromal/mesenchymal stem cells requires a three-dimensional environment and transforming growth factor β3." Matrix Biology **29**(8): 668-677.

Kartus, J., T. Movin and J. Karlsson (2001). "Donor-site morbidity and anterior knee problems after anterior cruciate ligament reconstruction using autografts." Arthroscopy: The Journal of Arthroscopic & Related Surgery **17**(9): 971-980.

Karve, T. M. and A. K. Cheema (2011). "Small changes huge impact: the role of protein posttranslational modifications in cellular homeostasis and disease." Journal of amino acids **2011:** 1-13.

Kastelic, J., A. Galeski and E. Baer (1978). "The multicomposite structure of tendon." Connect Tissue Res **6**(1): 11-23.

Keene, D. R., E. Engvall and R. W. Glanville (1988). "Ultrastructure of type VI collagen in human skin and cartilage suggests an anchoring function for this filamentous network." J Cell Biol **107**(5): 1995-2006.

Khan, K. M. and N. Maffulli (1998). "Tendinopathy: an Achilles' heel for athletes and clinicians." Clin J Sport Med **8**(3): 151-154.

Kiani, C., L. Chen, Y. J. Wu, A. J. Yee and B. B. Yang (2002). "Structure and function of aggrecan." Cell Res **12**(1): 19-32.

Kielty, C. M. (2006). "Elastic fibres in health and disease." Expert Reviews in Molecular Medicine **8**(19): 1-23.

Kielty, C. M. and M. E. Grant (1993). "The collagen family: structure, assembly, and organization in the extracellular matrix." Connective tissue and its heritable disorders: 159-221.

Kielty, C. M., M. J. Sherratt and C. A. Shuttleworth (2002). "Elastic fibres." Journal of cell science **115**(14): 2817-2828.

Kirkpatrick, D. S., S. A. Gerber and S. P. Gygi (2005). "The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications." Methods **35**(3): 265-273.

Kjaer, M. (2004). "Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading." Physiol Rev **84**(2): 649-698.

Knott, L. and A. J. Bailey (1998). "Collagen cross-links in mineralizing tissues: a review of their chemistry, function, and clinical relevance." Bone **22**(3): 181-187.

Kobayashi, S., H. Baba, K. Uchida, K. Negoro, M. Sato, T. Miyazaki, E. Nomura, K. Murakami, M. Shimizubata and A. Meir (2006). "Microvascular system of anterior cruciate ligament in dogs." J Orthop Res **24**(7): 1509-1520.

Koob, T. J. and K. G. Vogel (1987). "Site-related variations in glycosaminoglycan content and swelling properties of bovine flexor tendon." J Orthop Res **5**(3): 414-424.

Kostrominova, T. Y., S. Calve, E. M. Arruda and L. M. Larkin (2009). "Ultrastructure of myotendinous junctions in tendon-skeletal muscle constructs engineered in vitro." Histol Histopathol **24**(5): 541-550.

Kozel, B. A., C. H. Ciliberto and R. P. Mecham (2004). "Deposition of tropoelastin into the extracellular matrix requires a competent elastic fiber scaffold but not live cells." Matrix Biol **23**(1): 23-34.

Kraus-Hansen, A. E., G. Fackelman, C. Becker, R. Williams and F. Pipers (1992). "Preliminary studies on the vascular anatomy of the equine superficial digital flexor tendon." Equine veterinary journal **24**(1): 46-51.

Krstić, R. V. (1985). "Loose Connective Tissue". General Histology of the Mammal**:** 128-129. Springer Verlag: Berlin Heidelberg.

Kuo, C. K., J. E. Marturano and R. S. Tuan (2010). "Novel strategies in tendon and ligament tissue engineering: Advanced biomaterials and regeneration motifs." Sports Med Arthrosc Rehabil Ther Technol **2**: 1-20.

Kwon, H.-S., H.-S. Lee, Y. Ji, J. S. Rubin and S. I. Tomarev (2009). "Myocilin is a modulator of Wnt signaling." Molecular and cellular biology **29**(8): 2139-2154.

Latimer, C. A., M. Nelson, C. M. Moore and K. E. Martin (2014). "Effect of collagen and elastin content on the burst pressure of human blood vessel seals formed with a bipolar tissue sealing system." J Surg Res **186**(1): 73-80.

Legerlotz, K., E. R. Jones, H. R. Screen and G. P. Riley (2012). "Increased expression of IL-6 family members in tendon pathology." Rheumatology (Oxford) **51**(7): 1161-1165.

Lejard, V., F. Blais, M.-J. Guerquin, A. Bonnet, M.-A. Bonnin, E. Havis, M. Malbouyres, C. B. Bidaud, G. Maro and P. Gilardi-Hebenstreit (2011). "EGR1 and EGR2 involvement in vertebrate tendon differentiation." Journal of Biological Chemistry **286**(7): 5855-5867.

Lin, T. W., L. Cardenas and L. J. Soslowsky (2004). "Biomechanics of tendon injury and repair." Journal of biomechanics **37**(6): 865-877.

Lin, T. W., L. Cardenas and L. J. Soslowsky (2004). "Biomechanics of tendon injury and repair." J Biomech **37**(6): 865-877.

Little, D., J. W. Thompson, L. G. Dubois, D. S. Ruch, M. A. Moseley and F. Guilak (2014). "Proteomic Differences between Male and Female Anterior Cruciate Ligament and Patellar Tendon." PloS one **9**(5): e96526.

Liu, S. H., R. S. Yang, R. al-Shaikh and J. M. Lane (1995). "Collagen in tendon, ligament, and bone healing. A current review." Clin Orthop Relat Res(318): 265-278.

Lo, I. K., S. Chi, T. Ivie, C. B. Frank and J. B. Rattner (2002). "The cellular matrix: a feature of tensile bearing dense soft connective tissues." Histol Histopathol **17**(2): 523-537.

Lo, I. K. Y., Y. Ou, J. P. Rattner, D. A. Hart, L. L. Marchuk, C. B. Frank and J. B. Rattner (2002). "The cellular networks of normal ovine medial collateral and anterior cruciate ligaments are not accurately recapitulated in scar tissue." J Anat **200**(3): 283-296.

Lohmander, L.S., Ostenberg, A., Englund, M., and Roos, H. (2004) "High prevalence of knee osteoarthritis, pain, and functional limitations in female soccer players twelve years after anterior cruciate ligament injury". Arthritis Rheum **50**: 3145-3152

Lorda-Diez, C. I., A. Canga-Villegas, L. Cerezal, S. Plaza, J. M. Hurle, J. A. Garcia-Porrero and J. A. Montero (2013). "Comparative transcriptional analysis of three human ligaments with distinct biomechanical properties." J Anat **223**(6): 593-602.

Lorda-Diez, C. I., J. A. Montero, J. A. Garcia-Porrero and J. M. Hurle (2014). "Divergent differentiation of skeletal progenitors into cartilage and tendon: lessons from the embryonic limb." ACS Chem Biol **9**(1): 72-79.

Ma, J, Smietana, M.J, Tatiana Y. Kostrominova, T.Y., Wojtys, E.M., Larkin, L.M., M. Arruda, E.L. (2012). "Three-Dimensional Engineered Bone–Ligament–Bone Constructs for Anterior Cruciate Ligament Replacement". Tissue Engineering Part A. **18** (1-2): 103-116.

Maffulli, N., P. M. Binfield and J. B. King (2003). "Articular cartilage lesions in the symptomatic anterior cruciate ligament-deficient knee." Arthroscopy: The Journal of Arthroscopic & Related Surgery **19**(7): 685-690.

Maffulli, N., F. Spiezia, V. Testa, G. Capasso, U. G. Longo and V. Denaro (2012). "Free gracilis tendon graft for reconstruction of chronic tears of the Achilles tendon." J Bone Joint Surg Am **94**(10): 906-910.

Maffulli, N., J. Wong and L. C. Almekinders (2003). "Types and epidemiology of tendinopathy." Clinics in sports medicine **22**(4): 675-692.

Marieb, E. N. and K. Hoehn (2007). Chapter 4. "Tissue: the living fabric" Human anatomy & physiology: 118-149. Pearson Education: Harlow, Essex.

Martin RB, Burr DB, Sharkey NA (1998). "Mechanical properties of ligament and tendon". Skeletal tissue mechanics: 309-346. Springer: New York.

Mastrokalos, D. S., J. Springer, R. Siebold and H. H. Paessler (2005). "Donor Site Morbidity and Return to the Preinjury Activity Level After Anterior Cruciate Ligament Reconstruction Using Ipsilateral and Contralateral Patellar Tendon Autograft A Retrospective, Nonrandomized Study." The American journal of sports medicine **33**(1): 85-93.

Matthiesen, R. and O. N. Jensen (2008). "Analysis of mass spectrometry data in proteomics." Methods Mol Biol **453**: 105-122.

McNeilly, C. M., A. J. Banes, M. Benjamin and J. R. Ralphs (1996). "Tendon cells in vivo form a three dimensional network of cell processes linked by gap junctions." J Anat **189**(Pt 3): 593-600.

Meller, R., F. Schiborra, G. Brandes, K. Knobloch, T. Tschernig, S. Hankemeier, C. Haasper, A. Schmiedl, M. Jagodzinski and C. Krettek (2009). "Postnatal maturation of tendon, cruciate ligament, meniscus and articular cartilage: A histological study in sheep." Annals of Anatomy-Anatomischer Anzeiger **191**(6): 575-585.

Mienaltowski, M. J., S. M. Adams and D. E. Birk (2013). "Regional differences in stem cell/progenitor cell populations from the mouse achilles tendon." Tissue Eng Part A **19**(1-2): 199-210.

Mienaltowski, M. J., S. M. Adams and D. E. Birk (2014). "Tendon proper- and peritenon-derived progenitor cells have unique tenogenic properties." Stem Cell Res Ther **5**(4): 86.

Mienaltowski, M. J. and D. E. Birk (2014). "Structure, physiology, and biochemistry of collagens." Adv Exp Med Biol **802**: 5-29.

Mikawa, Y., H. Hamagami, J. Shikata and T. Yamamuro (1986). "Elastin in the human intervertebral disk. A histological and biochemical study comparing it with elastin in the human yellow ligament." Arch Orthop Trauma Surg **105**(6): 343-349.

Millar, N. L., J. H. Reilly, S. C. Kerr, A. L. Campbell, K. J. Little, W. J. Leach, B. P. Rooney, G. A. Murrell and I. B. McInnes (2012). "Hypoxia: a critical regulator of early human tendinopathy." Annals of the rheumatic diseases **71**(2): 302-310.

Miller, P. J. (1971). "An elastin stain." Med Lab Technol **28**(2): 148-149.

Mithieux, S. M. and A. S. Weiss (2005). "Elastin." Adv Protein Chem **70**: 437-461.

Miyashita, H., M. Ochi and Y. Ikuta (1997). "Histological and biomechanical observations of the rabbit patellar tendon after removal of its central one-third." Arch Orthop Trauma Surg **116**(8): 454-462.

Molloy, T., Y. Wang and G. A. Murrell (2003). "The roles of growth factors in tendon and ligament healing." Sports Medicine **33**(5): 381-394.

Moshiri, A. and A. Oryan (2013). "Tendon and ligament tissue engineering, healing and regenerative medicine." J Sports Med Doping Stud **3**(126): 2161-0673.1000126.

Movin, T., A. Gad, F. P. Reinholt and C. Rolf (1997). "Tendon pathology in long-standing achillodynia. Biopsy findings in 40 patients." Acta Orthop Scand **68**(2): 170-175.

Murray, M., S. Martin, T. Martin and M. Spector (2000). "Histological Changes in the Human Anterior Cruciate Ligament After Rupture*." The Journal of Bone & Joint Surgery **82**(10): 1387-1387.

Murray, M. M. (2009). "Current status and potential of primary ACL repair." Clin Sports Med **28**(1): 51-61.

Murray, M. M. and M. Spector (1999). "Fibroblast distribution in the anteromedial bundle of the human anterior cruciate ligament: the presence of alpha-smooth muscle actin-positive cells." J Orthop Res **17**(1): 18-27.

Murray, M. M., A. Weiler and K. P. Spindler (2004). "Interspecies variation in the fibroblast distribution of the anterior cruciate ligament." Am J Sports Med **32**(6): 1484-1491.

Myllyharju, J. and K. I. Kivirikko (2004). "Collagens, modifying enzymes and their mutations in humans, flies and worms." Trends Genet **20**(1): 33-43.

Myllyharju, J. and K. I. Kivirikko (2004). "Collagens, modifying enzymes and their mutations in humans, flies and worms." TRENDS in Genetics **20**(1): 33-43.

Naba, A., S. Hoersch and R. O. Hynes (2012). "Towards definition of an ECM parts list: An advance on GO categories." Matrix Biology **31**(7): 371-372.

Nagineni, C. N., D. Amiel, M. H. Green, M. Berchuck and W. H. Akeson (1992). "Characterization of the intrinsic properties of the anterior cruciate and medial collateral ligament cells: an in vitro cell culture study." J Orthop Res **10**(4): 465-475.

Nakagawa, H., Y. Mikawa and R. Watanabe (1994). "Elastin in the human posterior longitudinal ligament and spinal dura. A histologic and biochemical study." Spine (Phila Pa 1976) **19**(19): 2164-2169.

Nakama, K., M. Gotoh, T. Yamada, Y. Mitsui, H. Yasukawa, T. Imaizumi, F. Higuchi and K. Nagata (2006). "Interleukin-6-induced activation of signal transducer and activator of transcription-3 in ruptured rotator cuff tendon." J Int Med Res **34**(6): 624-631.

Narama, I., M. Masuoka-Nishiyama, T. Matsuura, K. Ozaki, M. Nagatani and T. Morishima (1996). "Morphogenesis of degenerative changes predisposing dogs to rupture of the cranial cruciate ligament." J Vet Med Sci **58**(11): 1091-1097.

Newton, P. O., S. L. Woo, L. R. Kitabayashi, R. M. Lyon, D. R. Anderson and W. H. Akeson (1990). "Ultrastructural changes in knee ligaments following immobilization." Matrix **10**(5): 314-319.

O'Brien, M. (1992). "Functional anatomy and physiology of tendons." Clinics in sports medicine **11**(3): 505-520.

O'Brien, M. (2005). "Anatomy of tendons." Tendon injuries: basic science and clinical medicine: 3-13. Springer: USA.

Okuda, Y., J. P. Gorski, K. N. An and P. C. Amadio (1987). "Biochemical, histological, and biomechanical analyses of canine tendon." J Orthop Res **5**(1): 60-68.

Pankov, R. and K. M. Yamada (2002). "Fibronectin at a glance." Journal of cell science **115**(20): 3861-3863.

Parkinson, J., T. Samiric, M. Z. Ilic, J. Cook and C. J. Handley (2011). "Involvement of proteoglycans in tendinopathy." J Musculoskelet Neuronal Interact **11**(2): 86-93.

Patel, V. J., K. Thalassinos, S. E. Slade, J. B. Connolly, A. Crombie, J. C. Murrell and J. H. Scrivens (2009). "A comparison of labeling and label-free mass spectrometry-based proteomics approaches." Journal of proteome research **8**(7): 3752-3759.

Paxton, J. Z., L. M. Grover and K. Baar (2010). "Engineering an in vitro model of a functional ligament from bone to bone." Tissue Eng Part A **16**(11): 3515-3525.

Paxton, J. Z., P. Hagerty, J. J. Andrick and K. Baar (2012). "Optimizing an intermittent stretch paradigm using ERK1/2 phosphorylation results in increased collagen synthesis in engineered ligaments." Tissue Eng Part A **18**(3-4): 277-284.

Paxton, J. Z., U. N. Wudebwe, A. Wang, D. Woods and L. M. Grover (2012). "Monitoring sinew contraction during formation of tissue-engineered fibrin-based ligament constructs." Tissue Engineering Part A **18**(15-16): 1596-1607.

Pearse, R. V., D. Esshaki, C. J. Tabin and M. M. Murray (2009). "Genome-wide expression analysis of intra-and extraarticular connective tissue." Journal of Orthopaedic Research **27**(4): 427-434.

Peffers, M. J., C. T. Thorpe, J. A. Collins, R. Eong, T. K. Wei, H. R. Screen and P. D. Clegg (2014). "Proteomic analysis reveals age-related changes in tendon matrix composition, with age-and 1 injury-specific matrix fragmentation." Journal of Biological Chemistry: jbc. M114. 566554.

Petersen, W., G. Hohmann, V. Stein and B. Tillmann (2002). "The blood supply of the posterior tibial tendon." Journal of Bone & Joint Surgery, British Volume **84**(1): 141-144.

Petersen, W. and B. Tillmann (1999). "Structure and vascularization of the cruciate ligaments of the human knee joint." Anat Embryol (Berl) **200**(3): 325-334.

Petersen, W. and B. Tillmann (2002). "Anatomy and function of the anterior cruciate ligament." Orthopade **31**(8): 710-718.

Petersen, W. and T. Zantop (2007). "Anatomy of the anterior cruciate ligament with regard to its two bundles." Clin Orthop Relat Res **454**: 35-47.

Plaas, A. H., S. Wong-Palms, T. Koob, D. Hernandez, L. Marchuk and C. B. Frank (2000). "Proteoglycan metabolism during repair of the ruptured medial collateral ligament in skeletally mature rabbits." Arch Biochem Biophys **374**(1): 35-41.

Polacek, M., J. A. Bruun, O. Johansen and I. Martinez (2010). "Differences in the secretome of cartilage explants and cultured chondrocytes unveiled by SILAC technology." J Orthop Res **28**(8): 1040-1049.

Posthumus, M., A. V. September, M. Keegan, D. O'Cuinneagain, W. Van der Merwe, M. P. Schwellnus and M. Collins (2009). "Genetic risk factors for anterior cruciate ligament ruptures: COL1A1 gene variant." Br J Sports Med **43**(5): 352-356.

Proctor, C. S., D. W. Jackson and T. M. Simon (1997). "Characterization of the repair tissue after removal of the central one-third of the patellar ligament. An experimental study in a goat model*." The Journal of Bone & Joint Surgery **79**(7): 997-1006.

Provenzano, P. P., C. Hurschler and R. Vanderby (2001). "Microstructural morphology in the transition region between scar and intact residual segments of a healing rat medial collateral ligament." Connective tissue research **42**(2): 123-133.

Provenzano, P. P. and R. Vanderby (2006). "Collagen fibril morphology and organization: implications for force transmission in ligament and tendon." Matrix Biology **25**(2): 71-84.

Pryce, B. A., S. S. Watson, N. D. Murchison, J. A. Staverosky, N. Dünker and R. Schweitzer (2009). "Recruitment and maintenance of tendon progenitors by TGFβ signaling are essential for tendon formation." Development **136**(8): 1351-1361.

Ralphs, J. R., M. Benjamin, A. D. Waggett, D. C. Russell, K. Messner and J. Gao (1998). "Regional differences in cell shape and gap junction expression in rat Achilles tendon: relation to fibrocartilage differentiation." J Anat **193**(2): 215-222.

Ralphs, J. R., M. Benjamin, A. D. Waggett, D. C. Russell, K. Messner and J. Gao (1998). "Regional differences in cell shape and gap junction expression in rat Achilles tendon: relation to fibrocartilage differentiation." J Anat **193 (Pt 2)**: 215-222.

Raspanti, M., M. Viola, M. Sonaggere, M. E. Tira and R. Tenni (2007). "Collagen fibril structure is affected by collagen concentration and decorin." Biomacromolecules **8**(7): 2087-2091.

Raymond, A., M. A. Ensslin and B. D. Shur (2009). "SED1/MFG-E8: A Bi-Motif protein that orchestrates diverse cellular interactions." Journal of cellular biochemistry **106**(6): 957-966.

Rees, S., C. Dent and B. Caterson (2009). "Metabolism of proteoglycans in tendon." Scandinavian journal of medicine & science in sports **19**(4): 470-478.

Rees, S. G., C. R. Flannery, C. B. Little, C. E. Hughes, B. Caterson and C. M. Dent (2000). "Catabolism of aggrecan, decorin and biglycan in tendon." Biochemical Journal **350**: 181-188.

Rees, S. G., A. D. Waggett, B. C. Kerr, J. Probert, E. C. Gealy, C. M. Dent, B. Caterson and C. E. Hughes (2009). "Immunolocalisation and expression of keratocan in tendon." Osteoarthritis Cartilage **17**(2): 276-279.

Riley, G. (2004). "The pathogenesis of tendinopathy. A molecular perspective." Rheumatology (Oxford) **43**(2): 131-142.

Riley, G. (2005). "Chronic tendon pathology: molecular basis and therapeutic implications." Expert Rev Mol Med **7**(5): 1-25.

Riley, G. (2008). "Tendinopathy--from basic science to treatment." Nat Clin Pract Rheumatol **4**(2): 82-89.

Riley, G. P. (2004). "Tendon and ligament biochemistry and pathology". Sport Injuries: 3-39 Oxford University Press: Oxford.

Riley, G. P., V. Curry, J. DeGroot, B. van El, N. Verzijl, B. L. Hazleman and R. A. Bank (2002). "Matrix metalloproteinase activities and their relationship with collagen remodelling in tendon pathology." Matrix Biol **21**(2): 185-195.

Riley, G. P., M. J. Goddard and B. L. Hazleman (2001). "Histopathological assessment and pathological significance of matrix degeneration in supraspinatus tendons." Rheumatology (Oxford) **40**(2): 229-230.

Riley, G. P., R. L. Harrall, C. R. Constant, M. D. Chard, T. E. Cawston and B. L. Hazleman (1994). "Glycosaminoglycans of human rotator cuff tendons: changes with age and in chronic rotator cuff tendinitis." Ann Rheum Dis **53**(6): 367-376.

Ritty, T. M., K. Ditsios and B. C. Starcher (2002). "Distribution of the elastic fiber and associated proteins in flexor tendon reflects function." Anat Rec **268**(4): 430-440.

Ritty, T. M., R. Roth and J. E. Heuser (2003). "Tendon cell array isolation reveals a previously unknown fibrillin-2-containing macromolecular assembly." Structure **11**(9): 1179-1188.

Robertson, A., R. Nutton and J. Keating (2006). "Current trends in the use of tendon allografts in orthopaedic surgery." Journal of Bone & Joint Surgery, British Volume **88**(8): 988-992.

Rodrigues, M. T., R. L. Reis and M. E. Gomes (2013). "Engineering tendon and ligament tissues: present developments towards successful clinical products." Journal of tissue engineering and regenerative medicine **7**(9): 673-686.

Rokito, A. S., F. Cuomo, M. A. Gallagher and J. D. Zuckerman (1999). "Long-Term Functional Outcome of Repair of Large and Massive Chronic Tears of the Rotator Cuff*." The Journal of Bone & Joint Surgery **81**(7): 991-997.

Ronel, D. N., M. I. Newman, L. B. Gayle and L. A. Hoffman (2004). "Recent advances in the reconstruction of complex Achilles tendon defects." Microsurgery **24**(1): 18-23.

Rosenberg, L. C., H. U. Choi, A. R. Poole, K. Lewandowska and L. A. Culp (1986). "Biological roles of dermatan sulphate proteoglycans." Ciba Found Symp **124**: 47-68.

Ross, M. and W. Pawlina (2006). Chapter six: "Connective tissue" Histology: A Text and Atlas: With Correlated Cell and Molecular Biology: 146-181. Lippincott Williams & Wilkins: USA.

Royer-Zemmour, B., M. Ponsole-Lenfant, H. Gara, P. Roll, C. Lévêque, A. Massacrier, G. Ferracci, J. Cillario, A. Robaglia-Schlupp and R. Vincentelli (2008). "Epileptic and developmental disorders of the speech cortex: ligand/receptor interaction of wild-type and mutant SRPX2 with the plasminogen activator receptor uPAR." Human molecular genetics **17**(23): 3617-3630.

Ruiz-Romero, C. and F. J. Blanco (2010). "Proteomics role in the search for improved diagnosis, prognosis and treatment of osteoarthritis." Osteoarthritis Cartilage **18**(4): 500-509.

Rumian, A. P., A. L. Wallace and H. L. Birch (2007). "Tendons and ligaments are anatomically distinct but overlap in molecular and morphological features—a comparative study in an ovine model." Journal of orthopaedic research **25**(4): 458-464.

Samiric, T., M. Z. Ilic and C. J. Handley (2004). "Characterisation of proteoglycans and their catabolic products in tendon and explant cultures of tendon." Matrix Biology **23**(2): 127-140.

Santra, M., C. C. Reed and R. V. Iozzo (2002). "Decorin binds to a narrow region of the epidermal growth factor (EGF) receptor, partially overlapping but distinct from the EGF-binding epitope." J Biol Chem **277**(38): 35671-35681.

Sardone, F., F. Traina, F. Tagliavini, C. Pellegrini, L. Merlini, S. Squarzoni, S. Santi, S. Neri, C. Faldini, N. Maraldi and P. Sabatelli (2014). "Effect of mechanical strain on the collagen VI pericellular matrix in anterior cruciate ligament fibroblasts." J Cell Physiol **229**(7): 878-886.

Schonherr, E., P. Witsch-Prehm, B. Harrach, H. Robenek, J. Rauterberg and H. Kresse (1995). "Interaction of biglycan with type I collagen." J Biol Chem **270**(6): 2776-2783.

Screen, H. R. C. (2009). "Hierarchical approaches to understanding tendon mechanics." Journal of Biomechanical Science and Engineering **4**(4): 481-499.

Serpell, B. G., J. M. Scarvell, N. B. Ball and P. N. Smith (2012). "Mechanisms and risk factors for noncontact ACL injury in age mature athletes who engage in field or court sports: a summary of the literature since 1980." J Strength Cond Res **26**(11): 3160-3176.

Sharma, P. and N. Maffulli (2005). "Basic biology of tendon injury and healing." Surgeon **3**(5): 309-316.

Sharma, P. and N. Maffulli (2005). "Tendon injury and tendinopathy: healing and repair." J Bone Joint Surg Am **87**(1): 187-202.

Sharma, P. and N. Maffulli (2006). "Biology of tendon injury: healing, modeling and remodeling." J Musculoskelet Neuronal Interact **6**(2): 181-190.

Shen, Z., S. Gantcheva, B. Mansson, D. Heinegard and Y. Sommarin (1998). "Chondroadherin expression changes in skeletal development." Biochem J **330 (Pt 1)**: 549-557.

Singer, V. L., L. J. Jones, S. T. Yue and R. P. Haugland (1997). "Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation." Anal Biochem **249**(2): 228-238.

Smith, K., D. (2010). The distrubution and function of elastin and elastic fibres in the canine cruciate ligament complex. PhD, University of Liverpool.

Smith, K. D., P. D. Clegg, J. F. Innes and E. J. Comerford (2013). "Elastin content is high in the canine cruciate ligament and is associated with degeneration." The Veterinary Journal **199**: 169-174.

Smith, K. D., A. Vaughan-Thomas, D. G. Spiller, P. D. Clegg, J. F. Innes and E. J. Comerford (2012). "Variations in cell morphology in the canine cruciate ligament complex." Vet J **193**(2): 561-566.

Smith, K. D., A. Vaughan-Thomas, D. G. Spiller, J. F. Innes, P. D. Clegg and E. J. Comerford (2011). "The organisation of elastin and fibrillins 1 and 2 in the cruciate ligament complex." J Anat **218**(6): 600-607.

Sodersten, F., K. Hultenby, D. Heinegard, C. Johnston and S. Ekman (2013). "Immunolocalization of collagens (I and III) and cartilage oligomeric matrix protein in the normal and injured equine superficial digital flexor tendon." Connect Tissue Res **54**(1): 62-69.

Soroceanu, A., F. Sidhwa, S. Aarabi, A. Kaufman and M. Glazebrook (2012). "Surgical Versus Nonsurgical Treatment of Acute Achilles Tendon RuptureA Meta-Analysis of Randomized Trials." The Journal of Bone & Joint Surgery **94**(23): 2136-2143.

Spindler, K. P., J. T. Andrish, R. R. Miller, K. Tsujimoto and D. I. Diz (1996). "Distribution of cellular repopulation and collagen synthesis in a canine anterior cruciate ligament autograft." J Orthop Res **14**(3): 384-389.

Spindler, K. P., J. E. Kuhn, K. B. Freedman, C. E. Matthews, R. S. Dittus and F. E. Harrell (2004). "Anterior cruciate ligament reconstruction autograft choice: Bone-tendon-bone versus hamstring does it really matter? a systematic review." The American Journal of Sports Medicine **32**(8): 1986-1995.

Steiglitz, B. M., D. R. Keene and D. S. Greenspan (2002). "PCOLCE2 encodes a functional procollagen C-proteinase enhancer (PCPE2) that is a collagen-binding protein differing in distribution of expression and post-translational modification from the previously described PCPE1." Journal of Biological Chemistry **277**(51): 49820-49830.

Stoll, C., T. John, C. Conrad, A. Lohan, S. Hondke, W. Ertel, C. Kaps, M. Endres, M. Sittinger and J. Ringe (2011). "Healing parameters in a rabbit partial tendon defect following tenocyte/biomaterial implantation." Biomaterials **32**(21): 4806-4815.

Streich, N.A., Zimmermann, D., Bode, G., and Schmitt, H. (2011). "Reconstructive versus non-reconstructive treatment of anterior cruciate ligament insufficiency. A retrospective matched-pair long-term follow-up". Int Orthop **35**: 607-613

Strickland, S. M., J. D. MacGillivray and R. F. Warren (2003). "Anterior cruciate ligament reconstruction with allograft tendons." Orthopedic clinics of North America **34**(1): 41-47.

Strocchi, R., V. De Pasquale, S. Guizzardi, P. Govoni, A. Facchini, M. Raspanti, M. Girolami and S. Giannini (1991). "Human Achilles tendon: morphological and morphometric variations as a function of age." Foot Ankle **12**(2): 100-104.

Styrer (1996). Chapter five: "Carbohydrates". Biochemistry: 59-66. W.H. Freeman: New york.

Svensson, L., A. Aszodi, F. P. Reinholt, R. Fassler, D. Heinegard and A. Oldberg (1999). "Fibromodulin-null mice have abnormal collagen fibrils, tissue organization, and altered lumican deposition in tendon." J Biol Chem **274**(14): 9636-9647.

Tashiro, T., H. Kurosawa, A. Kawakami, A. Hikita and N. Fukui (2003). "Influence of Medial Hamstring Tendon Harvest on Knee Flexor Strength after Anterior Cruciate Ligament Reconstruction A Detailed Evaluation with Comparison of Single-and Double-Tendon Harvest." The American journal of sports medicine **31**(4): 522-529.

Thakkar, D., T. M. Grant, O. Hakimi and A. J. Carr (2014). "Distribution and expression of type VI collagen and elastic fibers in human rotator cuff tendon tears." Connect Tissue Res **55**(5-6): 397-402.

Thomopoulos, S., F. L. Harwood, M. J. Silva, D. Amiel and R. H. Gelberman (2005). "Effect of Several Growth Factors on Canine Flexor Tendon Fibroblast Proliferation and Collagen Synthesis In Vitro." The Journal of hand surgery **30**(3): 441-447.

Thornton, G. M., G. P. Leask, N. G. Shrive and C. B. Frank (2000). "Early medial collateral ligament scars have inferior creep behaviour." J Orthop Res **18**(2): 238-246.

Thorpe, C., P. Clegg and H. Birch (2010). "A review of tendon injury: why is the equine superficial digital flexor tendon most at risk?" Equine veterinary journal **42**(2): 174-180.

Thorpe, C. T. (2010). "Extracellular matrix synthesis and degradation in functionally distinct tendons". PhD, University College London.

Thorpe, C. T., H. L. Birch, P. D. Clegg and H. R. Screen (2013). "The role of the non-collagenous matrix in tendon function." Int J Exp Pathol **94**(4): 248-259.

Thorpe, C.T., Godinho, M.C., Riley, G.P, Birch, H.L., Clegg, P.D., Screen, H.R. (2015) "The interfascicular matrix enables fascicle sliding and recovery in tendon, and behaves more elastically in energy storing tendons". Journal of the Mechanical Behavior of Biomedical Materials **1**: 1-10

Thorpe, C. T., I. Streeter, G. L. Pinchbeck, A. E. Goodship, P. D. Clegg and H. L. Birch (2010). "Aspartic acid racemization and collagen degradation markers reveal an accumulation of damage in tendon collagen that is enhanced with aging." J Biol Chem **285**(21): 15674-15681.

Thorpe, C. T., C. P. Udeze, H. L. Birch, P. D. Clegg and H. R. Screen (2012). "Specialization of tendon mechanical properties results from interfascicular differences." Journal of The Royal Society Interface: rsif20120362.

Tischer, T., S. Vogt, S. Aryee, E. Steinhauser, C. Adamczyk, S. Milz, V. Martinek and A. B. Imhoff (2007). "Tissue engineering of the anterior cruciate ligament: a new method using acellularized tendon allografts and autologous fibroblasts." Archives of orthopaedic and trauma surgery **127**(9): 735-741.

Tortora, G. J. and B. Derrickson (2013). Chapter four: " The tissue level organization". Essentials of anatomy and physiology: 113-152. Wiley & Sons: Asia.

Toth, A. and F. Cordasco (2000). "Anterior cruciate ligament injuries in the female athlete." The journal of gender-specific medicine: JGSM: the official journal of the Partnership for Women's Health at Columbia **4**(4): 25-34.

Tozer, S. and D. Duprez (2005). "Tendon and ligament: development, repair and disease." Birth Defects Res C Embryo Today **75**(3): 226-236.

Uhorchak, J. M., C. R. Scoville, G. N. Williams, R. A. Arciero, P. S. Pierre and D. C. Taylor (2003). "Risk factors associated with noncontact injury of the anterior cruciate ligament a prospective four-year evaluation of 859 west point cadets." The American journal of sports medicine **31**(6): 831-842.

Uitto, J. (1979). "Biochemistry of the elastic fibers in normal connective tissues and its alterations in diseases." J Invest Dermatol **72**(1): 1-10.

Valiyaveettil, M., J. S. Mort and C. A. McDevitt (2005). "The concentration, gene expression, and spatial distribution of aggrecan in canine articular cartilage, meniscus, and anterior and posterior cruciate ligaments: a new molecular distinction between hyaline cartilage and fibrocartilage in the knee joint." Connective tissue research **46**(2): 83-91.

Van de Graaff, K., R. Rhees and S. Palmer (2010). "Schaum's Outlines Human Anatomy and Physiology."

van der Rest, M. and R. Garrone (1991). "Collagen family of proteins." FASEB J **5**(13): 2814-2823.

Vasseur, P. B., R. R. Pool, S. P. Arnoczky and R. E. Lau (1985). "Correlative biomechanical and histologic study of the cranial cruciate ligament in dogs." Am J Vet Res **46**(9): 1842-1854.

Vogel, K. G., A. Ordog, G. Pogany and J. Olah (1993). "Proteoglycans in the compressed region of human tibialis posterior tendon and in ligaments." J Orthop Res **11**(1): 68-77.

Vogel, K. G., A. Ördög, G. Pogany and J. Oláh (1993). "Proteoglycans in the compressed region of human tibialis posterior tendon and in ligaments." Journal of orthopaedic research **11**(1): 68-77.

Vogel, K. G. and J. A. Peters (2005). "Histochemistry defines a proteoglycan-rich layer in bovine flexor tendon subjected to bending." J Musculoskelet Neuronal Interact **5**(1): 64-69.

Waggett, A. D., J. R. Ralphs, A. P. Kwan, D. Woodnutt and M. Benjamin (1998). "Characterization of collagens and proteoglycans at the insertion of the human Achilles tendon." Matrix Biol **16**(8): 457-470.

Wallace, C. D. and D. Amiel (1991). "Vascular assessment of the periarticular ligaments of the rabbit knee." Journal of orthopaedic research **9**(6): 787-791.

Wan, C., Z. Hao, S. Wen and H. Leng (2014). "A quantitative study of the relationship between the distribution of different types of collagen and the mechanical behavior of rabbit medial collateral ligaments." PLoS One **9**(7): e103363.

Wang, I. E., S. Mitroo, F. H. Chen, H. H. Lu and S. B. Doty (2006). "Age-dependent changes in matrix composition and organization at the ligament-to-bone insertion." J Orthop Res **24**(8): 1745-1755.

Wang, S.-H., S.-C. Kuo and S.-C. Chen (2003). "High-performance liquid chromatography determination of methionine adenosyltransferase activity using catechol-O-methyltransferase-coupled fluorometric detection." Analytical biochemistry **319**(1): 13-20.

Weatherholt, A. M., R. K. Fuchs and S. J. Warden (2012). "Specialized connective tissue: bone, the structural framework of the upper extremity." J Hand Ther **25**(2): 123-131; quiz 132.

Wendel, D. P., D. G. Taylor, K. H. Albertine, M. T. Keating and D. Y. Li (2000). "Impaired distal airway development in mice lacking elastin." Am J Respir Cell Mol Biol **23**(3): 320.

Whitehair, J. G., P. B. Vasseur and N. H. Willits (1993). "Epidemiology of cranial cruciate ligament rupture in dogs." J Am Vet Med Assoc **203**(7): 1016-1019.

Wiberg C., Hedbom E., Khairullina A. et al. (2001). "Biglycan and decorin bind close to the N-terminal region of the collagen VI triple helix". J Biol Chem **276:** 18947–18952.

Wiberg, C., D. Heinegard, C. Wenglen, R. Timpl and M. Morgelin (2002). "Biglycan organizes collagen VI into hexagonal-like networks resembling tissue structures." J Biol Chem **277**(51): 49120-49126.

Wight, T. N. (2002). "Versican: a versatile extracellular matrix proteoglycan in cell biology." Curr Opin Cell Biol **14**(5): 617-623.

Wilkins, M. R., J.-C. Sanchez, A. A. Gooley, R. D. Appel, I. Humphery-Smith, D. F. Hochstrasser and K. L. Williams (1996). "Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it." Biotechnology and genetic engineering reviews **13**(1): 19-50.

Woo, S. L., S. D. Abramowitch, R. Kilger and R. Liang (2006). "Biomechanics of knee ligaments: injury, healing, and repair." J Biomech **39**(1): 1-20.

Woo, S. L., R. E. Debski, J. Zeminski, S. D. Abramowitch, S. S. Saw and J. A. Fenwick (2000). "Injury and repair of ligaments and tendons." Annu Rev Biomed Eng **2**: 83-118.

Woo, S. L., K. Hildebrand, N. Watanabe, J. A. Fenwick, C. D. Papageorgiou and J. H. Wang (1999). "Tissue engineering of ligament and tendon healing." Clin Orthop Relat Res **367**: S312-S323.

Xie, J., W. Huang, J. Jiang, Y. Zhang, Y. Xu, C. Xu, L. Yang, P. C. Chen and K. Sung (2013). "Differential expressions of lysyl oxidase family in ACL and MCL fibroblasts after mechanical injury." Injury **44**(7): 893-900.

Yahia, L. H. and G. Drouin (1989). "Microscopical investigation of canine anterior cruciate ligament and patellar tendon: collagen fascicle morphology and architecture." J Orthop Res **7**(2): 243-251.

Yamada S, Tomoeda M, Ozawa Y, Yoneda S, Terashima Y, et al. (2007). "PLAP-1/asporin, a novel negative regulator of periodontal ligament mineralization". J Biol Chem **282** 23070–23080.

Yang, C., A. C. Park, N. A. Davis, J. D. Russell, B. Kim, D. D. Brand, M. J. Lawrence, Y. Ge, M. S. Westphall and J. J. Coon (2012). "Comprehensive mass spectrometric mapping of the hydroxylated amino acid residues of the α1 (V) collagen chain." Journal of Biological Chemistry **287**(48): 40598-40610.

Yang, C. H., G. J. Culshaw, M. M. Liu, C. C. Lu, A. T. French, D. N. Clements and B. M. Corcoran (2012). "Canine tissue-specific expression of multiple small leucine rich proteoglycans." Vet J **193**(2): 374-380.

Yang, G., B. B. Rothrauff and R. S. Tuan (2013). "Tendon and ligament regeneration and repair: clinical relevance and developmental paradigm." Birth Defects Res C Embryo Today **99**(3): 203-222.

Yao, Z., H. Nakamura, K. Masuko-Hongo, M. Suzuki-Kurokawa, K. Nishioka and T. Kato (2004). "Characterisation of cartilage intermediate layer protein (CILP)-induced arthropathy in mice." Annals of the rheumatic diseases **63**(3): 252-258.

Ye, Q., G. Zünd, P. Benedikt, S. Jockenhoevel, S. P. Hoerstrup, S. Sakyama, J. A. Hubbell and M. Turina (2000). "Fibrin gel as a three dimensional matrix in cardiovascular tissue engineering." European Journal of Cardio-Thoracic Surgery **17**(5): 587-591.

Yin, Z., X. Chen, J. L. Chen and H. W. Ouyang (2010). "Stem cells for tendon tissue engineering and regeneration." Expert Opin Biol Ther **10**(5): 689-700.

Yoon, J. H. and J. Halper (2005). "Tendon proteoglycans: biochemistry and function." J Musculoskelet Neuronal Interact **5**(1): 22-34.

Yoshida, M. and K. Fujii (1999). "Differences in cellular properties and responses to growth factors between human ACL and MCL cells." J Orthop Sci **4**(4): 293-298.

Young, R., A. Vaughan-Thomas, R. Wardale and V. C. Duance (2002). "Type II collagen deposition in cruciate ligament precedes osteoarthritis in the guinea pig knee." Osteoarthritis and cartilage **10**(5): 420-428.

Zantop, T., W. Petersen and F. H. Fu (2005). "Anatomy of the anterior cruciate ligament." Oper Tech Orthop **15**(1): 20-28.

Zantop, T., W. Petersen, J. K. Sekiya, V. Musahl and F. H. Fu (2006). "Anterior cruciate ligament anatomy and function relating to anatomical reconstruction." Knee Surg Sports Traumatol Arthrosc **14**(10): 982-992.

Zhang, G., B. Young, Y. Ezura, M. Favata, L. Soslowsky, S. Chakravarti and D. Birk (2005). "Development of tendon structure and function: regulation of collagen fibrillogenesis." J Musculoskelet Neuronal Interact **5**(1): 5-21.

Zhang, G., B. B. Young, Y. Ezura, M. Favata, L. J. Soslowsky, S. Chakravarti and D. E. Birk (2005). "Development of tendon structure and function: regulation of collagen fibrillogenesis." J Musculoskelet Neuronal Interact **5**(1): 5-21.

Zhang, H., S. D. Apfelroth, W. Hu, E. C. Davis, C. Sanguineti, J. Bonadio, R. P. Mecham and F. Ramirez (1994). "Structure and expression of fibrillin-2, a novel microfibrillar component preferentially located in elastic matrices." The Journal of cell biology: 855-863.

Zhang, J., T. Pan, H.-J. Im, F. H. Fu and J. H. Wang (2011). "Differential properties of human ACL and MCL stem cells may be responsible for their differential healing capacity." BMC medicine **9**(1): 68.

Zhang, J., L. Yang, Z. Tang, R. Xue, Y. Wang, Z. Luo, W. Huang and K. L. Sung (2009). "Expression of MMPs and TIMPs family in human ACL and MCL fibroblasts." Connect Tissue Res **50**(1): 7-13.

Zhang, Y., Y. Wu, L. Cao, V. Lee, L. Chen, Z. Lin, C. Kiani, M. E. Adams and B. B. Yang (2001). "Versican modulates embryonic chondrocyte morphology via the epidermal growth factor-like motifs in G3." Exp Cell Res **263**(1): 33-42.

Zhu, J.-L., K. Zhang, X.-W. HE and Y.-K. Zhang (2010). "New Developments of Quantitative Mass Spectrometry-based Proteomics." Chinese Journal of Analytical Chemistry **38**(3): 434-441.

Zhu, J., X. Zhang, Y. Ma, C. Zhou and Y. Ao (2012). "Ultrastructural and morphological characteristics of human anterior cruciate ligament and hamstring tendons." Anat Rec (Hoboken) **295**(9): 1430-1436.

Zhu, W., J. W. Smith and C.-M. Huang (2009). "Mass spectrometry-based label-free quantitative proteomics." BioMed Research International **2010**: 1-6.