THE INFLUENCE OF MENISCECTOMY AND OVARIECTOMY ON
THE OVINE ANTERIOR CRUCIATE LIGAMENT

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This thesis is presented for the degree of Doctor of Philosophy of Murdoch University

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I declare that this thesis is my own account of my research, and contains as its main content work which has not previously been submitted for degree at any tertiary educational institution.

Alison Daniel

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ABSTRACT

Current research supports a link between osteoarthritis (OA) and the decline in oestrogen levels at menopause. It is not known whether altered hormone levels exert their effect primarily on articular cartilage, or whether associated degeneration of other oestrogen-responsive joint tissues such as cruciate ligaments may significantly contribute. This study investigated the influence of ovariectomy and/or the presence of concurrent osteoarthritis (bilateral meniscectomy model) on the fibrillar structure of collagen within the anterior cruciate ligament (ACL).

Sheep (n=24) were treated experimentally with one or more of the following treatments: ovariectomy (OVX), meniscectomy (MENX) or non-operated control (NOC). ACLs were examined using transmission and scanning electron microscopy (TEM & SEM), gene expression, biochemical analysis and histology.

TEM studies showed OVX and MENX affect collagen fibril size and arrangement, while the combination of prior OVX and concurrent osteoarthritis (MENX) produced a different pattern of derangement to either treatment alone and may indicate a synergistic effect. Observed structural changes complemented molecular findings of altered mRNA expression, and changes in the collagen and sulphated glycosaminoglycan content of ACL tissue.

This study demonstrates that ovariectomy significantly affects ACL collagen fibril structure, and influences the response of the ACL to surgical OA (MENX). These results show the potential for changes in ACL structure post-menopause to influence joint integrity.
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CONFERENCE PRESENTATIONS


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<tr>
<td>ACL</td>
<td>anterior cruciate ligament</td>
</tr>
<tr>
<td>AM</td>
<td>anteromedial</td>
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<tr>
<td>Basic FGF</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>BMD</td>
<td>bone mineral density</td>
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<td>CaCL</td>
<td>caudal cruciate ligament</td>
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<td>cranial cruciate ligament</td>
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<td>cDNA</td>
<td>complimentary deoxyribonucleic acid</td>
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<td>COX</td>
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<td>CTGF</td>
<td>connective tissue growth factor</td>
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<td>DC</td>
<td>dendritic cells</td>
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<td>dimethylaminobenzaldehyde</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
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<td>FSH</td>
<td>follicle stimulating hormone</td>
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<td>additional globular domain</td>
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<td>G3</td>
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<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>haematoxylin and eosin</td>
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<td>HRT</td>
<td>hormone replacement therapy</td>
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<tr>
<td>IDO</td>
<td>indoleamine 2-3 dioxygenase</td>
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<tr>
<td>IFN-</td>
<td>interferon</td>
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<td>IgG</td>
<td>serum immunoglobulin G</td>
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<tr>
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<td>interleukin</td>
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<td>IL-β</td>
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<td>IVD</td>
<td>intervertebral disc</td>
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<td>LAPs</td>
<td>large aggregating proteoglycans</td>
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<td>lateral collateral ligament</td>
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<tr>
<td>LH</td>
<td>luteinising hormone</td>
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<tr>
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<td>medial collateral ligament</td>
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<td>MENX</td>
<td>meniscectomised</td>
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<tr>
<td>mg</td>
<td>milligram</td>
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<td>Full Form</td>
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<tr>
<td>MGA</td>
<td>middle genicular artery</td>
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<tr>
<td>ml</td>
<td>millilitre</td>
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<tr>
<td>MMPs</td>
<td>matrix metalloproteinases</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>NaCl</td>
<td>sodium chloride</td>
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<td>nm</td>
<td>nanometres</td>
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<tr>
<td>NOC</td>
<td>non-operated control</td>
</tr>
<tr>
<td>NTC</td>
<td>non-template control</td>
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<tr>
<td>O+M</td>
<td>ovariectomised and meniscectomised</td>
</tr>
<tr>
<td>OA</td>
<td>osteoarthritis</td>
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<tr>
<td>OH</td>
<td>hydroxyproline</td>
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<td>O VX</td>
<td>ovariectomised</td>
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<td>PCL</td>
<td>posterior cruciate ligament</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>platelet derived growth factor</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>PL</td>
<td>posterolateral</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
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<td>ribonuclease</td>
</tr>
<tr>
<td>SABC</td>
<td>state agricultural biotechnology centre</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<tr>
<td>S-GAG</td>
<td>sulfated glycosaminoglycan</td>
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<tr>
<td>SLRPs</td>
<td>small leucine rich proteoglycans</td>
</tr>
<tr>
<td>SYSADOA</td>
<td>symptomatic slow-acting drugs in OA</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
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<tr>
<td>Tol Blue</td>
<td>toluidine blue</td>
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<tr>
<td>TPA</td>
<td>tibial plateau angle</td>
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<tr>
<td>ul</td>
<td>microlitre</td>
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<tr>
<td>um</td>
<td>micrometers</td>
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<td>β-actin</td>
<td>beta actin</td>
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PREFACE

This study was conducted to investigate the influence of knee osteoarthritis on other important joint structures, specifically the anterior cruciate ligament, and determine what effect the decline in ovarian hormones associated with menopause has on ligament tissue. It was conducted concurrently with another study investigating cartilage changes in ovariectomised and meniscectomised sheep. The author was involved both studies, including all surgical procedures and tissue harvesting, and is personally responsible for all ligament investigations presented in this thesis. Prof Rick Read and his research team, in co-operation with the Raymond Purves Bone and Joint Research Laboratories (Royal North Shore Hospital, Sydney) generated the cartilage results, some of which have been included to confirm the occurrence of osteoarthritis as induced by the meniscectomy model.

For efficiency, and to minimise the number of animal subjects used, several hypotheses were tested within the design of a single large trial. The reader is initially provided with a general introduction relevant to all subsequent chapters (Chapter 1) followed by details of the methods and materials used in the study (Chapter 2). The thesis then discusses the effect of each treatment individually by chapter, first investigating the effect of osteoarthritis alone (Chapter 3), and then the effect of ovariectomy with or without concurrent osteoarthritis (Chapter 4). Chapters 3-4 are presented each with their own specific introductory information, results and discussion, while the combined results, providing the opportunity to compare and contrast the results from all test groups, are available to view in the appendices. For each method of investigation, all samples from the four groups were processed as a single batch to avoid variation between groups and therefore allow them to be directly compared. Finally, Chapter 5 presents a review of all hypotheses and potential areas for future research are discussed.
CHAPTER 1

GENERAL INTRODUCTION TO CONNECTIVE TISSUE AND THE CRUCIATE LIGAMENTS

1.1 CONNECTIVE TISSUE

There are four traditional classes of living tissue which together comprise the human (or animal) body, namely epithelial, muscle, nervous and connective tissue \[1, 2\]. Of these, connective tissue encompasses the widest variety of tissues, exhibiting significant differences in structure, form and function. The main characteristic shared by all types of connective tissue is that it consists of cells surrounded by an extracellular matrix of fibres, fluid and ground substance. Variations in the characteristics of this extracellular matrix provide a basis for further classification of connective tissue into subgroups and reflect the functional requirements of each type of connective tissue \[1-4\].

In general, the key functions of the connective tissues are to provide a fibrous container for soft tissues, to function as a medium for the transmission of mechanical forces, to provide structure and support to the body, to provide transport for substances such as nutrients, oxygen and hormones around the body \[3, 5\].

1.1.1 Classification of Connective Tissues

Connective tissue is further classified into subgroups based on differences in the structure of the extracellular matrix and its functional properties. Differentiation of connective tissue into subgroups can be difficult due to a degree of overlap between characteristics of the many tissues which fall into this category. Therefore, any classification system is somewhat subjective and open to dispute. The following is one
The three main subgroups of connective tissue may be described as: embryonic connective tissue, connective tissue proper and specialised connective tissue [1, 3, 6].
1.1.1.1  *Embryonic Connective Tissue*

Embryonic connective tissue is derived from the mesoderm, which is the middle layer of the trilaminar germ disc of the embryo. Almost all of the connective tissues of the body originate from this embryonic germ layer. An exception to this is the head where some progenitor cells are derived from the ectoderm \[^3, 6\].

The formation of connective tissue begins through proliferation and migration of the mesodermal cells and specific neural crest cells. This leads to the formation of a primitive connective tissue known as mesenchyme \[^1, 3\]. Mesenchyme is primarily found in the embryo and consists of small spindle-shaped, as yet unspecialised (pluripotent) cells. The mesenchymal cells are progenitors of the various types of connective tissues as well as other tissues such as muscle, and the vascular system \[^7, 8\]. The cells are loosely packed and suspended in a viscous extracellular matrix. Extending from these cells are processes which enable contact between neighbouring cells via gap junctions, thereby forming a cellular network. Fine reticular (collagen) fibres are sparsely dispersed throughout the mesenchyme \[^3, 6\].

Mesenchyme is one of two embryonic tissue subtypes. The other subtype is mucous connective tissue which is found in the umbilical cord. It is similar to mesenchyme but more space exists between cells and fewer reticular fibres are present. Mucous connective tissue consists largely of a specialised type of extracellular matrix which has a gelatinous or mucous-like consistency. The ground substance of this extracellular matrix is also known as 'Wharton's jelly' \[^1, 6\].
1.1.1.2 Connective Tissue Proper

This connective tissue subtype is comprised of cells and an extracellular matrix consisting of protein fibres and a gelatinous or viscous ground substance \[\text{[2, 3, 5]}\]. Within this category the tissues can be further divided into two subtypes:

1.1.1.2.1 Loose Connective Tissue (or Areolar Tissue)

This is a cellular connective tissue in which the protein fibres within the extracellular matrix are thin and form a sparse meshwork. There are numerous spaces between the cells and fibres, which are filled by an abundance of viscous ground substance \([2-4]\). This ground substance serves as a medium for diffusion of oxygen & nutrients between blood vessels and connective tissues, and facilitates the exchange of waste products such as carbon dioxide and metabolites. For this reason, loose connective tissue is often found surrounding small blood vessels \([2, 3]\). Another function of this subtype is as a loose space-filling or packing material between organs or other structures in the body. It permits motion to occur between structures while still providing support due to its ability to shorten and elongate when necessary (within limits) \([4, 5]\), and the viscous nature of its ground substance also facilitates the free migration of many cell types such as fibroblasts lymphocytes and macrophages \([3, 4]\).

1.1.1.2.2 Dense Connective Tissue

This subtype contains an abundance of fibres which are packed tightly together, and it has fewer cells and ground substance than loose connective tissue. A majority of the fibres present are collagenous, as well as some reticular and elastic fibres. Its main functions are to support and connect body parts \([1, 2]\). Dense connective tissue can be further divided in two subcategories:
Dense Irregular Connective Tissue – in this tissue type the abundant fibres are arranged in a random meshwork. The cells present are typically fibroblasts and are relatively few in number, with little ground substance surrounding them. This tissue tends to occur in sheets and is resilient, able to withstand pulling forces exerted in many different directions. Examples include the dermis of the skin, the pericardium surrounding the heart and the submucosa of the intestinal tract [1-4]. This type of connective tissue is also produced in the development of scars [9].

Dense Regular Connective Tissue – like dense irregular connective tissue, fibres are the prominent feature of dense regular connective tissue and there are fewer cells and little ground substance present. The major difference between these two types of dense connective tissue lies in the arrangement of their fibres, with the fibres of dense regular connective tissue being arranged in a parallel manner, and are very densely packed into fibre bundles. This arrangement provides the tissue with great tensile strength and enables it to withstand the greatest forces along the axis of its fibres, reflecting the mechanical requirements of the tissue. Fibroblasts, which produce and maintain the fibres, are positioned between the fibre bundles. This tissue tends to occur as thick bands or cord-like structures. Examples of dense regular connective tissue are the tendons joining muscle to bone, and the ligaments which join bone to bone [1-4].

1.1.1.3 Specialised Connective Tissue

This category encompasses the more individual or atypical connective tissues, which serve various important and specific functions throughout the body. Like all connective tissues, they consist of cells surrounded by a non-living extracellular matrix of fibres and ground substance, while also possessing certain individual structural characteristics enabling them to perform a specific function [6]. Blood and bone are two forms of
specialised connective tissue with significant structural differences. Blood has a fluid ground substance which allows it to travel around the body and function as a transport medium for nutrients, wastes, respiratory gases, hormones etc, while in contrast bone has ground substance which is completely mineralised and rigid, thereby providing exceptional support and protection for other body structures \(^{[2, 6]}\). Specialised connective tissues also contain varying cell types depending on their location, and each tissue type has some fibres (collagenous, elastic or reticular) present but to varying degrees \(^{[1-3]}\). Types of specialised connective tissues found in the body are adipose tissue, blood, bone, cartilage, lymphatic tissue and haemopoietic tissue \(^{[3]}\).

1.2 COMPOSITION OF LIGAMENT

As previously discussed, ligament is a form of dense, regular connective tissue. Most ligaments are found in the musculoskeletal system where they join bone to bone \(^{[3, 10]}\), although the body also contains a number of suspensory ligaments which support various organs in the body such as the eyeball and ovary \(^{[6, 11]}\). Ligaments of the musculoskeletal system function across joints with both a wide range of motion such as the hip, and with very little movement such as the sacroiliac joint \(^{[10, 11]}\). They are named based on their bony attachments, their gross function, their position in relation to the joint, their shape and their arrangement with respect to other ligaments \(^{[5, 10]}\).

In addition attaching articulating bones to one another, skeletal ligaments serve a number of other roles which are important to correct joint function. They are responsible for accurately guiding the movement of joints and restricting joint movement to within set limits, and often contain mechanoreceptors that act as position
sensors for joints. Ligaments also provide maintenance of joint congruency and stability, therefore preventing wear and tear of the articulating surfaces [11].

Grossly, skeletal ligaments vary in shape, length and thickness depending on their location. They appear as glossy white, firm, fibrous tissue which may be arranged in bands, sheets or thickened strips of joint capsule and are composed of a complex extracellular matrix (ECM) of fibres and ground substance, with cells interspersed throughout. The composition of the ECM may vary slightly between ligaments, or even between different regions of the same ligament [10-12].

As ligament (in particular the cruciate ligaments of knee) is the focal tissue of this project, the individual structural components of this connective tissue will be discussed in further detail.

1.2.1 The Cells of Ligament

It is the specific cell populations present which give each connective tissue type its unique characteristics. In the case of ligament, the predominant cell type is the fibroblast [10, 13-15]. The morphological appearance of the fibroblast may vary and it is thought that the shape variation represents different metabolic states of the same cell [14]. Most often it appears as an elongated or spindle-shaped cell [3, 5, 8], but may also appear as ovoid or spheroid. Many fibroblasts have very long, thin and sheet-like cytoplasmic processes which extend between the collagen fibrils of the ECM [3, 10]. During periods of active growth or repair, the cytoplasm of the cell is more extensive with an abundant rough endoplasmic reticulum (ER) (Figure 1.2). The nucleus is either elliptical or disc-shaped and may have multiple nucleoli [1, 10].
Fibroblasts are responsible for the formation of the collagen, elastic and reticular fibres and complex carbohydrates found in ground substance (Figure 1.3) [8, 11]. They are solitary cells and are found anchored to the parallel collagen fibrils [13], but also have the ability to migrate through the connective tissue, especially in response to trauma [5]. In addition, the fibroblast is responsible for the maintenance process during which it enzymatically breaks-down and removes old collagen from the matrix [8]. During periods of inactivity where the cell is completely surrounded by matrix, it may be termed a fibrocyte [6, 12].
The concentration of cells synthesising new matrix is naturally higher in younger ligaments or in ligaments undergoing repair, however with increasing age, the fibroblasts become less active. Despite this they do retain the ability to grow and synthesise new matrix elements throughout life, with each individual fibroblast being capable of producing all of the components found in the ECM \cite{10}. This ability to regenerate is essential for maintaining structural and functional integrity of the ligament, thus preventing joint instability.
While ligament has relatively few cells compared with most other tissues, the cells do not exist in isolation from one another. Resting fibroblasts (fibrocyes), have a spider-like appearance, with long cell processes that join adjacent cells together in an extensive interconnected cellular network [16] (Figure 1.5). Located on the cell bodies and their processes are special gap junctions, which allow communication between adjacent fibrocytes via diffusion of small molecules (<1000Da) such as metabolites, ions, second messengers, water and electrical impulses between cells [17-19].

At the gap junction are proteins called connexins which mediate cell to cell coupling. Six connexin subunits join together to form a conduit known as a hemi-channel (also called a connexon) in the plasma membrane. This can dock with another connexon in the plasma membrane of an adjacent cell, thereby forming a complete gap junction channel [17, 18] (Figure 1.4). Currently there are 21 known connexin genes in the human genome [18, 20]. Of these, connexin 43 (Cx43) is the most ubiquitous, being found in 46 different tissue types in the body, including ligament. However, Cx43 is not the only connexin found in ligament, with a second type Cx32 also being isolated, suggesting that there are two distinct communication networks present [16, 17]. The ability of cells to communicate via gap junction coupling is essential for maintaining ligament homeostasis and its ability to remodel in response to mechanical stimuli [19, 21].

Figure 1.4: Schematic drawing of gap junction channels. Each apposed cell contributes a hemi-channel to the gap junction. The six connexin subunits may change configuration to allow the channel to open or close the hemi-channel. (Image from Sohl & Willecke, 2004.)
Figure 1.5: (TEM 850x) Transmission electron micrograph of sheep anterior cruciate ligament, showing the interconnection of fibroblasts and fibrocytes via long cellular processes to form a cellular network (arrow heads). (Image A. Daniel).
In addition to the fibroblast, several other cell types may be present in ligament in low numbers. One such cell type is the chondrocyte, a cell normally native to cartilage, but which is also found in certain ligaments such as the anterior cruciate ligament (ACL) near its tibial insertion where the ligament consists of fibrocartilage \(^{[13]}\). Endothelial cells may also be found in ligament due to the presence of small blood vessels and nerve cell processes \(^{[10]}\).

### 1.2.2 The Extracellular Matrix

The ECM is a complex, non-living structural network that is secreted by and surrounds the widely spaced fibroblasts, and gives each connective tissue its physical qualities. The ECM of ligament is strong and fibrous, yet flexible and in some cases elastic. It consists of protein fibres, water and a proteoglycan rich ground substance. Together these elements allow the ligament to withstand the tensile forces applied to it \(^{[1, 12]}\).

#### 1.2.2.1 Fibres

In dense regular connective tissues such as ligament, the fibres are the predominant feature \(^{[3]}\). There are three main types of fibres present in connective tissues; collagen, elastic and reticular \(^{[2, 4]}\). Reticular fibres, common in loose connective tissue, are not typically found in ligament: therefore, only collagen and elastic fibres will be discussed further.

##### 1.2.2.1.1 Collagen Fibres

Collagen is the predominant macromolecule of the ECM of ligament, providing it with its form and high tensile strength. It is estimated that collagen contributes 70-80% of the dry weight of ligament \(^{[22]}\).
Collagen is a form of secretory protein produced by fibroblasts. Each collagen molecule is actually a right-handed triple-helix of three polypeptides known as α-chains\textsuperscript{[11, 23]}. The amino acids that make up the α-chain generally follow a particular sequence, in which every third amino acid is a glycine. This glycine is usually followed by a proline, which is succeeded by either a hydroxyproline or a hydroxylysine. In short it is a repeating sequence of glycine – \(\chi\) – \(\gamma\) [where \(\chi\) = proline, \(\gamma\) = hydroxyproline or hydroxylysine]. This repeating sequence is essential for the triple-helix formation\textsuperscript{[8, 11, 24]}. Weak covalent intramolecular cross-links are present between the three α-chains of the triple-helix (Figure 1.6). These cross-links contribute to the strength of the tissue\textsuperscript{[11, 25]}.

![Figure 1.6](Image A. Daniel)

The triple-helix molecules are also known as tropocollagen, and are aligned head to tail in staggered rows (overlapping by one quarter of a molecule), to form a collagen fibril (Figure 1.7). Gaps are present between the tropocollagen in each row. Strong covalent intermolecular cross-links occur between the adjacent rows of tropocollagen, rather than between the heads and tails of the molecules in a row. These cross-links are formed by enzymatic reactions between the amino acids lysine and hydroxylysine. This specific staggered arrangement of tropocollagen imparts the greatest mechanical and
biochemical stability to the fibril \cite{8, 11, 25}. The intramolecular and intermolecular cross-links are also essential for imparting strength and stability to the ligament tissue. Without these cross-links, the tissue would be extremely weak and friable \cite{8, 25}.

The assembly of collagen fibres is complex, with a number of recognisable hierarchical levels of organisation between the tropocollagen and the fibril (Figure 1.8). These are outlined below in ascending order:

**Microfibril** – tropocollagen molecules are tightly packed into groups of 5, forming microfibrils. These are approximately 3.5nm in diameter.

**Subfibril** – the microfibrils assemble together to form subfibrils of 10-20nm.
Fibrils — are formed from the aggregation of subfibrils. They have a diameter of 50-500 nm. It is these fibrils which ultimately go on to form the collagen fibres [12, 14, 23].

The production of fibrillar collagen involves a series of intracellular and extracellular events. It begins within the cell, where the polyribosomes of the rough ER produce the initial polypeptide chains mediated by messenger ribonucleic acid (mRNA). The newly synthesised polypeptides are then released simultaneously into the cisternae of the rough ER. It is here, and in the Golgi apparatus, that a number of post-translational modifications are made to the polypeptides, including the congregation of three polypeptides to form a triple-helix molecule. These modifications lead to the production of procollagen, the precursor of the collagen molecule.
The procollagen is packed into secretory vesicles and is moved to the outside of the cell via exocytosis. During its movement out of the cell, it comes into contact with procollagen peptidase, an enzyme associated with the cell membrane. This enzyme cleaves the uncoiled ends of the procollagen molecule, resulting in a collagen molecule (or tropocollagen). It is outside the cell that the tropocollagen molecules are organised into microfibrils, subfibrils, fibrils and finally fibres [3, 11, 22].

To date, there are at least 27 different types of collagen in the body, with new types being discovered regularly [24]. Each collagen type is designated a Roman numeral, reflecting the chronological order in which they were discovered [11]. The different types of collagen arise from differences in the $\alpha$-chains that make up the triple-helices. Collagen I, for example, consists of 2 identical $\alpha_1$ chains, and 1 different $\alpha_2$ chain. It is designated the collagen nomenclature $[\alpha_1(I)]_2 \alpha_2(I)$. Using collagen III as a further example, this consists of three identical $\alpha_1$ chains, and so is designated $[\alpha_1(III)]_3$. Each collagen type has its own unique characteristics, functions and locations throughout the body [24].

Collagen types I, II and III are the most abundant in the musculoskeletal system, with collagen I being the most common type by far. In fact, type I collagen constitutes approximately 90% of the body's total collagen, being present in skin, bone, dentin, sclera, fascia, tendon and ligament [11, 13]. As much as 90% of the collagen of ligaments is type I collagen, which is the principal tensile-resistant collagen. Less that 10% is type III collagen, which is found in the loose connective tissue that separates the type I collagen bundles. Collagen types V, VI, X, XII may also be present in small amounts [15, 25, 26]. Type II collagen normally does not occur in ligament, it is typically found in
cartilage. However, type II collagen has been identified in the fibrocartilaginous zones of some ligament insertions [13].

1.2.2.1.2 Elastic Fibres

Ligament may also contain elastic fibres, which allow the tissue to stretch and distend. Most ligaments contain less than 5% elastic fibres by dry weight, but a few have higher concentrations, and so are known as elastic ligaments, e.g. the nuchal ligament and the ligamentum flavum [4, 11]. Elastic fibres are produced by the same cells that produce collagen, though they are typically thinner in diameter and have a different structural composition to collagen. They are arranged in a branching pattern to form a three-dimensional network that is interwoven with the collagen fibres to limit the distensibility of the tissue [2, 6].

Elastic fibres are comprised of a central core of elastin with surrounding fibrillin microfibrils. The elastin is rich in glycine and proline like collagen, but is poor in hydroxyproline and lacks hydroxylysine [3]. Elastin may form layers or fibres of variable thickness. When not under stress, elastin fibres assume the confirmation of random coils. These coils allow them to undergo a degree of deformation without rupturing or tearing, and then return to their original size and shape when unloaded [10, 11]. The elastin itself is a highly insoluble and stable protein due to its extremely hydrophobic nature [11].

The fibrillin component of elastic fibres is a glycoprotein that forms fine microfibrils. These microfibrils are formed first, and then the elastin is deposited onto their surface. The absence of these fibrillin microfibrils during elastogenesis results in the development of elastin sheets rather than fibres [1, 3]. The synthesis of elastin by
fibroblasts parallels that of collagen and both processes may occur simultaneously in the cell. The procollagen and proelastin (the precursor to elastin) molecules remain separate throughout the series of synthetic events, due to the activity of signal peptides. These are built into the beginning of the polypeptide chains of each molecule [3].

1.2.2.2 Water

Approximately 60-80% of the wet weight of most ligaments is water [10, 25]. Water fills the spaces between the cells and the macromolecular network of fibres and ground substance. The presence of water in ligament is important for allowing oxygen, nutrients and metabolites to diffuse through the extracellular matrix to the ligament cells. This is essential because the cells may lie at some distance from the blood vessels and water provides a medium through which cellular communication can occur [10, 27]. Water is attracted to the extracellular matrix due to the presence of hydrophilic molecules called glycosaminoglycans in the ground substance [3].

1.2.2.3 Ground Substance

Ground substance is the component of the ECM that fills the space between the cells and fibres. It initially appears to be a substance with little morphological structure, but in fact contains molecules that are highly structured and complex combinations of polysaccharides and proteins. The consistency of ground substance ranges from fluid to calcified, depending on the amount of water present. The ground substance of ligament tends to be quite viscous to gelatinous in texture [5, 27].

Ground substance is not usually visible when viewed microscopically, as it is lost in the fixation and dehydration processes in routine H&E preparations [2]. Despite its outwardly amorphous appearance it serves a number of important roles. Its molecules
attract and hold water which, as mentioned previously, facilitates the diffusion of gases and nutrients, and allows cellular communication. It acts as a barrier to certain large molecules such as bacteria while allowing the passage of smaller, water soluble molecules \[^1\]. It also contributes to the mechanical properties of the tissue, especially in compressive loading and viscoelasticity. In addition to these roles, the ground substance helps to order, support and lubricate the other tissue components \[^11\].

1.2.2.3.1 Proteoglycans

An important component of ground substance is the non-collagenous molecules called proteoglycans. Proteoglycans contribute only a small portion of the framework of ligament, usually less than 1% of the total dry weight, but they play an important role in the organisation of collagen fibres and interaction with tissue fluid \[^10, 22\]. In addition, proteoglycans interact with the collagen fibres to yield the viscoelastic properties of the ligament \[^12\] and are involved in the lubrication of ligaments \[^25\].

Proteoglycans represent a special class of glycoproteins that are heavily glycosylated. They can be very large macromolecules comprising a core protein to which one or more glycosaminoglycan side chains are covalently bound \[^2, 12\]. Glycosaminoglycans (GAGs) are long chained polysaccharides of repeating disaccharide units. They are so named for the hexosamine sugar glycosamine, which is present in each disaccharide \[^3, 11\]. GAGs possess a high number of negative charges that repel one another, therefore enabling the proteoglycans they form to extend through a greater volume than if they were uncharged. This negative charge also allows the GAGs to interact with positively charged molecules such as water. As a result, proteoglycans have a high affinity for water, which determines the viscosity of the ECM and maintains the correct homeostatic environment for the cells of the ligament tissue \[^1, 11\].
GAGs form the major carbohydrate component of all proteoglycans. Currently a family of 7 distinct GAGs are recognised \[3\]. They are:

1. Keratan sulfate
2. Chondroitin 4-sulfate
3. Chondroitin 6-sulfate
4. Dermatan sulfate
5. Hyaluronic acid
6. Heparan sulfate
7. Heparin

Of these, keratan sulfate, chondroitin sulfate, dermatan sulfate and hyaluronic acid predominate \[1, 5\]. Keratan sulfate is a GAG that is commonly found in bone, cartilage and the cornea, while chondroitin sulfate provides support and adhesiveness in bone, cartilage, skin and blood vessels and dermatan sulfate is found in the ground substance of tendons, ligaments, heart valves and blood vessels \[1\]. Hyaluronic acid is a viscous slippery substance that binds cells together and lubricates joints. Unlike the other GAGs, hyaluronic acid is not directly associated with the proteoglycan's protein core \[2\]. Instead, special link proteins indirectly bind proteoglycan monomers to the hyaluronic acid. It also differs from other GAGs because it is a much longer and more rigid molecule, being composed of a carbohydrate chain of thousands of sugars, rather than the hundreds (or less) of other GAGs \[5\].

Like collagen and elastin fibres, the proteoglycans of ligaments are produced by the fibroblasts. The process involves the synthesis of a protein core by the fibroblast's rough ER. Extension and sulfation of the GAG chains then commence in the smooth
ER and are completed and transported in the Golgi apparatus. The completed chains are
finally released into the ECM by the fibroblast where they assemble with the protein
core to form a proteoglycan monomer. Proteoglycans also contain within their
structure, a number of binding sites for growth factors such as transforming growth
factor $\beta$ (TGF-$\beta$). The binding of such growth factors to proteoglycans leads to either
their local aggregation or dispersion. This can in turn inhibit or enhance the movement
of migrating macromolecules or micro-organisms in the ECM \cite{3}. There are two known
classes of proteoglycans; small leucine rich proteoglycans, and large aggregating
proteoglycans.

Small Leucine Rich Proteoglycans (SLRPs) assist in the formation, organisation and
stability of the ECM, including the formation and diameter of the collagen fibrils during
fibrillogenesis. They tend to lie directly on the surface of the collagen fibrils \cite{28, 29}. A
distinguishing feature of SLRPs is the presence of a central domain containing 6-10
leucine-rich repeats in their protein core. It is this domain that is responsible for the
functional activity of proteoglycans \cite{30-32}. In addition to their role in fibrillogenesis,
SLRPs also protect the collagen fibrils from degradation by collagenases by limiting
access to their cleavage site \cite{33}. Four principle SLRPs found in ligament are decorin,
biglycan, fibromodulin and lumican.

Decorin and biglycan (which have the highest homology) and are both class I SLRPs.
These proteoglycans contain an N-terminal domain that is usually substituted with
either one (decorin) or two (biglycan) dermatan/chondroitin sulfate side chains and 10
leucine-rich repeats \cite{34, 35}. Decorin is the most abundant proteoglycan in the matrix of
ligament. It is estimated that it constitutes as much as 90% of the total ligament
proteoglycan content [36, 37]. Decorin is involved in several biological functions including modulation of cell adhesion, regulation of growth factor activity and inhibition of fibroblast proliferation [38-40]. It plays an important role in regulating collagen fibril organisation by retarding the rate and degree of collagen fibrillogenesis and delaying fibril maturation [41, 42]. It limits the diameter of collagen fibrils produced [40, 43], and as the GAGs of decorin extend laterally from adjacent collagen fibrils, it also assists in maintaining fibrillar spacing and prevents lateral fusion of fibrils [44]. Decorin preferentially binds to collagen types I and II collagen fibrils but can also bind to types III, VI and XIV [41]. Collagen fibrils may be linked together through the binding of GAG chains of adjacent decorin molecules resulting in a collagen-decorin-decorin-collagen linkage. While the structure of decorin holds the collagen molecule tightly within its concave surface, the interaction between adjacent GAGs is not as strong as that between the collagen and decorin’s core protein. Therefore the bond between decorin molecules is possibly the mechanically weakest point between collagen fibrils, and may be a breaking point during stretching [44].

Biglycan is closely related to decorin and the two compete for the same binding site on collagen I fibrils [31, 45]. Like decorin, it is involved in matrix organisation and regulation of fibrillogenesis, and can interact with a variety of other proteins and growth factors [30, 37]. Biglycan is normally present in ligament at lower levels than decorin [15, 36], but is increased during ACL healing when it contributes to the rate of collagen fibril formation [37, 46, 47]. During this time decorin expression may be reduced, suggesting the two SLRPs have opposite expression patterns and play contrasting roles in ligament [48]. In addition, biglycan plays a role in the events of healing through regulation of cell migration, adhesion, signal transduction and proliferation [36, 38].
Lumican and fibromodulin are termed class II SLRPs. Members of this class share an identical cysteine-rich region before the leucine rich repeats, and contain clusters of tyrosine sulfate residues in contrast to the N-terminal region of class I SLRPs. They are primarily substituted with keratan sulfate chains \cite{34, 35}. Lumican can bind to type I collagen and is normally expressed early in fibril development, with expression decreasing by 60-70% as fibrillogenesis progresses to the later stages \cite{49}. It regulates fibrillogenesis with other SLRPs such as decorin and fibromodulin \cite{31}, and modulates the assembly of collagens into higher order fibrils \cite{50}. Like decorin, it acts to inhibit collagen type I fibrillogenesis, reduces lateral fusion of fibrils, and limits ultimate fibril diameter, however the two SLRPs bind to separate site on the collagen fibril surface \cite{43}. It is a key modulator of ligament strength and enhances collagen fibril stability \cite{51, 52}, as well as functioning in wound healing and inflammatory responses \cite{50}.

Fibromodulin shares a common binding site with lumican \cite{52, 53}. It is required for the maturation of collagen fibrils and it works in co-ordination with lumican in fibril assembly \cite{31}. Fibromodulin contains up to four keratan sulfate chains and maintains a substantial interaction with the formed fibrils \cite{51}. It has a higher affinity for the binding site than lumican and the two SLRPs have differential expression patterns during normal ligament development \cite{49, 53, 54}. As fibrillogenesis progresses, lumican decreases to barely detectable levels whereas fibromodulin increases significantly indicating a more prominent regulatory role during the later stages, and determination of the mature fibril’s structural phenotype \cite{43, 53}.

*Large Aggregating Proteoglycans LAPs* - maintain water within the tissue due to their long chains of negative charges. In doing so, they contribute to the mechanical properties of the tissue such as the ability to withstand compressive loading. The
proteoglycans swell as they attract water until they reach their maximum expansion and are restrained by the collagen fibril network, filling the regions between the collagen fibrils \[10\]. Ligament however, does not contain appreciable amounts of the huge proteoglycan aggregates that are characteristic of cartilage. This is due to the fact that ligament is more subject to tensile forces rather than compressive loading \[5, 15\]. More recently, LAPs have also been identified as acting as a lubricant between collagen fibres due to their hydrophilic nature \[15\]. This class of proteoglycans tends to contain the GAGs chondroitin and keratan sulfate. Aggrecan and versican are two primary examples of LAPs.

1.3 THE CRUCIATE LIGAMENTS

1.3.1 Structure and Function

The cruciate (meaning crossed) ligaments are the main stabilisers of the knee joint \[13\]. Their anatomy and function in humans is shown to be comparable to a number of animal species such as dogs \[55, 56\], sheep \[57\], goats \[58\], pigs \[59\], and rabbits \[60\]. This similarity across species is invaluable as it permits animal models to be utilised in the study of knee joint disease in humans, as well as facilitating the crossover of research from veterinary and medical disciplines.

The knee joint contains two cruciate ligaments that course between the tibia and the femur (Figure 1.9). They are known as the anterior cruciate ligament (ACL) and posterior cruciate ligament (PCL) in humans \[13, 61\], and the cranial cruciate ligament (CCL) and caudal cruciate ligament (CaCL) in animal species such as the dog \[55, 56, 62\]. In this discussion it should be assumed that comments relating the ACL and PCL in humans also pertain to the CCL and CaCL in animals unless otherwise stated.
Both cruciate ligaments are intra-articular but extra-synovial structures as they are covered by a mesentery-like fold of synovium that is thought to protect the ligaments from the synovial fluid [13, 63, 64]. The PCL is the strongest ligament of the knee joint with approximately twice the strength of the ACL, and rupture of this ligament is less common [65, 66]. The superior strength of the PCL most likely stems from its larger cross-sectional area and its extensive femoral attachment [66, 67]. The main function of the PCL is to prevent the posterior dislocation of the tibia in relation to the femur [65], also
referred to as tibial posterior tibial draw \[^{66}\]. The PCL is made up of two functional fibre bundles known as the anterolateral (AL), and the posteromedial (PM), which become taught at different angles of knee flexion \[^{67}\]. The AL bundle attaches to the femur at the roof of the femoral intercondylar notch, while the PM bundle attaches to the femoral intercondylar notch on the medial side. Attachment of both bundles to the tibia occurs at the posterior intercondylar fossa \[^{55, 66}\].

Ligaments are attached to bones via direct or indirect insertions (or entheses), with direct insertions being the most common. With indirect insertions, the ligament passes obliquely along the surface of the bone. They tend to cover more bone surface than direct insertions and their boundaries are difficult to define \[^{11, 68}\]. Direct insertions are where the ligament seems to pass directly into the bone cortex. These regions are more easily defined and possess four morphologically distinct layers, namely ligament, uncalcified fibrocartilage, calcified fibrocartilage and bone. There is a gradual transition through these layers at direct insertions. The cruciate ligaments of the knee attach to the bone via direct insertion \[^{13, 68, 69}\]. Ligaments transfer force to and from the skeleton, and distribute the loads applied to them dynamically in order to execute movement patterns. Stress is concentrated in the region where they attach to bone, and these areas may be vulnerable to acute or overuse injuries \[^{70}\].

The ACL is the most significant ligament of interest in this study. It is the smaller of the two cruciate ligaments and is much more prone to injury. In both humans and dogs, ACL injury is well reported to be one of the most common causes of pelvic limb pain and joint instability \[^{64, 71}\]. The ACL crosses the knee joint from the medial aspect of the lateral femoral condyle to the anterior aspect of the middle portion of the tibial plateau (also referred to as the intercondylar area) \[^{72, 73}\]. Its main functions are the resistance of
anterior translation of the tibia with respect to the femur (anterior draw) and to limit hyperextension of the knee joint. Secondary functions include resistance of internal rotation of the tibia during flexion of the knee, and resistance of valgus rotation of the tibia. It also plays a minor role in preventing medial displacement of the tibia along with the collateral ligaments [64].

Like the posterior ligament, the ACL is separated into two functional bundles, known as the anteromedial (AM) and the posterolateral (PL) based on the anatomic positions of the fascicular attachments of the tibial insertions. Observations have shown the fibres of the AM bundle originate most anteriorly on the femoral side and insert anteriorly and medially at the tibial point of attachment. Therefore, as the name suggests, the fibres of the PL bundle course from the posterior part of the femoral attachment to the posterior and lateral aspect of the tibial attachment site. Furthermore, the two bundles spiral 90 degrees laterally between their attachments [74]. The AM and PL bundles are thought to have reciprocal functions, as the AM bundle is taut during flexion of the knee, and the PL bundle is taut in extension [75-77]. From a functional perspective, the AM bundle is the primary restraint against anterior tibial translation, while the larger PL bundle serves to stabilise the knee against rotary loads when the knee is near full extension [71, 78].

Within these bundles are fascicles of wavy or undulating collagen fibres with a complex ultra-structural organisation and abundant elastic system, making it different to most other ligaments and tendons [72, 79]. The ACL is primarily comprised of type I collagen fibrils, orientated parallel to the longitudinal axis of the ligament, and which are chiefly responsible for the ligament’s tensile strength [13]. Type II collagen, although not typically found in ligament, is present in the ACL within the fibrocartilaginous regions of its tibial and femoral sites of attachment [64]. Type III collagen is found throughout
the ACL in the loose connective tissue dividing the type I collagen bundles, and in higher concentrations near the ligament’s attachment sites \[13, 64\]. Collagen types IV and VI may also be found in ACL in low concentrations. Like other ligaments, the ACL contains GAGs and glyco-conjugates such as laminin, tenascin and fibronectin, which function to attract key elements in normal, healing and growing tissues. Finally, ACL contains elastic components which protect the ligament from damage during times of extreme tension throughout normal joint motion \[73\].

### 1.3.2 Blood Supply and Innervation

Compared to other connective tissues such as skin and bone, ligament is poorly vascularised \[62\]. Major blood supply to the cruciate ligaments comes from branches of the middle genicular artery (MGA) which arises from the anterior aspect of the popliteal artery \[13, 64\]. The distal regions of both cruciates are also supplied by branches of the middle and lateral inferior geniculate arteries \[62, 68\]. During its extra-capsular course, the MGA is immersed in the fat of the popliteal space. It then pierces the posterior capsule of the joint, passing through an aperture in the oblique popliteal ligament \[64, 68\]. Within the joint the artery branches into the soft tissues lodged in the intercondylar notch such as the ACL and PCL.

The cruciate ligaments themselves are covered by a layer of loose connective tissue called synovium where the blood vessels penetrate and form an epiligamentous network \[14\]. Anastamoses exist between the extra and intra-ligamentous blood networks where the epiligamentous blood vessels penetrate the transversely into the cruciate ligaments \[62\]. The blood vessels penetrate the ligament substance via small diameter vascular channels which lie parallel to the collagen bundles \[80\]. No intra-ligamentous vessels transverse the femoral or tibial attachment site of either cruciate ligament \[64\]. In
addition to this vascular contribution, the cruciate ligaments may also receive
nourishment by passive permeation from the synovial fluid \cite{80, 81}.

Blood vessel distribution within the cruciate ligaments is not homogenous \cite{73}. The mid
section or central core of both the ACL and PCL is generally less vascular than the
proximal and distal ends \cite{14}. Furthermore, blood supply to the central core of the ACL
is further compromised by pressure exerted by the PCL as the two ligaments twist upon
each other during the course of normal joint movement. Research has shown blood
supply to the ACL in general tends to be less than the PCL, possibly contributing to the
poor healing capacity of this ligament \cite{13}. A number of avascular regions have also
been identified within the cruciate ligaments, including the fibrocartilaginous zones of
the femoral and tibial insertion sites of the ligaments, as well as a region located in the
distal zone of fibrocartilage adjacent to the roof of the intercondylar fossa \cite{73}.

The nerves supplying the cruciate ligaments come from the saphenous, tibial and
common peroneal nerves. The main trunk of the nerve bundle can be found at the
femoral end of the cruciates where greater numbers of mechanoreceptors are located \cite{62}.
Within the cruciate ligaments the nerves course alongside the blood vessels in the
interfascicular areolar spaces \cite{62}. Their function is thought to be primarily associated
with the autonomic nervous regulation of blood supply to the ligaments and in the
perception of pain signalling injury \cite{82}. Free nerve endings have been discovered in the
cruciates which are activated by an increase in ligament tension. This sensory
mechanism provides the central nervous system with information about proprioception
and when stimulated, these nerve endings signal to muscles to adjust their tension and
stabilise the joint, preventing possible injury \cite{83}.
1.3.3 Cruciate Ligament Rupture

The ACL is one of the most frequently injured ligaments of the knee\textsuperscript{[84, 85]}. Together with the MCL it accounts for 95% of all multi-ligament injuries in the knee joint, leading to significant functional impairment, joint instability and degenerative damage\textsuperscript{[86]}. To date, the precise pathophysiology of ligament rupture is not known but is thought to be multifactorial, with joint inflammation, mechanical loading, musculature, gender, age, ligament microinjury and ischaemia all considered to be contributing factors\textsuperscript{[87]}.

1.3.3.1 Inflammation

Inflammatory changes, involving multiple knee joint structures such as the synovial membrane, epiligament and synovial fluid, are typical in patients with ACL rupture\textsuperscript{[88]}. It is not known whether this inflammation precedes or follows ligament rupture, but it has been hypothesised that in dogs it develops in the early phase of cruciate disease\textsuperscript{[89]}. It is well established that rupture of the cruciate ligaments, in particular the anterior ligament, leads to joint instability and subsequent development of osteoarthritis (OA) with associated inflammation\textsuperscript{[90]}. Even if the ligament is not completely ruptured, degeneration of normal ligament structure causes increased laxity and can lead to OA\textsuperscript{[89, 91-94]}. While we know in human subjects OA and associated inflammation is a consequence of ACL rupture following trauma, less is known about the influence of pre-existing inflammation (such as from pre-existing knee OA or synovitis) on the development of ligament disease in the absence of trauma.

Cushner et al. (2003) histologically evaluated the ACLs from 19 human knees with OA\textsuperscript{[95]}. They found these ACLs showed degenerative changes that were not observed in normal control knees. The authors identify osteophyte formation in the intercondylar notch leading to mechanical destruction of the ACL as a possible cause; however the
ligaments exhibited widespread histological changes throughout the ligament, not just at the intercondylar notch. This indicates the degenerative changes in the ligament could be due in part to some other aspect of the inflammatory process.

Mullaji et al. (2008) again studied the histology of ACLs and PCLs from 45 osteoarthritic human knee joints and evaluated the frequency of and severity of degenerative changes in the ligaments [96]. They found the ACL was severely degenerated or absent in knees with more advanced radiologic OA (higher than grade 3). The PCL was only moderately affected regardless of the severity of OA. They concluded that OA causes degeneration of both cruciates, with the ACL being more severely degenerated than the PCL in OA. These results were consistent with the findings of a similar study performed Allain et al. (2001) [97].

Hill et al. (2005) evaluated the prevalence of ACL and PCL rupture among human subjects with established OA of the knee [91]. Results showed ACL rupture is more common among those with symptomatic knee OA, but fewer than half of subjects with rupture of this ligament recalled a specific knee injury. This may suggest chronic degeneration of the ligament leading to eventual rupture, or that OA preceded ligament rupture.

A number of canine studies have identified immune-mediated responses may play a role in CCL degeneration. Muir et al. (2007) recently hypothesised that chronic synovitis induces CCL degeneration and a significant reduction in the tensile strength of the ligament. From the results of their study they conclude that inflammation likely develops in the early phase of cruciate disease before the development of stifle instability. Their findings suggest that immune responses within the stifle may be
involved in the persistent synovitis and associated joint degradation in dogs with arthritis and degenerative CCL rupture [89]. Several other studies support the hypothesis that inflammation within the synovium in dogs with CCL rupture is at least in part immune mediated. The inflammatory process is thought to weaken ligament collagens due to the activity of biochemical factors such as collagenases and gelatinases (known as matrix metalloproteinases). Anti-inflammatory treatment therefore has the potential to slow degradation of the ligament and could even prevent ligament rupture [14, 98-100].

It has been established in both humans and animals that ligament injury does more than just affect the articular cartilage. It also puts strain on other ligaments of the joint leading to a breakdown of normal architecture, and can affect other joint components such as the menisci, synovium and subchondral bone [86]. Funakoshi et al. (2007) used an ovine model to demonstrate that intact ligaments in joints with an injured ACL are also impacted by the injury. In particular, the intra-articular structures such as the PCL were more significantly affected than the extra-articular MCL and LCL. The intact ligaments showed an increase in water content and cell density, and collagen fibril diameter distribution was significantly altered. Furthermore, Nelissen et al. (2001) showed that intact ligaments, such as the PCL, in human knees with ACL rupture are architecturally damaged [101]. As the PCL is rarely ruptured under normal conditions, its degeneration could be a result of inflammatory mediators associated with the rupture of the ACL and/or subsequent OA, or changes in mechanical loading due to joint instability.

1.3.3.2 Mechanical loading

Degeneration due to repetitive micro-trauma may also be the cause of cruciate rupture. Micro-trauma leads to disruption of normal architecture of the ligament such as loss of
‘crimp’ and destruction of ligament fascicles \(^{56, 100}\). Crimp is an accordion-like wave pattern of ligament cells, fibres and matrix, which provides a buffer against mechanical loading, so that any rapid or excessive load will stretch out the undulations without damaging the tissue (Figure 1.10) \(^{11}\). Abnormal loading conditions can arise for a number of reasons. These include excessive weight of the subject \(^{74}\), the influence of surrounding musculature \(^{102, 103}\), excessive physical activity such as sport \(^{104}\). Although acute ligament injury can occur due to a single overload from trauma, ACL rupture usually results from progressive adaptive or degenerative changes within the core of the ligament \(^{87}\). Ligament degeneration and injury during sporting activity may occur via a combination of number of different mechanisms such as internal and external tibial torque (with the foot planted and the knee near full extension), straight anterior tibial force (e.g. landing after jumping), hyperextension and hyper-flexion, and frontal-plane or transverse-plane loading (e.g. during rapid deceleration) \(^{103, 105-108}\).

Figure 1.10: ACL (Tol Blue 200x) longitudinal section, showing the wave-pattern or crimp in the collagen fibres of the ligament. This stain has a high affinity for the glycosaminoglycans and elastin fibres (which stain a darker blue) present in ligament \(^{109}\). (Image A. Daniel).
Another factor that has been identified as contributing to abnormal loading in dogs is the angle of the tibial plateau. A steeper tibial plateau angle (TPA) is thought to increase cranial tibial thrust and contribute to CCL rupture [110-112]. In humans, an excessively steep TPA does not ordinarily occur, but can if the patient has undergone an open wedge high tibial osteotomy. This purpose of this procedure is to alter the weight-bearing axis of the knee joint to relieve pressure on damaged joint surfaces in patients with osteoarthritis. In some patients however, an unintended outcome of this procedure is an increase in TPA [113]. As in dogs, the increased TPA can lead to increased cranial tibial thrust and result in cruciate rupture [114].

1.3.3.3 Age

Throughout life, the biomechanical properties of the cruciate ligaments change. As the subject reaches maturation, there is an increase in the quality and quantity of the cross-linkages and collagen content of the ligaments. Then with increasing age, the amount of cross-linkages and collagen content begins to decrease. Therefore the risk of cruciate rupture may be greater in older subjects, as the ligaments are less capable of withstanding mechanical load [115].

1.3.3.4 Gender

Gender can be another risk factor affecting ligament rupture in both humans and dogs. It is well reported in human studies that females have a much higher incidence of ACL injury than do males engaged in similar activities [116-119]. A number of theories have been suggested to explain why this disparity exists, but as yet the exact reason has not been elucidated. Studies in dogs have revealed a similar predisposition in females [120-122]. This literature concerning the role of sex hormones on ligament structure and function is reviewed in detail in Chapter 4.
1.3.4  Cruciate Ligament Healing Capacity

Healing in the knee joint is ligament specific. Some ligaments such as the MCL have good healing potential whereas others such as the ACL have a very poor chance of healing, especially in the case of complete rupture [88, 123]. Ligament healing depends on the response of the tissue to injury. The normal stages of healing in ligament can be outlined as follows:

*The Bleeding and Inflammatory Phase* - lasts for up to 3 days post injury. Bleeding and oedema occur and there is an acute inflammatory response involving polymorphonuclear leukocytes, monocytes/macrophages, lymphocytes, mast cells, and the release of inflammatory mediators. Platelets are also activated and growth factors such as PDGF are up-regulated. Blood escaping from the injured blood vessels forms a clot at the injury site. Fibrin accumulates within the clot and platelets bind to fibrillar collagen providing haemostasis. The clot provides a framework for vascular and fibroblast cell invasion. Endothelial cells accumulate near the injury site forming new capillaries [68, 124-126].

*The Repair Phase* – lasts from 1 to 2 days up to 6 weeks post injury. Early in this phase the water content of ligament is high. The rate of new collagen synthesis continues to rise until it peaks at around 3 weeks post injury. Fibroblasts within the wound begin to proliferate rapidly and synthesise new matrix. The clot and any necrotic tissues are replaced with a soft, loose, fibrous matrix consisting of water, GAGs and type II collagen. At this stage, any movement such as premature exercise is extremely detrimental to the normal orientation of the new collagen fibres, and can result in bulky scar tissue and adhesions. After the 3-week stage, gradual mobilisation of the joint can
assist in increasing the tensile of the repairing tissue. The water and GAG content of the ligament decreases and type I collagen replaces the type II collagen [29, 68, 126].

The Remodelling Phase – lasts from approximately 6 weeks to 6 months post injury. By this stage the water content of the ligament has returned to normal while the collagen content remains slightly increased. The tissue is still highly cellular with limited tensile strength. As the scar tissue slowly matures, the number of cells decreases and the fibrils become orientated in the direction of greatest load. The maturing ligament gradually acquires the properties of normal tissue, however the repaired tissue will never regain the tensile strength of uninjured ligament tissue. Usually repaired ligament has approximately 50-70% of normal tensile strength [68, 125-127].

Importantly, injuries to extra-capsular structures tend to repair successfully via the above stages of healing. In contrast, intra-capsular structures such as the ACL often fail to produce a successful repair response [128]. The exact reason for the poor healing capacity of the ACL remains unclear may be due to a number of factors. One cause may be the disruption of any local clot due to the synovial fluid environment and the presence of proteases. This combined with non-contact between the two ends of the injured ligament may contribute to the lack of repair [72]. A study by Murray et al. in 2000 evaluated healing in 23 ruptured human ACLs. Results revealed no evidence of tissue bridging between the femoral and tibial remnants of the ACL at any time after rupture. They observed that the remnants of ACL appeared to retract after rupture thus preventing a bridging scar from forming. They also noted the formation of a layer of synovial tissue over the ruptured surface, which may further impede repair of the ligament [129].
When Murray et al. (2006) examined the healing of ruptured CCLs in dogs, they too found that no stabilised fibrin clot formed between the ligament remnants, even after induced bleeding into the joint. They found that a gap persisted at the rupture site despite attempts at suture repair or gross reapproximation of the tissue edges \cite{130}. Hayashi et al. (2003) also found a complete lack of bridging scar between the ends of ruptured CCL \cite{87}. This may be explained by the activity of collagenases. Amiel et al. (1989) investigated acute ACL injury in a rabbit model and observed a rapid degenerative process within the ligament, with loss of cellularity and matrix organisation. This was associated with a significant increase in collagenase activity and a decrease in total collagen content, which may explain the rapid degeneration of the remnants of the injured ligament and the persistence of a gap that cannot be bridged \cite{131}. Muir et al. (2006) reported similar findings in a canine model of CCL rupture \cite{100}.

Other explanations for the poor healing capacity of ACL relate to differences in collagen synthesis and cell migration to the injury site. Wiig et al. (1991) compared the levels of procollagen type I mRNA in normal and healing MCL and ACL in rabbits \cite{132}. Results showed that procollagen mRNA levels were significantly higher in MCL than ACL, which may contribute to the differences in the healing capacities of these ligaments. Chen et al. (2002) reported similar findings in their study of fibroblast cells from rabbit ACL and MCL \cite{133}. Although they found ACL and MCL cells exhibited similar morphology, there were significant differences in cellular growth and the synthesis of collagen. Studies by Nagineni et al. (1992) and Geiger et al. (1994), also studied cultured cells from explants of ACL and MCL \cite{134,135}. Results from both studies revealed that ACL cells demonstrated a much lower migration potential than MCL cells. The poor ability of ACL cells to migrate to the site of injury again may contribute to the reduced healing potential of the ACL.
1.4 STUDY OVERVIEW

From this literature review, it is clear that little is known about changes in ligaments in OA, and what influences ligament cell metabolism. The ligament of major interest in humans is the ACL, which is a major stabiliser of the knee joint, and is commonly injured. Ligament damage associated with trauma can contribute to OA through destabilisation of the joint and subsequent abnormal wear and tear, but the role of ligament degeneration in the pathogenesis of OA in the absence of trauma remains unclear.

In addition, current literature suggests there is an association between OA and the decline in oestrogen levels at menopause. It is uncertain whether changes in hormone levels primarily affect articular cartilage, or whether the degeneration of other joint tissues which are oestrogen-responsive, such as cruciate ligaments, may contribute significantly to the pathogenesis of OA. In a recent review of literature on the role of hormones in the modulation of connective tissues and the associated risk of knee OA, Boyen et al. (2013) highlighted that numerous gaps exist in our knowledge in this area. They identified a lack of research into sex-based differences in knee osteoarthritis involving age-appropriate female models, and that many studies fail to recognise the importance of sexual heterogeneity in the hormonal control of knee connective tissues.

An important objective of this study was to expand on current knowledge in this area.

The overall aim of this thesis was to investigate the influence of ovariectomy and/or the presence of concurrent OA on the fibrillar structure of collagen within the anterior cruciate ligament. Chapter 2 outlines the research tools and methods used in both main studies (Chapters 3 & 4), and also describes a preliminary trial which was conducted to
determine if there were any significant molecular differences between the anterior and posterior cruciate ligaments, and whether or not the results of ACL testing would be representative of both cruciate ligaments.

Chapter 3 presents the results of a study of the effect of OA separately on the structure and gene expression in ACL using a bilateral meniscectomy model of OA. Chapter 4 investigates the effect of oestrogen deficiency on ACL tissue, using an ovariectomy model to simulate menopause. It also looks at the effect of oestrogen deficiency in combination with OA (combined meniscectomy and ovariectomy), simulating the knee joint of a postmenopausal subject with OA.

The major hypotheses examined in Chapters 3 & 4 are listed here, and are repeated at the conclusion of the introduction of each chapter.

Chapter 3: THE INFLUENCE OF MENISCECTOMY ON THE ANTERIOR CRUCIATE LIGAMENTS OF SHEEP

**Hypothesis 1:** that osteoarthritis (meniscectomy) alters gene expression and collagen fibril structure and organisation in ACL.

**Hypothesis 2:** animals with worse osteoarthritic changes have more severe ligament derangement.

Chapter 4: THE INFLUENCE OF OVARIECTOMY ON THE ANTERIOR CRUCIATE LIGAMENTS IN NORMAL AND OSTEOARTHRITIC (MENISCECTOMISED) SHEEP

**Hypothesis 3:** that the decrease in ovarian hormone levels associated with menopause (ovariectomy) alters the normal composition and organisation of cruciate ligament tissue, in particular collagen fibril structure, which has the potential to influence the mechanical properties of the ligament, as observed in other connective tissue types post-menopause.
**Hypothesis 4:** that the combined effects of ovariectomy and meniscectomy have an even greater influence on ligament composition and structure than either meniscectomy or ovariectomy alone, and that these changes reflect those of postmenopausal women with concurrent osteoarthritis.

Finally, Chapter 5 provides an overview of the results of this project, by reviewing each of the four hypotheses and whether each was proved/supported or not. Avenues for possible further investigation are also suggested.
CHAPTER 2

METHODS AND MATERIALS

2.1 EXPERIMENTAL GROUPS

All animal experiments were approved by the Murdoch University Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes - 7th Edition. Animals were selected for uniformity of size, conformation, soundness of health and for absence of lameness. On introduction to the Murdoch University farm, the sheep were ear tagged for identification, and vaccinated against clostridial diseases and Corynebacterium pseudotuberculosis [Glanvac 6, CSL Australia]. All sheep were also treated prophylactically for endo- and ectoparasites [Ivomec Liquid for sheep (ivermectin), MSD Agvet, Australia; Vanquish (alphamethrin 50g/L), SmithKline Beecham, Australia] and to prevent footrot [Footrite (zinc sulphate 224g/L), Hardman, Australia]. All sheep were kept on the same pasture at the Murdoch University farm with supplementary feed given when necessary.

2.1.1 Preliminary Study

12 size-matched, four year old ewes were randomly separated into two groups of six sheep. The first group underwent ovariectomy (OVX1) and the second served as non-operated control group (NOC1). Six months (26 weeks) post surgery, both groups were sacrificed and samples of both ACL and PCL were harvested for PCR analysis of gene expression. The purpose of this preliminary study was to test the PCR protocols and primers, and to assess whether there were any differences in gene expression between the anterior and posterior cruciate ligaments.
It is evident that study groups OVX1 & NOC1 are similar to groups I & K described in the main study (2.1.2). It was necessary to replicate these groups in the main study for three reasons. Firstly, to confirm that the results obtained in the preliminary study were repeatable. Secondly, for all samples in a study to be directly comparable, they had to be batch processed all at the same time. This removes any influence that slight differences in processing or reagents may introduce; therefore it was necessary to repeat the OVX and NOC groups in the main study. Finally, the main study was conducted over a longer experimental period than the preliminary study (36 weeks vs. 24 weeks) in order to determine whether any outcomes increased or decreased over time. The results of the preliminary study are presented in Chapter 4 along with those of the main study.

Sheep tag numbers:

<table>
<thead>
<tr>
<th>OVX1</th>
<th>439</th>
<th>445</th>
<th>448</th>
<th>453</th>
<th>1049</th>
<th>1060</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOC1</td>
<td>409</td>
<td>414</td>
<td>432</td>
<td>433</td>
<td>437</td>
<td>442</td>
</tr>
</tbody>
</table>

Table 2.1: Ear tag numbers of the sheep assigned to each treatment group; OVX1 and NOC1.

2.1.2 Main Study

This larger study involved a total of 24 size-matched, four year old ewes. They were randomly assigned to four experimental groups (see table 2.2), consisting of six sheep each.

Sheep tag numbers:

<table>
<thead>
<tr>
<th>Group H</th>
<th>03</th>
<th>08</th>
<th>10</th>
<th>21</th>
<th>27</th>
<th>58</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>69</td>
<td>70</td>
<td>71</td>
<td>72</td>
<td>77</td>
<td>94</td>
</tr>
<tr>
<td>Group J</td>
<td>68</td>
<td>76</td>
<td>78</td>
<td>79</td>
<td>95</td>
<td>/</td>
</tr>
<tr>
<td>Group K</td>
<td>45</td>
<td>52</td>
<td>60</td>
<td>63</td>
<td>91</td>
<td>92</td>
</tr>
</tbody>
</table>

Table 2.2: Ear tag numbers of the sheep assigned to each treatment group in the main study. One sheep died prematurely of bloat during the course of the experiment (groups J). This individual was therefore excluded from the study.
The four experimental groups were assigned the following treatment protocols:

H. Ovariectomised, meniscectomised, sacrificed at 36 weeks.
I. Non-operated control
J Meniscectomised only, sacrificed at 36 weeks.
K. Ovariectomised only, sacrificed at 36 weeks.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>Sacrificed 36 weeks</th>
<th>Focus of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX: MENX:</td>
<td>(May 07)</td>
<td>H</td>
<td>(Feb 08)</td>
</tr>
<tr>
<td>No OVX No MENX</td>
<td>-</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>No OVX MENX:</td>
<td>(June 07)</td>
<td>J</td>
<td>(Mar 08)</td>
</tr>
<tr>
<td>OVX: No MENX</td>
<td>(Nov 06)</td>
<td>K</td>
<td>(Aug 07)</td>
</tr>
</tbody>
</table>

Table 2.3: Summary of main study, 24 sheep were divided into 6 treatment groups as indicated, H,I,J,K. OVX = Ovariectomised, MENX = Meniscectomised, NOC = non-operated control.

### 2.2 SURGICAL PROCEDURES

#### 2.2.1 Anaesthesia

All surgical procedures were performed under general anaesthesia in aseptic conditions in a surgical setting. Food and water was withheld from the evening prior to surgery. Immediately prior to surgery, general anaesthesia (GA) was induced using intravenous injection of diazepam (5mg/ml) /ketamine (100mg/ml), [Pamlin, Parnell Labs Aust Pty Ltd] [Ketamine, as ketamine hydrochloride, Parnell Labs Aust Pty Ltd], 50:50 mixture administered at 1ml/10kg and maintained with 2% inhaled Isoflurane (1ml/ml) [I.S.O. VCA Veterinary Companies of Aust Pty Ltd] delivered via a Fluotec 3 vaporiser, and a circle circuit with a size 11 endotracheal tube. Analgesia was provided with carprofen
anti-inflammatory injection (50mg/ml) at 3mg/kg, [Tergive, Parnell Labs Aust Pty Ltd] and 1ml of morphine sulfate (10mg/ml), given prior to recovery. Post-operative analgesia was maintained for the first three days post surgery with morphine sulfate and/or carprofen injection as required.

2.2.2 Meniscectomy

Bilateral medial meniscectomy was performed on groups B, C, G, H & J, under general anaesthesia in a sterile surgical setting. Both knee joints were clipped free of wool and aseptically prepared using alternating swabs of 2% Chlorhexidine surgical skin cleanser [Tasman Chemicals Pty Ltd, Aust] and 70% alcohol. The surgical approach was the same for both knees, with a medial skin incision made over the joint allowing access to the medial meniscus. The medial meniscus was removed using a combination of scalpel (#15 blade) and a meniscotome, which allowed the meniscal ligaments to be cut without damaging the joint surface. The joint was then flushed with sterile saline and closed by routine surgical methods using absorbable suture; 3/0 polydioxanone [PDS II, Ethicon, USA] for the retinacular and facial layers, and 3/0 poliglecaprone [Monocryl, Ethicon, USA] for the skin layers. Both joints were treated in an identical manner. A prophylactic intravenous injection of 1g cephazolin [as cephazolin sodium, Hospira Australia Pty Ltd] diluted in 10ml sterile saline was administered during surgery. The sheep were housed indoors, in pens with non-slip flooring, for an observation period of three days post surgery before being returned to pasture.

2.2.3 Ovariectomy

Ovariectomy was performed on sheep in groups H & K in the main study, and in group OVX1 in the preliminary study. Surgery was again performed under general anaesthesia in aseptic conditions in a sterile surgical setting. Food and water was
withheld from the evening prior to surgery. The sheep were ovariectomised via a 5cm ventral midline coeliotomy through the linea alba, placed approximately 5cm forward of the cranial border of the mammary gland. Both ovaries were located and externalised from the body cavity, the ovarian pedicle clamped and then ligated using 3/0 polydioxanone [PDS II, Ethicon, USA], and all ovarian tissue was removed. The uterus was left intact. The incision was closed using 3/0 polydioxanone for the linea alba, 3/0 poliglecaprone [Monocryl, Ethicon, USA] for the subcutaneous tissues and the skin. The skin sutures were dissolvable and placed intradermally, thus avoiding the need for suture removal. Recovery from anaesthesia was monitored for two days post surgery, with the sheep housed in pens inside the barn, after which they were released back onto the pasture.

2.3 SACRIFICE AND TISSUE COLLECTION

2.3.1 Preliminary Study

After six months the sheep were sacrificed by intravenous injection of 20ml of pentobarbitone sodium (300mg/ml) [Valabarb, Jurox Pty Ltd, Australia] and the right anterior (cranial) and posterior (caudal) cruciate ligaments were immediately harvested and snap frozen in liquid nitrogen. Tissues were processed for RT-PCR analysis only.

2.3.2 Main Study

At the time of sacrifice for all groups, the sheep were killed by intravenous injection of 20ml of pentobarbitone sodium (300mg/ml) [Valabarb, Jurox Pty Ltd, Australia]. Both the left and right femorotibial joints were immediately dissected and the anterior (cranial) cruciate ligaments harvested under aseptic conditions. The ligaments were dissected free of all other tissues and separated from the joint. The more fibrocartilaginous segments of
ligament near the origins and insertions were not included. Of the harvested ligament, the proximal $\frac{2}{3}$ of tissue was snap frozen in liquid nitrogen and stored at -80 degrees C. The remaining distal $\frac{1}{3}$ of the ligament tissue was fixed in 10% neutral buffered formalin for 48 hours, before being transferred to 70% ethanol for storage. Frozen tissue from the proximal $\frac{2}{3}$ of the ligament was processed for RT-PCR analysis & biochemistry, and formalin-fixed tissue from the distal $\frac{1}{3}$ of the ligament was processed for histology and electron microscopy. The articular surfaces of the femur and tibia were photographed for morphological assessment and samples of cartilage and synovium taken for histological analysis. This was done to confirm the presence of OA in the meniscectomised sheep.

2.4 MOLECULAR BIOLOGY

All details of the methods used, and the reagents & equipment required for the homogenisation of the ligament, extraction of RNA, and reverse transcription of RNA to cDNA are provided in the following:

2.4.1 Tissue Homogenisation

2.4.1.1 Equipment Required:

- Sterile Petri dishes
- Single-sided razor blades
- Tissue forceps
- RNase-free 1.5ml tubes
- Dewar flask with liquid nitrogen
- Dry ice
- Stainless steel plate
- Gloves
- Kim wipes
• Absorbent pads
• Tube rack
• 1000ul pipette with sterile, RNase-free filter tips
• UltraSpec® RNA Isolation Reagent (Fisher Biotec BL-10200/WS)
• RNase AWAY™ Reagent (Invitrogen SKU# 10328-001)
• Mikro-dismembrator laboratory ball mill (with 3ml Teflon chambers, o-rings and stainless steel ball bearings)
• Polystyrene tray (Esky lid)
• 2 x 250ml Glass beakers with milli-Q water
• Electronic scale (3 decimal places)

2.4.1.2 Preparations for Tissue Homogenisation:

• Ensure gloves are worn at all times to prevent contamination by RNases.
• Using RNase AWAY, wipe over bench-tops and soak the forceps, Teflon chambers, o-rings, ball bearings and Petri dishes. Then rinse in distilled water and pat dry with Kim wipes.
• Fill polystyrene tray with dry ice and place metal plate on top to cool.
• Fill one beaker with pure milli-Q water and the other with RNase AWAY Place absorbent pads beside beakers.
• Place mikro-dismembrator on a hard, stable surface and plug in to power.
• Fill Dewar flask with liquid nitrogen and add samples to be homogenised. This will keep the tissue frozen prior to processing.
• Place Petri dish on scale and tare.
• Label the 1.5ml tube with group, tag #, tissue type and date.

2.4.1.3 Homogenising the Tissue:

• Place the ball bearing and o-ring in the lid of the Teflon chamber.
• Ensure the UltraSpec is well mixed as it can separate during storage. Pipette 1ml of UltraSpec into the bottom of the chamber.
• Weigh out 100±20mg of ligament or cartilage for homogenisation. This is the appropriate amount of tissue for 1ml of UltraSpec and for adequate RNA yield.
• If the tissue requires chopping, place a Petri dish on the cold metal plate and cut the tissue samples using a single sided razor blade. A Kim-wipe placed over the tissue sample while chopping will prevent it flicking out of the Petri dish. It is essential to work quickly and keep the tissue frozen at all times.

• Drop the frozen tissue into the chamber with the UltraSpec, place on the lid, insert into the mikro-dismembrator and shake at 1800 rpm for 90 seconds.

• Remove the ball bearing and immediately wipe with a Kim-wipe and rinse in beaker with milli-Q water as UltraSpec corrodes metal.

• Using a new pipette tip, collect the homogenate and place in a labelled 1.5ml tube.

• Wipe the chamber, o-ring and lid clean with a Kim-wipe and place them, along with the ball bearing, into in the RNase AWAY. After a few minutes rinse again in milli-Q water and allow to drain on an absorbent pad. Pat dry with Kim wipe before use again. Repeat procedure between each sample.

• Let tubes with homogenate sit at room temperature for 30-45 minutes before storing at -80°C.

2.4.2 RNA Extraction

2.4.2.1 Equipment Required:

• Qiagen RNeasy Mini Kit spin columns
• Qiagen RNase-Free DNase Set
• Molecular Biology (Analytical) Grade Ethanol 70%
• Pure RNase-free water
• Chloroform
• RNAse-free 1.5ml tubes
• Incubator or water bath at 37°C.
• Centrifuge
• Tube rack
• Gloves
• Ice
• Kim wipes
• RNase AWAY™ Reagent (Invitrogen SKU# 10328-001)
2.4.2.2  *Preparations for RNA Extraction:*

- Ensure gloves are worn at all times to prevent contamination by RNases.
- Using RNase AWAY, wipe over bench-tops and surface of centrifuge.
- Thaw frozen tubes containing tissue/UltraSpec homogenate at room temperature. The maximum capacity of the centrifuge is 24 samples.
- Label a set RNAse-free 1.5ml tubes to match the homogenate tubes.
- Label a set of collection tubes from Qiagen kit to match original samples.
- Label a set of spin columns from Qiagen kit to match original samples.

2.4.2.3  *Retrieving the Aqueous Phase:*

- Add 300ul of chloroform/ml UltraSpec to each tube and vortex for 15 seconds.
- Allow tubes to stand at room temperature for 3-5 minutes.
- Spin tubes at 12,000 rpm for 15 minutes (room temperature).
- Transfer the aqueous phase to the pre-labelled 1.5ml tubes using a separate pipette tip for each sample. Recovery should be about 600ul per ml UltraSpec.
- Discard the tubes with the pink phenol phase in the contaminated waste.
- Add equal volumes of 70% ethanol to each aqueous phase.
- Seal lids and mix gently by inversion.
- Tubes may sit at room temperature for up to 30mins at this stage.

2.4.2.4  *Loading the Spin Columns:*

- Ensure spin columns have their collections tubes underneath them at all times during extraction process. Failure to do so will likely cause contamination of the final RNA preparation.
- Load 600ul of the aqueous/EtOH phase onto spin column membrane. This step should be performed in a tube rack not the centrifuge to prevent contamination of this instrument. If the total volume of the aqueous/EtOH phase exceeds 600ul, the remainder is loaded at a later stage.
- Transfer the spin columns with collection tubes to centrifuge and spin at 12,000 rpm for 15 seconds.
• Empty each collection tube into waste container and place back under spin column.
• Load any remaining aqueous/EtOH phase into appropriate spin column and spin again at 12,000 rpm for 15 seconds.
• Empty each collection tube into waste container and place back under spin column.

2.4.2.5 Washing the Spin Columns:

• Load 350ul of RW1 (from kit) into each spin column and let sit for 5 minutes.
• Spin at 12,000 rpm for 15 seconds.
• Empty and discard collection tube.
• Place a new collection tube underneath spin column.
• Remove DNase set from freezer and make up a master mix containing 10ul DNase and 70ul DNase buffer (from kit) per sample. For every 10 spin columns make up enough master mix for 11. This will allow for pipette wastage.
• Add 80ul of the DNase/buffer master mix to each spin column using a new tip for each sample.
• Spin at 12,000 rpm for 15 seconds to saturate the column membrane with DNase. Then re-apply the DNase/buffer mix from the collection tube onto the spin column again using a new tip per sample. Ensure the DNase is applied directly onto the column membrane.
• Incubate at 37°C for 1 hour.
• After the incubation period, spin again at 12,000 rpm for 15 seconds.
• Load another 350ul of RW1 (from kit) into each spin column.
• Spin at 12,000 rpm for 15 seconds.
• Empty each collection tube into waste container and place back under spin column.
• Load 500ul of RPE buffer (from kit) into each spin column.
• Spin at 12,000 rpm for 15 seconds.
• Empty each collection tube into waste container and place back under spin column.
• Load another 500ul of RPE buffer into each spin column.
• Spin at 12,000 rpm for 15 seconds.
• Empty each collection tube into waste container and place back under spin column.
• Spin at 12,000 rpm for a further 2 minutes to evaporate the EtOH and dry the column.
• Empty and discard the collection tubes.
• Transfer the spin columns to the pre-labelled 1.5ml tubes supplied with the kit.

2.4.2.6 Eluting the RNA:
• Depending on the expected yield, load 30-100ul of RNase free water into each column. For this study 32ul of water was loaded into each column.
• A new pipette tip should be used for each sample to prevent cross-contamination.
• Ensure the water is placed directly onto the column membrane.
• Incubate the tubes at room temperature for 10-15 minutes.
• Spin at 12,000 rpm for 1 minute.
• Discard spin columns.
• Place tubes containing eluted RNA on ice.
• Measure the RNA yield using the Nanodrop ND1000 spectrophotometer (in SABC). This spectrophotometer requires only 1ul of sample to quantify RNA content.
• Freeze tubes at -80°C if not continuing to reverse transcription stage.

2.4.3 Reverse Transcription of RNA to cDNA

2.4.3.1 Equipment Required:
• Qiagen Omniscript RT kit
• Bioline Random Hexamer Primers 50ng/ul
• Promega RNasin® Plus RNase Inhibitor 40units/ul
• 1000ul, 200ul, 20ul pipette with sterile, RNase-free filter tips
• RNAse-free 1.5ml tubes
• RNAse-free water
2.4.3.2  Preparations for Reverse Transcription of RNA:

- Ensure gloves are worn at all times to prevent contamination by RNases.
- Using RNase AWAY, wipe over bench-tops.
- Label a set of tubes to match RNA sample.
- Check incubator is set to 37°C.
- Check water bath or heating block is set to 93°C.
- Dilute RNase inhibitor to 10U/ul using 1xRT buffer.
- The amount of RNA to be reverse transcribed must be the same for all samples.
- All samples must be Reverse transcribed at the same time.
- Total RT volume is 40ul = 10ul master mix + RNA and water up to 30ul.
- Determine the volume of sample required for 1ug of RNA (volume required to yield 1ug must be less than 30ul).
- Calculate the volume of water (if any) required for total volume to = 30ul.
- Check stocks of all reagents required for the RT master mix. For 50 reactions the master mix volumes required are as follows:

  - 200ul 10x RT Buffer (supplied in Omniscript kit)
  - 100ul dNTP (supplied in Omniscript kit)
  - 100ul random hexamer primers
  - 50ul RT enzyme (supplied in Omniscript kit)
  - 50ul RNase inhibitor 10U/ul
2.4.3.3 Procedure for Reverse Transcription of RNA:

- Pipette the appropriate amounts of RNA/water into each pre-labelled tube as per calculations.
- In a separate tube, make up RT master mix as previously described. Ensure contents of the tube are well mixed. Store on ice until required.
- Using a new tip for each sample, pipette 10ul of RT master mix into each sample tube containing RNA/water.
- Spin samples quickly in the centrifuge (12,000 rpm for 15secs).
- Incubate samples for 1 hour at 37°C.
- Then incubate samples for 5 minutes in a water bath or heating block at 93°C.
- Following this incubation, cool samples quickly on ice for 5 minutes.
- For real time PCR analysis add 150ul of water per ug of original RNA to each sample. This gives a total volume of 190ul of cDNA for analysis.
- cDNA samples are stored at 4°C while in use, and at -20°C or -80°C long term.

2.4.4 Real Time PCR Analysis

2.4.4.1 Equipment Required:

- Bioline ImmoMix™ reaction mix
- Primers – forward and reverse
- Sybrgreen I working dilution 10x
- cDNA
- Molecular Biology RNase-free water grade water
- RNAse-free 1.5ml tubes
- RNAse-free 25ul Rotor Gene strip tubes
- 1000ul, 200ul, 20ul pipette with sterile, RNase-free filter tips
- Rotor-Gene 6000 PCR analyser
- Tube rack
- Ice
- Gloves
- Kim wipes
- RNase AWAY™ Reagent (Invitrogen SKU# 10328-001)
2.4.4.2 Experimental Procedure:

- Ensure gloves are worn at all times to prevent contamination by RNases.
- Using RNase AWAY, wipe over bench-tops.
- If frozen thaw cDNA samples. Make a pooled sample by taking a small volume from each cDNA sample. Using this pooled sample created a set of cDNA standards (4 fold dilutions labelled, 1:10, 1:40, 1:160 and 1:640).
- Stock Sybrgreen I at a concentration of 10,000x must be diluted to the correct working dilution. Make 1ml of 1000x solution by adding 1ul stock to 1000ul molecular biology grade water. Protect from light by wrapping tube in foil. Any remaining working dilution can be stored in the -20 freezer but should be discarded after 2 days.
- Dilute primers in molecular biology grade water to a working dilution of 10uM.
- Total PCR volume is 25ul = 20ul PCR master mix + 5ul cDNA or standards.
- Create a PCR master mix by combining ImmoMix, Sybrgreen, forward and reverse primers, and water according to the following ratio per sample:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>3.50ul</td>
</tr>
<tr>
<td>ImmoMix</td>
<td>12.50ul</td>
</tr>
<tr>
<td>Sybrgreen</td>
<td>2.50ul</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.75ul</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.75ul</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>20.00ul</strong></td>
</tr>
</tbody>
</table>

Table 2.4: PCR master mix ingredients totalling 20ul, for a final PCR volume of 25ul with standards or sample added.

- Combine PCR master mix by gentle inversion and spin down for 10 seconds.
- Calculate how many tubes you will need. Use 1 per sample and 2 per standard with 2 non-template controls (NTC).
- Place the correct number of tubes in rack and carefully pipette 20ul of PCR master mix into each. Avoid bubbles and place solution well within tube to allow room for the cDNA.
- The same tip can be used to pipette out the master mix.
• Make sure the standards and cDNA are homogenous and all at the base of the tube, especially if they were frozen. If there is condensation in the tubes, mix by inversion and spin down.
• Add 5ul of cDNA, standard or NTC to appropriate tube. Use a new pipette tip for each addition (including replicates).
• Pipette the standards first (starting with the highest standard), as the machine uses the first tube to automatically set the gain.
• Cap the tubes tightly. No need to mix or invert the tubes.
• Load the tubes into the rotor in the correct order and fill any vacant spaces with empty balance tubes.
• Place the locking ring firmly on rotor and insert the rotor into machine, lining up pin correctly. Close lid.
• Using Rotor-Gene software, set annealing temperature (according to primer used), times and cycle number. Check gain (usually 8-9) and start run.

Appendix 1 shows an example of a quantitation report and melt curve analysis generated by the rotor-gene software, including the run settings used. Appendix 2 is a list of all primer sequences used in this study and their annealing temperatures.

2.5 BIOCHEMICAL ANALYSIS
Total sulfated glycosaminoglycan (S-GAG) and collagen content was assayed using separate protocols. For SGAG a dimethylmethylene blue dye binding technique was used (Farndale method). Collagen was analysed using a papain digest in combination with a dimethylaminobenzaldehyde reagent for colour formation. Details of these protocols and the equipment used is provided below.

2.5.1 Sulfated Glycosaminoglycan (S-GAG)
2.5.1.1 Equipment Required:
• Microcentrifuge tubes
• Pipette tips (VistaLab, Ovation)
• Single pipette (20ul, 200ul, 1000ul)
• Multichannel pipette (VistaLab, Ovation 12 channel, 5-250ul)
• Analytical balance
• 96 well micro-titre plates (Sarstedt, Round Bottom Wells, single use)
• Microcentrifuge
• Tube racks
• pH meter
• Micro-titre plate reader (Opsys MR™ manufacturer Dynex Technologies USA, distributor DKSH Australia Pty Ltd)
• Computer software (Microsoft Excel 2007)
• Papain
• Milli-Q H₂O
• Sodium Formate
• Formic Acid
• 1,9 Dimethylmethylene Blue
• Glycine
• NaCl
• 0.1M HCl
• Chondroitin Sulfate

2.5.1.2  Tissue Preparation: Papain Digest

• Freeze dry all tissue samples prior to weighing.
• Accurately weigh out 5-10mg (dry weight) of each tissue sample and put into a 1.5ml screw top tube.
• Set incubator to 100°C and prepare papain digestion buffer as follow for 1000ml.
• Dissolve 3.72g of EDTA disodium salt in enough PBS to make up to 1000ml then adjust pH to 7.4. This pre-papain buffer can be stored on the shelf until required. Immediately before use add 0.79g L-cysteine HCL and readjust pH to 7.4. For each ml of buffer add 2ul of papain suspension. If smaller volumes of papain digestion buffer are required, scale volumes accordingly.
• Add 1ml of prepared papain digestion buffer per 5-10mg of tissue sample.
• Place samples into a sealed container with milli-Q soaked gauze to maintain humidity. Cartilage samples should be incubated overnight (16 hours) and
checked to ensure complete digestion has taken place. Ligament samples may take up to 3 days, with daily checking to assess if digestion is complete.

- Digests may be frozen at -80°C for some time prior to assaying.
- Prior to use, frozen digest must be thawed, vortexed vigorously to resuspend the precipitated material, then centrifuged at high speed for 2-5 minutes. The clear supernatant should be used for analysis.

2.5.1.3 Experimental Procedure:

A. 0.2% Sodium Formate pH 3.0
1.00g of Sodium Formate dissolved in 200ml of Milli-Q H_2O and pH adjusted to 3.0 using Formic acid. Transfer solution into 500ml volumetric flask; fill to the 500ml mark with Milli-Q H_2O.

B: Farndale Reagent
Dissolve 3.2mg of 1,9 Dimethylmethylene Blue (DMMB) (Sigma Chemical Co) in 19ml of 0.1M HCl, add 608mg of Glycine and 474mg of NaCl. Make up to 200ml with Milli-Q H_2O and adjust the pH to 3.0. Protect the reagent from light in an amber bottle or cover with aluminium foil and store at room temperature. Under these conditions the reagent lasts for up to 3 months.

C: Standard Solutions
Prepare a stock solution of Chondroitin Sulfate (ChS) (Sigma Chemical Co) by dissolving 10.0mg of ChS in 10ml of Milli-Q H_2O.
200ul of this stock ChS solution is placed in a 1.5ml microcentrifuge tube and 800ul of 0.2% Sodium Formate pH 3.0 is added.
A standard curve is prepared using the following ChS dilutions in triplicate in a 96 well plate.

<table>
<thead>
<tr>
<th>CSA conc. (ug/well)</th>
<th>Working CSA solution (ul)</th>
<th>0.2% Na Formate (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>20.0</td>
</tr>
<tr>
<td>0.4</td>
<td>2.0</td>
<td>18.0</td>
</tr>
<tr>
<td>0.8</td>
<td>4.0</td>
<td>16.0</td>
</tr>
<tr>
<td>1.2</td>
<td>6.0</td>
<td>14.0</td>
</tr>
<tr>
<td>1.6</td>
<td>8.0</td>
<td>12.0</td>
</tr>
<tr>
<td>2.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>2.4</td>
<td>12.0</td>
<td>8.0</td>
</tr>
<tr>
<td>2.8</td>
<td>14.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Table 2.5: Chondroitin Sulfate standard curve dilutions for a 96 well plate.
D: Sample Preparation

If tissue digests are frozen allow samples to defrost at room temperature then vortex vigorously to resuspend precipitated material then spin sharply for 2-5min in a microcentrifuge at full speed. This ensures the cloudy/insoluble material will settle at the bottom and the clear supernatant containing the soluble S-GAGs will read correctly on the plate reader. Prepare a 1:10 dilution of the clear supernatant in a labelled microcentrifuge tube using Milli-Q H₂O. This solution may be further diluted in 0.2% Na Formate (1:10, 1:20, 1:100 etc) depending on the anticipated S-GAG content of the samples.

E: Generation of the Chromophore

In triplicate, pipette 10ul of each of the dilutions of unknowns (as prepared above) into each well followed by 10ul of 2.0% Na Formate pH 3.0. To each well (standards and unknowns) add 200ul of the prepared Farndale reagent using the multichannel pipette. Immediately read the absorbance at 540nm using the micro-titre plate reader.

Calculate the S-GAG content of each unknown sample from the standard curve using the absorbance and the plate reader software. Convert values expressed as ug/well to ug/ml and calculate the % GAG contained in the unknown samples from these values using the appropriate dilution conversions. Ref: Farndale RW, Buttle DJ, Barrett AJ, Improved quantitation and discrimination of sulfated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta., 1986. 883(2): p. 173-7 1986 [137]).

2.5.2 Collagen

2.5.2.1 Equipment Required:

- Microcentrifuge tubes
- Pipette tips (VistaLab, Ovation)
- Single pipette (20ul, 200ul, 1000ul)
- Multichannel pipette (VistaLab, Ovation 12 channel, 5-250ul)
- Analytical balance
- 96 well micro-titre plates (Sarstedt, Round Bottom Wells, single use)
- Adhesive plate covers (temp safe up to 110°C)
- Microcentrifuge
- Tube racks
- Micro-titre plate reader (Opsys MR™ manufacturer Dynex Technologies USA, distributor DKSH Australia Pty Ltd)
- Computer software (Microsoft Excel 2007)
- Milli-Q H₂O
- pH meter.
- Citric acid monohydrate
- Glacial acetic acid
- Sodium acetate trihydrate
- Sodium hydroxide
- Hydrated chloramine T
- N-propanol
- NaCl salt
- P-dimethylaminobenzaldehyde
- 60% perchloric acid
- 6M hydrochloric acid solution
- 6M sodium hydroxide solution
- Hydroxyproline

2.5.2.2  **Tissue Preparation: Acid Digest**

- Take 200ul of papain digest and pipette into a screw-top 1.5ml tube.
- Place tubes into incubator oven at 90°C overnight without the lids to allow samples to dry.
- When completely dry, add 100ul of 6M HCl and seal tubes tightly with a lined screw cap.
- Place tubes in 110°C oven for 16 hours (overnight). Samples may become discoloured (brown) after acid digestion.
- Allow tubes to cool to room temperature then add 100ul of 6M NaOH to the hydrolysate and mix well.
- Allow tubes to stand on bench to settle.
2.5.2.3 Experimental Procedure:

A. Stock Buffer
Combine 5.0g of citric acid monohydrate, 1.2ml of glacial acetic acid, 12.0g sodium acetate trihydrate and 3.4g of sodium hydroxide in 100ml of aqueous solution (pH approx 5.75).

B. Assay buffer
Prepare assay buffer by diluting the stock buffer tenfold with Milli-Q water.

C. Chloramine T reagent
Dissolve 1.41g of chloramine T (hydrated form) in 20.7 ml of Milli-Q water. Add 26ml of n-propanol and 53.3ml of stock buffer. This makes 100ml of reagent. To make up enough reagent for one plate use: 0.282g chloramine T, 4.14ml Milli-Q water, 5.2ml n-propanol and 10.6ml of collagen assay buffer. Prepare reagent on the day required.

D. Dimethylaminobenzaldehyde (DMAB) reagent
Suspend 1.5g of p-dimethylaminobenzaldehyde in 6.0ml of n-propanol, followed by the slow addition of 2.6ml of 60% perchloric acid. This reagent must be freshly prepared.

E. Standard Curve:
Prepare a set of standards using 0 - 3.5ug of hydroxyproline (use 0 - 35ul of a 10mg OH-pro/100ml solution made up with Milli-Q water).

The following table outlines the volumes of each standard required per well. For each assay, standards are pipetted out in triplicate so volumes will need to be multiplied by 3.5 (allowing for wastage) to produce a full standard curve for each plate. The total volume of each standard = 40ul per well.

<table>
<thead>
<tr>
<th>OH-pro conc. (ug/well)</th>
<th>OH-pro solution (ul)</th>
<th>Milli-Q water (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>40.0</td>
</tr>
<tr>
<td>0.5</td>
<td>5.0</td>
<td>35.0</td>
</tr>
<tr>
<td>1.0</td>
<td>10.0</td>
<td>16.0</td>
</tr>
<tr>
<td>1.5</td>
<td>15.0</td>
<td>14.0</td>
</tr>
<tr>
<td>2.0</td>
<td>20.0</td>
<td>12.0</td>
</tr>
<tr>
<td>2.5</td>
<td>25.0</td>
<td>10.0</td>
</tr>
<tr>
<td>3.0</td>
<td>30.0</td>
<td>8.0</td>
</tr>
<tr>
<td>3.5</td>
<td>35.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Table 2.6: Hydroxyproline standard curve volumes.
**F: Generation of the Chromophore**

Pipette 40ul of each standard out in triplicate. To each standard add 10ul of 3M NaCl to bring the total volume of each standard to 50ul. Dilute unknown samples as required and pipette out 50ul in triplicate.

Add 50ul of assay buffer to all wells followed by 50ul of freshly prepared chloramine T reagent using a multi-channel pipette. Shake the plate and allow to stand for 20 minutes.

During the 20 minute waiting period the DMAB colour reagent is freshly prepared. 50 ul of DMAB is added to each well, the plate is shaken again, sealed with an adhesive plate cover and incubated at 60°C for 15 minutes. After the incubation period allow plate to sit at room temperature for a further 15 minutes, then read at 562nm.

NB during determination of the collagen content of the unknowns, a conversion factor of 7.4 for OH-pro must be incorporated into the calculations. Appendix 3 shows an analysis report produced by the Opsys MR™ micro-titre plate reader, from which the statistical analysis was generated.

---

**2.6 TRANSMISSION ELECTRON MICROSCOPY (TEM)**

For examination using TEM, the ligament was processed in the following way:

**2.6.1 Processing of Biological Tissue:**

- Transfer small (1mm x 3mm) cut tissue samples into sample vials.
- Fix tissue in 5% glutaraldehyde overnight at 4°C.
- Replace glutaraldehyde with enough 1% Dalton’s chrome OsO₄ to cover the tissue well. This step should be performed in a fume hood.
- Shake bottles to increase tissue exposure and keep in refrigerator for approximately 1½ hours. Shake at regular intervals during exposure time.
- Wash tissue in several changes of 90% alcohol over 5 minutes.
- Wash in 95% alcohol for 5 minutes.
- Wash in 3 changes of absolute alcohol over 10 minutes.
- Wash in 2 changes of propylene oxide over 10 minutes.
• Leave in 60:40 solution of propylene oxide/epoxy (Epon) resin for 1 hour.
• Leave in epoxy resin overnight on a rotator with lids off at room temperature.
• Embed tissue in capsules using fresh epoxy resin and bake at 60°C for 24 hours until hardened.
• Trim blocks to a rhomboid shape using a razor blade to expose tissue.
• Cut thin sections at approximately 0.5µ thickness and place onto a 200 mesh copper grid.
• Stain grids, first with uranyl acetate then with lead citrate.

2.6.2 Image Analysis

• Examine grids under transmission electron microscope. Two images are captured of each tissue sample at 37000x from different regions of the grid. All grids are examined blind. Images are recorded on negatives, developed then printed in the darkroom.
• Hard copy images from the microscope are then digitally scanned as JPEG images. The digital images are modified using Adobe Photoshop CS to enable analysis using Image J64 (National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). An example of a modified image is shown below.

![Figure 2.1: A) an original TEM image of ACL showing collagen fibrils in transverse section. B) the same image after Adobe Photoshop CS modification for image analysis by Image J64.](image-url)
Figure 2.2: Adjusting the threshold in the Image J64 image analysis program to establish which pixels of the TEM image will be analysed.

Figure 2.3: After applying the threshold, the dimensions of the collagen fibrils are clearly recognisable, and measurements can be obtained for individual surface area and Feret diameter (diameter at the widest point).
2.7 SCANNING ELECTRON MICROSCOPY (SEM)

Small cubes (approx 4mm³) of formalin fixed ACL tissue were cut with the fibril bundles in cross-section using a scalpel. 2 samples were taken from each test group. Samples were washed in several changes of alcohol at 30%, 50%, 70%, 80%, 90%, 95%, and 100%, and then washed in 50:50 super-dry:amylacetate for 30 minutes. Samples were then washed in 2 changes of amylacetate over 2 hours after which they were placed in the critical point dryer for several hours. Following tissue processing, the samples were mounted with the cut surface of the tissue facing up, on a metal pin-type SEM specimen stub using double sided carbon adhesive tape. The base of each stub was labelled with the specimen number and then placed in a Balzers Union SCD 020 sputtering device, in which each sample was coated with gold/palladium for a period of 2 minutes. Once coated, the samples were viewed using a Phillips XL-20 scanning electron microscope and TIF images captured on computer at a magnification of 170x and 2000x.

2.8 GROSS MORPHOLOGICAL ASSESSMENT

After sacrifice, each joint was removed and dissected free of surrounding soft tissues such as muscles and tendons (shown below).

Figure 2.4: Removal of surrounding structures and separation of the femorotibial joint.
At this time, samples of cruciate ligament and synovium were harvested for further examination. Both the femoral and tibial articular surfaces were visibly assessed for signs of cartilage degradation and the presence of osteophytes, in order to confirm the presence or absence of osteoarthritis. Prior to cartilage sample collection, both articular surfaces were digitally photographed and separately scored for cartilage damage and osteophyte formation by a single blinded operator. Each surface is assigned a score of 0 to 3 for osteophyte formation and 0 to 4 for morphological cartilage changes according to the following grading system:

<table>
<thead>
<tr>
<th>Osteophyte development – score each separately</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assessment of joint margin of:</td>
<td></td>
</tr>
<tr>
<td>Femoral articular surface</td>
<td></td>
</tr>
<tr>
<td>Tibial articular surface</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Mild osteophyte development (&lt;2mm outgrowth or</td>
<td>1</td>
</tr>
<tr>
<td>&lt;20% of joint margin)</td>
<td></td>
</tr>
<tr>
<td>Moderate osteophyte development (2-4mm outgrowth</td>
<td>2</td>
</tr>
<tr>
<td>or 20-50% of joint margin)</td>
<td></td>
</tr>
<tr>
<td>Large osteophyte development (&gt;4mm outgrowth or</td>
<td>3</td>
</tr>
<tr>
<td>&gt;50% of joint margin)</td>
<td></td>
</tr>
<tr>
<td>Add to give osteophyte score</td>
<td>0-6</td>
</tr>
</tbody>
</table>

Table 2.7: Macroscopic scoring of osteophytes (reproduced with permission, Little et al, 2010).

<table>
<thead>
<tr>
<th>Gross articular cartilage damage – score each separately</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assessment of joint margin of:</td>
<td></td>
</tr>
<tr>
<td>Femoral articular surface</td>
<td></td>
</tr>
<tr>
<td>Tibial articular surface</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Surface roughening</td>
<td>1</td>
</tr>
<tr>
<td>Fibrillation and fissures</td>
<td>2</td>
</tr>
<tr>
<td>Small erosions down to subchondral bone (&lt;5mm)</td>
<td>3</td>
</tr>
<tr>
<td>Large erosions down to subchondral bone (&gt;5mm)</td>
<td>4</td>
</tr>
<tr>
<td>Add to give cartilage damage score</td>
<td>0-8</td>
</tr>
</tbody>
</table>

Table 2.8: Macroscopic scoring of cartilage (reproduced with permission, Little et al, 2010).
An average score was calculated for osteophytes and cartilage degradation for both joint surfaces for each group.

Figure 2.5: Photographs demonstrating the morphological appearance of both joint surfaces at either extreme of the cartilage integrity scale. A= Grade 0 femoral surface; B= Grade 0 tibial surface; C= Grade 4 femoral surface; D= Grade 4 tibial surface. Note the Grade 0 surfaces exhibit normal architecture with no signs of disruption to the smooth and glossy articular cartilage. This is compared to the Grade 4 surfaces which show large areas of cartilage degradation with deep lesions and fissures (arrows). Note the scale at the bottom of each photo is 1mm for small graduations.
2.9 HISTOLOGY

Details of the protocol for the processing of ligament for histological section, and the special stains used are outlined below.

2.9.1 Processing of Ligament Tissue

- Fix the ligament samples in 10% neutral buffered formalin for 48 hours, using 10-20 times the amount of fixative to tissue.
- Soften ligament in 10% formic acid/2.5% formalin for 48 hours.
- Transfer to 70% ethanol (tissue can be stored indefinitely in 70% alcohol).
- Trim tissue samples to fit cassettes.
- Tissue must spend at least 24 hours in 70% ethanol.
- Soak in 80% ethanol for 5 hours.
- Soak in 95% ethanol for 5 hours.
- Soak in 100% ethanol for 3 hours.
- Change 100% ethanol and soak for further 3 hours.
- Change 100% ethanol and soak for further 3 hours.
- Change 100% ethanol and soak for further 3 hours.
- Soak in methyl benzoate for 3 days. (Note methyl benzoate is noxious and must be used in sealable containers in a fume hood at all times).
- Change methyl benzoate and soak for 3 days.
- Soak in methyl benzoate and soak for 3 weeks.
- Rinse with 3 changes of chloroform - 15 min each.
- Wax infiltration with Paraplast XTRA for 4 days under vacuum, with 2 wax changes per day.
- Wax embedding in Paraplast Plain.
- To cut the block, position it so that the cutting edge of the blade is parallel to the direction of the ligament fibres.
- Soften blocks for at least 30 min in 5% formic acid/45% ethanol/50% glycerol on a cold plate.
- Rinse in cold water then keep on cold plate.
- Cut sections at 5 microns on a microtome with Feather blades N35.
- Float sections on water bath filled with milli-Q water at 50 degrees C.
• Attach sections to SuperFrost Ultra Plus slides (Menzel) and immediately place slide in incubator at 85 degrees C.
• After 30 min, reduce temperature to 55 degrees C and leave overnight.
• Cut two sections for each sample, one for standard haematoxylin & eosin (H&E) staining and one for special staining with toluidine blue (Tol Blue).

(Method - S. Smith, Raymond Purves Bone and Joint Research Laboratory, Royal North Shore Hospital, Sydney).

2.9.2 Special Stains – Toluidine Blue

• Add sections to 70% ethanol for 15 min.
• Stain in 0.04% Toluidine Blue O (C152040)/0.1M sodium acetate buffer pH 4.0 for 10 min.
• Rinse quickly in tap water.
• Stain in 0.1% Fast Green FGF (C142053) for 2 min.
• Rinse quickly in tap water.
• Dehydrate quickly in 99% isopropyl alcohol, 2 changes, and clear in 2 changes of xylene.
• Mount with Eukitt's mounting medium.


2.9.3 Processing of Cartilage Tissue

After gross morphological assessment, histological slides were prepared from each of the four regions: MFC, LFC, MTP and LTP. The following method was used to prepare the sections.

• Obtain osteochondral slices (approximately 3mm thick) from each region by bandsaw cuts in the medio-lateral plane, perpendicular to the articular surface.
• Cuts should be positioned in the femoral condyles at the point of tibial contact in normal stance, and in the tibial plateaux at the level of the intercondylar eminences.

• Fix bone slices in 10% neutral buffered formalin for 48 hours, then decalcify in 10% formic acid / 5% formaldehyde for seven days.

• Specimens are then washed and dehydrated in increasing alcohol concentrations from 70-100%.

• Double-embed slices in methyl benzoate/celloidin and paraffin wax, and cut 4µm sections using a rotary microtome.

• Adhere slices to glass slides (Superfrost Plus, Menzel Gläcer, USA), and deparaffinised in 70% alcohol for 15 minutes.

• Stain in 0.04% toluidine blue / 0.1M sodium acetate buffer (10 minutes), then 0.1% fast green (2 minutes), dehydrate in isopropanol then xylene then apply coverslip.

2.9.4 Processing of Synovial Membrane

Synovial specimens were fixed in 10% neutral buffered formalin for 24 hours, washed and dehydrated in increasing alcohol concentrations from 70-100%. They were then cleared in chloroform, infiltrated and embedded in paraffin wax. 4µm sections were cut using a rotary microtome, adhered to glass slides (Superfrost Plus, Menzel Gläcer, USA), and stained with haematoxylin and eosin (H&E).

2.9.5 Histopathology

The histological sections of synovium and cartilage were scored according to the following scoring systems.
<table>
<thead>
<tr>
<th>Criteria</th>
<th>Score</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intimal hyperplasia</td>
<td>0</td>
<td>1-2 layers only</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3-4 layers, focal</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>≥ 5 layers, focal</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&gt; 5 layers, diffuse</td>
</tr>
<tr>
<td>Lymphocytic/plasmacytic infiltration</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>One focus of infiltration</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2-5 foci of infiltration</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Diffuse infiltration or &gt; 5 foci</td>
</tr>
<tr>
<td>Subintimal fibrosis (loose connective tissue areas only)</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Light focal collagenous staining up to 30%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Heavy focal or total light diffuse collagenous staining</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Heavy diffuse collagenous staining</td>
</tr>
<tr>
<td>Vascularity</td>
<td>0</td>
<td>0-2 vascular elements per 100x field</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3-4 vascular elements per 100x field</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5-8 vascular elements per 100x field</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&gt;8 vascular elements per 100x field</td>
</tr>
<tr>
<td>Aggregate score (joint)</td>
<td>0-12</td>
<td>Sum of the scores obtained for the four criteria above</td>
</tr>
</tbody>
</table>

Table 2.9: Microscopic scoring of ovine synovial histopathology (reproduced with permission) \[^139^].
<table>
<thead>
<tr>
<th><strong>Structure</strong> (score the worst area in field of view)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Slight surface irregularities (surface barely disturbed)</td>
<td>1</td>
</tr>
<tr>
<td>Moderate surface irregularities (surface roughened)</td>
<td>2</td>
</tr>
<tr>
<td>Severe surface irregularities (disruption, fissuring/fibrillation to &lt; 10% depth)</td>
<td>3</td>
</tr>
<tr>
<td>Fissures to transitional zone (1/3 depth)</td>
<td>4</td>
</tr>
<tr>
<td>Fissures to radial zone (2/3 depth)</td>
<td>5</td>
</tr>
<tr>
<td>Fissures to calcified zone (full depth)</td>
<td>6</td>
</tr>
<tr>
<td>Erosion or severe fibrillation to mid zone (1/3 depth)</td>
<td>7</td>
</tr>
<tr>
<td>Erosion or severe fibrillation to deep zone (2/3 depth)</td>
<td>8</td>
</tr>
<tr>
<td>Erosion or severe fibrillation to calcified zone (full depth)</td>
<td>9</td>
</tr>
<tr>
<td>Erosion or severe fibrillation to subchondral bone</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Chondrocyte density</strong> (“average” score for whole field of view in non-calcified cartilage)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Increase or slight decrease</td>
<td>1</td>
</tr>
<tr>
<td>Moderate decrease</td>
<td>2</td>
</tr>
<tr>
<td>Severe decrease</td>
<td>3</td>
</tr>
<tr>
<td>No cells</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Cell cloning</strong> (score the whole field of view)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Several doublets</td>
<td>1</td>
</tr>
<tr>
<td>Many doublets</td>
<td>2</td>
</tr>
<tr>
<td>Doublets and triplets</td>
<td>3</td>
</tr>
<tr>
<td>Multiple cell nests or No cells in section</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Interterritorial Toluidine blue</strong> (score the worst area in field of view working from AC surface down)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Decreased staining to mid zone (1/3 depth)</td>
<td>1</td>
</tr>
<tr>
<td>Decreased staining to deep zone (2/3 depth)</td>
<td>2</td>
</tr>
<tr>
<td>Decreased staining to calcified zone (full depth)</td>
<td>3</td>
</tr>
<tr>
<td>No staining</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Tidemark/calcified cartilage/subchondral bone</strong> (score the worst area in field of view)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact subchondral bone plate + single tidemark</td>
<td>0</td>
</tr>
<tr>
<td>Intact subchondral bone plate + duplicated tidemark</td>
<td>1</td>
</tr>
<tr>
<td>Blood vessels penetrate through subchondral bone plate to calcified cartilage</td>
<td>2</td>
</tr>
<tr>
<td>Tidemark penetrated by blood vessels</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2.10: Microscopic scoring of ovine cartilage (modified Mankin system). See Figure 2.6 for representative examples of each score. (reproduced with permission) [140]
Figure 2.6: Representative toluidine blue/fast green stained sections of cartilage from sheep with meniscectomy-induced OA, to demonstrate the features associated with the pathology scores for the different parameters outlined in Table 2.9. A higher magnification image is included for cell cloning to demonstrate doublets, triplets and multiple cell nests (clusters). Reproduced with permission M. Cake.\textsuperscript{[140]}
2.10 STATISTICAL METHODS

Cartilage and Synovium

Cartilage and synovium samples for all treatment groups were scored by a single blinded reader and the results collated. Statistical analysis for treatment effect was performed using Kruskal-Wallis nonparametric analysis and for specific between group comparisons using Mann Whitney U nonparametric analysis with p<0.05 considered significant.

Ligament

Measurements of ACL fibril diameter, fibril area, fibril density and number of fibrils for each group were carried out using an image analysis program (Image J64, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/), and the data collated using an Excel spreadsheet (Microsoft Office Excel 2007). Statistical analysis for treatment effect was performed using a one-way analysis of variance (ANOVA) across treatment groups, with Fisher’s protected least significant difference (PLSD) pairwise comparisons tests to compare treatment group means. For molecular and biochemical analysis, result reports were generated by device software programmes (Rotor-Gene Real-time Analysis Software 6.1© Corbett Research, and Revelation Quicklink Assays Statistics DLL Version 4.24 respectively). Gross morphology scoring was again performed by a single blinded reader and the results presented in an Excel spreadsheet. For each method of investigation, statistical analysis for treatment effect was performed using a paired, one-tailed, T-test. A significance level of p<0.05 was applied.

The association of OA score with decorin and elastin concentrations, and fibril diameter, within the MENX treatment group was explored using simple regression. The
R square was used as a measure of association with R square greater than 0.80 considered strong association. SAS 9.3 (SAS Institute, Cary, NC).

In all analyses, the left and right joints of each sheep were treated as independent events rather than repeated measures of the same event. From a pure statistical standpoint, this could be regarded as a weakness in the statistical methodology used in these studies. This decision was made for the following reasons:

1. Meniscectomy (MENX) represents a significant surgical insult to the joint. Although ovine MENX is recognized as providing a repeatable model of OA, the response to surgery within an individual joint does vary in severity. The magnitude of this variation was considered to be greater than that caused by genetic variation between individual sheep, which supports the view that the data from each joint can be regarded as individual events.

2. Large animal experimental models require large budgets, and are associated with significant animal welfare issues. Minimising the number of animals used is an economic as well as a welfare issue. To minimize the number of animals in these experiments, bilateral surgery was performed, providing the possibility of two joints for analysis per animal. Unilateral surgery decreases the tissue available for analysis and provides no advantage in reduction of animal numbers by providing an internal control to replace a separate control group. The contralateral limb cannot be considered a non-operated control due to the effects of abnormal loading in response to surgery on the contralateral limb (ref).
3. Three different treatment effects were examined in these studies – MENX, OVX and the combined effect of MENX+OVX. Having resolved that left and right joints could be regarded as individual events due to variation in surgical response, the structure of the studies required that the joints from the OVX alone groups were treated likewise for the purpose of statistical analysis.

4. The ACL tissue from each joint was analysed using biochemical and molecular techniques, as well as for structural analysis (histopathology, TEM and SEM), requiring significant amounts of tissue that are only available from a large animal model, which then creates the conflicts outlined above.

The impact of this potential weakness in statistical methodology on the interpretation of the results of these experiments will be discussed in Chapter 5.
CHAPTER 3

THE INFLUENCE OF MENISCECTOMY ON THE ANTERIOR CRUCIATE LIGAMENTS OF SHEEP

3.1 INTRODUCTION

3.1.1 The Structure of Cartilage

Cartilage is a tissue of mesenchymal origin and is found at numerous sites throughout the body. Cartilaginous tissues are classified histologically as being elastic, fibrocartilaginous or hyaline depending on their molecular composition [141-143]. Here the focus will be placed on a specialised type of hyaline cartilage known as articular cartilage, which is found covering the articular surfaces of synovial joints. It functions to provide a smooth, low-friction, gliding surface for the articulating bones of the joint, and enables the joint to withstand compressive loads. This shock absorbing ability minimises peak pressures on the subchondral bone, preventing wear of the joint's underlying structures under normal conditions [142, 144].

3.1.1.1 Chondrocytes

Structurally articular cartilage is a dynamic, highly organized tissue, comprised of a single cell type, the chondrocyte, located within an extracellular matrix (ECM) [145-147]. Chondrocytes make up 1-5% of the wet weight of cartilage and are only sparsely spread throughout the ECM, generally with no cell-to-cell contact. These spheroid-shaped cells are responsible for the synthesis and maintenance of the matrix infrastructure [142, 145]. As cartilage is an aneural, alymphatic and avascular tissue, the chondrocytes receive their nutrition and hormones by diffusion through the ECM from the synovial fluid, and survive on a low oxygen concentration via anaerobic metabolism. The
metabolic function of these cells is influenced by mechanical loading forces placed on the joint \[142, 145, 147\].

### 3.1.1.2 Extracellular Matrix

The ECM itself is comprised of a complex network of collagen fibres, elastin, hyaluronic acid, proteoglycans (mostly aggrecan and some other small leucine-rich proteoglycans), cartilage link proteins, fibronectin and water \[142, 144, 146\]. 65-80% of the wet weight of cartilage is water, depending on the depth from the articular surface. This high water content facilitates the transport of nutrients through the matrix and is a medium for lubrication, thus creating a low-friction gliding surface. The water content is fixed within the tissue and allows deformation of the cartilage in response to compressive loading, therefore protecting the underlying joint surfaces from trauma \[141, 142, 148\].

### 3.1.1.3 Collagen

The tensile strength of articular cartilage is provided by its collagen content, which forms 10-20% of the tissue’s wet weight. Type II collagen is the principle type present and forms a major component (90-95%) of the macrofibrilar framework of the ECM. Collagens III, VI, IX, X, XI, XII and XIV are all present in much lower amounts and also contribute to the structure of the mature matrix. These minor collagens help to organise and stabilise the larger collagen II fibril network \[142, 143, 147, 149\].

### 3.1.1.4 Proteoglycans

Another major component of the ECM are the proteoglycans, which are synthesised and secreted into the matrix by chondrocytes \[142, 147\]. Proteoglycans are protein polysaccharide molecules which comprise 10-20% of the tissues wet weight. They are a
family of glycoconjugates with a central core protein to which one or more glycosaminoglycan (GAG) side chains are covalently bonded [141, 142]. There are two major classes of proteoglycans, large aggregating proteoglycans (LAPs) and small leucine-rich proteoglycans (SLRPs). The principle LAP present is aggrecan which forms large aggregates in the ECM [150, 151]. These aggregates are comprised of numerous monomers that are non-covalently bound to a hyaluronic acid (HA) chain and stabilised by small glycoprotein link proteins [144, 148, 150, 151].

Aggrecan molecules consist of a core protein of ~2300 amino acids. At one end is an amino-terminal globular domain (G1) which is responsible for its interaction with the HA chain. This is followed by a short interglobular domain which separates G1 from an additional globular domain (G2). A third carboxy-terminal globular domain (G3) is separated from G2 by a keratan sulfate (KS) attachment domain, and two chondroitin sulfate (CS) attachment domains. Up to 100 covalently attached KS or CS chains may be present in these three GAG attachment domains resembling a bottle-brush-like configuration (Figure 3.1) [146, 147, 150].

When fully ionized the GAG chains attribute a charge negative density of ~9000 negative charges/aggrecan to each aggrecan molecule. This high density of fixed negative charges draws water into the tissue, therefore yielding a high osmotic pressure which is responsible for more than 50% of the compressive modulus of articular cartilage [144, 146, 150, 151].
Other LAPs present in articular cartilage include versican and perlecan but these are expressed at much lower levels. Like aggrecan, versican has the ability to bind with HA and is involved in the modulation of cell proliferation, adhesion and migration, and plays a role in early joint morphogenesis \cite{152, 153}. Perlecan is usually found in the basement membrane of tissues, however as cartilage is devoid of a basement membrane, its role here is unclear \cite{150}.

A number of different SLRPs may be found in cartilage tissue such as decorin, biglycan, fibromodulin and lumican. Their function depends on their core protein and the type of GAG side chains present. The core protein of the SLRP allows it to interact with the collagen fibrils that form the tissue framework, enabling them to perform a

Figure 3.1: Schematic representation of the structure of aggrecan and its interaction with hyaluronate (hyaluronic acid), and the link protein. (KS), keratan sulfate chains attached at the KS attachment domain; (CS), chondroitin sulfate chains attached at the two CS attachment domains; (G1), amino-terminal globular domain; (G2), additional globular domain; (G3), carboxy-terminal globular domain. (Image modified from Heinegard & Oldberg, 1989).
number of functions such as regulation of fibril diameter during fibrillogenesis, and assisting in fibril-fibril interactions in the ECM. They may also limit the access of collagenases to their cleavage sites on the collagen molecules therefore protecting them from degradation [141].

3.1.1.5 Ultrastructure
Articular cartilage can be further classified into four morphologically distinct zones [68] (Figure 3.2). Each zone is characterised by variation in the organisation of the collagen network, and by differences in the amounts and types of proteoglycans present [142, 143, 145, 147].

1. The Superficial Zone: is the thinnest of all the zones, 10-20% of the total cartilage volume, and is located closest to the surface. The chondrocytes present here are flattened or disc-shaped [145], and are covered by a thin film of synovial fluid which acts to lubricate the gliding surface [147, 148]. This zone contains high levels of collagen (primarily types II and IX) which are packed tightly and arranged parallel to the articular surface. The high levels of collagen give this layer the greatest tensile strength, enabling it to resist the sheer, tensile and compressive forces of the articular surface. Relatively low levels of GAG are present at this level [154].

2. The Transitional Zone: is immediately deep to the superficial zone and represents 40-60% of the total cartilage volume. This level has a lower type II collagen content, however the collagen fibrils are thicker, and the amount of GAG increases [147, 154]. The chondrocytes become more spherical in shape and lower in density. In this zone the collagen fibrils are arranged more obliquely to further resist compressive forces [145, 147].
3. *The Deep Zone*: is deep to the transitional zone and represents ~30% of the total cartilage volume. It provides the greatest resistance to compressive forces, with the collagen fibrils arranged perpendicular to the articular surface. Collagen levels in this region are the lowest while GAG levels are the highest. The spherical chondrocytes are aligned in columns parallel to the collagen fibres [147, 154].

4. *The Calcified Cartilage Zone*: this zone is distinguished from the deep zone by a line known as the 'tide mark' and it contains high levels of type X collagen as the cartilage integrates with the subchondral bone. In this zone the cell population is very low and the chondrocytes are hypertrophic [147, 154].

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**Figure 3.2**: cross sectional diagram of the normal structure of articular cartilage outlining: A, the cellular organisation in the different zones of articular cartilage; B, the orientation of collagen fibres throughout the various cartilage zones. (Image modified from Buckwalter *et al.*, 1994).
3.1.2 Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis and is a leading cause of chronic disability, particularly in the hips, knees and hands of human sufferers [155-158]. Current estimates by the World Health Organisation state that the OA is a cause of disability in at least 10% of the population over the age of 60 years [155, 159]. Furthermore, the disorder is not limited to human populations, with all animals that fuse epiphyses of synovial joints, both primate and non-primate, being capable of developing OA. While this fact greatly increases the scope of the problem, it also provides us with the opportunity to increase our understanding of the pathophysiology of the disorder through the study of animal models [156].

3.1.2.1 Pathophysiology

OA is characterised by focal areas of articular cartilage loss within the synovial joints that may progress to fibrillation of the cartilage matrix (especially in weight bearing zones), fissure appearance, ulceration and eventually full thickness loss of the joint surface (Figures 3.3 and 3.4) [157, 159-161].

Figure 3.3: A schematic representation of the progression of OA. A), Healthy cartilage demonstrating the intact superficial layer with a smooth lubricated surface; B), Early stage OA with loss of the superficial layer and exposure of the transition layer, resulting in surface irregularities such as deamination, fibrillation, fissures and erosions; C), Late stage OA with the appearance of deep erosions and sclerosis of the subchondral bone. (Image from Tummala et al 2011).
Associated with the loss of cartilage, is remodelling of the calcified cartilage zone (also known as the tidemark), which becomes less distinct on histological section and is characterised by infiltration of blood vessels in the later stages of OA. In addition, sclerosis of the subchondral bone occurs, with disordered bone remodelling (particularly in non-weight bearing regions) which may result in microfractures as OA progresses[162].

Figure 3.4: A, a histological section (Tol Blue) of normal sheep articular cartilage showing normal ultrastructure. Note the evenly distributed chondrocytes, homogeneous proteoglycan staining and smooth, even surface layer; B, an osteoarthritic section of sheep articular cartilage showing disruption of normal structure, with loss of blue staining reflecting depletion of proteoglycans. Note the focal loss of cellularity, the uneven cartilage thickness and the distinct areas of fibrillation (Image courtesy M. Cake).
Another hallmark of OA is the onset of chondrocyte cloning, where usually solitary chondrocytes appear in clusters as a result of increased focal mitotic activity (Figure 3.5). These proliferating chondrocytes associated with erosive lesions have been found to contain unstable DNA with tetraploidy\textsuperscript{[162]}. 

![IMAGE REMOVED DUE TO COPYRIGHT RESTRICTIONS](image)

Figure 3.5: Histological section of cartilage from an osteoarthritic human knee showing proliferation of chondrocytes in clusters, also known as cloning (see arrows and enlarged inset). (Image from Pauli et al., Osteoarthritis and Cartilage, 20, 2012)\textsuperscript{[163]}.  

Cartilage breakdown is accompanied by hypertrophy of the bone (osteophyte formation and subchondral bone sclerosis), and may include inflammation of the joint capsule and synovial membrane\textsuperscript{[157, 159-161]}. In addition, there is a decrease in the concentration and viscosity of the synovial fluid of patients with OA, which may reduce joint lubrication and decrease the capacity to withstand compressive force\textsuperscript{[164]}.  

The most prominent symptom in most people with OA is joint pain, ranging from mild to severe, and it is the most important determinant of the degree of disability in patients. Other general features of OA include limited joint movement, crepitus, joint effusion and distension of the joint capsule, wasting of the muscles that act across the affected joint, instability of the joint and in severe cases joint locking [157,165,166].

### 3.1.2.2 Epidemiology

The cause of OA is likely to be multifactorial, with the joint having a limited capacity to react to various insults, the OA lesion may reflect a common end point [160, 161, 167]. The OA process is thought to involve a complex interaction between cells and soluble mediators such as cytokines, growth factors, inflammatory mediators, matrix-metalloproteinases and chondrodegradative enzymes [164]. Pro-inflammatory enzymes such as IL-β and TNF-α, also have a significant influence on OA. They act to increase MMP synthesis and reduce the production of the major physiological inhibitors of these enzymes. They may also inhibit the synthesis of matrix constituents such as collagen and proteoglycans [161, 168].

OA can be classified as primary (idiopathic) or may be secondary as a consequence of trauma, surgery, infection or some other disease process [155, 164]. There are a host of risk factors that have been identified which potentially increase the likelihood of developing OA. These include age, gender, obesity, occupation, nutrition, genetics, ethnicity, anatomical abnormalities, proprioception, injury, bone density and muscle weakness [156, 166, 169]. Of these, age, gender and injury are among the most detrimental.

- Age - reduces the joint's ability to protect itself from biomechanical stress due to thinning of the non-calcified cartilage, increased joint laxity, aberrant joint
loading, oxidative damage, wear and tear and accumulated past injuries [155-157, 167, 170]. In addition, proteoglycan content falls sharply with advancing age and may decrease by up to 50% [166, 171].

- Gender - the incidence of OA in women and men is similar until about the age of 50 yrs. Thereafter the disease becomes more prevalent, severe and generalised in women, particularly involving the hands and knees [158]. The incidence of OA in older women post-menopause exceeds the expected increase in prevalence that would occur simply from increasing age [167, 169, 172-175]. Oestrogen affects joint tissues directly via oestrogen receptors which have been found on human articular chondrocytes, or indirectly by secondary messengers [172], and potentially influences collagen, cytokines, MMPs and may be beneficial by improving tissue metabolism [176-178]. Studies suggest ovarian hormone replacement therapy may have a protective effect on joints and decrease the development OA [172, 176, 179-183]

- Injury - this is of particular significance in the knee joint. There is a very high prevalence of OA, with pain and functional limitation, following anterior cruciate ligament (ACL) rupture or meniscal damage, either due to sport injury or some other trauma [184-188]. Injury to these structures predisposes the subject to OA due to disruption of normal joint biomechanics and instability of the joint. This results in aberrant wear on the articular surfaces and can cause further degeneration of other joint structures. ACL reconstruction surgery and techniques aim to restore long term function and joint-stability in order to prevent OA development. It is difficult however to reproduce 100% normal joint function after injury and studies have demonstrated that degenerative
changes often progress over time, even in patients with a good surgical outcome.

This suggests that OA development is not preventable after joint insult\(^{189-193}\).

### 3.1.2.3 Diagnosis

Radiographic, symptomatic and self-reported (via questionnaire) are the most common terms used in the diagnosis of OA, with most epidemiological studies using one or a combination of these\(^ {159}\). Radiography is the often the first-line diagnostic tool in OA, but it is insensitive to change and doesn't depict the soft tissue changes sufficiently\(^ {194, 195}\), and has limited ability to detect OA in the early stages\(^ {196}\). Furthermore, radiographic findings do not always correlate with clinical symptoms\(^ {197, 198}\). Several grading systems have been developed such as the Kellgren-Lawrence, Brandt and Ahlback based on the presence or absence of features such as osteophytes and joint space narrowing\(^ {199, 200}\). There is dispute however, among researchers, about whether there is some inconsistency in the interpretation of these scales between studies\(^ {171, 198}\), and others conclude a more sensitive method of imaging such as magnetic resonance imaging (MRI) should be utilised\(^ {187, 194, 195}\). In general a diagnosis of osteoarthritis is made when there is a combination of radiographic changes and the presence of clinical pain\(^ {197}\).

### 3.1.2.4 Treatment

OA remains an irreversible disease with main treatment goals being the reduction of pain and the maintenance, if not improvement of joint mobility\(^ {199}\). There are a number of pharmacologic and non-pharmacologic treatments available. Non-pharmacologic options should, where possible, be the first step in the treatment of the disorder. These include specific exercises to improve muscle strength and improve joint stability,
modification of lifestyle to reduce joint loading and the discontinuation of exacerbating activities. Other suggestions may include weight loss in obese patients, improving posture, the wearing of supportive splints or braces and the use of orthotics etc [166].

If these treatments do not ameliorate symptoms, pharmacologic options may need to be utilised. These include acetaminophen, topically and orally administered non-steroidal anti-inflammatory drugs, COX-2 inhibitors, and intra-articular injection of long-acting steroids. A new group of drugs known as symptomatic slow-acting drugs in OA (SYSADOAs) including chondroitin sulfate, diacereine, glucosamine sulfate, avocado/soybean unsaponifiables and hyaluronic acid have demonstrated improvement in pain and physical function with very low toxicity [166, 197, 201].

The final option available is surgery and in most cases should be reserved until other options have proven ineffective. Surgical options include arthroscopy to remove damaged articular cartilage, tidal irrigation to remove fibrin and debris, osteotomy to remove osteophytes and total joint replacement (usually in the knee and hip) [166, 197].

3.1.3 The Role of Ligament in OA
As previously discussed, there is strong evidence that ligament damage (commonly ACL rupture), may result in OA of a joint rendered unstable by the disruption of normal joint biomechanics [93]. In fact, surgical transection of the ACL (the Pond-Nuki technique) is a commonly utilised model to induce OA in animals for the purpose of research [202]. While the cause of OA in these instances is clear, occurring as a consequence of significant ACL disruption, there are often times when the initiating factors are unknown. It may also be that the characteristic bone and cartilage changes
commonly associated with OA are features of the later stages of the disease, with other less detectible changes occurring earlier in other joint structures such as the ligaments [203]. The exact role of ligament in the aetiology of idiopathic OA is still unclear, but has been the focus of a number of animal and human studies in recent years.

3.1.3.1 Animal Studies

Being that age is a significant risk factor for OA, one of the earlier animal studies utilised a rabbit model to determine the effect of age on the tensile properties of medial collateral ligament (MCL). Results indicated that the tensile strength of the MCL increased steadily until maturation and after a brief plateau, began to decline as age increased. If ligament tensile strength decreases with advancing age, this could potentially have a detrimental effect on normal knee biomechanics in older subjects, predisposing them to OA [204].

Anderson-MacKenzie et al. (1999) [203] proposed that biochemical cruciate ligament alteration may play a role in early primary, idiopathic OA. Using an STR/ORT mouse model of spontaneous OA, they discovered that active collagen metabolism in the cruciate ligament was up-regulated in the STR/ORT strain. They determined that active collagen remodelling was occurring in the STR/ORT ligament due to the higher levels of MMP-2 (responsible for ligament catabolism), and the presence of higher levels of keto-imine immature collagen crosslinks (evidence of ligament anabolism) compared to controls. In addition, levels of MMP-9 were not significantly higher in the STR/ORT ligament compared to controls suggesting that the remodelling is not being mediated by inflammation. These changes, which were associated with significantly weaker ACLs in the STR/ORT strain, were detected before radiological signs of OA developed,
suggesting that biomechanical cruciate ligament alteration may be an important factor in early osteoarthritis.

Studies using the Dunkin-Hartley (DH) guinea pig model of spontaneous OA have also detected changes in ligament metabolism prior to the onset of cartilage changes. Young et al. (2002) [205], examined the posterior cruciate ligaments (PCLs) in DH guinea pigs, finding aberrant synthesis of cartilage-like matrix in the mid-ligament prior to the appearance of OA (cartilage and bone changes). Deposition of type II collagen in ligament is usually restricted to the ligamentous attachment sites; therefore it is reasonable to expect that the abnormal presence of fibrocartilage in mid-ligament would alter the mechanical properties of the ligament, reducing its elasticity. As the cruciate ligaments are the main interconnecting and stabilising elements of the knee joint, changes in their composition and biomechanical function could lead to instability and predispose the joint to OA.

Quasnichka et al. (2005) [92], also used the DH guinea pig OA model to examine the structure and metabolism of the cruciate ligaments, revealing the DH strain had significantly laxer ACLs than the control strain. This increased laxity was linked to uncontrolled collagen turnover. In a subsequent study, Quasnichka et al. (2006) [206] found the DH strain had again significantly increased ACL laxity than the control strain at all experimental time-points. This increased laxity was again associated with increased remodelling of the cruciate ligaments, based on markers of collagen turnover, and it occurred prior to bone and cartilage pathology, demonstrating the fundamental role of ligament in the aetiology of OA.
Comerford et al. (2005) \cite{25} presented further evidence of abnormal ligament metabolism influencing joint stability. They compared ACLs from Labradors (a breed predisposed to cruciate rupture) with Greyhounds (not predisposed) and found differences in the metabolism of the collagens matrix (specifically increased collagen turn-over). In addition, they discovered anterior-posterior (AP) laxity was significantly greater in the Labrador knee joints and their ACLs tended to be weaker (lower ultimate tensile strength). Inferior mechanical properties of the ACL and increased joint laxity could increase the potential for non-contact ligament rupture, and eventual knee OA \cite{207}.

\subsection{3.1.3.2 Human Studies}

Like animal based studies, human research into the aetiology of OA has identified changes in ligament as a potential causative factor. Silman et al. (1987) \cite{208} revealed strong evidence that adolescent women had significantly greater joint mobility than men for the lower limb (knee) and fore-arm rotation. In the knee, this may contribute to the female susceptibility to cruciate rupture and compound the risk of OA in older women. A predisposition of older females to OA is demonstrated by the findings of Woo et al. (1991) \cite{209}, which showed the structural properties of the female femur-ACL-tibia complex (i.e. linear stiffness, ultimate load, and energy absorbed) all decreased significantly with advancing age. This may explain in part the predisposition of older females to OA.

Sharma et al. (1999) \cite{210}, further investigated the correlation between age, gender and knee laxity, with results also demonstrating that women had a greater knee laxity than men (especially valgus-varus), and that ligaments become more compliant with age. In addition, they showed that there was greater laxity in the uninvolved knees of patients
with OA versus age matched control subjects without OA, supporting the concept that some portion of increased laxity may predate the disease \[211\].

Multiple authors have investigated the role of increased joint laxity in degenerative joint disease, particularly in the knee. Results identified increased joint laxity (identified and measured prior to ACL injury) as a significant risk factor for non-contact ACL injury and subsequent development of OA \[212-215\]. It is possible that even in the absence of complete rupture, the ligament may sustain multiple 'micro-injuries' over time which, in the long term, could contribute to the degeneration of the ligament structure and loss of biomechanical integrity.

In 2010, Zhang et al. \[169\] stated that knee laxity was also greater in the as yet non-arthritic knees of patients with idiopathic OA compared to a control group. Like Sharma's 1999 study, this suggests that a portion of the increased laxity in knee OA precedes disease development and may exacerbate its progress. While it is likely that ligament plays a key role in knee laxity, muscles and other structures such as the joint capsule may also play a part.

Kleinbart et al. (1996) \[216\] provided more evidence of ligament's involvement in joint laxity in humans in their study of PCLs from arthritic knees of patients at the time of total knee arthroplasty (TKA). They histologically compared the PCLs from severely OA affected knees to non-OA control PCLs. Ligaments were considered histologically abnormal and degenerative if loose, mucoid, myxoid, or cystic changes were noted. The magnitude of degeneration was defined as normal, slight, mild, moderate, or marked, based on the amount of tissue demonstrating change. Of PCLs in the control group,
45% were normal, 33% showed slight changes and 22% showed mild changes. Interestingly no control PCLs exhibited marked degenerative changes. PCLs from the OA knees were very different, with 63% showing marked degenerative changes (± 17% normal and 20% mild-moderate). The authors concluded that the PCL is not spared the degenerative changes involved in OA of the knee. It is not possible to ascertain from this study whether these changes in the PCLs precede OA or if they are a result of it. It has been well established that damage to the ACL is often involved in the OA process, so it would be common to find the ACL in a degenerated state at the time of TKA. Acute PCL injury occurs far less frequently and this is one of few studies that have examined the fate of the PCL in OA. Interestingly the PCL was also found to be degenerate in the arthritic knee joint, potentially as a result of disruption to normal knee biomechanics, exposure to inflammatory mediators or possibly due to abnormal ligament metabolism as has been identified in animal models. Foos et al. (2001) [217], cited tissue remodelling within the ACL of females due to abnormal metabolism as a contributing factor to their predisposition to rupture.

While there is mounting evidence that increased knee laxity due to compromised ligament integrity is common preceding the development of disease and in the early stages of joint degeneration, this may change as the disease progresses to the late stages. Laxity of the knee in late stage OA may in fact be due to other factors such as loss of cartilage and/or bone height, chronic capsuloligamentous stretch or combinations of ligamentous, meniscal muscular and capsular pathology [218, 219]. Brage et al. (1994) [220] identified differences in knee laxity in early and late stage OA, finding that knees of severe OA patients displayed less laxity than normal knees. This may be due to a
combination of factors such as contracture of the ligaments, and pressure of osteophytes against joint structures.

More recently, Ragusa & Hill (2011) [221] investigated the role of the elastic fibre system in the pathogenesis of OA. The elastic fibre system gives the knee ligaments their elastic property which allows them to withstand stretch and deformation. Without this system in place, ligaments would become lax, and joints unstable. This study found a correlation between the elastic fibre content of ligament and the development of OA. The elastic fiber density, measured as the concentration of elastic fibers per unit area, was correlated with the severity of OA, which was graded on a 0–16 scale using histologic and macroscopic markers. Knees with the highest OA scores exhibited the lowest elastic fibre content in the collateral ligaments (medial and lateral).

Much has been written about ligament and OA of the knee, but what of other joints in the body? As previously mentioned the hand is another region prone to development of OA. Several authors have examined the effect of ligament insufficiency and the occurrence of hand OA. Tan et al. (2005) [222], used high resolution MRI to show ligamentous and tendon abnormalities were a feature of hand OA at even the earliest stages. These findings were repeated in a subsequent 2006 study from these authors in which ligament and tendons showed degenerative changes, while cartilage appeared normal and inflammation was minimal [225]. Hunter et al. (2005) [224] provided longitudinal radiographic evidence supporting their hypothesis that instability of the trapeziometacarpal joint with radial subluxation due to ligament laxity leads to OA of the thumb.
3.1.4 The Meniscectomy Model of Osteoarthritis

Osteoarthritis (OA) is a naturally occurring condition in a variety of animal species such as mice, guinea pigs, dogs and macaques. Despite this, it is difficult to establish colonies of animals with spontaneous OA that are suitable for experimental studies. This is due to the influence of ill-defined environmental factors, the time required to collect adequate sample numbers (often years) and the difficulty in matching study groups e.g. for age, sex, weight, stage of disease [225]. Therefore in order to examine the effect of osteoarthritis on the ligaments of the knee joint, the disease must be experimentally induced under controlled conditions.

Transection of the anterior cruciate ligament (ACL) is a well-established technique for surgically inducing OA in the knee joint [226-228], but this would render the ligament useless for the purposes of this study. Furthermore, while this method has proven effective in other species (such as the dog, rat or rabbit), cruciate ligament transection alone appears to induce very limited or mild cartilage damage in sheep [140]. An alternative method of inducing OA while leaving the ACL intact is to perform a meniscectomy, a procedure that involves surgical removal of the medial or lateral meniscus of the knee [225, 229, 230].

Many studies have successfully utilised the meniscectomy model in small animals such as rats, guinea pigs and dogs to produce a progressive pattern of cartilage damage similar to that seen in human OA [231, 232]. Small animals such as rodents may be ideal when very large study numbers are required, but there are several disadvantages associated with some small animal models. Firstly, dogs, rabbits, rats and mice stand with their knees flexed and therefore experience different biomechanical loading to

...
humans \cite{233, 234}. Secondly, as mentioned previously, the amount of tissue available for biochemical and histological studies is limited in animals such as rats, mice, guinea pigs and rabbits \cite{225, 234}. Thirdly, the short lifespan of rodents may preclude them from use in long-term OA studies. Finally, there is a social stigma associated with the use of small animals like dogs, cats and rabbits for research purposes \cite{235}.

A number of authors have identified the sheep meniscectomy model as an ideal alternative for the study of OA. Ghadially et al. (1986), Turner et al. (1997) and Burger et al. (2007) stated that the anatomy and biomechanics of the sheep knee joint closely resemble that of the human knee \cite{57, 233, 236}. Although they are quadrupeds, sheep knee joints are relatively extended, much like the knee joint of humans. The sheep knee may be smaller than that of a human, but there are few other anatomical differences between the knees of the two species. Little et al. (1997) and Oakley et al. (2004) concluded that the cartilage changes in meniscectomised sheep were analogous to those seen in the knee cartilage of humans with OA, and that the ovine meniscectomy model is valid means for evaluating the initiation and progression of the disease \cite{237, 238}. 
3.2 AIMS & HYPOTHESES

The evidence presented in Section 3.1 shows the effects of OA on articular cartilage and subchondral bone are well documented. Less is known however about the collateral damage OA may impose on other intracapsular joint structures such as the cruciate ligaments. It may be that the inflammatory process associated with OA induces deterioration of these major stabilisers of the joint, and therefore may alter normal knee biomechanics, further contributing to the disease process. This aim of this study was to show that the meniscectomy model leads to degenerative changes in the knee joint which parallel naturally occurring OA, and using this model, investigate the influence of OA on the structure and organisation of the anterior cruciate ligament.

**Hypothesis 1:** that osteoarthritis (meniscectomy) alters gene expression and collagen fibril structure and organisation in ACL.

**Hypothesis 2:** animals with worse osteoarthritic changes have more severe ligament derangement.
### 3.3 RESULTS

#### 3.3.1 Molecular Biology

#### EXPRESSION NORMALISED TO TOTAL RNA

<table>
<thead>
<tr>
<th>TARGET GENE</th>
<th>NOC</th>
<th>MENX</th>
<th>FOLD DIFFERENCE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (CI)</td>
<td>Mean (CI)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggrecan</td>
<td>279.3 (0 – 686.2)</td>
<td>130.6 (44.2 – 217.0)</td>
<td>2.14</td>
<td>0.436</td>
</tr>
<tr>
<td>Basic FGF</td>
<td>329.9 (138.2 – 521.6)</td>
<td>164.7 (121.7 – 207.7)</td>
<td>2.00</td>
<td>0.147</td>
</tr>
<tr>
<td>Biglycan</td>
<td>164.3 (93.7 – 234.9)</td>
<td>165.3 (100.6 – 230.0)</td>
<td>0.99</td>
<td>0.985</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>65.1 (28.6 – 101.6)</td>
<td>48.4 (37.0 – 59.8)</td>
<td>1.34</td>
<td>0.439</td>
</tr>
<tr>
<td>Collagen type II</td>
<td>355.2 (0 – 728.1)</td>
<td>252.0 (84.2 – 419.8)</td>
<td>1.41</td>
<td>0.649</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>34.8 (22.2 – 47.3)</td>
<td>36.0 (24.7 – 47.3)</td>
<td>0.97</td>
<td>0.888</td>
</tr>
<tr>
<td>CTGF</td>
<td>307.2 (98.8 – 515.5)</td>
<td>131.5 (71.7 – 191.3)</td>
<td>2.34</td>
<td>0.084</td>
</tr>
<tr>
<td>Decorin</td>
<td>395.4 (213.9 – 577.0)</td>
<td>150.1 (109.2 – 191.0)</td>
<td>2.63</td>
<td>0.008</td>
</tr>
<tr>
<td>Elastin</td>
<td>579.5 (301.2 – 857.8)</td>
<td>234.5 (168.5 – 300.5)</td>
<td>2.47</td>
<td>0.042</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>298.7 (161.1 – 436.2)</td>
<td>209.4 (149.8 – 269.0)</td>
<td>1.43</td>
<td>0.289</td>
</tr>
<tr>
<td>iNOS</td>
<td>338.9 (113.0 – 564.9)</td>
<td>266.9 (90.5 – 443.3)</td>
<td>1.27</td>
<td>0.638</td>
</tr>
<tr>
<td>Lubricin</td>
<td>219.3 (51.9 – 386.7)</td>
<td>100.2 (69.9 – 130.5)</td>
<td>2.19</td>
<td>0.224</td>
</tr>
<tr>
<td>Lumican</td>
<td>200.2 (70.7 – 329.6)</td>
<td>167.8 (81.6 – 254.0)</td>
<td>1.19</td>
<td>0.700</td>
</tr>
<tr>
<td>MMP-1</td>
<td>35.2 (11.7 – 58.8)</td>
<td>123.1 (66.8 – 179.4)</td>
<td>0.29</td>
<td>0.006</td>
</tr>
<tr>
<td>PDGF</td>
<td>138.4 (38.7 – 238.1)</td>
<td>95.5 (64.6 – 126.4)</td>
<td>1.45</td>
<td>0.466</td>
</tr>
</tbody>
</table>

Table 3.1: Results for target gene expression in ACL tissue in meniscectomised (MENX) and non-operated control (NOC) sheep, shown as value of total RNA.

#### EXPRESSION NORMALISED TO β-actin

<table>
<thead>
<tr>
<th>TARGET GENE</th>
<th>NOC</th>
<th>MENX</th>
<th>FOLD DIFFERENCE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (CI)</td>
<td>Mean (CI)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggrecan</td>
<td>2.501 (0.6 - 4.4)</td>
<td>2.084 (0.5 - 3.7)</td>
<td>1.20</td>
<td>0.750</td>
</tr>
<tr>
<td>Basic FGF</td>
<td>3.631 (2.4 - 4.9)</td>
<td>2.389 (1.8 - 3.0)</td>
<td>1.52</td>
<td>0.114</td>
</tr>
<tr>
<td>Biglycan</td>
<td>1.766 (1.4 - 2.1)</td>
<td>2.334 (1.4 - 3.2)</td>
<td>0.76</td>
<td>0.234</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>0.701 (0.3 - 1.1)</td>
<td>0.678 (0.5 - 0.8)</td>
<td>1.03</td>
<td>0.927</td>
</tr>
<tr>
<td>Collagen type II</td>
<td>3.539 (0.9 - 6.2)</td>
<td>4.090 (1.0 - 7.2)</td>
<td>0.87</td>
<td>0.793</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>0.393 (0.3 - 0.5)</td>
<td>0.479 (0.4 - 0.6)</td>
<td>0.82</td>
<td>0.288</td>
</tr>
<tr>
<td>CTGF</td>
<td>2.648 (1.9 - 3.4)</td>
<td>1.734 (1.1 - 2.3)</td>
<td>1.53</td>
<td>0.078</td>
</tr>
<tr>
<td>Decorin</td>
<td>4.282 (2.8 - 5.7)</td>
<td>2.132 (1.6 - 2.7)</td>
<td>2.01</td>
<td>0.020</td>
</tr>
<tr>
<td>Elastin</td>
<td>6.280 (3.8 - 8.7)</td>
<td>3.216 (2.6 - 3.8)</td>
<td>1.95</td>
<td>0.042</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>3.172 (2.5 - 3.8)</td>
<td>3.124 (1.8 - 4.4)</td>
<td>1.02</td>
<td>0.947</td>
</tr>
<tr>
<td>iNOS</td>
<td>3.577 (1.2 - 6.0)</td>
<td>3.744 (1.2 - 6.3)</td>
<td>0.96</td>
<td>0.927</td>
</tr>
<tr>
<td>Lubricin</td>
<td>2.466 (1.2 - 3.7)</td>
<td>1.409 (0.9 - 1.9)</td>
<td>1.75</td>
<td>0.162</td>
</tr>
<tr>
<td>Lumican</td>
<td>2.426 (1.1 - 3.7)</td>
<td>2.178 (1.2 - 3.2)</td>
<td>1.11</td>
<td>0.776</td>
</tr>
<tr>
<td>MMP-1</td>
<td>0.416 (0.2 - 0.7)</td>
<td>1.586 (1.0 - 2.2)</td>
<td>0.26</td>
<td>0.001</td>
</tr>
<tr>
<td>PDGF</td>
<td>1.324 (0.9 - 1.8)</td>
<td>1.323 (0.9 - 1.7)</td>
<td>1.00</td>
<td>0.997</td>
</tr>
</tbody>
</table>

Table 3.2: Results for target gene expression in ACL tissue in meniscectomised (MENX) and non-operated control (NOC) sheep, with data normalised to β-actin.
3.3.1.1 Gene Expression Normalised to Total RNA

Results showed that decorin expression was significantly reduced in ACLs from MENX sheep compared to NOC sheep \((P \leq 0.028)\), as was expression of elastin \((P \leq 0.042)\). MMP-1 expression was significantly higher in MENX sheep than in NOCs \((P \leq 0.006)\). There was no statistical difference between the NOC and MENX groups for all other genes tested. See Appendix A4.1.2 (page 245) for charted molecular biology results of NOC vs. MENX and comparison with other experimental groups in Chapter 4. A number of other genes were tested but only produced a weak signal that could not be further analysed. These genes were MMP-3, MMP-13, TNF-\(\alpha\), IL-1 and TGF-\(\beta\).

3.3.1.2 Gene Expression Normalised to \(\beta\)-actin

When data was normalised to a housekeeping gene (\(\beta\)-actin), results showed expression of decorin \((P \leq 0.045)\) and elastin \((P \leq 0.043)\) was significantly lower in MENX sheep than in NOC sheep, and that expression of MMP-1 was significantly higher in MENX vs. NOC sheep \((P \leq 0.001)\).

3.3.2 Biochemistry

3.3.2.1 Collagen Content

Total collagen content in the ACL of MENX sheep was not significantly different to the ACL of NOC sheep \([\text{mean } \pm \text{ SE}; \text{MENX } 60.58 \pm 1.4 \text{ vs. NOC } 57.16 \pm 1.1 \% \text{ of dry weight}; \ P = 0.07]\). Full results and charts for all biochemical analyses comparing NOC vs. MENX and including comparison with the other experimental groups (Chapter 4) are available in Appendix A4.2.1 (page 257).
3.3.2.2 Sulfated Glycosaminoglycans (S-GAG) Content

Results showed the amount of S-GAG in ACL from the MENX group was significantly lower than in the ACL of the NOC group, [mean ± SE; MENX 9.35 ± 0.6 vs. NOC 11.78 ± 0.7 % of dry weight; \( P = 0.07 \)].

![BIOCHEMICAL ANALYSIS](image)

Figure 3.6: Results of biochemical analysis of collagen and S-GAG content in ACL, comparing meniscectomised and non-operated control groups.

3.3.3 Transmission Electron Microscopy (TEM)

3.3.3.1 Fibril Size and Arrangement

In NOC sheep, fibrils were of similar size and regularly arranged. The majority of fibrils in the NOC ACLs were found to be within a close size range of 100-200nm. By contrast, MENX had a significantly smaller average fibril size than NOC [mean fibril diameter (nm) ± SE, 128.64 ± 5.14 vs. 151.05 ± 2.28 \( P = 0.013 \)] and showed a greater size range with many small fibrils and a less regular arrangement (Figures 3.7-3.9).
Figure 3.7: TEM (37000x) of ACL collagen fibrils. Image 8666 is a sample representative of the NOC group with fibrils that are relatively uniform in size and regularly arranged. Image 8607 is a sample from the MENX group with fibrils that are more variable in size and more erratic in their arrangement. To view all NOC and MENX sample TEM images examined in this study refer to Appendix A4.3 (page 259).

Figure 3.8: Distribution of collagen fibrils in NOC ACL tissue based on fibril size (diameter in nm).
Figure 3.9: Distribution of collagen fibrils in MENX vs. NOC ACL tissue based on fibril size (diameter in nm), as determined by TEM analysis. Note the wider size distribution profile of the MENX group.

Table 3.3: Combined fibril diameter data for sheep ACL. Mean fibril counts (per two TEM fields) for MENX sheep were compared to the NOC sheep for each fibril size range and calculated as a percentage of the total count.
Data for fibril density was calculated as a % of total field area. MENX showed no significant difference in fibril density compared to NOC [mean % of field ± SE; MENX 47.3 ± 1.4 vs. NOC 48.1 ± 1.3]. Charts of all TEM results are provided in Appendix A4.3 (page 259).

3.3.4 Scanning Electron Microscopy (SEM)

At a low power of magnification the architecture of the MENX ligament was variable with multiple large gaps and what appears to be loose connective tissue between the fibre bundles (Figure 3.10). This was in contrast to the NOC tissue which had fibre bundles that were closely packed and more uniform in appearance (Figure 3.11). At a higher power of magnification, the images showed conspicuous electron dense bands on the cut surface of the ACL of the MENX sheep (Figure 3.12), which were not readily apparent in the NOC sheep (Figure 3.13).
Figure 3.10: SEM (170x) ACL tissue from a MENX sheep viewed in cross-section. Note the degree of separation between fibre bundles (arrow heads) and loose connective tissue between bundles (arrows).

Figure 3.11: SEM (170x) ACL tissue from a NOC sheep viewed in cross-section. The fibril bundles within the mid-substance ligament are tightly packed with little space between them, and the tissue is uniform in appearance (arrow heads).
Figure 3.12: SEM (2000x) ACL tissue from a MENX sheep viewed in cross-section. Note the clearly visible light and dark striations in the tissue.

Figure 3.13: SEM (2000x) ACL tissue from a NOC sheep viewed in cross-section. The tissue is very homogeneous, lacking the clearly striated appearance of the MENX sample.
### 3.3.5 Gross Morphology

#### 3.3.5.1 Articular Cartilage OA Scores

<table>
<thead>
<tr>
<th>REGION</th>
<th>NOC</th>
<th>MENX</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage</td>
<td>Mean (CI)</td>
<td>Mean (CI)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FEMUR</td>
<td>0.08 (0 - 0.2)</td>
<td>3.10 (2.9 - 3.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TIBIA</td>
<td>0.17 (0 - 0.4)</td>
<td>2.80 (2.2 - 3.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WHOLE JOINT</td>
<td>0.13 (0 - 0.3)</td>
<td>2.95 (2.6 - 3.3)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 3.4: Results of macroscopic scoring of ovine knee articular cartilage, comparing meniscectomised and non-operated control groups.

---

**MACROSCOPIC SCORING OF ARTICULAR CARTILAGE DEGENERATION IN OVINE KNEE JOINTS**

![Chart](chart.png)

Figure 3.14: Charted data for macroscopic scoring of articular cartilage, comparing meniscectomised and non-operated control groups.
### 3.3.5.2 Osteophytes

<table>
<thead>
<tr>
<th>REGION</th>
<th>NOC</th>
<th>MENX</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteophytes</td>
<td>Mean (CI)</td>
<td>Mean (CI)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FEMUR</td>
<td>0.08 (0 - 0.2)</td>
<td>1.30 (1.0 - 1.6)</td>
<td></td>
</tr>
<tr>
<td>TIBIA</td>
<td>0.08 (0 - 0.2)</td>
<td>1.90 (1.5 - 2.3)</td>
<td></td>
</tr>
<tr>
<td>WHOLE JOINT</td>
<td>0.08 (0 - 0.2)</td>
<td>1.60 (1.3 - 1.9)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5: Results of macroscopic scoring of osteophyte formation in ovine knee joints, comparing meniscectomised and non-operated control groups.

Macroscopic evaluation of joints from the meniscectomised and non-operated control groups revealed that the meniscectomised sheep scored higher for cartilage degradation and osteophyte formation compared to the controls. This was evident on both the tibial and femoral surfaces, and for the combined joint data.
3.3.6 Histology

3.3.6.1 Ligament

Haematoxylin & Eosin

General observations of the NOC and MENX samples showed the two groups were comparable in regards to the number of cells present (cellularity) and cell appearance (morphology) (Figure 3.16 A&B). The fibres of the NOC group appeared slightly more regular and parallel in their arrangement.

Toluidine Blue

Tol Blue stain revealed the distribution and density of elastic fibres within the ligament. Elastin fibres in the NOC group samples were abundant and evenly distributed throughout the ligament sections. In comparison, the density of elastin in the MENX sections was lower and the fibres were more randomly distributed (Figure 3.16 C&D).

Figure 3.16:
A and B = H&E 200x sections of ACL tissue. A, NOC sample showing normal cellularity and morphology, with parallel collagen fibril arrangement; B, MENX sample demonstrating less ordered collagen fibril arrangement.

C and D = Tol Blue 200x sections of ACL tissue. C, NOC sample with abundant and evenly distributed elastin fibres; D, MENX sample with a lower density of elastin which is less regular in its distribution. All histological sections, can be viewed in Appendix A4.5 (pages 289-296).
### 3.3.6.2 Cartilage

Histological examination of cartilage revealed the meniscectomised group scored significantly higher than the control group for all markers of OA except tidemark, and received a much greater total score than NOC. This was consistent for the femoral and tibial surfaces, and the total joint data.

#### Table 3.6: Results of microscopic scoring of OA changes in ovine knee articular cartilage, comparing meniscectomised and non-operated control groups.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Mean (CI)</th>
<th>Mean (CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Femoral AC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Structure</td>
<td>1.46 (0.68 - 2.24)</td>
<td>3.17 (2.14 - 4.20)</td>
<td>0.015</td>
</tr>
<tr>
<td>Cell Number</td>
<td>0.79 (0.48 - 1.10)</td>
<td>1.38 (1.00 - 1.75)</td>
<td>0.029</td>
</tr>
<tr>
<td>Cloning</td>
<td>0.46 (0.26 - 0.66)</td>
<td>2.71 (2.27 - 3.15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IT TB</td>
<td>0.79 (0.30 - 1.28)</td>
<td>1.71 (1.28 - 2.13)</td>
<td>0.011</td>
</tr>
<tr>
<td>Tidemark</td>
<td>1.04 (0.96 - 1.13)</td>
<td>1.08 (0.97 - 1.20)</td>
<td>0.557</td>
</tr>
<tr>
<td>Total</td>
<td>4.54 (2.93 - 6.15)</td>
<td>10.04 (8.35 - 11.73)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Tibial AC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Structure</td>
<td>0.58 (0.06 - 1.11)</td>
<td>5.63 (4.48 - 6.77)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cell Number</td>
<td>0.42 (0.21 - 0.62)</td>
<td>2.42 (2.02 - 2.81)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cloning</td>
<td>0.21 (0.02 - 0.40)</td>
<td>3.42 (3.06 - 3.78)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IT TB</td>
<td>0.29 (0.04 - 0.55)</td>
<td>2.96 (2.68 - 3.24)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tidemark</td>
<td>1.13 (0.95 - 1.30)</td>
<td>1.13 (1.00 - 1.25)</td>
<td>1.000</td>
</tr>
<tr>
<td>Total</td>
<td>2.63 (1.79 - 3.46)</td>
<td>15.54 (13.79 - 17.29)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Joint AC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Structure</td>
<td>2.04 (1.14 - 2.95)</td>
<td>9.00 (6.94 - 11.06)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cell Number</td>
<td>1.21 (0.82 - 1.60)</td>
<td>3.79 (3.18 - 4.40)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cloning</td>
<td>0.67 (0.36 - 0.97)</td>
<td>6.13 (5.51 - 6.74)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IT TB</td>
<td>1.08 (0.57 - 1.59)</td>
<td>4.67 (4.21 - 5.12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tidemark</td>
<td>2.17 (1.98 - 2.35)</td>
<td>2.21 (2.06 - 2.35)</td>
<td>0.731</td>
</tr>
<tr>
<td>Total</td>
<td>7.17 (5.44 - 8.89)</td>
<td>25.58 (22.49 - 28.67)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

#### Figure 3.17: Chart of total joint data for the meniscectomised and non-operated control groups.
3.3.6.2.1 Correlation Between Cartilage Degeneration & Ligament Changes in Individuals

Figure 3.18: Simple regression analysis plot comparing OA score (total joint cartilage structure score) with ACL decorin expression.

Figure 3.19: Simple regression analysis plot comparing OA score (total joint cartilage structure score) with ACL elastin expression.
Figure 3.20: Simple regression analysis plot comparing OA score (total joint cartilage structure score) with ACL fibril size.
3.3.6.3 Synovium

Table 3.7: Results of microscopic scoring of synovium in ovine knee articular cartilage, comparing meniscectomised and non-operated control groups.

<table>
<thead>
<tr>
<th>SYNOVIUM</th>
<th>NOC Mean (CI)</th>
<th>MENX Mean (CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTIMAL HYPERPLASIA</td>
<td>0.83 (0.35 - 1.32)</td>
<td>0.67 (0.42 - 0.92)</td>
<td>0.557</td>
</tr>
<tr>
<td>CELL INFILTRATION</td>
<td>0.29 (0.01 - 0.57)</td>
<td>0.29 (0.04 - 0.55)</td>
<td>1.000</td>
</tr>
<tr>
<td>SUBINTIMAL FIBROSIS</td>
<td>1.63 (1.26 - 1.99)</td>
<td>2.67 (2.45 - 2.89)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VASCULARITY</td>
<td>2.96 (2.88 - 3.04)</td>
<td>3.00 (0.00 - 0.00)</td>
<td>0.328</td>
</tr>
<tr>
<td>AGGREGATE SCORE</td>
<td>5.71 (4.62 - 6.80)</td>
<td>6.63 (6.13 - 7.12)</td>
<td>0.147</td>
</tr>
</tbody>
</table>

Figure 3.21: Chart of ovine synovium scores for the meniscectomised and non-operated control groups.

Microscopic scoring of synovium from the meniscectomised and NOC groups showed they did not differ significantly for any criteria except for fibrosis, in which MENX scored higher than NOC. The aggregate scores for the two groups were not considerably different.
3.4 DISCUSSION

The gross morphological assessment of the articular surfaces clearly demonstrates the presence of major osteoarthritic changes in the knees of the meniscectomised group, which were not observed in the non-operated controls. The MENX group showed significant cartilage degradation and osteophyte formation compared to the NOC sheep, which exhibited negligible morphological change. Histological examination of the articular cartilage also revealed greater changes consistent with OA in the knee joints of meniscectomised sheep compared to NOC. Furthermore, examination of the synovial membrane showed significantly greater subintimal fibrosis in the knees of MENX sheep, a prominent feature of chronic synovitis associated with OA [139]. It is possible that other synovial changes such as vascularity and cellular infiltration may have occurred in the acute stages of the disease process but were more transitory. As the MENX group was treated identically to the NOC group in all other respects, this indicates that meniscectomy was successful in inducing OA. This supports the premise that the ligament changes identified within the joints of meniscectomised sheep in this study are analogous to those occurring in the joints of individuals with naturally occurring osteoarthritis.

Investigation of gene expression in the cruciate ligaments revealed that the meniscectomy group did not exhibit many differences in ligament gene expression when compared to the NOC group. One noteworthy difference that was observed however was the significantly reduced expression of decorin (the most abundant proteoglycan in ligament [15]) in the ACL samples of the MENX group, which has the potential to affect the structure of the tissue considerably. Decorin is a SLRP involved in regulating collagen I fibrillogenesis (the main structural element in ligament) [9, 41], it
can interact with a variety of proteins such as growth factors and other collagens such as type III \(^{[30, 40]}\), and it modulates cell/matrix interactions, thus influencing the metabolism and integrity of the ECM \(^{[37]}\). Furthermore, an increase in the expression of MMP-I (collagenase I) which primarily degrades type I collagen, could accelerate the breakdown of collagen fibrils in the ligament tissue. As there was no increase in type I collagen mRNA expression which would counteract this loss of collagen, the overall effect would likely be a decrease in the integrity of the tissue and potentially loss of biomechanical function. It was noted that the ligament itself produced no measurable increase in the expression of other key inflammatory mediators such as MMP-3, MMP-13, TNF-\(\alpha\) or IL-1, suggesting that the ACL was exposed to cytokines produced by other structures within the joint capsule such as synovium. Finally, a reduction in elastin expression, which gives ligament its elastic properties, may decrease the ability of the ligament to stretch adequately when subjected to biomechanical forces. This in turn may contribute to an increased risk of ACL rupture or over time contribute to an increase in the laxity of the ligament and joint instability.

The results for decorin, elastin and MMP-I outlined above were obtained through analysis of the total mRNA data. Although normalised data revealed similar findings, there is some doubt regarding the validity of the normalised results due to highly variable mRNA expression in the chosen housekeeping gene across all study groups. Theoretically, a chosen housekeeping gene should be a stably expressed reference which is representative of the cDNA concentration of the sample groups \(^{[239, 240]}\), although this is not always the case for all biological specimens \(^{[241]}\). A number of commonly used housekeeping genes were tested in this study and none were found to be stably expressed in ligament, and were therefore found to be unsuitable as internal references,
potentially introducing large unpredictable error into the results analysis. Results are shown as normalised to β-actin which showed the least variation of housekeeping genes tested, but was still believed to be too influenced by the effects of the various test parameters (e.g. OVX, MENX, O+M) to serve as a suitable housekeeping gene. Figures 3.22 and 3.23 display the variable expression of two commonly used housekeeping genes across the test groups. The outcome of normalising all molecular biology results to a housekeeping gene (β-actin) is shown graphically in Appendix A4.1.2 (page 245).

Figure 3.22: mRNA expression of beta actin in ACL tissue varied significantly across the study groups.

Figure 3.23: mRNA expression of GAPDH in ACL tissue was also highly variable across the study groups and therefore is unsuitable as a housekeeping gene.
Based on the simple regression analyses comparing individual OA scores with ligament changes (as indicated by ligament fibril size, and decorin & elastin content) it was not possible to prove an association between osteoarthritic changes and the severity of ligament derangement. These findings may be influenced however, by the consistent manner in which the meniscectomy model induced OA in the MENX sheep. Because this model is so reliable in generating OA, as has been demonstrated by numerous studies of knee pathology in sheep and mice [242-247], variation in the cartilage scores of individuals in the MENX group was insufficient to correlate with ligament change.

Biochemical analysis of the collagen and S-GAG content of the ACL samples from the NOC vs. MENX sheep corresponds to the findings of the RT-PCR analysis. Results showed there was no significant reduction in the collagen content of the MENX sheep compared to the NOC sheep, which would fit with the lack of change in the expression of collagen types I, II or III. Decorin is the most abundant SLRP found in ligament, therefore if expression is significantly reduced in MENX sheep, it is likely that there would be an overall reduction in ligament S-GAG content. This fits with findings of the ACL biochemical analysis which found there was a significant reduction in S-GAG content in MENX sheep compared to NOC.

These findings correspond with structural changes observed using TEM. Calculation of fibril density (as a % of the total field) did not reveal any significant difference between the MENX and the NOC groups, however this does not reflect the differences seen in the size of fibrils and their arrangement. The fibrils of ACL tissue from NOC sheep were uniform in size and arranged in a regular fashion. This was in contrast to those of MENX sheep which had a significantly smaller average fibril size and showed a greater
size range with many small fibrils and a less regular arrangement. Similar findings have been made in studies involving decorin deficient mice which exhibited connective tissues (such as skin and tendon) with more loosely and irregularly packed fibrillar networks and reduced mechanical function \cite{31, 42, 248}. Danielson et al. (1997) \cite{42} examined the properties of skin and tendon in decorin deficient mice, finding they produced fibrils with abrupt increases and decreases in mass along their axes, therefore producing fibres with coarser, cross-sectional outlines that were irregular in size and shape. Furthermore, the fibrils were more haphazard in their arrangement, with increased interfibrillar spacing, allowing uncontrolled lateral fusion of collagen fibrils and reduced tensile strength of the tissue. While this current study observed similar changes in fibril size and arrangement in the decorin deficient MENX group, we did not observe the lateral fusion seen by Danielson et al. (1997). This may be due to the fact that the mice in Danielson's study were devoid of decorin (knockout mice), whereas the sheep in this study were deficient although not completely lacking in decorin.

Amaye et al. (2002) \cite{49} found decorin deficient mice produced abnormal fibrils in tendon and skin with significant variation in the range, mean and distribution profile of collagen fibril diameters. They also found changes in collagen fibril orientation, with fibrils adopting a more random organisation instead of the usual parallel orientation of wild type mice. Connective tissues in these decorin deficient mice were more fragile with markedly reduced tensile strength. Similarly, Matheson et al. (2005) \cite{31} examined the effect of decorin deficiency in the periodontal ligament of decorin deficient mice, and discovered they produced fibril bundles that were heterogeneous in size, with a decreased average fibril diameter, and were non-uniform in shape. Also, the
interfibrillar spacing was more variable than in the control mice and the fibrils were more disorganised.

In contrast, Birk et al. (1995) [249], demonstrated that fibril growth was associated with a significant decrease in fibril associated decorin. These findings fit with the common knowledge that decorin limits the size of collagen fibrils during fibrillogenesis [9, 34, 40, 48], therefore it's reasonable to assume a reduction in decorin would result in larger fibrils; however this does not correlate with the findings of this study or those mentioned previously. It has been suggested by a number of authors that different SLRPS are able to perform similar functions, and that one SLRP may be able to compensate for a deficiency in another. Zhang et al. (2006) [250] noted this in their study of decorin deficiency in mouse periodontal ligament and tail tendon. They discovered decorin deficiency was associated with increased protein levels of biglycan, a SLRP that is structurally related to decorin and competes for the same binding site [38]. Analysis of tendon and ligament showed dysfunctional regulation of fibril growth in the early stages of linear and lateral growth from immature fibril intermediates, resulting in premature progression of fibrillogenesis and lateral fusion of fibrils, which were abnormally arranged and had decreased biomechanical strength. This shows that while biglycan may be able to compensate for a lack of decorin in part, it was unable to delay fibril assembly and inhibit fibril maturation until the latter stages of development, two functions normally performed by decorin.

Young et al. (2003) [251] also showed that biglycan and decorin are highly homologous and co-expressed in various connective tissues. Results of their study revealed that biglycan & decorin double knockout mice had more profoundly disturbed connective
tissue than single decorin or biglycan knockout mice which appeared to be tissue specific. The same was observed in biglycan & fibromodulin double knockout mice vs. single biglycan or fibromodulin knockout mice, suggesting a compensatory or synergistic function amongst these SLRPs in some tissues. Neame et al. (2000) [43] also showed SLRPs can have a synergistic effect in modulating collagen fibrillogenesis in their in vitro study of decorin and lumican. They analysed the effects of these two SLRPs, finding individually both were able to limit collagen I fibril diameter, while in combination they had an even greater effect on fibril size than either SLRP alone, suggesting they act on different binding sites on the fibril.

This compensatory ability demonstrated by SLRPs may explain why this study (and others) found smaller rather than larger fibrils in decorin deficient tissues. This study did not show a significant increase in the expression of other SLRPs, however this may be due to the levels of decorin being reduced rather than completely absent, and other proteoglycans may have been able to compensate without having to greatly increase their levels.

While it appears clear that other SLRPs may act to modulate fibril size under decorin deficient conditions, they seem less able to compensate for decorin in its other role as a stabiliser and organiser of the collagen fibril network [42, 44]. Haphazard fibril organisation with increased interfibrillar spacing was common to all previously mentioned studies, with a number finding disruption was so severe that adjacent fibrils were able to fuse together [42, 250]. This implies that other SLRPs may be able to compensate for decorin in some but not all of its functions.
Histological comparison of the MENX and NOC sheep showed the collagen fibrils of NOC sheep were more regular and parallel in their arrangement. This reflects the findings of the TEM analysis which found collagen fibrils in the MENX sheep were irregularly arranged, and again this may be influenced by a deficiency in decorin which plays a key role in maintaining the regular arrangement of the collagen network. In addition, while the NOC sheep demonstrated an abundance of elastin fibres, evenly distributed throughout the tissue, the MENX sheep showed relatively few elastin fibres which were more randomly distributed. This evidence further suggests a disruption to the regular arrangement of the fibre network, and supports the molecular findings that showed a significant reduction in the expression of elastin in the MENX sheep. As previously stated, this may compromise the ability of the ligament to stretch in response to biomechanical forces and may contribute to ligament degeneration over time.

Of further interest were the unusual electron dense bands seen on the cut surface of the MENX ACL samples that were examined in cross-section via scanning electron microscopy (SEM 2000x). These striations were prominent on the MENX samples but were not as obvious on the NOC samples (Figures 3.10 - 3.13). It is unclear what causes these striations but it is possible they reflect the underlying disruption to normal ligament structure and potential loosening of interlamellar structure. This is supported by the lower power (170x mag.) images of the same samples in which the MENX tissue appears loosely arranged with large spaces separating the bundles of collagen fibres. In comparison, the fibre bundles in the NOC sample are tightly packed with little or no space between them.
In conclusion, the significant differences observed in the size and arrangement of MENX collagen fibrils demonstrates a disruption in normal ligament structure, which may affect the biomechanical properties of the tissue. The reduced expression of decorin may contribute to the altered fibrillar arrangement observed in the MENX ligament tissue and the reduction in elastin could negatively influence the ability of the tissue to stretch in response to mechanical forces, predisposing it to injury. Furthermore, the increased expression of MMP-I may further affect the integrity of the tissue by degrading collagen fibrils at a faster than normal rate.
4.1 INTRODUCTION

4.1.1 The Ovaries

The ovaries are a pair of ovoid shaped structures located on either side of the uterus and connected by the fallopian tubes \[252, 253\]. Their primary functions in the reproductive system are to house and expel the ova, beginning at menarche and continuing until menopause, and the production and secretion of hormones \[254\].

Structurally each ovary is made up of several layers (see Figure 4.1). The outermost layer is the ovarian surface epithelium (formerly known as the germinal epithelium), which overlies the tunica albuginea, a tough and fibrous protective layer which covers a deeper dense region known as the cortex \[253\]. Located in the cortex are the primary ovarian follicles, each containing an oocyte (a potential or immature ovum). Surrounding the oocyte are the follicle cells (granulosa) which secrete oestrogen \[252, 255\]. Oestrogen and follicle stimulating hormone (FSH) from the pituitary gland stimulate the follicle to mature, at which time it is called the Graafian follicle. Luteinising hormone (LH), also from the pituitary gland, causes the release of the ovum (ovulation) leaving a ruptured follicle that subsequently becomes the corpus luteum. The innermost layer of the ovary is known as the medulla and contains blood vessels, lymphatic vessels, and nerves \[252, 253\].
4.1.2 The Ovarian Hormones

The major ovarian hormones are divided in two classes, the steroids (oestrogen and progesterone), and the peptides (particularly the inhibins and activins) \cite{256}. The ovaries (and the testis in males) secrete cholesterol-derived hormones under the influence of the hypothalomo-pituitary axis, although activity of the ovary is cyclical unlike the testis which is maintained in a more or less constant state of activity. The hormonal secretions of the ovary vary according to the phase of the menstrual cycle and are subject to both negative and positive feedback systems \cite{257}.

4.1.2.1 Oestrogen

Oestrogen is one of the major hormones that controls the development and maintenance of the female phenotype, and regulates critical signalling pathways involved in the control of cell proliferation and differentiation in reproductive and non-reproductive
tissues such as bone, cartilage, and blood vessels \cite{252, 254, 258}. The primary sources of oestrogen are the ovaries (a secretory product of the granulosa cells of the ovarian follicle) and the placenta of pregnant females, with additional small amounts being produced by the adrenal glands and adipose tissue \cite{259}.

The ovary requires both LH and FSH to produce sex hormones such as oestrogen. LH stimulates the thecal cells surrounding the follicle to produce androgenic precursors (testosterone and androstenedione), which diffuse across the basement membrane to the granulosa cell layer. Here the action of FSH facilitates their aromatisation to oestrogens via aromatase \cite{255, 257}. There are three principle types of oestrogen produced, oestradiol, oestrone and oestriol \cite{259-261}. Oestradiol (E2) is the predominant oestrogen produced during the premenopausal period, and is mainly secreted by the ovaries. 17β oestradiol is the most potent and abundant naturally occurring oestrogen in humans and is synthesised from testosterone \cite{258, 261}. Oestrone (E1) is the main oestrogen present after menopause. It is weaker than oestradiol and is the least abundant of the three oestrogen types. Oestradiol can be reversibly oxidised to form oestrone, but its major precursor is androstenedione. In post-menopausal women 95% of androstenedione is produced by the adrenal glands with only 5% produced by the ovaries. Androstenedione is converted to oestrone in the peripheral adipose tissue \cite{259, 261, 262}. Oestriol (E3) is the primary oestrogen during pregnancy and is produced in large quantities by the placenta \cite{263}. In some women oestriol is secreted by the ovaries, especially during the luteal phase, but this is less common \cite{264}. Both oestradiol and oestrone can be irreversibly converted to oestriol which is the weakest of the three oestrogens \cite{258, 261}.

Once secreted into the blood, most oestrogen circulates reversibly bound to sex hormone-binding globulin (which inhibits its function), with a smaller fraction
remaining free or unbound in the serum (as biologically active hormone). When oestrogen reaches its target tissues, free oestrogen penetrates the cell surface and binds to specific proteins known as oestrogen receptors [255, 259].

4.1.2.2 **Progesterone**

Progesterone, also known as antioestrogenic hormone, is another major steroid-derived hormone produced by the ovaries, placenta and adrenal glands [254, 255]. In the ovary, the site of production is the corpus luteum tissue after ovulation [259]. If fertilisation occurs, the placenta produces progesterone for the duration of the pregnancy, which functions to prepare and maintain the uterine wall, and inhibit muscular contractions. If the egg is unfertilised, the ovaries continue to produce progesterone until a few days before menstruation [253, 259]. In addition to regulating normal female reproductive function, progesterone also plays an important role in non-reproductive tissues such as the cardiovascular system, bone, brain, liver, skin, gut and bladder [265, 266]. Progesterone receptors have also been identified in ligament [267-270].

4.1.2.3 **Peptide Hormones**

The peptide hormones are secretory products of the ovarian granulosa cell and include inhibin, activin and relaxin. Inhibin and activin are structurally related dimeric protein complexes that are functionally diverse with opposing biological effects [271, 272]. These proteins were first identified for their role in modulating pituitary FSH secretion (inhibin down-regulates and activin stimulates) but there is now evidence of their involvement in a local autocrine/paracrine regulatory role in ovarian follicles [273, 274]. Investigation into the potential role of inhibins and activins in a variety of non-reproductive tissues is ongoing, but they have been implicated in the regulation of cell proliferation and wound healing [275].
Relaxin is another peptide hormone produced by the corpus luteum of the ovary. It is produced in the highest amounts during pregnancy, when it stimulates the breakdown of collagen in preparation for parturition. However relaxin is not exclusively a hormone of pregnancy, and is also produced in non-pregnant females and males. Its biological effects on the structure of collagen are far reaching, affecting a diverse range of tissues \cite{276, 277}. It has been shown to reduce collagen deposition in the airways via stimulation of collagen-degrading enzymes (MMPs) and inhibition of TGF-β induced matrix protein production \cite{277}, and it modulates collagen metabolism in the ECM of ligaments throughout the body \cite{276, 278-280}. Researchers have also revealed effects on the cardiovascular system \cite{281}, on regulation of the activity of mature osteoclasts \cite{282}, and demonstrated its ability to stimulate healing in ischemic wounds suggesting that relaxin may also have angiogenic effects \cite{283}.

4.1.3 The Influence of Sex Hormones on Ligament

4.1.3.1 Hormone Receptors

In order for a hormone to stimulate a biological response in a target tissue, the tissue must possess specific hormone receptors. Therefore an important step in determining whether the sex hormones affect ligament is establishing the presence of the appropriate receptors in that tissue. Researchers have identified the expression of oestrogen and progesterone receptors in ligaments such as the human periodontal ligament \cite{270, 284, 285}, and the uterosacral ligament of women \cite{267, 286, 287}, but it was Liu et al. (1996) \cite{268} who first identified the presence of oestrogen and progesterone receptors in the human ACL. Using immunohistochemistry, they localised these hormone receptors to the synoviocytes in the synovial lining, fibroblasts in the ACL stroma, and cells in the blood vessel walls of the ligament. This establishes that the ACL is responsive to the
female sex hormones, and that these hormones may have an effect on its structure and composition.

Sciore et al. (1998) further investigated the presence of female sex hormone receptors in human and rabbit knee ligaments using RT-PCR. Their investigations stemmed from existing research showing a higher frequency of knee ligament injury and joint hyper-mobility in females compared to males, and that the occurrence of degenerative joint osteoarthritis is more common in older females. These findings lead Sciore et al. (1998) to hypothesise that the connective tissue components may be regulated differently in males and females, and that these tissues may be responsive to oestrogen and progesterone. Results of their study confirmed that oestrogen and progesterone receptor transcripts are expressed in ligament tissue in both humans and rabbits, therefore suggesting that ligament is a hormone-responsive tissue. Sciore et al. concluded that the rapid induction and reversal of the depression in oestrogen receptor transcript levels observed in their study in response to changing hormone concentrations, likely indicates that they serve an important function in ligament tissue.

As outlined previously, relaxin is another ovarian hormone believed to play a major role in modulating collagen metabolism in the ECM of ligaments. In pregnant women it is well known that relaxin contributes to loosening of the pelvic ligaments and cervical changes that occur before childbirth, however less is known of its effects on other ligaments in non-pregnant females and males. In 2003, two research groups postulated that relaxin may play a role in the female predisposition for non-contact ACL injury, and sought to establish the presence of relaxin receptors in the ACL. Dragoo et al. (2003) tested for the presence of relaxin receptors in the remnants of ruptured ACLs
of men and women undergoing reconstructive surgery. They discovered all female ACLs showed specific relaxin binding, suggesting the presence of relaxin receptors in the ACL of the female subjects. This binding exhibited hormone specificity as it was only noted with relaxin, and not with relaxin's structural homolog, insulin. In contrast, there was no binding of relaxin in the ACL tissues taken from male subjects. As serum relaxin levels in non-pregnant females range from 20-150 pg/ml \[^{[298-300]}\], they concluded that women expressing relaxin receptors with higher relaxin levels may be at increased risk of ACL injury due to increased exposure to this collagenolytic hormone.

The same year, Galey et al. (2003) \[^{[301]}\] also investigated the presence of relaxin receptors in ACL specimens from eight female and four male reconstruction patients using immunohistology techniques. All 12 ACL samples stained positive for relaxin binding, although staining was more intense in female subjects. Their data suggests that relaxin binding to the ACL is specific, indicative of a receptor-mediated event.

The effect of testosterone on the female ACL was also investigated by Lovering et al. (2005) \[^{[302]}\]. The findings of their study identified the presence of androgen receptors on the ACL of female subjects suggesting that it is an androgen responsive tissue, however testosterone fluctuations did not correlate with changes in ACL stiffness. This indicates that ACL stiffness is likely influenced by another sex hormone, or a combination of hormones.

\subsection{Animal Studies}

Animal research may be conducted to further knowledge about that species or may serve as a model for human disease. A number of research groups have made use of animal models to explore the influence of differing ovarian hormone concentrations on
ligament, including during pregnancy, through variation in the oestrus cycle and after ovariectomy. There are multiple benefits to using animal models, especially in respect to controlling experimental parameters, minimising environmental influences, and obtaining adequately matched study groups. Conversely, for the animal model to be valid in human research, it must bear adequate relevance to the anatomy and physiology of the human disease process.

As with human populations, there is evidence that gender may play a part in ligament injury in animals. Several studies investigating the epidemiology of cranial cruciate rupture (equivalent to human ACL) in canine populations have identified female gender among the significant risk factors \[120, 121, 303-305\]. A recent study of dogs in the United Kingdom by Adams et al. (2011) \[306\] found that females were twice as likely to suffer cranial cruciate rupture compared to males. Yet another study by Harasen (1995) \[307\] reported a considerable 70% of their study population of dogs with cranial cruciate rupture were female. In the absence of any known differences in other risk factors, this suggests that the female hormonal state influences susceptibility to injury, presumably through modulation of structure or cellular function.

During animal pregnancy, the levels of sex hormones such as oestrogen, progesterone and relaxin increase steadily until shortly before birth, paralleling that of humans \[308-312\]. Several authors have used animal models to investigate the increase in joint laxity known to occur in pregnancy \[313\]. In 1998, Hart et al. \[314\] used a rabbit model to examine the effect of pregnancy on gene expression in knee ligaments (specifically the ACL and MCL). Results of their study demonstrated that hormonal fluctuations occurring during pregnancy resulted in changes in the patterns of gene expression in knee ligaments. Messenger RNA (mRNA) transcripts for both collagen types tested (I
& III) were depressed in RNA isolated from the MCL. Likewise transcripts for collagen type III were significantly depressed in ACL, however in contrast to the MCL, transcripts for collagen type I were not depressed. Expression for proteoglycans was more variable, with biglycan depressed and decorin increased in MCL, while ACL exhibited no alteration in transcript levels. With regard to proteinase expression, MCL showed an increase in MMP-1 (collagenase) expression but not its inhibitor TIMP-1. ACL showed no difference in MMP-1 or TIMP-1 expression compared to non-pregnant controls. These results suggest that the response of knee ligaments to pregnancy at the mRNA level is complex and may be ligament specific. Further investigation is required to confirm that these mRNA changes translate to biochemical alterations at a tissue level.

Hart *et al.* (1998b) \(^{315}\) conducted a similar study, again investigating the effect of the mRNA changes in knee ligaments induced by pregnancy in a rabbit model. Results of this subsequent study showed that mRNA for collagen types I & III were depressed in both MCL and ACL during pregnancy. Expression of mRNA for biglycan, decorin and TIMP-1 was also significantly depressed in MCL and ACL. Transcript levels for collagenase remained unchanged compared to non-pregnant controls. While these results vary to some degree from those of their previous study, they still indicate that the relationship between increased ligament laxity and molecular changes during pregnancy is complex and may warrant further study.

Ohtera *et al.* (2002) \(^{316}\) examined the effect of pregnancy on joint contracture in the rat knee. During pregnancy oestrogen and relaxin levels are elevated which affects fibroblast function \(^{290}\), which in turn is associated with altered soft tissue structure and increased joint laxity, particularly due to the effect of oestrogen in reducing type I
collagen synthesis and fibroblast proliferation [317]. Furthermore, relaxin also decreases the synthesis and secretion of interstitial collagen, while increasing the expression of MMP procollagenase and decreasing TIMP production by fibroblasts [318, 319]. Given evidence supporting the influence of female hormones on ligament laxity, Ohtera et al. hypothesised that the hormonal changes occurring during pregnancy could have a therapeutic role in preventing joint contracture. Results of their study demonstrated a trend towards reduced contracture in the knee joints of pregnant subjects compared to non-pregnant subjects, however these findings were not statistically significant. The authors identified a number of limitations in this study which may have influenced the results and so suggest further research should be conducted in this area in order to draw more accurate conclusions.

Further evidence to suggest an increase in female sex-hormone levels alters the properties of knee ligament is provided by Liu et al. (1997) [317]. Having previously identified the presence of oestrogen and progesterone receptors in fibroblasts of human ACL, they investigated the affect of oestradiol on the proliferation of fibroblasts and collagen synthesis in rabbit ACL. After confirming the presence of oestrogen receptors in rabbit ACL fibroblasts, they exposed cultured ACL fibroblasts to increasing concentrations of oestrogen for a period of 2 weeks. Results revealed collagen synthesis and fibroblast proliferation were significantly reduced with increased oestradiol concentration. The authors propose that these alterations in ACL cellular metabolism may lead to changes in ligament composition, therefore rendering it more susceptible to injury.

Slauterbeck et al. (1999) [320] investigated the effect of oestrogen levels on the failure load of the ACL in rabbits. Based on evidence that female athletes tear their ACL more
frequently than male athletes \cite{288}, they hypothesised that increasing the level of circulating serum oestrogen would decrease the load at failure for rabbit ACL. Their study involved two groups of ovariectomised (OVX) rabbits, one group treated with oestrogen and the other a non-treated control group. While results showed that specimens treated with oestrogen had a significantly reduced load at failure compared with the non-treated controls, suggesting that oestrogen may influence ligament strength, the investigators conclude that further research is required to clearly establish the exact mechanism responsible for ACL failure in women.

In a similar study Komatsuda et al. \cite{321} hypothesised that serum oestrogen levels may play some role in the discrepancy in injury rates between the sexes. Various serum oestrogen levels were tested in OVX rabbits using 17\textbeta{} oestradiol. Their findings showed a decrease in ultimate tensile strength and linear stiffness occurred in specimens with high serum oestrogen levels. This suggests that high serum levels of oestrogen may be one of the factors in the multifactorial pathogenesis of ACL rupture. In a subsequent study from this research group, Hattori et al. \cite{322} continued investigations into the effect of supraphysiologic levels of oestrogen on the tissue properties of the ACL. Again using an OVX rabbit model, they administered various doses of 17\textbeta{} oestradiol over a 4-week period, however this study utilised different methods to evaluate changes in the ACL. Scanning acoustic microscopy was used to measure tissue sound speed in ligament samples as an indicator of the elastic modulus of the tissue. Results indicated that there was a significant, negative correlation between increasing serum oestrogen levels and the elasticity of the tissue.
Lee et al. (2004) ³23 examined whether mechanical tensile forces affect oestrogen regulation of collagen synthesis by porcine ACL fibroblasts in vitro. Results revealed that oestrogen alone stimulated synthesis of collagen types I and III at the mRNA level. When oestrogen and mechanical loading were combined, expression of collagen types I & III was decreased at all tested oestrogen levels. This suggests that oestrogen may directly regulate ligament structure and function through alteration of collagen synthesis, dependent on mechanical loading.

In a subsequent study, Lee et al. (2004b) ³24 studied the combined effects of oestrogen and mechanical loading on the gene expression of three major ligament components; type I collagen, type III collagen and biglycan. Results revealed that cyclic tensile loading alone increased the expression of type I collagen but not type III collagen or biglycan. Oestrogen alone increased the mRNA expression of collagen types I & III but not biglycan. Combined oestrogen and cyclic tensile loading however, inhibited the mRNA expression of all three genes. This suggests that the inhibition of all three matrix molecules resulting from a combination of oestrogen and mechanical loading is unique to females and may explain the high incidence of ACL injury in females.

Woodhouse et al. (2006) ³25 studied the effects of reproductive hormones on the strength of rat ACL. The material properties of ACL in rats with normal oestrus cycles were compared with rats administered oral contraceptives. After 12 oestrus cycles, those rats influenced by oral contraceptives displayed greater ACL toughness and had significantly lower serum progesterone levels. The investigators do not believe differences in the mechanical characteristics of the two groups were the result of short-term changes in the ACL structure due to hormone fluctuations, suggesting that this was
unlikely due to the short 5-day cycle of rats. Rather, they believe the differences in
ACL structure are due to exposure to differing hormone levels over time. They
concluded that chronic exogenous hormone treatment in rats, dosed to mimic oral
contraceptive use in humans, is associated with changes in the mechanical
characteristics of the ACL.

In contrast to the findings of these studies, a number of other authors dispute the notion
that changes in sex-hormone levels alter the composition of ligament tissue or fibroblast
activity. Seneviratne et al. (2004) [326], examined the effect of various levels of
oestrogen on ACL fibroblast proliferation and collagen synthesis in vitro in an ovine
model. They found no significant difference in ACL fibroblast proliferation or collagen
synthesis regardless of oestradiol concentration. They comment that collagen has a low
turnover in ligament and therefore it is unlikely that a 2 to 3-day per month increase in
serum oestrogen would result in significant alterations in the material properties of the
ACL. Rather, they believe the aetiology of non-contact ACL injury is complex and
multifactorial in nature. One suggestion is that oestrogen or relaxin may have an effect
on the expression and activation of metalloproteinases in ACL tissue. Despite the
results of this study, which indicate oestrogen alone does not directly affect fibroblast
activity, oestrogen cannot be excluded as a contributing factor in ACL injury.

Rau et al. (2003) [327] studied the failure properties of the ACL during the oestrous cycle.
Their theory was that oestrogen may play a role in the mechanism underlying female
ACL injury, based on the presence of oestrogen receptors in human and rabbit ACL.
They examined the mechanical properties of the ACL from the right hind limbs of 60
female rats. The rats were divided into four groups according to their stage of the
oestrus cycle. Results showed no difference in the mechanical properties of the ACL
between the groups, suggesting that fluctuations in the oestrous cycle during the normal oestrus cycle do not alter the failure properties of the tissue. Again this study may demonstrate a lack of influence of hormonal fluctuation on the properties of rat ACL, but it cannot rule out the presence of the female sex hormones as a contributing factor in female ACL injury.

Strickland et al. (2003) hypothesised that although female athletes injure their ACL at a much higher rate than males, oestrogen does not directly affect ligament ultimate stress and knee laxity. Using an ovine model, the effect of ovariectomy, ovariectomy plus oestrogen implant, and a selective oestrogen agonist (raloxifene) were tested against a sham control. Difference in the ultimate stress between ram and ewe ACL was also tested. From the results of this study they concluded that ultimate stress differences do exist between the ACL of rams and ewes, but this decrease in ligament strength could not be attributed to the effects of oestrogen or oestrogen receptor agonists at physiologic levels. There are however, aspects of their scientific method which may call into question these findings. Primarily, no explanation is given why the ligament is dissected down to a single bundle, which presumably would not represent the true biomechanical strength of the ligament. In addition, the joint angle used during testing was not equal to that of normal stance. Again no explanation was provided as to why this was done, raising the question as to whether the results are representational of normal (sheep) knee biomechanics.

Warden et al. (2006) tested the viscoelastic and tensile mechanical properties of MCL and ACL in male rats administered either oestrogen, or an oestrogen agonist to avoid the influence of endogenous oestrogen. Data indicated that neither oestrogen nor pharmacological stimulation of oestrogen receptors has a major direct effect on
ligament mechanical properties. This study is limited however, in that it is unclear whether the expression of oestrogen receptors in male ligament is the same as it is in female ligament, or whether male oestrogen receptors exhibit the same degree of sensitivity as females, which could affect the results.

4.1.3.3  Human Studies

It is well documented that there is a higher incidence of ligament injury involving the knee joints of females compared to males \([330-332]\). In addition, it is widely reported that hormonal fluctuations associated with pregnancy or the menstrual cycle lead to an increase in the knee joint laxity \([333-337]\). This evidence suggests that the female sex hormones, in particular oestrogen, may have some influence on the function of knee joint structures in women and that this may predispose them to ligament injury. Pollard et al. (2004) \([332]\) investigated whether gender differences in hip and knee joint mechanics in male and female athletes would explain the higher incidence of injury in females. They concluded that male and female athletes demonstrate similar hip and knee joint mechanics while performing a randomly-cued cutting (side-step) manoeuvre. This suggests that basic hormonal differences are a potential cause for the injury discrepancy.

Slauterbeck et al. (2001) \([338]\) stated that while the reasons for this discrepancy between injury rates in males and females is unknown, possible causative factors include size, strength, anatomic, social and hormonal differences. Their study examined gender differences in metalloproteinases (MMPs) and their inhibitors in order to establish whether normal tissue remodelling events play a role in ACL injury. Tissue remodelling occurs continuously in normal and repairing tissue, with a balance existing between the degradative and biosynthetic arms of tissue remodelling, modulated by
MMPs and tissue inhibitors of MMPs (TIMPs). Gender differences may play a part in this control because oestrogen and progesterone regulate the transcription of several MMP and TIMP genes. Result of this study showed that one parameter of tissue remodelling, the MMP3/TIMP1 gene expression ratio, is higher in the ACL of women than in men. This provides support for the hypothesis that that tissue remodelling in the human ACL varies by gender, and that such gender differences may explain the high injury rate in females.

Chandrashekar et al. (2005) [339] examined differences in the mechanical properties of ACL tissue in order to elucidate whether the female predisposition to injury was due to anatomical, neuromuscular or hormonal differences. They performed a cadaveric study to determine if any difference exists between the tensile properties of the ACL of males and females. When accounting for age and body anthropometric measurements as covariates, results revealed the female ACL was found to have lower mechanical properties such as strain at failure, stress at failure, strain energy density at failure and modulus of elasticity. Although this study did not identify the cause of the gender difference in mechanical properties, it does show that there is variation between males and females, and hormonal differences between the sexes cannot be ruled out as an influence.

The highest levels of endogenous oestrogen, progesterone and relaxin occur in the female body during pregnancy [340-343]. Dumas and Reid (1997) [344] stated that a pregnancy-related increase in the laxity of knee joint ligaments may result in joint instability, and presumably renders the joint more susceptible to injury. They assessed knee laxity changes in healthy female subjects during pregnancy using a KT-100 clinical arthrometer, and observed that knee laxity was increased during the second half
of pregnancy and significantly decreased by 4 months after birth. They also tested the
effect of exercise on the same group of pregnant women and found that increased
weight-bearing exercise had no additional influence on the laxity of the joint.

Charlton et al. (2001) \[^{339}\] performed another study examining a correlation between
endogenous oestradiol in pregnancy and ACL laxity. In this study patients were
initially examined during the third trimester of pregnancy, and again post partum.
Results showed that high serum oestradiol levels present during the third trimester of
pregnancy correlated with increased ACL laxity. Laxity was found to significantly
decrease as serum oestradiol returned to non-pregnant levels. This evidence supports
the theory that oestrogen may affect laxity of the ACL in female subjects. If joint laxity
predisposes the ACL to injury, then the effects of female hormones such as oestrogen
may prove a significant factor in the higher incidence of non-contact ACL injury in
females. The authors are however, careful not to conclude that oestrogen directly
affects laxity of the ACL, stating, “…the hormonal milieu in female subjects is
complex.” Rather, oestrogen fluctuations may induce changes in ACL collagen and
elastin composition which in turn leads to changes in the mechanical properties of the
ligament tissue. They also identified relaxin, particularly active during pregnancy, as
also being likely to have an effect on collagen remodelling to reduce soft tissue tension,
including that of ligaments. Oestrogen however, is thought to influence relaxin
regulation.

Many researchers have focussed on investigating oestrogen fluctuations associated with
the menstrual cycle as a potential cause for the female predisposition to knee joint laxity
and ACL injury, as it is well reported that non-traumatic ACL injuries are 4-8 times
greater in women compared to men \[^{288, 345-348}\]. Wojtys et al. (1998) \[^{336}\] postulated that
oestrogen has a direct effect on collagen metabolism and behaviour, and investigated the association between the menstrual cycle and the occurrence of ACL injury in female athletes. They found a significant correlation between the ovulatory phase of the cycle and ACL injury, with statistically more than expected injuries occurring during the ovulatory phase, and significantly fewer injuries occurred during the follicular phase. Wojtys et al. (1998) infer that the surge of oestrogen just prior to the ovulatory phase suggests that the increased incidence of non-contact ACL injury may be hormonally induced. The investigators make reference to previous studies showing oestrogen acutely decreases the total collagen content in rat facia, and reduces collagen synthesis in rat periodontal tissue $^{[349,350]}$.

Since the results of this study were published a number of issues have been raised regarding its validity. In a letter to the Editor of the American Journal of Sports Medicine, Wolman (1999) $^{[351]}$ outlined three major flaws in this study. Firstly he stated that five of the 28 subjects in the study were taking oral contraceptives which would give them a very different hormone profile to the others, and that the ovulatory phase of the cycle would not occur in these five women. The article assumes that women taking an oral contraceptive and those who menstruate naturally have a similar cyclical hormone profile, which Wolman (1999) believes is wrong. Secondly, in their study Wojtys et al. (1998) assumed a 28-day cycle for all subjects in their chi-square test, however they state that cycle lengths for the study group actually ranged from 21 to 32 days. We are not told what proportion of subjects had the ideal 28-day cycle. Those subjects that differ from the 28-day ideal will alter the expected frequency, especially those with very short cycle lengths, and dramatically change the result of the study. Finally the study involved subjects with widely varying ages, namely 11 to 42 years.
Wolman (1999) stated that hormonal profiles in menstruating women differ across age groups, with anovulatory cycles being common in the first few years after menarche and approaching menopause. He believes this group is heterogeneous regarding their hormone profiles and should not be considered together in a study drawing conclusions on a hormonal basis. In 2000 McShane et al. [352], in a second letter to the editor, detected errors in the statistical analyses of the data proving Wojtys et al. (1998) result for the correlation between ovulatory phase and ACL injury was not significant as claimed.

Yu et al. (1999) [353] studied the effects of 17β oestradiol on cell proliferation and procollagen levels, which serve as an indicator of collagen synthesis in human ACL fibroblasts. They hypothesised that fluctuation in the serum oestrogen levels may in turn translate to changes in the metabolism of ACL cells, and consequently may affect ligament structure and composition. Results showed a significant dose dependent effect of oestrogen on ACL fibroblasts during the early period of hormone exposure. Type I procollagen synthesis and fibroblast proliferation were significantly reduced with increased oestradiol concentrations. Therefore, cumulative or acute fluctuations in serum oestrogen concentrations, such as those occurring during the menstrual cycle, may induce changes in ACL fibroblast metabolism. Yu et al. (1999) conclude that this in vitro study highlights the important influence of the female sex hormones (in particular oestrogen) on collagen metabolism, and comment that further in vivo studies should be undertaken in the future.

Heitz et al. (1999) [354] also examined the correlation between hormonal changes during the menstrual cycle and ACL laxity, to determine whether surges of oestrogen and progesterone during a normal menstrual cycle affect ACL laxity. They state that
increased laxity may increase the risk of ACL injury, but also comment that this may also be a protective mechanism allowing the ligament to elongate rather than rupture. They do not elaborate however, on why this “protective mechanism” would need to fluctuate with hormone changes. Results of the study showed a significant increase in knee joint laxity in subjects throughout the menstrual cycle compared to baseline measurements. They were unable to determine if the increased laxity was greatest during a particular phase of the cycle, or if it was due to surges in oestrogen, progesterone or a combination of both. The investigators concluded that further research was required to establish whether the circulating hormones simply increase joint laxity or if they contribute to the increased incidence of ACL injury in women.

Yu et al. (2001) [335], studied the combined effects of oestrogen and progesterone on cell proliferation and procollagen synthesis in human ACL fibroblasts. Results showed fibroblast proliferation and type I procollagen synthesis are significantly reduced with increasing oestrogen concentrations. These effects are mitigated by increasing levels of progesterone, which subsequently increases fibroblast proliferation and type I procollagen synthesis. Yu et al. (2001) suggest that cumulative or acute fluctuations in these hormones such as those occurring during the female menstrual cycle could result in structural and compositional changes. This in turn could decrease the strength of the ligament and render it more susceptible to injury.

In 2002, Slauterbeck et al. [355] examined the predisposition for ACL injury in female athletes. They outline gender differences regarding the ACL; with females having increased risk of ACL graft rupture and unsuccessful outcomes post ACL reconstruction [356]. They reiterate that a possible explanation for these observations is sex specific differences in ligament remodelling. Based on the premise that as oestrogen and
progesterone regulate the expression of tissue remodelling proteins (MMPs and TIMPs), they hypothesised that ACL injury would be more likely at a certain time or times in the menstrual cycle. Therefore the aim of their study was to determine whether ACL injuries in females occur randomly or correlate with a specific phase of the menstrual cycle. Results showed that a significantly greater number of ACL injuries occurred on days 1 and 2 of the menstrual cycle. In commentary on this article, Kirk (2002), raised some doubts on the reliability of the study results. She stated that the authors relied on the subjects to identify the phase of the cycle they believed themselves to be in at the time of injury, which can be unreliable. The authors attempted to correct for this problem by using salivary oestrogen and progesterone measurements, however surges in hormones can occur at multiple times during the cycle making determination of cycle stage using this method difficult [357].

Deie et al. (2002) [358] examined changes in anterior knee joint laxity across different stages of the menstrual cycle, and found the anterior displacement values varied significantly between the follicular and ovulatory phase, and the follicular and luteal phase. There were no statistical differences in the anterior displacement of knee joints in a similar, age-matched group of men. This lead the investigators to conclude that ACL laxity in women might be dependent on the concentration of hormones, with further study required to establish whether increased joint laxity is linked to the high injury risk in women.

Wojtys et al. (2002) [359] theorised that there was an association between the distribution of ACL tears and menstrual cycle phase. Results of hormone assays indicated that women taking part in the study had a significantly greater than expected percentage of ACL injuries during the midcycle (ovulatory) phase, and a less than expected
percentage occurring during the luteal phase. It was not clear however, if female hormones such as oestrogen act directly on ACL collagen metabolism or via indirect action.

Dragoo et al. (2003) \(^{297}\) investigated the mechanism for increased non-contact ACL injuries in women by examining ACL remnants from men and women undergoing reconstruction. Results showed relaxin exhibits specific saturable binding in the female ACL where specific relaxin receptors were present. These receptors were not present in men. Dragoo et al. (2003) suggest that the high incidence of ACL rupture in females may be partially explained by the effects of relaxin, which reduces the density and organization of collagen bundles, observed using histology and immunohistochemistry techniques. This leads to a marked local decrease in the total collagen content. Gender differences also play a role because the expression of relaxin receptors appears to be under the control of oestrogen, and oestrogen priming also increases the response of target organs to relaxin. Furthermore, during the luteal phase when relaxin levels are highest, there may be simultaneous binding of oestrogen and relaxin in ACL tissue. The investigators found that these hormones have a synergistic effect on the collagen degradation in the ACL. Dragoo et al. conclude that the effects of these hormones could increase the risk of ACL rupture in females.

In 2003 Romani et al. \(^{360}\) investigated the high ratio of ACL rupture in females by examining the correlations between the female sex hormones and ACL stiffness in healthy females. They tested ACL stiffness and assessed concentrations of oestradiol, oestriol, oestrone and progesterone during the three phases of the menstrual cycle. Results indicated that there was a significant correlation between oestradiol, oestrone and progesterone and ACL stiffness near ovulation. ACL stiffness correlated positively
with progesterone and negatively with oestradiol concentrations near ovulation. These two hormones appear to have an antagonistic effect on ACL. They conclude that future studies should examine the physical properties of the ACL near the ovulatory phase.

Shultz et al. (2004) \[361\] similarly examined the notion that ACL laxity is affected by fluctuating hormones levels associated with the menstrual cycle. They measured changes in knee joint laxity and hormone levels across the menstrual cycle in 25 female subjects. Results showed that changes in knee joint laxity in response to changes in serum hormone levels are not immediate, but occur a few days later. A relationship was also evident between hormone concentration and knee laxity. Oestrogen, progesterone and testosterone each contributed to changes in knee laxity across the cycle. The effects of oestrogen and progesterone combined contributed to more of the variance in knee laxity that any of the hormones acting alone. The conclusions made from this study were that changes in sex hormones may mediate knee laxity across the menstrual cycle, and that the relative contribution of each hormone and the associated time delay is highly variable among women.

Shultz et al. (2005) \[362\] further investigated whether absolute sex hormone concentrations (oestrogen, progesterone and testosterone) predict the magnitude of knee joint changes. Results showed that the absolute minimum concentrations of progesterone and oestrogen in the early follicular phase are important factors in determining the sensitivity of the knee joint to changing hormone levels. Lower oestrogen levels and higher progesterone levels during the early follicular phase correlated to women experiencing a greater increase in knee laxity when oestrogen and testosterone levels eventually peaked. The study also revealed that knee laxity changes are fairly transient and vary between individuals. The investigators found it difficult to
identify a day or even a range of days that represents the same point in the menstrual cycle for all females. In addition, there was a delay of 3 to 4 days from a change in hormone levels to when knee laxity altered.

Shultz et al. (2006) [363] continued the investigation into sex differences in knee joint laxity across the menstrual cycle. In this study they comment that sex differences in knee laxity appear to be restricted to adults. This theory is based on research by Flynn et al. (2000) [364], which measured knee laxity in children. They found no statistical difference in knee laxity between girls and boys of a similar age. Shultz et al. (2006) suggest that this leads to the hypothesis that it is the influence of sex hormones that begin circulating after puberty that contributes to ACL rupture in women. The aim of this study was to investigate hormonal influences on joint behaviour in males versus females across the menstrual cycle. Results revealed sex differences in knee laxity were dependent on the menstrual cycle, with increased laxity coinciding with significant increases in oestradiol. Females were found to have greater knee joint laxity than males at all measured stages. Females were also found to have variable joint laxity at different stages of their cycle. These results suggest sex hormones may be a primary mediator of sex-based differences in knee laxity.

Pollard et al. (2006) [333] examined the collective effects of gender, oestrogen and exercise on knee laxity, and showed females exhibited greater knee laxity than males across all three stages of the menstrual cycle (follicular, ovulatory and luteal), both pre and post exercise. They did not find that oestrogen fluctuations during the menstrual cycle altered knee laxity, rather a general increase in knee laxity was observed in females, possibly leading to decreased joint stability and increased ACL injury.
Beynnon et al. (2006) [334] collected serum samples and self-reported menstrual history information from skiers immediately after ACL injury (within 2 hours). Skiers were found to be three times more likely to rupture their ACL in the preovulatory phase than the postovulatory phase. These findings suggest cycle phase may be one of the risk factors that influence knee ligament injury in females. It is still unclear from this study whether oestradiol and progesterone act directly on the ACL, or if other hormones associated with the menstrual cycle may modulate injury risk.

Zazulak et al. (2006) [365] and Hewitt et al. (2007) [366] reviewed current literature on the effects of the menstrual cycle on ACL injury and concluded that women are more susceptible to ACL injury during the preovulatory phase of the menstrual cycle. In addition, Hewitt et al. stated that it is difficult to perform a controlled study in which a “normal” cycle is clearly defined. They also outlined the limitations of some studies that relied on the use of questionnaires to collect data.

In contrast to many of the afore mentioned studies, which agree to some degree that fluctuations in the female sex hormones effect the laxity and injury rate of the female ACL, a number of investigators have concluded that fluctuations in these hormones are unlikely to effect the mechanical properties of the ligament. Karageanes et al. (2000) [367] studied the knee joints of female athletes over an 8-week period using arthrometry. Results showed no statistical difference in knee joint laxity across the three phases of the menstrual cycle. The investigators concluded that no single phase of the menstrual cycle clinically affects the female ACL more than another. This study may suggest that fluctuations in the levels of hormones such as oestrogen do not alter joint laxity, however it cannot rule out that the presence of the female sex hormones may indeed
predispose females to ACL injury. The fact that laxity doesn’t fluctuate with changing hormone levels does not mean that oestrogen plays no part in the aetiology of ACL disease in females.

Van Lunen et al. (2003) [368] used an arthrometer to measured the knee joint laxity of 12 female subjects and correlated the results with reproductive hormone levels across their menstrual cycles. The results indicated that there was no significant difference in knee laxity across the menstrual cycle, therefore suggesting that oestrogen fluctuations do not directly alter ACL laxity. Similarly, Belanger et al. (2004) [369] examined the laxity of the ACL of 18 females and concluded that ACL laxity is not significantly different across the three phases of the menstrual cycle. However, both of these studies are limited by their small sample size and the variation in the menstrual cycles of women.

A study by Beynnon et al. (2005) [370] also failed to show a significant correlation between hormone fluctuations during the menstrual cycle and cyclic fluctuations in joint laxity. However this does not rule out the possibility that these hormones contribute to the increased risk of injury observed in females. This same study also found that women had significantly greater knee and ankle joint laxity values compared to men. The investigators concluded that only a single time-point joint laxity measurement is required to assess an individual for injury risk.

Wolman (2009) [331] in a review of knee injuries in females athletes comments that results regarding the timing of injury and the phase of the menstrual cycle are inconclusive, with different studies each identifying risk during a different phase of the cycle. Often these studies involve subjects taking oral contraceptives, when the effect
of oral contraceptives on injury risk is unknown, and many utilise questionnaires to
determine the subject’s stage of cycle, which may be unreliable.

Changes in tissues other than ligament may also affect knee joint laxity and should not
be overlooked. Oestrogen receptors are present within skeletal muscle, leading Bell et
al. (2011) [371] to hypothesise that muscle stiffness in the hamstrings may limit ACL
loading. There is substantial evidence to suggest that muscle strength does not change
across the menstrual cycle [372-375] but it has been shown that muscle stiffness does
change during the menstrual cycle [376]. Bell et al. (2011) evaluated the muscle
properties of the hamstrings and reproductive hormone levels in men and women with
no history of knee injury. Male subjects showed no affect of the selected hormones
(17β oestradiol, free testosterone and progesterone) on muscle properties, while females
demonstrated a negative correlation between muscle stiffness and 17β oestradiol & free
testosterone. Given the low turnover of collagen in normal ligament [326, 377-379] it seems
improbable that the short-term fluctuations in hormone levels would cause significant
changes in the material properties of the ACL. In light of Bell et al.’s results, it may be
possible that other structures such as muscles may modulate knee joint laxity in
females, particularly in response to hormone fluctuations.

4.1.3.4 Conclusions

From this review of current literature on the effects of female sex hormones and
ligament a number of summations can be made. It seems clear that oestrogen has an
influence on the mechanical properties of knee ligament, however it is not clear whether
it is via direct or indirect action. Furthermore oestrogen may not act alone in its effects
on the mechanical properties of ligament. What seems likely is that the aetiology of
physiologic changes in knee ligament, leading to an increased risk of knee injury, is multifactorial involving sex hormones as well as other molecules such as MMPs and their inhibitors which can modulate collagen turnover. Also a number of challenges seemed to arise when conducting studies of the hormonal effects on ligament. One difficulty encountered by a number of studies was identifying the effect of a particular hormone amongst the hormonal milieu in vivo. In addition, conducting a controlled study using live subjects is made difficult by the variation in hormone levels and hormonal fluctuation of individuals that exists within a group of subjects (inter-subject variability). It is also difficult to isolate the exact same time-point in the menstrual cycle of a number of subjects, or even identify what exactly is a “normal” menstrual cycle. To account for the degree of variability that exists, much larger sample groups would be appropriate, with adequate controls. The same limitations in current research were also highlighted by Smith et al. (2012), in their recent review of the risk factors for anterior cruciate ligament injury involving female hormone levels [380].

This represents a large volume of information available regarding the effects of physiologic and supraphysiologic levels of female hormones such as oestrogen on the properties of knee joint ligament. In contrast there is relatively little information of the effect of a hormonal deficiency on knee ligament. Future studies focussing on the effects of ovariectomy (or menopause) on the physical properties and metabolism of knee joint ligament could clarify the interaction between ligament and the female sex hormones. A study such as this would presumably be less affected by inter-subject and cyclic hormonal variability. It is well known that menopause (for which ovariectomy is a common model) affects the metabolism of other connective tissues in the body such as cartilage, skin and bone, therefore it is likely that ligament could also be affected in some way. A number of unanswered questions and conflicting opinions remain
surrounding the subject of sex hormones and ligament, which compel further research in this area.

4.1.4 The Influence of Sex Hormone Deficiency on Connective Tissue

The female sex hormones, in particular oestrogen, play an important role in the normal maintenance and turnover of connective tissues throughout the body. With age and the onset of menopause, a decline and eventually cessation in oestrogen production by the ovaries occurs, leading to a number of changes in the structure and integrity of the body’s connective tissues. A number of biological changes signify the menopause transition in women. These include a critically low level of oocytes, disruption of the normal menstrual cycle, rising gonadotrophin levels, declining oestrogen levels and a host of associated clinical symptoms [381, 382].

4.1.4.1 Effects on Bone

The decline in endogenous oestrogen is well documented to affect the turnover and integrity of bone tissue in postmenopausal women. The decline in oestrogen levels leads to increased bone turnover, resulting in a high rate of bone loss and decreased bone mineral density (BMD). This leads to a condition known as osteoporosis, and predisposes sufferers to pathological bone fractures [383-385]. Decreased bone mineral density measurements and evidence of increased bone resorption, which can be assessed by measurement of the urinary excretion of bone type I collagen degradation products, are indicative of osteoporosis [386]. The following evidence exists to support the role of oestrogen deficiency in the disease mechanism of osteoporosis.
Pacifici et al. (1991) [383] emphasised that the bone-sparing effect of oestrogen is related to an inhibitory effect on bone resorption and importantly, outlined the existence of oestrogen receptors in the osteoclasts and osteoblasts of bone. This is significant because it indicates bone is an oestrogen responsive tissue.

Satoh et al. (1993) [387] also demonstrated the importance of oestrogen in the maintenance of normal bone structure in their study comparing the BMD of women with hysterectomy and unilateral or bilateral oophorectomy (OVX). In the bilateral OVX group accelerated bone changes were obvious immediately following surgery, and the rapid bone loss continued throughout the follow-up period. In contrast, the unilateral OVX group exhibited no initial signs of accelerated bone loss post surgery, with a slow phase of bone loss during the follow-up period. Therefore bone loss was greater in the bilateral OVX group in which oestrogen had been removed completely.

A prospective study of bone loss in menopausal Australian women was conducted by Guthrie et al. in (1998) [388], demonstrating the influence of menopause on bone loss. BMD of the hip and lumbar spine was assessed in 224 women; 74 pre-menopausal, 90 peri-menopausal and 60 post-menopausal. Results showed BMD loss was accelerated during the transition from peri to post-menopause, indicating that menopause and the associated oestrogen deficiency is a catalyst for osteoporosis.

In 1999, Meunier et al. [389] again highlighted the important role of oestrogen in prevention of osteoporosis. They studied the effect of raloxifene, a selective oestrogen receptor modulator, on the bone density of post-menopausal women. Post-menopausal women were tested because they are at high risk of osteoporosis, with an estimated 23% of post-menopausal women over 50 yrs being affected. Results showed raloxifene
increased bone density and decreased bone turnover, therefore may prove an effective treatment for osteoporosis in post-menopausal women. This conclusion was reiterated by Ringa (2000) [390], who stated that osteoporosis is directly associated with hormonal changes at menopause, and that hormone replacement therapy is proven to be effective in the prevention of bone loss.

A study by Picard et al. (2000) [391] assessed the bone density of the lumbar spine in 141 women pre-menopause, and again 10 years later post-menopause. Multiple regression analyses of bone mineral density results and information gained from questionnaires revealed bone density pre-menopause, time without oestrogen, weight and vitamin D intake were the only significant predictors of present bone mass or bone loss. This study emphasises the importance of good bone mass prior to menopause, and the early commencement of oestrogen replacement therapy to minimise bone loss in the years post-menopause, therefore reducing the risk of osteoporosis.

In order to assess the influence of other hormones on bone density, Guthrie et al. (2004) [392] performed a study of 159 Australian born women during the menopause transition. They tested the association of a number of hormones with bone loss at the lumbar spine and femoral neck. In addition to oestrogen, free testosterone, sex-hormone binding globulin and dehydroepiandrosterone were also tested. Results showed oestrogen was the only hormone tested to have a significant effect on BMD during the menopause transition.

Zecchin et al. (2004) [393] examined the association between osteoporosis and oestrogen deficiency, utilising the post-dental extraction healing process as a model for the
disease. Their study focussed on the essential role of MMPs play in the remodelling of the bone extracellular matrix after tooth extraction. They compared the healing process in 2 groups of rats; ovariectomised to simulate menopause versus sham operated controls. The OVX animals showed a significant delay in the healing process. This was attributed to a decrease in the gelatinolytic activities and expression of MMPs and collagen types I & III, which was observed in the OVX group. Therefore it can be concluded from this study that the absence of oestrogen contributes to delayed healing by interfering with bone extracellular matrix turnover.

In 2005, Jochems et al. [394] studied the link between rheumatoid arthritis (RA) and osteoporosis. In postmenopausal RA, osteoporosis is caused both by the deficiency in oestrogen and the inflammation associated with the arthritis. Statistically, the peak incidence of RA in women occurs around the time of menopause, with 50% of sufferers having generalised osteoporosis. In this study, non-arthritic OVX mice were compared to OVX and RA induced mice. At completion of the trial period, the OVX group showed trabecular BMD reduction of 22%, whereas the OVX and RA group showed BMD reduction of 58%. This study proves interesting because it examines the compounding effect inflammation has on osteoporosis. A similar situation may exist in the knee joint, where inflammation associated with osteoarthritis may compound degeneration of ligament tissue.

Akhter et al. (2007) [395] examined the effect of menopause on the structure of trabecular bone. Their study focussed on the excessive remodelling of bone that results from oestrogen deficiency in postmenopausal women. This results in a deficit of bone which compromises bone structure, and is characteristic of osteoporosis. They showed that measuring the activation frequency of new bone remodelling sites is a more powerful
predictor of bone strength and bone fragility fractures than BMD alone. Their study showed that at the onset of menopause, the remodelling rate of bone increases to twice the rate of that pre-menopause, highlighting the important role oestrogen plays in the regulation of normal bone turnover.

Mas et al. (2007) [396] demonstrated that OVX is associated with systemic bone loss in a baboon model. Their study showed bone loss following acute oestrogen deficiency takes up to 5 years to plateau, with the maximal deleterious effect on bone density occurring in the first 12 months. Another 2007 study conducted by van Essen et al. [385] suggested that increased osteocyte apoptosis, as a result of oestrogen deficiency, could play a role in the decreased bone strength and bone mass seen in postmenopausal osteoporosis. Furthermore, a study by Taxel et al. (2008) [397], suggests that oestrogen deficiency at menopause causes an increase in osteoclast formation and activation, therefore leading to excessive bone resorption and osteoporosis.

4.1.4.2 Effects on Cartilage

The evidence provided by the studies listed in Section 4.1.4.1 supports a clear link between oestrogen deficiency and bone loss. Similarly, current research also supports a link between the degeneration of articular cartilage associated with osteoarthritis (OA), and the decline in oestrogen levels at menopause [398-401]. Statistically, the prevalence of OA in women and men is similar until the age of 50 years, when it becomes more prevalent, more severe and more generalised in women [167, 169, 173]. The increased prevalence of arthritic symptoms coincides with the onset of menopause, and with joint symptoms being experienced by up to 50% of women with menopausal symptoms, hormonal changes are the likely modulators of the disease [172, 181, 233, 402]. Spector &
Campion (1989) \cite{398} quoted population studies that showed the incidence of OA in women over the age of 70 was even higher at 90%.

Oestrogen receptors have been isolated in human articular chondrocytes, indicating cartilage is an oestrogen responsive tissue \cite{233, 402, 403}. These oestrogen changes are thought to play a role in triggering the initial changes in cartilage proteoglycans, either directly or indirectly via cytokines. Furthermore, studies have shown less severe OA symptoms occur in individuals treated with oestrogen replacement therapy \cite{172, 399, 402, 404-406}.

Evidence to support the correlation between menopause and OA is provided by a number of previous studies. Dai \textit{et al.} (2005) \cite{403} investigated oestrogen receptor expression in cartilage, and osteoarthritis induced by OVX in a guinea pig model. Two groups were studied, ovariectomised to simulate menopause, and sham operated control. Results showed that oestrogen receptor expression in joint cartilage in the OVX group was significantly lower than in the control group at the same time-point post surgery. In addition, examination of joint articular cartilages using scanning electron microscopy and transmission electron microscopy revealed severe degenerative lesions in the OVX group, but not in the control group, at 12 weeks post-op. This suggests that bilateral OVX in the guinea pig leads to development of severe OA, and this is likely to be related to lower serum oestrogen levels and a reduction in oestrogen receptor expression.

Song \textit{et al.} (2004) \cite{406} studied the protective effect of hormone replacement therapy (HRT) in postmenopausal women with OA. A group of 64 women with radiographic evidence and symptoms of knee OA were randomly divided in two groups, those receiving HRT, and those receive a placebo control. A significant reduction in
symptoms such as tenderness around the knee, pain during walking and morning stiffness was observed in the in the HRT group compared to the control group after 1 month. The improvement in symptoms was still significantly greater in the HRT group compared to the control group after 6 months. This indicates that HRT (combined oestrogen and progestin) can provide relief from OA symptoms in postmenopausal women.

Claassen et al. (2005) [401] state that OA is aggravated in menopausal women, and the likely cause is a change in serum oestrogen levels. They studied the effect of oestradiol on cultured articular chondrocytes. They postulated that oestrogen protects chondrocytes cells from oxidative stress induced by oxygen free radicals. Results of their study supported this hypothesis, indicating that oestradiol protects articular chondrocytes from oxygen free radical damage, and that decreased oestrogen levels contribute to menopausal OA.

Christgau et al. (2005) [400] investigated the association between post-menopausal OA and oestrogen deficiency by studying the effects of OVX on cartilage erosion in rats. Histology and analysis of cartilage-specific type II collagen degradation products were utilised to assess cartilage erosion. Results showed OVX rats had significantly increased urinary excretion of type II collagen degradation products compared to controls. This subsequently manifested as increased surface erosion of knee articular cartilage in the OVX group compared to controls. This leads to the conclusion that oestrogen-deficiency induced changes in type II collagen are correlated with cartilage surface erosion and postmenopausal OA. Ma et al. (2007) [407] also demonstrated the role of sex hormones in cartilage degradation and progression of OA using a murine
model. OVX females developed significantly more severe OA than control females following transection of the meniscotibial ligament of the medial meniscus (a technique used to accelerate OA development). This result led the investigators to conclude that ovarian hormones decrease the severity of OA in females [404].

Wluka et al. (2001) [399] tested the hypothesis that oestrogen replacement therapy, administered for a period of 5 years or more, is associated with increased cartilage in the knee joints of postmenopausal women. Two test groups of postmenopausal women over the age of 50 years were examined, those administered oestrogen supplementation and those without. The study showed women on long-term oestrogen replacement therapy had increased knee cartilage thickness compared to controls, therefore indicating that oestrogen may prevent cartilage loss in women. Similarly, a study by Ham et al. (2002) [404] demonstrated significantly less severe cartilage lesions developed in the knee joints of OVX cynomolgus monkeys administered oestrogen replacement therapy compared to those receiving no treatment.

Hoegh-Andersen et al. (2003) [408] examined the validity of an OVX rat model in the assessment of postmenopausal OA, with the aim to assess the influence of OVX on the degradation and turnover of cartilage. Urinary excretion of collagen degradation products was measured, and histological analyses of articular cartilage were performed to assess cartilage metabolism and erosion. Results showed surface erosion of cartilage was more severe in the knees of OVX rats than the sham controls, and that OVX also significantly increased the excretion of type I and type II collagen degradation products. The study also showed that administration of oestrogen or a selective oestrogen receptor modulator inhibits the degradative effects of OVX on cartilage.
In 2006, Sowers et al. \cite{178} aimed to establish a correlation between endogenous oestrogen and an increased risk of knee OA in women. In their study they examined the serum oestrogen levels and incidence of knee OA and concluded that there was a significant correlation between lower baseline serum oestradiol levels and development of knee OA.

Cake et al. (2005) \cite{409} examined the effect of OVX on the structure of articular cartilage in an ovine model. Two groups of ewes were tested, an OVX group and a non-operated control group, using dynamic biomechanical indentation testing and histology to assess articular cartilage. This study concluded that oestrogen depletion associated with OVX resulted in thinning of the femoro-tibial cartilage. Biomechanical and histological examination of the cartilage also revealed changes indicating the structural organization of the cartilage tissue was altered. In addition, the study suggests an observed up-regulation in cartilage iNOS could be responsible for the matrix changes.

Finally, Oestergaard et al. (2006) \cite{410} investigated the influence of oestrogen supplementation on type II collagen turnover and integrity in the articular cartilage of OVX rats. The results indicated that oestrogen mitigates the degradation of type II collagen and related alterations in cartilage structure, therefore suggesting oestrogen replacement therapy may prove beneficial in the prevention of OA post menopause. The investigators also deduced from their research that the protective effect of oestrogen is related to a direct inhibition of the catabolic function of chondrocytes.

As in bone, there appears to be a large volume of evidence to support the theory that oestrogen deficiency has significant effects on the structure and metabolism of articular
cartilage, and this may predispose women to the development of OA in the years post-menopause.

### 4.1.4.3 Effects on Other Connective Tissues

In addition to bone and articular cartilage, other connective tissues such as the skin, uterus, urogenital system, intervertebral disks and blood vessel walls are also adversely affected by the decline in oestrogen accompanying menopause in women. The skin is comprised of two primary layers, the epidermis containing keratin producing cells, and the underlying dermis containing collagen and elastin fibres. Collagen makes up the main mass of the dermis, with collagen types I, II, III, V, XI all being present in skin. The collagen is produced by dermal fibroblasts, which have been shown to contain oestrogen receptors on their surface. This indicates that skin dermis is also an oestrogen responsive tissue \(^{411, 412}\). Oestrogen has been shown to have a profound effect on the structure of skin, and this is particularly evident in postmenopausal females \(^{413}\). A decline in oestrogen levels occurring with the onset of menopause leads to a reduction in skin thickness and collagen content. As the skin atrophies, it becomes wrinkled and fragile, is easily bruised, and exhibits delayed wound healing. The skin also tends to become dry, itchy and flaky due to loss of water content \(^{414-416}\).

The effects on skin brought about by declining oestrogen levels can be arrested and even reversed by the administration of oestrogen replacement therapy \(^{411, 416}\). Studies have demonstrated that oestrogen replacement therapy can increase skin thickness by 10-20\% \(^{414}\). Replacing depleted oestrogen increases the dermal content of GAGs and collagen, and improves the hydroscopic properties of the dermis, which likely occurs via enhanced synthesis of HA. It is postulated that oestrogen increases the rate of collagen production by altering the degree of polymerisation of the GAGS in the
extracellular matrix. In addition, collagen fibrils have been found to be less fragmented in the dermis of women taking hormone replacement therapy compared to non-users [411].

Degeneration in the intervertebral discs (IVDs) of the spine is also linked to declining oestrogen levels at menopause. Garnero et al. (2004) [417] investigated the link between IVD degeneration and the increased urinary excretion of type II collagen degradation products in postmenopausal women, with results showing a positive correlation exists.

Gambacciani et al. (2007) [418] further evaluated the effect of menopause on IVD height in 464 normal menopausal women, between the 12th thoracic and 4th lumbar vertebrae. In postmenopausal women, data showed a significant correlation between years since menopause (i.e. years without oestrogen) and IVD height, but no correlation between age and IVD height. In conclusion they stated that IVD height shows a progressive decrease after menopause, in particular during the first 10 years, suggesting oestrogen decline may rapidly alter the metabolism of IVD connective tissue.

Menopause is also associated with late onset of visceral prolapse and urinary incontinence due to weakening of connective tissues. Reay-Jones et al. (2003) [419] assessed the resilience of the uterosacral ligament in order to establish a link between menopause and uterine mobility and/or uterocervical prolapse. Results showed that a decrease in pelvic connective tissue resilience was related to menopause, and may progress to pelvic visceral collapse. In addition, Lang et al. (2003) [420] reported serum oestrogen levels and the number of oestrogen receptors present were significantly lower in the uterine ligaments of women with pelvic organ collapse.
Dyer et al. (1980) \cite{349} also indicated a link between oestrogen and uterine collagen in OVX rats. They examined the effect of 17β oestradiol on uterine collagen synthesis in two groups, OVX versus OVX plus oestradiol supplementation. The oestradiol treated group showed a 2 to 3-fold increase in newly-synthesised collagen, and a 8-fold increase in insoluble collagen in the uterine connective tissue when compared to the non-treated group. Results showed oestradiol stimulates the synthesis of both type I and type II collagens.

Galea & Brincat, (2000) \cite{411} showed that the female urinary and genital tracts are both oestrogen sensitive, and undergo changes following menopause. With the withdrawal of oestrogen, the vagina becomes less vascularised, the tissue is thinned and inflamed, and the cervix atrophies and retracts. The genital atrophy that accompanies menopause is associated with dyspareunia and urinary incontinence. An estimated 40% of women over the age of 60 years suffer from incontinence to some degree, with oestrogen being key to the modulation of urogenital symptoms. This is supported by the role oestrogen deficiency plays in the onset of symptoms, and by the fact that oestrogen replacement therapy can mitigate these symptoms by increasing synthesis of collagen types I & II, and reducing incontinence by increasing urethral resistance \cite{411, 421}.

Oestrogen can also influence the cardiovascular system. Di Carlo et al. (2007) \cite{422} showed that postmenopausal oestrogen deficiency increases arterial vascular tone via alterations in the neuropeptide content of the arterial walls. This can have negative effects, such as hypertension, on the cardiovascular system. Derby (2000) \cite{423} hypothesised that the depletion of ovarian hormones with menopause is associated with increased cardiovascular disease, and that the risk is reduced with hormone replacement therapy. Results of their study showed post-menopausal women exhibited more severe
arteriosclerosis, which impedes blood flow, when compared to those post-menopausal women who took oestrogen replacement therapy. Oestrogen receptors are present in the vessels of the cardiovascular system and therefore oestrogen may influence the blood flow within these vessels. It is thought that oestrogen inhibits arteriosclerosis via action on the structure of vascular connective tissue. For example, oestrogen inhibits smooth muscle cell proliferation, thus helping maintain normal vessel wall thickness and function.

4.1.4.4 Effects on Ligament

In contrast to the numerous studies examining the effect of oestrogen fluctuation on human knee ligaments (reviewed in Section 4.1.3), little is known about the effect of menopause on the ligaments of human synovial joints, in particular the knee. It is known that aging is linked to changes in the properties of ligament in the body, which are independent of gender. These include an increase in collagen crosslinking, which is postulated to lead to an increase in ligament and tendon stiffness, a reduction in collagen fibril diameter, a decrease in ECM turnover (due to a decreased load) and a decrease in water content \[^{424-426}\]. However, studies of the epidemiology of cruciate rupture in dogs have yielded some evidence that sterilisation (castration or ovariohysterectomy) may predispose them to injury. Boute et al. (2009) \[^{427}\] conducted a study of the prevalence of cranial cruciate ligament rupture in a group of 94 Labrador retrievers. Of this group with initial cruciate rupture, 32 (34%) were castrated males, 50 (53.2%) were spayed females, 7 (7.4%) were intact males and 5 (5.3%) were intact females. Of these 94 dogs, 45 (48%) went on to rupture the CCL in the contra-lateral limb; 16 (35.5%) castrated males, 23 (51.1%) spayed females, 2 intact males (4.4%) and 4 (8.9%) intact females. Sterilised individuals (in particular females) are over-represented in these figures, indicating an increased risk in this group. This is possibly
due to hormonal effects on the ligament, although other factors such as increased body weight cannot be ruled out.

Scavelli et al. (1990)\cite{121} studied the signalment for CCL injury in 25 dogs with partial rupture of the CCL. Of these 25 dogs a larger proportion were female (n = 17), compared to male (8), and a majority of the females were sterilised (14). Again these figures demonstrate a particular predisposition for injury in sterilised females. As there was no history of trauma in 23 of the 25 cases, a degenerative or immune mediated process may possibly be responsible. Hayashi et al. (2004)\cite{14} suggest inflammatory changes within the joint tissues and increased expression of collagenolytic proteases may contribute to the progressive degradation of CCL collagen and lead to progressive rupture of the ligament.

Whitehair et al. (1993)\cite{120} carried out a much larger study of 10,769 dogs with CCL rupture, comparing them to data from a control population of 591,548 dogs. For the effect of gender, analysis showed females had a significantly higher prevalence than males and that sterilisation of either gender increased the prevalence of ACL injury over intact dogs. Furthermore, they found the difference in prevalence between spayed females and intact females was statistically greater than the difference between castrated males and entire males, suggesting the sex hormones affect the prevalence of ACL injury, especially in females. Similarly, Slauterbeck et al. (2004)\cite{428} conducted a retrospective study of 3218 dogs CCL rupture, and Duval et al. (1999)\cite{122} performed a smaller study of 201 dogs with CCL rupture. Both studies found that the frequency of injury was significantly higher in sterilised male and female dogs compared with sexually intact dogs, with sterilised females demonstrating the highest prevalence overall.
4.1.5 The Ovine Oestrous Cycle

Sheep provide a useful model to study the effects of menopause in women, as they exhibit similarities in oestrous periodicity, breeding and metabolic rate \[^{429}\]. Although most breeds of ewe are seasonally polyoestrous, with an annual anoestrous period of 1-2 months, during the remainder of the year oestrogen production is relatively high, with levels similar to those of women \[^{430, 434}\]. Sheep have up to 20 oestrous cycles during the breeding period of the year \[^{431, 432}\], with some breeds of sheep such as the Merino (the breed used in this study) able to continue to cycle almost year-round \[^{434}\]. It is thought that an ovariectomised animal model with more frequent oestrous cycles than, for example, the dog, may prove to be more sensitive to estrogen deficiency \[^{433}\], and therefore more representational of menopause in women.

The oestrous cycle of the sheep lasts 17 days on average, which more closely approximates that of women than other animal species such as the rat, with an oestrous cycle of just 4 days \[^{430, 431}\]. Sheep are also reproductively similar to humans in that they are generally limited to one offspring at a time. This is in contrast to other animal species commonly used as human models such as rats, mice and dogs, which are far more prolific breeders \[^{435}\]. Furthermore, it has been shown that sheep, like humans, are born with a limited number of primordial ovarian follicles that declines throughout the life of the animal \[^{430, 435}\]. Similarly, older ewes experience similar changes to women with declining oestrogen levels, including a loss of bone density \[^{432, 434, 436}\], an increase in coronary artery disease \[^{234, 437}\], changes in temperature regulation (hot flashes) \[^{438, 439}\] and changes in articular cartilage \[^{233, 234, 409}\].
4.2 AIMS & HYPOTHESES

With so much evidence to support the assumption that a deficiency in oestrogen causes significant changes in the structure and integrity of connective tissue throughout the body, it seems likely that similar changes could occur in ligaments. There is much written about the effect of variable oestrogen levels on ligaments such as the ACL, but there appears to be a deficit in knowledge about the effects of oestrogen loss. While cruciate ligament rupture is thought to be multifactorial with no single factor responsible for all aspects of its pathogenesis [62, 440], the female predisposition for injury, apparent in both animals and humans, cannot be overlooked. It is also unclear whether the coexistence of other joint conditions such as OA amplifies degeneration of ligament tissue such as is the case with arthritis and osteoporosis. It may be that ligament degeneration precedes the onset of OA, with destabilisation of the joint contributing to OA development. With so many types of connective tissues in the body being responsive to the same hormone, it could be that the same disease mediators affect multiple tissue structures within the same joint.

As previously described, ligament is certainly an oestrogen responsive tissue with much research dedicated to investigating the effect of fluctuating oestrogen levels on knee biomechanics and ligament function. Despite this, researchers have failed to come to any common conclusions about oestrogen's influence on ligament, with some studies utilising questionable methodology to obtain their results. Furthermore, ligament has been largely overlooked in the field of research into the effects of menopause on the body, regardless of its important role in the body's biomechanical function. It has also been shown that the incidence of OA increases with the onset of menopause, although
the pathogenesis remains unclear. The aim of this study was to elucidate what affect a
deficit in ovarian hormones (menopause) has on the major stabilising ligaments of the
knee, particularly the commonly injured anterior cruciate ligament, and whether these
effects (if any) may contribute to the development of OA. It also examined the effects
of concurrent OA and menopause on the structure and metabolism of ligament tissue, in
order to better understand the possible synergistic relationship between these two
conditions.

**Hypothesis 3:** that the decrease in ovarian hormone levels associated with menopause
(ovariectomy) alters the normal composition and organisation of cruciate ligament
tissue, in particular collagen fibril structure, which has the potential to influence the
mechanical properties of the ligament, as observed in other connective tissue types
post-menopause.

**Hypothesis 4:** that the combined effects of ovariectomy and meniscectomy have an even
greater influence on ligament composition and structure than either meniscectomy or
ovariectomy alone, and that these changes reflect those of postmenopausal women with
concurrent osteoarthritis.
## 4.3 RESULTS

### 4.3.1 Molecular Biology

#### 4.3.1.1 Preliminary OVX Study

Table 4.1: Results of the preliminary study of RNA expression in target genes in ACL and PCL tissue. Results are normalised to total RNA. OVX1 = ovariectomised, NOC1 = non-operated control, ACL = anterior cruciate ligament, PCL = posterior cruciate ligament. Results shown compare treated ACL and PCL with their relevant non-operated controls to demonstrate the effect of OVX on ligament gene expression. Comparison is also made between ligament types (ACL and PCL) to determine if any difference exists between either the treated groups, or the controls.

<table>
<thead>
<tr>
<th>TARGET GENE</th>
<th>NOC1 ACL Mean (CI)</th>
<th>OVX1 ACL Mean (CI)</th>
<th>FOLD DIFFERENCE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type I</td>
<td>245.0 (135.4 - 354.6)</td>
<td>93.0 (76.7 - 109.3)</td>
<td>2.6 0.023</td>
<td></td>
</tr>
<tr>
<td>Collagen type II</td>
<td>263.5 (154.7 - 372.3)</td>
<td>212.5 (123.1 - 301.9)</td>
<td>1.2 0.494</td>
<td></td>
</tr>
<tr>
<td>Collagen type III</td>
<td>524.7 (432.0 - 617.4)</td>
<td>250.2 (173.0 - 327.4)</td>
<td>2.1 0.001</td>
<td></td>
</tr>
<tr>
<td>Decorin</td>
<td>582.3 (522.9 - 641.7)</td>
<td>166.2 (133.1 - 199.3)</td>
<td>3.5 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>570.7 (518.0 - 623.4)</td>
<td>201.0 (103.6 - 298.4)</td>
<td>2.8 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Biglycan</td>
<td>202.5 (158.8 - 248.2)</td>
<td>231.2 (137.3 - 325.1)</td>
<td>0.9 0.602</td>
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<tr>
<th></th>
<th>NOC1 PCL Mean (CI)</th>
<th>OVX1 PCL Mean (CI)</th>
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<tr>
<td>Collagen type I</td>
<td>345.8 (243.5 - 448.1)</td>
<td>112.2 (88.9 - 135.5)</td>
<td>3.1 0.001</td>
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</tr>
<tr>
<td>Collagen type II</td>
<td>188.6 (9.5 - 367.7)</td>
<td>270.5 (153.7 - 387.3)</td>
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</tr>
<tr>
<td>Collagen type III</td>
<td>508.5 (370.1 - 646.9)</td>
<td>222.3 (164.9 - 279.7)</td>
<td>2.3 0.004</td>
<td></td>
</tr>
<tr>
<td>Decorin</td>
<td>549.2 (452.6 - 645.8)</td>
<td>278.3 (197.7 - 358.9)</td>
<td>2.0 0.002</td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>Biglycan</td>
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<td>258.6 (199.6 - 317.6)</td>
<td>0.9 0.654</td>
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<tr>
<th></th>
<th>NOC1 ACL Mean (CI)</th>
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<td>556.3 (448.1 - 664.5)</td>
<td>1.1 0.810</td>
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<td>1.0 0.590</td>
<td></td>
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<table>
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<tr>
<th></th>
<th>OVX1 ACL Mean (CI)</th>
<th>OVX1 PCL Mean (CI)</th>
<th>FOLD DIFFERENCE</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Collagen type I</td>
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<td>112.2 (88.9 - 135.5)</td>
<td>0.9 0.081</td>
<td></td>
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<tr>
<td>Collagen type II</td>
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<td>270.5 (153.7 - 387.3)</td>
<td>0.8 0.555</td>
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<td>Collagen type III</td>
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<td>0.8 0.605</td>
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</tr>
<tr>
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<td>1.1 0.027</td>
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<tr>
<td>Fibromodulin</td>
<td>201.0 (103.6 - 298.4)</td>
<td>312.0 (207.9 - 416.1)</td>
<td>0.6 0.123</td>
<td></td>
</tr>
<tr>
<td>Biglycan</td>
<td>231.2 (137.3 - 325.1)</td>
<td>258.6 (199.6 - 317.6)</td>
<td>0.6 0.639</td>
<td></td>
</tr>
</tbody>
</table>
Results are presented normalised to total RNA. GAPDH was selected as a housekeeping gene, however its expression proved too variable for this purpose. Type I collagen expression for ACL was significantly lower in OVX1 sheep vs. NOC1 sheep \( (P \leq 0.023) \). Type I collagen expression in PCL was also significantly lower in OVX1 sheep vs. NOC1 sheep \( (P \leq 0.001) \). There was no significant difference between ACL and PCL for type I collagen expression in the NOC1 group \( (P = 0.220) \) or the OXV1 group \( (P = 0.081) \). See Appendix A4.1.1 for charted results (page 241).

No significant difference was found between NOC1 and OVX1 for collagen type II expression in ACL \( (P = 0.494) \) or PCL \( (P = 0.471) \). There was also no significant difference between ACL and PCL for NOC1 \( (P = 0.440) \) or OVX1 \( (P = 0.555) \).

Type III collagen expression in ACL was significantly lower in OXV1 compared to NOC1 \( (P \leq 0.001) \), and also for PCL in OXV1 compared to NOC1 \( (P \leq 0.004) \). No significant difference was found between ACL and PCL for NOC1 \( (P = 0.700) \) or OVX1 \( (P = 0.605) \).

Decorin expression for ACL was significantly lower in OVX1 sheep compared to NOC1 sheep \( (P \leq 0.00001) \), and also for PCL in OVX1 sheep vs. NOC1 sheep \( (P \leq 0.002) \). There was no significant difference between ACL and PCL for decorin expression in the NOC1 group \( (P = 0.520) \), however expression was significantly lower in ACL compared to PCL in the OXV1 sheep \( (P = 0.027) \).

Expression of fibromodulin for ACL was significantly lower in OVX1 sheep vs. NOC1 sheep \( (P \leq 0.0001) \). Fibromodulin expression in PCL was also significantly lower in OVX1 sheep vs. NOC1 sheep \( (P \leq 0.010) \). No significant difference was found for fibromodulin expression between ACL and PCL in the NOC1 group \( (P = 0.810) \) or the OXV1 group \( (P = 0.123) \).
Biglycan expression was not significantly different for NOC1 vs. OVX1 in ACL ($P = 0.602$) or PCL ($P = 0.654$). There was also no significant difference between ACL and PCL for biglycan expression in NOC1 sheep ($P = 0.590$) or OVX1 sheep ($P = 0.639$).

### 4.3.1.2 Main Study

**EFFECTS OF OVX**

<table>
<thead>
<tr>
<th>TARGET GENE</th>
<th>NOC Mean (CI)</th>
<th>OVX Mean (CI)</th>
<th>FOLD DIFFERENCE</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecan</td>
<td>279.3 (0 – 686.2)</td>
<td>66.2 (32.2 – 100.2)</td>
<td>4.22</td>
<td>0.159</td>
</tr>
<tr>
<td>Basic FGF</td>
<td>329.9 (138.2 – 521.6)</td>
<td>155.3 (110.7 – 200.0)</td>
<td>2.12</td>
<td>0.096</td>
</tr>
<tr>
<td>Biglycan</td>
<td>164.3 (93.7 – 234.9)</td>
<td>104.2 (58.5 – 149.9)</td>
<td>1.58</td>
<td>0.175</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>65.1 (28.6 – 101.6)</td>
<td>16.4 (10.0 – 22.9)</td>
<td>3.96</td>
<td>0.017</td>
</tr>
<tr>
<td>Collagen type II</td>
<td>355.2 (0 – 728.1)</td>
<td>23.8 (10.6 – 36.9)</td>
<td>14.95</td>
<td>0.096</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>34.8 (22.2 – 47.3)</td>
<td>14.4 (7.9 – 24.3)</td>
<td>2.41</td>
<td>0.010</td>
</tr>
<tr>
<td>CTGF</td>
<td>307.2 (98.8 – 515.5)</td>
<td>152.7 (91.9 – 235.3)</td>
<td>2.01</td>
<td>0.152</td>
</tr>
<tr>
<td>Decorin</td>
<td>395.4 (213.9 – 577.0)</td>
<td>102.8 (54.6 – 150.9)</td>
<td>3.85</td>
<td>0.001</td>
</tr>
<tr>
<td>Elastin</td>
<td>579.5 (301.2 – 857.8)</td>
<td>236.2 (182.1 – 290.2)</td>
<td>2.45</td>
<td>0.027</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>298.7 (161.1 – 436.2)</td>
<td>94.7 (67.4 – 122.0)</td>
<td>3.15</td>
<td>0.009</td>
</tr>
<tr>
<td>INOS</td>
<td>338.9 (113.0 – 564.9)</td>
<td>258.6 (182.8 – 434.4)</td>
<td>1.31</td>
<td>0.588</td>
</tr>
<tr>
<td>Lubricin</td>
<td>219.3 (51.9 – 386.7)</td>
<td>152.9 (114.3 – 191.5)</td>
<td>1.43</td>
<td>0.457</td>
</tr>
<tr>
<td>MMP-1</td>
<td>35.2 (11.7 – 58.8)</td>
<td>42.4 (8.9 – 83.9)</td>
<td>0.83</td>
<td>0.765</td>
</tr>
<tr>
<td>PDGF</td>
<td>138.4 (38.7 – 238.1)</td>
<td>77.0 (44.3 – 109.7)</td>
<td>1.80</td>
<td>0.264</td>
</tr>
</tbody>
</table>

Table 4.2: RNA expression of target genes in ACL tissue, shown as value of total RNA. NOC = non-operated control, OVX = ovariectomised.

**EFFECT OF PRIOR OVX ON OA**

<table>
<thead>
<tr>
<th>TARGET GENE</th>
<th>O+M Mean (CI)</th>
<th>MENX Mean (CI)</th>
<th>FOLD DIFFERENCE</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggrecan</td>
<td>420.6 (181.1 – 660.1)</td>
<td>130.6 (44.2 – 217.0)</td>
<td>3.22</td>
<td>0.069</td>
</tr>
<tr>
<td>Basic FGF</td>
<td>523.3 (372.5 – 674.1)</td>
<td>164.7 (121.7 – 207.7)</td>
<td>3.18</td>
<td>0.001</td>
</tr>
<tr>
<td>Biglycan</td>
<td>749.6 (419.7 – 1079.4)</td>
<td>165.3 (100.6 – 230.0)</td>
<td>4.53</td>
<td>0.798</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>477.0 (219.3 – 734.7)</td>
<td>48.4 (37.0 – 59.8)</td>
<td>9.86</td>
<td>0.008</td>
</tr>
<tr>
<td>Collagen type II</td>
<td>1167.4 (184.0 – 2150.9)</td>
<td>252.0 (84.2 – 419.8)</td>
<td>4.63</td>
<td>0.116</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>466.5 (225.3 – 707.7)</td>
<td>36.0 (24.7 – 47.3)</td>
<td>12.96</td>
<td>0.005</td>
</tr>
<tr>
<td>CTGF</td>
<td>361.8 (215.4 – 508.2)</td>
<td>131.5 (71.7 – 191.3)</td>
<td>2.75</td>
<td>0.015</td>
</tr>
<tr>
<td>Decorin</td>
<td>699.1 (412.4 – 985.8)</td>
<td>150.1 (109.2 – 191.0)</td>
<td>4.66</td>
<td>0.003</td>
</tr>
<tr>
<td>Elastin</td>
<td>667.7 (330.9 – 1004.5)</td>
<td>234.5 (168.5 – 300.5)</td>
<td>2.85</td>
<td>0.035</td>
</tr>
<tr>
<td>Fibromodulin</td>
<td>900.1 (474.5 – 1325.5)</td>
<td>209.4 (149.8 – 269.0)</td>
<td>4.30</td>
<td>0.009</td>
</tr>
<tr>
<td>iNOS</td>
<td>240.0 (91.0 – 389.0)</td>
<td>266.9 (90.5 – 443.3)</td>
<td>0.90</td>
<td>0.820</td>
</tr>
<tr>
<td>Lubricin</td>
<td>217.7 (159.7 – 275.6)</td>
<td>100.2 (69.9 – 130.5)</td>
<td>2.17</td>
<td>0.003</td>
</tr>
<tr>
<td>Lumpercan</td>
<td>513.9 (261.2 – 766.6)</td>
<td>167.8 (81.6 – 254.0)</td>
<td>3.06</td>
<td>0.029</td>
</tr>
<tr>
<td>MMP-1</td>
<td>224.9 (74.7 – 375.2)</td>
<td>123.1 (66.8 – 179.4)</td>
<td>1.83</td>
<td>0.627</td>
</tr>
<tr>
<td>PDGF</td>
<td>268.4 (164.0 – 372.8)</td>
<td>95.5 (64.6 – 126.4)</td>
<td>2.81</td>
<td>0.010</td>
</tr>
</tbody>
</table>

Table 4.3: RNA expression of target genes in ACL tissue, shown as value of total RNA. O+M = ovariectomised & meniscectomised, MENX = meniscectomised.
4.3.1.2.1 Gene Expression Normalised to Total RNA

Total RNA results showed that the OXV group had significantly lower expression of collagen type I ($P \leq 0.017$), collagen type III ($P \leq 0.01$), decorin ($P \leq 0.006$), fibromodulin ($P \leq 0.009$), and elastin ($P \leq 0.027$) than the NOC group. Charts comparing gene expression for all experimental groups are shown in Appendix A4.1.2 (page 245).

The O+M group showed significantly higher expression of collagen type I ($P \leq 0.005$), collagen type III ($P \leq 0.002$), biglycan ($P \leq 0.002$), lumican ($P \leq 0.041$) and MMP-I ($P \leq 0.029$) when compared to NOC sheep.

Expression in the OVX sheep was significantly lower than in sheep from the O+M group for most genes tested; collagen type I ($P \leq 0.002$), collagen type II ($P \leq 0.033$), collagen type III ($P \leq 0.001$), decorin ($P \leq 0.001$), biglycan ($P \leq 0.001$), fibromodulin ($P \leq 0.001$), lumican ($P \leq 0.005$), elastin ($P \leq 0.021$), aggrecan ($P \leq 0.007$), MMP-1 ($P \leq 0.048$), basic FGF ($P \leq 0.0001$), CTGF ($P \leq 0.017$), and PDGF ($P \leq 0.002$).

4.3.1.2.2 Compared to MENX Results (Chapter 3)

Total RNA results comparing MENX and OVX groups showed expression of collagen type I ($P \leq 0.0001$), collagen type II ($P \leq 0.008$), collagen type III ($P \leq 0.003$), fibromodulin ($P \leq 0.002$) and MMP-I ($P \leq 0.034$) was significantly lower in OVX sheep than MENX.

Expression of collagen type I ($P \leq 0.008$), collagen type III ($P \leq 0.005$), decorin ($P \leq 0.003$), biglycan ($P \leq 0.005$), fibromodulin ($P \leq 0.009$), lumican ($P \leq 0.029$), lubricin ($P \leq 0.003$), elastin ($P \leq 0.035$), basic FGF ($P \leq 0.001$), CTGF ($P \leq 0.015$) and PDGF ($P \leq 0.01$) was significantly lower in MENX sheep compared to O+M sheep.
4.3.2  Biochemistry

4.3.2.1  Collagen

Results were calculated as a % of the dry weight of tissue. Results showed total collagen in the ACL of OVX sheep was significantly lower than in the NOC sheep [mean ± SE, OVX 53.55 ± 1.33 vs. NOC 57.16 ± 1.07, $P \leq 0.03$] and O+M sheep [67.30 ± 2.51, $P \leq 0.0001$]. Collagen in the O+M group was significantly higher than in the NOC group ($P = 0.001$).

4.3.2.1.1  Compared to MENX Results (Chapter 3)

The OVX group had significantly less collagen than the MENX group ($P = 0.002$), while the O+M group had significantly higher collagen content than the MENX group ($P \leq 0.039$). Appendix A4.2 shows results comparing all experimental groups (page 257).

4.3.2.2  Sulfated Glycosaminoglycans (S-GAG)

Results were calculated as a % of the dry weight of tissue.

Results showed S-GAG content in the ACL of OVX sheep was significantly lower than in the NOC sheep [mean ± SE, OVX 9.43 ± 0.40 vs. NOC 11.78 ± 0.70, $P \leq 0.02$] but not O+M sheep [10.11 ± 0.32, $P = 0.196$]. ACLs from O+M sheep contained significantly less S-GAG than those from NOC sheep ($P = 0.013$).

4.3.2.2.1  Compared to MENX Results (Chapter 3)

There was no significant difference between the OVX and MENX groups ($P = 0.837$), or the O+M and MENX groups ($P = 0.196$) for S-GAG content.
4.3.3 Transmission Electron Microscopy (TEM)

4.3.3.1 Fibril Size and Arrangement

NOC fibrils were of similar size and regularly arranged. The majority of fibrils in the NOC ACLs were found to be within a close size range of 100-200nm.

OVX fibrils had a significantly smaller average fibril diameter than NOC [96.87 ± 1.79 vs. 151.05 ± 2.28; \( P \leq 0.0001 \)] and a lower fibril size range. The fibrils in the OVX group were much more haphazard in their arrangement (Figure 4.3 & 4.4).

The O+M group was not significantly different to NOC for average fibril diameter, however this does not reflect the abnormal bimodal distribution with unusually small and large fibrils, or the irregular fibril arrangement (Figure 4.5 & 4.6). See Appendix A4.3 for graphical presentation of TEM results comparing all experimental groups (page 259) and to view all TEM images from each group (pages 264-286).
Figure 4.3: TEM images (37000x) of ACL collagen fibrils. Image 8666 is from the NOC group displaying uniform sized fibrils that are regularly arranged. Image 8700 is a typical sample from the OVX group with smaller sized fibrils that are variable in their arrangement.

Figure 4.4: Chart showing ACL fibril diameter (size) distribution for the OVX group compared to the NOC group, as determined using TEM analysis. Note the shift towards smaller sized fibrils in the OVX group.
Figure 4.5: TEM images (37000x) of ACL collagen fibrils, comparing fibril size and arrangement in NOC (image 8666) with O+M (image 8584) samples. Note the abnormal bimodal distribution with unusually small and large fibrils in the O+M sheep compared to the more uniform fibrils in the OVX sheep.

Figure 4.6: Distribution of collagen fibrils in O+M vs. NOC ACL tissue based on fibril size (diameter in nm), as determined using TEM analysis. Note the bimodal size distribution in the O+M group with many unusually small and large sized fibrils.
Table 4.4: Combined fibril diameter data for sheep ACL. Fibril counts for OVX and O+M sheep were compared to the NOC sheep for each fibril size range. P-values are calculated at 5% significance level. MENX data is included for comparison.

<table>
<thead>
<tr>
<th>FIBRIL SIZE (nm)</th>
<th>Mean number of fibrils per two TEM fields, approx. 5µm² (% of total in brackets)</th>
<th>NOC (n=12)</th>
<th>OVX (n=12)</th>
<th>P (vs NOC)</th>
<th>O+M (n=12)</th>
<th>P (vs NOC)</th>
<th>MENX (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;100</td>
<td></td>
<td>1.2 (&lt;1%)</td>
<td>94.8 (48%)</td>
<td>&lt;0.001</td>
<td>54.6 (29%)</td>
<td>&lt;0.001</td>
<td>1.2 (&lt;1%)</td>
</tr>
<tr>
<td>100-200</td>
<td></td>
<td>154.4 (99%)</td>
<td>102.2 (52%)</td>
<td>&lt;0.001</td>
<td>98.6 (53%)</td>
<td>&lt;0.001</td>
<td>154.4 (99%)</td>
</tr>
<tr>
<td>&gt;200</td>
<td></td>
<td>0.2 (&lt;1%)</td>
<td>0.3 (&lt;1%)</td>
<td>0.255</td>
<td>33.4 (18%)</td>
<td>&lt;0.001</td>
<td>0.2 (&lt;1%)</td>
</tr>
</tbody>
</table>

Figure 4.7: Comparison of distribution of collagen fibrils in all groups for ACL tissue, based on fibril size (diameter in nm), as determined using TEM analysis. All experimental groups displayed a pattern of fibril size distribution that varied greatly from that of the NOC group.
Data for fibril density was calculated as a % of total field. Results showed OVX had significantly lower fibril density than all other groups; NOC [mean % of field ± SE, OVX 27.3 ± 0.9% vs. NOC 48.1 ± 1.3%; \( P \leq 0.0001 \)], MENX [47.3 ± 1.4%; \( P \leq 0.0001 \)] and O+M [59.9 ± 1.8%; \( P \leq 0.0001 \)]. O+M had greater total fibril density than all other groups, NOC (\( P \leq 0.0001 \)), MENX (\( P \leq 0.0001 \)).

4.3.4 Scanning Electron Microscopy (SEM)

Cross-sectional SEM images were taken of ACL from NOC, OVX and O+M sheep. The OVX and O+M images showed structural differences and obvious electron dense bands or striations across the cut surface of the tissue that weren’t clearly seen in the NOC sheep (Figures 4.8 - 4.14). Similar striations were observed in MENX ACL tissue (Chapter 3).

Figure 4.8: SEM (170x) ACL tissue from a NOC sheep viewed in cross-section. The fibril bundles within the mid-substance ligament are tightly packed with little space between them, and the tissue is uniform in appearance.
Figure 4.9: SEM (170x) ACL tissue from an OVX sheep viewed in cross-section. The bundles within the ligament are more open with multiple cracks appearing throughout (arrows).

Figure 4.10: SEM (2000x) ACL tissue from a NOC sheep viewed in cross-section. The tissue is more homogeneous, lacking the striated appearance seen in the MENX sample (Chapter 3).
Figure 4.11: SEM (2000x) ACL tissue from an OVX sheep viewed in cross-section. The tissue has striated appearance similar to that seen in the MENX sheep.

Figure 4.12: SEM (3016x) ACL tissue from an OVX sheep. Viewed at a higher power of magnification this striated section of tissue appears to have multiple areas of separation between groups of collagen fibres.
Figure 4.13: SEM (2000x above, 170x below) ACL tissue from an O+M sheep viewed in cross-section. In this ovariectomised and meniscectomised sample, the spaces between the fibre bundles were not as pronounced as in the OVX and MENX only samples, however there were a number of areas where the mid-fascicular architecture appeared distracted. This was not seen in the NOC samples.
Figure 4.14: SEM (2000x) ACL tissue from an O+M sheep viewed in cross-section. Like the OVX and MENX only samples, the tissue had obvious light and dark banding across the cut surface.
### 4.3.5 Gross Morphology

#### 4.3.5.1 Articular Cartilage

<table>
<thead>
<tr>
<th>REGION</th>
<th>NOC   (Mean (CI))</th>
<th>MENX (Mean (CI))</th>
<th>OVX   (Mean (CI))</th>
<th>O+M   (Mean (CI))</th>
<th>P (vs. NOC)</th>
<th>P (vs. NOC)</th>
<th>P (vs. OVX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage</td>
<td>0.08 (0.0 - 0.2)</td>
<td>3.10 (2.9 - 3.3)</td>
<td>0.08 (0.0 - 0.2)</td>
<td>1.00 (2.8 - 3.7)</td>
<td>&lt;0.0001</td>
<td>0.564</td>
<td>0.191</td>
</tr>
<tr>
<td>FEMUR</td>
<td>0.17 (0.0 - 0.4)</td>
<td>2.80 (2.2 - 3.4)</td>
<td>0.50 (0.2 - 0.8)</td>
<td>0.09 (2.9 - 3.7)</td>
<td>&lt;0.0001</td>
<td>0.156</td>
<td>0.191</td>
</tr>
<tr>
<td>TIBIA</td>
<td>0.13 (0.0 - 0.3)</td>
<td>2.95 (2.6 - 3.3)</td>
<td>0.30 (0.1 - 0.5)</td>
<td>0.10 (2.9 - 3.5)</td>
<td>&lt;0.0001</td>
<td>0.191</td>
<td>0.191</td>
</tr>
</tbody>
</table>

Table 4.5: Results of macroscopic scoring of ovine knee articular cartilage, comparing the non-operated control group with the ovariectomised group and the ovariectomised & meniscectomised group.

![MACROSCOPIC SCORING OF CARTILAGE DEGRADATION IN OVINE KNEE JOINTS](image)

Figure 4.15: Charted data for macroscopic scoring of articular cartilage, comparing the non-operated control group with the ovariectomised, meniscectomised and the ovariectomised & meniscectomised group.
4.3.5.2 Osteophytes

<table>
<thead>
<tr>
<th>REGION</th>
<th>NOC</th>
<th>MENX</th>
<th>OVX</th>
<th>O+M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage</td>
<td>Mean (CI)</td>
<td>Mean (CI)</td>
<td>Mean (CI)</td>
<td>Mean (CI)</td>
</tr>
<tr>
<td>FEMUR</td>
<td>0.08 (0.0 - 0.2)</td>
<td>1.30 (1.0 - 1.6)</td>
<td>0.41 (0.1 - 0.7)</td>
<td>0.06</td>
</tr>
<tr>
<td>TIBIA</td>
<td>0.08 (0.0 - 0.2)</td>
<td>1.90 (1.5 - 2.3)</td>
<td>0.08 (0.0 - 0.3)</td>
<td>1.00</td>
</tr>
<tr>
<td>JOINT</td>
<td>0.08 (0.0 - 0.2)</td>
<td>1.60 (1.3 - 1.9)</td>
<td>0.26 (0.1 - 0.4)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Table 4.6: Results of macroscopic scoring of the presence of osteophytes in ovine knee joints, comparing the non-operated control group with the ovariectomised group and the ovariectomised & meniscectomised group.

MACROSCOPIC SCORING OF OSTEOPHYTE FORMATION
IN OVINE KNEE JOINTS

Figure 4.16: Charted data for macroscopic scoring of osteophyte formation, comparing the non-operated control group with the ovariectomised, meniscectomised and the ovariectomised & meniscectomised group.
4.3.6  Histology

4.3.6.1  Ligament

OVX - Haematoxylin & Eosin

While the NOC and OVX groups (Figure 4.17 A&C) appeared similar in their cellularity, the cells of the NOC samples were arranged in straight lines along the fibre bundles, whereas those of the OVX samples were more randomly distributed. The two groups also appeared comparable for cell morphology, although in regions of some OVX sections, the cells appeared slightly more rounded due to a more transverse than longitudinal orientation when sectioned. This was also observed in the arrangement of collagen fibres of the OVX samples, with some fibre bundles orientated transversely while adjacent bundles were longitudinal. In general, the collagen fibres of the OVX samples were more irregular in their arrangement and much less parallel in their alignment than the NOC samples.

OVX - Toluidine Blue

The density of elastin fibres in the OVX samples was reduced compared to the NOC samples (Figure 4.17 B&D). The few darkly stained elastin fibres observed in the OVX samples were more haphazard in their distribution than those of NOC samples.

O+M - Haematoxylin & Eosin

The cellularity of the O+M samples did not appear to differ greatly from that of the NOC samples, and they appeared similar in their morphology (Figure 4.17 A&E). The arrangement of collagen fibres in the O+M samples was variable, with some sections having fibres that were parallel in their alignment and regularly arranged, while others were more random in their organisation. Like the OVX samples, some sections
exhibited fibres that were both longitudinal and transversely orientated, despite being sampled from the same region of the ligament and transversely embedded in the block.

O+M - Toluidine Blue

The density of the elastin fibres in the O+M group was greater than in the OVX only group and appeared similar to that of the NOC group, however the elastin fibres were less evenly distributed and more irregular in their alignment (Figure 4.17 B&F).

Figure 4.17:
A and B = NOC ACL tissue. A, H&E 200x sample showing normal cellularity and morphology, with parallel collagen fibril arrangement; B, Tol Blue 200x sample demonstrating abundant and evenly distributed elastin fibres. All histological sections can be viewed in Appendix A4.5 (pages 285-292).

C and D = OVX ACL tissue. C, H&E 200x showing fibre bundles that are less regular in their arrangement, with some bundles orientated transversely while adjacent bundles are longitudinal; D, Tol Blue 200x sample showing the reduced density of elastin fibres in the OVX tissue, with less regular arrangement.

E and F = O+M ACL tissue. E, H&E 200x arrangement of collagen fibres was variable with some sections having fibres that regularly arranged, while others were more random in their organisation as shown here; F, elastin fibre density was greater than in the OVX only group but was less evenly distributed than in NOC.
### 4.3.6.2 Cartilage

<table>
<thead>
<tr>
<th></th>
<th>NOC</th>
<th>MENX</th>
<th>O VX</th>
<th>O+M</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FEMORAL CARTILAGE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STRUCTURE</td>
<td>1.46 (0.68 - 2.24)</td>
<td>3.17 (2.14 - 4.20)</td>
<td>3.25 (2.14 - 4.36)</td>
<td>3.17       (2.14 - 4.20)</td>
</tr>
<tr>
<td>CELL NUMBER</td>
<td>0.79 (0.48 - 1.10)</td>
<td>1.38 (1.00 - 1.75)</td>
<td>0.92 (0.64 - 1.19)</td>
<td>0.79       (0.48 - 1.10)</td>
</tr>
<tr>
<td>CLONING</td>
<td>0.46 (0.26 - 0.66)</td>
<td>2.71 (2.27 - 3.15)</td>
<td>1.67 (0.98 - 2.35)</td>
<td>0.46       (0.26 - 0.66)</td>
</tr>
<tr>
<td>IT TB</td>
<td>0.79 (0.30 - 1.28)</td>
<td>1.71 (1.28 - 2.13)</td>
<td>1.25 (0.88 - 1.62)</td>
<td>0.79       (0.30 - 1.28)</td>
</tr>
<tr>
<td>TIDEMARK</td>
<td>1.04 (0.96 - 1.13)</td>
<td>1.08 (0.97 - 1.20)</td>
<td>1.38 (1.02 - 1.73)</td>
<td>1.04       (0.96 - 1.13)</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>4.54 (2.93 - 6.15)</td>
<td>10.04 (8.35 - 11.73)</td>
<td>8.46 (6.19 - 10.73)</td>
<td>8.46       (6.19 - 10.73)</td>
</tr>
</tbody>
</table>

| **TIBIAL CARTILAGE** |      |      |      |      |
| STRUCTURE          | 0.58 (0.06 - 1.11) | 5.63 (4.48 - 6.77) | 5.67 (4.57 - 6.77) | 5.67       (4.57 - 6.77) |
| CELL NUMBER        | 0.42 (0.21 - 0.62) | 2.42 (2.02 - 2.81) | 1.96 (1.55 - 2.37) | 1.96       (1.55 - 2.37) |
| CLONING            | 0.21 (0.02 - 0.40) | 3.42 (3.06 - 3.78) | 3.00 (2.34 - 3.66) | 3.00       (2.34 - 3.66) |
| IT TB              | 0.29 (0.04 - 0.55) | 2.96 (2.68 - 3.24) | 2.08 (1.52 - 2.65) | 2.08       (1.52 - 2.65) |
| TIDEMARK           | 1.13 (0.95 - 1.30) | 1.13 (1.00 - 1.25) | 1.58 (1.25 - 1.92) | 1.58       (1.25 - 1.92) |
| **TOTAL**          | 2.63 (1.79 - 3.46) | 15.54 (13.79 - 17.29)| 14.29 (11.64 - 16.96)| 14.29      (11.64 - 16.96)|

| **WHOLE JOINT** |      |      |      |      |
| STRUCTURE          | 2.04 (1.14 - 2.95) | 9.00 (6.94 - 11.06) | 8.92 (7.00 - 10.83) | 8.92       (7.00 - 10.83) |
| CELL NUMBER        | 1.21 (0.82 - 1.60) | 3.79 (3.18 - 4.40) | 2.88 (2.29 - 3.46) | 2.88       (2.29 - 3.46) |
| CLONING            | 0.67 (0.36 - 0.97) | 6.13 (5.51 - 6.74) | 4.67 (3.48 - 5.86) | 4.67       (3.48 - 5.86) |
| IT TB              | 1.08 (0.57 - 1.59) | 4.67 (4.21 - 5.12) | 3.33 (2.60 - 4.07) | 3.33       (2.60 - 4.07) |
| TIDEMARK           | 2.17 (1.98 - 2.35) | 2.21 (2.06 - 2.35) | 2.96 (2.39 - 3.53) | 2.96       (2.39 - 3.53) |
| **TOTAL**          | 7.17 (5.44 - 8.89) | 25.58 (22.49 - 28.67)| 22.75 (18.57 - 26.93)| 22.75      (18.57 - 26.93)|

Table 4.7: Microscopic scoring of articular cartilage degeneration in ovariectomised joints with and without concurrent OA, compared to controls.

#### MICROSCOPIC SCORING OF ARTICULAR CARTILAGE DEGENERATION IN OVINE KNEE JOINTS

Figure 4.18: Charted data for microscopic scoring of articular cartilage, examining the effect of ovariectomy in joints with and without concurrent OA (O+M).
### 4.3.6.3 Synovium

<table>
<thead>
<tr>
<th>SYNOVIUM</th>
<th>NOC Mean (CI)</th>
<th>MENX Mean (CI)</th>
<th>OVX Mean (CI)</th>
<th>P (vs. NOC)</th>
<th>O+M Mean (CI)</th>
<th>P (vs. NOC)</th>
<th>P (vs. MENX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTIMAL HYPERPLASIA</td>
<td>0.83 (0.35 - 1.32)</td>
<td>0.67 (0.42 - 0.92)</td>
<td>0.54 (0.26 - 0.82)</td>
<td>-</td>
<td>0.67 (0.39 - 0.95)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CELL INFILTRATION</td>
<td>0.29 (0.01 - 0.57)</td>
<td>0.29 (0.04 - 0.55)</td>
<td>0.25 (0.00 - 0.51)</td>
<td>-</td>
<td>0.58 (0.35 - 0.82)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SUBINTIMAL FIBROSIS</td>
<td>1.63 (1.26 - 1.99)</td>
<td>2.67 (2.45 - 2.89)</td>
<td>0.83 (0.49 - 1.18)</td>
<td>0.014</td>
<td>1.71 (1.38 - 2.04)</td>
<td>0.300</td>
<td>0.0017</td>
</tr>
<tr>
<td>VASCULARITY</td>
<td>2.96 (2.88 - 3.04)</td>
<td>3.00 (0.00 - 0.00)</td>
<td>2.08 (1.71 - 2.46)</td>
<td>0.002</td>
<td>3.00 (0.00 - 0.00)</td>
<td>0.400</td>
<td>1.000</td>
</tr>
<tr>
<td>AGGREGATE SCORE</td>
<td>5.71 (4.62 - 6.80)</td>
<td>6.63 (6.13 - 7.12)</td>
<td>3.71 (2.65 - 4.77)</td>
<td>0.014</td>
<td>5.96 (5.39 - 6.53)</td>
<td>0.042</td>
<td>0.130</td>
</tr>
</tbody>
</table>

Table 4.8: Microscopic scoring of synovial tissue in ovariectomised joints with and without concurrent OA, compared to controls. MENX data is also presented for comparison.

![Microscopic Scoring of Synovial Tissue in Ovine Knee Joints](image)

Figure 4.19: Charted data for microscopic scoring of synovial tissue, examining the effect of ovariectomy in joints with and without concurrent OA (O+M).
4.4 DISCUSSION

4.4.1 Ovariectomy

Gross morphological examination of the knee joints of the ovariectomised and NOC sheep found no statistical difference between the groups for either cartilage degradation or osteophyte formation. This was also the case when the articular cartilages were viewed on histological section. These findings indicate that ovariectomy alone does not appear to have any adverse affect on the cartilaginous surfaces of the knee joint, within the experimental time frame of this study. It was also interesting to note, that the synovium of the OVX group actually scored better than the NOC group for fibrosis and vascularity. These findings are in contrast to those of a number of other studies, that found ovariectomy induced oestrogen deficiency had a detrimental effect on cartilage, synovium and subchondral bone \cite{408, 409, 441-444}. It should be noted that some of these studies were performed using small animals such as rats or rabbits, and the disease progression may differ in these models. Furthermore, the findings of the present study do not fit with the body of evidence that shows an increase in the incidence of OA in females associated with the onset menopause, and may represent an anomaly. An alternative explanation is that the six-month experimental period was not a long enough for the development of perceivable arthritic changes in a large animal model following the decrease in oestrogen.

Results of the preliminary molecular study showed that ovariectomy altered the expression of key structural genes similarly in both the ACL and PCL tissue. The only notable difference between the two ligaments was that ACL exhibited even lower decorin expression that PCL in the ovariectomised groups. As the two ligaments were so similar in their expression profiles, it was determined that the main study should
focus on further investigating the ACL, which is the more frequently injured \[85, 445\], and macroscopically has been shown to be more severely degenerated than the PCL in OA \[96\].

In the ACLs, molecular findings for OVX vs. NOC sheep were comparable in the preliminary and main studies, with analysis in both studies showing the same molecular changes in OVX ligament, involving some of the main structural components of the ECM. Collagen types I & III were significantly down-regulated, and as these two collagens comprise 70-80% of the dry weight of ligament \[11, 25\] this has the potential to significantly alter ACL collagen fibril structure, and effect a change in ACL biomechanical function post-menopause. These findings are consistent with results of other studies using an animal OVX model to examine the effects of oestrogen deficiency on connective tissues such as bone \[393\], cartilage \[410, 441\], and uterine tissue \[349\].

Furthermore, there was a significant decrease in fibromodulin and decorin, which bind to different sites on collagen I fibrils and are involved in regulating the normal progression of fibrils through the stages of assembly and growth to maturity \[446\]. They also assist in the organisation of the collagen network, acting to regulate interfibrillar spacing and prevent lateral fusion of fibrils \[34, 53\]. The reduction in fibromodulin expression is noteworthy as a lack of fibromodulin has been associated with increase osteoarthritis in the knees of mice \[54\]. Gill and Oldberg (2002) \[54\] found fibromodulin deficient mice have a higher propensity towards degenerative changes in their knee joints than comparable wild type animals although this was not attributed to underlying ultra-structural or fibril abnormalities within the articular cartilage. They concluded that alterations in ligaments, and possibly other tissues within the knee, are of considerable importance in the pathogenesis of the observed articular cartilage degeneration, a conclusion supported by this ligament study. Furthermore, in addition to the reduction
in fibromodulin and decorin, results of the main study showed a reduction in the expression of elastin (not tested in the preliminary study). This may decrease the ability of the ligament to stretch adequately when subjected to biomechanical forces and in turn may contribute to an increased risk of ACL injury.

These molecular findings complement structural changes observed with TEM. Decorin deficiency was shown to promote fibrils which had a greater size range, with a tendency towards smaller fibrils, and an irregular fibre arrangement in MENX sheep (Chapter 3). In OVX sheep, the deficiency of decorin in combination with decreased fibromodulin expression had an even greater effect on ACL collagen fibril structure. They had a significantly smaller average fibril diameter than NOC with a lower fibril size range, and were much more haphazard in their arrangement exhibiting increased spacing between fibrils. A number of authors made similar findings in studies of fibromodulin deficient mice, discovering fibrils with irregular profiles, increased interfibrillar spacing and a greater frequency of small diameter fibrils [49, 50, 52, 53, 446, 447].

In some fibromodulin knockout studies, a deficiency in fibromodulin was associated with a significant increase in lumican [53, 446, 447], however this was not observed in the OVX sheep. Interestingly, in contrast to fibromodulin, lumican knockout mice were found to produce fibrils that were much larger in size, indicating that these two SLRPs work in co-ordination to modulate different phases of fibrillogenesis, while sharing the same collagen I binding site [53, 448]. While lumican is expressed in the early development period during fibril assembly, fibromodulin is expressed later, promoting the maturation of collagen fibrils and their growth from a small to large diameter [50, 52, 447]. Therefore in the absence of fibromodulin, there is prevalence of small sized fibrils, reflecting the
appearance of the OVX ACL, presumably due to failure to progress through the stages of assembly and growth to maturation. Other studies have also attributed the presence of small diameter fibrils in the absence of fibromodulin to a failure of intermediate fibrils to mature, and found it resulted in tissue with reduced strength and aberrant healing \footnote{449, 450}.

In the absence of fibromodulin, an up-regulation of lumican may represent a compensatory response which attempts to maintain normal fibrillogenesis, however studies have found collagen fibrils still exhibit abnormal morphology \footnote{446}. Fibrillogenesis is a finely regulated process in which each SLRP has a specific role to play, and while lumican can fulfil some of the roles of fibromodulin, it has a very different overall function \footnote{446}. When Hedbom \textit{et al.} (1993) \footnote{451} identified that decorin and fibromodulin are structurally related, share some common functions and have a similar affinity for collagen I via separate binding sites, it seemed reasonable to assume that these two SLRPs may be able to compensate for each other. This assumption however was proven incorrect by Svensson \textit{et al.} (1999) \footnote{446}, who showed that decorin expression was unchanged in their study of fibromodulin-null mice, and Forslund \textit{et al.} (2002) \footnote{452}, found that the two proteins failed to substitute for each other in their study of tendon mechanics in knockout mice. Furthermore, Forslund \textit{et al.} found a lack of both fibromodulin and decorin, inhibited fibril maturation in regards to both strength and stiffness.

As OVX sheep exhibited a deficiency in both fibromodulin and decorin in ACL tissue, and significant structural changes (as observed via TEM), normal biomechanical function may be compromised in these ligaments, potentially predisposing them to
injury and/or joint instability. The observation of these ligament changes in the absence of cartilage pathology suggests that disruption of the ligaments could be a primary event in the pathogenesis of OA. Further investigation should focus on assessing the physical properties of OVX ACL compared to NOC. In addition, if these molecular changes are present in both cruciate ligaments as the preliminary study suggests, the potential for disruption of joint stability would be even greater.

At a biochemical level, there was a significant decrease in OVX ACL collagen and S-GAG content compared to NOC sheep. This fits with expected results based on the molecular findings, and suggests that mRNA expression may correspond to actual protein levels, although immunohistochemical analysis is required to confirm this. These results do raise an interesting question however. If OVX sheep exhibit a decrease in both S-GAG and collagen content, as well as a decrease in collagen fibril area, what remains in the space that exists between fibrils? One possibility is fibrillin, a glycoprotein which is involved in the formation of elastic fibres in connective tissues \cite{453}, and which has been shown to be abundant in canine cruciate ligaments \cite{454}. Further analysis is required to confirm what other substances comprise the extracellular matrix of OVX ligament tissue in the absence of collagen and S-GAG.

*Normalising Molecular Data to a Housekeeping Gene:* As outlined in Chapter 3, there is some doubt regarding the suitability of β-actin as a stably expressed housekeeping gene to which results could be normalised. As β-actin showed the least variability of the commonly housekeeping genes tested in this study, for completeness normalised results are presented in Appendix A4.1.2 (page 245), however they do not fit with findings of the biochemical and TEM analysis.
4.4.2 Combined Ovariectomy and Meniscectomy

Macroscopic examination of the articular surfaces of the femur and tibia revealed that the O+M joints had greater cartilage erosion and osteophyte formation than the NOC group. The severity was similar to that observed in the MENX group. When the O+M joints were viewed on histological section, similar OA changes were noted. The femurs of the O+M group displayed worsening of the cartilage structure and increased cloning and tidemark scores. The tibial surfaces in O+M joints scored higher for all OA parameters, with significantly higher total joints scores overall compared to NOC. The synovial changes were less marked, however the O+M group did receive a higher aggregate score than NOC. These findings indicate that the meniscectomy model was successful in inducing OA in the O+M group.

In contrast to the findings of OVX sheep and MENX sheep, the combination of ovariectomy and concurrent osteoarthritis (meniscectomy) showed a very different pattern of disruption to either treatment alone. Molecular results for O+M sheep showed a significant increase in the expression of collagen types I and III. This combined with the up-regulation of MMP-1 may indicate an increase in collagen turnover, and is characteristic of a healing or remodelling response in ligament [455]. In support of this, Ireland et al. (2001) [456] found significantly increased expression of type I collagen mRNA in degenerate samples of human Achilles tendon tissue, and Jarvinen et al. (1997) [457] found increased amounts of type I and III collagen, and proteoglycans in tendon samples with chronic tendonitis. In addition, Provenzano et al. (2005) [9] showed sub-failure injury in ligament coincides with up-regulation of proteoglycan and type I and III collagen, indicating fibrillogenesis is occurring to remodel the tissue and restore its mechanical properties. Corps et al. (2004) [458] also found an 8-fold increase
in the expression of type I collagen in Achilles tendons with tendinopathies, while Bramono et al. (2005) [47] found collagens I and III and MMP-I were increased during the healing of injured human ACL tissue.

Changes in proteoglycan expression were also very different in O+M sheep than in either MENX or OVX when compared to NOC, with analysis showing a significant increase in lumican and biglycan expression. Lumican is responsible for regulating the assembly of collagens into higher order fibrils [50] and its expression is found to be significantly increased in healing tissue [45]. It has also been shown that lumican is up-regulated in some disease processes where there is a high cell turnover, such as in the growth and metastasis of some cancers [50, 459, 460].

Like lumican, biglycan also tends to be up-regulated in healing tissue [45, 47] and both SLRPs are able to modulate cell growth, cell adhesion and migration following injury [38, 461]. Plaas et al. (2000) showed that biglycan accumulated in rabbit MCL following injury and remained elevated in the repairing ligament for over two years [36]. Ordinarily decorin is more abundant in ligament, however during healing biglycan is expressed in higher concentrations [45, 47]. The fact that expression of these two proteoglycans was increased in O+M sheep combined with the increased collagen turnover and up-regulation of MMP-I indicates that there is some disruption to the normal metabolism of the ligament and may lead to a disruption in the normal functional properties of the tissue.

TEM analysis also revealed that O+M sheep displayed a very different fibril configuration than was seen in either the OVX or MENX groups. Calculations revealed the O+M group had a significantly greater fibril density (as a % of total field) than the
NOC group, yet there was no significant difference between the O+M and the NOC groups for average fibril diameter. This result does not reflect the abnormal bimodal fibril size distribution with unusually small and large fibrils, or the irregular fibril arrangement. It is not clear precisely what causes this fibril pattern, but it may be the combined result of increased collagen metabolism and disruption to the finely coordinated process of fibrillogenesis.

SEM examination of both the OVX and O+M groups showed the same striations that were prominent on the MENX samples but were not as obvious on the NOC samples. Again it is unclear what causes these striations but they may reflect a disruption to normal ligament structure.

Results of the biochemical analysis showed there was a significant increase in the collagen content of O+M sheep compared to NOC. This result is expected because although the large and small sized fibrils averaged to be the same size as the more consistent NOC fibrils, the O+M group had a greater fibril density than the NOC sheep. Results for S-GAG content however differed from expected in the O+M group. While it was shown that mRNA expression of lumican and biglycan were increased in these sheep compared to controls, it was not reflected by an increase in S-GAG content at a biochemical level. This may be due to a number of factors. Firstly, the abnormally increased mRNA expression of these SLRPS may not translate to actual protein levels in this tissue, which would need to be confirmed via immunohistochemistry. Secondly, the amount of S-GAG bound to the SLRPs may be reduced which could compromise their normal function and predispose them and their associated collagen fibrils to cleavage by degradative enzymes. Thirdly, the increase in lumican and biglycan could be countered by a decrease in some other SLRP which was not revealed in this study.
In conclusion, the effects of OVX, MENX and combined O+M on ligament tissue are varied and complex. The combination of ovariectomy and concurrent osteoarthritis (meniscectomy) showed a very different pattern of derangement to either treatment alone which may indicate a synergistic effect resulting in over-expression of certain genes with an equally disruptive effect on the normal structural arrangement of the ligament (as highlighted by the abnormal bimodal distribution of fibrils). While these changes in gene expression and collagen structure have been observed, further biomechanical tests are required to elucidate what affect they have on the tensile strength of the tissue.
CHAPTER 5

REVIEW OF HYPOTHESES & CONCLUSIONS

_Hypothesis 1:_ that osteoarthritis (meniscectomy) alters gene expression and collagen fibril structure and organisation in ACL.

In order to prove this hypothesis, it must first be verified that meniscectomy did indeed induce osteoarthritis in the joints of the MENX sheep. Gross morphology and histopathology results confirmed the presence of cartilage changes consistent with OA. In addition, subintimal fibrosis of the synovial tissue, a feature characteristic of chronic synovitis associated with OA \[139, 245\], was significantly greater in the MENX sheep compared to the non-operated controls. These results demonstrate that osteoarthritis was present in the knees of the MENX sheep.

Having established the presence of OA, the hypothesis that osteoarthritis alters gene expression and collagen fibril structure and organisation in ACL can be addressed. Gene expression of decorin and elastin, two major structural components of ligament, were significantly reduced in the ACL of MENX sheep. As these molecules are fundamental to the development of normal ligament structure and integrity, their deficiency leads to the formation of tissue that is structurally abnormal. Furthermore, expression of the collagenase MMP-1 was increased, which is typical of OA, and is a clear indication that tissue remodelling was occurring in the ligament \[462, 463\]. Biochemical analysis showed that the content of both collagen and S-GAG were significantly reduced in ACL in the presence of OA. These changes in gene expression, and the reduction in the content of structural molecules in ACL correspond to changes in the structural appearance of the collagen fibrils. TEM and SEM analysis, and
histological sections of ACL from MENX sheep displayed an abnormal fibril size profile and a pattern of arrangement that differed greatly from that seen in the ACLs of non-operated sheep. Therefore, from this collective data, it can be concluded that the above hypothesis was proven to be correct.

**Hypothesis 2:** animals with worse osteoarthritic changes have more severe ligament derangement.

From the results of the regression analysis comparing osteoarthritic changes (as determined by cartilage degradation score), and ligament derangement (indicated by decorin and elastin expression) within individuals, it was not possible to prove or disprove this hypothesis. A major influencing factor is the reliability of the meniscectomy model, which generated a consistent grade of OA across the study group. This dependability of this model in generating OA is shown repeatedly in studies of knee pathology in mice and sheep \(^{245, 247}\). Due to this, there was inadequate variation in the degree of OA amongst individuals in the MENX group to relate to differences in the composition of the ligament. Further information may be gained by examining the relationship between variables (ligament and cartilage changes) within individuals at different time-points. It may be that variation between individuals in the progression of OA is more apparent in the earlier stages, but becomes less marked as the pathogenesis continues.
Hypothesis 3: that the decrease in ovarian hormone levels associated with menopause (ovariectomy) alters the normal composition and organisation of cruciate ligament tissue, in particular collagen fibril structure, which has the potential to influence the mechanical properties of the ligament, as observed in other connective tissue types post-menopause.

The results of the ligament studies from OVX sheep presented in Chapter 4, support this hypothesis. Molecular analysis revealed significant reductions in the expression of the main structural proteins of ligament, collagen types I & III, which constitute the majority of the tissue substance. There were also significant reductions in the expression of multiple key SLRPs which are integral to normal collagen fibril formation and organisation of the collagen matrix, and elastin which provides ligament with the ability to undergo a degree of deformation without rupturing or tearing (elastic deformation) [464]. These findings (except elastin which was only tested in the main study) were repeatable in two separate studies, and were present at both six and nine month time-points post ovariectomy. Molecular results were corroborated by biochemical assays measuring the collagen and S-GAG (SLRP) content of the ACL from OVX sheep, which showed levels were significantly reduced compared to non-operated controls.

TEM, SEM and histological examination provided confirmation that these molecular and biochemical results were associated with physical changes in ligament structure and organisation. The collagen fibrils of ACL from OVX sheep were markedly narrower in diameter than non-operated controls, and much more haphazard in their arrangement. This was consistent with histological sections of the same ligament, which showed irregularly orientated fibre bundles, and a reduction in the density of elastin fibres. Furthermore, SEM examination revealed that OVX ligament fibre bundles had an unusual striated appearance and were much more loosely packed than those of non-operated controls. Changes in the composition and organisation of ligament as a result of
ovariectomy would be expected to result in alteration of the normal mechanical properties of the tissue.

In 2011 J. Gilmore, a member of our research group, conducted a study into the effects of ovariectomy on the biomechanical properties of the ovine stifle joint \(^{465}\). The results of this study provided some insight into what effect the ligament changes observed in the present study may have on the biomechanical function of the knee joint (see Appendix 5 for results). The study utilised an Instron 5566 universal mechanical test frame to assess intact knee joints, and generate force displacement plots, from which posterior and anterior drawer were calculated, and ligament stiffness gradients determined. Results showed no difference between OVX and NOC knee joints for anterior drawer displacement, however posterior drawer displacement and total displacement were significantly reduced in OVX knee joints. This corresponded to an increase in ligament stiffness during posterior drawer testing. These findings do not fit with the theory that the structural changes observed in the present study could lead to increased ligament laxity, which would increase the risk of developing knee OA. It is difficult to draw definitive conclusions about the effect of OVX on specific ligaments from this test as multiple other joint structures are involved, which may influence the results. However, an increase in ligament stiffness would have the potential to disrupt normal joint function. The present study showed that ovariectomy was associated with a decrease in elastin expression, which would reduce the ability of the ligament to deform under strain. This could predispose the ligament to multiple micro-ruptures, which over time could lead to degeneration and biomechanical failure of the ligament. This biomechanical study showed that OVX is associated with significant alteration in the biomechanical properties of the knee joint, however to further clarify the involvement of the cruciate ligaments in the mechanism of this alteration, additional studies should investigate these structures more specifically.
It is therefore concluded that the evidence presented here supports the hypothesis that ovariectomy alters the normal composition and organisation of cruciate ligament tissue, in particular collagen fibril structure, which has the potential to influence the mechanical properties of the ligament. Further biomechanical studies would be required to define the exact nature of these changes.

**Hypothesis 4:** that the combined effects of ovariectomy and meniscectomy have an even greater influence on ligament composition and structure than either meniscectomy or ovariectomy alone, and that these changes reflect those of postmenopausal women with concurrent osteoarthritis.

The combination of ovariectomy and meniscectomy resulted in ACLs with a markedly disturbed gene expression profile compared to ligaments of non-operated controls, and that differed considerably from ACLs of either single treatment group (meniscectomy or ovariectomy alone). When compared to those of NOC sheep, ACLs from the O+M group showed a general increase in the expression of major structural proteins (collagen types I and III) and up-regulation of MMP-1, indicating an increase in collagen turnover \[47, 466, 467\]. This result, combined with the increased expression of proteoglycans biglycan and lumican, is indicative of an attempted healing or remodelling response in ligament \[36, 46\]. In contrast, the MENX and OVX groups exhibited a general decrease in collagen and proteoglycan expression.

In addition, microscopic analysis revealed a marked disturbance in normal collagen organisation. TEM examination of O+M ACL showed increased numbers of abnormally large and small collagen fibrils (bimodal size distribution), which were irregular in their arrangement.
While it is clear from these results that O+M ACL differs from that of non-operated controls in regard to composition and structure, it is difficult to determine whether the combination of ovariectomy and meniscectomy exerts a greater influence than either treatment alone. Because the pattern of derangement resulting from the combined treatment is not simply a worsening of the effects of the single treatment groups, it is not clear whether the influence is lesser or greater, it is simply different. To determine which treatment (OVX, MENX or O+M) has the greatest influence on ligament composition and structure, it would be valuable to compare ligament function between groups, utilising biomechanical studies and load to failure testing.

Regarding whether or not these changes reflect those of postmenopausal women with concurrent osteoarthritis, it is reasonable to assume that they do. The meniscectomy model successfully induces OA in sheep knee joints, as confirmed by the cartilage results, and the surgical removal of the ovaries induces a deficiency in ovarian hormone levels, that mimics the cessation of ovarian function at menopause. To definitively affirm that the changes observed in sheep ligament accurately reflect those of postmenopausal women with OA, it would be necessary to conduct similar tests in human ACL samples. Difficulty may arise however, in obtaining sufficient sample numbers and ensuring adequate control of variables within sample groups.

REVIEW OF THE MAJOR FINDINGS AND STATISTICAL METHODOLOGY

As stated in Chapter 2, the experimental design of the experiments in this study involved harvesting the ACL from both knee joints from each sheep and subjecting them to the same array of analyses. This represents a potential weakness in
the power of the statistical analysis of the results. Whilst it can be argued that the response of each knee joint to meniscectomy is an individual event, and unaffected by the surgical procedure on the contralateral limb, this argument cannot be made for the effect of ovariectomy. The reasons behind this decision have been outlined in Chapter 2 in the presentation of statistical methods, and related to economic factors as well as the need for large volumes of tissue to complete the multiple analyses planned on each ACL. In retrospect, separating the analyses into two groups and allocating biochemistry and molecular studies to the left ACL and microscopy (TEM, SEM and histopathology) to the right ACL may have been preferable.

While acknowledging this weakness in the statistical analysis, the consistency of the results across the various experimental methods used in this study provides strong support for the interpretation that the tissue of the ACL responds to both the presence of OA and the absence of ovarian hormones in a consistent manner in which collagen fibrillogenesis is disturbed resulting in structural change in the ligament. This finding adds significantly to current knowledge of knee joint function in response to life-stage changes. Repeating the experiments conducted in this study with a larger number of animals to increase the power of the statistical analysis was beyond the financial resources of this project. Given the consistency of the results of these experiments, such a study would be unlikely to add substantially to the results of the current study or their interpretation.

This prediction is supported by limited additional statistical analysis performed retrospectively. A number of key results were re-analysed using the same statistical methods but pooling the results from the two limbs of individual sheep, thus halving the
number of samples in the analysis. In all cases, the resultant p value was virtually identical to that in the original analysis.

Another limitation of this study is that it utilises a single time point for analysis. It would certainly strengthen the findings of the study if larger (n) numbers were used and if repeat test groups at a second time point were added, however the cost of surgery, housing the sheep and performing the analyses was excessive. The time it would require to duplicate the entire study at a second time point was also prohibitive.

CONCLUSIONS

Results of this study suggest that the inflammatory milieu that exists within an osteoarthritic knee joint may have an effect on the expression of key structural genes and the organisation of the ACL microstructure. The ACL itself did not show an increase in the expression of inflammatory mediators, however it would have been exposed to cytokines present within the joint capsule that originated from other soft tissues such cartilage and synovium. Biomechanical changes in loading and joint stability would have provided further stimulus to the response seen in the ACL tissue. As a result of these factors, the organisation of ACL microstructure was diminished, and the expression of important ligament components was reduced.

To further this research, it would be valuable to perform similar studies of joint structures located outside the joint capsule, such as the LCL or MCL. These structures are separated from the internal inflammatory environment by the joint capsule, and therefore by comparing their results to those of the ACLs it could provide further information about the direct influence of cytokines. Additional studies could also be
performed in wethers to determine if similar ligament changes occur with a decrease in male gonadal hormones.

Of notable interest were the findings of ACL studies from ovariectomised sheep. These ligaments exhibited an even greater disruption to normal structural organisation and expression of major ligament components than those of the meniscectomised sheep. Currently there is a volume of research describing the effect of oestrogen deficiency on a number of connective tissues such as bone, skin, cartilage and blood vessels, but there is little information available about its effects on ligament, particularly in human subjects. This study provides some insight, by demonstrating that a reduction in ovarian hormones has an influence on the organisation and composition of ligament. This statement is supported by molecular, biochemical and microscopic analyses. To confirm that gene expression relates to an actual reduction in protein production, it would be of value to perform immunohistochemistry studies on ACL samples from ovariectomised sheep.

Contrary to expectations, the combination of ovariectomy and meniscectomy in sheep produced a pattern of collagen fibril derangement and a gene expression profile that was notably different from the single treatment groups. It was anticipated that combining the treatments would exacerbate the effects of either single treatment, but this was not reflected in the results. Normal structure and composition was indeed disrupted but it was due to an increase in expression of fundamental genes. Furthermore, the appearance of the collagen fibrils was significantly different with an unusual bimodal size distribution. This indicates that the modulation of normal fibril size was disrupted, with the multiple small fibrils suggestive of an increase in immature fibrils (due to more
rapid ligament turnover). While these ligaments were not the same as those of the single treatment groups, they were clearly not representative of normal ACL. The two treatments combined seem to have exerted a synergistic effect, producing results that were dissimilar to either treatment alone. Previous studies into the combined effect of prior OVX and subsequent MENX in sheep showed a similar response in the knee joint, in which OVX resulted in thinning of the subchondral bone plate which was not exacerbated by subsequent MENX \[468\].

Finally, to complete the picture, it is important to confirm a link between the observed structural and compositional changes described in these studies, and the physical function of the ligaments. Further investigation should focus on examining the biomechanical performance and load to failure strength of ACL from similar experimental groups.
REFERENCES


233. Roos, H., Exercise, knee injury and osteoarthritis. 1994, University of Lund Sweden


APPENDIX 1  Rotor-Gene Analysis Reports & Profile Settings

Quantitation Report

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## APPENDIX 2  LIST OF PRIMER SEQUENCES USED IN RT-PCR

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Table A2.1  List of Ovis aries & Bos taurus primer sequences utilised for RT-PCR analysis of ligament in this study.
APPENDIX 3 BIOCHEMICAL ASSAY REPORT

Statistics DLL Version 4.24
File Name gp ACL collagen 1.40.TXT
File Format Table
File Time 26/01/2010 4:54:47 PM

[Assay]
Assay title COLLAGEN AD
Read Time 26/01/2010 4:54:22 PM
Operator AD
Kit Lot Data
Plate Lot Data
Wells A1 - D9
OD RESULTS Units OD
CURVE FITTING equation Lin/Lin Linear regression with data extrapolation
R-Squared 0.9998 Units ug/well

\[ y = 0.5845x + 0.054 \]
\[ R^2 = 0.9998 \]
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End of results
A4.1 MOLECULAR BIOLOGY

A4.1.1 Preliminary OVX Study: RT-PCR Results

A4.1.1.1 ACL & PCL target gene expression for NOC1 and OVX1 groups

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Table A4.1: Results for the preliminary study OVX1 vs. NOC1 for ACL and PCL, showing mean RT-PCR expression and standard error (SE) for each target gene. Results presented are from total RNA data.
Figure A4.1: Collagen type I total RNA expression for NOC1 vs. OVX1 groups, ACL & PCL.

Figure A4.2: Collagen type II total RNA expression for NOC1 vs. OVX1 groups, ACL & PCL.

Figure A4.3: Collagen type III total RNA expression for NOC1 vs. OVX1 groups, ACL & PCL.

Figure A4.4: Decorin total RNA expression for NOC1 vs. OVX1 groups, ACL & PCL.
Figure A4.5: Fibromodulin total RNA expression for NOC1 vs. OXV1 groups, ACL & PCL.

Figure A4.6: Biglycan total RNA expression for NOC1 vs. OXV1 groups, ACL & PCL.

Figure A4.7: GAPDH total RNA expression for NOC1 vs. OXV1 groups, ACL & PCL. The expression of this gene was constant enough to serve as a housekeeping gene.
### A4.1.1.2 Statistical analysis data for OVX1 vs. NOC, ACL & PCL

**RT-PCR STATISTICAL DATA - PRELIMINARY OVX STUDY**

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<td>0.010</td>
<td>0.810</td>
<td>0.123</td>
</tr>
<tr>
<td>BIGLYCAN</td>
<td>0.602</td>
<td>0.654</td>
<td>0.590</td>
<td>0.639</td>
</tr>
</tbody>
</table>

*P value (5% level of significance)*

Table A4.2: RT-PCR statistical analysis (T-test) figures for NOC1 and OVX1 sheep ACL & PCL samples, calculated from total RNA results at a significance level of 5%.
### A4.1.2 Main Study: RT-PCR Results

#### A4.1.2.1 ACL target gene expression for all study groups

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>AGGRECAN</th>
<th>BASIC FGF</th>
<th>BIGLYCAN</th>
<th>COLLAGEN TYPE I</th>
<th>COLLAGEN TYPE II</th>
<th>COLLAGEN TYPE III</th>
<th>CTGF</th>
<th>DECORIN</th>
<th>ELASTIN</th>
<th>FIBROMODULIN</th>
<th>INOS</th>
<th>LUBRICIN</th>
<th>LUMICAN</th>
<th>MMP-1</th>
<th>PDGF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NOC</strong></td>
<td>279.3 (207.6)</td>
<td>329.9 (97.8)</td>
<td>164.33 (36.0)</td>
<td>65.1 (18.6)</td>
<td>355.2 (190.3)</td>
<td>34.8 (6.4)</td>
<td>232.2 (43.6)</td>
<td>345.4 (58.3)</td>
<td>579.5 (141.8)</td>
<td>298.7 (70.2)</td>
<td>338.9 (115.3)</td>
<td>219.3 (85.4)</td>
<td>219.3 (85.4)</td>
<td>35.2 (12.1)</td>
<td>138.4 (50.9)</td>
</tr>
<tr>
<td><strong>MENX</strong></td>
<td>130.6 (44.1)</td>
<td>164.7 (21.9)</td>
<td>165.3 (33.0)</td>
<td>48.4 (5.8)</td>
<td>252.0 (85.6)</td>
<td>36.0 (5.7)</td>
<td>131.5 (30.5)</td>
<td>150.1 (20.9)</td>
<td>234.5 (33.7)</td>
<td>209.4 (30.4)</td>
<td>266.9 (90.0)</td>
<td>100.2 (15.5)</td>
<td>100.2 (15.5)</td>
<td>123.1 (28.7)</td>
<td>95.5 (15.8)</td>
</tr>
<tr>
<td><strong>OVX</strong></td>
<td>66.2 (17.3)</td>
<td>155.3 (22.8)</td>
<td>104.2 (23.3)</td>
<td>16.4 (3.3)</td>
<td>23.8 (6.7)</td>
<td>14.4 (3.3)</td>
<td>152.7 (31.0)</td>
<td>102.7 (24.6)</td>
<td>236.2 (27.6)</td>
<td>94.7 (13.9)</td>
<td>258.6 (89.7)</td>
<td>152.9 (19.7)</td>
<td>152.9 (19.7)</td>
<td>42.4 (21.2)</td>
<td>77.0 (16.7)</td>
</tr>
<tr>
<td><strong>O+M</strong></td>
<td>420.6 (122.2)</td>
<td>523.3 (76.9)</td>
<td>749.6 (168.3)</td>
<td>477.0 (131.0)</td>
<td>1167.4 (501.8)</td>
<td>466.5 (123.1)</td>
<td>361.8 (74.7)</td>
<td>699.1 (146.3)</td>
<td>667.7 (171.8)</td>
<td>900.0 (217.1)</td>
<td>240.0 (76.0)</td>
<td>217.7 (29.6)</td>
<td>217.7 (29.6)</td>
<td>224.9 (76.7)</td>
<td>268.4 (53.3)</td>
</tr>
</tbody>
</table>

**Table A4.3:** Collective results for all groups, showing mean RT-PCR expression and standard error (SE) for each target gene. Results presented are from total RNA data. Values represent an expression copy number.
<table>
<thead>
<tr>
<th>AGGREGAN</th>
<th>BASIC FGF</th>
<th>BIGLYCAN</th>
<th>COLLAGEN TYPE I</th>
<th>COLLAGEN TYPE II</th>
<th>COLLAGEN TYPE III</th>
<th>CTFG</th>
<th>DECORIN</th>
<th>ELASTIN</th>
<th>FIBROMODULIN</th>
<th>INOS</th>
<th>LUBRICIN</th>
<th>LUMICAN</th>
<th>MMP-1</th>
<th>PDGF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NOC</strong></td>
<td>2.50</td>
<td>0.00</td>
<td>1.77</td>
<td>0.70</td>
<td>3.54</td>
<td>3.90</td>
<td>2.65</td>
<td>4.28</td>
<td>6.28</td>
<td>3.17</td>
<td>3.58</td>
<td>2.47</td>
<td>2.43</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>(1.0)</td>
<td>(0.2)</td>
<td>(0.2)</td>
<td>(0.6)</td>
<td>(1.4)</td>
<td>(0.06)</td>
<td>(0.4)</td>
<td>(0.7)</td>
<td>(1.3)</td>
<td>(0.3)</td>
<td>(1.2)</td>
<td>(0.6)</td>
<td>(0.7)</td>
<td>(0.1)</td>
</tr>
<tr>
<td><strong>MENX</strong></td>
<td>2.08</td>
<td>0.00</td>
<td>1.31</td>
<td>0.68</td>
<td>4.09</td>
<td>0.48</td>
<td>1.73</td>
<td>2.13</td>
<td>3.22</td>
<td>3.12</td>
<td>3.74</td>
<td>1.41</td>
<td>2.18</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td>(0.8)</td>
<td>(0.3)</td>
<td>(0.3)</td>
<td>(0.1)</td>
<td>(1.6)</td>
<td>(0.05)</td>
<td>(0.3)</td>
<td>(0.3)</td>
<td>(0.3)</td>
<td>(0.7)</td>
<td>(1.3)</td>
<td>(0.2)</td>
<td>(0.5)</td>
<td>(0.3)</td>
</tr>
<tr>
<td><strong>OVX</strong></td>
<td>1.77</td>
<td>0.00</td>
<td>2.38</td>
<td>0.41</td>
<td>0.69</td>
<td>0.32</td>
<td>4.50</td>
<td>2.78</td>
<td>6.95</td>
<td>2.57</td>
<td>9.18</td>
<td>4.79</td>
<td>2.37</td>
<td>1.57</td>
</tr>
<tr>
<td></td>
<td>(0.6)</td>
<td>(0.9)</td>
<td>(0.2)</td>
<td>(0.1)</td>
<td>(0.3)</td>
<td>(0.03)</td>
<td>(1.1)</td>
<td>(0.7)</td>
<td>(1.2)</td>
<td>(0.5)</td>
<td>(4.6)</td>
<td>(1.2)</td>
<td>(0.3)</td>
<td>(0.8)</td>
</tr>
<tr>
<td><strong>O+M</strong></td>
<td>1.86</td>
<td>0.00</td>
<td>2.46</td>
<td>1.55</td>
<td>2.71</td>
<td>0.36</td>
<td>1.33</td>
<td>2.29</td>
<td>2.19</td>
<td>2.70</td>
<td>3.88</td>
<td>0.65</td>
<td>1.70</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td>(0.7)</td>
<td>(0.4)</td>
<td>(0.2)</td>
<td>(0.4)</td>
<td>(0.8)</td>
<td>(0.19)</td>
<td>(0.2)</td>
<td>(0.2)</td>
<td>(0.4)</td>
<td>(0.2)</td>
<td>(2.4)</td>
<td>(0.2)</td>
<td>(0.2)</td>
<td>(0.3)</td>
</tr>
</tbody>
</table>

Table A4.4: Collective results for all groups, showing mean RT-PCR expression and standard error (SE) for each target gene listed. Results are shown normalised to β-actin. Values represent an expression copy number.
Figure A4.8: Aggrecan total RNA expression for all study groups. (NOC = non-operated control; O/M = ovariectomy + meniscectomy; M = meniscectomy; O = ovariectomy).

Figure A4.9: Aggrecan expression normalised to β-actin for all study groups. (NOC = non-operated control; O/M = ovariectomy + meniscectomy; M = meniscectomy; O = ovariectomy).

Figure A4.10: Basic FGF total RNA expression for all study groups.

Figure A4.11: Basic FGF expression normalised to β-actin for all study groups.
Figure A4.12: Biglycan total RNA expression for all study groups.

Figure A4.13: Biglycan expression normalised to $\beta$-actin for all study groups.

Figure A4.14: Type I collagen total RNA expression for all study groups.

Figure A4.15: Type I collagen expression normalised to $\beta$-actin for all study groups.
Figure A4.16: Type II collagen total RNA expression for all study groups.

Figure A4.17: Type II collagen expression normalised to β-actin for all study groups.

Figure A4.18: Type III collagen total RNA expression for all study groups.

Figure A4.19: Type III collagen expression normalised to β-actin for all study groups.
Figure A4.20: CTGF total RNA expression for all study groups.

Figure A4.21: CTGF expression normalised to β-actin for all study groups.

Figure A4.22: Decorin RNA expression for all study groups.

Figure A4.23: Decorin expression normalised to β-actin for all study groups.
Figure A4.24: Elastin RNA expression for all study groups.

Figure A4.25: Elastin expression normalised to β-actin for all study groups.

Figure A4.26: Fibromodulin RNA expression for all study groups.

Figure A4.27: Fibromodulin expression normalised to β-actin for all study groups.
Figure A4.28: iNOS RNA expression for all study groups.

Figure A4.29: iNOS expression normalised to $\beta$-actin for all study groups.

Figure A4.30: Lubricin RNA expression for all study groups.

Figure A4.31: Lubricin expression normalised to $\beta$-actin for all study groups.
Figure A4.32: Lumican RNA expression for all study groups.

Figure A4.33: Lumican expression normalised to β-actin for all study groups.

Figure A4.34: MMP-1 RNA expression for all study groups.

Figure A4.35: MMP-1 expression normalised to β-actin for all study groups.
Figure A4.36: PDGF RNA expression for all study groups.

Figure A4.37: PDGF expression normalised to β-actin for all study groups.
### A4.1.2.2 Statistical analysis data for all study groups

#### RT-PCR STATISTICAL DATA - NORMALISED TO TOTAL RNA

<table>
<thead>
<tr>
<th>STUDY GROUPS (A vs. B)</th>
<th>AGGRECAN</th>
<th>BASIC FGF</th>
<th>BIGLYCAN</th>
<th>COLLAGEN TYPE I</th>
<th>COLLAGEN TYPE II</th>
<th>COLLAGEN TYPE III</th>
<th>CTGF</th>
<th>DECORIN</th>
<th>ELASTIN</th>
<th>FIBROMODULIN</th>
<th>INOS</th>
<th>LUBRICIN</th>
<th>LUMICAN</th>
<th>MMP-1</th>
<th>PDGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOC vs. MENX</td>
<td>0.436</td>
<td>0.147</td>
<td>0.985</td>
<td>0.439</td>
<td>0.649</td>
<td>0.888</td>
<td>0.084</td>
<td>0.008</td>
<td>0.042</td>
<td>0.289</td>
<td>0.638</td>
<td>0.224</td>
<td>0.700</td>
<td>0.006</td>
<td>0.466</td>
</tr>
<tr>
<td>NOC vs. OVX</td>
<td>0.159</td>
<td>0.096</td>
<td>0.175</td>
<td>0.017</td>
<td>0.096</td>
<td>0.010</td>
<td>0.152</td>
<td>0.001</td>
<td>0.027</td>
<td>0.009</td>
<td>0.588</td>
<td>0.457</td>
<td>0.186</td>
<td>0.765</td>
<td>0.264</td>
</tr>
<tr>
<td>NOC vs. O+M</td>
<td>0.539</td>
<td>0.134</td>
<td>0.003</td>
<td>0.005</td>
<td>0.144</td>
<td>0.002</td>
<td>0.148</td>
<td>0.035</td>
<td>0.696</td>
<td>0.015</td>
<td>0.481</td>
<td>0.985</td>
<td>0.041</td>
<td>0.029</td>
<td>0.092</td>
</tr>
<tr>
<td>MENX vs. OVX</td>
<td>0.137</td>
<td>0.773</td>
<td>0.137</td>
<td>0.0001</td>
<td>0.008</td>
<td>0.003</td>
<td>0.636</td>
<td>0.167</td>
<td>0.970</td>
<td>0.002</td>
<td>0.949</td>
<td>0.055</td>
<td>0.199</td>
<td>0.034</td>
<td>0.436</td>
</tr>
<tr>
<td>O+M vs. OVX</td>
<td>0.007</td>
<td>0.0001</td>
<td>0.002</td>
<td>0.033</td>
<td>0.001</td>
<td>0.017</td>
<td>0.001</td>
<td>0.021</td>
<td>0.001</td>
<td>0.876</td>
<td>0.082</td>
<td>0.005</td>
<td>0.048</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>O+M vs. MENX</td>
<td>0.069</td>
<td>0.001</td>
<td>0.005</td>
<td>0.008</td>
<td>0.116</td>
<td>0.005</td>
<td>0.015</td>
<td>0.003</td>
<td>0.035</td>
<td>0.009</td>
<td>0.820</td>
<td>0.003</td>
<td>0.029</td>
<td>0.310</td>
<td>0.010</td>
</tr>
</tbody>
</table>

Table A4.5: Statistical analysis (T-test) results for total RNA expression of the listed target genes. Significance was calculated at a level of 5%. All significant values are highlighted in yellow.
<table>
<thead>
<tr>
<th>STUDY GROUPS</th>
<th>AGGRECAN</th>
<th>BASIC FGF</th>
<th>BIGLYCAN</th>
<th>COLLAGEN TYPE I</th>
<th>COLLAGEN TYPE II</th>
<th>COLLAGEN TYPE III</th>
<th>CTFG</th>
<th>DECORIN</th>
<th>ELASTIN</th>
<th>FIBROMODULIN</th>
<th>INOS</th>
<th>LUBRICIN</th>
<th>LUMICAN</th>
<th>MMP-1</th>
<th>PDGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOC vs. MENX</td>
<td>0.750</td>
<td>0.114</td>
<td>0.234</td>
<td>0.927</td>
<td>0.793</td>
<td>0.288</td>
<td>0.078</td>
<td>0.020</td>
<td>0.042</td>
<td>0.947</td>
<td>0.927</td>
<td>0.162</td>
<td>0.776</td>
<td>0.001</td>
<td>0.997</td>
</tr>
<tr>
<td>NOC vs. OVX</td>
<td>0.513</td>
<td>0.499</td>
<td>0.054</td>
<td>0.203</td>
<td>0.052</td>
<td>0.293</td>
<td>0.112</td>
<td>0.160</td>
<td>0.703</td>
<td>0.312</td>
<td>0.250</td>
<td>0.094</td>
<td>0.944</td>
<td>0.170</td>
<td>0.091</td>
</tr>
<tr>
<td>NOC vs. O+M</td>
<td>0.607</td>
<td>0.073</td>
<td>0.036</td>
<td>0.064</td>
<td>0.007</td>
<td>&lt;0.0001</td>
<td>0.007</td>
<td>0.017</td>
<td>0.005</td>
<td>0.235</td>
<td>0.063</td>
<td>0.028</td>
<td>0.311</td>
<td>0.298</td>
<td>0.105</td>
</tr>
<tr>
<td>MENX vs. OVX</td>
<td>0.754</td>
<td>0.076</td>
<td>0.921</td>
<td>0.013</td>
<td>0.030</td>
<td>0.013</td>
<td>0.031</td>
<td>0.442</td>
<td>0.011</td>
<td>0.498</td>
<td>0.306</td>
<td>0.018</td>
<td>0.729</td>
<td>0.986</td>
<td>0.076</td>
</tr>
<tr>
<td>O+M vs. OVX</td>
<td>0.925</td>
<td>0.045</td>
<td>0.817</td>
<td>0.008</td>
<td>0.028</td>
<td>&lt;0.0001</td>
<td>0.008</td>
<td>0.520</td>
<td>0.001</td>
<td>0.796</td>
<td>0.090</td>
<td>0.004</td>
<td>0.083</td>
<td>0.343</td>
<td>0.002</td>
</tr>
<tr>
<td>O+M vs. MENX</td>
<td>0.839</td>
<td>0.781</td>
<td>0.798</td>
<td>0.054</td>
<td>0.424</td>
<td>0.0006</td>
<td>0.310</td>
<td>0.672</td>
<td>0.068</td>
<td>0.517</td>
<td>0.045</td>
<td>0.119</td>
<td>0.362</td>
<td>0.077</td>
<td>0.063</td>
</tr>
</tbody>
</table>

Table A4.6: Statistical analysis (T-test) results for RNA expression normalised to B-actin. Significance was calculated at a level of 5%. All significant values are highlighted in yellow.
A4.2 BIOCHEMICAL ANALYSIS

A4.2.1 ACL Collagen Content

A4.2.1.1 Results for all study groups

<table>
<thead>
<tr>
<th>GROUP</th>
<th>NOC Mean</th>
<th>MENX Mean</th>
<th>OVX Mean</th>
<th>O+M Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN</td>
<td>57.16</td>
<td>60.58</td>
<td>53.56</td>
<td>67.3</td>
</tr>
<tr>
<td>SE</td>
<td>1.07</td>
<td>1.39</td>
<td>1.33</td>
<td>2.51</td>
</tr>
</tbody>
</table>

Table A4.7: ACL collagen content - results for all study groups, mean and SE. (Value shown as % of dry weight).

Figure A4.38: ACL collagen content - charted results for all study groups.

A4.2.1.2 Statistical significance for all study groups

<table>
<thead>
<tr>
<th>GROUP X vs. Y</th>
<th>P value (*5% sig.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOC vs. MENX</td>
<td>0.070 *</td>
</tr>
<tr>
<td>NOC vs. OVX</td>
<td>0.039 *</td>
</tr>
<tr>
<td>NOC vs. O+M</td>
<td>0.001 *</td>
</tr>
<tr>
<td>O+M vs. MENX</td>
<td>0.039 *</td>
</tr>
<tr>
<td>O+M vs. OVX</td>
<td>0.0001 *</td>
</tr>
<tr>
<td>MENX vs. OVX</td>
<td>0.002 *</td>
</tr>
</tbody>
</table>

Table A4.8: Listing of statistical differences between all study groups, calculated using a T-test analysis at a significance level of 5%. * denotes significant values.
A4.2.2 ACL S-GAG Content

A4.2.2.1 Results for all study groups

<table>
<thead>
<tr>
<th>GROUP</th>
<th>NOC</th>
<th>MENX</th>
<th>OVX</th>
<th>O+M</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN</td>
<td>11.78</td>
<td>9.35</td>
<td>9.43</td>
<td>10.11</td>
</tr>
<tr>
<td>SE</td>
<td>0.7</td>
<td>0.59</td>
<td>0.4</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Table A4.9: ACL S-GAG content - results for all study groups, mean and SE. (Value shown as % of dry weight).

Figure A4.39: ACL S-GAG – charted results for all study groups.

A4.2.2.2 Statistical significance for all study groups

<table>
<thead>
<tr>
<th>GROUP X vs. Y</th>
<th>P value (*5% sig.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOC MENX</td>
<td>0.012 *</td>
</tr>
<tr>
<td>NOC OVX</td>
<td>0.005 *</td>
</tr>
<tr>
<td>NOC O+M</td>
<td>0.027 *</td>
</tr>
<tr>
<td>O+M MENX</td>
<td>0.249</td>
</tr>
<tr>
<td>O+M OVX</td>
<td>0.196</td>
</tr>
<tr>
<td>MENX OVX</td>
<td>0.907</td>
</tr>
</tbody>
</table>

Table A4.10: Listing of statistical differences between all study groups for S-GAG content, calculated using a T-test analysis at a significance level of 5%. * denotes significant values.
A4.3   TRANSMISSION ELECTRON MICROSCOPY (TEM)

A4.3.1   Average Number of Fibrils

<table>
<thead>
<tr>
<th>GROUP</th>
<th>NOC</th>
<th>MENX</th>
<th>OVX</th>
<th>O+M</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN</td>
<td>77.88</td>
<td>101.75</td>
<td>98.58</td>
<td>94.79</td>
</tr>
<tr>
<td>SE</td>
<td>2.77</td>
<td>7.32</td>
<td>4.94</td>
<td>6.65</td>
</tr>
</tbody>
</table>

Table A4.11: TEM Results for ACL, average number of fibrils, for all study groups, mean and SE.

A4.3.2   Average Fibril Diameter

<table>
<thead>
<tr>
<th>GROUP</th>
<th>NOC</th>
<th>MENX</th>
<th>OVX</th>
<th>O+M</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN</td>
<td>151.05</td>
<td>128.64</td>
<td>96.87</td>
<td>148.14</td>
</tr>
<tr>
<td>SE</td>
<td>2.28</td>
<td>5.15</td>
<td>1.79</td>
<td>5.97</td>
</tr>
</tbody>
</table>

Table A4.12: TEM results for ACL, average fibril diameter (in nanometers), for all study groups, mean and SE.

Figure A4.40: TEM ACL average number of fibrils - charted results for all study groups.

Figure A4.41: TEM ACL average fibril diameter (nm) - charted results for all study groups.
A4.3.3 Average Fibril Area

<table>
<thead>
<tr>
<th>GROUP</th>
<th>NOC</th>
<th>MENX</th>
<th>O VX</th>
<th>O+M</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN</td>
<td>16.92</td>
<td>13.00</td>
<td>7.46</td>
<td>17.80</td>
</tr>
<tr>
<td>SE</td>
<td>0.49</td>
<td>1.05</td>
<td>0.28</td>
<td>1.39</td>
</tr>
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</table>

Table A4.13: TEM results for ACL, average fibril area (µm²) - charted results for all study groups, mean and SE.

A4.3.4 Total Fibril Area

<table>
<thead>
<tr>
<th>GROUP</th>
<th>NOC</th>
<th>MENX</th>
<th>O VX</th>
<th>O+M</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEAN</td>
<td>2607.94</td>
<td>2517.26</td>
<td>1451.98</td>
<td>3188.68</td>
</tr>
<tr>
<td>SE</td>
<td>37.90</td>
<td>77.12</td>
<td>50.36</td>
<td>96.97</td>
</tr>
</tbody>
</table>

Table A4.14: TEM results for ACL, total fibril area (µm²) - charted results for all study groups, mean and SE.

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Figure A4.42: TEM ACL average fibril area (µm²) - charted results for all study groups.

Figure A4.43: TEM ACL total fibril area (µm²) - charted results for all study groups.
A4.3.5 Fibril Density

### Table A4.15: TEM results for ACL fibril density (% of total field) - charted results for all study groups, mean and SE.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>MEAN</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOC</td>
<td>48.1</td>
<td>1.3</td>
</tr>
<tr>
<td>MENX</td>
<td>47.3</td>
<td>1.4</td>
</tr>
<tr>
<td>OVX</td>
<td>27.3</td>
<td>0.9</td>
</tr>
<tr>
<td>O+M</td>
<td>59.9</td>
<td>1.8</td>
</tr>
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</table>

### Table A4.16: Anova statistical analysis data for TEM fibril results, average number of fibrils, average fibril diameter, average fibril area, total fibril area and fibril density. Analysis was performed at a 5% level of significance.

<table>
<thead>
<tr>
<th>STUDY GROUPS (x vs. y)</th>
<th>AVERAGE NUMBER OF FIBRILS</th>
<th>AVERAGE FIBRIL DIAMETER</th>
<th>AVERAGE FIBRIL AREA</th>
<th>TOTAL FIBRIL AREA</th>
<th>FIBRIL DENSITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOC vs. MENX</td>
<td>0.0084</td>
<td>0.0013</td>
<td>0.0072</td>
<td>0.4315</td>
<td>0.6772</td>
</tr>
<tr>
<td>NOC vs. OVX</td>
<td>0.0158</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NOC vs. O+M</td>
<td>0.0466</td>
<td>0.6438</td>
<td>0.5155</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MENX vs. OVX</td>
<td>0.7172</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>O+M vs. MENX</td>
<td>0.4273</td>
<td>0.0045</td>
<td>0.0012</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>O+M vs. OVX</td>
<td>0.6494</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Figure A4.44: TEM ACL fibril density (% of total field) - charted results for all study groups.
A4.3.6  Fibril Diameter Distribution

<table>
<thead>
<tr>
<th>GROUP (x)</th>
<th>n=</th>
<th>FIBRILS &lt;100 (nm)</th>
<th>p (x vs. NOC)</th>
<th>FIBRILS 100-200 (nm)</th>
<th>p (x vs. NOC)</th>
<th>FIBRILS &gt;200 (nm)</th>
<th>p (x vs. NOC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOC</td>
<td>12</td>
<td>1.2 (1)</td>
<td></td>
<td>154.4 (99)</td>
<td></td>
<td>0.2 (&lt;1)</td>
<td></td>
</tr>
<tr>
<td>MENX</td>
<td>10</td>
<td>63.5 (31) &lt;0.0001</td>
<td>133.2 (65)</td>
<td></td>
<td>6.8 (3) 0.9981</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVX</td>
<td>12</td>
<td>94.8 (48) &lt;0.0001</td>
<td>102.2 (52)</td>
<td></td>
<td>0.3 (0) 0.2551</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O+M</td>
<td>12</td>
<td>54.6 (29)</td>
<td>98.6 (53)</td>
<td></td>
<td>33.4 (18) &lt;0.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table A4.17: TEM results for ACL fibril diameter distribution for all study groups. Fibril counts for each size range (nm) were compared to NOC for each experimental group.

Figure A4.45: Chart showing ACL fibril diameter (size) distribution for all test groups compared to the NOC group, as determined using TEM analysis.
A4.3.7  Fibril Arrangement

Figure A4.46: TEM 37000x images of ACL tissue from NOC (8666), MENX (8607), OVX (8700) and O+M (8584) sheep, demonstrating obvious differences in fibril size and arrangement between groups.

All TEM images used in this study are presented on pages 264-286; NOC figures A4.47-58, MENX figures A4.59-68, OVX figures A4.69-80, O+M figures A4.81-92.
Figure A4.47: TEM 37000x image of NOC ACL, sheep # 69L; image A-8736, B-8737.

Figure A4.48: TEM 37000x image of NOC ACL, sheep # 69R; image A-8641, B-8732.
Figure A4.49: TEM 37000x image of NOC ACL, sheep # 70L; image A-8696, B-8712.

Figure A4.50: TEM 37000x image of NOC ACL, sheep # 70R; image A-8649, B-8650.
Figure A4.51: TEM 37000x image of NOC ACL, sheep # 71L; image A-8717, B-8718.

Figure A4.52: TEM 37000x image of NOC ACL, sheep # 71R; image A-8593, B-8664.
Figure A4.53: TEM 37000x image of NOC ACL, sheep # 72L; image A-8611, B-8612.

Figure A4.54: TEM 37000x image of NOC ACL, sheep # 72R; image A-8693, B-8694.
Figure A4.55: TEM 37000x image of NOC ACL, sheep # 77L; image A-8723, B-8724.

Figure A4.56: TEM 37000x image of NOC ACL, sheep # 77R; image A-8678, B-8679.
Figure A4.57: TEM 37000x image of NOC ACL, sheep # 94L; image A-8685, B-8686.

Figure A4.58: TEM 37000x image of NOC ACL, sheep # 94R; image A-8666, B-8667.
Figure A4.59: TEM 37000x image of MENX ACL, sheep # 68L; image A-8637, B-8638.

Figure A4.60: TEM 37000x image of MENX ACL, sheep # 68R; image A-8710, B-8711.
Figure A4.61: TEM 37000x image of MENX ACL, sheep # 76L; image A-8734, B-8735.

Figure A4.62: TEM 37000x image of MENX ACL, sheep # 76R; image A-8598, B-8599.
Figure A4.63: TEM 37000x image of MENX ACL, sheep # 78L; image A-8672, B-8673.

Figure A4.64: TEM 37000x image of MENX ACL, sheep # 78R; image A-8590, B-8591.
Figure A4.65: TEM 37000x image of MENX ACL, sheep # 79L; image A-8596, B-8597.

Figure A4.66: TEM 37000x image of MENX ACL, sheep # 79R; image A-8606, B-8607.
Figure A4.67: TEM 37000x image of MENX ACL, sheep # 95L; image A-8681, B-8705.

Figure A4.68: TEM 37000x image of MENX ACL, sheep # 95R; image A-8635, B-8636.
Figure A4.69: TEM 37000x image of OVX ACL, sheep # 45L; image A-8699, B-8700.

Figure A4.70: TEM 37000x image of OVX ACL, sheep # 45R; image A-8703, B-8704.
Figure A4.71: TEM 37000x image of OVX ACL, sheep # 52L; image A-8727, B-8728.

Figure A4.72: TEM 37000x image of OVX ACL, sheep # 52R; image A-8674, B-8675.
Figure A4.73: TEM 37000x image of OVX ACL, sheep # 60L; image A-8585, B-8586.

Figure A4.74: TEM 37000x image of OVX ACL, sheep # 60R; image A-8668, B-8669.
Figure A4.75: TEM 37000x image of OVX ACL, sheep # 63L; image A-8640, B-8722.

Figure A4.76: TEM 37000x image of OVX ACL, sheep # 63R; image A-8671, B-8701.
Figure A4.77: TEM 37000x image of OVX ACL, sheep # 91L; image A-8663, B-8714.

Figure A4.78: TEM 37000x image of OVX ACL, sheep # 91R; image A-8680, B-8706.
Figure A4.79: TEM 37000x image of OVX ACL, sheep # 92L; image A-8613, B-8615.

Figure A4.80: TEM 37000x image of OVX ACL, sheep # 92R; image A-8731, B-8733.
Figure A4.81: TEM 37000x image of O+M ACL, sheep # 03L; image A-8604, B-8605.

Figure A4.82: TEM 37000x image of O+M ACL, sheep # 03R; image A-8583, B-8584.
Figure A4.83: TEM 37000x image of O+M ACL, sheep # 08L; image A-8738, B-8739.

Figure A4.84: TEM 37000x image of O+M ACL, sheep # 08R; image A-8725, B-8726.
Figure A4.85: TEM 37000x image of O+M ACL, sheep # 10L; image A-8691, B-8692.

Figure A4.86: TEM 37000x image of O+M ACL, sheep # 10R; image A-8683, B-8684.
Figure A4.87: TEM 37,000x image of O+M ACL, sheep #21L; image A-8658, B-8659.

Figure A4.88: TEM 37,000x image of O+M ACL, sheep #21R; image A-8647, B-8648.
Figure A4.89: TEM 37000x image of O+M ACL, sheep # 27L; image A-8587, B-8588.

Figure A4.90: TEM 37000x image of O+M ACL, sheep # 27R; image A-8656, B-8657.
Figure A4.91: TEM 37000x image of O+M ACL, sheep # 58L; image A-8682, B-8715.

Figure A4.92: TEM 37000x image of O+M ACL, sheep # 58R; image A-8676, B-8677.
Figure A4.93: SEM 2000x images of ACL tissue samples. A = NOC; B = MENX; C = OVX; D = O+M. Note the unusual light and dark striations in the SEM cross-sections, which were conspicuous on the OVX, MENX and O+M samples but were not as obvious on the NOC sample.
Figure A4.94: SEM 170x images of ACL tissue samples. A = NOC; B = MENX; C = OVX; D = O+M. Note the differences in the arrangement of the fibre bundles between groups.
Figure A4.95: H&E 200x longitudinal sections of ACL tissue from NOC sheep.
Figure A4.96: Tol Blue 200x longitudinal sections of ACL tissue from NOC sheep.
Figure A4.97: H&E 200x longitudinal sections of ACL tissue from MENX sheep.
Figure A4.98: Tol Blue 200x longitudinal sections of ACL tissue from MENX sheep.
Figure A4.99: H&E 200x longitudinal sections of ACL tissue from OVX sheep.
Figure A4.100: Tol Blue 200x longitudinal sections of ACL tissue from OVX sheep.
Figure A4.101: H&E 200x longitudinal sections of ACL tissue from O+M sheep.
Figure A4.102: Tol Blue 200x longitudinal sections of ACL tissue from O+M sheep.
APPENDIX 5  BIOMECHANICAL STUDY RESULTS

Presented below are a brief description of the methods and results of J. Gilmore’s (2011) study, which examined the effect of ovariectomy on the biomechanical properties of the ovine knee joint. Results are reproduced with the author’s permission.

METHODS

Whole ovine knee joints from OVX (n=6) and NOC (n=6) sheep were dissected free of surrounding soft tissues, leaving the collateral ligaments and joint capsule intact. The tibia was secured to the upper plate of an Instron 5566 universal mechanical test frame, with the femur mounted in a vice secured to the base of the frame. Posterior and anterior drawer tests were performed at 250N for 10 cycles, 1 cycle per second, and the data collected for analysis. An example of a force displacement plot produced by the Instron 5566 is show below in Figure A5.1. From the acquired data, anterior & posterior drawer, and total deformation were calculated. Stiffness gradients (σ) were generated from each drawer test to determine changes in ligament stiffness.

![Displacement Curve OVX 263](image)

Figure A5.1: Force displacement curves of anterior drawer (AD) and posterior drawer (PD) tests in OVX 263. AD σ=277N/mm; AD displacement = 1.371 mm; PD σ=252 N/mm; PD displacement = 1.753 mm; combined displacement = 3.124 mm (J. Gilmore, 2011).
RESULTS

<table>
<thead>
<tr>
<th></th>
<th>NOC  n=6 Mean (mm) ± [SE]</th>
<th>OVX n=6 Mean (mm) ± [SE]</th>
<th>P=</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANTERIOR DRAWER</td>
<td>1.61 [0.038]</td>
<td>1.60 [0.122]</td>
<td>0.928</td>
</tr>
<tr>
<td>POSTERIOR DRAWER</td>
<td>1.87 [0.053]</td>
<td>1.51 [0.091]</td>
<td>0.006</td>
</tr>
<tr>
<td>TOTAL DISPLACEMENT</td>
<td>3.38 [0.067]</td>
<td>3.00 [0.143]</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Table A5.1: Summary of biomechanical testing - joint displacement

Figure A5.2: results of biomechanical testing showing effect of ovariectomy on joint displacement in anterior and posterior drawer tests. Data represent total displacement (mm) +/- se. * p=0.006; ** p=0.011
Table A5.2: Summary of biomechanical testing - ligament stiffness

<table>
<thead>
<tr>
<th></th>
<th>NOC  n=6 Mean (N/mm) ± [SE]</th>
<th>OVX n=6 Mean (N/mm) ± [SE]</th>
<th>P=</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANTerior DRAWER</td>
<td>220.46 [8.014]</td>
<td>242.85 [12.429]</td>
<td>0.161</td>
</tr>
<tr>
<td>POSTerior DRAWER</td>
<td>202.16 [15.346]</td>
<td>259.12 [7.449]</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Figure A5.3: results of biomechanical testing showing the effect of ovariectomy on ligament stiffness (N/mm) +/- se. * p =0.007.