A RADIOLOGICAL AND BIOCHEMICAL PERSPECTIVE ON AGEING AND DEGENERATION OF THE HUMAN THORACIC INTERVERTEBRAL DISC

Celia I C Tan
Masters of Applied Science (Physiotherapy)
Graduate Diploma in Applied Science (Paediatric Physiotherapy)
Diploma in Physiotherapy

This thesis is presented for the degree of Doctor of Philosophy at
The University of Western Australia
Centre for Musculoskeletal Studies
School of Surgery and Pathology

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ABSTRACT

Disc degenerative changes are directly or indirectly associated with spinal pain and disability. Literature revealed a high prevalence of disc degeneration in the thoracic region, however thoracic MRI degeneration trends and information on disc biochemical matrix constituents are limited for thoracic discs compared to lumbar and cervical discs.

The objective of this thesis was to use MRI to investigate the prevalence of disc degenerative changes affecting the human thoracic spine, and to determine the factors affecting spinal disc biochemical matrix. A 3-point subjective MRI grading scale was used to grade the films. The feasibility of using archived formalin-fixed cadaver material was investigated to analyse collagen and elastin crosslinks.

The prevalence of degenerative changes in human thoracic discs and vertebrae (T1 to T12) was determined retrospectively from an audit of 216 MRI cases, using sagittal T1- and T2-weighted MR images. In a subsequent series of ex-vivo studies, human thoracic discs and LF from 26 formalin-fixed and two fresh spines, involving all thoracic levels, were examined macroscopically to determine the degeneration status. Subsequently, disc and ligament tissues were analysed biochemically for collagen (pyridinoline and deoxypyridinoline) and elastin (desmosine and isodesmosine) crosslinks. These crosslinks were extracted from hydrolysed samples by cellulose partition chromatography, and analysed by reverse-phase HPLC. Collagen content was determined using its hydroxyproline content, and proteoglycan content was assayed using a modified DMB assay for chondroitin sulphate. Finally the MRI and macroscopic assessments of thoracic discs, were compared with the biochemical data from two fresh cadaver thoracic spines.

The 3-point MRI grading scale had a high inter- (k = 0.57 to 0.78) and intra-rater (k = 0.71 to 0.87) reliability. There were no significant differences in the collagen and elastin content and extent of collagen crosslinks between formalin fixed and unfixed ligament and disc tissues, after 25 weeks of formalin fixation.

From the in-vivo MRI series of investigations (n = 216 MRI films), the prevalence of thoracic disc degenerative and vertebral morphological changes revealed significant age, gender and spinal level trends (p < 0.05). Generally, males had a higher propensity for disc degeneration in contrast to females, especially older females, where the trend showed a higher prevalence of osteophytes and vertebral body changes. In particular, the mid and lower thoracic levels have a higher prevalence of degenerative changes, except for osteophytes and anterior
vertebral wedging. With increased age, there was a concomitant increase in anterior wedging and bi-concavity and disc degenerative changes except for end-plates.

The biochemical investigations on the ex-vivo series of formalin-fixed thoracic discs ($n = 303$) also revealed significant changes in the disc matrix due to degeneration status, age, gender and spinal regional factors. With increased age, normal disc matrices have significantly lower collagen content and extent of pyridinoline ($p < 0.001$). In contrast, the degenerated disc matrix revealed significantly higher collagen content and extent of deoxypyridinoline ($p < 0.05$). These findings suggest that an altered matrix existed in normal ageing discs, which render the disc prone to injury and degeneration over the life span. The higher collagen and deoxypyridinoline in degenerated disc matrices reflects an increase in chondrocyte synthesis, and is also a novel finding, suggesting that they may be used as markers of ageing and degeneration processes.

The biochemical investigations on another series of ex-vivo spinal LF tissues ($n = 364$), revealed that this had a lower collagen and pyridinoline, but significantly higher elastin and deoxypyridinoline compared to spinal discs ($p < 0.05$). Elastin crosslinks however were difficult to detect in spinal discs, being present in negligible amounts in a few lumbar discs. The elastin crosslinks in the LF were not significantly affected by age, but were significantly higher in calcified, and female ligamentum tissues, and also in the lumbar region ($p < 0.05$).

These MRI prevalence findings enhanced our knowledge of vertebral body and disc degeneration trends in the thoracic region and contributed to the interpretation of MR images for pathology in the human thoracic spine. Information on the associated collagenous and elastic changes in the disc and ligamentum matrices provide original data and insight on the pathogenesis of degeneration in the disc matrix from a biochemical perspective, highlighting gender, age and spinal level influences on the matrix tensile strength and cellular synthetic activities.
STATEMENT OF ORIGINALITY

This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia. Studies were undertaken between September 1998 and March 2004, through the Centre for Musculoskeletal Studies, School of Surgery and Pathology, in association with the following:

1. Department of Neuropathology and Department of Imaging, Royal Perth Hospital
2. Department of Diagnostic Radiology, Sir Charles Gairdner Hospital
3. Department of Diagnostic Radiology, Singapore General Hospital
4. Clinical Pathology Division, The Western Australian Centre for Pathology and Medical Research (PathCentre), QEII Medical Centre

This series of research studies were developed in association with my thesis supervisors, who were also involved in editing both this thesis and associated publications. I have independently performed all the experimental work and analysis of results except for the following:

1. Methodology for the isolation of collagen (pyridinoline, deoxypyridinoline) and elastin (desmosine and isodesmosine) crosslinks, from spinal tissues using HPLC, and routine biochemical assays for collagen and chondroitin sulphate were developed by biochemical scientists at the Clinical Pathology Division, PathCentre. These assays were utilised in Studies 5.1 to 5.6 in Chapter 5, and detailed in Appendix B.
2. Thoracic vertebral body morphometry and corresponding statistical analyses described in Study 4.3 in Chapter 4, were performed by a co-investigator (S Goh).

I declare that all material presented in this thesis is original, apart from the work which has been acknowledged from other sources within the text. Review of relevant literature to the thesis has been included up to December, 2003.

Celia I C Tan
Dated March 2004
We, the undersigned, declare that the statement of originality, as stated above regarding the development of the biochemical assays, is true and accurate. We acknowledge our assistance in the development of the above assays, which were employed in this thesis.

Dr G Neil Kent  
Managing Scientist  
Clinical Pathology Division  
PathCentre, Queen Elizabeth II Medical Centre  
Nedlands, Western Australia.  
Dated: 8/11/2001

Mr Andrew G Randall  
Section Manager, Special Chemistry  
Clinical Pathology Division  
PathCentre, Queen Elizabeth II Medical Centre  
Nedlands, Western Australia.  
Dated: 22/10/02
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This thesis is dedicated to my husband, Alvin and my son, Jeremy.

With all my love and adoration.
THIS THESIS IS BASED ON THE FOLLOWING PUBLICATIONS


PRESENTATIONS ARISING FROM THIS PROGRAM

In addition to the papers prepared for the compilation of this thesis, parts of this research study have been presented at the following conferences:

Conference presentations


Other publications:

Letter to the Editor: Tan CIC, Kent GN, Randall, AG and Singer KP. Comment on “Collagen crosslinks in human lumbar intervertebral disc ageing”. Spine 1999;24;1271.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAF</td>
<td>Anterior anulus</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycosylation end-products</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BAAW</td>
<td>Butanol: acetic acid:water (ratio of 4:1:1)</td>
</tr>
<tr>
<td>C</td>
<td>Cervical</td>
</tr>
<tr>
<td>Chlor-T</td>
<td>Chloramine T reagent</td>
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<tr>
<td>CML</td>
<td>N-carboxymethyllysine</td>
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<tr>
<td>Coll</td>
<td>Collagen</td>
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<tr>
<td>CS</td>
<td>Chondroitin sulphate</td>
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<tr>
<td>CT</td>
<td>Computer tomography</td>
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<tr>
<td>Des</td>
<td>Desmosine</td>
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<tr>
<td>DMB</td>
<td>1, 9 dimethylmethylene blue-chloride</td>
</tr>
<tr>
<td>DMBR</td>
<td>p-Dimethyl amino-benzaldehyde reagent</td>
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<tr>
<td>Dpd</td>
<td>Deoxypyridinoline</td>
</tr>
<tr>
<td>EDTA</td>
<td>di-sodium ethylene di-aminetetra-acetic acid</td>
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<tr>
<td>ER</td>
<td>Rough endoplasmic reticulum</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
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<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<tr>
<td>HFBA</td>
<td>n-Heptafluorobutyric acid</td>
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<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
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<tr>
<td>Isodes</td>
<td>Isodesmosine</td>
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<tr>
<td>KS</td>
<td>Keratan sulphate</td>
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<tr>
<td>LF</td>
<td>Ligamentum flavum</td>
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<tr>
<td>L</td>
<td>Lumbar</td>
</tr>
<tr>
<td>ml</td>
<td>milliLitre</td>
</tr>
<tr>
<td>mPa</td>
<td>milliPascal</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MR</td>
<td>Magnetic resonance</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleus pulposus</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>PA</td>
<td>Perchloric acid 17.5%</td>
</tr>
<tr>
<td>PAF</td>
<td>Posterior anulus</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycan</td>
</tr>
<tr>
<td>Pyd</td>
<td>Pyridinoline</td>
</tr>
<tr>
<td>RPH</td>
<td>Royal Perth Hospital</td>
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<tr>
<td>SCGH</td>
<td>Sir Charles Gairdner Hospital</td>
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<td>SGH</td>
<td>Singapore General Hospital</td>
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<tr>
<td>T</td>
<td>Thoracic</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
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<tr>
<td>Wt</td>
<td>Weight</td>
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<tr>
<td>X-Ray</td>
<td>Plain radiographic imaging</td>
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### GLOSSARY OF TERMS

<table>
<thead>
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<tr>
<td>Bi-concavity</td>
<td>Ratio of the mid vertebral height (Hm) to posterior vertebral diameter (Hp). It is an index of the bi-concavity or curvature of the spinal column used in Study 4.3.</td>
</tr>
<tr>
<td>DIW</td>
<td>Deionised water of 18 MΩ resistivity, passed through a 0.22 μm filter.</td>
</tr>
<tr>
<td>Ha/Hp</td>
<td>Ratio of anterior (Ha) to posterior (Hp) vertebral height measure using MR films. It is an index of anterior wedging of the spinal column used in Study 4.3.</td>
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<tr>
<td>Lower Thoracic</td>
<td>This region refers to the thoracic vertebrae and discs from T9 to T12.</td>
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<tr>
<td>Mid Thoracic</td>
<td>This region refers to the thoracic vertebrae and discs from T5 to T8.</td>
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<tr>
<td>Disc region</td>
<td>Refers to the different regions of the disc, ie anterior anulus, posterior anulus or nucleus.</td>
</tr>
<tr>
<td>Spinal region</td>
<td>Refers to different regions of the spinal column, eg cervical, lumbar, upper, mid or lower thoracic regions.</td>
</tr>
<tr>
<td>T1-T2</td>
<td>Refers to the thoracic intervertebral disc below the T1 vertebra. Similarly T2-T3, refers to the disc below T2.</td>
</tr>
<tr>
<td>T1-weighted</td>
<td>Refers to the MRI spin echo sequence in which the repetition times (TR) is short, ranging from 500 to 700ms, and echo times (TE) ranging from 12 to 20ms</td>
</tr>
<tr>
<td>T2-weighted</td>
<td>Refers to the MRI spin echo sequence in which the TR and TE is long (usually TR 2000ms, TE 90ms)</td>
</tr>
<tr>
<td>Upper Thoracic</td>
<td>This region refers to the thoracic vertebrae and discs from T1 to T4.</td>
</tr>
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Figure 2.6 Schematic representation of a typical intervertebral disc showing the alternating layers of anular lamellae with fibers titled at a 70° angle to the axis. Figure taken from Boden et al (1991).

Figure 2.7 Photograph of transverse horizontal sections of human thoracic discs from a 78-year old male subject showing all levels from T1-T2 to T12-L1 (reading from right to left, and top to bottom). In the upper thoracic region, the discs are small and elliptical, gradually changing to circular or triangular in the mid thoracic region and becoming more elliptical in the lower segments. The size of the discs also increases from cranial to caudal.
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Figure 2.9 Schematic diagram of the transfer of axial compression forces in the nucleus to the anulus. Disc internal pressure (P) in the nucleus is balanced axially by an applied compressive force (F), and radially by a circumferential tangential force (T) in the anulus. Figure taken from Hukins (1988).

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CHAPTER 1   INTRODUCTION

Much of the information on human spinal discs has focussed primarily on the lumbar and cervical regions and the pathogenesis of spinal disc degeneration is usually generalised from lumbar disc studies. Such generalisation is acceptable especially in terms of basic spinal anular and nuclear morphology and biomechanical function, however the location of the thoracic discs between the cervical and lumbar regions predisposes them to a unique set of biomechanical influences. The biomechanical environment in the thoracic region is primarily due to the kyphotic curvature (Schmorl and Junghanns 1971), thinner and more circular discs (Pooni et al 1986), and the attachment of the ribs and sternum (Andriacchi et al 1974). The major spinal deformity in the thoracic region is senile or osteoporotic kyphosis, especially in aged men (Schmorl and Junghanns 1971) and women (Goh et al 1999), respectively, leading to high costs in management and associated disability (Melton et al 1993).

Much of the existing literature on the thoracic spine has been derived from cadaver (Nathan 1962, Schmorl and Junghanns 1971, Malmivaara et al 1987, Singer 1997) and radiological (Lawrence 1977, Singer 1997) studies. Traditionally, the thoracic region is presumed to be rigid and stable, with relatively less disc pathology compared to the lumbar and cervical regions. However thoracic MRI studies have reported a higher incidence of asymptomatic degenerative changes, which has resulted in a greater awareness of the need to be more cautious in interpreting degenerative changes on MR films (Williams et al 1989, Wood et al 1995). In addition, T1- and T2- weighted MRI films can provide objective measurement for vertebral morphology (Goh et al 2000a), as well as subjective grading of the disc degeneration status, based on the MR signal intensity, respectively (Thompson et al 1988, Eyre et al 1989, Pfirrmann et al 2001). However, there is a need to survey the prevalence of disc degenerative changes on MR images over the life span, particularly for the thoracic region, not only because such information is limited, more importantly, MRI is currently the preferred method of diagnostic imaging for spinal degenerative changes (Pfirrmann et al 2001).

Age, gender and spinal level influences have been implicated as important factors resulting in degeneration of thoracic discs (Lawrence 1977, Singer 1997), however how these factors influence the biochemical constituents of the disc matrix, in particular the collagen and proteoglycan content, is also important. A review of spinal biochemical studies, again drawn largely from lumbar disc studies, suggests that the tensile strength of the disc is more accurately reflected by the extent of mature, non-reducible post-translational collagen crosslinks, pyridinoline (Duance et al 1998, Pokharna and Phillips 1998) and
deoxypyridinoline (Eyre 1979, 1988). Traditionally these crosslinks are measured as markers of bone collagen turnover in joint diseases (Eyre and Wu 1995), such as Paget’s disease (Randall et al 1996) and osteoarthritis (Takahashi et al 1998).

Another important element in connective tissue matrix is the elastic fiber, which is essential in conferring elasticity to the collagenous fibers in the disc (Eyre 1979, Johnson et al 1985, Yu et al 2002). Studies generally report that elastic fibers are present in small amounts and are difficult to detect in the disc, even in lumbar discs (Buckwalter et al 1976, Eyre 1979, Yu 2002). Elastin crosslinks are however, found abundantly in spinal ligamentous tissues (Chen et al 2000). There is currently a lack of comprehensive data on collagen and elastin crosslink content in human spinal discs and ligamentum flavum, and how different spinal regions, gender, ageing and degree of degeneration influence its content in the matrix. Such biochemical information may provide insights into the pathogenesis of degenerative changes in spinal discs and ligaments from a molecular perspective.

Due to formalin induced crosslinks with protein molecules in the matrix, biochemical studies have traditionally utilised fresh or unfixed tissues (Hickey and Hukins 1979, Boskey et al 1982, Toledo et al 1996). Formaldehyde is reported to form crosslinkages in the extracellular matrix by reacting with unbonded or free lysine residues on the exterior of the protein molecule (Leong 1994). As access to fresh human tissues is often restricted, stored tissues are usually the preferred alternative. There is a need to determine the feasibility of using formalin-fixed spinal tissues for biochemical analysis of collagen and elastin crosslinks because such information is still limited.

From the literature, it is evident that there is still a lack of studies detailing the trend of degenerative changes in thoracic discs, due to the various factors such as age, gender and spinal level. In particular, studies correlating disc degenerative changes with the biochemical matrix constituents are also few. This thesis sought to add to the limited information on the prevalence of thoracic disc degenerative changes over the life span using MRI investigations. In particular, the association and influence of degeneration and other factors, such as age, gender and spinal regions on spinal disc and ligamentous biochemical matrix is investigated. Such correlative information provides insight into the sequel of the pathogenesis of spinal disc degeneration. It is hoped that data from these studies will contribute to future research aimed at restoring or maintaining the biochemical integrity of the disc and ligament tissues to sustain their function, hence reduce degeneration, pain, disability and deformity, in particular for the thoracic spine.
CHAPTER 2 LITERATURE REVIEW

This review presents a summary of the literature relating to the anatomy of thoracic intervertebral discs, and the available information describing the development and mechanism of thoracic disc degeneration. As the number of studies on the thoracic discs are fewer compared to the lumbar and cervical discs, throughout the review, reference has been made to relevant literature on lumbar and cervical spine from human and animal studies, to contrast with or add to, the information for the thoracic spine. This is necessary especially for the background on the growth and development of the intervertebral disc, the mechanism of disc degeneration and its related influence on the disc biochemical matrix. Information on the biochemical changes in the disc was also scarce, even for lumbar discs; therefore some of the biochemical information on proteoglycans and collagen crosslinks also draw from studies on articular cartilage. In order to evaluate further the distribution of elastin crosslinks in soft tissue matrix, a review of the biomechanical function and biochemical matrix constituents of the spinal ligamentum flavum is also included. The use of MRI for disc degenerative grading, and formalin-fixed tissues for biochemical analysis is also described.

2.1 ANATOMY AND BIOMECHANICS OF THE SPINE

A review of the gross anatomy and the functional biomechanics of the spine is important in understanding the possible mechanisms involved in the degeneration of intervertebral discs, in particular the discs in the thoracic region. The human spine normally comprises 33 vertebrae. Of these, 24 are presacral, that is 7 cervical, 12 thoracic and 5 lumbar vertebrae, and these make up the mobile segment of the spine. The 23 intervening discs between the presacral vertebrae account for 20% to 33% of the entire spinal length (De Puky 1935, Taylor 1975). There are no discs between the first two cervical vertebrae and in the fused sacrum and coccyx (Butler 1988, Hukins 1988). The thoracic spine usually consists of 12 vertebrae, with occasional variations of 11 (5% of 125 human specimens) or 13 (1%) according to Schultz (1961).

The upright human spine is characterised by four natural curves in the sagittal plane; lordotic or convex forward in the cervical and lumbar regions and kyphotic or concave forward in the thoracic and sacro-coccygeal regions (White 1969) (Figure 2.1). According to Schmorl and Junghann (1971) and De Puky (1935), the shape of the disc is the main determinant of the lordosis in the cervical and lumbar regions. In contrast, the thoracic kyphosis is mainly determined by the morphology of the vertebral bodies (Goh et al 1999), as the ratio of
thoracic disc to vertebral body height is much smaller compared to the lumbar and cervical regions (Pooni et al 1986). Therefore the thoracic kyphosis is less influenced by postural changes (De Puuy 1935, White 1969, Edmundston and Singer 1997). However Schmorl and Junghanns (1971) further proposed that the progression of thoracic kyphosis develops in two ways; senile kyphosis, which is due predominantly to thoracic disc degeneration; and osteoporotic kyphosis, which is caused by the collapse of the thoracic vertebral body and wedging.

![Diagram of Spinal Regions and Curves]

**Figure 2.1** Diagram demonstrating the lordotic curves of the cervical and lumbar regions and the kyphotic curves of the thoracic and sacro-coccygeal regions. Adapted from Singer et al (1989).

### 2.2 Biomechanics of the Thoracic Spine

In the newborn, the whole spine is flexed forward or kyphotic; however once erect standing is achieved, the spine assumes an adaptive S-shape curvature (Snijders 1969), with the cervical and lumbar regions convexed anteriorly (Schmorl and Junghanns 1971, Panjabi et al 1991). The thoracic region however, maintains the same flexed posture as the newborn, even in the erect posture and this is the primary curvature (Schmorl and Junghanns 1971, Taylor 1975, Maiman and Pintar 1990). The thoracic sagittal curve forms part of the S-shaped curvature of the vertebral column (Snijders 1969). These spinal curvatures are an adaptation of the spine to
the upright posture, providing for efficient transmission of gravitational forces (Pal and Routal 1986, Panjabi et al 1991), as well as increasing the flexibility and shock absorbing ability of the spine (White 1969, White and Panjabi 1990a).

The typical thoracic vertebrae is broader and stronger than the cervical vertebrae, and increases in size caudally, as it bears more body weight (Panjabi et al 1991, Edmondston et al 1994b) (Figure 2.2). The spinous process of the thoracic vertebra is long, pointed, oblique and inclined caudally (White 1969). The vertical height of the thoracic vertebra and the disc is smaller anteriorly than posteriorly, and both structures contribute to the thoracic kyphosis (Pooni et al 1986, Goh et al 1999, Singer and Goh 2000). The spinal canal is narrowest in the mid thoracic region, which is also the critical vascular zone for the spinal cord (Roaf 1980, Bogduk and Twomey 1991). According to Pal and Routal (1986), most of the body weight in the thoracic region is borne anteriorly by the vertebrae and disc, with less weight borne by the zygapophyseal joints (20 to 30%). They reported a gradual increase in axial weight taken by the vertebral body and disc from 70% in the upper thoracic region (Pal and Routal 1986), to 76% in the mid and 80% in the lower thoracic regions (Pal and Routal 1987).

**Figure 2.2** Schematic diagram of the thoracic cavity formed by the sternum anteriorly (arrow), diaphragm caudally, the thoracic vertebrae posteriorly and the first rib cranially. Figure taken from Moore (1985).

Located between the cervical and lumbar regions, the thoracic spine has two transitional zones, with gradual changes in curvature and orientation of the zygapophyseal joints between the lower cervical and upper thoracic regions (Pal and Routal 1986, Boyle et al 1996) and between the lower thoracic and upper lumbar regions (Malmivaara et al 1987, Singer et al
The thoracic discs therefore experience a unique set of biomechanical forces compared to the discs in the cervical and lumbar regions, and is structured for stability rather than mobility (Singer and Goh 2000, Stokes 2000). Even among the thoracic joints, the biomechanical function in each thoracic region is different and these are summarised in Figure 2.3.

**Figure 2.3** Schematic representation of the thoracic spine, adapted from Singer and Goh (2000), with dashed lines demarcating the upper, mid and lower thoracic regions (on the left) and a summary of biomechanical forces that influence each of these regions (on the right).

### 2.2.1 The Upper Thoracic Region

Biomechanically the thoracic spine may be divided into three regions: upper, mid and lower thoracic regions (Veleanu et al 1972, Panjabi et al 1991, Edmondston and Singer 1997)(Figure 2.3 and Table 2.1). The upper thoracic region includes the first 4 thoracic vertebrae, where the discs are shaped similarly to those in the lower cervical region (Pal and Routal 1986, Boyle et al 1996). This region is characterised by a marked decrease in flexion and extension range compared to the adjacent lower cervical region (White and Panjabi 1978), due to the articulation with the ribs and sternum (Veleanu et al 1972, Andriacchi et al...
1974, White and Panjabi 1978, Boyle et al 1998) (Figure 2.2); and the coronal orientation of the zygapophyseal joints (Maiman and Pintar 1990, Stokes 2000). Andriacchi et al (1974) modelled a two-fold increase in stiffness of the spine and three-fold increase in load-bearing capacity of the spine, due to the presence of the rib cage. Conversely, the range of spinal flexion is increased when the sternum is removed (White 1969, Andriacchi et al 1974).

The costo-transverse and costo-vertebral joints also contribute to restrict the range of thoracic rotation as well as to transfer axial load postero-anteriorly (Pal and Routal 1987, Stokes 2000) (Figure 2.4). The ribs articulate with the transverse process of the same thoracic vertebrae and the vertebral body above from T2 to T9 (Roaf 1980, Moore 1985). Exceptions to this rib-vertebral body articulation exists in T1 and T10 to T12, where the ribs articulate with the corresponding vertebral body only (Moore 1985).

Figure 2.4 Schematic diagram showing the costo-sternal and vertebral ligaments and the attachment of the 3rd rib to the inferior costo-vertebral facet on T2 and the superior costo-vertebral facet on T3. Figure taken from Moore (1985).

2.2.2 The Mid Thoracic Region
The mid thoracic region consists of the next 4 thoracic vertebrae (T5 to T8), which also articulate with the ribs. According to White and Panjabi (1978), the sagittal and rotational range of motion in this region is similar to that in the upper thoracic region (Table 2.1), however the difference between these two regions is that the mid thoracic region is also the apex of the thoracic kyphotic curvature (Veleanu et al 1972), found typically between T6 and T8 (Schmorl and Junghanns 1971, Singer et al 1990). Due to the kyphotic curvature of the mid thoracic region, there are generally greater compression forces anteriorly, both on the discs and the vertebral bodies (Schmorl and Junghanns 1971, Pal and Routal 1987, Goh et al
1999). The kyphotic curve is accentuated with increased age and osteoporosis, especially in older women (Schmorl and Junghanns 1971, De Smet et al 1988).

Unlike the upper thoracic region, the rotation range in the mid thoracic region is facilitated by the coronal and near vertical orientation of the thoracic zygapophyseal joints which offer very little resistance to rotation, instead limiting flexion and extension movements (Gregerson and Lucas 1967, Singer and Goh 2000) (Figure 2.5). Gregerson and Lucas (1967) also reported a higher frequency of torsional forces occurring in this region, as it is the pivot for spinal rotation movements during locomotion. In addition, the overlapping, almost vertical orientation of the thoracic spinous processes from T5 to T8 also contribute to the reduction of extension range in this region (White 1969) (Figure 2.2).

Table 2.1 Spinal range of motion in the upper, mid and lower thoracic regions and zygapophyseal (Z) joint orientation, with units in degrees (deg), adapted from White (1969) and White and Panjabi (1978), with Z joint comments for the lower thoracic region also adapted from *Malmivaara et al (1987) and Singer et al (1989).

<table>
<thead>
<tr>
<th>Thoracic Region</th>
<th>Rotation (deg)</th>
<th>Flexion/ Extension (deg)</th>
<th>Lateral Flexion (deg)</th>
<th>Z joint orientation</th>
<th>Remarks on Z joints</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper T1-T4</td>
<td>10 to 12</td>
<td>5</td>
<td>7</td>
<td>Coronal &amp; oblique</td>
<td>similar to lower cervical joint</td>
</tr>
<tr>
<td>Mid T5-T8/9</td>
<td>8 to 10</td>
<td>8</td>
<td>5 to 7</td>
<td>Coronal &amp; vertical</td>
<td>Unique to thoracic region</td>
</tr>
<tr>
<td>Lower T9/10-T12</td>
<td>4 to 5</td>
<td>9 to 12</td>
<td>7 to 10</td>
<td>*Sagittal and vertical</td>
<td>*similar to upper lumbar joint</td>
</tr>
</tbody>
</table>

2.2.3 The Lower Thoracic Region

The lower thoracic region (T9 to T12), especially the last two thoracic vertebrae, is different from the rest of the thoracic spine (Moore 1985). The last two thoracic vertebrae articulate with the ribs only at the vertebral body and do not attach to the sternum, therefore they are called floating ribs (Moore 1985, Panjabi et al 1991). Movement of these vertebrae is therefore not as restricted as the upper 10 thoracic vertebrae (Markolf 1972, Roaf 1980, Malmivaara et al 1987).
The lower thoracic region is also the transition zone from the thoracic to lumbar region, and is characterised by a gradual change in the orientation of the zygapophyseal joints, from coronal to sagittal, especially in T11 and T12 vertebrae (Malmivaara et al 1987, Singer et al 1989). This region bears greater axial loading compared to the upper and mid thoracic regions (White 1969, Pal and Routal 1987, Edmondston et al 1994b). It has greater flexion, extension and lateral flexion ranges (White and Panjabi 1978) due to the sagittal orientation of the zygapophyseal joints and the lack of bony restriction from rib articulations with the sternum (Markolf 1972, Malmivaara et al 1987, Singer et al 1989). The sagittally orientated zygapophyseal joints however limit rotation in the lower thoracic region, in particular T11-T12 and T12-L1 joints. (Malmivaara et al 1987, Singer et al 1989) (Table 2.1).

![Figure 2.5 Schematic lateral view of a spinal motion segment consisting of two adjacent thoracic vertebrae, the intervening intervertebral disc with the anulus enclosing the nucleus, and the zygapophyseal or facet joint, formed by the articular processes. The anular lamellae are attached to the end-plates superiorly and inferiorly. Figure adapted from Moore (1985).](image)

### 2.3 ANATOMY AND GROWTH OF THE INTERVERTEBRAL DISC

The review of the anatomy and growth of the intervertebral disc is drawn predominantly from lumbar disc studies (Peacock 1951, Walmsley 1953, Taylor and Twomey 1988), with a few studies reporting specifically on developmental changes in the thoracic region (Bardeen 1904, Taylor 1975, 1988). The basic anatomy of the spinal intervertebral disc consists of a fibrous anulus, attached to the hyaline cartilaginous end-plates at the superior and inferior borders, and at the periphery of the vertebral body by fibers termed Sharpey’s fibers (Schmorl and...
Junghanns 1971, Johnson et al 1982). The inner anulus and end-plates encapsulate the gel-like mucoid nucleus pulposus in the centre of the disc (Figure 2.5).

2.3.1 The nucleus pulposus
The nucleus pulposus is a gelatinous mucoid tissue occupying about 50 to 60% of the disc volume, and located in the centre of the lumbar disc (Markolf and Morris 1974, Taylor 1975); is smaller for thoracic discs (Taylor 1975); and occupies only 25% of the cervical disc volume (Mercer and Jull 1996). In the prenatal and infant stage, the boundary between the nucleus and the innermost anular layers is distinct, however when the disc matures this clear demarcation is reduced (Walmsley 1953, Taylor and Twomey 1988). This morphological change is probably due to modifications in the tissue matrix with age and degeneration (Vernon-Roberts and Pirie 1977, Twomey and Taylor 1987, Thompson et al 1990).

The intervertebral disc, adjacent vertebrae and cartilaginous end-plates are derived from the sclerotome and the notochord in the embryo (Peacock 1951, Walmsley 1953, Roaf 1980). Notochordal cells are important in the growth and development of the intervertebral disc, especially in the prenatal stages (Butler 1988). Postnatally, notochordal cells are replaced by chondrocytes in the vertebral body, however in the disc, the notochordal cells continue to multiply to form the nucleus pulposus (Peacock 1951, Walmsley 1953). There is much controversy in the early literature as to whether the nucleus is a remnant of the notochord. However it is generally accepted that the notochord, in conjunction with perichordal cells, form the matrix of the nucleus pulposus (Peacock 1951, Walmsley 1953, Krämer 1990). As the nucleus develops, these notochordal cells are eventually replaced by chondrocytes and fibroblasts in mature discs, and are usually non-existent by 10 years of age (Walmsley 1953, Eyre et al 1989).

According to Walmsley (1953), expansion of the nucleus pulposus occurs with multiplication of the notochord cells and liquefaction of the inner most anular lamellae, to form loose collagen fibers, randomly orientated in the extracellular matrix, hence the gelatinous, less rigid structure of the nucleus compared to the anulus. Postnatally, the rate of growth of the nucleus exceeds that of the anulus and the end-plates until the age of 3, when it is reversed (Walmsley 1953, Taylor 1975). Due to the assumption of the upright posture, the nuclear position also changes during growth and development (Walmsley 1953, Taylor 1975). According to Taylor (1975), the nucleus moves from an anterior to a postero-central position in lumbar discs but remained antero-central in thoracic discs.
The matrix of the nucleus consists primarily of proteoglycans (65% dry weight), collagen (15-20% dry weight) and water (70-90% wet weight) (Hallen 1962, Adams et al 1977, Eyre and Muir 1977, Eyre 1988, Scott et al 1994). With increased age, the water content in the nucleus decreases at a faster rate compared to the anulus (Olczyk 1994c, Scott et al 1994). Thereafter in adulthood (> 60 years old), there is little change in water content in both the anulus and the nucleus (Twomey and Taylor 1987). However, most pressure studies on the disc still consider the nucleus as behaving as a fluid, redistributing the axial compression load equally in all directions, to the anulus and the end-plates (Nachemson 1965b). The water content in the nucleus is directly related to the proteoglycan content, which is a highly hydrophilic component, drawing water into the nuclear matrix (Adams and Muir 1976, Urban and Maroudas 1980, Bayliss et al 1988, McDevitt 1988). This uptake of water into the nucleus enables it to maintain the height of the disc, which is important for the flexibility of the spinal segment. (Markolf and Morris 1974, Pooni et al 1986).

2.3.2 The Anulus Fibrosus

While the nucleus is formed predominantly from the notochord cells, the anulus is developed from mesodermal cells of the anterior and posterior sclerotomes (Bardeen 1904, Peacock 1951, Walmsley 1953). In the infant disc, the inner anulus or perichordal zone of the disc has a hyaline cartilage appearance, however the presence of collagen fibers in the outermost part of the perichordal tissue gives it a more fibrous appearance (Walmsley 1953, Butler 1988, Taylor and Twomey 1988). It is the anular cells in the innermost layers near the nucleus pulposus that contribute to the growth of both the anulus and the nucleus pulposus (Peacock 1951, Walmsley 1953). In the outer anulus, growth is seen mainly as an increase in lamella thickness and not an increase in lamellae numbers (Herbert et al 1975), probably because it is the diameter of collagen fibers that increases with age (Hickey and Hukins 1982, Guiot and Fessler 2000). According to Walmsley (1953), the anterior lumbar anulus in the prenatal stage grows at a faster rate compared to the posterior anulus, hence pushing the nucleus posteriorly in the lumbar discs. These nuclear positional changes in the lumbar discs were also observed by Taylor (1975).

The outermost layer of the anulus fibrosus of the lumbar disc consists of 12 to 16 concentric lamellae of collagen fibers which run obliquely from the vertebra above to the one below (Butler 1988, Hukins 1988, Krämer 1990) (Figure 2.6). The collagenous fibers in each lumbar anular lamellae are tilted obliquely at a 65º to 70º angle to the vertical axis, and are orientated in opposite directions in each alternate lamella, in a criss-cross manner (Galante 1967, Pooni et al 1986, Hukins 1988).
It has been assumed that the anular morphology in the lumbar region would be similar for the thoracic and cervical regions, however studies have shown that there are slight morphological variations between the three spinal regions. For example, the tilt angle of the fibers in thoracic (White 1969) and cervical discs (Mercer and Bogduk 1999) is 60º to 65º to the vertical, which is slightly less than that of the lumbar region; and there are also less concentric lamellae (approximately 6 to 9) in the thoracic discs (Galante 1967). According to Mercer et al (1999), the anular fibers in the cervical discs are interwoven in a criss-cross manner instead of in distinct laminae; and the anterior anular fibers are crescentric, not concentric as in lumbar discs, being thicker anteriorly and tapering posterior-laterally. Mercer and Bogduk (1999) also report that the posterior anular fibers of cervical discs are not oblique as in lumbar discs, instead are vertically orientated and interwoven. They also suggest that this posterior arrangement may be similar for thoracic discs. In addition, the concentric layers of the lumbar anulus do not form complete rings around the nucleus, instead 40 to 60% of the layers usually attenuate and bifurcate to enclose the termination of another lamina (Walmsley 1953, Marchand and Ahmed 1990). Marchand and Ahmed (1990) reported a higher frequency of bifurcation at the posterior lateral parts of the lumbar disc, hence this region is prone to fissuring.

Krämer (1990) suggested that all structures of the intervertebral disc that are important for mechanical function are present from the time of birth. The presence of these alternating criss-cross lamellar patterns and the oblique angle of the collagen fibers are established in the

Figure 2.6 Schematic representation of a typical intervertebral disc showing the alternating layers of anular lamellae with fibers titled at a 70° angle to the axis. Figure taken from Boden et al (1991).
spinal disc even before birth, and do not change significantly with age, hence they are not formed as a response to mechanical or gravitational forces (Peacock 1951, Walmsley 1953, Hickey and Hukins 1982). According to Naylor (1954) and Galante (1967), the ability of the anulus to control spinal torsional and bending movements and to resist the outward pressure of the nucleus during compression loading is dependent not only on the number of collagen fibers but more importantly, on the tilt angle of these fibers in the anulus.

2.3.3 Gender-related differences in disc development
The disc grows rapidly in the first 2 years of life, with another growth spurt at age 12 years (Brandner 1970, Taylor 1975). The growth of the intervertebral disc is gradual and slower than the vertebral body, probably due to the poor blood supply to the disc, reaching their final shape in the second decade of life, when they eventually stop growing (Brandner 1970, Taylor 1975, Krämer 1990). Taylor and Twomey (1988) reported that gender dimorphism exists in the development of the vertebral body and discs, especially after adolescence (Taylor 1975). They observed that the transverse growth of the vertebral body and discs was faster in males compared with females, and vice versa for vertical growth. This difference in growth rates probably resulted in the marked lordosis and greater lumbar spinal range of movement in adolescent females compared to males (Nachemson et al 1979, Van Herp et al 2000). Generally, the thoracic (Singer and Goh 2000) and lumbar (Nachemson et al 1979) vertebral bodies are thicker in males compared to females (Brandner 1970). According to Brandner (1970), the adolescent female is reported to be more prone to spinal deformity due to the smaller and more slender (long and slim) vertebral body. Singer and Goh (2000) also found that the thoracic discs in adult males are generally thicker compared to females.

2.4 Anatomy and biomechanics of thoracic intervertebral disc
Reports by Peacock (1951), Hukins (1988) and McDevitt (1988) have shown that the matrix of the spinal disc is similar in composition to hyaline articular connective tissue in the body. However unlike the articular cartilage, which provides a mainly lubricating function, the morphology of the intervertebral disc is structured to bear large mechanical loads while at the same time providing flexibility to a semi-rigid spinal column (Eyre 1988, Hukins 1988, Bogduk and Twomey 1991, Buckwalter 1995). Eyre (1988) suggests that the morphological arrangement of the collagen fibers in the anulus, and the different biochemical composition of the anulus and nucleus, makes it a specialised and unique cartilaginous connective tissue,
enabling it to bear large mechanical loads and to withstand bending and twisting forces during spinal motion (Hukins 1988, Adams and Dolan 1995). Motion of the spine can be described as relating to either: a single motion segment, a series of motion segments (spinal region), or as movement of the entire spine (Maiman and Pintar 1990). A mobile spinal segment refers to the intervertebral disc, with adjacent vertebral bodies superiorly and inferiorly, the supporting ligaments and zygapophyseal or facet joints (Moore 1985, Hukins 1988) (Figure 2.5).

### 2.4.1 Disc mechanics - Stability and flexibility

The anatomy and growth patterns of the intervertebral discs in the different spinal regions are generally similar, however the shape and size of the discs in each spinal region is distinct and is an important factor to consider in spinal biomechanics. Thoracic discs are typically narrower and more circular compared to the elliptical shape of cervical and lumbar discs (Peacock 1951, Kapandji 1975, Pooni et al 1986) (Figure 2.7). In the upper thoracic region, the discs are small and elliptical, similar to cervical discs, and the shape gradually changes to circular or triangular in the mid thoracic region, becoming more elliptical again in the lower segments (Pooni et al 1986, Singer and Goh 2000). The cross-section and height of thoracic discs and vertebrae increases craniocaudally, in tandem with the increasing axial loading (Edmondston et al 1994b). According to Goh et al (1999) the anterior thoracic disc height varies from 4 mm in the upper thoracic region to 6 mm in the lower thoracic region, and is generally thicker posteriorly, resulting in a wedge-shape configuration especially in the mid thoracic region. The lowest anterior disc height however, is reported at T4-T5 disc, measuring approximately 2.5 mm (Pooni et al 1986, De Smet et al 1988, Goh et al 1999), which is the apex of the thoracic kyphosis.

According to Schmorl and Junghann (1971) and Goh et al (1999), the thoracic discs do not usually contribute to the thoracic kyphosis as much as the thoracic vertebrae, especially in older women, where the kyphosis is accentuated by osteoporosis and anterior wedging of the vertebrae in the mid thoracic region (Figure 2.8). The same authors also suggest that in older men, the progression of age-related or senile kyphosis is more commonly due to a reduced anterior disc height in the mid thoracic region. Probably the thicker thoracic discs in males compared to females (Singer and Goh 2000), is more prone to disc wedging with increased age.

It was mentioned above, that the ability of the anular fibers to resist torsional forces is dependent on the tilt angle of the lamellae. In addition to this, Farfan et al (1970) and Pooni et al (1986) proposed that the shape and size of the intervertebral disc were also important
factors as well. The amount of stability and flexibility that is provided by the intervertebral disc within a spinal motion segment, is dependent on the cross-section and height of the disc, respectively (Pooni et al 1986). The combination of a smaller thoracic disc height to cross-section ratio (Pooni et al 1986) and a circular disc shape, especially in the mid thoracic region, confers stability and optimal support during torsional stresses (Markolf 1972, Horst and Brinckmann 1981, Hukins 1988 Pooni, 1986 #87). Conversely, the elliptical shape and greater disc height seen in upper and lower thoracic, cervical and lumbar discs, confer better stability during flexion of the spine but offers little resistance to rotation (Markolf 1972, Pooni et al 1986, Hukins 1988).

![Image of transverse horizontal sections of human thoracic discs](image)

**Figure 2.7** Photograph of transverse horizontal sections of human thoracic discs from a 78-year old male subject showing all levels from T1-T2 to T12-L1 (reading from right to left, and top to bottom). In the upper thoracic region, the discs are small and elliptical, gradually changing to circular or triangular in the mid thoracic region and becoming more elliptical in the lower segments. The size of the discs also increases from cranial to caudal.
Figure 2.8 MR image of an 83-year old female showing anterior osteophytes at T5-T6 vertebral edge [A], anterior disc wedging at T6-T7 level [B] and an anterior vertebral wedge deformity with posterior osteophytes at T7-T8 [C], which is the apex of the kyphosis.

2.4.2 Disc mechanics – Weight bearing or compression loading

Besides providing stability and flexibility to the spine, the intervertebral disc is also important in resisting compressive forces (Adams and Hutton 1988). Lumbar spine literature indicates that the intact nucleus is important in dissipating the compression force hydrostatically to the surrounding anular fibers during axial loading (Nachemson 1965b, Galante 1967, Hukins 1988). Adams and Hutton (1980) reported that the thicker the intervertebral disc, the greater the proportion of weight borne by the discs compared with the zygapophyseal joints. Therefore in thinner thoracic discs, 25% of the axial load is borne by the zygapophyseal joints and 75% by the discs (Pal and Routal 1986), compared to 18% and 82%, respectively for larger lumbar discs (Pal and Routal 1987).

During axial compression, the gel-like nucleus transfers these forces radially to the annular lamellae, resulting in a circumferential or tangential hoop stress (Hukins 1988) (Figure 2.9). The ability of the intervertebral disc to resist compressive loading is evidenced by experiments on lumbar discs, where the vertebral end-plates fractured under high compressive loading, with no prolapse or damage to the discs (Roaf 1960, Shirazi-Adl et al 1984, Adams...
and Dolan 1995). Even when the nucleus is removed, the ability of the disc to withstand compression force is not severely affected, and the only difference is that there is increased bulging or distention of anular lamellae (Markolf and Morris 1974, Brinckmann 1986).

Figure 2.9 Schematic diagram of the transfer of axial compression forces in the nucleus to the anulus. Disc internal pressure (P) in the nucleus is balanced axially by an applied compressive force (F), and radially by a circumferential tangential force (T) in the anulus. Figure taken from Hukins (1988).

Injury to the anular fibers usually occurs when compression forces are applied in combination with spinal flexion or rotation (Adams and Hutton 1988, Adams and Dolan 1995). Flexion and rotation forces are resisted predominantly by the zygapophyseal joints, interspinous ligaments and the collagenous fibers in the anulus (Farfan et al 1970, Adams and Hutton 1988), and in the thoracic region, by the attachment of the ribs and sternum (Andriacchi et al 1974). In the flexed, extended or rotated positions, usually only 50% of the anular fibers are positioned to resist the bending or twisting forces (Hukins 1988). The zygapophyseal joints and joint capsules together can provide up to 45% of resistance to torsional forces in the lumbar region (Farfan et al 1970). As mentioned above, in the upper and mid thoracic regions, the zygapophyseal joints have a predominantly coronal orientation which restricts thoracic flexion, or shear, but allows thoracic rotation instead (Gregerson and Lucas 1967, Singer and Goh 2000). Although the thoracic rotation range is small (< 12°) however Farfan et al (1970) reported that even small rotation ranges of 2° or 3°, are sufficient to inflict anular tears in lumbar discs if the force is high or repeated often enough.
Literature on lumbar disc degeneration suggests that a change in the anular morphology, compared to the nucleus, is a major contributor to disc degenerative changes (Roaf 1960, Gregerson and Lucas 1967, Farfan et al 1970, Markolf and Morris 1974). Animal studies have shown that a cut or injury to the anular fibres results in fibrous tissue replacement, with collapse of the anular lamellae (Osti et al 1990), followed by disc narrowing and osteophyte formation (Melrose et al 1992). An anular tear in the lumbar disc is also associated with reduced spinal or stability or an abnormally increased range of spinal movement, especially in rotation (Schmidt et al 1998).

Although axial compression forces alone do not result in anular tears or nuclear prolapses (Roaf 1960, Shirazi-Adl et al 1984, Adams and Dolan 1995), however discs exposed to sustained or repeated axial compression loading or twisting movements, such as during occupational or recreational activities involving constant sitting or lifting, can interrupt disc nutrition and potentially disrupt matrix synthesis, leading to cell apoptosis (Lotz and Chin 2000) and disc degeneration (Ohshima et al 1995, Handa et al 1997, Hutton et al 1999). Hutton et al (1999) observed disc degenerative changes in canine spinal discs after 8 months of prolonged axial compression forces. Studies have shown that sustained activities, such as carrying school bags (Wedderkopp et al 2001) or long periods of sitting (Kelsey and White 1980) are associated with increased spinal pain. More importantly, it is the speed, frequency and combination of the lifting and twisting forces that will determine the extent of damage to the disc anular fibers (Kellgren and Lawrence 1958, Hickey and Hukins 1980, Adams and Hutton 1986b, Masset et al 1998).

### 2.5 Degenerative Changes in the Intervertebral Disc

The intervertebral disc is considered to be one of the chief structures implicated in painful conditions of the spine (Vanharanta et al 1987, Krämer 1990, Salminen et al 1999). In spite of the increased stability and reduced range of spinal movement in the thoracic region, degenerative changes in thoracic discs are not uncommon. A review of the radiological and cadaver studies suggest that thoracic discs are prone to anular and nuclear degenerative changes (Lawrence 1977, Singer 1997), and herniation (Awwad et al 1992, Brown et al 1992, Videman et al 1995a, Wood et al 1995, Singer 2000), osteophyte formation (Nathan 1962, Lawrence 1977, Brown et al 1992, Videman et al 1995a, Wood et al 1997, O'Neill et al 1999, Singer 2000), and end plate lesions or Schmorl’s nodes (Hilton et al 1976, Lawrence 1977, Awwad et al 1992, Singer 2000, Pfirrmann and Resnick 2001) (Figure 2.10). In addition, the spinal canal in the thoracic region is relatively small compared to the cervical and lumbar
regions, especially between T3 to T9, therefore small anatomical or pathological disturbances, such as disc herniations in the mid to lower thoracic regions, may induce profound neurologic consequences (Panjabi et al 1991, Skubic and Kostuik 1991, Brown et al 1992).

Unlike the lumbar disc, where disc degeneration is reported as a definite cause of low back pain (Cassinelli et al 2001), the clinical relevance of these morphological disc degenerative changes in thoracic region is tenuous, especially in the light of MRI thoracic disc studies (Videman et al 1995a, Wood et al 1995). Literature reporting on symptomatic degenerative changes in the thoracic region usually relates to surgically operated thoracic disc herniation, the incidence of which is low (0.5 to 5% per thousand disc herniations) (Love and Schorn 1965, Russell 1989). With more advanced imaging techniques, the incidence of asymptomatic thoracic disc herniation and degeneration has been found to be markedly higher than previously reported.

![Figure 2.10](image)

**Figure 2.10** Disc degenerative changes in the lower thoracic spine showing nuclear clefts and discoloration [A], anular clefts [B], Schmorl’s nodes [C] at T12-L1, and osteophytes (Singer et al ) at T11-T12.

Using T2- and T1-weighted thoracic MRI, Wood et al (1995) found that 66 of 90 asymptomatic subjects (73%) had at least one or more characteristic features of disc degeneration and 37% had thoracic disc herniation. This incidence of asymptomatic disc degeneration in the thoracic region is higher than that reported for the cervical (37% to 44%)
(Teresi et al 1987, Boden et al 1990b) and lumbar (23% to 58%) regions (Boden et al 1990a, Weishaupt et al 1998, Elfering et al 2002). Longitudinal studies on asymptomatic discs found that these thoracic nuclear herniations may remain the same, reduce or enlarge in size (Wood et al 1997). Similarly, lumbar discs studies also revealed that degenerated discs may subsequently develop herniations (Elfering et al 2002), become symptomatic (Boos et al 2000) after 2 to 5 years (Elfering et al 2002), or remain asymptomatic (Wood et al 1997, Borenstein et al 2001). The implications of these findings suggest that while degenerative disc changes in the thoracic region are not uncommon, painful syndromes requiring surgery are not as common compared to the lumbar and cervical regions (Singer 2000). Nevertheless, when symptomatic, thoracic disc herniations may result in painful and disabling motor and autonomic dysfunctions (Skubic and Kostuik 1991), and often mimic cervical (Chok and Wong 2000) and lumbar disc herniation symptoms (Ito et al 1999, Lyu et al 1999).

2.6 MECHANISM OF DISC DEGENERATION IN THE THORACIC REGION

2.6.1 Spinal regional and mechanical factors

Much of the thoracic spine literature suggests a high prevalence of disc degeneration in the mid and lower thoracic regions (Nathan 1962, Hilton et al 1976, Lawrence 1977, Wood et al 1995), with less disc degeneration in the upper thoracic region (Ten Have and Eulderink 1980, Boyle et al 1998). As discussed above, spinal discs that are exposed to prolonged or cyclical compression loading combined with extreme spinal ranges are predisposed to injury and degenerative changes. Therefore reasons for the degeneration trend in the thoracic spine may be due to the lesser axial load (Pal and Routal 1986), greater stability and reduced flexibility (Andriacchi et al 1974, Pooni et al 1986) in the upper thoracic region compared to the mid and lower regions (White and Panjabi 1978) (See Table 2.1).

The higher prevalence of disc degeneration in the mid thoracic region is probably due to the increased and prolonged axial loading due to the kyphotic curvature (Schmorl and Junghanns 1971, Goh et al 1999), and a higher frequency of rotational movements especially during walking (Gregerson and Lucas 1967). The discs and vertebral bodies in the mid thoracic region, especially the anterior aspect, are exposed to sustained compression forces as they lie at the apex of the concavity of the thoracic kyphosis (T6 to T8) (Pal and Routal 1987, Singer et al 1990, Goh et al 1999). A number of studies have reported a high prevalence of anterior wedge fractures in the mid thoracic region, usually from T6 to T8, especially in osteoporotic elderly females (De Smet et al 1988, Hedlund et al 1989, Melton et al 1993). In the absence of osteoporosis and osteopenia, an increased kyphotic curvature probably results in excessive
compression on the anterior aspect of the anulus in the mid thoracic region, hence a higher potential for disc degeneration, especially in older males (Schmorl and Junghanns 1971).

The mid thoracic region, in particular T7, is the pivot or fulcrum during locomotion, hence is exposed to a higher frequency of torsional forces (Gregerson and Lucas 1967), which is potentially damaging to the anular fibers (Snijders 1969, Farfan et al 1970). As mentioned above, the predilection of disc degeneration in the mid thoracic region is probably due to the combination of sustained compression loading due to kyphosis (Schmorl and Junghanns 1971) and the higher frequency of torsional movements (Gregerson and Lucas 1967).

Anterior osteophytes in the mid to lower thoracic regions usually occur on the right side, as the abdominal aorta lies on the left side of the thoraco-lumbar spine (Nathan 1962, Schmorl and Junghanns 1971, O'Neill et al 1999). According to Nathan (1962) osteophytes are formed due to stress and strain from the disc pulling on the rim of the vertebral body where the anular fibers are attached. Osteophytes occur especially in regions with prolonged compression loading or high ranges of spinal movement or instability, and are more common in males compared to females (Nathan 1962, Schmorl and Junghanns 1971, O'Neill et al 1999). It is not common to find osteophytes in the sacrum, as they usually develop in spinal levels with an intervertebral disc and spinal movement (Nathan 1962, O'Neill et al 1999). Calcification of the disc secondary to degeneration and herniation, is also common in the mid thoracic region leading to fusion of the disc and a further reduction in thoracic range of motion (Schmorl and Junghanns 1971, Chin et al 1987).

Similarly the discs in the lower thoracic region are also prone to disc degeneration as it is exposed to increased spinal flexion and extension ranges (White and Panjabi 1978) and a higher axial load (Pal and Routal 1987). In particular Singer et al (1989) and Malmivaara et al (1987) reported a higher potential for rotational injuries at the thoraco-lumbar junction or transition zone due to the abrupt change in the orientation of the zygapophyseal joints from coronal in the thoracic region to sagittal in the lumbar region.

2.6.2 Age and gender related factors

It is also apparent from the literature that age is a significant contributing factor to disc degeneration in the human spine (Lawrence 1977, Miller et al 1988, Videman et al 1995a, Boyle et al 1998, Singer 2000). During the life span, the vertebral column is exposed to a variety of compression and rotational forces as mentioned above, which, if repeated or
sustained over long periods of time, can result in disc and vertebral pathological changes (Schmorl and Junghanns 1971, Adams and Dolan 1995).

In attempting to trace the sequelae of disc degeneration over the life span, most lumbar disc studies suggest a series of changes in the disc, commencing with a loss of proteoglycan and water in the nucleus with increasing age (Adams et al. 1996b, Prescher 1998). There is a reduction of water in the disc, especially the nucleus from 90% at birth to 74% in the first decade of life (Krämer 1990). Roaf (1960) found that high compression axial load in a normal hydrated disc does not damage the anulus, instead leads to end-plate ruptures. In a dehydrated aged disc however, the hydrostatic change in the nucleus disrupts the biomechanical relationship between the nucleus and the anulus (McNally and Adams 1992). A dehydrated nucleus is not able to resist the axial load during compression, therefore this load is transferred to the anulus, resulting in fissures (Roaf 1960, Adams et al 1996a), or to the vertebral body, resulting in end-plate disruption (Roaf 1960).

Dehydration in the disc may not always be accompanied by a loss of disc height, which is commonly seen in most degenerated lumbar discs (Twomey and Taylor 1987). Therefore a possible differentiating feature between a pathological or traumatic disc degeneration and that due to ageing per se, is the loss of disc height in the former (Twomey and Taylor 1987, Sether et al 1990, Prescher 1998). In the absence of trauma, a normal aged disc is characterised by a dehydrated disc, with fibrosed nucleus and anulus, with little or no loss of disc height (Vernon-Roberts and Pirie 1977, Modic et al 1984, Twomey and Taylor 1987). It is the marked loss in disc height secondary to anular fissures and loss of nuclear material that eventually leads to an increase in the stiffness of the spine (Hickey and Hukins 1980).

Various thoracic disc studies support the association between disc degenerative changes (Videman et al 1995a) and osteophytic changes (Nathan 1962, O'Neill et al 1999) with increased age. End-plate lesions or Schmorl’s nodes and disc herniations in the thoracic region however, have not been shown to be associated with increased age (Schmorl and Junghanns 1971, Hilton et al 1976, Wood et al 1995). The prevalence of thoracic disc herniations tends to peak in individuals between the fourth to sixth decade, with a low incidence in the elderly (Russell 1989), and the prevalence of end-plate lesions are found equally in all age groups (Hilton et al 1976, Saluja et al 1985, Pfirrmann and Resnick 2001).

The reason for the higher prevalence of Schmorl’s nodes in the younger age group may be due to the lesser viscosity of the nucleus, which is still fluid enough to herniate through the weakened end plates (Schmorl and Junghanns 1971). End-plates are weakened either
congenitally, due to remnant or persistent vascular channels (Schmorl and Junghanns 1971, Resnick and Niwayama 1978, Saluja et al 1985, Pfirrmann and Resnick 2001), or by trauma, when the end-plates fail or burst due to sudden high compression loading (Roaf 1960, Resnick and Niwayama 1978, Shirazi-Adl et al 1984, Adams and Dolan 1995).

Studies of gender-related influences on the incidence of disc degeneration have not been conclusive. From an epidemiological radiographic study, Lawrence (1977) reports a high incidence of thoracic disc degeneration in women compared to men especially in the mid thoracic region. Females, especially in older women, are more prone to osteoporotic changes in the mid thoracic vertebrae, which results in a higher prevalence of anterior wedge fractures and increased kyphosis, with a lesser incidence of disc degenerative changes (De Smet et al 1988, Hedlund et al 1989, Melton et al 1993). Gender-related musculoskeletal changes especially for women more than 50 years of age, are probably related to osteoporosis, which is associated with hormonal changes after menopause (Kanis and McCloskey 1992).

Thoracic cadaver (Singer 1997), radiological (Tovi and Strang 1960, Arce and Dohrmann 1985) and MRI studies (Videman et al 1995a, Wood et al 1995) generally found a higher prevalence of thoracic anular tears, disc bulge and herniation in males compared to females instead. There are also more frequent osteophytes and Schmorl’s nodes in males compared to females (Schmorl and Junghanns 1971, Scoles et al 1991, Prescher 1998, O’Neill et al 1999). Males tended to have a higher prevalence of concentric tears in the anterior margin of the anulus fibrosus (Schmorl and Junghanns 1971), probably due to the increased compression and rotational forces during occupational and recreational activities (Gregerson and Lucas 1967, Schmorl and Junghanns 1971, Wood et al 1995).

2.7 BIOCHEMISTRY OF THE DISC

The two morphologically distinct regions of the intervertebral disc are also biochemically different. Apart from a few thoracic studies, much of the literature review on the biochemical aspect of the intervertebral disc is derived from lumbar disc studies, both on human and animal subjects. Much is known about the two main protein components in the disc matrix, collagen and proteoglycan, which is found closely bound together and are responsible for the tensile and hydrostatic properties of the disc (Urban and Maroudas 1980, Hukins 1982, Eyre 1988, Buckwalter 1995). However the factors that influence the biochemical change in the disc resulting in disc ageing and degeneration, remains an enigma.
Recent biochemical studies on spinal discs further suggest that the tensile strength of collagen fibers may be more accurately reflected by the presence of mature, non-reducible post-translational collagen crosslinks in the disc matrix (Eyre 1979, Kivirikko and Myllyla 1982, Eyre et al 1988, Duance et al 1998, Pokharna and Phillips 1998). The ability to isolate and detect the presence of mature collagen and elastin crosslinks in various human tissues has been facilitated with recent advances in high performance liquid chromatography (HPLC) (Eyre et al 1984a, Takahashi et al 1995, Chen et al 2000).

2.8 COLLAGENS IN THE DISC

Collagen is the most abundant macromolecular protein and is found in virtually all tissues of the body, being responsible for the structural and functional integrity of tissues such as bone, cartilage, tendon, ligament, skin and spinal discs (Prockop et al 1979, Eyre 1980). There are currently at least 21 different collagen types reported in the literature, and the spinal disc matrix has 11 of these collagen types (Eyre 1988, Boos et al 1997b, Eyre et al 2002) (Table 2.2). The main collagen types in the anulus and the nucleus are collagen Type I and II fibers, which make up 80% of the dry weight of the disc (Eyre and Muir 1976, Eyre 1988). The significance of the proportion of different collagens in the disc matrix has a bearing on the type and the extent of post-translational collagen crosslinks formed (Eyre 1988, Eyre et al 1989).

Collagen Type V is usually found closely bound with Type I collagen fibers, especially in the anulus, and Type IX collagen with Type II fibers in the nucleus (Eyre 1988, Eyre et al 1989, Eyre et al 1991, Eyre 1995). The close association of the different collagen types is important to stabilise the collagen network and to regulate the size of the collagen fibers in the matrix (Eyre et al 1989). The nuclear matrix consists predominantly of Type II collagen which is responsible for load bearing, compared to the anulus which has more Type I collagen, hence is more suited to resist tension and torsional stresses (Roaf 1980, Eyre 1988).

The anulus consists of densely packed, alternating layers of collagen fibers (44 –70% of disc dry weight) (Hallen 1962, Eyre and Muir 1977, Eyre 1988) separated by elastin and proteoglycan molecules, hence the matrix is fibrocartilaginous (Hallen 1962, Galante 1967, Krämer 1990, Scott et al 1994). Although the anular matrix has slightly more Type II (65%) compared with Type I (30 to 40%) (Herbert et al 1975, Eyre and Muir 1976), however the portion of each collagen type varies for different regions of the anulus. The outer anulus has a higher proportion of Type I (84%) and less Type II (16%) fibers, while a reverse trend is
reported for the inner anular tissues (Eyre and Muir 1976). Type I collagen fibers are long, with the ability to resist high tensile forces, therefore a high concentration of these fibers is usually found in fibrous tissues such as the anulus, bone, tendon, ligaments and joint capsules (Adams et al 1977, Hutton et al 1999). In contrast, Type II collagens are shorter and characteristic of the hyaline connective tissues of articular cartilages which bear sustained compression loads, therefore they are found in the inner anular region and in the nucleus (Adams et al 1977, Eyre 1988, Hutton et al 1999).

**Table 2.2** Collagen types in the nucleus (NP) and anulus (AF) over the life span with different degeneration grades (0-V). Data adapted from †Nerlich et al (1997) and †Eyre (1988) and ††Eyre et al (Eyre et al 2002).

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<th>Adolescent Moderate (II-III)</th>
<th>Young Adult</th>
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<th>Adolescent Moderate (II-III)</th>
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- = absent in tissues  ( ) = minimally present  */**/*** = present in increasing amounts

The nucleus, in contrast to the anulus, is gelatinous with a high water (70 to 90% wet weight) and proteoglycan (65% dry weight) content, and fewer collagen fibers (15 to 25% of dry
weight) (Hallen 1962, Adams et al 1977, Eyre and Muir 1977, Eyre 1988, Scott et al 1994). The nucleus consists of mainly collagen Type II fibers, which are not orientated in any direction (Naylor et al 1954), and with thinner fibers (Eyre and Muir 1977), unlike the anulus. Bayliss et al (1988) proposed that the function of the few collagen fibrils in the nucleus is to interact with the proteoglycan molecules, therefore stabilise the nuclear matrix.

### 2.8.1 Collagen synthesis

Collagen Types I, II and III monomers share similar molecular dimensions and are known as interstitial or fibrillar collagens (Eyre 1988). Each fibrillar collagen molecule consists of three alpha chains wound together into a right-handed triple helical structure along most of the length of the molecule, with two non-helical telopeptide sites at its amino and carboxy terminals (Prockop et al 1979, Eyre 1980) (Figure 2.11[A]). Each alpha chain consists of three amino acids per turn with each alpha chain twisted in a left-handed helix as well (Prockop et al 1979, Eyre 1980). Collagen Type I consists of two $\alpha_1$(I) chains and one $\alpha_2$(I) chain, and collagen Type II consists of three identical $\alpha$(II) chains (Eyre and Muir 1976, Prockop et al 1979).

There are approximately 1,000 amino acids per chain, and the three major amino acids in the alpha chain are (G) glycine (30%), (X) proline (12%) and (Y) hydroxyproline (12%), which appear as a repeating sequence, Gly-X-Y (Prockop et al 1979, Krämer 1990). The triple helical structure is stabilised post-translationally by the hydroxyl group from the hydroxyproline amino acid (Prockop et al 1979). The majority of hydroxyproline in animal tissues are found in the collagen molecule, therefore it is used to analyse collagen content in the tissues (Kivirikko et al 1967).

### 2.8.2 Intracellular processes

Collagen is synthesised in chondrocytes and fibroblast cells, the process of which is highly regulated by genetic expression (Eyre 1988) (See Figures 2.11 [B] and 2.12). The fibroblast cells in the disc tend to form collagen Types I, III, V and VI, and hyaline chondrocytes tend to form Types II, IX and XI (Eyre 1988). The synthesis of collagen fibers consists of a series of complex processes that take place first intracellularly followed by extracellularly (Eyre 1980, Burgeson and Nimni 1992) (Figure 2.12). Within the fibroblast or chondroocyte cell, the collagen gene transcribes the type of collagen to be synthesised and this message is transferred to the messenger or mRNA (Burgeson and Nimni 1992). The mRNA for each
alpha chain is found in the cytoplasm and translated in the rough endoplasmic reticulum (ER) on membrane bound ribosomes (Burgeson and Nimni 1992).

Once secreted within the ER, the pro alpha chain is modified by a series of post-translational modifications of the amino acid residues, such as hydroxylation of proline and lysine residues by prolyl (3- and 4-) hydroxylase and lysyl hydroxylase, respectively (Prockop et al 1979, Eyre 1980). Hydroxylation of the lysine residues is important in providing sites for carbohydrate attachment (Kivirikko and Myllyla 1982, Robins 1982), and for proline residues to provide thermal stability to the collagen triple helix structure at body temperature (Prockop et al 1979, Eyre 1980). The action of lysyl hydroxylase on lysine residues is important to prepare the collagen molecule for crosslinking (Robins 1982, Eyre et al 1984b, Bailey et al 1998). A lack of this enzyme, such as in Ehlers Danlos VI syndrome, can result in deficient collagen crosslink sites, hence reduced crosslinks and loss of mechanical strength in the tissue matrix (Kivirikko and Myllyla 1982, Robins 1982, Eyre et al 1984a, Eyre et al 1984b, Bailey et al 1998). In contrast, too much hydroxylation results in impaired triple helix formation in osteogenesis imperfecta (Bailey et al 1998).

Figure 2.11 [A] Schematic diagram of the collagen fibre showing the primary protein sequence, helical α chains and fibril formation. Figure taken from Eyre (1980).
Figure 2.11[B] Schematic diagram of the intracellular and extracellular processes involved in collagen and crosslink synthesis. Figure taken from Van der Rest (1989).
Collagen and crosslink synthesis

**Intracellular processes**

**Procollagen synthesis**
1. mRNA is transcribed to form procollagen.
2. Collagen chains are hydroxylated by lysyl hydroxylase.
3. Further glycosylated by galactose and glucose transferase.
4. Collagen chains wind into triple helix through disulphide bonds forming helical and non-helical globular domains at each end.
5. Procollagen is secreted by golgi complex extracellularly by exocytosis.

**Fibril formation**
1. Procollagen is cleaved by proteinases at the non-helical ends to form tropocollagen.
2. Cleaved collagen assemble spontaneously into fibril aggregates.
3. Collagen molecules oxidised by lysyl oxidase to form reducible crosslinks or undergo Maillard reactions to form AGE.

**Extracellular processes**

**Crosslink formation**
1. Enzymatic crosslinks have 2 pathways -
   - Hydroxyallysine
   - Allysine
   - Lysyl oxidase
   - Reducible Crosslinks
   - Pyd and Dpd
   - Des and Isodes
   - Non reducible crosslinks
2. Tropocollagen in high sugar concentrations undergo glycation to form 2 types of AGE: non-crosslinked CML and crosslinked Pentosidine

**Figure 2.12** Summary of the intracellular and extracellular processes in collagen synthesis and crosslink formation.

After hydroxylation, the procollagen chains undergo glycosylation before the triple helix is formed (Eyre 1980). Galactose is added to the hydroxylysine residues by galactosyltransferase, and glucose is subsequently added by glucosyltransferase. Collagen Type II fibrils are usually more glycosylated and have more hydroxylysine residues compared to collagen Type I (Eyre 1979, Eyre 1980). The number of hydroxylysine residues is important as it provides the side chains for crosslinking extracellularly (Eyre 1979, Bailey et al 1998).

The assembly of the triple helix is said to be an entropy-driven process, formed by intra-chain disulphide bridges between N-terminal non-helical propeptide regions (Eyre 1980). Further winding of the helix occurs through inter-chain disulfide linkages at the non-helical C-terminal and even in the helical region (Prockop et al 1979, Kivirikko and Myllyla 1982). A lack of disulphide bonds prevents the triple helix formation (Prockop et al 1979). The resultant procollagen moves out of the ER, migrates to the golgi apparatus and is secreted through the cytoplasmic membrane by exocytosis (Prockop et al 1979, Burgeson and Nimmi 1992).
2.8.3 Extracellular processes
The procollagens are secreted from the cells and converted to collagen extracellularly, via a series of enzymatic cleavages (Prockop et al 1979). The two enzymes responsible for cleavage of the non-helical C- and N- propeptides are procollagen aminoprotease at the N terminal, and procollagen carboxyprotease at the C terminal (Prockop et al 1979). These cleaved collagen molecules, or tropocollagens, assemble spontaneously into a staggered array of fibrils and aggregates (Eyre 1980, Kivirikko and Myllyla 1982). At the ends of the cleaved N- and C- terminals are short non-helical extensions or telopeptides (Eyre et al 1984a). These two telopeptides and two triple helical sites at the two ends of the collagen molecule are important sites to form mature collagen crosslinks, in the presence of the enzyme lysyl oxidase (Eyre 1980, Eyre et al 1984a). Tropocollagen matures either enzymatically to form tri-functional mature crosslinks or nonenzymatically to form advanced glycation end-products (AGE).

2.8.4 Collagen Crosslink Pathways
Collagen fibers, especially the fibrillar collagen Types I, II and III, are capable of forming mature, strong covalent inter- and intra-molecular bonds or crosslinks, which are responsible for the tensile strength in the disc collagenous matrix (Eyre 1980, Kivirikko and Myllyla 1982, Eyre et al 1989) (Figure 2.12). Collagen crosslinks are formed by oxidative deamination of amino groups from lysyl and hydroxylysyl residues to reactive aldehydes by lysyl oxidase (Prockop et al 1979). Lysyl oxidase requires copper and pyridoxyl as co-factors with molecular oxygen acting as the hydrogen acceptor, and acts mainly at the N- and C-terminal telopeptides of hydroxylysine and lysine residues (Kivirikko and Myllyla 1982).

These reactive aldehydes undergo a series of condensation reactions to form reducible crosslinks, which are easily solubilised by sodium borohydride (Eyre et al 1984b). During growth and development, the reducible crosslinks undergo spontaneous reactions to form mature collagen crosslinks which are non-reducible, that is, “insoluble” (Eyre et al 1984b, Bailey et al 1998). In human spinal discs, reducible crosslinks decrease with age and are not detectable after 25 years of age, when skeletal growth and development are completed, instead mature crosslinks increase and remain constant after that (Herbert et al 1975, Kivirikko and Myllyla 1982, Eyre et al 1988). At the same time, lysyl oxidase activity in most connective tissues also decreases with increased age (Bailey et al 1998).

Fibrillar collagens have two main aldehyde-mediated cross-linking pathways, allysine and hydroxyallysine aldehyde pathways (Eyre et al 1984b), of which the predominant pathway in
the anulus and nucleus is hydroxyallysine aldehydes (Eyre 1988). The hydroxyallysine pathway results in collagenous hydroxypyridinium crosslinks and the allysine pathway, on lysine derived aldehyde, is involved in the formation of collagen crosslink dehydrohydroxylysinonorleucine as well as elastin crosslinks, desmosine and isodesmosine (Eyre et al 1984b). Lysyl oxidase is responsible for crosslink formation in both hydroxyallysine and allysine pathways (Kivirikko and Myllyla 1982). According to Kivirikko and Myllyla (1982) and Eyre (1988), the hydroxyallysine route of collagen cross-linking are more stable crosslinks compared to lysine residues, and are commonly found in connective tissues that bear large mechanical loads, such as spinal discs, bone, tendon and articular cartilage.

Both Types I and II collagen form the same hydroxypyridinium crosslinks, however collagen Type II fibers are able to form twice the number of crosslinks compared to Type I fibers (Eyre 1987, Eyre 1988). Gibson et al (1982) suggested that collagen Type I can only form 1 mol of crosslinks per mol of collagen because of a lack of lysine at the carboxy-telopeptide crosslinking site of the \( \alpha2(I) \) chain. The nucleus, which has the highest concentration of collagen Type II fibers, therefore forms more hydroxypyridinium crosslinks compared to other vertebrate connective tissue (Eyre et al 1984b). Eyre (1988) proposed that the number of collagen fibers crosslinked in the nucleus can reach up to 3 mol/mol collagen in young mature tissues. This high density of collagen crosslinks in the nucleus may compensate for the low collagen content, and provides for matrix stability by withstanding frictional and shear forces during axial loading and spinal movements.

Various authors have proposed that any alteration of the collagen crosslink content in the disc matrix will impair disc function, in particular, the ability to withstand mechanical loading (Duance et al 1998, Pokharna and Phillips 1998), and may increase matrix susceptibility to degradation by collagenase (Vater et al 1979, Robins 1982). A lack of lysyl oxidase results in deficient collagen and elastin crosslinks in connective tissues which become loose with many folds, such as in Ehlers-Danlos V syndrome or cutis laxa (Eyre 1987). A lack of copper co-factors or excessive dietary intake of zinc, will also produce connective tissues that are brittle and easily damaged, for example, a high incidence of aortic aneurysm in Marfan syndrome (Kivirikko and Myllyla 1982, Eyre et al 1984a).

### 2.8.5 Pyridinoline and deoxypyridinoline

The primary two mature collagenous crosslinks present in cartilaginous and bony connective tissues are hydroxyllysyl pyridinoline or pyridinoline (Pyd) (Fujimoto 1977) and lysyl
pyridinoline, also known as deoxypyridinoline (Dpd) (Ogawa et al 1982), which are also commonly present in spinal discs (Eyre et al 1989) (Figure 2.13). They are formed from lysyl oxidase-mediated reactions on lysyl and hydroxylysyl amino group residues (Eyre 1987). Pyd was first discovered and named by Fujimoto (1977) using bovine tendon, and Dpd by Ogawa et al (1982) using bovine femoral bone. Pyd and Dpd are easily detected due to their acid stability and fluorescence at an excitation wavelength of 295 nm, and an emission wavelength of 395 nm on ion-exchange reverse-phase HPLC (Fujimoto 1977, Eyre et al 1984a, Bailey et al 1998).

![Pyridinoline and Deoxypyridinoline](image)

**Figure 2.13** Structure of the pyridinium crosslinks hydroxylysyl pyridinoline (Pyridinoline) and lysyl pyridinoline (Deoxypyridinoline). Figure taken from Eyre (1987).

Pyd and Dpd are mature, non-reducible trifunctional 3-hydroxypyridinium cross-links, derived from two hydroxyallysine and one hydroxylysine residue (Fujimoto 1977, Eyre 1988). Besides spinal discs, these crosslinks are also found in skeletal and cartilage tissues (Takahashi et al 1994), but have not been found in human skin or lens tissue as the crosslinks are denatured by ultraviolet light (Eyre et al 1984a, Eyre et al 1984b, Bailey et al 1998). Dpd in particular, is present in greater concentrations in dental and skeletal tissues, hence is commonly used as a urinary marker for bone collagen turnover in musculoskeletal and joint diseases (Eyre et al 1984a, Takahashi et al 1994, Eyre 1995, Randall et al 1996). The ratio of Pyd to Dpd varies for different connective tissues. Typical ratios for bone tissues are 3.5:1, and for cartilage > 40:1, which is similar to that for disc tissues (>50:1) (Eyre 1995). Pyd is therefore the major hydroxypyridinium crosslink in the disc tissue, compared with Dpd.

The extent of Pyd in the disc ranges from 2.0 to 2.8 mol/mol collagen in the nucleus and 1.6 to 1.9 mol/mol collagen in the anulus (Eyre 1995, Duance et al 1998) (Table 2.3). Dpd has
been quantified in bone with an extent of 0.05 mol/mol collagen (Eyre et al 1984b) and < 0.04 mol/mol collagen in spinal discs (Eyre et al 1984a). Recent studies have reported a decreasing trend for Pyd in lumbar disc tissues (Duance et al 1998, Pokharna and Phillips 1998) and an increase in Dpd in articular cartilage and meniscus tissues (Takahashi et al 1995) with increased age, however these changes were not statistically significant. Walters and Eyre (1983) found that skeletal or mineralised tissues inhibited the formation of Pyd and Dpd in the matrix. Various authors have also proposed that calcification in connective tissues inhibits spontaneous crosslinking reactions (Walters and Eyre 1983, Eyre et al 1984a, Hoshino et al 1995, Bailey et al 1998). Dpd is prominent in connective tissues of patients with Ehlers-Danlos syndrome Type VI, due to lysyl hydroxylase enzyme deficiency (Eyre 1987), which normally favours the production of Pyd (Bailey et al 1998).

Table 2.3 The extent of Pyd and Dpd in mol/mol collagen in the nucleus, anulus, articular cartilage and cortical bone tissues. Data adapted from Eyre et al (1984a) and Eyre (1995).

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Pyd</th>
<th>Dpd</th>
<th>Pyd:Dpd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus</td>
<td>2.0 to 2.8</td>
<td>&lt; 0.03</td>
<td>&gt;50.1</td>
</tr>
<tr>
<td>Anulus</td>
<td>1.6 to 1.9</td>
<td>&lt;0.03</td>
<td>&gt;50:1</td>
</tr>
<tr>
<td>Articular cartilage</td>
<td>1.5</td>
<td>&lt;0.03</td>
<td>&gt;40:1</td>
</tr>
<tr>
<td>Cortical bone</td>
<td>0.26</td>
<td>0.07</td>
<td>3.5:1</td>
</tr>
</tbody>
</table>

There is currently limited information regarding Pyd and in particular, Dpd, not only in thoracic discs but also in lumbar discs, although most of the information on collagen crosslinks to date is derived from the latter (Eyre 1995). There is even less information on the gender bias or spinal segmental influences on the distribution of collagen crosslinks in the disc biochemical matrix, which could assist in understanding the factors that influence the distribution of these collagen crosslinks in the disc matrix.

2.8.6 Advanced glycation end-products (AGE)

The slow matrix turnover of collagen in spinal disc tissues predisposes the tissues to accumulation of AGE from non-enzymic glycosylation (Eyre et al 1989, Hormel and Eyre 1991, Verzijl et al 2000). The half-life of collagen in cartilage is approximately 117 years and probably longer for spinal disc tissues, which are avascular (Verzijl et al 2000), therefore articular cartilage collagen are prone to form AGE in older individuals (Takahashi et al 1995, Verzijl et al 2000). Hormel and Eyre (1991) reported that glucose forms covalent bonds with
lysine and hydroxylysine residues on their amine side chains by Amadori rearrangement in a process known as browning or Maillard reactions. These reactions are spontaneous and do not require enzymes to proceed, however they require an aerobic environment for synthesis (Verzijl et al 2000). Non enzymatic reactions, usually take place at the triple helical portion of the collagen molecule (Paul and Bailey 1996), increase with age and may alter the mechanical strength of the tissue matrix (Verzijl et al 2000). In addition, these AGE may compete with lysyl oxidase for lysine and hydroxylysine residues (Last et al 1990).

There are two types of non-enzymatic glycation pathway, one requires gloxal and lysine to form crosslinked AGE, pentosidine (Sell and Monnier 1989, Paul and Bailey 1996, Pokharna and Phillips 1998) and pyrroles (Bailey et al 1998); and the other is a non-crosslinked AGE from metal ion induced oxidation breakdown product, N-carboxymethyllysine (CML) (Paul and Bailey 1996, Nerlich et al 1997). It has been suggested that the brown pigmentation of the disc tissues is not due to non-collagenous lipofuscin, but rather an accumulation of AGEs in collagen and other long-lived proteins components in the disc matrix (Eyre et al 1988, Hormel and Eyre 1991, Yang et al 1994). In addition, these AGE inhibit the formation of mature crosslinks, hence disrupts the collagen network arrangement in the matrix (Yang et al 1994). Wound healing in tissues is also impaired by the formation of AGE (Paul and Bailey 1996).

2.9 PROTEOGLYCANS IN THE DISC

The hydrostatic function of the nucleus to resist and transmit compression stresses is dependent on the high proteoglycan (PG) content in thoracic and lumbar nuclei (Scott et al 1994). The function of the nucleus is therefore similar to that of the hyaline cartilage tissue in articular joints (Urban and Maroudas 1980, Hukins 1988, McDevitt 1988, Eyre et al 1989). Besides collagen, PGs are the next most abundant protein component in the disc matrix (Eyre 1979). The amount and type of PG present in the matrix depends on the mechanical function of the tissue, such that tissues under high compression, such as the articular cartilages and nucleus, have a greater number of PG compared to tensile tissues (Urban and Maroudas 1980, Lyons et al 1981). Eyre (1979) reported that the PG in articular cartilage make up 8-10% dry weight, with a higher percentage of PG (50% dry weight) found in the nucleus, compared to 0.2% in tendonous tissues and 10 % in the anulus. Besides the hydrostatic function in the disc, PGs are also important in the regulation of cell function, growth and differentiation (Bushell et al 1977, McDevitt 1988).
Proteoglycans are a polydispersed, heterogeneous group of molecules which consist of a number of sulphated glycosaminoglycan (GAG) chains covalently bound to a protein core, giving it a bottle-brush appearance (Bushell et al 1977, Urban and Maroudas 1980) (Figure 2.14). Each GAG chain consists of disaccharide repeats of sulphated sugars, except for hyaluronic acid (Hukins 1988, McDevitt 1988). The common GAGs which exist in various tissues are keratan sulphate (KS), chondroitin sulphate (CS) 4 and 6, dermatan sulphate, hyaluronic acid (HA), and heparan sulphate (Eyre 1979, Heinegård and Sommarin 1987, McDevitt 1988, Eyre et al 1989). The major GAGs found in spinal discs are CS, KS and HA (Adams and Muir 1976), with small amounts of dermatan sulphate, therefore it is not a true fibrocartilage (Eyre 1979). The nucleus has the highest concentration of KS compared to other connective tissues (McDevitt 1988).

The CS and KS GAGs are clustered rather than distributed randomly along the core protein, with the shorter KS found near the attachment to the link protein and the longer CS located at the free ends of the PG chain (Urban and Maroudas 1980) (Figure 2.14). Tissues under high compressive forces have a greater concentration of CS (Culav et al 1999), probably because CS has twice the negative charge compared to KS and is more hydrophilic (Urban and Maroudas 1980). Although the function of the disc matrix and articular cartilage are almost similar, however their PG structure varies (McDevitt 1988), with a much shorter protein core in spinal disc compared to articular cartilage. The proportion of PG aggregates are fewer in the disc, similar amounts of KS but much less CS in the disc compared to the articular cartilage (Urban and Maroudas 1980, McDevitt 1988).

The synthesis of PG in the matrix is similar to that for collagen. According to Johnstone and Bayliss (1995) PG molecules are transcribed by mRNA in the nucleus of chondrocytes (intracellularly), translated into protein in the ER; glycosylated and sulphated in the golgi apparatus, where GAG chains are added to the protein core and secreted extracellularly as PG monomers. PGs form aggregates extracellularly, binding to HA with the help of link proteins.

2.9.1 Proteoglycan groups
Proteoglycans in the matrix exist in different forms. There are the large extracellular proteoglycans which form large aggregates of PGs such as aggrecan and versican (Bayliss et al 1988, Eyre et al 1989). Aggrecan consist of a number of PGs linked to a HA chain (containing 8 to 18 PGs) with the help of link proteins and is the major component of PGs in the intervertebral disc (Bayliss et al 1988, Eyre et al 1989). Link proteins function to stabilise the large aggregate and are also important to bind protein chains to the collagen network.
(Bayliss et al 1988, Eyre et al 1989). Sixty to 80% of PGs in hyaline articular cartilage exist as aggregates (Urban and Maroudas 1980, Hardingham and Fosang 1995). Versican is found more abundantly between the annular lamellae of fetal sheep discs, and enhances the viscoelastic properties of this tissue (Melrose et al 2001).

Figure 2.14 Schematic representation of a PG molecule and PG aggregate taken from Bushell et al (1977) [A]. Comparison of disc and hyaline cartilage PG. Figure taken from Stevens et al (1979) [B].

All GAGs are negatively charged, due to the sulphate and carboxyl groups (Handley and Buttle 1995), therefore when grouped together in a large aggregate they create a high negative charge density, and have a greater propensity to attract water from the surrounding area (Bushell et al 1977, Urban and Maroudas 1980). It is the negative charge that is responsible
for the swelling pressure in the nucleus, which is resisted by the collagen fibers in the anulus (Handley and Buttle 1995).

In newborns, the nucleus consists mainly of large aggregates, however in the adult discs, they are reduced to small PG aggregates both in the anulus and nucleus (McDevitt 1988, Johnstone and Bayliss 1995), probably due to ageing and pathology in the matrix (Adams et al 1977, Eyre et al 1989). Studies have shown that although the anulus has less PG compared to the nucleus (Adams et al 1977), large aggregates are more commonly found in the anulus (60%) compared to the nucleus (30%) (Bushell et al 1977, Eyre 1979, Urban and Maroudas 1980). The major PGs in the mature nucleus are therefore present as non-aggregable (10-40%) PGs (Bushell et al 1977, Heinegård and Sommarin 1987, McDevitt 1988, Johnstone and Bayliss 1995). According to Eyre et al (1989) non-aggregating PGs do not have HABR binding sites, therefore they have more KS compared to CS and dermatan sulphate, with a limited role in resisting mechanical loading. Therefore the reduction in large aggregates may imply that the PGs in matured or aged nucleus are not able to provide optimal resistance to compressive loading.

Besides the large extracellular proteoglycans, there are also groups of small leucine-rich proteoglycans (SLPG) such as decorin, biglycan, fibromodulin and lumican forming the smaller protein groups found in intervertebral discs (Johnstone and Bayliss 1995, Inkinen et al 1998). Unlike the large aggregating PGs, these SLPG are not present in significant proportions and have a limited role in the compression function of the matrix (Culav et al 1999). However these small proteoglycan groups may interact with other matrix components, such as collagen and contribute to the stability and development of the extracellular matrix of the disc (Culav et al 1999, Melrose et al 2001). The small leucine-rich PGs have an amino acid sequence that resembles each other, with collagen-binding sites at the C-terminal end. The other N-terminal shows a high variability in the amino acid sequence, and may be involved in protein-to-protein or cell-to-matrix interactions (Yamaguchi et al 1990). SLPGs have been found to have an important role in the organisation, assembly, transport and orientation of collagen fibrils in the extracellular matrix of the disc, during growth and development, and also during disc matrix repair (Melrose et al 2001).

Biglycan and decorin are small PGs which contain more dermatan and chondroitin sulphate chains, and are usually found in connective tissue matrix, in particular cartilage and bone (Inkinen et al 1998). Decorin has one GAG chain and is the smallest PG and biglycan has 2 GAG chains (Yamaguchi et al 1990, Culav et al 1999). It has been suggested that the function of decorin is to bind collagen fibrils, helping to stabilise fibrils and orient fibrillogenesis
(Inkinen et al 1998, Culav et al 1999). It therefore also regulates the thickness of collagen fibers and the spacing between collagen fibrils in the matrix, and have been found in degenerated anular tissues (Heinegård and Sommarin 1987, Inkinen et al 1998). In the disc matrix, PG is usually found bound to collagen fibers and help to increase the packing of collagen molecules in the anulus (Vater et al 1979, Krämer 1990). Keratan sulphate in particular is usually bound to collagen molecules (Eyre 1979). Both decorin and biglycan are also important in regulating cell growth, as its protein core has been shown to bind with or neutralise the transforming growth factor beta (TGF-β), retarding matrix growth (Yamaguchi et al 1990, Culav et al 1999). The main SLPG found in fetal sheep spinal discs are biglycan, and decorin is more abundant in the older sheep discs, with the highest level in the outer anulus (Melrose et al 2001). Both decorin and biglycan are reported to accumulate in degenerated human intervertebral discs relative to other uronic acid containing PGs, which significantly alters the disc matrix properties, hence contribute to the pathogenesis of further disc pathology (Inkinen et al 1998).

There is very little information on the function of SLPG fibromodulin and lumican, which are heavily glycosylated by keratan sulphate, and are closely related to each other but both competing for the same binding site on collagen Type I fibers (Svensson et al 2000). They are slightly different from decorin and biglycan, and are mainly involved in regulating the orientation of collagen fibers, especially collagen Type I fibers (Svensson et al 2000). Fibromodulin was detected in fetal sheep discs especially in the anulus, with some fibromodulin present in the nucleus (Melrose et al 2001). The amount of fibromodulin and biglycan was noted to decrease with increasing age of sheep discs (Melrose et al 2001).

### 2.10 ELASTIN CROSSLINKS - DESMOSINE AND ISODESMOSINE

Connective tissues, especially those from vascular and alveolar tissues, skin (Uitto 1979) and the ligaments, are a rich source of elastic fibers (Yong-Hing et al 1976). Elastic fibers consists of two distinct components, a central amorphous branching structure, which is the elastin protein, and an outer microfibrillar component (Uitto 1979, Rosenbloom 1987, Melrose and Ghosh 1988) found on the periphery of the elastin protein (Uitto 1979). The elastin protein has a very different amino acid composition of sequence compared to that of collagen (Anwar 1965, Uitto 1979) and is most hydrophobic (Yu 2002). The major amino acid groups in elastin are glycine, proline and alanine, but with little or no hydroxyproline and small amounts of crosslinked amino acids, desmosine (Des) and isodesmosine (Isodes) (Anwar 1965, Uitto 1979, Rosenbloom 1987) (Figure 2.15). Due to their unique presence in elastin
protein, these crosslinks, Des and Isodes, are commonly used to quantify and investigate the amount of elastin in the disc tissue (Thomas et al 1963, Anwar 1965, Rosenbloom 1987). Desmosine and Isodes were first named and isolated by Thomas et al (1963) using bovine ligamentum nuchae.

![Figure 2.15 Schematic structure of Desmosine [A] and Isodesmosine [B] Crosslinks. Figure taken from Thomas et al (1963).](image)

The synthesis of elastin is similar to that of collagen molecules (Uitto 1979). Elastin is an insoluble protein, synthesised from the soluble precursor molecule tropoelastin, transcribed genetically from elastin mRNA intracellularly (Uitto 1979, Rosenbloom 1987, Melrose and Ghosh 1988). Fibroblast cells in connective tissues assemble polypeptide chains in the ER (Uitto 1979, Davidson and Sephel 1987). Unlike collagen chains, these chains are hydroxylated but not glycosylated (Uitto 1979, Davidson and Sephel 1987). The polypeptide chains are then secreted by exocytosis from the golgi apparatus into the extracellular space as soluble tropoelastin (Uitto 1979, Eyre et al 1984b, Rosenbloom 1987).

In the extracellular space, tropoelastin is assembled into fibers which are capable of forming crosslinks via oxidative deamination of lysyl residues to form reactive aldehydes or allysine, which is catalysed by the enzyme lysyl oxidase (Uitto 1979, Eyre et al 1984b, Rosenbloom 1987). All the crosslinks in elastin are derived from the C-terminal lysine residues (Eyre et al 1984b, Rosenbloom 1987), unlike collagen crosslinks, which are derived from both lysine and hydroxylysine residues (Eyre et al 1984b, Melrose and Ghosh 1988). Desmosine and Isodes
crosslinks are released from elastin by elastase which cleave the peptide bonds (Partridge and Davis 1955, Rosenbloom 1987).

Desmosine and Isodes are crosslinks derived from amino acids, but have a different structural configuration (Partridge and Davis 1955, Thomas et al 1963). They are tetrafunctional with four amino and four carboxyl groups and are hydrophobic (Thomas et al 1963, Eyre et al 1984b), formed from condensation of four lysine residues (Ghosh et al 1977, Uitto 1979). Most elastic tissues in animals and man have equal amounts or slightly more Des compared to Isodes crosslinks (Anwar 1965, Starcher and Galione 1976). While Isodes is fluorescent, Des however, is not fluorescent, therefore both crosslinks are usually detected by ultraviolet absorption at 275 nm wavelength (Chen et al 2000, Abe et al 2003).

2.10.1 Elastin crosslinks in the disc
Elastic fibers in human spinal discs were thought to be non-existent until electron microscope and histological reports of small numbers of these fibers in the disc tissue (1 to 10% disc dry weight) (Buckwalter et al 1976, Hickey and Hukins 1982, Yu 2002). The role and function of elastic fibers in the disc has still not been established conclusively, although small amounts of elastic fibers are sufficient to provide the elastic function in the skin (Uitto 1979, Mikawa et al 1986, Yu 2002). A recent morphological study by Yu et al (2002) also suggested that the elastic fibers, although present in small amounts (< 5% of total dry disc weight), were present only in specific disc regions, and were highly organised, being coupled with other matrix components, especially collagen fibers. The highly organised structure of elastic fibers, despite the small quantities in the disc, provided a significant mechanical role in the disc mechanical function (Yu 2002, Yu et al 2002). Elastic fibers have been proposed to provide elasticity and resilience to the anular fibers, enabling the collagen fibers to recover after deformation (Buckwalter et al 1976, Ghosh et al 1977, Hickey and Hukins 1981, Olczyk 1994b). In addition, elastic fibers are also reported to maintain the collagen crimping, which protects the fibers during sudden impact loading (Hukins 1988).

The small amount of elastin in the disc matrix has also made it difficult to locate specifically in the disc tissue, and contradictory findings have been reported from various spinal disc studies. Buckwalter et al (1976) reported that elastic fibers are randomly distributed throughout the anular and nuclear matrix, while Sylvest et al (1977) noted a higher percentage of these fibers at the junction between the anulus and the nucleus. Olczyk et al (1994b) also reported a higher amount in the anulus compared to the nucleus. Johnson et al (1982) reported that elastic fibers are only seen in the superficial layers of the thoracic anulus and the nucleus,
where the lamellae is attached to the vertebral body or hyaline cartilaginous end-plate. They did not find any elastic fibers in the thoracic disc matrix in the bulk of the disc tissue. However in subsequent study, Johnson et al (1985) found elastic fibers throughout the lumbar disc matrix, especially where the lamellae is attached to the vertebrae. A recent study by Yu et al (2002), reported that the amount of elastic fibers in the disc varied depending on the regional loading patterns of the disc. Yu et al (2002) reported the presence of elastic fibers in the nucleus and anulus of bovine discs, especially within and between the anular lamellae, therefore enabling the collagen fibers to recover after stretching during spinal axial loading and movement.

In elastic tissues, elastic fibers were usually found parallel and closely packed with collagen fibers (Partridge et al 1955, Minns et al 1973, Buckwalter et al 1976, Sylvest et al 1977, Yu et al 2002). Both elastic and collagen fibers anchor the superficial lamellae of the anulus to the epiphysseal compact bone and hyaline cartilage plate (Sharpey’s fibers) (Johnson et al 1982). Within the anulus, these elastic fibers are also arranged obliquely, circularly and longitudinally, parallel to the collagen fibers in the outer layers of the anular lamellae (Johnson et al 1982, Yu et al 2002); more importantly, they form cross bridges between the lamellae (Yu et al 2002). In contrast, the fibers in the nucleus are randomly (Buckwalter et al 1976, Johnson et al 1982) or radially oriented (Yu 2002, Yu et al 2002). Olczyk (1994b) suggested that there are more elastic fibers in the anulus compared to the nucleus, except in prolapsed nuclear tissues, where the elastin content in the prolapsed nuclear material is increased.

2.11 NON-COLLAGENOUS PROTEINS

According to Ghosh et al (1977), non-collagenous proteins refers to all other proteins besides collagen and PGs which also exist in the disc matrix, such as plasma proteins, albumins, glycoproteins and enzymes. The most common non-collagenous proteins are glycoproteins. The common glycoproteins are fibronectin, tenascin, link proteins and laminin (Ghosh et al 1977, Culav et al 1999). Non-collagenous proteins make up 20 to 45 % dry weight in the nucleus and 5 to 25% dry weight in the anulus (Ghosh et al 1977). They are soluble, multifunctional macromolecules, and are integral in stabilising the matrix, as well as linking the matrix to cellular processes of synthesis and degradation (Ghosh et al 1977, Culav et al 1999). With ageing, the matrix has an increase in both collagen and non-collagenous proteins in the matrix, especially in the nucleus (Hallen 1962, Eyre 1979).
2.12 DISC METABOLISM AND TURNOVER

Compared to articular connective tissues, the turnover of the disc matrix is much slower, due to its avascularity (Walmsley 1953, Urban et al 1977, Liu et al 1991). One of the main factors determining the rate of synthesis and degradation of the disc matrix is disc nutrition, however other factors may affect matrix metabolism either directly or indirectly by disrupting disc nutrition. They are:

- Chondrocyte synthetic activity
- Oxygen concentration
- Matrix stimulators and inhibitors
- Nutrition
- Compression loading and trauma
- Age
- Gender

The biosynthesis of the extracellular matrix is a recurring cellular function of the chondrocytes and fibroblast cells, such that if matrix repair is slower than degradation processes, then degeneration of the matrix takes place (Krämer 1990).

2.13 CHONDROCYTE SYNTHETIC ACTIVITY

Disc metabolism and turnover is regulated by disc cells (Gray et al 1988, Bartels et al 1998, Gruber and Hanley 1998), which make up 20 to 30% of tissue volume (Krämer 1990). During matrix repair, these cells are responsible for the synthesis of the extracellular matrix from enzymes, lipids and inorganic ions, such as sodium, potassium and calcium, amino acids, salt, glucose and water (Krämer 1990, Horner and Urban 2001). Water, which forms 90% of the matrix volume (especially in young discs), is an important component in the disc matrix for metabolism and nutrition and most of it is extracellular, due to the small cell density (Urban and Maroudas 1980).

The distribution of cells is not uniform in the disc, being more concentrated near the end-plate (15,000 cells/µL) and outer anulus (9,000 cells/µL) and less concentrated in the nucleus (4,000 cells/µL) (Urban and Maroudas 1980, Burgeson 1982, Eyre et al 1989, Stairmand et al 1991). In the nucleus, disc cells are similar to chondrocyte, and in the anulus, to fibroblast cells (Krämer 1990, Ishihara et al 1996, Handa et al 1997, Guiot and Fessler 2000).
Chondrocyte cells predominate in the nucleus as they are more suited to the avascular environment (Guiot and Fessler 2000), however these cells can be increased if there is vascularisation in the damaged nucleus (Bartels et al 1998).

2.14 OXYGEN CONCENTRATION

Chondrocyte synthetic activity is affected not just by the cell number or hydration, but more importantly by the oxygen concentration and the nutrient supply (Bayliss et al 1988, Stairmand et al 1991, Bartels et al 1998, Guiot and Fessler 2000, Horner and Urban 2001). Therefore the concentration of oxygen in the disc matrix also determines the number of chondrocytes and the level of chondrocyte synthesis activity (Stairmand et al 1991). According to Stairmand et al (1991), the disc matrix, especially in the centre of the disc, has adapted to the low oxygen environment by reducing the number of chondrocytes, hence lower cellular activity. However, any disruption to the oxygen supply or diffusion of solutes in the disc will result in a high rate of glycolysis and accumulation of lactic acid, leading to cell death and a further reduction in the number of viable cells (Urban et al 1977, Stairmand et al 1991, Ohshima and Urban 1992, Bartels et al 1998, Horner and Urban 2001). Such an acidic condition will also reduce matrix synthesis (Horner and Urban 2001), decrease cellular metabolism and increase the action of degradative enzymes such as cathepsins (Maroudas 1988).

2.15 MATRIX STIMULATORS AND INHIBITORS

The maintenance and remodelling of the matrix in adult intervertebral discs occurs due to the presence of matrix cell stimulators such as cytokines (Interleukin-1) and growth factors (Culav et al 1999), and regulation or degradation by inflammatory enzymes, such as prostaglandin E$_2$, proteinases (trypsin and chymotrypsin) and finally, matrix metalloproteinases (MMPs) (Liu et al 1991, Kang et al 1995, Crean et al 1997, Takahashi et al 2001). MMPs are a large family of enzymes that contributes to both normal and pathological tissue remodelling, and have an important function to degrade and regulate changes in the extracellular matrix (Guiot and Fessler 2000). In normal physiology, MMPs can contribute to tissue remodelling during development or repair. During pathologic processes, MMPs are usually involved in breakdown of connective tissues especially in inflammatory diseases, or during injury and trauma (Guiot and Fessler 2000).
MMPs are enzymes and are usually synthesised and secreted in the extracellular matrix as inactive precursors under the regulation of growth factors and cytokines (Guiot and Fessler 2000). They may be activated after transcription by growth factors, noxious stimulus or inflammatory cytokines, especially in inflammatory or degenerative diseases (Guiot and Fessler 2000). Once activated, MMPs may be regulated or inactivated by tissue inhibitors of MMP (TIMPs) (Handa et al. 1997, Guiot and Fessler 2000). These inhibitors are the subject of much research in order to produce suitable drugs for therapy to reduce the inflammatory effects of tissue breakdown and degradation due to MMP activity. These MMPs are secreted by disc cells (Liu et al. 1991) and may be activated by nutritional and oxygen deficiency and a change in acidity, due to inflammation and trauma (Guiot and Fessler 2000, Cassinelli et al. 2001). Pentosidine, a senescent AGE, is also known to stimulate macrophages and chondrocytes to release cytokines and MMPs, resulting in disc degeneration or turnover (Buckwalter 1995) and decreased PG synthesis (DeGroot et al. 1999). Cell apoptosis is also accompanied by the release of inflammatory MMPs (Handa et al. 1997).

The four types of MMPs are the collagenase, gelatinase, stromelysin and membrane-type MMPs (MTI-MMP) (Pattison et al. 2001), and they are usually named according to the substrate that they act on, therefore collagenase acts on collagen, metalloelastase on elastin and stromelysin on PGs (Guiot and Fessler 2000). However it is also recognised in the literature that MMPs can degrade multiple substrates, for example, interstitial collagenase MMP-1, is capable of degrading collagen and other non-collagens proteins and PGs (Crean et al. 1997). Examples of MMPs found in spinal discs are collagenase, metalloelastase, gelatinase, stromelysin (Liu et al. 1991, Kang et al. 1995, Crean et al. 1997, Takahashi et al. 2001). The role of each MMP, cytokine and inflammatory product in the disc matrix is still not fully understood (Guiot and Fessler 2000, Cassinelli et al. 2001), although adult or aged discs tend to have a higher concentration of MMPs in the extracellular matrix (Liu et al. 1991). The contribution of MMPs to disc degeneration is due to the ability of MMPs to breakdown the biochemical substrates in the disc matrix (Kang et al. 1995).

Stromelysin is a key enzyme that degrades the extracellular matrix, especially in the nucleus (Eyre et al. 1991, Liu et al. 1991, Kang et al. 1995, Kang et al. 1996, Guiot and Fessler 2000, Takahashi et al. 2001), however collagenase and gelatinase are more active in the anulus, working together to break down collagen (Guiot and Fessler 2000). The fibrillar collagens in the disc are broken down mainly by the interstitial collagenase, MMP-1, which cleaves collagen Types I, II and III (Crean et al. 1997). The other common MMPs involved in disc matrix degradation are MMP-3 (stromelysin-1) and gelatinase (MMP-2 and 9) (Crean et al. 1997), and main tissue inhibitor is TIMP-1 (Handa et al. 1997). MMP-3 was found more
abundant in the nucleus and TIMP-1 was found mainly in the inner annulus of discs under high compressive load (Liu et al 1991, Handa et al 1997). An increase in MMP-2 and MMP-9 have also been reported in both the annulus and nucleus of scoliotic disc tissues, suggesting a major contribution of these enzymes in the degradation of disc tissues with increased or abnormal spinal loading (Crean et al 1997).

An interesting report by Ng et al (1986) found a novel collagenase that preferentially degraded Type II collagen in normal spinal discs. However in prolapsed discs, the collagenolytic enzyme activity was reversed, preferring to degrade Type I collagens and elastin instead. Similar findings were reported by Price et al (1999) where degradation of Type II collagen in the normal cartilage preceded or led to the onset of osteoarthritis in the knee. The preferential degradation of Type I collagen fibers in prolapsed discs reported by Ng et al (1986) is paradoxical to the process of matrix repair and synthesis, where the newly formed scar tissue consists predominantly of Type I fibers (Brickley-Parsons and Glimcher 1984, Hutton et al 1998). Reasons for this collagen type preference in the degenerated matrix is not known and may be genetic (Eyre et al 1989). However it is possible that if collagen Type II is degraded in normal cartilage (Price et al 1999) and disc (Ng et al 1986) matrices, it may suggest that the normal ageing process, apart from degeneration processes, may also initiate matrix degradative processes at the cellular level as well.

The turnover or ability to replace the matrix is slower in the disc compared to articular cartilage, probably due to the fewer disc cells and the poor circulation in the disc, which is highly dependent on diffusion for solute transport (Liu et al 1991). For the same reasons, the intervertebral disc is prone to accumulate degraded matrix molecules due to the slow diffusion of these macromolecules out of the disc matrix (Urban and Maroudas 1980, Liu et al 1991, Stairmand et al 1991). These degenerated products in the disc matrix may facilitate the release of more MMPs (Kang et al 1996, Crean et al 1997), hence the disc tends to degenerate more quickly than other cartilage tissues (Buckwalter 1982).

2.16 DISC NUTRITION

Much of the information on the in vivo nutrition and metabolism of the intervertebral disc is extrapolated from ex-vivo human lumbar or animal spine studies. Nutrition of the intervertebral disc is important as it determines the cellular metabolism in the disc matrix (Urban et al 1977, Crock et al 1988). Inadequate nutrition and accumulation of waste products
in the disc matrix are major factors leading to disc degeneration (Urban et al 1977, Maroudas 1988). Disc nutrition and consequently matrix metabolism depends on three main factors:

- Blood supply to the disc
- Diffusion rate through the matrix
- Mechanical loading history

### 2.16.1 Blood supply to the disc

The intervertebral disc, especially the lumbar disc, is the largest avascular connective tissue in the body (Maroudas 1988), with limited blood supply from only two sources. The main nutrient artery to the vertebral body sends out branches, called epihyseal vessels, to the cartilaginous end-plates supplying the disc superiorly and inferiorly (Walmsley 1953, Taylor and Twomey 1988). The second source of blood supply to the disc is found in the outer annulus, particularly in the anterior annulus (Urban et al 1977) (Figure 2.16). Lumbar disc studies report that the disc matrix is highly dependent on these blood supplies to ensure adequate nutrition and normal intra and extracellular metabolic activities (Urban et al 1977, Horner and Urban 2001).

![Figure 2.16](image.png)

Figure 2.16 Schematic diagram showing the blood supply to the disc via arterial branches supplying the end-plates through the vertebrae [A] and to the superficial annular lamellae [B]. Figure taken from Butler (1988).
In the newborn, the disc is supplied by blood vessels in the end-plates (Krämer 1990); however as early as 4 years of age, these blood vessels disappear leaving permanent perforations that allow diffusion of water and nutrients from the epiphyseal vessels in the vertebrae (Taylor and Twomey 1988, Roberts et al 1989, Krämer 1990). According to Krämer (1990) the disappearance of the blood vessels from the end-plates may coincide with the onset of sustained erect posture, probably due to the increased compression loading at the end plates and vertebral body.

The epiphyseal branches end in capillary plexuses at end-plates located centrally near the nucleus, and peripherally close to the anulus (Crock et al 1988, Taylor and Twomey 1988). The end-plates, consist of hyaline cartilage (0.1 to 1.6 mm thick), and are thinnest in the centre of the disc (Roberts et al 1989). The density of the capillary plexus and permeability (80 to 85%) of the end-plate is highest nearest the nucleus and lowest at the outer anulus (Urban et al 1977, Crock et al 1988, Maroudas 1988, Roberts et al 1989). Calcifications or destruction of the end-plate, such as occurs in Schmorl’s nodes, will disrupt nutrition to the disc, particularly in the nucleus, and accelerate disc degeneration (Urban et al 1977, Eyre et al 1989, Roberts et al 1989).

Besides losing its arterial vessels, the blood supply is also reduced during growth as the disc volume increases faster than the surface area, hence the rate of diffusion of nutrients to the nucleus is reduced (Taylor and Twomey 1988). In the lumbar disc, the cells in the nucleus may be 5 to 8 mm away from the blood supply, therefore the cells in the centre of the disc have a low oxygen and glucose concentration (Maroudas 1988). Taylor (1975) suggested that this growing period coincides with the change of notochordal cells in the nucleus to that of chondrocytes and fibroblasts, which have a higher viability in avascular conditions.

### 2.16.2 Diffusion rate through the matrix

The nucleus derives nutrition mainly from diffusion from the end-plates and in a limited way from the outer anulus (Urban et al 1977, Taylor and Twomey 1988). Diffusion in the disc matrix occurs either by molecular diffusion, which is faster, or by bulk flow or convective transport, being carried by the movement of fluid in the disc matrix during cyclic loading of the disc (Urban and Maroudas 1980, Eyre et al 1989). The latter form of diffusion is important for large solutes or macromolecules (Eyre et al 1989). Crock et al (1988) suggested that movement of solutes into and out of the disc occurs daily and within minutes, as seen in the immediate distribution of radio-opaque dyes injected into the disc tissue used in discography.
The rate of molecular diffusion of nutrients to the disc matrix is dependent on a number of factors such as, the water and GAG concentration, size of solutes and thickness of the disc (Urban et al 1977). The negatively charged PG or GAG molecules are able to selectively attract small positively charged solutes or cations, and exclude negatively charged anions, in particular the divalent sulphate ions and to a lesser extent the monovalent chloride anions (Urban et al 1977, Maroudas 1988). Uncharged small solutes diffuse into the disc equally via the end-plates and the outer anulus, whereas, negatively charged anions enter into the disc predominantly via the outer anulus (Urban et al 1977, Eyre et al 1989). The three-dimensional network of collagen fibres in the outer anulus also acts as a barrier to diffusion resisting the flow of large solutes, although it remains permeable to small solutes (Krämer 1990). Therefore diffusion through the nucleus is faster compared to the anulus, with an uptake of sulphate ions of 17.5 mmol/ml/hr and 10.4 mmol/ml/hr, respectively (Urban et al 1977).

The concentration of water in the matrix, particularly, extracellular water, is important in determining the osmotic pressure in the matrix, hence the diffusion gradient for different solute transport (Maroudas 1988). Diffusion is the main method of small solute transport such as urea, glucose, glycine, proline (Urban et al 1977). For large solutes, such as immunoglobulins, serum albumin, antibodies and enzymes, diffusion into the disc matrix is almost negligible, as it requires more than just a diffusion gradient (Urban and Maroudas 1980, Maroudas 1988). Maroudas (1988) and Urban et al (1977) suggested that large solutes, for example degradative enzymes, are usually excluded from the disc matrix and occasionally gain access when there is a disruption of the structural integrity such as during disc degeneration or trauma.

\[ \text{2.16.3 Mechanical loading history} \]

Posture or compression loading, creates a pumping action which has a significant effect in the diffusion or movement of large protein solutes into and out of the disc as well as within the disc matrix (Maroudas 1988), therefore the compression loading history is also a regulator of matrix nutrition, metabolism and synthesis (Ishihara et al 1996, Handa et al 1997). The change of spinal postures in daily activities are important in encouraging solute diffusion and disc nutrition (Adams and Hutton 1986a), facilitating fluid movement between intra and extra discal spaces (Krämer 1990). According to Urban et al (1977), prolonged or sustained axial compression on the disc increases the hydrostatic pressure within the disc, hence has a negative effect on the diffusion of water and large solutes into extracellular spaces of the disc.
matrix. Therefore compression loading history can also determine the biochemical changes and matrix synthetic activities in the spinal disc.

2.17 COMPRESSION LOAD AND TRAUMA

2.17.1 Compression load

Chondrocyte biosynthetic (Ohshima et al 1995, Ishihara et al 1996, Handa et al 1997) and disc enzyme activities (Krämer 1990, Crean et al 1997) are significantly affected by the amount of disc compression forces. As described above in Section 2.2, the mechanical forces in the spine are specific to each spinal region, therefore the biochemical matrix in each spinal level is also expected to be different. In the young spine, the biochemical constituent of the disc is similar at all spinal levels, especially in the first two decades of life, suggesting that the disc biochemical matrix is genetically determined (Urban and Maroudas 1980, Brickley-Parsons and Glimcher 1984). Only when there was degeneration or altered mechanical loading, is there a difference in the biochemical components in the disc (Urban and Maroudas 1980, Brickley-Parsons and Glimcher 1984). Changes in the disc biochemical constituents noted with increased age are probably due to a matrix response to the different mechanical loading in each spinal region (Brickley-Parsons and Glimcher 1984).

The collagen content in the anulus has been reported to increase craniocaudally with the thoracic and lumbar anuli having almost similar concentrations (Brickley-Parsons and Glimcher 1984, Li et al 1994, Scott et al 1994). In contrast, the total PG content in the anulus was found to decrease craniocaudally (Scott et al 1994). The differing high collagen content and low PGs in the thoracic and lumbar anulus is reflective of the high tensile and rotation forces exerted in these spinal regions compared to the cervical region (Li et al 1994, Scott et al 1994). Similarly more mature collagen crosslinks (Herbert et al 1975) and a higher collagen content (Eyre and Muir 1977) are found in the adult lower lumbar discs compared to the upper lumbar discs.

In the nucleus, the PG content increased craniocaudally instead, being highest in the lumbar region, with a contrasting trend for collagen content (Scott et al 1994). The high level of PG in the lumbar nuclei enables the matrix to support compression forces therefore is consistent with the higher axial loading observed in the lumbar region.

The disc matrix response to compression forces is varied, depending on the dosage and duration of compression. Lumbar and thoracic disc studies have shown that sustained
compression leads to disc degeneration and failure, however, a minimum amount of compression is required to stimulate matrix synthesis of collagen and PG (Handa et al 1997, Hutton et al 1999, Edwards et al 2001). Pressures of 1 to 7.5 milliPascal (mPa) for 20 seconds in bovine (Ishihara et al 1996) and canine (Hutton et al 1999) intervertebral discs provide optimum mechanical load to stimulate matrix synthesis. When carrying a load of 10 kg, the intradiscal pressure in man increases to 1.5 mPa (Nachemson 1981), therefore normal spinal loading is within the range to stimulate disc matrix synthesis. Pressures that are too high, that is more than 7.5 mPa for twenty seconds or more in bovine intervertebral discs (Ishihara et al 1996), or more than 3.0 mPa in canine spinal discs (Hutton et al 1999), have an opposite effect. Sustained high compression loads usually result in decreased cell metabolism and synthesis rate, and an increase in MMPs, leading to disc degeneration (Handa et al 1997, Edwards et al 2001).

In addition, the duration of the sustained compression is also an important determinant of matrix synthesis (Hutton et al 1999, Lotz and Chin 2000). Even small physiologic loads of 0 to 3 mPa, when sustained for periods of more than 12 hours, resulted in decreased PG synthesis in rat spinal discs (Lotz and Chin 2000) and bovine articular cartilage (Gray et al 1988). Therefore normal physiologic compression loading can stimulate matrix synthesis in spinal and articular cartilage; however, if the load is too high or of too long a duration, the opposite effect in matrix synthesis is observed (Gray et al 1988, Hall et al 1991, Ishihara et al 1996, Lotz and Chin 2000). This change in matrix synthesis and turnover is confirmed by cross-sectional spinal biochemical studies using scoliotic spines.

A number of studies have reported that human disc matrices which are exposed to asymmetrical compression forces in the growing adolescent spine, present with significantly different matrices even within the same disc (Bushell et al 1979, Brickley-Parsons and Glimcher 1984). Discs in scoliotic spines are exposed to both compression loading on the concave side, and tensile forces on the convex side of the curve (Crean et al 1997). The anulus of thoracic discs on the concave side of the scoliotic curve are reported to have a lower PG and collagen content compared to the anulus on the convex side (Bushell et al 1979, Crean et al 1997, Duance et al 1998). More interestingly, changes in collagen type were also observed, with collagen Type I fibers increased on the concave side but decreased on the convex side (Bushell et al 1979, Brickley-Parsons and Glimcher 1984). Similar increase in collagen Type I instead of Type II is also noted in the nucleus under compression loading (Hutton et al 1999). The response of the disc matrix within the same disc to asymmetrical compression and tensile forces, provide evidence that the disc is able to adapt biochemically
to the changing environment, as stated in Wolff’s law (Bushell et al 1979, Brickley-Parsons and Glimcher 1984). However, whether the change in the matrix is desirable is not known.

2.17.2 Trauma – the inflammatory and repair processes

In the absence of trauma (disruption of tissue integrity) or pathology (due to diseases), injury to the disc still occurs as normal biochemical age changes may lead to a weakened matrix, which succumbs to normal mechanical loading and stresses (Modic and Herfkens 1990). Alternatively, the disc may be injured by mechanical overload either from large compression forces or fatigue failure from normal stresses in repeated or sustained spinal movements or compression forces described above in Section 2.5.1. Repair after trauma is facilitated by inflammatory metabolites, ingrowth of fibrotic tissues and blood vessels (Eyre et al 1989, Kang et al 1996). Healing of the disc matrix after injury is possible only if there is proliferation of vascular tissues, therefore regeneration of the disc is not possible without nutrient and the disc matrix undergoes desiccation, fissuring and calcification (Schmorl and Junghanns 1971).

According to Kang et al (1995, 1996) surgically removed herniated lumbar and cervical discs were found to have a higher concentration of MMPs, nitric oxide, prostaglandin E2 and Interleukin 6 (cytokines). They suggested that nitric oxide facilitated the release of cytokines and MMPs, while prostaglandin E2 and Interleukin 6 act to suppress or inhibit the synthesis of PGs and to stimulate the action of collagenase. Therefore the combined effect of the MMPs, cytokines and inflammatory enzyme prostaglandin E2 in the herniated disc tissue probably results in a further degradation of the disc matrix and reduced matrix synthesis (Kang et al 1996, Crean et al 1997, Guiot and Fessler 2000).

Following trauma to the disc, matrix synthesis usually results in new granulation tissue, however the new collagen fibers does not stop eventual degeneration of the disc (Schmorl and Junghanns 1971, Osti et al 1990). Olczyk et al (1992) found that herniated nuclear tissues initially increased collagen synthesis, however after the age of 50 years, the total collagen content decreased sharply. There was also more collagen Type I and II in herniated and aged nuclear tissues. The combination of newly synthesised granulation tissues, increased inflammatory and vascular exudate may act as a barrier to diffusion of oxygen and nutrient supply, which has a negative impact on disc nutrition, leading to further matrix degradation and disc degeneration (Adams and Dolan 1995). In addition, Schmorl and Junghann (1971) suggested that even if injured tissues are replaced with new fibrous scar tissues, the disc
mechanics is still altered, due to a loss in disc height, fibre elasticity and tensile strength and a change in lamellae orientation of the anular fibers.

It is not known if normal biochemical age changes take place before degenerative changes or vice versa (Adams and Dolan 1995). The difficulty in determining the sequelae of disc degeneration especially biochemically is that most studies examining the effects of compression loading or injury do not take into account the time lag from the onset of trauma or biomechanical changes to biochemical matrix responses (Olsewski et al 1996, Hutton et al 1998). Therefore longitudinal studies are necessary to evaluate the biochemical changes due to mechanical forces over a long duration. Longitudinal animal spinal disc studies have shown that immediately after an injury to the disc tissue, the mechanical function may be affected, however the disc biochemical matrix requires some time to change (Olsewski et al 1996, Hutton et al 1998, 1999, Lotz and Chin 2000). Therefore disc degeneration is associated with a wide spectrum of biochemical changes, because the rate of matrix turnover depends on the synthesis rate and the time lapse from the onset of injury (Bayliss et al 1988). Olsewski et al (1996) and Hutton et al (1998) reported no matrix biochemical change in injured canine lumbar anular tissues 6 months after the onset of injury or prolonged compression in the latter study. However Melrose et al (1992) reported biochemical changes mainly in the nucleus of sheep discs 8 months after the onset of disc injury. In addition, Lotz and Chin (2000) further suggested that disc studies using smaller animals, such as rats, presented with biochemical matrix changes after a shorter duration of compression, as few as 7 days of compression and immobilisation.

Occupational and recreational activities involving joint motion or exercises are also important to maintain the matrix and to stimulate matrix biosynthesis. Studies on articular cartilage have shown positive matrix turnover and synthesis with regular exercise, and the reverse effect with immobilisation (Iatridis et al 1999). Short term running exercises increased collagen content in canine intervertebral discs (Säämänen et al 1993) and increased KS (Kuiper et al 1998), HA and sulphation of PGs in rabbit articular cartilage (Säämänen et al 1988). However long term, long distance running exercises had the opposite effect in canine discs (Puustjärvi et al 1993). Sahlman et al (2001) proposed that the disc matrix synthesis may be governed genetically, as mice with deficient Col2a1 gene for collagen Type II formation in spinal discs, tended to be less willing to exercise compared to control animals.
2.18 AGE AND GENDER INFLUENCES

As mentioned above, in the absence of trauma, normal aged disc is characterised by a dehydrated disc, with fibrosed nucleus and anulus and little or no loss of disc height (Vernon-Roberts and Pirie 1977, Modic et al 1984, Twomey and Taylor 1987). The injured disc instead is characterised by a loss of disc height and disrupted structural integrity such as anular fissures and nuclear prolapses, as described in Section 2.6.2. However biochemically, it may not be possible to differentiate between ageing and degeneration, as changes in the disc matrix are almost identical for these two processes (Lyons et al 1981). Ageing and degeneration are usually accompanied by a decrease in water, PG and an increase in collagen in the nucleus (Eyre 1979, Krämer 1990, Olczyk 1992, Olczyk 1994c). There is often a loss of distinction between the anulus and the nucleus in aged discs, both morphologically (Thompson et al 1990) and biochemically (Herbert et al 1975, Twomey and Taylor 1987, Pokharna and Phillips 1998). With increased age, water, magnesium, sulphate and proteoglycan decrease, while collagen (Olczyk 1992), calcium, non-collagenous protein and amyloid deposition increase (Krämer 1990).

With increased age, there is a change in concentration of collagen and collagen phenotype; that is, more Type I is present compared to Type II, especially in the nucleus (Brickley-Parsons and Glimcher 1984). In contrast, the collagen content in the anulus remains constant or decreases (Eyre and Muir 1977, Lyons et al 1981, Olczyk 1992). Naylor (1954) observed that the aged nucleus loses its gel structure and its collagen fibers undergo crystallisation and fibrillation. The turnover rate for collagen is much slower than for PG, ranging from 100 to 400 years (Bank et al 1998, Verzijl et al 2000), having a half life of approximately 117 years in articular cartilage (Verzijl et al 2000). Therefore the number of collagen fibers present at birth usually remains until death, unless it is degraded by injury or pathology (Eyre et al 1989). There is also no change in the amount of Type II collagen in the nucleus with age, however the proportion of Type II to Type I may change during matrix repair and remodelling, as a response to injury (Eyre and Muir 1977). In addition, collagen degradation may be affected if the number of PG is decreased, as a lack of PG makes the collagen fibril prone to degradation from collagenase (Burgeson 1982), and reduces fibrillogenesis (Olczyk 1994b).

The turnover rate for PGs in human lumbar discs takes approximately 2 to 3 years, which is similar to that in articular cartilage (Urban et al 1977, Maroudas 1988). The rate of PG synthesis is further reduced in conditions of high acidity or low oxygen and water concentration, and high levels of lactic acid in the matrix (Maroudas 1988). Most lumbar disc
studies usually report a reduction in the PG content with age, especially in the nucleus (Adams and Muir 1976, Adams et al 1977, Olczyk 1994a), however when combined with degeneration, PG reduction is observed in both the anulus and the nucleus (McDevitt 1988, Olczyk 1994a). The specific GAG that decreased with ageing in the disc matrix is CS, with an associated decrease in the number of PG aggregates as well (Adams and Muir 1976, Adams et al 1977, Urban and Maroudas 1980, Olczyk 1994a, Scott et al 1994). In contrast, HA and KS are reported to increase with age (Eyre 1979, Olczyk 1994a).

There appears to be a preferential synthesis of KS instead of CS with increased age in the disc matrix (Eyre 1979, Urban and Maroudas 1980, Scott et al 1994). Reasons for this differing trend are speculative. There may be more KS chains due to the shorter protein core with increased age (Urban and Maroudas 1980), or KS may be a functional substitute for CS in the matrix, especially in conditions of poor oxygenation (Taylor et al 1992, Scott et al 1994). Keratan sulphate does not require oxygen in its synthesis compared with CS (Taylor et al 1992) and is also more resistant to hyaluronidase (Mankin and Lippiello 1971). The implication of these GAG changes is an increased susceptibility to degeneration under mechanical loading (Adams and Muir 1976), as CS is usually more abundant in load bearing tissues (Culav et al 1999). Keratan sulphate is therefore sometimes analysed as a marker of disc degeneration and ageing (Kuiper et al 1998). In addition, there is also a loss of large aggregates in the disc with increasing age, which is not due to a lack of HA, instead the new PGs formed are not able to form aggregates (Adams and Muir 1976, Johnstone and Bayliss 1995). Therefore the molecular weight of the PG in the nucleus also decreases with increased age (Urban and Maroudas 1980).

The concentration of elastin in the disc does not seem to change with age (Herbert et al 1975, Hickey and Hukins 1982), however Johnson et al (1985) and Olczyk (1994b) found that with increased age (after the 4th decade), there is a gradual decrease in the elastin content. A similar decrease in the elastin content is also noted in herniated or degenerated nuclear matrix (Olczyk 1994b). The amount of elastic fibers are also decreased with increased age in vascular tissues (Fujimoto 1982).

Literature has not reported extensively on gender differences in the disc biochemical matrix. Zanze et al (1997) suggested that generally, girls appear to maintain a high degree of collagen degradation, in the body, up to 2 years of age despite a decrease in their rate of collagen formation, compared to boys. Therefore the timing of the changes in skeletal collagen resorption rate during the first two years of life may be gender related. Changes in the disc biochemical matrix may also be due to gender-related occupational and recreational or
physical activities and biomechanical stresses, as mentioned above in Section 2.6.2, which in turn influences disc matrix synthesis and degradation described in Section 2.17. Further studies are needed to investigate if there is a gender bias that influences the natural ageing or degeneration pattern of biochemical components in the spinal disc matrix.

2.19 Spinal Ligamentum Flavum

2.19.1 Anatomy and function of the ligamentum flavum

Elastic fibers are present in small amounts in spinal discs, but are found abundantly in spinal ligament tissues. In this thesis, spinal ligamentum flavum (LF) tissues were used to investigate the distribution of elastin crosslinks in the ligament matrix. In the thoracic region, the LF is thicker (White 1969) and is not paired as in the lumbar and cervical regions. Rather it exists as a single structure (measuring 2-3 mm laterally from the midline), which runs from the vertebral lamina below to the one above (Yong-Hing et al 1976). The upper portion of the LF attaches to the anterior inferior border of the vertebral lamina above; and the lower portion attaches to the posterior superior border of the vertebral lamina below (White 1969, Yong-Hing et al 1976, Mainman and Pintar 1990) (Figure 2.17). The LF in the lower thoracic region has the greatest tensile strength (White and Panjabi 1990a).

![Figure 2.17 Location of the ligamentum flavum (LF) on the posterior aspect of the spinal canal, indicated by arrows in [A] transverse plane, with ossification in [B] and passing over the joint capsule of the zygapophyseal joints in [C].](image-url)
Biomechanical studies have shown that the LF serves to pre-stress the intervertebral disc, exerting a disc pressure of about 0.70 kg/cm² or 0.07 MNm⁻² (Nachemson and Evans 1968). According to Nachemson and Evans (1968), the biological importance of pre-stressing the disc is not known, and may enhance the intrinsic stability of the spinal motion segment. It is also speculated that the location of the LF immediately behind the vertebral canal and its attachment to the vertebral lamina, enables the LF to assist the paravertebral muscles in restoring the spine from the flexed to the upright position (White 1969, Yong-Hing et al 1976, Johnson et al 1982), as well as to resist excessive separation of the vertebral lamina during spinal flexion and rotation (Yong-Hing et al 1976). In this way, the LF may also be important to protect the disc from injury (White 1969). Not surprisingly, Ponseti (1995) observed that animals with greater spinal mobility, such as in cats and monkeys, are also found to have a higher elastic content in the LF.

Unlike a non-elastic collagenous ligament, the LF is able to return to its original length after stretching, and does not buckle during compression of the vertebrae (Yong-Hing et al 1976, Bogduk and Twomey 1991). This is important especially in the thoracic region, where the spinal canal is relatively narrower (Roaf 1980) and any buckling of the LF will encroach on the spinal cord or nervous tissue (Bogduk and Twomey 1991), resulting in neurological symptoms or pain (van Oostenbrugge et al 1999).

The thickening or formation of calcifications in the LF has been reported in the thoracic region, especially in the mid to lower thoracic region (van Oostenbrugge et al 1999), which may be responsible for nerve entrapment and cauda equina symptoms (Yong-Hing et al 1976) (Figure 2.17 [B]). Occasionally there are some calcifications reported in the upper thoracic region, however where these calcifications occur, they have been known to produce symptoms of spinal stenosis, such as sensory impairment and motor paralysis (van Oostenbrugge et al 1999, Specchia et al 2001). Otani et al (1988) reported that 87% of surgically operated thoracic disc herniation in a Japanese population were also associated with ossification or calcification of the LF.

2.19.2 Biochemistry of the ligamentum flavum

The high elastin content (50 to 80% of dry weight), gives this tissue its characteristic elastic properties and yellow colour, hence it is also known as the yellow ligament (Partridge et al 1955, Yong-Hing et al 1976, Maiman and Pintar 1990). It is the most elastic tissue in the human body, even compared to other collagenous spinal ligament (Yong-Hing et al 1976, Maiman and Pintar 1990). The LF is therefore a rich source of elastic fibers having a ratio of
2:1 elastic to collagen fibers (Nachemson and Evans 1968). Injury to the LF usually results in a loss of elastic and collagen fibers, however during matrix repair, only the collagen and not the elastic fibers are synthesized (Yong-Hing et al 1976).

According to Mikawa et al (1986), the elastic fibers of the LF are not only more abundant but are also larger in diameter than those found in the disc. The amino acid composition of elastin are also different between the disc and the LF, with differing amounts of amino acid residues except for glycine, isoleucine and phenylalanine. Specchia et al (2001) reported that normal LF matrix is rich in collagen Type I fibers with no Type II collagen, however Types II and X collagen were observed only in degenerated and calcified LF tissues. The combination of collagen and elastin in LF is important in enabling the ligament to behave in a viscoelastic manner (Nachemson and Evans 1968), where the collagen fibers resists high tensile loads, and the elastic component enables the return to their original length after deformation (Minns et al 1973). Mikawa et al (1986) also found no significant gender differences in the amount of elastin in LF tissues, although females tended to have more elastin compared to males.

To date, there are limited quantitative studies of the biochemical constituents of the LF in the different spinal regions. A HPLC study by Chen et al (2000) on 32 human LF (18 to 78 years), found that there was no significant age-related change in the distribution of collagen crosslinks, Pyd and Dpd or elastin crosslinks, Des and Isodes in the LF. Instead they found an increase in pentosidine content with age. This age-related finding is supported by reports from Mikawa et al (1986) and Yong-Hing et al (1976). Studies on elastic fibers however showed a contrasting trend where Nachemson and Evans (1968) and Kashiwagi (1993) reported a decrease in elastic fibers and an increase in collagen fibers in human lumbar LF with increased age. In particular, collagen Type II fibers increased in the dorsal and central portion of lumbar LF with age (Kashiwagi 1993) and in degenerated LF tissues (Specchia et al 2001). One explanation for the different findings between the studies may be due to the increased accuracy of biochemical analysis and quantification of collagen crosslinks by HPLC (Chen et al 2000), which forms only part of and not the whole collagen and elastic fibers stained histologically (Kashiwagi 1993, Specchia et al 2001). It was pointed out by Nachemson and Evans (1968) that with increased age, the LF loses its extensibility from 70% to 30%. In view of the current biochemical findings, the increased stiffness may not be accompanied by a loss of elastic fibers or elastin crosslinks in the LF instead it might be due to the increased collagen in the LF matrix.
2.20 MRI ASSESSMENT OF THE DISC

Magnetic resonance imaging is currently the preferred choice for radiological spinal diagnosis and assessments, due to its greater accuracy, reliability and non-ionising radiation (Blumenkopf 1988, Boden et al 1991). In addition to the subjective evaluation of the disc, MRI may also provide a means of objectively measuring the morphological dimensions in the disc and vertebrae (Boos et al 1996), as well as the biochemical content of the disc (Weidenbaum et al 1992, Boos et al 1994). Degenerative changes in spinal discs are often described by changes in the MRI signal intensity (Aguila et al 1985, Yu et al 1988a, Eyre et al 1989, Yu et al 1989), which is more clearly observed by heavy T2-weighted MRI pulse sequences (Teresi et al 1987, Thompson et al 1988, Kaiser and Ramos 1990, Maravilla and Cohen 1991). The MR signal intensity of T2-weighted disc images is reported to be highly correlated to the gross morphology, with kappa coefficients ranging from 0.76 to 0.87 (Videman et al 1994), as well as to the biochemical constituent in lumbar disc matrices (Thompson et al 1988, Pearce et al 1991, Boos et al 1994).

Magnetic resonance imaging also provides improved soft-tissue delineation compared with previous radiological imaging techniques (Modic et al 1984, Gibson et al 1986, Maravilla and Cohen 1991, Martin et al 1992, Boos et al 1995). The sensitivity of MRI in detecting early disc degenerative changes is reported to be higher than X-Rays (Modic et al 1984, Paajanen et al 1989), CT scans (Modic et al 1984, Ridenour et al 1993), myelography (Blumenkopf 1988, Ridenour et al 1993) and discography (Gibson et al 1986, Schneiderman et al 1987, Videman et al 1994). Russell (1989) reported that the accuracy in diagnosing surgically treated thoracic disc herniation is reported to be advanced with MRI (100%) compared with plain radiography, which yielded only 55% positive results and contrast myelography with 95% accuracy. Magnetic resonance imaging was 100% positively correlated with CT scans after myelography (Ross et al 1987, Blumenkopf 1988, Ridenour et al 1993) and 99% accurate in predicting lumbar disc degeneration, confirmed on discography (Schneiderman et al 1987).

The MRI investigation system consists mainly of 4 components; a strong homogenous magnetic field with a field strength ranging from 0.5 to 2.0 Tesla; gradient coils which are used during scanning to generate small-gradient magnetic fields; Radio frequency coils to transmit and receive radio waves at a pre-determined frequency; and a computer system, which analyses the signals received to reconstruct an image (Kaiser and Ramos 1990). According to Kaiser and Ramos (1990), it is the hydrogen nuclei or protons, in the human body that is magnetically activated to align itself along the long axis of the surrounding magnetic field. The radio frequency coils subsequently transmit short radio waves that cause
the protons to deviate, which when the transmission is stopped, the protons re-align in the plane of the magnetic field, releasing small pockets of energy signals which are picked up by the receiving coils. These latter signals constitute the MRI signal.

The signal intensity of the MR image formed is dependent on the density of protons and macromolecules in the tissue matrix (Maravilla and Cohen 1991, Pearce et al 1991). Initially the signal intensity was assumed to reflect the content of water in the disc matrix (Boden et al 1991), however Pearce et al (1991) have found that the signal intensity was more closely related to the PG and collagen content. Lumbar disc studies correlating the MRI signal intensity and the biochemical matrix have found that the loss of water and proteoglycans and increased collagen content in the nucleus is associated with low signal intensity (Thompson et al 1988, Yu et al 1988a, Pearce et al 1991, Tertti et al 1991, Martin et al 1992). Areas of high signal intensity are instead correlated with increased water or inflammatory exudate, and usually indicate the presence of nuclear material due to the higher water and proteoglycan content (Modic et al 1988, Yu et al 1988b, Sether et al 1990, Pearce et al 1991). However Tertti et al (1991) and Gunzburg et al (1992) cautioned that changes in MRI signal intensity can occur due to biochemical changes with no observable morphological changes. Therefore MRI is able to provide detection of early disc degenerative changes before morphological changes appear, especially if there are associated biochemical changes occurring.

The imaging protocol for human spine studies usually include both sagittal T1- and T2-weighted images. T1-weighted images are acquired using short repetition times (300 to 800 msec) and short echo times (20-40 msec) (Kaiser and Ramos 1990), and provide better definition of spinal morphology such as vertebral body, facet joints, intra-spinal bone mass, tumour or inflammation (Maravilla and Cohen 1991). Conversely T2-weighted images provide the best definition for spinal disc morphology, delineating the anulus, nucleus, bulging disc and herniated disc fragments, and spinal cord (Maravilla and Cohen 1991). This MRI sequence also provides a high contrast between the cerebro-spinal fluid and detection of tumours in the spinal cord (Kaiser and Ramos 1990, Maravilla and Cohen 1991), and is acquired using long repetition (2500 to 3000 msec) and echo times (75 to 120 msec) (Kaiser and Ramos 1990).

Using MRI, disc degenerative changes are often graded according to changes in the T2-weighted signal intensity in the disc components. A 5-point scale for grading lumbar disc degenerative changes on MR images, using T2-weighted sequences, has been developed by Thompson (unpublished) cited in Eyre et al (1989). This grading scale has been found to be correlated with the biochemical constituents of the disc matrix and morphological status of
the disc \((k = 0.62)\) (Thompson et al 1988, Pearce et al 1991). The intra-observer reliability of grading lumbar and thoracic discs using other conventional subjective MRI disc parameters is reported to be good \((k = 0.7)\) (Brant-Zawadzki et al 1995, Raininko et al 1995, Pfirrmann et al 2001), with better consistency grading lumbar than thoracic discs (Raininko et al 1995). The discs in the middle and upper thoracic region are more difficult to evaluate reliably due to the small disc size and background noise from respiration and cardiac movement (Raininko et al 1995, Videman et al 1995a).

The main disadvantages in using MRI are;

1. Movement artefacts due to respiration, cardiac motion, pulsatile cerebro-spinal fluid, swallowing and movement of the blood in the aorta (Modic et al 1984, Goldberg et al 1988, Raininko et al 1995). This is especially relevant to MRI of the thoracic region.

2. The MRI acquisition sequences are often quite long and may cause claustrophobia for some patients, especially children (Modic et al 1984).

3. High false-positive results, especially in asymptomatic individuals, can occur, therefore there is a need to correlate MRI findings with clinical assessment and history (Maravilla and Cohen 1991, Wood et al 1995).

Recent MRI studies have also reported on the use of quantitative MRI parameters to correlate with spinal morphology (Southern et al 2000, Kerttula et al 2001, Vaithianathar et al 2003), although biochemical content correlation in spinal disc is still limited (Weidenbaum et al 1992, Boos et al 1997a). The T2-weighted signal intensity has been quantified and correlated with disc degeneration in lumbar discs (Southern et al 2000, Kerttula et al 2001), and quantitative T1 relaxation times have been used to assess cervical cord atrophy (Vaithianathar et al 2003). However Stafira et al (2003) reported that the ability of MRI signal intensities to objectively assess cervical spinal stenosis was still variable.

### 2.21 EFFECTS OF FORMALIN FIXATION IN SPINAL TISSUE

Living tissues undergo autolysis or self-destruction upon cell death (Leong 1994). If these tissues are not fixed or preserved, the proteins will degrade and the matrix will disintegrate, making the handling and analysis of tissues difficult (Leong 1994). Formalin fixation arrests cellular autolysis and is used to preserve the cellular structure and tissue constituents, with minimal alteration from the living state (Chapman et al 1990, Leong 1994). However fixation, and its attendant induced crosslinks, renders certain biochemical analysis invalid due to its stabilising effect on the extracellular matrix (Hickey and Hukins 1979, Boskey et al 1982,
Brooks et al 1998). With the increased legal regulations and restrictions on access to fresh human cadaver for research purposes, and the potential risk of infection from handling these tissues, there is a need for alternative means of investigating biochemical changes in human tissues, such as using formalin-fixed archived tissues (Cavanaugh and King 1990).

Formaldehyde (CH$_2$O) or its hydrated form methylene glycol (CH$_2$(OH)$_2$), commonly used as 4% buffered formaldehyde or 10% buffered formalin, is widely used as a universal tissue fixative (Leong 1994). Formaldehyde produces formalin induced non-reducible chemical cross-links with proteins and glycoproteins in the tissues, to prevent deterioration of the tissue matrix (French and Edsall 1945, Walker 1964, Brooks et al 1998). It is commonly used in tanning and preserving collagen and tissue components (French and Edsall 1945). Formalin interacts with a number of different kinds of proteins, forming crosslinks which stabilise the tissue matrix (Gustavson 1956, Chapman et al 1990). In spite of the new crosslinks formed, some biochemical parameters may be measured in formalin-fixed tissues, as fixation does not result in crosslinking for all tissue components (French and Edsall 1945), or they may be released for analysis by cleavage of the formalin induced crosslinks (Dwek et al 1996, Brooks et al 1998).

Studies on the effect of formalin fixation on bony and soft tissue matrices are limited. The main function of formalin fixation on tissues is to preserve tissue morphology (Brooks et al 1998), evidenced by the unchanged collagen morphology in fixed anterior cruciate ligament and tendon tissues (Viidik and Lewin 1966); collagen fibre orientation and arrangement in fixed heart valve (Lee et al 1984) and myocardium tissues (Yoshikane et al 1992) and mummified tissues (Montes et al 1985). Inspite of the preservation of tissue morphology, certain biomechanical functions, such as tensile strength, was instead found to be reduced in porcine aortic valve (Lee et al 1984) and in rabbit ligament (Viidik and Lewin 1966). Formalin fixation also increased collagenous packing in disc tissues (Hickey and Hukins 1979) resulting in hardening or tanning of soft tissues (Gustavson 1956) and increased stiffness in rabbit ligaments (Viidik and Lewin 1966) and spinal ligaments (Wilke et al 1996). However Edmondston et al (1994a) reported minimal change in the density and mechanical strength of formalin-fixed sheep and human vertebrae.

Formalin produces extensive crosslinks by deamination of protein or collagen molecules at the free amino acid groups on lysine and hydroxylysine residues, to form methylene crosslinkages (Gustavson 1956, Walker 1964, Harris and Farrell 1972, Puchtler and Meloan 1985), which are mainly non-polymeric (Chapman et al 1990). These monomeric formalin induced crosslinks are however resistant to bacterial and synovial collagenase (French and
Edsall 1945, Walker 1964) and insoluble in acetic acid (Harris and Farrell 1972). However they could be denatured by collagenase at temperatures of more than 37°C (Harris and Farrell 1972). Formalin crosslinks have a thermal resistance up to 86°C (Chapman et al 1990, Sung et al 1997). These induced crosslinks may be cleaved on acid digestion or hydrolysis and are quantitatively accounted for during chemical analysis (French and Edsall 1945, Gustavson 1956, Puchtler and Meloan 1985). Therefore formaldehyde is sometimes referred to as an “innocuous fixative” (Puchtler and Meloan 1985) or a “forgiving fixative” (Leong 1994). Abe et al (2003) reported no significant differences in the collagen content and collagen crosslinks Pyd and pentosidine, between fresh and formalin-fixed human LF and cartilage tissues. They however reported significantly different lower amounts of Des and Isodes in formalin-fixed tissues. Fung and Sobin (1981) reported that the elastic fibers of ligamentum nuchae did not lose the elasticity and was not altered by fixation.

Toledo et al (1996) also reported significantly lower PGs in formalin-fixed connective tissues. Formalin induced crosslinks with PG molecules usually result in a decrease of PGs during chemical analysis, due to a change in reactivity of functional groups or inability to extract PG after treatment with formalin (Boskey et al 1982, Chapman et al 1990). Even after a year, there was no change in the amount of PG that could be extracted from fixed cortical bone tissues (Boskey et al 1982). Adipose tissue and lipids however are not affected by formalin fixation unless stored for more than a year (Boskey et al 1982, Leong 1994). Therefore besides the type of tissue to be fixed, the duration of fixation is also an important factor to consider. Short exposure of tissues to formalin (< 10 minutes) produces a change in the number of collagen crosslinks (Chapman et al 1990). According to Leong (1994) the depth of penetration of formalin is estimated to be 0.78 mm after one hour, therefore tissues of approximately 2 mm thickness should be exposed for at least four to eight hours for full penetration of formalin. Other matrix components such as heavy metals, for example aluminium and manganese, were reportedly altered only after 1 year of fixation in rat liver tissues (Bush et al 1995).

The influence of formalin fixation on collagen content and crosslinks Pyd and pentosidine, appear to be unaffected by fixation in tissue matrices. However evidence on the effect of formalin on elastin crosslinks needs further confirmation despite findings by Abe et al (2003), as histological and biomechanical studies report no alteration of the elastic fiber morphology and function with fixation.
2.22 SUMMARY OF THE LITERATURE REVIEW

The thoracic region is reported to have less disc degenerative changes compared to the cervical and lumbar regions, however MRI studies do provide evidence that disc degenerative changes are present in this spinal region. The limited cadaver, radiological and MRI thoracic disc studies demonstrate that degenerative changes are found predominantly in the mid and lower thoracic regions. The major mechanical forces accentuating disc degeneration in the mid thoracic region are the combination of increased compression loading due to the kyphotic curvature; and the higher frequency of rotational forces in the mid thoracic region during daily activities. In the lower thoracic region, the mechanical forces that perpetuate disc degeneration are the increased spinal range of motion especially in the last two thoracic vertebrae; and the larger compression loading in these discs. In addition to these biomechanical factors, disc degeneration in the mid and lower thoracic regions is also exacerbated by gender and age-related occupational and recreational activities.

Biomechanical lumbar discs studies also confirm that sudden and excessive torsion or combined compression and torsion or flexion stresses compromise the integrity of the disc structures, in particular that of the anulus, and are the primary cause of disc degeneration. Even normal physiological stresses on the disc if repeated often enough during occupational or daily activities throughout the life span, can predispose the disc to injury. In addition, sustained compression loading in the disc may cause poor nutrition and matrix synthesis, which also renders the disc prone to degenerative changes.

Even more limited than morphological studies are spinal biochemical studies, especially for the thoracic discs. The main extracellular matrix fibers in the disc are collagen and PG, and to a lesser amount, elastic fibers, non-collagenous proteins and water (Figure 2.18). Lumbar disc studies provide sufficient evidence that water and PG are decreased with ageing, especially in the nucleus. Turnover of the matrix is usually slow, due to the avascularity of the disc, unless there is injury and inflammatory changes, which release enzymes facilitating matrix repair or breakdown. In such circumstances, the disc matrix turnover is faster however, the new matrix synthesised may be of poor quality and quantity (Krämer 1990, Bartels et al 1998, Gruber and Hanley 1998); with decreased cell viability and increased susceptibility to further degeneration (Cassinelli et al 2001, Horner and Urban 2001). The disc matrix is therefore not static but is capable, to a limited extent, of altering its metabolic and biosynthetic activities to meet the changing demands due to mechanical forces, injury and disease.
Recent biochemical studies have identified the presence of mature stable post-translational crosslinks, in particular collagen (Pyd and Dpd) and elastin (Des and Isodes) crosslinks, which are essential for the tensile and viscoelastic strength of spinal disc and ligament matrices (Eyre et al 1989). Although elastic fibers are present in small amounts, lumbar disc studies have shown that they are important to support the function of the collagen fibers (Yu et al 2002). The collagen crosslink, Pyd, may be influenced negatively by age and degenerative changes, therefore it may be a biochemical marker of disc degeneration. There is currently not much information on the changes in Dpd or elastin crosslinks, Des and Isodes, due to age, degeneration, gender and spinal segmental influences. A loss or degradation of collagen and elastin crosslinks in the disc matrix may impact negatively on the mechanical ability of the disc to bear compression load and to withstand tensile forces, resulting in accelerated disc matrix degeneration.

![Diagram showing ANULUS and NUCLEUS with their biochemical constituents](image)

**Figure 2.18** Summary of the different biochemical constituents of the anulus and the nucleus within a typical spinal intervertebral disc.

Elastic fibers are more abundant in spinal ligament tissues, therefore, biochemical investigations to analyse the changes of elastin crosslinks should preferably analyse these tissues. To date information on the normative biochemical data for human spinal discs and LF in particular, the collagen and elastin crosslinks, is limited, not just for thoracic discs but in lumbar discs as well. Such information may assist our understanding of normal biochemical changes due to spinal region and gender-related biomechanical forces over the life span.

In addition, there is limited information on the effect of formaldehyde on collagen and elastin crosslinks in spinal discs and LF. The recent study by Abc et al (2003) suggested that formaldehyde does not change or mask the collagen content or the extent of Pyd in spinal disc ligament tissues, however they found that elastin crosslinks were significantly affected by fixation. Proteoglycans in particular were also denatured by formalin crosslinks, which are
not retrievable for biochemical analysis (Toledo et al 1996). Further studies to provide more information on the effects of formalin fixation in the biochemical matrices of spinal soft tissues will improve our understanding of the crosslinking properties of formalin, and enable the use of archived cadaver material as an alternative source for biochemical analyses.

Based on the current literature, there is also a paucity of correlative radiological, morphological and biochemical information on the thoracic disc, which warrants further research. MRI is particularly suited to investigate and correlate the morphological changes in spinal discs, as the MRI signal intensity is contingent on the matrix biochemical constituents. Information that correlates the disc morphology and biochemical matrix is required to advance our knowledge on the pathogenesis and sequel of thoracic disc degenerative processes, in particular, from a biochemical perspective.

In summary, the main contributors to spinal disc degeneration are age, spinal region, gender-related mechanical loading history and trauma or disease (Figure 2.19). Studies provide evidence for a causative influence of these various factors perpetuating disc degeneration either through injury or by altering the spinal mechanics and disc biochemical matrix. These factors influence the biochemical matrix synthesis and turnover, such that if repair is slower than degradation, disc matrix degeneration occurs. Disc degenerative changes in turn lead to further alterations in the disc mechanics, accentuating the sequence of degeneration. The extent to which disc degeneration is a pathologic process or an inevitable consequence of ageing is not conclusive, however the two processes collectively accentuate the demise of the disc matrix.
Figure 2.19 Schematic diagram to show putative interactions and influences of age, gender, spinal level and trauma, on the mechanical loading, disc nutrition and matrix metabolism, leading to disc degeneration. Dashed lines represent responses that further accentuate degenerative causative factors.
CHAPTER 3  AIMS OF INVESTIGATION

This thesis reports investigations on \textit{ex-vivo} and \textit{in-vivo} human thoracic spine subjects, to survey the prevalence of thoracic disc and vertebral body degenerative changes across the life span using MRI and macroscopic examination. Using a second series \textit{ex-vivo} cadavers, biochemical analyses were performed to determine the distribution of collagen and elastin crosslink content in thoracic discs and spinal ligamentum flavum matrices, due to the influence of age, gender and spinal level factors.

3.1 MAIN OBJECTIVES

The following issues were examined in this thesis:

1. To investigate the reliability of using subjective assessment scales to grade disc degenerative changes in thoracic discs using sagittal T2-weighted MRI.

2. To survey the prevalence and associations of degenerative disc and vertebral changes in the thoracic spine, using sagittal T1- and T2-weighted MR images. In addition, the influence of age, gender and spinal regional factors on the distribution of these degenerative changes were also examined.

3. To determine the effect of formalin fixation on the collagen and elastin crosslinks in the extracellular matrix of human spinal discs and ligamentum flavum.

4. To measure the distribution of collagen and collagen crosslinks, pyridinoline and deoxypyridinoline, and elastin crosslinks, isodesmosine and desmosine, in human thoracic discs and ligamentum flavum, and to examine for age, degeneration, gender and spinal regional influences.

5. To correlate MRI and macroscopic assessments of thoracic disc and ligamentum flavum degeneration with the biochemical distribution of proteoglycans, collagen and elastin crosslinks in fresh cadaver tissues.

MR images of \textit{in vivo} spine cases were graded for degenerative changes in thoracic discs and used to measure the morphological changes of the thoracic vertebral body. In a separate series of \textit{ex-vivo} cadaver spinal cases, thoracic discs and spinal LF were harvested and
examined macroscopically before being analysed for biochemical changes. Various biochemical assays were utilised to extract and determine collagen, collagen and elastin crosslinks in disc and ligamentum matrices. The final case study report in Study 5.6, correlated the MRI and macroscopic degeneration grading with the biochemical constituents of collagen, elastin and proteoglycans in the disc and ligamentum matrices from two fresh human thoracic spine cadavers.

3.2 ETHICAL CONSIDERATIONS

Approval for the proposed research studies had been obtained by the University’s Human Research Ethics Committee. Consent was also obtained from the various Heads of Department of Radiology, at RPH, SCGH and SGH to extract thoracic MRI films from their archives. Approval for the use of the laboratories for biochemical tests was obtained from the Head of Clinical Pathology Division, The Queen Elizabeth II Medical Centre, PathCentre, and approval to perform morphological macroscopic examinations on post-mortem vertebral columns was obtained from the Head of Neuropathology, RPH.
CHAPTER 4 INVESTIGATIONS USING THORACIC MRI FILMS

This chapter summarises the methods used in this thesis to examine in vivo spine cases using MRI films. MR images of spine cases were graded to examine for degenerative changes in thoracic discs and correlated with morphological measurements of the thoracic vertebral body. As the 5-point MRI grading scale described by Thompson published in Eyre et al (1989) was originally designed and evaluated to grade lumbar discs, it was necessary to determine the reliability of this scale to evaluate thoracic discs.

In vivo MRI films were used for the following investigations:

4.1 Reliability of a modified grading scheme for MRI assessment of thoracic intervertebral disc (Tan et al 2000b).

4.2 Patterns of thoracic disc degeneration using MRI: Age, gender and spinal level influences (Tan et al 2001).

4.3 Age and gender influences on thoracic vertebral body shape and disc degeneration: An MR investigation of 169 cases (Goh et al 2000b).
STUDY 4.1 RELIABILITY OF A MODIFIED GRADING SCHEME FOR MRI ASSESSMENT OF THORACIC INTERVERTEBRAL DISC

Various subjective MRI grading schemas have been developed and used to assess disc degeneration changes in spinal discs, based on examinations of human lumbar discs (Thompson et al. 1988, Boden et al. 1990a, Sether et al. 1990, Tertti et al. 1991, Boos et al. 1997a). The 5-point MRI disc grading system developed by Thompson (unpublished) but reported in Eyre et al. (1989), showed a high correlation to the macroscopic grading scale by the same authors (Thompson et al. 1988), on human lumbar discs ($k = 0.62$ and correlation coefficient $= 0.84$, $p<0.0001$). Grading of the signal intensity from MR images were found to be closely associated with changes in the collagen and proteoglycan content of the disc matrix (Tertti et al. 1991). The Thompson MRI 5-point scale however, had not been reported for the evaluation of thoracic discs, which were more difficult to assess because of their reduced size and signal intensity compared to the lumbar discs (Raininko et al. 1995).

Brant-Zawadzki et al. (1995) suggested that the reliability of any standardised MRI nomenclature might not be influenced as much by the experience of the reader, as by the morphologic descriptions. They suggested that a binary form of nomenclature was likely to be more reliable than a grading system that might not include descriptions of all possible stages of disc degeneration. A modified 3-point MRI grading scale was therefore designed to evaluate the smaller thoracic discs, by collapsing grades 2 and 3 criteria as grade II, and grades 4 and 5 as grade III, and using grade 1 as I for non-degenerate discs (Table 4.1).

The objective of the study was to compare the intra-observer variability of grading thoracic disc degenerative changes using the 5-point MRI Thompson grading scale and a 3-point modification of this scale, on sagittal T2-weighted MR images. The inter-observer variability of these two scales within the thoracic region was also examined as a preliminary to a larger thoracic MRI audit.

4.1.1 MATERIALS AND METHODS

4.1.1.1 Sample selection
A survey of human thoracic MR spine films taken between 1995 and 1998 were reviewed retrospectively from MR archives of three metropolitan teaching hospitals, RPH and SCGH in Western Australia and SGH in Singapore. The audit identified 300 cases for review. This series of films comprised MRI investigations of the spine for various systemic and musculoskeletal investigations of the thoracic region. Both T1- and T2-weighted sagittal
image sequences and accompanying diagnostic reports were audited to exclude cases demonstrating scoliosis deformity, severe localised vertebral deformation, pathological fracture, spinal metastases, or evidence of spinal surgery. Cases were selected to ensure that a spectrum of normal to severe degenerative changes in the discs was evaluated. Films were excluded from the study if the image quality was poor, especially at the periphery, or if the images were contrast-enhanced. Thoracic discs were evaluated from all available sagittal sequences.

A total of 46 T2-weighted sagittal thoracic MRI films (18 males and 28 females, aged 7 to 73 years, mean age = 41 ± 16.3 years) were randomly selected from the 300 MRI cases. An independent observer, who did not participate in the study, selected the 46 MRI cases. All films were numbered and the first 30, comprising 13 males and 17 females, (7 to 73 years, mean age = 39 ± 18 years) were graded using the modified 3-point scale (Table 4.1, Figure 4.1). On completion of the 3-point grading, the films were returned to the original pool of 46 films, and another 30 MR cases were randomly selected for grading, comprising of 12 males and 18 females, (13 to 73 years, mean age = 44 ± 15 years). These were subsequently graded using the 5-point grading scale (Table 4.1, Figure 4.1).

The second reading of the MR images, using both grading scales, was conducted using the same set of 30 films for each grading scale, but in a random order from the first reading. The second reading was conducted at least 3 months after the first reading. T2-weighted MR images were used to examine for thoracic disc degeneration status and all grading of the discs using T2-weighted MR images was conducted by myself, investigator C Tan.

### 4.1.1.2 Review of vertebral disc morphology using T2-weighted MR images

MRI units used were Picker Vista 1.0 Tesla (Cleveland, Ohio) at the RPH, the 1.5 Tesla Siemens Magnetom Vision Plus (Erlangen, Germany) at SCGH and 1.5 Tesla Siemens Magnetom Vision (Erlangen, Germany) at SGH. The MRI parameters for examining vertebral disc morphological changes comprised T2-weighted turbo or fast-spin echo sequences, with repetition time ranging from 3000 to 4700 msec, and echo time ranging from 90 to 112 msec. The slice thickness ranged from 3 to 4 mm with a field of view of 30 to 35 mm and an acquisition matrix range of 192X256 (RPH), 270X512 (SCGH) and 225X300 (SGH).

All sagittal T2-weighted thoracic sequences were reviewed for disc changes at all available thoracic levels (T1 to T12). Using the Thompson MRI grading scale (Table 4.1), grading for the anulus referred to the signal intensities identified from both anterior and posterior anular
components of the disc. The higher degenerative grade from either the anterior or posterior anulus was taken as the overall grade for the anulus of that disc. Progressive grades for end-plates represented gradual disruption of end-plates culminating in distinct herniations into the vertebral body; often referred to as Schmorl’s nodes (Schmorl and Junghanns 1971, Resnick and Niwayama 1978). Grading for the presence of osteophytes examined for the increasing size of bony osteophytes at the anterior disc margins only.

No attempt was made to differentiate superior or inferior osteophytes, or end-plate lesions. Multiple lesions at a particular thoracic disc level, such as the presence of superior and inferior end-plate lesions, were counted as one occurrence for end-plates. Thoracic discs were numbered below the level of the thoracic vertebra, that is, T1-T2 disc referred to the intervertebral disc below the first thoracic vertebral body. Thoracic levels were confirmed with the help of location markers in the sagittal “scout” image.

4.1.1.3 MRI training sessions

The investigator (C Tan) was trained by an experienced MRI radiologist (S Song), to use both the 3- and 5-point MRI grading scales to evaluate degenerative changes in thoracic discs. Training sessions were conducted using MR images, which included normal and degenerated thoracic discs from spinal cases of different ages.

One month after the last training session, an inter-rater reliability evaluation was conducted between both investigators. The first ten randomly selected MRI cases from the 46 cases \( n = 120 \) discs) were evaluated using the 3-point scale and returned to the pool. Another 10 films were randomly selected from the same pool for the evaluation using the 5-point scale. An independent observer selected the MRI cases. Both examiners evaluated the ten thoracic MRI films in random order and independently for each grading scale. The MR images were only evaluated once by each of the investigators.
Figure 4.1 Sagittal T2-weighted thoracic MR image of a 46-year old female showing examples of different grades for the spinal disc components. Using the 3-point scale: (A) shows a T2-T3 disc with grade I nucleus, anulus, end-plates and no osteophyte. (B) shows a T6-T7 disc with grade II nucleus and grade III osteophyte. (C) is a T7-T8 disc with grade III nucleus, and grade II anulus and osteophyte and (D) is a T10-T11 disc with grade II nucleus and anulus, and grade III end-plate and osteophyte. Using the 5-point scale: (A) the T2-T3 disc is similar to the 3-point scale, whereas (B) T6-T7 disc has grade II nucleus and grade IV osteophyte. (C) is a T7-T8 disc with grade IV nucleus, grade III anulus and grade II osteophyte and (D) T10-T11 disc has grade II nucleus and anulus, grade V end-plate and grade IV osteophyte.
Table 4.1 Description of the 5-point and the modified 3-point grading scales used for assessing thoracic anulus, nucleus, end-plate and osteophytes, based on the unpublished Thompson* MRI grading criteria cited in Eyre et al (1989).

<table>
<thead>
<tr>
<th>3-point scale</th>
<th>5-point scale</th>
<th>Nucleus</th>
<th>Anulus</th>
<th>End-plate</th>
<th>Osteophytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (normal)</td>
<td>I</td>
<td>Homogeneous; bright; demarcation distinct</td>
<td>Homogenous dark gray</td>
<td>Single dark line</td>
<td>Margins rounded</td>
</tr>
<tr>
<td>II (Moderate)</td>
<td>II</td>
<td>Horizontal dark bands extend across the anulus centrally</td>
<td>Areas of increased signal intensity</td>
<td>Increase in central concavity</td>
<td>Tapering of margins</td>
</tr>
<tr>
<td>III</td>
<td>III</td>
<td>Signal intensity diminished; gray tone with dark and bright stippling</td>
<td>Indistinguishable from nucleus</td>
<td>Line less distinct</td>
<td>Small dark projections from margins &lt; 2 mm size</td>
</tr>
<tr>
<td>III (Severe)</td>
<td>IV</td>
<td>Proportion of gray signal reduced; bright and dark regions larger</td>
<td>Indistinguishable from nucleus; some bright and dark signals contiguous with nucleus and anulus</td>
<td>Focal defects in line</td>
<td>Projections &gt; 2 mm with same intensity as marrow</td>
</tr>
<tr>
<td>V</td>
<td>V</td>
<td>Gross loss of disc height; bright and dark signals dominant</td>
<td>Signals contiguous with nucleus</td>
<td>Defects and areas of thickening</td>
<td>Projections &gt; 2 mm with projections from both superior and inferior meeting</td>
</tr>
</tbody>
</table>

*Based on T2-weighted spin-echo images TR 2,000 msec and TE 90 msec for lumbar discs.
4.1.2 DATA REDUCTION AND STATISTICAL ANALYSIS

The kappa coefficient ($k$), was used to assess the intra-rater reliability of using both the 3- and 5-point MRI grading scales. The kappa coefficient values below 0.40 were rated as “poor”; between 0.41 and 0.74 as “fair”; between 0.75 to 0.90 as “good” and above 0.90 as “excellent” for intra-rater reliability. Raw data is recorded in Appendix A.

The percentage of agreements on degeneration grades (that is, grades II and III for the 3-point scale and grades II to V for the 5-point scale) and the percentage of disagreements between the two readings, were also reported. The percentage of degeneration agreement between the two readings was calculated as total number of agreements on degeneration grades divided by total number of degeneration readings. The percentage of disagreement between the two readings was calculated as the total number of disagreements divided by the total number of readings.

To analyse for thoracic regional influences on the intra-rater reliability of MR grading, data from each thoracic regional level were regrouped to form upper (T1 to T4), mid (T5 to T8) and lower (T9 to T12) thoracic regions.

4.1.3 RESULTS

4.1.3.1 Inter-rater reliability

The inter-rater reliability between the investigator and the expert was higher using the 3-point ($k$ range = 0.64 to 0.88) scale compared to the 5-point scale ($k$ range = 0.54 to 0.83) except for the rating on end-plates, which was slightly higher for the 5-point scale (Table 4.2). Using the 3-point scale, the inter-rater reliability was highest or “good” for the nucleus ($k = 0.88$, $p<0.001$) and lowest or “fair” for the osteophytes ($k = 0.64$, $p<0.001$). For both scales, the $k$ rating for the nucleus and the end-plate were “good” and for the anulus and osteophyte, “fair”.

4.1.3.2 Intra-rater reliability

The intra-rater $k$ coefficient for the 5-point and 3-point grading scales was highest for the nucleus (0.78 and 0.87, $p<0.0001$, respectively) and lowest for osteophytes (0.57 and 0.71, $p<0.0001$, respectively). For the modified 3-point scale, the $k$ coefficient was rated “good” for the nucleus, anulus and end-plates, except for osteophytes, which rated “fair”. Using the 5-point scale, only the nucleus was rated as “good”, while the anulus, end-plates and
Table 4.2 The Inter-rater $k$ coefficient for the four spinal disc components using the MRI 3- and 5-point grading scales.

<table>
<thead>
<tr>
<th>Disc component</th>
<th>3-point scale</th>
<th>5-point scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus</td>
<td>0.88</td>
<td>0.83</td>
</tr>
<tr>
<td>Anulus</td>
<td>0.72</td>
<td>0.70</td>
</tr>
<tr>
<td>End-plate</td>
<td>0.75</td>
<td>0.81</td>
</tr>
<tr>
<td>Osteophyte</td>
<td>0.64</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Osteophytes were rated as “fair” (Tables 4.3 and 4.4). The percentage of disagreement was also lower for the 3-point scale (4 to 18%) compared to the 5-point scale (6-24%). The intra-rater $k$ coefficients were generally higher compared to the inter-rater $k$ coefficients for the 3-point scale. However for the 5-point scale, this was reversed, with the inter-rater $k$ coefficients being slightly higher for the nucleus, anulus and end-plate readings compared to the intra-rater $k$ coefficients.

4.1.3.3 Intra-rater reliability for different thoracic levels

Generally, the intra-rater $k$ coefficient was highest for the upper thoracic region and lowest for the lower thoracic region for both scales (Figure 4.2). There were however, a few exceptions. Using the 5 point-scale, the $k$ coefficient for end-plate lesions was higher in the lower thoracic (0.76) compared to the mid thoracic region (0.61); and the $k$ coefficient for osteophytes was higher in the mid thoracic region (0.58) compared to the upper thoracic region (0.55, Table 4.4). For the 3-point scale, the only exception was the $k$ coefficient for anular grading in the mid thoracic (0.96), which was higher than the upper thoracic region (0.76).

The percentage of agreements on degeneration findings was highest for the upper thoracic level and lowest for the lower thoracic region using both 3- and 5-point scales (Tables 4.3 and 4.4). The percentage of disagreement in the mid thoracic region was similar (3-point scale) or slightly higher (5-point scale) compared with the lower thoracic region.
Table 4.3 The Intra-rater observer $k$ coefficient, percentage of agreements (% Agreement) for degeneration grading, and percentage of disagreements (% Disagreements) between the two readings, for the spinal components at different thoracic regions ($n = 360$ discs), using the modified 3-point grading scale.

<table>
<thead>
<tr>
<th>Level</th>
<th>Disc</th>
<th>$k$</th>
<th>%Agreement</th>
<th>%Disagreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper</td>
<td>Nucleus</td>
<td>0.88</td>
<td>86</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Anulus</td>
<td>0.76</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>End-plate</td>
<td>1.00</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Osteophytes</td>
<td>0.80</td>
<td>70</td>
<td>8</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>0.76 - 1.00</td>
<td>64 - 100</td>
<td>0 – 8</td>
</tr>
<tr>
<td>Mid</td>
<td>Nucleus</td>
<td>0.85</td>
<td>83</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Anulus</td>
<td>0.96</td>
<td>93</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>End-plate</td>
<td>0.92</td>
<td>88</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Osteophytes</td>
<td>0.71</td>
<td>69</td>
<td>18</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>0.71 - 0.96</td>
<td>69 - 93</td>
<td>2 – 18</td>
</tr>
<tr>
<td>Lower</td>
<td>Nucleus</td>
<td>0.85</td>
<td>88</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Anulus</td>
<td>0.74</td>
<td>67</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>End-plate</td>
<td>0.89</td>
<td>82</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Osteophytes</td>
<td>0.59</td>
<td>49</td>
<td>19</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>0.59 - 0.89</td>
<td>49 - 88</td>
<td>4 – 19</td>
</tr>
<tr>
<td>Thoracic spine</td>
<td>Nucleus</td>
<td>0.87</td>
<td>85</td>
<td>8</td>
</tr>
<tr>
<td>(All levels)</td>
<td>Anulus</td>
<td>0.83</td>
<td>75</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>End-plate</td>
<td>0.85</td>
<td>75</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Osteophytes</td>
<td>0.71</td>
<td>57</td>
<td>18</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>0.71 - 0.87</td>
<td>57 - 85</td>
<td>4 – 18</td>
</tr>
</tbody>
</table>
Table 4.4 The Intra-rater observer $k$ coefficient, percentage of agreements (% Agreement) for degeneration grading, and percentage of disagreements (% Disagreements) between the two readings, for the spinal components at different thoracic regions ($n = 360$ discs), using the 5-point grading scale.

<table>
<thead>
<tr>
<th>Level</th>
<th>Disc</th>
<th>$k$</th>
<th>%Agreement</th>
<th>%Disagreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper</td>
<td>Nucleus</td>
<td>0.88</td>
<td>86</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Anulus</td>
<td>0.71</td>
<td>55</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>End-plate</td>
<td>1.00</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Osteophytes</td>
<td>0.55</td>
<td>43</td>
<td>21</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>0.55 - 1.00</td>
<td>43 - 100</td>
<td>0 - 21</td>
</tr>
<tr>
<td>Mid</td>
<td>Nucleus</td>
<td>0.75</td>
<td>72</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Anulus</td>
<td>0.71</td>
<td>60</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>End-plate</td>
<td>0.61</td>
<td>39</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Osteophytes</td>
<td>0.58</td>
<td>49</td>
<td>37</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>0.58 - 0.75</td>
<td>49 - 76</td>
<td>9 – 37</td>
</tr>
<tr>
<td>Lower</td>
<td>Nucleus</td>
<td>0.68</td>
<td>76</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Anulus</td>
<td>0.49</td>
<td>35</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>End-plate</td>
<td>0.76</td>
<td>63</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Osteophytes</td>
<td>0.41</td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>0.41 - 0.76</td>
<td>29 - 76</td>
<td>8 - 22</td>
</tr>
<tr>
<td>Thoracic spine (All)</td>
<td>Nucleus</td>
<td>0.78</td>
<td>77</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Anulus</td>
<td>0.63</td>
<td>49</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>End-plate</td>
<td>0.72</td>
<td>53</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Osteophytes</td>
<td>0.57</td>
<td>45</td>
<td>24</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>0.57 - 0.78</td>
<td>45 - 77</td>
<td>6 - 24</td>
</tr>
</tbody>
</table>
4.1 Reliability of MRI grading scale

**Figure 4.2** The intra-rater $k$ coefficient for the 5-point scale and the 3-point scale, for the anulus (AF), end-plates (EP), nucleus (NP) and osteophytes (OS) at different thoracic regions. Overall, the $k$ coefficient showed a decreasing craniocaudal trend, except for the end-plates and osteophytes for the 5-point scale, and the anulus for the 3-point scale. The intra-rater $k$ coefficients were higher for the 3-point scale compared to the 5-point scale for all spinal components.

### 4.1.4 DISCUSSION

The inter- and intra-rater reliabilities of the MRI grading scale to assess thoracic disc degeneration, were found to be highest for soft tissue changes in the nucleus and lowest for osteophytes, regardless of the 3- or 5-point grading scale used. This finding was not surprising as MRI was particularly suited to evaluate early soft tissue degenerative changes in spinal disc. MRI was found to demonstrate changes in the signal intensity of the anulus and nucleus, which are indicative of early degenerative or old age disc changes, without any evidence of obvious morphological changes, such as, diminished disc height or the presence of disc herniation (Aguila et al 1985, Sether et al 1990, Pearce et al 1991). The single band of low signal intensity in the nucleus, or intra-nuclear cleft noted on MR images have been reported to reflect an area of increased collagen content (Pearce et al 1991). These tissues were also noted to be histologically similar to anular lamellar material (Aguila et al 1985). Therefore, the grade II signal intensity on MRI films in the nucleus may reflect early histological nuclear degeneration.
The difference in the mean age of the cases selected for the evaluation of the two scales, \(39 \pm 18\) years for the 3-point scale and \(44 \pm 15\) years for the 5-point scale, was not expected to bias the reading as studies have reported a high prevalence of disc degenerative changes even at the age of 20 years (Tertti et al 1991, Videman et al 1995a). Both scales appear appropriate for use in MRI studies across the age span as there was relatively little change in signal intensity from the immature to the normal aged disc (Sether et al 1990). It was acknowledged that subtle variations in image intensity might have been present across this MRI series, due to the different MRI systems used in the 3 hospitals, which might have influenced the comparisons. However, the primary nature of the evaluation sought to test for reliability in grading, as opposed to a comparison of signal intensities between films, therefore the use of MRI films taken from different MRI systems should not bias the results.

The “fair” intra-rater reliability of grading osteophytes using the 3-point MRI scale \((k\ range = 0.59\ for\ lower\ thoracic\ to\ 0.71\ for\ mid\ thoracic\ regions)\) was slightly higher than that reported by Raininko et al (1995) for the mid \((k = 0.49)\) thoracic region, and almost similar for the lower thoracic \((k = 0.66)\) region. The fair intra-rater reliability might be due to the low signal intensity of calcified material on T2-weighted MR images (Martin et al 1992), which made it difficult to compare the true extent of osteophytic formation against the dark anular tissues (Maravilla and Cohen 1991). Osteophyte and calcifications are usually not well defined on T2-weighted MRI (Ross et al 1987), and were better viewed on T1-weighted MRI (Enzmann 1990, Kaiser and Ramos 1990). Computed tomography and X-rays were also suggested to be more reliable than MRI for identifying the presence and size of osteophytes (Maravilla and Cohen 1991), although such correlative MRI and CT or X-ray data had not been reported.

The higher intra-rater \(k\) coefficient for the modified 3-point scale compared to the original 5-point scale was not an unexpected finding due to the higher variability of observations when using the 5-point scale. This high variability might be due to the inability to define all possible disc degeneration stages from normal to abnormal (Tertti et al 1991, Brant-Zawadzki et al 1995). The correlation of the 5-point scale to pathologic macroscopic disc changes (Thompson et al 1990) had yet to be verified for the modified 3-point scale, however, it would be expected to be similar, if not improved, given the fewer decisions.

Consistency of overall MRI grading was found to be highest in the upper thoracic region compared to the mid and lower thoracic regions. There are currently no other studies which had reported the reliability of MRI grading of the upper thoracic discs for comparison. The observation for the mid and lower thoracic regions were however, in contrast to that reported by Raininko et al (1995). They reported high intra-observer agreement for the lower thoracic
and lumbar regions and poor agreement for the mid-thoracic region due to the smaller disc size and high prevalence of chemical shift artefact and background noise in the mid thoracic region. The different findings between the current study and that of Raininko et al (1995) might be due to the different criteria used to grade disc changes on T2-weighted images. It was expected that the different criteria used for MRI assessments in different studies might not be comparable and further, it was recommended that combining evaluations from two or more readers from different studies, should be done cautiously, bearing in mind the low inter-rater reliability for some spinal components (Raininko et al 1995). In the current study, the percentage of disagreements was variable and not necessarily higher in the lower thoracic region compared to the mid thoracic region.

Consistent with the report by Raininko et al (1995), the inter-rater reliability for all disc components was lower compared with the intra-rater reliability using both scales, except for the 5-point scale, where the inter-rater reliability was higher than the intra-rater reliability for the end-plate scoring. The inter and intra-rater kappa coefficient were both rated as ‘good’ for the nucleus, although the actual score was lower, and ‘fair’ for the anulus and osteophytes. This finding for the end-plate lesion was unexpected and may be due to the smaller number of discs ($n = 120$ discs) graded for the inter-rater study compared to that used in the intra-rater ($n = 360$) reliability, which potentially contributes to greater error with more discs evaluated in the latter evaluation.

**4.1.5 CONCLUSION**

The inter- and intra-rater reliabilities of the modified 3-point scale to assess T2-weighted thoracic MR images were found to be higher and with less disagreements, compared to the 5-point scale. The intra-rater reliability for the two scales was also highest for soft tissues changes especially in the nucleus compared to osteophytes, and lowest for discs in the lower thoracic region as compared to the upper and mid thoracic regions, with the exception for end-plate lesions. The inter-rater reliability was found to be lower than the intra-rater reliability using the 3-point scale but not for the 5-point scale.
STUDY 4.2 PATTERNS OF THORACIC DISC DEGENERATION USING MRI: AGE, GENDER AND SPINAL LEVEL INFLUENCES

Prevalence studies on disc degenerative changes, end-plate lesions and osteophytes in the thoracic region were few compared to the published literature on the lumbar and cervical regions. Prior to MRI, surveys on thoracic disc degeneration and associated degenerative changes were conducted using radiography, (Hilton et al 1976, Lawrence 1977, Resnick and Niwayama 1978, O’Neill et al 1999, Singer 2000), histological (Hilton et al 1976, Singer 2000) and cadaver examinations (Nathan 1962, Resnick and Niwayama 1978, Saluja et al 1985, Singer 2000). These earlier studies suggested a higher prevalence of thoracic disc degeneration with age, occurring principally in the mid and lower thoracic regions.

As MRI became the preferred choice of imaging for diagnosing spinal problems, it was pertinent to determine the prevalence of disc degenerative changes in the thoracic region to facilitate more accurate diagnostic assessment using MRI. Apart from the studies by Wood et al (1995) and Videman et al (1995a) surveys on thoracic disc degenerative changes using MRI were relatively few compared to lumbar disc studies. The present MRI study sought to define patterns of disc degenerative changes in the thoracic spine according to age, gender and thoracic spinal region, which were not examined in the former two studies.

4.2.1 MATERIALS AND METHODS
4.2.1.1 Sample Selection
Of the 300 MRI cases audited, 216 cases were selected, consisting of 101 males and 115 females, aged 1 to 85 years (mean age = 42 ±19.7 years). Sample selection and MRI assessment parameters were described in detail in Study 4.1 (sections 4.1.1.1 and 4.1.1.2). In 27 cases some upper or lower thoracic levels were not visualised, which resulted in 70 unrecorded disc segments. Therefore, a total of 2,522 discs were examined and graded from the 216 MRI cases selected. Degenerative changes in the nucleus, annulus and end-plates, and the presence of osteophytes, were graded using a modified 3-point grading scale described in Study 4.1 (Table 4.1 and section 4.1.1.2).

4.2.2 DATA REDUCTION AND STATISTICAL ANALYSIS
Descriptive statistics were used to present and report age, gender and thoracic regional frequencies for each disc variable. To facilitate trend analysis, data were categorised into 8 age groups with a 10-year interval (Figure 4.3), except for the last age group, which included
all cases above 70 years of age. The percentage of degeneration findings, that is, grades II and III, for the four disc components ranged from 5% to 43%, and was particularly low for grade III findings (Table 4.5). Therefore, grades II and III data for each gender were pooled for meaningful statistical and descriptive comparisons.

A test-against-trend analysis (Pfanzagl 1960) was used to determine the level of significance for trends due to age and thoracic regional variables. The level of significance for all statistical tests was accepted at \( p < 0.05 \). Data summaries are recorded in Appendix C.

![Distribution of the number of MRI cases according to gender and age groups (n = 216 cases). Each age group is an interval of 10 years, except for the last age group, which included any cases above 70 years old.](image)

**Figure 4.3** Distribution of the number of MRI cases according to gender and age groups (\( n = 216 \) cases). Each age group is an interval of 10 years, except for the last age group, which included any cases above 70 years old.

### 4.2.3 RESULTS

The number of male and female MRI cases were approximately equal for each age group except for age groups 11–20, 21–30 and 31–40 (Figure 4.3). The percentage of degenerative changes was highest in the nucleus (86% of 216 cases) and lowest in the end-plates (63%, Table 4.5). Males showed a higher percentage of these degenerative changes compared to females, except for osteophytes which was higher in females.
4.2.3.1 Age and Gender Influence on Disc Degeneration

The prevalence of degenerative changes (grades II and III) in the nucleus and anulus and the presence of osteophytes was found to increase significantly with age ($p < 0.05$). End-plate lesions did not show this age trend, instead grades I, II and III findings had approximately equal prevalence in each age group, particularly above 21 years of age (Figure 4.4).

The prevalence of degenerative changes (grades II and III) in the nucleus, anulus and end-plates was higher in males than females for each age group. Fifty percent of males had degenerative changes in the nucleus (grades II and III) by the third decade, which was earlier compared to females (fourth decade). There were no gender differences for grade III osteophytes in each age group, except for grade II osteophytes, which was slightly higher in females.

4.2.3.2 Thoracic Regional and Gender Influence on Disc Degeneration

The prevalence of degenerative changes (grades II and III) in the nucleus, anulus and end-plates increased significantly in a craniocaudal direction ($p < 0.01$). These trends were similar for both males and females, with males having a higher prevalence in each thoracic level compared to females except for the lower two thoracic levels (Figure 4.5). In contrast, the prevalence of osteophytes was highest in the mid thoracic region (T3–T4 to T8–T9).

The peak occurrence of osteophytes was at T5–T6 for both males and females, with females showing a higher prevalence (63%) compared with males (53%). Osteophytes were higher for females in the mid thoracic region (T4–T5 to T9–T10) and for males in the lower thoracic region (T10–T11 to T12–L1). Degeneration in the nucleus peaked in the lower thoracic region, and anular degeneration at the mid to lower thoracic levels (T6–T7 to T11–T12) for both males and females. End-plate lesions were also most commonly located in the lower thoracic region, and the prevalence was higher for males (53%) compared to females (32%). In contrast, the percentage of end-plate lesions in the upper thoracic region (T1–T2 to T3–T4) was less than 1%.
Table 4.5 Number and percentage of grade I, II and III discs in the nucleus, anulus, end-plates and osteophytes, according to gender (n = 216 cases).

<table>
<thead>
<tr>
<th>Spinal component</th>
<th>Grade</th>
<th>Total discs (n = 2,522)</th>
<th>% of all discs</th>
<th>Male discs (n = 1,185)</th>
<th>% of male discs</th>
<th>Female discs (n = 1,337)</th>
<th>% of female discs</th>
<th>No. of spines (n = 216) with at least one grade II or III disc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus</td>
<td>I</td>
<td>1,059</td>
<td>42</td>
<td>466</td>
<td>39</td>
<td>593</td>
<td>44</td>
<td>186 (86)</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>1,056</td>
<td>42</td>
<td>496</td>
<td>42</td>
<td>560</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>407</td>
<td>16</td>
<td>223</td>
<td>19</td>
<td>184</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Anulus</td>
<td>I</td>
<td>1,745</td>
<td>69</td>
<td>787</td>
<td>66</td>
<td>958</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>639</td>
<td>25</td>
<td>325</td>
<td>27</td>
<td>314</td>
<td>23</td>
<td>161 (75)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>138</td>
<td>5</td>
<td>73</td>
<td>6</td>
<td>65</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>End-plates</td>
<td>I</td>
<td>2,062</td>
<td>82</td>
<td>921</td>
<td>78</td>
<td>1,141</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>282</td>
<td>11</td>
<td>161</td>
<td>14</td>
<td>121</td>
<td>9</td>
<td>135 (63)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>178</td>
<td>7</td>
<td>103</td>
<td>9</td>
<td>75</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Osteophytes</td>
<td>I</td>
<td>1,651</td>
<td>65</td>
<td>806</td>
<td>68</td>
<td>845</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>529</td>
<td>21</td>
<td>220</td>
<td>19</td>
<td>309</td>
<td>23</td>
<td>175 (81)</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>342</td>
<td>14</td>
<td>159</td>
<td>13</td>
<td>183</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>
4.2.4 DISCUSSION

Using MRI archives, the present study examined degeneration trends in thoracic discs in relation to age, gender and thoracic region. The prevalence of thoracic disc degeneration and osteophyte development increased with age. These findings were consistent with thoracic surveys reported from radiographs (Lawrence 1977, Singer 2000) and MRI (Wood et al 1995), and for thoracic and lumbar histological and cadaver examinations (Lawrence 1977, Miller et al 1988, Boyle et al 1998, Singer 2000) and MRI studies (Videman et al 1995a, Wood et al 1995). A higher prevalence of low signal intensity, disc bulge, and disc space narrowing was noted in the thoracic discs with increasing age (Videman et al 1995a, Wood et al 1995). The early changes noted in the nucleus is probably associated with the greater susceptibility of the nuclear matrix to early degenerative processes, due to decreases in water and proteoglycan concentration, and number of viable cells, as opposed to the more stable and resilient nature of the annular matrix (Schmorl and Junghanns 1971, Eyre et al 1989, Buckwalter 1995).

A less prominent age trend was noted for end-plate findings, especially > 21 years of age, which is also reported in previous radiological and cadaver thoracic studies (Hilton et al 1976, Saluja et al 1985, Singer 2000). The authors of these studies had suggested that the development of end-plate irregularities or Schmorl’s nodes might not represent an age-related event, as such lesions were reported as frequently in the young as the older subjects (Schmorl and Junghanns 1971, Hilton et al 1976, Videman et al 1995a). This observation supported the suggestion that the susceptibility of the hyaline cartilaginous end-plate, which allowed the nucleus to herniate into the vertebral body (Schmorl and Junghanns 1971), was probably due to factors such as congenital weakness or trauma (Schmorl and Junghanns 1971, Resnick and Niwayama 1978), with a lesser contribution from age-related degeneration of the end-plate. The nuclear material would be expected to have a certain amount of turgor in order to herniate through the end-plates (Resnick and Niwayama 1978), which in the aged nucleus, this turgor might be reduced, hence the lower prevalence of Schmorl’s nodes in aged discs noted in the present survey.
Figure 4.4 The percentage of discs in males and females in each age group, with grades I (λ), II (□) and III (x) changes in the nucleus, annulus, end-plates and osteophytes. The percentage of grades II and III degenerative changes in the nucleus, annulus and osteophytes increased with age in both males and females, whereas end-plate changes did not show this trend. Males tended to have a higher prevalence of all degenerative changes compared to females in each age group, except in the older age groups. Data points had been linked to demonstrate trends.
Figure 4.5 The percentage of degenerated (grades II or III) nucleus and anulus, end-plate lesions and osteophytes for males and females in each thoracic level. The prevalence of degeneration in the nucleus, anulus and end-plates increased craniocaudally, and was higher in males compared to females for each thoracic level. In contrast, the prevalence of osteophytes peaked in the mid thoracic region (T4–T5 to T8–T9), and was higher in females compared to males.
Besides the lack of an age trend, the significant regional and gender patterns for the prevalence of thoracic end-plate lesions were also consistent with cadaver and radiological studies. The prevalence of Schmorl’s nodes was found to be predominantly higher in males (Sahuja et al 1985, Scoles et al 1991), and had an increasing craniocaudal trend (Hilton et al 1976, Scoles et al 1991, Singer 2000). The prevalence of end-plate lesions in the upper thoracic region (T1–T2 to T3–T4) was very low (< 1%) which is generally similar with the cadaver survey of disc degeneration reported at the cervico-thoracic junction (Boyle et al 1996).

The prevalence of disc degeneration demonstrated significant craniocaudal trends, especially for annular, nuclear and end-plate lesions. However, osteophytes appeared to peak in the mid thoracic region instead. There appeared to be two distinct distributions of thoracic disc degeneration, localised within the mid and lower thoracic regions. The higher pathology in the annulus and nucleus was consistent with other thoracic studies (Lawrence 1977, Videman et al 1995a, Singer 1997, 2000), as these regions were also commonly associated with thoracic disc herniations, especially in the lower thoracic region (Awbad et al 1992, Brown et al 1992, Ridenour et al 1993, Singer 2000). A higher prevalence of low signal intensity in the mid thoracic region was also reported in a study of 232 males aged 35-69 by Videman et al (1995a).

In the present study, the distribution of osteophytes did not demonstrate a craniocaudal trend, instead appeared to peak in the mid thoracic region (T4–T5 to T7–T8). While a similar finding was noted by Singer (1997), other studies reported a higher prevalence at the lower thoracic level, predominantly at the T9 to T11 level (Nathan 1962, Malmivaara et al 1987, Videman et al 1995a, O’Neill et al 1999). From a skeletal review of 346 spinal columns, Nathan (1962) reported the peak prevalence between T8 to T11. The predominance of osteophytes in the mid thoracic region however was not unexpected as these discs lie in the concavity of the thoracic kyphosis, therefore were exposed to high anterior compression loading (Nathan 1962, Schmorl and Junghanns 1971, Scoles et al 1991, Singer 1997). With increasing age and osteoporosis, the thoracic kyphotic curve was accentuated (Schmorl and Junghanns 1971, De Smet et al 1988), which might account for the present higher prevalence of osteophytes found between T4–T5 to T9–T10 in females. Goh et al (Goh et al 2000a), found an increased kyphotic curvature at the mid thoracic region to be associated with a higher prevalence of osteophytes in females.

Early biomechanical studies of Gregersen and Lucas (1967) and Farfan et al (1970), suggested that torsional stresses, rather than compressive loads, induced degenerative
damages in spinal intervertebral discs. In particular, Gregersen and Lucas (1967) proposed that the mid thoracic region was the site where the pivot of trunk rotation during locomotion was located. It was therefore a potential site for increased disc degeneration due to the higher frequency of torsional stresses imposed on the discs during trunk rotation.

In the lower thoracic region, disc degenerative changes, particularly in the annulus and the nucleus, occurred as a result of both increasing axial compression load (Pal and Routal 1987) and the relative lack of resistance to torsional movements by the zygapophyseal joints immediately above the thoraco-lumbar transitional junction (Malmivaara et al 1987, Singer et al 1989). The combination of high load, increased torsion and lack of protection from the zygapophyseal joints presents as a potential site of structural weakness for injuries (Malmivaara et al 1987, Singer et al 1989).

The higher prevalence of thoracic end-plate lesions and disc degenerative changes in males compared to females was consistent with the MRI survey by Wood et al (1995) and the post-mortem cadaver investigation by Singer (2000). The male bias for end-plate disruption, or presence of Schmorl's nodes was also well documented (Schmorl and Junghanns 1971, Hilton et al 1976, Scoles et al 1991, O'Neill et al 1999). However, the prevalence for osteophyte formation in the mid thoracic region was higher in females instead of males. This finding may be consistent with the epidemiological review by Lawrence (1977), who based on radiographs, reported a higher prevalence of thoracic disc degeneration in females. Predominance of disc degeneration in males were also reported for lumbar (Lawrence 1977, Miller et al 1988) and cervical discs (Lawrence 1977).

From the limited literature evidence, there appeared to be general consensus that more frequent exposure to higher levels of repetitive stress imposed on the vertebral column in males, may account for the higher prevalence of disc abnormalities (Schmorl and Junghanns 1971, Wood et al 1995, O'Neill et al 1999). It might be postulated that the male tendency to intervertebral disc degeneration was due to the larger disc size, hence poorer disc nutrition (Urban et al 1977, Miller et al 1988), in addition to the greater exposure to occupational and recreational stresses (Miller et al 1988, Swärd et al 1991, Videman et al 1997, 1999). In the present study however, Grade II or III mid thoracic osteophytes (disc margin) were more prevalent in females, probably due to hormonal changes (Hui et al 1999) and subsequent osteoporosis and kyphotic stress in the mid thoracic region (Melton et al 1993), instead of occupational stress.
4.2.5 CONCLUSION

This study provides prevalence data on thoracic disc degeneration in relation to age, gender and thoracic region from an audit of thoracic MRI investigations. The prevalence of thoracic disc degenerative changes and osteophytes, was significantly associated with age. In contrast, end-plate lesions did not show an age-related association. Significant cranial to caudal trends, from T1 to T12, were also noted for degenerative changes in the nucleus and anulus, and end-plate lesions. Osteophytes however, tended to peak instead in the mid thoracic region. The trend for a higher prevalence of thoracic disc degenerative changes in the mid to lower thoracic regions and increased age was demonstrated. Generally males had a higher prevalence of thoracic disc degeneration, except for osteophytes which were higher in females compared to males. The clinical relevance of degenerative changes observed on thoracic MRI might need to be interpreted according to these regional influences.
STUDY 4.3  AGE AND GENDER INFLUENCES ON THORACIC VERTEBRAL BODY SHAPE AND DISC DEGENERATION: AN MR INVESTIGATION OF 169 CASES

Commonly, the thoracic vertebral bodies were affected by progressive shape changes, which often manifest as vertebral deformity or fracture in individuals with osteoporosis. The identification and classification of vertebral deformity had gradually evolved from qualitatively descriptive methods to morphometric approaches, including the definition of reference ranges for vertebral body shapes (Eastell et al 1991, O'Neill et al 1994, Cheng et al 1998, Ismail et al 1999). A majority of vertebral morphometry studies relied on radiological surveys, from which vertebral shape indices based on height ratios were calculated. Common indices included those used to distinguish between anterior wedge, bi-concave, and compression deformities (O'Neill et al 1994). From the morphometry literature, it appeared that limited data were available on the pattern of thoracic vertebral shape changes across the life span. Reference data for males remained scarce, except for a few documented studies which provided limited information (O'Neill et al 1994, Burger et al 1997, Cheng et al 1998).

In particular, recent literature reports reflected growing interest in the association between vertebral body deformity and disc degeneration (Verstraeten et al 1991, Dai 1998). While these had primarily concerned the lumbar spine, investigations of this nature might be applied to the thoracic region. Anecdotally, it was suggested that age-associated changes in males usually involve degeneration of the thoracic discs, in contrast with the progressive wedge deformation of the thoracic vertebrae commonly seen in older females (Schmorl and Junghanns 1971). Investigation of these changes within the thoracic column would no doubt yield more than an improved understanding of their patterns of age-related progression. In clinical terms, the outcome might be of significance in the management of degenerative conditions in the elderly, such as spinal osteoarthritis and senile osteoporosis. Furthermore, serial investigations might be favoured by the non-ionising nature of MR imaging.

The aims of this study were to examine age-related changes in vertebral body shape and the prevalence of disc degenerative findings, from a retrospective investigation of thoracic spine MR images involving a sample of convenience. Associations between disc findings and vertebral shape measurements were also examined. Data were collected from individuals across a broad age range, and comparisons between the upper, middle and lower thoracic regions were performed.
4.3.1 MATERIALS AND METHODS

4.3.1.1 Sample selection
Of the 300 cases audited, a total of 169 cases (88 females, 81 males) were eventually selected for vertebral morphometric analysis and examination of disc degenerative changes. The MR imaging protocols and procedures employed for assessing disc morphological changes using T2-weighted MR images are detailed in section 4.1.1.2 in Study 4.1, and the MR imaging protocol for T1-weighted MR image is detailed below.

4.3.1.2 Vertebral morphometry
Morphometric analysis was performed on T1-weighted mid sagittal digital images following their transfer onto a personal computer, using the image processing The National Institutes of Health (NIH) Image software (Version 1.61, National Institutes of Health, USA). Mid sagittal images were determined from the presence of the spinous processes and clear demarcation of the spinal cord. For all T1-weighted images, MR imaging was performed utilising a surface coil, with repetition times ranging from 500 to 700ms, and echo times ranging from 12 to 20ms. The acquisition matrix ranged between 192 x 256 and 300 x 512, with a field of view of 30 or 35cm. Slice thickness was 3 to 4mm, with a slice gap of 0.3 or 1mm.

Using the NIH Image software, six anatomical landmarks were marked on each vertebral body, representing the four corners and mid points of the superior and inferior end-plates (Figure 4.6 B,D). Landmarks were defined at the end-plate/bone junction, thus excluding the thickness of the end-plates for deriving morphometric parameters. The uncinate-like process at the superior-posterior corner was excluded by selecting a point below this prominence (Genant et al 1996). In the case of marked osteophytic formation, corner landmarks were selected to best represent the point of intersection of the anterior vertebral border with the end-plates. For each vertebral level between T1 and T12, the anterior (Ha), mid (Hm), and posterior heights (Hp) were calculated. The antero-posterior diameter (D) was defined by the distance between the mid points of the lines used to determine Ha and Hp.

Three indices of vertebral body shape were then derived from these measurements: anterior wedge index (Ha/Hp), bi-concavity index (Hm/Hp), and compression index (Hp/D). The Hp/D ratio was based on the specific compression index utilised by Nicholson et al (1993). This ratio provides an indication of the extent of compression deformity that is not reliant on measurements of vertebrae above or below the deformity. All image processing and morphometric analyses were performed by co-investigator, S Goh.
4.3.1.3 Vertebral disc analysis
All T2-weighted sagittal MR images of the 169 cases were reviewed by investigator C Tan, and graded for degenerative changes in the thoracic discs using the 3-point MRI grading scale described in Table 4.1 and section 4.1.1.2 in Study 4.1. The method and reliability of disc grading using the 3-point MRI grading scale is reported in Study 4.1.

4.3.1.4 Reliability for vertebral height measurements using T1-weighted MR images
Preliminary studies were conducted to examine the intra-observer reliability of vertebral height measurements derived from the six anatomical landmarks, and disc analysis using the modified MR grading scheme.

Repeatability of vertebral heights was examined by co-investigator S Goh, using 10 mid sagittal images selected at random. For each case, identification of the six landmarks from a randomly selected segment was repeated on five occasions over varying time intervals, to reduce bias associated with recollection of landmark selection. Mean standard deviation values for repeated measurement of anterior, mid, and posterior vertebral heights were 0.13mm, 0.15mm, and 0.20mm respectively, with coefficients of variation of 1.6%, 2.1%, and 2.4% respectively. Intraclass correlation coefficient values for the three variables ranged from 0.99 to 1.0.

4.3.2 DATA REDUCTION AND STATISTICAL ANALYSIS
All morphometry and disc data were grouped into upper (T1-T4), mid (T5-T8), and lower (T9-T12) thoracic regions. For each thoracic region, age trends were examined across five age cohorts (Table 4.6). Gender differences in thoracic vertebral body shape were analysed using unpaired t-tests. One factor analysis of variance (ANOVA) with post-hoc polynomial contrast analyses was used to examine the influence of age on vertebral shape. Data summaries are recorded in Appendix C.
Table 4.6 Distribution of 169 cases by age cohort and gender, utilised in an MR investigation of thoracic vertebral body shape and disc degeneration.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Age range (years)</th>
<th>Female ($n = 88$)</th>
<th>Male ($n = 81$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$n$</td>
<td>Mean age (SD)</td>
<td>$n$</td>
</tr>
<tr>
<td>One</td>
<td>1 - 20</td>
<td>15</td>
<td>12.3 (5.0)</td>
</tr>
<tr>
<td>Two</td>
<td>21 - 35</td>
<td>15</td>
<td>30.5 (4.2)</td>
</tr>
<tr>
<td>Three</td>
<td>36 - 50</td>
<td>35</td>
<td>43.1 (4.6)</td>
</tr>
<tr>
<td>Four</td>
<td>51 - 65</td>
<td>13</td>
<td>56.8 (4.3)</td>
</tr>
<tr>
<td>Five</td>
<td>66 - 85</td>
<td>10</td>
<td>72.3 (6.6)</td>
</tr>
</tbody>
</table>

Disc degenerative findings were reported as a percentage of total female or male discs involved within an age cohort, for a particular thoracic region. Each disc was considered as a single statistical unit. The association between vertebral shape and disc degeneration was analysed by comparing mean vertebral shape index values of adjacent vertebral bodies for the three disc degenerative grades. One factor analysis of variance (ANOVA) and analysis of covariance (ANCOVA), with age as a covariate, were used to determine statistical associations. For all analyses, statistical significance was defined by a probability level of $p < 0.05$.

### 4.3.3 RESULTS

#### 4.3.3.1 Vertebral shape indices

In both genders, a significant linear age-related decline in the Ha/Hp ratio was noted in the mid and lower thoracic regions (Figure 4.7a). This trend was particularly evident in females in the mid thoracic region, whereby a sharp decline was demonstrated between Age Cohorts Three (Age 36-50) and Five (Age >65). Within Cohort Five in the mid thoracic region, the mean value was significantly lower in females than males ($p < 0.01$).

A similar age-associated linear decline in the Hm/Hp ratio was demonstrated in females in all regions of the thoracic column, and in the mid and lower regions for males (Figure 4.7b). In the lower thoracic spine, the mean index was significantly lower in females within Cohort Five ($p < 0.0001$).
Figure 4.6 (A) Vertebral morphometry measurements were performed on mid sagittal T1-weighted MR images of the thoracic spine. In all cases, scout images, which included C2, were used to identify segmental levels. (B) Illustration of vertebral landmarks from which vertebral shape parameters were defined. Marked points represent the four vertebral corners and mid points of the end-plates, while 'D' represents the antero-posterior vertebral diameter. (C) A T2-weighted MR image illustrating examples of disc degenerative changes, based upon a 3-point MRI disc grading system described in Table 4.1. (i) disc margin Grade III, (ii) end-plate Grade III, (iii) nucleus Grade III, (iv) anulus Grade II, (v) disc margin Grade II, (vi) anulus Grade I.
Lower mean Hp/D values were observed in the mid and lower thoracic regions (Figure 4.7c). The effects of age were quadratic in nature, with mean values increasing during the first few decades of the life span, then decreasing thereafter. In both genders, statistical significance was noted in all regions, except for the mid thoracic region in females.

4.3.3.2 Disc analysis

Figure 4.8 illustrates the age and gender trends in the distribution of disc degenerative changes within the upper, mid and lower thoracic spine, with Figure 4.8a representing the trends for females, and Figure 4.8b for males. Values shown indicate the percentage of total discs involved for each gender and age cohort.

(i) Nucleus
An age-related increase in the prevalence of disc degeneration (grades II or III) was noted in all regions of the thoracic column. These findings were prominent from Cohort Two onwards (Age 21 to 35) and demonstrated a craniocaudal trend, with the greatest prevalence noted in the lower thoracic region. Within Cohort Two, the percentage of normal discs (grade I) in the mid thoracic region was 63.3% and 53.6% for females and males, respectively, and 31.7% and 40% respectively in the lower thoracic region. The prevalence of normal disc findings declined sharply with increasing age, reaching zero or near-zero values for Cohort Five mid and lower thoracic discs (Age >65). For Cohort Five lower thoracic discs, severe degenerative findings (grade III) were noted in one-third of female discs, and one-half of male discs.

(ii) Anulus
The regional distribution of degenerative findings in the anulus was similar to the nucleus, with a more prominent age-related increase in prevalence noted in the mid and lower thoracic regions. In general, grade II or III findings were less frequently noted in the anulus than the nucleus. These findings were also less prevalent in females. For Cohort Five mid and lower thoracic discs, degenerative findings were noted in 72.5% and 82.5% of male discs respectively, while 52.5% of female discs were involved in both regions. Within the same age cohort, severe lower thoracic degenerative findings (grade III) were noted in 35% of male discs, and 12.5% of female discs.
Figure 4.7 Age and gender trends in (a) mean Ha/Hp (anterior wedge index), (b) Hm/Hp (bi-concave index), and (c) Hp/D (compression index), for the upper, mid, and lower thoracic regions. Error bars represent 95% confidence intervals. A significant linear age-related decline was noted in the mid and lower thoracic regions for Ha/Hp (* denotes a linear trend, $p < 0.05$). For Hm/Hp, similar age-associated trends were noted in the mid and lower thoracic spine for both genders, and in females only for the upper thoracic region. Significant quadratic age trends were noted for Hp/D, except for females in the mid thoracic region (** denotes a quadratic trend, $p < 0.05$).
(iii) End-plate
No degenerative changes were noted in the upper thoracic end-plates. In the mid and lower thoracic regions, there was a slight age-related increase in grade II and III discs in females, although the prevalence was low compared to males. An increasing craniocaudal trend was also noted. Within Cohort Five, degenerative findings were demonstrated in 27.5% and 40% of mid and lower thoracic discs respectively, in females, and 45% and 62.5% respectively, in males. In general, the prevalence of severe end-plate defects (grade III) was low in both genders.

(iv) Disc margin
An age-related increase in the prevalence of osteophytic changes was noted for both genders, particularly in the mid thoracic spine. For Cohort Five mid thoracic discs, 77.5% and 65% of female and male discs, respectively, were graded II or III. In the lower thoracic region, the prevalence was greater in Cohort Five male discs, whereby 55% demonstrated grade II or III changes, compared to 27.5% of female discs.

4.3.3.3 Associations between vertebral morphometry and disc findings
(i) Mean Ha/Hp
For comparisons involving the nuclei, anuli, and end-plates, there was a corresponding decrease in mean Ha/Hp values of adjacent vertebral bodies with increasing disc degenerative grades in the mid and lower thoracic regions (Figure 4.9a). A similar reduction in mean Ha/Hp was noted for increasing osteophytic degenerative grades in females. Using one factor ANOVA, differences in mean Ha/Hp values were noted to be statistically significant ($p < 0.05$). However, further analysis using one factor ANCOVA, with age as a covariate, indicated no significant trends, with the exception of comparisons involving the anuli of lower thoracic discs in females.

(ii) Mean Hm/Hp
Mean Hm/Hp values were significantly lower for increasing grades of disc degeneration involving the nuclei and anterior disc margins of the mid thoracic discs in females (Figure 4.9b). With age as a covariate, one factor ANCOVA demonstrated no significant differences in the mean Hm/Hp values. No other distinct trends were noted.
4.3 Thoracic vertebral body and disc influences on disc degeneration

Figure 4.8a

<table>
<thead>
<tr>
<th>Upper thoracic (T1-T4)</th>
<th>Mid thoracic (T5-T8)</th>
<th>Lower thoracic (T9-T12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Graph]</td>
<td>![Graph]</td>
<td>![Graph]</td>
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</table>

Figure 4.8 Results of MR analysis of thoracic disc degenerative changes (O Grade I or normal v Grade II or moderate Δ Grade III or severe), indicating the distribution of disc grades according to age cohort and thoracic region, for (a) females, and (b) males (next page). Data are expressed as a percentage of total discs involved for each gender and age cohort, within a thoracic region.
Figure 4.8b

Upper thoracic (T1-T4)  Mid thoracic (T5-T8)  Lower thoracic (T9-T12)

Nucleus % of discs

Anulus % of discs

End-plate % of discs

Disc margin % of discs

One  Two  Three  Four  Five
One  Two  Three  Four  Five
One  Two  Three  Four  Five
One  Two  Three  Four  Five
(iii) Mean Hp/D
Using ANOVA, mean Hp/D values were significantly lower for increasing grades of disc degeneration involving the nuclei and anuli of the mid thoracic discs (Figure 4.9c, \( p < 0.05 \)).

In the lower thoracic region, a similar trend was demonstrated for comparisons involving the anuli and end-plates. Significant differences in the lower thoracic Hp/D index were also noted for increasing degenerative grades involving the nuclei. Mean values were highest for grade II disc findings, and lowest for grade III findings.

**4.3.4 DISCUSSION**

Compared to the lumbar and cervical regions, the pattern of age-associated changes to the thoracic vertebral bodies and intervertebral discs was not fully understood. This study reports the nature of vertebral shape changes and the prevalence of disc degenerative findings across the life span, utilising thoracic MR images involving a sample of convenience.

An age-related decline in Ha/Hp and Hm/Hp was noted, particularly in the mid and lower thoracic regions, corresponding with an increase in the degree of vertebral wedge configuration and vertebral bi-concavity respectively. These findings were consistent with population-based evidence demonstrating a predilection for age- and osteoporosis-related vertebral deformity in the mid thoracic and thoraco-lumbar regions (Eastell et al 1991, Melton et al 1993, Ismail et al 1999). To some extent, these changes might be explained from a biomechanical perspective, in terms of the differential functional loads imposed on the thoracic column. In the mid thoracic region, the vertebrae were typically predisposed to the cumulative effects of accentuated loads imposed on the apex of the kyphosis, resulting in gradual shape adaptation of these vertebrae. In contrast, the lower thoracic segments formed the transition between the stiffer thoracic column and the freely mobile lumbar segments, therefore maximising compression forces within this region and increasing their susceptibility to fracture or deformity when higher loads were transmitted through the region (Levine and Edwards 1987, Ismail et al 1999).

As noted in the present study, vertebral shape changes might be accelerated in females, in response to the normal rapid decline in axial bone mass during the menopausal and post-menopausal periods (Hui et al 1999). In the mid and lower thoracic regions, a greater rate of increase in the degree of anterior wedge and bi-concavity configuration was evident in females between Cohort Three (Age 36-50) and Five (Age >65). Furthermore, gender differences for these shape parameters were more pronounced within Cohort Five (Age >65),
supporting the findings of Ismail et al (1999) who noted an increased frequency of wedge and bi-concave deformities in females over 65 years. From these results, it might perhaps be suggested that the pattern of normal age-associated changes occurring in the thoracic region in older females reflect the nature of biomechanical loads acting on the thoracic column, and the influence of accelerated spinal bone loss.

The quadratic nature of age-associated changes in the Hp/D index might provide an indication of the pattern of vertical change in vertebral dimension throughout the life span. Using a similar index in a radiographic study, Brandner (1970) suggested that the accelerated rate of vertical growth and a corresponding slowing down of the increase in the sagittal vertebral dimension during the adolescent growth spurt resulted in a rapid increase in this vertebral shape index. The present study extended the data, indicating a pattern of gradual decline in the ratio throughout the adult life span. This decline might well be associated with the natural cessation of skeletal growth prior to adulthood and the eventual gradual loss in height stature during the later years of life. The mid and lower thoracic trends for lower Hp/D values appeared to mirror distribution patterns of vertebral compression fracture or deformity in the thoracic spine, where an increased prevalence is commonly reported in these regions (Hedlund et al 1989, Melton et al 1993, Ismail et al 1999). Consistent with this, Hedlund et al (1989) in their study of 139 females aged 45 to 90, reported an increase in mid and lower thoracic antero-posterior vertebral dimension was evident in patients with two or more vertebral fractures, compared to normal controls. Ross et al (1995) also noted an increase in vertebral fracture risk with increasing vertebral depth, or the distance between the antero-superior and postero-superior aspects of the vertebral body. These findings reflected the nature of changes in vertebral body diameter, in addition to posterior height reductions associated with vertebral compression fracture or deformity. It also highlighted the advantages of the Hp/D compression index, compared to the more commonly utilised Hp/Hp' index which compares posterior height of a nominated vertebra against posterior height of the level below or above. Calculation of the Hp/D ratio was independent of shape changes in adjacent segments, therefore providing greater sensitivity in examining vertebral shape changes at individual levels, particularly when reductions in posterior vertebral height were evident in a number of adjacent segments.
4.3 Thoracic vertebral body and disc influences on disc degeneration

ANTERIOR WEDGE INDEX

Figure 4.9a

Figure 4.9 Comparison of (a) anterior wedge index or Ha/Hp, (b) bi-concavity index or Hm/Hp (next page), and (c) compression index or Hp/D, of adjacent vertebral bodies with increasing disc degenerative grades, analysed by gender and thoracic region (* significant difference at p < 0.05). Error bars represent 95% confidence intervals. Legend for disc grades: $\theta$ Grade I  $\theta$ Grade II  $\theta$ Grade III.
Chapter 4 4.3 Thoracic vertebral body and disc influences on disc degeneration

Figure 4.9b

BI-CONCAVITY

Nucleus

Mean Hm/Hp

End-plate

Disc margin

Upper thoracic (T1-T4)
Mid thoracic (T5-T8)
Lower thoracic (T9-T12)
The distribution pattern of thoracic degenerative disc changes indicated a higher prevalence of mid and lower thoracic degenerative grades, increasing with age and more common in males than females. These results were consistent with findings in the previous data in study 4.2. Bearing in mind the cross-sectional nature of the present study, these findings reflected typical patterns of age-related changes in the thoracic discs and shape adaptation of the thoracic vertebral bodies. The high prevalence of abnormal discs conform to other thoracic MR studies of asymptomatic individuals (Videman et al 1995a, Wood et al 1995). While the association of the present findings with clinical symptoms was not determined, the spectrum of disc and vertebral changes might provide a useful reference for determining the clinical relevance of degenerative or pathological findings noted on thoracic MR images.

Further analyses of morphometry and disc findings, using ANOVA, indicated significant increases in adjacent vertebral shape changes with increasing disc degeneration, particularly in the mid and lower thoracic regions. From ANCOVA results however, these findings appeared to be related to the age factor, as a common denominator or covariate, suggesting that age-related changes to the thoracic vertebral bodies and intervertebral discs might co-exist. This might be particularly true in older individuals, as suggested by Schmorl and Junghans (1971), who also proposed different gender trends in the patterns of age-associated changes within the thoracic vertebral column. As noted in the current study, age-related changes to the male thoracic column were thought to predominantly involve the intervertebral discs, while in females, the vertebral bodies were more likely to undergo progressive shape deformation, particularly anterior wedging (Schmorl and Junghans 1971). In older females, the co-existence of osteoarthritis and osteoporosis was reported in a study by Verstraeten et al (1991), where the mean age of subjects was 71.5 years. Furthermore, Roaf (1960) demonstrated that compressive loading of older disc specimens resulted in collapse of vertebral bodies in addition to tearing of anular fibres, with a combination of flexion and rotational forces inducing greater changes.

In view of studies suggesting an antagonism, or inverse relationship between primary osteoporosis and osteoarthritis (Verstraeten et al 1991, Dai 1998), it might be of interest to further analyse the nature of the relationship between disc degenerative processes and vertebral shape changes within the thoracic column. While limited by the current study design, further insight might be gained from these previously reported findings, which had primarily been derived from studies of the lumbar spine. From the clinical study of Dai (1998), it was suggested that in spines with normal discs, the vertebral bodies were subjected to higher stresses, increasing their likelihood of deformity or fracture. Furthermore, in a histological study of sand rats, Silberberg (1988) proposed that disc degeneration and
herniation induced local stresses upon vertebral spongiosa and promoted an increase in vertebral bone mass, while Verstraeten et al (1991) suggested that osteoarthritis might be a negative risk factor for the development of osteoporosis.

While speculative in nature, these preliminary reports are deserving of further attention, given their potential clinical implication for the prevention and management of common degenerative conditions affecting the vertebral column. In the thoracic region, these included senile osteoporosis and age-related spondylosis. Clearly, further studies were necessary to enhance the understanding of these age-related processes, their progression across the life span, and clinical correlation with patient history. The enhanced sensitivity of MR imaging had no doubt provided greater appreciation of the spectrum of abnormalities in the thoracic spine, particularly early age-related changes in the intervertebral discs.

**4.3.5 CONCLUSION**

A linear age-related decline in the Ha/Hp and Hm/Hp indices was noted. The Hp/D index increased during the first few decades of life, then decreased gradually thereafter. The prevalence of abnormal findings in the anuli, nuclei and disc margins increased with increasing age, particularly in the mid and lower thoracic discs. Greater disc degenerative changes were observed in males. These findings provide further insight into the nature of thoracic vertebral shape changes across the lifespan, and the typical patterns of degeneration of the thoracic intervertebral discs.
OVERALL CONCLUSION FOR CHAPTER 4

Study 4.1 examined the inter- and intra-rater reliability of the modified 3-point and 5-point scales to examine T2-weighted thoracic MR images.

1. The intra- and inter-rater reliability was highest for soft tissue changes especially in the nucleus (0.87, 0.88 respectively) compared to osteophytes (0.78, 0.64 respectively) using the 3-point scale.

2. The inter-rater reliability was higher using the 3-point ($k$ range = 0.64 to 0.88) scale compared to the 5-point scale ($k$ range = 0.54 to 0.83) except for the rating on end-plates, which was slightly higher for the 5-point scale of Thompson in Eyre et al (1989).

3. The intra-rater $k$ coefficient was highest for the upper thoracic region and lower for the mid and lower thoracic regions for both scales. There were however, a few exceptions. Using the 5 point-scale, the $k$ coefficient for end-plate lesions was highest in the lower thoracic (0.76). For the 3-point scale, the only exception was the $k$ coefficient which was highest for anular grading in the mid thoracic (0.96).

Study 4.2 provided prevalence data on thoracic disc degeneration in relation to age, gender and thoracic region, from an audit sample of 216 thoracic T2 weighted MR images. In addition, Study 4.3 examined the association of these degenerative trends with the changes in vertebral morphology, using 169 corresponding T1 weighted MR images.

4. The prevalence of thoracic disc degenerative changes and osteophytes was significantly associated with increased age ($p < 0.05$). In contrast, end-plate lesions did not show an age-related trend.

5. There was a trend for a higher prevalence of thoracic disc degenerative changes from the mid to lower thoracic regions. A significant increasing craniocaudal trend from T1 to T12 was noted for degenerative changes in the nucleus and anulus, and for end-plate lesions ($p < 0.05$). Osteophyte location however tended to peak in the mid thoracic region.

6. There was also a significant linear age-associated decrease in the antero-posterior and mid-posterior vertebral height ratios, reflecting an increase in anterior wedging, associated with an increase in the bi-concavity configuration of the vertebral body ($p < 0.05$). Age-related changes in anterior wedge deformation were particularly prevalent in the mid-thoracic region of older female cases.

7. Vertebral deformity and osteophyte formation were predominant in the older female cases, however, disc degenerative changes were more commonly found in males compared to females.
CHAPTER 5 BIOCHEMICAL INVESTIGATIONS ON SPINAL DISCS AND LIGAMENTUM FLAVUM

This chapter summarises the methods used in this thesis to examine ex vivo spine cases using biochemical analysis. A series of ex vivo spinal cases, thoracic and lumbar discs and spinal LF were harvested from formalin-fixed human cadavers, and examined macroscopically before being analysed for biochemical changes. In a second series of two ex vivo spine cases, thoracic discs and LF were harvested from human cadavers, and examined radiologically, macroscopically and biochemically. Non-fixed lumbar discs and LF were also utilised to evaluate the effect of formalin fixation on the biochemical matrix of spinal soft tissues. Various biochemical assays were utilised to extract and determine collagen, proteoglycan, collagen and elastin crosslinks in disc and ligamentum matrices in the following investigations:

5.1 Biochemical investigations of fresh and formalin-fixed thoracic intervertebral discs and spinal ligamentum flavum (Tan et al 2002a).
5.2 Distribution of collagen and collagen crosslinks in the thoracic anulus (Tan et al 2000a).
5.3 Age-related changes in collagen, pyridinoline and deoxypyridinoline in normal human thoracic intervertebral discs (Tan et al 2003).
5.4 Pyridinoline and deoxypyridinoline in degenerate human thoracic intervertebral discs over the life span (Tan et al 2000a).
5.5 Collagen and elastin crosslinks in human intervertebral discs and ligamentum flavum: Age, gender and spinal level influences (Tan et al 2002b).
5.6 Biochemical and MRI evaluation of aged human thoracic intervertebral discs and ligamentum flava: Two case studies.

The collagen content was determined using a modified method from Kivirikko et al (1967), and the collagen and elastin crosslinks were analysed using HPLC assay modified from Randall et al (1996). The evaluation of CS for proteoglycan was evaluated using a modified DMB assay, outlined in detail in Appendix B3.
CHAPTER 5

5.1 Effects of formalin on spinal tissues

STUDY 5.1 BIOCHEMICAL INVESTIGATIONS OF FRESH AND FORMALIN-FIXED THORACIC INTERVERTEBRAL DISCS AND SPINAL LIGAMENTUM FLAVUM

With limited access to fresh human tissues for research, the use of formalin-fixed archived tissues had become an important alternative. Formaldehyde, either as 4% formaldehyde or 10% buffered formalin, was commonly used to preserve the morphology of human tissues for review and research purposes (Walker 1964, Leong 1994, Brooks et al 1998). Formaldehyde produced formalin induced non-reducible chemical cross-links with proteins and glycoproteins in the tissues, especially if there were free amino acid groups at the ends of the chain (Leong 1994). However the extent to which proteins were crosslinked with formaldehyde was still not fully understood or reported. In particular, a recent study by Abe et al (2003) reported that formalin fixation did not affect the release of collagen crosslinks and pentosidine in spinal ligament tissues, however, elastin crosslinks, Des and Isodes, were significantly reduced with formalin fixation. Explanations for these biochemical changes were not conclusive, and did not investigate changes for Dpd and warrants further investigations with fresh and formalin-fixed spinal disc and LF tissues.

The objective of the study was to determine if the collagen and elastin crosslink content and the extent of collagen crosslinks could be evaluated in formalin-fixed spinal disc and ligament tissues. The effect of formalin fixation in the disc and ligament matrices was monitored over a 25-week period to determine if the duration of fixation had any effect on the release of these biochemical constituents in the disc and ligament matrices for biochemical analysis.

5.1.1 MATERIAL AND METHODS

5.1.1.1 Tissue collection and preparation

Five fresh human lumbar disc tissues and 22 thoracic and lumbar ligamentum flavum samples from T1 to T12, and L4-5, were removed from two fresh human cadaver spines: a 67-year old female and an 85-year old male, following routine post-mortem procedures. The selected discs did not show any degeneration changes, however some of the LF tissues harvested had variable amounts of calcification. For this study, care was taken to remove only non-calcified portions of the ligament tissues for biochemical evaluation, to ensure sufficient material for matched comparisons. Where the ligament was too severely calcified, tissues could not be harvested.
The discs were removed axially at the mid section from the two fresh lumbar spines, with the anterior portion of the anulus and the nucleus intact. Each slice, approximately 1.5 to 2 mm thick, was subsequently divided into equal left and right halves [Figure 5.1(A)]. Half of the disc was stored untreated in the freezer at -20°C, and the other half was fixed in 10% buffered formalin solution (pH 7), made from formaldehyde concentrate (Ramprie Laboratories, Welspool, WA, Australia). Formalin specimens were refrigerated at 4°C.

**Figure 5.1** Paired spinal intervertebral disc (A) and LF samples [tissues within the dotted lines] (B) were removed for biochemical assay. In an attempt to control for biological variation, tissues were selected to reflect mirror images in terms of geographical location (black and white rectangles).
Chapter 5

5.1 Effects of formalin on spinal tissues

In total, 22 LF samples were harvested from the two fresh spines. Twenty LF tissues were removed from thoracic levels T1 to T12 from both spines (except for two thoracic levels where the ligament was too calcified to harvest sufficient tissue for testing), and two ligament tissues taken from lumbar levels L4 and L5 of one spine only (67-year old female). Each LF tissue was divided in the midline into left and right halves [Figure 5.1(B)]. One half was kept fresh in the freezer (-20°C), and the corresponding half stored in buffered formalin (4°C), similar to the disc preparation above.

The first assay for both fresh and formalin-fixed disc samples started after one week of fixation, and for ligament samples, at week 2. One to two mm³ pieces of anular, nuclear and LF tissues were removed from each half of the stored fresh and formalin-fixed tissues, for biochemical analysis. At all times, samples removed from each half were selected so that they were approximate mirror images in terms of geographic location within the disc and ligament (Figure 5.1). That is, left outer anterior anular samples were compared with right outer anterior anular samples. This principle sought to minimise the effect of normal biological (and biochemical) variations within the respective tissue samples (Crean et al 1997, Duance et al 1998).

Further analyses of the fresh and formalin-fixed disc samples were conducted initially at weekly intervals for 5 weeks initially, thereafter at 2 or 4 weeks interval until week 25 (Table 5.1). With smaller LF tissues harvested, most of the tissues were only sufficient for one assay at week 2 of formalin fixation. Where tissues were large enough for further analyses, especially lumbar LF, these ligament tissues continued to be assayed at longer 3 to 4 weekly intervals, until 25 weeks of fixation, until the tissues were depleted. In total, 76 anterior anulus, 22 nuclei and 58 LF fresh and formalin-fixed tissues were tested over the duration of 25 weeks.

5.1.1.2 Sample preparation for biochemical analysis

For biochemical investigations, 1 mm³ of the tissue sample was removed from each half of the spinal disc and LF for Pyd, Dpd, Des and Isodes and collagen assays. The disc and LF tissue was diced finely and weighed to determine the wet weight. Tissues were then dried in an oven at 80°C for at least 24 hours. Drying for longer than 24 hours yielded no further loss in tissue weight. The dried tissue sample was re-weighed, then hydrolysed in 2 ml of 6M hydrochloric acid at 105°C for 16 to 20 hours, to hydrolyse peptide bonds.
Table 5.1 The number of formalin-fixed samples tested from the five discs and 22 LF tissues and the duration of formalin fixation in weeks.

<table>
<thead>
<tr>
<th>Weeks of formalin fixation</th>
<th>Anulus</th>
<th>Nucleus</th>
<th>LF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>23</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>25</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>12</td>
<td>29</td>
</tr>
</tbody>
</table>

5.1.1.3 Extraction procedure for Pyd, Dpd, Des and Isodes

The assay for Pyd, Dpd, Des and Isodes was performed using a modification of the method by Randall et al (1996), with the reagent preparations and column set-up described in detail in Appendix B1. The cooled sample hydrolysate (200 μl for ligament hydrolysate and 500 μl for the disc hydrolysate) was added to glacial acetic acid (2 ml), cellulose slurry (0.5 ml) and butanol (8 ml). Collagen and elastin crosslinks were then separated from potential interfering compounds by precipitating them in a mixture of Butanol: Acetic Acid: Water (4:1:1) and isolating them on a cellulose mini column (Alltech Extract-Clean filter columns with 20 μfrit, Alltech Australia, Sydney, Australia). The column was washed with 1ml of THF. After washing, the crosslinks were eluted from the column with an ion-pairing agent, 0.75 ml of 0.5% HFBA (Sequanal/HPLC grade from Pierce Biochemicals, Rockford, Illinois, USA), ready for injection onto the HPLC column. 100 μl of the resulting eluate was injected into reversed-phase HPLC.

5.1.1.4 Detection and calculation for Pyd, Dpd, Des and Isodes using method HPLC (I)

The HPLC (I) system consisted of a Waters model 700 Satellite WISP, Waters 600E system controller and pump (Waters, Corp. Milford, USA), a Shimadzu RF551 Spectrofluorometric
Detector (Tokyo, Japan) and a LKB 2151 UV detector (LKB, Sweden) with a 275 nm filter. The column was a Supelco-Spelcosil LC-18DB (3 μm, 4.0 mm X 7.5 cm cartridge PN58992, Pennsylvania, Bellefonte, USA) column. The mobile phase A was a mixture of 5% methanol, and 0.12% HFBA at pH = 2.0, and mobile phase B was a mixture of 80% methanol and 0.12% HFBA.

Each chromatogram took approximately 33 minutes to complete, with Pyd and Dpd identified with a fluorescent detector at approximately 7 and 9 minutes, respectively, and Isodes and Des crosslinks detected at 16 and 17.5 minutes, respectively (Figure 5.2). Fluorescence detection of Pyd and Dpd was set at excitation 295 nm and emission 395 nm wavelength, and UV detection of Des and Isodes at excitation 275 nm wavelength. Data was calculated using a Waters Millennium 32 Chromatography Manager (Waters, Corp. Milford, USA). All Pyd, Dpd, Isodes and Des values were calculated against the concentrations of the standards used in the beginning and end of each test run:

\[
\begin{align*}
\text{Pyd} & = 571 \text{ nmol/L} \\
\text{Dpd} & = 288 \text{ nmol/L} \\
\text{Isodes} & = 76 \text{ nmol/L} \\
\text{Des} & = 74 \text{ nmol/L}
\end{align*}
\]

The extent of Pyd and Dpd in the discs was expressed as number of moles of crosslinks per mole of collagen. The amount of Des and Isodes crosslinks was calculated as nmol/mg dry weight of tissue.

5.1.1.5 Collagen assay

The collagen content was measured in terms of hydroxyproline content. Hydroxyproline was assayed using a modified method of Kivirikko et al (1967), with the reagent preparation and sampling technique described in detail in Appendix B2. The tissue hydrolysate was mixed with a cationic resin (H⁺ form) which binds peptides and amino acids. The amino acids were then eluted from the resin and the eluant was assayed to determine the hydroxyproline content. The hydroxyproline was oxidised with chlor-T and the product reacted with DMBR in isopropanol at 65°C to develop a pink colour, which was measured at 550 nm, using the automated colourimeter - Technicon AAII analyser (Appendix B2). Collagen content was calculated assuming 300 moles of hydroxyproline are equivalent to 1 mole of collagen (Eyre et al 1984a). The mean inter-assay coefficient of variance of hydroxyproline was 4%.
5.1.1.6 Preparation of standard crosslinks

The Pyd and Dpd working standard was prepared as an aqueous solution from kangaroo bone, and assigned values using standards provided by The Bath Institute of Rheumatic Diseases, Bath, UK. Details of the standard preparation are described in Appendix B1. Des and Isodes standards were bovine neck ligament, purchased from ICN Biomedicals Incorporated (USA). Hydroxyproline standards were trans-4-hydroxyproline-L-proline (Sigma Chemicals, St Louis, USA).

![Figure 5.2](image)

**Figure 5.2** Representative chromatogram using a fluorescence detector to identify Pyd and Dpd, at 7 and 9 minutes, respectively [A]. Representative chromatogram using an ultraviolet detector to identify Isodes and Des peaks at 16 and 17.5 minutes, respectively [B].
5.1.1.7 HPLC (I) limitations and recovery of spiked crosslinks

The limitations of the HPLC procedure was determined by monitoring the inter-assay coefficient of variance and the recovery of spiked standard amounts of crosslinks. The inter-assay coefficient of variance for the control standards were Pyd = 6\%, Dpd = 12\%, Isodes = 5\% and Des = 22\%.

Recovery of the standards from extraction and HPLC (I) analyses was determined by adding low, medium and high concentrations of the crosslink standards to 10 unfixed ligamentum, anular and nuclear samples, therefore a total of 30 samples. The detailed methodology for spiking tests is described in Appendix B4. The lowest and highest spiked concentrations tested for each crosslink standard is shown in Table 5.2. The mean percentage recovery of spiked standard crosslinks using the HPLC method for the medium concentrations of standard Pyd, Dpd, Des and Isodes, were 571 nmol/L for Pyd concentrations, 288 nmol/L for Dpd, 12 µmol/L for Des and 30 µmol/L for Isodes. The mean percentage of recovery ranged from 76\% to 123\% (Table 5.2). The medium concentrations of the standards were used as they were within the average values detected for spinal disc and ligament tissues.

5.1.2 DATA REDUCTION AND STATISTICAL ANALYSIS

The preparation of the samples for HPLC (I) and the data analysis of the chromatograph trace were conducted by two investigators (C Tan and S Dunn, respectively). When evaluating the chromatograph trace, S Dunn was blind to the status of the injected eluate, whether it was fresh or formalin-fixed.

Paired $t$-tests were used to compare the collagen, Des and Isodes content, and extent of Pyd, Dpd between the fresh and formalin-fixed disc and LF tissues. One factor ANOVA was used to determine any significant changes in the biochemical parameters over the 25 weeks of storage and formalin fixation for fresh and formalin-fixed tissues, respectively. A probability of $p < 0.05$ was used to distinguish significant differences in all statistical evaluations. Demographic and raw data are recorded in Appendix D.
Table 5.2  Recovery rates for spiked Pyd, Dpd, Des and Isodes standards, added to fresh anular, nuclear and LF samples \( (n = 30) \), after extraction and analysis using the HPLC (I) assay. For Des and Isodes results, only LF tissues were used.

<table>
<thead>
<tr>
<th>Spiked concentrations</th>
<th>Crosslink standards</th>
<th>No of disc &amp; LF samples</th>
<th>Volume of standard added ( \mu l )</th>
<th>Concentration of standards</th>
<th>Mean % recovery of standards</th>
<th>± SD % recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Pyd</td>
<td>5</td>
<td>400</td>
<td>285.5 nmol/L</td>
<td>152</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Dpd</td>
<td>5</td>
<td>400</td>
<td>144 nmol/L</td>
<td>79</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Isodes</td>
<td>10</td>
<td>100</td>
<td>6 ( \mu )mol/L</td>
<td>138</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Des</td>
<td>10</td>
<td>100</td>
<td>14.8 ( \mu )mol/L</td>
<td>85</td>
<td>11</td>
</tr>
<tr>
<td>Medium</td>
<td>Pyd</td>
<td>5</td>
<td>800</td>
<td>571 nmol/L</td>
<td>123</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Dpd</td>
<td>5</td>
<td>800</td>
<td>288 nmol/L</td>
<td>89</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Isodes</td>
<td>10</td>
<td>200</td>
<td>12 ( \mu )mol/L</td>
<td>106</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Des</td>
<td>10</td>
<td>200</td>
<td>29.6 ( \mu )mol/L</td>
<td>76</td>
<td>12</td>
</tr>
<tr>
<td>High</td>
<td>Pyd</td>
<td>5</td>
<td>1000</td>
<td>713.8 nmol/L</td>
<td>114</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Dpd</td>
<td>5</td>
<td>1000</td>
<td>360 nmol/L</td>
<td>71</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Isodes</td>
<td>10</td>
<td>350</td>
<td>21 ( \mu )mol/L</td>
<td>102</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Des</td>
<td>10</td>
<td>50</td>
<td>74 ( \mu )mol/L</td>
<td>91</td>
<td>3</td>
</tr>
</tbody>
</table>

5.1.3  RESULTS

A frequency distribution assessment of the formalin-fixed and fresh data revealed a few outliers; subsequently data points within the 10th and 90th percentile were employed in subsequent statistical analyses. In total, 2 Pyd and 1 Dpd data points were omitted from statistical analyses for the anulus and 1 collagen and 1 Dpd data points were omitted for the nucleus. Two Dpd data were also not calculated for the nucleus as the chromatograph traces were too low for adequate detection (< 5 nmol/L). All data points for the disc and LF were normally distributed and used for statistical analysis (Table 5.3).

The mean dry weight of the fresh and formalin-fixed samples was recorded in Table 5.3. Des and Isodes could only be detected in the LF samples. Lumbar disc samples showed minute traces of Des and Isodes however, the traces were too low for reliable quantification (< 2 \( \mu \)mol/L).
There were no significant differences in the collagen and elastin crosslink content or extent of collagen crosslinks between pooled fresh and formalin-fixed anular, nuclear and ligament tissues. There were also no significant changes in the mean value for the biochemical parameters for all tissues, over the 25-week duration of storage and formalin fixation. Using data from the fresh tissues as controls for the formalin-fixed tissues, a plot of the data from the formalin-fixed tissues over the weeks of fixation showed that 99% to 100% of the formalin data were within 2 standard deviations of the mean value from the fresh samples (Figures 5.3 to 5.5).

5.1.4 DISCUSSION

Although fresh and formalin tissues assayed were essentially mirror-images, however minor differences in the tissue biochemical matrix might be expected due to the geographical variability even within the same tissue sample, therefore samples for this study were taken only from the anterior anulus. Even then some variation is to be expected. Biochemical differences between left and right sides of the discs had so far been detected in cases of spinal scoliosis (Crean et al. 1997, Duance et al. 1998), which suggested a mechanical loading influence on changes in tissue matrix biochemistry. The cadavers selected for the study did not have a history or obvious presentations of spinal deformity or scoliosis, which should minimise tissue location sampling bias.

The penetration of formalin into the tissue depended on the thickness and type of specimen (Leong 1994, Wilke et al. 1996). Generally, formaldehyde usually takes about 1 to 3 days to form crosslinkages with proteins and the penetration rate was estimated at 0.78 mm/hr (Leong 1994). According to Leong (1994), the crosslinkages formed in the tissues were still reversible within 24 hours. The sectioned discs and ligaments in the present study were not more than 2mm thick, therefore penetration of formalin into the disc and ligament tissues should be adequate after one week of storage in buffered formalin. Studies have reported formalin effects as early as 24 hours after fixation for rat liver tissues (Brooks et al. 1998), 7 days in human spinal ligaments (Wilke et al. 1996), taking longer for bony tissues, 3 to 4 weeks for human and sheep vertebrae (Edmondston et al. 1994a).
Figure 5.3 A time plot of the collagen, Des and Isodes content, extent of Pyd and Dpd, from formalin-fixed anular tissues over the 25 weeks of formalin fixation. All data points from the formalin anular samples were within the shaded area, except for 2 points for Pyd and 1 point for Dpd. The shaded area represents the mean (± 2 SD) from the pooled fresh anular samples.
Figure 5.4 A time plot of the collagen, Des and Isodes content, extent of Pyd and Dpd, from formalin-fixed nuclear tissues over the 25 weeks of formalin fixation. All data points from the formalin nuclear samples were within the shaded area, except for 3 points for collagen and 3 points for Dpd. The shaded area represents the mean ($\pm 2$ SD) from the pooled fresh nuclear samples.
Figure 5.5 A time plot of the collagen, Des and Isodes content, extent of Pyd and Dpd, from formalin-fixed LF tissues over the 25 weeks of formalin fixation. All data points from the formalin ligamentum samples were within the shaded area, except for Pyd 1 point. The shaded area represents the mean (± 2 SD) from the pooled fresh ligamentum flava samples.
Table 5.3 The mean and standard deviation (SD) of the dry weight, collagen content, extent of Pyd, Dpd, Isodes and Des content in fresh and formalin-fixed (FF) anulus, nucleus and LF tissues.

<table>
<thead>
<tr>
<th></th>
<th>Dry weight</th>
<th>Collagen</th>
<th>Pyd</th>
<th>Dpd</th>
<th>Isodes</th>
<th>Des</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>nmol/mg dry wt</td>
<td>mol/mol collagen</td>
<td>mol/mol collagen</td>
<td>nmol/mg dry wt</td>
<td>nmol/mg dry wt</td>
</tr>
<tr>
<td>Fresh</td>
<td>FF</td>
<td>Fresh</td>
<td>FF</td>
<td>Fresh</td>
<td>FF</td>
<td>Fresh</td>
</tr>
<tr>
<td>Anulus</td>
<td>n</td>
<td>36</td>
<td>36</td>
<td>36</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>Mean</td>
<td>34.6</td>
<td>34.9</td>
<td>1.4</td>
<td>1.3</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>SD</td>
<td>24.5</td>
<td>27.2</td>
<td>0.6</td>
<td>0.5</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Nucleus</td>
<td>n</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Mean</td>
<td>20.6</td>
<td>20.4</td>
<td>0.73</td>
<td>0.75</td>
<td>2.2</td>
<td>2.1</td>
</tr>
<tr>
<td>SD</td>
<td>6.3</td>
<td>13.9</td>
<td>0.15</td>
<td>0.31</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>LF</td>
<td>n</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Mean</td>
<td>14.1</td>
<td>16.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>SD</td>
<td>10</td>
<td>11.5</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>
5.1 Effects of formalin on spinal tissues

Formalin fixation had been reported to increase the tensile strength of tendon (Viidik and Lewin 1966) and the stiffness of spinal ligament (Wilke et al 1996), however, it did not seem to alter the extent of mature and insoluble collagen and elastin crosslinks, in spinal disc and ligament tissue matrices, as found in the present study. There were no significant differences noted in the collagen content, the extent of collagen crosslinks and the elastin crosslink content in the anulus and nucleus between fresh and formalin-fixed spinal discs and the LF. These original data for spinal soft tissues were in contrast to the osseous findings reported by Boskey et al (1982), who reported significantly higher collagen content in human cortical bones fixed in formalin for 3 to 11 days. According to Boskey et al (1982) the high collagen content in formalin-fixed compared to fresh bone tissues was due to a greater loss of collagen in fresh bone tissues from processing and handling procedures.

Results from this study on collagen crosslink Pyd were similar to that of Abe et al (2003) for spinal ligament tissues, however the latter study reported significantly reduced elastin crosslinks, Des and Isodes, in formalin-fixed tissues compared to fresh tissues. They suggested that the elastin crosslinks were masked or altered by formalin fixation. Reasons for this contrasting finding in elastin crosslink for formalin fixed tissues is not known and not reported in the literature, however it is probable that if mature collagen crosslinks, Pyd and Pentosidine are not affected by formalin fixation, that mature elastin crosslinks should be similarly affected too. Other reasons for the different findings may be due to methodological differences in the sampling, storage and preparation of the samples for HPLC analyses.

When preserving tissues, formaldehyde forms monomeric crosslinks with protein molecules in the tissues by reacting with unbonded or free lysine residues on the exterior of the protein molecule (Chapman et al 1990, Leong 1994). These free lysine residues might not be available on matured collagen and elastin crosslinks, which were formed as end-products of a series of hydroxylation reactions from lysine and hydroxylysine residues (Baurain et al 1976, Laurent et al 1983, Eyre 1987, Rosenbloom 1987), to form new formalin induced crosslinks. Even if there were some free lysines available on the collagen molecule, formaldehyde did not appear to form more crosslinks or to destroy existing mature collagen and elastin crosslinks in spinal disc and ligament tissues. The non-significant findings for collagen and elastin crosslinks provided evidence that formalin fixation may not affect the structure, hence content of mature collagen and elastin crosslinks. The possibility of formaldehyde reacting with or breaking down mature pyridinium crosslinks was unlikely because these crosslinks were stable and resistant to most collagenase (Liu et al 1991). The stability of these mature collagen and elastin crosslinks was one of the reasons that enabled them to be identified following the determination of the method to separate them from other reducible crosslinks.
(Starcher and Galione 1976, Fujimoto 1977). The findings from this study suggested that formalin induced crosslinks might be hydrolysed to release collagen, collagen and elastin crosslinks for biochemical analysis in spinal tissues.

Findings from this study, despite the contrasting elastin findings by Abe et al (2003), suggest that formalin-fixed tissues might be utilised for biochemical analysis of collagen and mature collagen and elastin crosslinks in human spinal and LF tissues. Such information is useful as archival tissues are usually more easily handled and accessible compared to fresh tissues, and have a lower risk of infection (Cavanaugh and King 1990).

5.1.5 CONCLUSION
Human spinal discs and LF fixed in 10% buffered formalin for 25 weeks (approximately 6 months), did not have significantly different collagen, Des and Isodes content or extent of Pyd and Dpd, compared with control fresh samples. Biochemical analysis of these matrix constituents is validated for formalin-fixed tissues.
STUDY 5.2 DISTRIBUTION OF COLLAGEN AND COLLAGEN CROSSLINKS IN THORACIC ANULUS

Cross-sectional biochemical studies using spinal discs removed from scoliotic spines reported that human disc matrices exposed to asymmetrical biomechanical stresses in the growing adolescent, presented with significantly different matrices within the same disc (Crean et al 1997, Duance et al 1998). The anulus of thoracic discs on the concave side of the scoliotic curve were reported to have a lower PG and collagen content compared to the anulus of the same discs on the convex side (Bushell et al 1979, Crean et al 1997, Duance et al 1998). In contrast, discs on the convex side, had an increase in PG (Crean et al 1997) and collagen content (Bushell et al 1979). To determine if there were differences in the biochemical constituent from different anular regions of the same disc in the absence of scoliosis, a pilot study was conducted with thoracic disc tissues from two formalin-fixed female spines, aged 77 and 81 years.

5.2.1 MATERIALS AND METHODS

5.2.1.1 Tissue collection
Two fixed thoracic spines, which had no obvious spinal deformity from posture, trauma or surgery, were selected from the archive. The spine was sectioned at each thoracic level (T1 to T12) through the mid transverse plane, and the anulus was examined for any abnormality, such as clefts, fissures, nuclear herniation or calcification. Only anuli observed as normal, were selected for investigation.

After examination one mm$^3$ of disc tissue was removed from four different regions of the anulus (anterior and posterior left and right quadrants). In total 24 thoracic discs were removed from the two spines, which yielded 96 anular samples, four from each anular quadrant. In addition, anular samples were also removed from the mid sagittal and mid coronal position of the thoracic disc at level T6, to observe for regional variation within the same disc.

5.2.1.2 Biochemical assay for Collagen, Pyd and Dpd
The preparation of disc tissues for biochemical analysis and the extraction of crosslinks for identification on the HPLC were similar to that described in Study 5.1. However, the HPLC procedure used to detect and analyse the collagen crosslinks was different from that described in Study 5.1 (see section 5.2.1.3 below). In Study 5.2, elastin crosslinks were not analysed,
therefore Isodes was used as an internal standard in each run. The chromatogram run time was also shorter, and the UV detector was not required as in the HPLC (I) method. The collagen assay for analysing hydroxyproline was similar as in Study 5.1.

### 5.2.1.3 Detection and calculation for Pyd and Dpd using HPLC (II)

The HPLC (II) unit consisted of a Waters model 700 WISP Model 600E pump and controller, a Waters 441 Absorbance Detector fitted with a 280 nm filter set (Waters, Corp. Milford, USA), and a Shimadzu FR535 Fluorescence Detector (Tokyo, Japan). The column was a Supelco-Spelcosil LC-18DB (3 μm 4.0 mm X 7.5 cm cartridge PN58992, Pennsylvania, Bellefonte, USA) column. Detection of Pyd and Dpd was set at excitation 295 nm and emission at 395 nm wavelength. Absorbance of the internal standard, Isodes (bovine neck ligament, ICN Biomedicals Inc. Cat No. 191379), was set at 280 nm. The mobile phase A was a mixture of 5% methanol, and 0.12% HFBA at pH = 2.0, and mobile phase B was a mixture of 80% methanol and 0.12 % HFBA.

Each chromatogram took approximately 25 minutes to complete, with Pyd and Dpd identified with a fluorescent detector at approximately 7 and 9 minutes, respectively, and Isodes crosslinks detected at 12 minutes. The extent of Pyd and Dpd in the discs was expressed as number of moles of crosslinks per mole of collagen. Data was calculated using a Waters Maxima 825 chromatography system (Waters, Corp. Milford, USA). All Pyd and Dpd results were corrected for internal standard recovery and calculated against the concentration of the working standard used:

\[
\text{Pyd} = 571 \text{ nmol/L} \quad \text{Dpd} = 288 \text{ nmol/L}
\]

The inter-assay variation between each laboratory run was monitored by using high, medium, low and thoracic disc controls in each run for Pyd, Dpd and collagen content. The inter-assay coefficient of variance of the standards used between runs averaged at 11% for Pyd, 17% for Dpd and 5% for hydroxyproline (HOP) assays (Table 5.4). In addition, an internal standard, Isodes, was added to the hydrolysate to ensure consistency between HPLC runs. The preparation for the run standards is similar to that described in Study 5.1 (section 5.1.1.6).

### 5.2.2 RESULTS

The collagen content and extent of Pyd and Dpd were not significantly different between the left and right quadrants of the anterior and posterior anuli (Table 5.5). There was also no significant difference in the collagen content and extent of Dpd between the anterior and
Chapter 5

5.2 Collagen crosslink distribution in anulus

However the extent of Pyd was significantly higher in the posterior anulus compared to the anterior anulus ($p < 0.05$). A comparison of the tissues taken from different regions of T2-T3, T6-T7 and T10-T11 showed that the T6-T7 (mid thoracic region) had the highest %CV compared to T2-T3 and T10-T11 (Table 5.6). Raw data is recorded in Appendix D.

**Table 5.4** The inter-assay coefficient of variation (% CV) between each laboratory run using high, medium and low Pyd and Dpd standards and thoracic disc (T9-T10) control for HPLC (II) assay, and Urine and thoracic disc controls for hydroxyproline (HOP) assay.

<table>
<thead>
<tr>
<th></th>
<th>High</th>
<th>Medium</th>
<th>Low</th>
<th>Urine</th>
<th>T9-T10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HOP</strong> Mean</td>
<td>0.400</td>
<td>0.204</td>
<td>0.432</td>
<td>0.175</td>
<td>758.12</td>
</tr>
<tr>
<td>SD</td>
<td>0.003</td>
<td>0.022</td>
<td>0.011</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>% CV</td>
<td>0.6</td>
<td>10.7</td>
<td>2.5</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td><strong>Pyd</strong> Mean</td>
<td>1031.90</td>
<td>308.70</td>
<td>55.29</td>
<td>112.40</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>104.84</td>
<td>41.55</td>
<td>5.48</td>
<td>14.8</td>
<td></td>
</tr>
<tr>
<td>% CV</td>
<td>10.1</td>
<td>13.5</td>
<td>9.9</td>
<td>14.8</td>
<td></td>
</tr>
<tr>
<td><strong>Dpd</strong> Mean</td>
<td>425.17</td>
<td>76.60</td>
<td>18.29</td>
<td>21.69</td>
<td>29.4</td>
</tr>
<tr>
<td>SD</td>
<td>58.54</td>
<td>13.02</td>
<td>4.17</td>
<td>6.37</td>
<td></td>
</tr>
<tr>
<td>% CV</td>
<td>13.7</td>
<td>17</td>
<td>22.8</td>
<td>29.4</td>
<td></td>
</tr>
</tbody>
</table>

5.2.3 DISCUSSION

Studies have shown that the biochemical content of discs taken from scoliotic spines were different between the convex or concave side, that is, left and right sides (Bushell et al 1979, Crean et al 1997, Duance et al 1998). The spinal discs in this study were taken from normal spines, which supports the non-significant finding between left and right quadrants of the anterior and posterior anuli.

The significantly different extent of Pyd between the anterior and posterior halves of the anuli may suggest a regional influence in the thoracic disc due to non-symmetrical compression loading of the discs, as mentioned for scoliotic spines above. The disc regional difference may be due to the influence of increased compression loading in the anterior aspect of the thoracic disc, especially in the mid thoracic region, which is the apex of the thoracic kyphosis.
(Schmorl and Junghanns 1971, Singer 1997). Variation of Pyd density in the different parts of the disc in the mid thoracic region, is supported by the high %CV of samples taken from the T6-T7 disc, where most of the asymmetrical compression loading is experienced.

It is noted that the sample size of this preliminary study is small therefore generalisation is limited. However, the significant difference of the extent of Pyd between anterior and posterior anuli suggests that disc tissues would be more accurately analysed if the region of the disc sampled is taken into consideration, especially if the spine has associated vertebral body deformities.

### 5.2.4 CONCLUSION

Results from this study suggest that when investigating the biochemical changes in spinal thoracic discs, the location of disc samples is important. Sample selection should take into consideration the disc regional differences not only between the anulus and the nucleus, but also between the anterior and posterior anulus.

**Table 5.5** The mean collagen content and extent of Pyd and Dpd in the different regions of the anulus from two thoracic spines (n = 24 thoracic discs).

<table>
<thead>
<tr>
<th>Annular region</th>
<th>Anterior (n = 24)</th>
<th>Posterior (n = 24)</th>
<th>Total Anterior (n = 48)</th>
<th>Total Posterior (n = 48)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left</td>
<td>Right</td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td>Collagen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nmol/mg dry wt</td>
<td>1.29</td>
<td>1.27</td>
<td>1.3</td>
<td>1.47</td>
</tr>
<tr>
<td>Pyd</td>
<td>1.67</td>
<td>1.69</td>
<td>1.93</td>
<td>1.8</td>
</tr>
<tr>
<td>mol/mol collagen</td>
<td>0.033</td>
<td>0.035</td>
<td>0.038</td>
<td>0.040</td>
</tr>
</tbody>
</table>

* = p <0.05 between pooled anterior and posterior data.

**Table 5.6** The coefficient of variation (%CV) for Pyd, Dpd and Collagen in the different regions of each anulus from thoracic levels T2-T3, T6-T7 and T10-T11 from one spine.

<table>
<thead>
<tr>
<th>n</th>
<th>Pyd</th>
<th>Dpd</th>
<th>Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2-T3</td>
<td>4</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>T6-T7</td>
<td>8</td>
<td>50</td>
<td>34</td>
</tr>
<tr>
<td>T10-T11</td>
<td>4</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>
STUDY 5.3 AGE-RELATED CHANGES IN COLLAGEN, PYRIDINOLINE AND DEOXYPYRIDINOLINE IN NORMAL HUMAN THORACIC INTERVERTEBRAL DISCS

The pyridinium crosslinks formed by Types I and II collagen fibers are Pyd and Dpd (Takahashi et al 1994). Pyd and Dpd are mature, non-reducible trifunctional 3-hydroxypyridinium cross-links, representing two of the end products of lysyl oxidase-mediated reactions on lysyl and hydroxylysyl amino group residues (Eyre 1988). It had been hypothesised that these collagenous crosslinks were essential in maintaining the structure and tensile strength of the collagen fibrillar network (Takahashi et al 1994). Alteration of the collagen crosslink content in the disc matrix has been suggested to impair disc function, in particular, the disc's ability to withstand mechanical loading (Duance et al 1998, Pokharna and Phillips 1998).

Studies investigating the presence of these crosslinks in human spinal discs are still limited with a few studies reporting on lumbar discs. Duance et al (1998) and Eyre (1988) found a higher distribution of Pyd in the nucleus compared to the anulus of lumbar discs. Pokharna and Phillips (1998) also reported a decrease in the levels of Pyd with increasing age and degeneration in lumbar discs. Similar data on thoracic discs have not been reported in the literature.

Studies on the influence of gender differences in distribution of the disc collagenous matrix were even fewer. There were studies suggesting that gender-related occupation might be associated with a higher predominance of disc degeneration in lumbar discs, particularly in males (Miller et al 1988, Videman et al 1990, Riihimaki et al 1998). However information on gender differences in the collagen and crosslink composition of spinal discs is still limited. In view of the association between collagen crosslinks and spinal disc function, this study examined the influence of age, gender and spinal level differences on the collagen content and the extents of Pyd and Dpd in normal human thoracic intervertebral discs.

5.3.1 MATERIALS AND METHODS
5.3.1.1 Tissue Collection and Preparation
Thoracic human cadaver spines, which had been removed following routine post-mortem procedures and fixed in 4% buffered formalin, were used for biochemical analysis following macroscopic examination. A sample of convenience of 26 thoracic spines was selected for
investigation if they had no history of spinal trauma, surgery or frank pathology to the thoracic spine (Table 5.7). This resulted in a total of 303 thoracic discs, of which nine discs were not available for investigation because 1 spine was incomplete, with only the upper four thoracic levels available, and another case had a completely fused T7-8 disc. The study sample comprised 13 males and 13 females with a mean age of 45.8 ± 28.2 years (age range from 1 to 90 years, Table 5.7).

5.3.1.2 Macroscopic grading of thoracic intervertebral discs
The macroscopic examination of formalin-fixed thoracic intervertebral discs, used in the following investigations, was performed using the mid-sagittal section of the hemisected thoracic cadaver spine. A modified 3-point grading scale from I to III, based on the original 5-point criteria reported by Thompson et al (1990), was selected to grade the discs, as the latter was found to be correlated to the 5-point MRI grading scale (Thompson et al 1988).

Grade I indicated normal or non-degenerate discs, similar to Grade I on the Thompson scale. Grade II, reflected moderate degenerative changes, which consisted of Grades II and III criteria for the anulus and the nucleus from the Thompson scale. Grade III, indicated severely degenerate disc changes, which consisted of Grades IV and V criteria from the Thompson scale (Figure 5.6 and Table 5.8). Investigator, C Tan, graded all the discs. For this study, only discs graded as normal or grade I (n = 209), were selected for investigation. One mm$^3$ of disc tissue was removed from the anterior and posterior anulus and nucleus of the hemisected spine, and prepared for biochemical analysis.

The intra-rater reliability of investigator C Tan in disc grading was evaluated using 10 hemisected thoracic spines (n =120 discs). Discs from all the thoracic levels were graded with the modified 3-point scale described in Table 5.8, and data recorded in Appendix A5. The second repeat reading was conducted after a three-month period. The kappa $\kappa$ correlation coefficient for intra-rater reliability of disc grading was excellent for both nucleus (0.91) and anulus (0.81).

5.3.1.3 Biochemical assay for Collagen, Pyd and Dpd using HPLC (II)
The sample preparation for biochemical analysis and the extraction of crosslinks for the HPLC (II) procedure is similar to that described in Study 5.2, section 5.2.1.3, to determine the extent of Pyd and Dpd. Collagen content was evaluated using the method in Study 5.1.
Table 5.7 Demographic details and cause of death for all thoracic cadaver cases ($n = 26$).

<table>
<thead>
<tr>
<th>Subject Code</th>
<th>Age yrs</th>
<th>Age group</th>
<th>Gender</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAT</td>
<td>77</td>
<td>Old</td>
<td>M</td>
<td>Pituitary tumour and cerebral swelling</td>
</tr>
<tr>
<td>ABT</td>
<td>77</td>
<td>Old</td>
<td>F</td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>ACT</td>
<td>90</td>
<td>Old</td>
<td>M</td>
<td>Myocardial infarct and bronchopneumonia</td>
</tr>
<tr>
<td>ADT</td>
<td>75</td>
<td>Old</td>
<td>M</td>
<td>Myocardial infarct and emphysema</td>
</tr>
<tr>
<td>AET</td>
<td>79</td>
<td>Old</td>
<td>F</td>
<td>Bronchopneumonia and myocardial infarct</td>
</tr>
<tr>
<td>AFT</td>
<td>2</td>
<td>Child</td>
<td>M</td>
<td>Cerebral swelling and lung congestion</td>
</tr>
<tr>
<td>AT</td>
<td>81</td>
<td>Old</td>
<td>F</td>
<td>Metastatic adenoma</td>
</tr>
<tr>
<td>BT</td>
<td>52</td>
<td>Mid</td>
<td>M</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>CT</td>
<td>18</td>
<td>Young</td>
<td>M</td>
<td>Spinal cord with autolysis</td>
</tr>
<tr>
<td>DT</td>
<td>29</td>
<td>Young</td>
<td>M</td>
<td>Cerebral trauma</td>
</tr>
<tr>
<td>ET</td>
<td>42</td>
<td>Mid</td>
<td>F</td>
<td>Cerebral haemorrhage from head injury</td>
</tr>
<tr>
<td>FT</td>
<td>77</td>
<td>Old</td>
<td>F</td>
<td>Myocardial infarct</td>
</tr>
<tr>
<td>GT</td>
<td>63</td>
<td>Old</td>
<td>F</td>
<td>Pulmonary congestion, death from asphyxia</td>
</tr>
<tr>
<td>HT</td>
<td>90</td>
<td>Old</td>
<td>M</td>
<td>Meningioma, death by myocardial infarct</td>
</tr>
<tr>
<td>IT</td>
<td>46</td>
<td>Mid</td>
<td>F</td>
<td>Undetermined cause of death</td>
</tr>
<tr>
<td>JT</td>
<td>33</td>
<td>Young</td>
<td>M</td>
<td>Undetermined cause of death</td>
</tr>
<tr>
<td>KT</td>
<td>23</td>
<td>Young</td>
<td>F</td>
<td>Post traumatic epilepsy</td>
</tr>
<tr>
<td>LT</td>
<td>7</td>
<td>Child</td>
<td>F</td>
<td>Undetermined cause of death</td>
</tr>
<tr>
<td>MT</td>
<td>7</td>
<td>Child</td>
<td>M</td>
<td>Intracranial haemorrhage</td>
</tr>
<tr>
<td>NT</td>
<td>32</td>
<td>Young</td>
<td>F</td>
<td>Cervical fracture and massive haemorrhage</td>
</tr>
<tr>
<td>OT</td>
<td>23</td>
<td>Young</td>
<td>M</td>
<td>Undetermined cause of death</td>
</tr>
<tr>
<td>PT</td>
<td>43</td>
<td>Mid</td>
<td>F</td>
<td>Suicide – Burn injuries</td>
</tr>
<tr>
<td>QT</td>
<td>40</td>
<td>Mid</td>
<td>F</td>
<td>Cerebral swelling and pulmonary congestion</td>
</tr>
<tr>
<td>RT</td>
<td>48</td>
<td>Mid</td>
<td>M</td>
<td>Undetermined cause of death</td>
</tr>
<tr>
<td>ST</td>
<td>37</td>
<td>Mid</td>
<td>M</td>
<td>Hypoxic encephalopathy by hanging</td>
</tr>
<tr>
<td>TT</td>
<td>1</td>
<td>Child</td>
<td>F</td>
<td>Undetermined cause of death</td>
</tr>
</tbody>
</table>
Figure 5.6 Mid sagittal view of hemisected thoracic discs showing grade I anulus and nucleus of a 29-year old male subject [A]. Grade II anulus and nucleus of a 63-year old female subject [B]. Grade III anulus and nucleus of a 77-year old female subject [C].

Table 5.8 Criteria for the 3-point macroscopic grading scale used to examine the anulus and nucleus of spinal intervertebral discs, modified from Thompson et al (1990).

<table>
<thead>
<tr>
<th>Grade</th>
<th>Anulus</th>
<th>Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Discrete lamellae, white in appearance</td>
<td>Gel-like, bulging, blue-white in appearance</td>
</tr>
<tr>
<td>II</td>
<td>Chondroid or mucinous material between the lamellae</td>
<td>Fibrous tissue bands or consolidated tissue</td>
</tr>
<tr>
<td>III</td>
<td>Clefts extending throughout</td>
<td>Clefts extending throughout</td>
</tr>
</tbody>
</table>
5.3.2 DATA REDUCTION AND STATISTICAL ANALYSIS

Paired t-tests were applied to examine for intra-disc or disc regional differences, and ANOVA was used to examine the age, gender and thoracic spinal level influences on the collagen content and extents of Pyd and Dpd. For statistical comparison, each disc was considered as a single statistical unit. However, due to the small number of spines in the study spanning a wide age range (1 – 90 years), samples were grouped according to age ranges for statistical analysis, in order to determine age trends. The age groups consist of Child (1 to 15 years), Young (16 to 35 years old), Mid (36 to 60 years old) and Old (> 60 years old) (Table 5.9) were employed. Similarly thoracic levels were grouped to form upper (T1 to T4), mid (T5 to T8) and lower (T9 to T12) thoracic regions in order to determine spinal level trends.

To determine the correlation coefficient between age and biochemical variables, partial correlation coefficient analysis were performed. All statistical tests were performed using the Statview statistical software packages (version 4.1 Abacus Concept Inc, USA). A probability level of $p < 0.05$ was accepted as representing a meaningful difference in all tests of statistical significance. Where multiple t-tests comparisons were performed, Bonferroni correction was applied to determine the probability level. Therefore $p < 0.0167$ and $p < 0.008$, were accepted as representing a meaningful difference in tests of statistical significance for spinal levels and age groups, respectively. Raw data is recorded in Appendix E.

5.3.3 RESULTS

5.3.3.1 Influence of disc region

Significant differences in collagen content and extents of Pyd and Dpd were found between the nucleus and the anulus of normal or grade I non-degenerate thoracic discs (Figure 5.7). The anterior anulus had significantly lower extents of Pyd and Dpd compared with the nucleus and posterior anulus ($p < 0.001$). The nucleus however, had significantly lower collagen content ($p < 0.001$) compared with both anuli, but significantly higher extents of Pyd, compared to the anterior anuli ($p < 0.001$), and significantly higher extent of Dpd compared to both anuli ($p < 0.001$).

There were no significant differences in the collagen content between the anterior and posterior anulus. These differences in the collagen content and extent of collagen crosslinks in the anulus and nucleus, were also observed when data were examined for each age group.
**Table 5.9** Information on 26 cadaver thoracic spines showing number of discs (anulus and nucleus) graded I (non-degenerate) according to age groupings.

<table>
<thead>
<tr>
<th>Age groups</th>
<th>Age Range (years)</th>
<th>No of thoracic spines</th>
<th>Mean Age (yrs ± SD)</th>
<th>Female</th>
<th>Male</th>
<th>No of grade I discs</th>
<th>Total no of discs graded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Child</td>
<td>1-15</td>
<td>4</td>
<td>4 ± 3.2</td>
<td>2</td>
<td>2</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Young</td>
<td>16-35</td>
<td>5</td>
<td>25 ± 5.5</td>
<td>2</td>
<td>4</td>
<td>52</td>
<td>72</td>
</tr>
<tr>
<td>Mid</td>
<td>36-60</td>
<td>7</td>
<td>44 ± 5.1</td>
<td>4</td>
<td>3</td>
<td>69</td>
<td>84</td>
</tr>
<tr>
<td>Old</td>
<td>&gt;60</td>
<td>10</td>
<td>78 ± 8.0</td>
<td>5</td>
<td>4</td>
<td>48</td>
<td>107</td>
</tr>
<tr>
<td>Total</td>
<td>1-90</td>
<td>26</td>
<td>45.8 ± 28.2</td>
<td>13</td>
<td>13</td>
<td>209</td>
<td>303</td>
</tr>
</tbody>
</table>

**Figure 5.7** The collagen content and extent of Pyd and Dpd in the nucleus, anterior and posterior anulus (AF) of grade I (non-degenerate) thoracic discs (*n* = 209). The collagen content was significantly higher in the anterior and posterior anulus compared with the nucleus (*p* < 0.001). However the extent of Pyd and Dpd were highest in the nucleus compared to both anuli. Error bars represent one standard deviation.

* = *t*-test between anterior AF and nucleus where *p* < 0.001.

** = *t*-test between both anuli and nucleus where *p* < 0.001.
5.3.3.2 Influence of spinal level
The collagen content and extent of collagen crosslinks in the disc in different thoracic levels showed significant spinal level trends mainly in the anterior anulus (Figure 5.8). In the mid thoracic region of the anterior anulus, the collagen content was lower, while the extent of Pyd was higher, compared to the upper and lower thoracic regions. These quadratic trends were statistically significant when tested using ANOVA ($p < 0.05$). When data was grouped under the 3 thoracic regions, the collagen content in the upper thoracic region of the anterior anulus, was higher than the mid and lower thoracic regions ($p < 0.001$). The anterior anulus of the lower thoracic region had the lowest extent of Pyd, compared to the upper and mid (statistically significant at $p < 0.001$) thoracic regions. Similarly, the extent of Dpd in the anterior anulus of the lower thoracic region was also significantly lower compared to the upper and middle thoracic regions ($p < 0.001$).

In the nucleus, only the extent of Dpd was lowest in the lower thoracic region compared to the upper and mid thoracic regions ($p < 0.0167$). The extent of Pyd also had a significant decreasing craniocaudal trend in the nucleus ($p <0.05$, Figure 5.8), however, when comparing between spinal regions, it was not statistically significant. The lower thoracic region therefore had a low collagen content and extent of Pyd and Dpd in the anterior anulus and nucleus, but not in the posterior anulus. There were no significant spinal level trends in the collagen content, extent of Pyd and Dpd for the posterior anulus.

5.3.3.3 Age Influence
With increasing age, the collagen content and extent of Pyd decreased significantly in all disc regions ($p < 0.001$, Figure 5.9). In contrast, the extent of Dpd increased with age, but this trend was only significant in the nucleus ($p < 0.001$). However the correlation coefficients between age and the biochemical variables were fair to good, ranging from -0.5 to -0.7 between age and collagen content for all disc regions ($p < 0.001$); and -0.4 to -0.5 for the extent of Pyd ($p < 0.001$). The only significant positive partial correlation coefficient for the extent of Dpd was 0.3 ($p < 0.001$) for nuclear samples. The highest correlation with age is the collagen content, especially in the nucleus (-0.7).
Figure 5.8 The collagen content [A] and extent of Pyd [B] and Dpd [C] in the nucleus, anterior and posterior anulus of grade I thoracic discs in the different thoracic levels (T1 to T12, n = 209). Significant decreasing craniocaudal trends were noted in all disc regions for the extent of Dpd (p < 0.05) [C]. The dip in the collagen content [A] and the peak in the extent of Pyd crosslink in the mid thoracic region (T5 to T8) of the anterior anulus [B] were also statistically significant (p < 0.05). In the nucleus, the extent of Pyd in the lower thoracic region had significantly lower values compared to the upper thoracic region (p < 0.05). Error bars represent one standard deviation.

*= ANOVA test for comparison of means of each thoracic level from T1 to T12, where p < 0.05.

**= t-tests comparison between lower and upper thoracic segments, where p < 0.0167.
Figure 5.9 Changes in the collagen content [A] and extent of Pyd [B] and Dpd [C] in the different age groups, Child (C), Young (Y), Mid (M) and Old (O) for all grade I thoracic discs ($n = 209$), in the different disc regions. In all disc regions, the collagen content and the extent of Pyd was significantly lower with increased age ($p < 0.001$) [A,B]. In contrast, the extent of Dpd was higher with age but was only significant in the nucleus ($p < 0.001$) [C]. Error bars represent one standard deviation.

* = ANOVA tests for comparison of means of each age group, where $p < 0.001$. 
5.3.3.4 Gender influence

The overall extent of Pyd were significantly higher in male subjects compared to females in all disc regions ($p < 0.001$, Figure 5.10). When data were ordered according to age groups, the extent of Pyd in male discs dropped below that of females in the Old age group in all the disc regions. However this gender difference in the Old age group was not statistically significant.

![Collagen Content and Extent of Pyd](image)

**Figure 5.10** The collagen content [A] and extent of Pyd [B] and Dpd [C] in the nucleus, anterior and posterior anulus of grade I male ($n = 86$) and female ($n = 123$) thoracic discs. The collagen content was significantly higher in males compared to females for both the anterior and posterior anulus ($p < 0.05$) [A]. The extent of Pyd was also significantly higher in male disc samples compared with female samples in all discs regions and decreased significantly with age ($p < 0.001$) [B]. There was no significant gender difference for the extent of Dpd [C]. Error bars represent one standard deviation.

$* = t$-tests comparison of means of male and female samples, where $p < 0.05$. 

The collagen content was also significantly higher in males compared to females but only for the anterior and posterior anulus ($p < 0.05$). In contrast, there were no significant gender differences in the extent of Dpd in all disc regions and in all age groups. Similar differences in gender results were noted for collagen content, and extent of Pyd and Dpd, when data were regrouped according to thoracic levels, that is, a higher value in males compared with females.

5.3.4 DISCUSSION

Literature on the biochemical crosslinks in human thoracic discs was limited when compared to studies on lumbar discs (Crean et al 1997, Duance et al 1998, Pokharna and Phillips 1998). The extent of Pyd reported in the literature ranged from 0.7 to 1.66 mol/mol collagen in the anulus and 1.0 to 1.59 mol/mol collagen in the nucleus of lumbar discs (Eyre 1995, Duance et al 1998). Interestingly, Pokharna and Phillips (1998) reported Pyd values of 3.4 to 4.2 mol/mol collagen in lumbar discs, despite Eyre’s (1995) contention that collagen fibers in the disc were only able to form 3 moles of crosslink per mole of collagen. An explanation of the high values reported by Pokharna and Phillips (1998) was not given. The present study on thoracic discs found a mean of 1.83 mol Pyd per mol collagen in the anulus and 1.95 mol/mol collagen in the nucleus. This value was considerably lower than that of Pokharna and Phillips (1998) but slightly higher than that of Eyre (1995), and Duance et al (1998). The variation in the extent of Pyd in the different lumbar studies, could be due to the age and degenerative status of the disc used in the study (Duance et al 1998). The results of the present study for the collagen content and extents of Pyd and Dpd using only normal thoracic discs, provided a basis for future comparison with degenerated thoracic discs.

Significant regional differences in the collagenous matrix were found between the anulus and the nucleus of non-degenerate thoracic discs. The present study found higher collagen content in the anulus compared to the nucleus, but a higher extent of Pyd and Dpd in the nucleus, which was consistent in all the age groups. This regional difference in collagen content and extent of collagen crosslinks is similar to most lumbar disc studies (Eyre 1988, Olczyk 1992, Scott et al 1994, Duance et al 1998). The higher extent of collagen crosslinks in the nucleus observed in this study was not surprising because of the abundance of Type II collagen fibers in the nucleus compared to the anulus (Eyre and Muir 1977). According to Eyre (1988), Type II collagen had the potential to form up to 3 moles of crosslinks per mole of collagen, while Type I collagen was able to form half the number of crosslinks as Type II collagen.
Pokharna and Phillips (1998) however found no significant difference in the extent of Pyd between the anulus and the nucleus in lumbar discs. Their non-significant result might be due to the inclusion of degenerated lumbar discs in their study (Pokharna and Phillips 1998). With disc degeneration, the proportion of Type II collagen fibers in the nucleus had been reported to decrease, and Type I fibers to increase, being the main collagen fibers formed in new granulation tissue (Nerlich et al 1997).

This study found that the collagen content and extent of Pyd was significantly lower with increased age, for all disc regions. The lower collagen content was consistent with the observations of Crean et al (1997), however Olczyk (1992) and Scott et al (1994) found an increase in collagen content with increasing age in lumbar discs instead. A plausible explanation for these conflicting reports could be the inclusion of degenerated discs in the samples of the latter two studies. Degeneration may stimulate and encourage new collagen synthesis in the disc matrix, which was part of the tissue repair and regeneration process (Crean et al 1997). This tissue repair process might explain the high collagen content in lumbar disc studies, as opposed to the low collagen content noted in the present group of normal disc samples. Although Olczyk (1992) reported an increase in collagen with age initially, she also showed a decline in the collagen content in nuclear and anular tissues, especially after the 4th decade of life. The lower collagen content seen in these normal aged discs, might also be due to an increase in other matrix constituents, such as keratan sulphate and other non-collagenous components (Urban and Maroudas 1980, Pearce et al 1991, Duance et al 1998), which were not measured in the present study.

The lower extent of Pyd with increasing age was consistent with that reported by Pokharna and Phillips (1998) and Duance et al (1998). However, these two studies did not separate the effects of degeneration and age on the discs. The results from the present study, although using a sample of convenience, selected only non-degenerate discs from a wide age range, suggesting that the natural ageing process might have a negative influence on the collagen content and the extent of Pyd in the disc matrix, hence resulting in a reduced tensile strength (Eyre and Wu 1995). The possible consequence of a low collagen content and extent of Pyd in a normal aged disc, was a disc matrix that was susceptible to injury under normal load bearing conditions (Pokharna and Phillips 1998, Cassinelli et al 2001). Whether degenerative processes would expedite or alter the normal age changes on the disc collagenous matrix was worthy of further investigation.

The thoracic spinal level trends for Pyd and Dpd had not been reported previously. Scott et al (1994) found a decreasing craniocaudal trend in the collagen content for thoracic discs. The
present study provides preliminary findings on the distribution of collagen, Pyd and Dpd in the different levels of the thoracic spine, which generally also showed decreasing craniocaudal trends. However the only significant findings were a lower collagen content and higher extent of Pyd in the mid thoracic region of the anterior anulus, and a lower extent of Dpd in the nucleus and anterior anulus of the lower thoracic region compared to the upper and mid thoracic regions.

The significantly lower collagen and higher extent of Pyd noted in the mid thoracic region of the anterior anulus is an interesting finding. The anterior thoracic region, especially with increasing age, was subjected to sustained compressive loading, due to the natural kyphotic posture (Schmorl and Junghanns 1971, White and Panjabi 1990b), which was associated with lower anterior disc heights in the mid thoracic region (Pooni et al 1986, Goh et al 1999). The greater loading experienced by the anterior anulus might be the reason for the different disc matrix composition compared with the posterior anulus. Similar stress reactions were seen in middle thoracic vertebral bodies as a function of the spinal configuration and habitual kyphosis (Goh et al 1999), and thoracic disc of scoliotic spine (Bushell et al 1979, Crean et al 1997). However, in order to test this hypothesis, an investigation correlating biomechanical stress with the disc collagenous matrix would be necessary.

Few studies have described gender differences in the collagen content or collagen crosslinks in spinal discs. In the present study, significantly higher collagen content and extent of Pyd was observed in male thoracic discs compared to females, in all disc regions. In the Old age group, this gender difference was reversed. Aged female disc samples tended to have a higher extent of Pyd, possibly due to a faster rate of collagen and crosslink degradation in males with increasing age. The accelerated collagenous changes in the aged male disc matrix might be associated with the higher exposure to biomechanical stresses from gender-related occupational and recreational activities (Miller et al 1988, Swärd et al 1991, Videman et al 1995b, 1997, Riihimäki et al 1998), which might also potentially increase wear and tear at a faster rate.

The low extent of Dpd compared to Pyd in the thoracic disc matrix seen in the present study was consistent with Eyre’s (1995) report. The present study found an average ratio of Pyd to Dpd of 50:1, comparable with the >50:1 ratio by Eyre (1995). Similar to Pyd, the extent of Dpd was highest in the nucleus and lowest in the anterior anulus of non-degenerate thoracic discs. With increasing age however, in contrast to collagen and Pyd, the extent of Dpd was significantly higher, but only in the nucleus (Figure 5.9). This finding differed from that reported by Takahashi et al (1995), who found no significant change in the extent of Dpd in
human bone, cartilage, ligament, tendon, meniscus and muscle, with increasing age. Although this age difference was small, the mean difference being 0.04 mol/mol collagen, however the physiological significance was not known, as this crosslink was present in very small amounts in the disc matrix (ranges from 0.02 to 0.08 mol/mol collagen). A possible reason for the higher extent of Dpd with age was that in the aged non-degenerate nuclei, the enzyme lysyl hydroxylase, which had been suggested as favouring the formation of Pyd, was not as active as in the younger nuclear matrix (Bailey et al 1998). In clinical syndromes resulting from a deficiency of lysyl hydroxylase, a higher extent of Dpd is found in the tissues (Eyre 1987). This differing age influence on Pyd and Dpd in thoracic discs was an unexpected finding and worthy of further investigation.

5.3.5 CONCLUSION

In conclusion, this study provides baseline data on the distribution of the collagenous matrix in non-degenerate thoracic discs in relation to disc region, thoracic spinal level, age and gender factors, and serves for future comparisons with degenerative thoracic discs. The collagen content was lower and the extent of Pyd and Dpd were significantly higher in the nucleus compared to the anulus, and similarly these collagenous variable were higher in the posterior anulus compared to the anterior. The mid thoracic region of the anterior anulus showed significant trends with a lower collagen content and higher extent of Pyd, however the lower thoracic region generally had the lowest collagen content and extents of Pyd and Dpd compared to the upper and mid thoracic regions. The collagen content and extent of Pyd were significantly lower with increasing age in the anulus and nucleus. Young male discs had a significantly higher extent of Pyd compared with females ($p < 0.001$). Age, gender and disc region differences were found to have a significant influence on the biochemical composition of the normal disc extracellular matrix.
The previous study had shown that in non-degenerate thoracic discs, the collagen content and extent of Pyd decreased significantly with increased age. However changes in aged and degenerate thoracic discs had not been investigated in the literature. Studies had reported a higher collagen content (Olczyk 1992) and decreased concentrations of Pyd in aged and degenerated human lumbar discs, however this change was not statistically significant (Duance et al 1998, Pokharna and Phillips 1998). Macroscopic degenerative changes in spinal discs might be confounded by a number of factors, such as age, mechanical loading history and gender, all of which might have an impact on the biochemical constitution in the disc matrix (Buckwalter 1995, Crean et al 1997, Duance et al 1998, Hutton et al 1998). This study sought to determine the collagen content and the extent of Pyd and Dpd in a sample of normal and degenerated human thoracic intervertebral discs, and to investigate the influence of age, gender and spinal level on the degenerated disc matrix.

5.4.1 MATERIALS AND METHODS

5.4.1.1 Tissue Collection.

The same 26 formalin-fixed human thoracic spines sampled in Study 5.3 were used to investigate the influence of gender, age, spinal level and degeneration status on thoracic disc biochemical matrix in this study. In total 303 thoracic discs from 13 males and 13 females, with an age range of 1 to 90 years, mean age of 45.8 years (SD ± 28.2 years), were graded and removed for biochemical analysis (Table 5.10). Nine discs were not available for testing, as one spine had a fused disc at T7-T8, and another spine had only four thoracic levels (T1-T2 to T4-T5) available for examination. The disc samples were grouped according to age groups: Child (1 to 15 years), Young (15 to 35 years old), Mid (36 to 60 years old) and Old (>60 years old).
Table 5.10 Information on 26 cadaver thoracic spines showing number (no) of disc samples graded I to III, according to age, gender and disc regions.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Age Range</th>
<th>Mean Age years (SD)</th>
<th>No of female spines</th>
<th>No of male spines</th>
<th>Total no of discs samples</th>
<th>No of grade I AF/NP</th>
<th>No of grade II AF/NP</th>
<th>No of grade III AF/NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Child</td>
<td>1-15</td>
<td>4 (3.2)</td>
<td>2</td>
<td>2</td>
<td>40</td>
<td>40/40</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Young</td>
<td>16-35</td>
<td>25 (5.5)</td>
<td>2</td>
<td>3</td>
<td>72</td>
<td>54/60</td>
<td>18/11</td>
<td>0/1</td>
</tr>
<tr>
<td>Mid</td>
<td>36-60</td>
<td>44 (5.1)</td>
<td>4</td>
<td>3</td>
<td>84</td>
<td>80/70</td>
<td>4/13</td>
<td>0/1</td>
</tr>
<tr>
<td>Old</td>
<td>&gt;60</td>
<td>78 (8.0)</td>
<td>5</td>
<td>5</td>
<td>107</td>
<td>55/51</td>
<td>35/39</td>
<td>17/17</td>
</tr>
<tr>
<td>Total</td>
<td>1-90</td>
<td>45.8 (28.2)</td>
<td>13</td>
<td>13</td>
<td>303</td>
<td>229/221</td>
<td>57/63</td>
<td>17/19</td>
</tr>
</tbody>
</table>

AF = anulus fibrosus and NP = nucleus pulposus
5.4.1.2 Disc grading and biochemical analysis
The mid-sagittal section of the 26 hemisected thoracic spines were examined macroscopically and graded, as described in Study 5.3 (section 5.3.1.2). After grading, one mm$^3$ disc tissues were selectively sampled from the nucleus, anterior and posterior anulus in the mid-sagittal plane, from each thoracic level (T1 to T12). The tissue preparation and extraction procedure for HPLC (II) biochemical analysis of collagen content and extent of Pyd and Dpd was similar to that described in Study 5.2 (section 5.2.3.1).

5.4.2 DATA REDUCTION AND STATISTICAL ANALYSIS
Paired $t$-tests were used to examine for disc regional differences in the collagen content and extent of Pyd and Dpd. ANOVA and unpaired $t$ tests were applied to examine for differences in the biochemical variables due to age, spinal level, gender and degeneration status. Analysis of covariance (ANCOVA) using age groups as a covariate, was then applied to determine any significant differences due to degeneration status per se. All statistical tests were performed using the SPSS (v 6.1, SPSS Inc, Illinois, USA) and Statview (v 4.1, Abacus Concept Inc, USA) statistical software packages. A probability level of $p < 0.05$ was accepted as representing a meaningful difference in all tests of statistical significance. Where multiple $t$-tests were performed, Bonferroni correction was applied, therefore for degeneration status and thoracic disc regions, $p < 0.0167$ was accepted as statistically significant. For the purposes of statistical analysis, each disc was treated as a separate case. Raw data is recorded in Appendix E.

5.4.3 RESULTS
5.4.3.1 Macroscopic examination
Approximately 25 percent of the 303 disc samples (anular $n = 74$ and nuclear $n = 82$) had some degree of degeneration (grade II or III). Signs of disc degeneration were noted, as early as 18 years old, in both the anulus and the nucleus (Figure 5.11). Grade III anuli were seen only in the Old age group, whereas severely degenerated nuclei were seen as early as the Young age group. Approximately 50% of the 107 disc samples from the Old age group (anular = 49% and nuclear = 52%) had some degree of disc degeneration (that is, grades II or III, Table 5.10).
5.4 Collagen crosslinks in degenerate thoracic discs

5.4.3.2 Spinal level and disc regional differences

Grade I and II nuclear samples had significantly lower collagen content but significantly higher extent of Pyd and Dpd compared to grade I and II anterior anular samples ($p < 0.001$, Figure 5.12). In contrast, there were no significant differences in any of the biochemical variables for grade III or severely degenerated anular or nuclear samples. There was also no significant difference in the collagen content between the anterior and posterior anuli for all degeneration grades. However, the posterior anulus had significantly higher extent of Pyd and Dpd compared to anterior anulus for grade I and II samples ($p < 0.001$).

The decreasing craniocaudal spinal level trends for the non-degenerate samples were similar to results in Study 5.3, where the lower thoracic region had lower collagen content and extent of Pyd an Dpd compared to the mid and upper thoracic regions (Figure 5.13). In the degenerate samples, only the extent of Pyd and Dpd had significant decreasing craniocaudal trends in the anterior anulus ($p < 0.05$), which was significantly lower in the lower thoracic region compared to the upper and mid thoracic regions ($p < 0.0167$).

**Figure 5.11** The percentage of disc samples in each age group, with grade I, II and III samples in the anulus and the nucleus. In the anulus, grade II samples were observed in the Young age group and grade III samples, only in the Old age group. In contrast, grade II and III samples in the nucleus were found in the Young age group.
Figure 5.12 The collagen content [A] and extent of Pyd [B] and Dpd [C] in the nucleus, anterior and posterior anulus, with grade I to III degenerative changes. No significant differences in the collagen content and extent of Pyd and Dpd were found between grade III anular and nuclear disc samples [A, B, C]. However all grade I and II nuclear samples had significantly lower collagen content but significantly higher extent of Pyd and Dpd compared to grade I and II anular samples ($p < 0.0167$). ANCOVA tests, with age as the covariate factor, revealed only significantly higher collagen content in grade III nuclear samples compared to grade I ($p < 0.001$), and a higher extent of Dpd in grade III anular samples compared to grade I ($p < 0.001$) [A, C]. Error bars represent one standard deviation. $* = \text{ANCOVA tests, with age as covariate, where } p < 0.001$. 

Chapter 5  
5.4 Collagen crosslinks in degenerate thoracic discs
Figure 5.13 The collagen content [A] and extent of Pyd [B] and Dpd [C] in the nucleus, anterior and posterior anulus, in degenerate and non-degenerate disc samples in the Upper (U), Mid (M) and Lower (L) thoracic regions. In the degenerate (grade II and III) samples, only the extent of Pyd and Dpd in the anterior anulus had significant decreasing craniocaudal trends ($p < 0.05$). Error bars represent one standard deviation.

* = ANOVA tests comparing the means in each thoracic level, where $p < 0.05$.

** = $t$-test comparing the means between thoracic regions, where $p < 0.0167$.

This trend is similar to that seen in non-degenerate samples. The degeneration trends for the nucleus and posterior anulus had an increase in collagen and extent of Dpd instead, while the extent of Pyd continued to show a decreasing trend. These changes in the degenerated nucleus and posterior anulus were however not statistically significant.
5.4.3.3 Age versus degeneration influences
The age trends were similar with results for normal disc samples, with lower collagen content and extent of Pyd and higher extent of Dpd in all disc regions. However with increasing degeneration status, particularly in the Old age group, the collagen content was significantly higher in the nucleus instead. This was statistically significant, even when taking the age factor as covariate \((p < 0.001, \text{Figure 5.14})\). Using ANCOVA tests, the extent of Dpd was also significantly higher with increasing degeneration grade but only in the anulus \((p < 0.001)\).

No consistent degeneration trends for the biochemical variables were noted for all age groups in all disc regions (Figure 5.14). This may be due to the small number of grade III anular and nuclear samples, especially for the anulus, where grade III samples were observed only in the Old Age group. Taking into consideration only the disc samples in the Old age group, the changes in the collagenous matrix of the aged anulus and nucleus did show some interesting degeneration trends in Figure 5.14. The collagen content in the nucleus was higher with increased degeneration, and this trend was statistically significant \((p < 0.001)\). The only significant changes in the anulus was a higher extent of Dpd in both the anterior and posterior anuli \((p < 0.001)\). Even though the extent of Pyd was lower with degeneration, this trend was not statistically significant.

The ratio of Pyd to Dpd was also evaluated to determine the influence of Dpd in the disc matrix (Figure 5.15). The ratio of Pyd/Dpd was significantly lower for the nucleus and posterior anulus with increasing age \((p < 0.001)\) and degeneration status \((p < 0.01)\), which indicates an increase in the extent of Dpd in the matrix.

5.4.3.4 Degeneration and gender interactions
When changes in the biochemical variables in the discs were separated into gender groups (Figure 5.16), the trends in all biochemical variables, due to increasing degeneration grade, were different for both male and female samples. The collagen content was higher in degenerated female anular samples compared to males \((p < 0.05)\). In contrast, the collagen content was higher in degenerated male nuclear samples compared to females \((p < 0.05)\). The extent of Pyd and Dpd were not significantly different between degenerated male and female anular samples, except in the nucleus, where the extent of Pyd increased with degeneration in female samples.
Figure 5.14 The collagen content [A] and extent of Pyd [B] and Dpd [C] in the nucleus, anterior and posterior anulus, with grade I to III degeneration grades in the Child, Young, Mid and Old age groups. The collagen content and extent of Pyd were significantly lower ($p < 0.001$) and the extent of Dpd was significantly higher ($p < 0.001$) with increasing age, for all disc regions [A,B,C]. Error bars represent one standard deviation.
Figure 5.15 The ratio of Pyd to Dpd in the anterior and posterior anulus, and nucleus showing the change due to age groups [A] and degeneration status [B]. The change in Pyd/Dpd was statistically significant with increased age ($p < 0.001$) in the nucleus and posterior anulus [A], and with increasing degeneration status ($p < 0.01$) in all disc regions [B]. Error bars represent one standard deviation from the mean.

* = ANOVA tests comparing the means, where $p < 0.05$.

Generally, the collagen, Pyd and Dpd degeneration trends for males were similar to that observed in Figure 5.12, that is, a lower collagen content, extent of Pyd and a higher extent of Dpd. The degeneration trends for female samples were however different from male samples especially in the nucleus, where there was a lower collagen content, but a higher extent of Pyd and Dpd instead (Figure 5.16). These changes were however not statistically significant.
Figure 5.16 The collagen content [A] and extent of Pyd [B] and Dpd [C] in the nucleus, anterior and posterior anulus, with grade I to III degeneration grades in male and female groups. Using ANCOVA tests, with age as a covariate, significant trends were noted in all disc regions in males with increasing degeneration grade ($p < 0.05$), except for the extent of Dpd in the nucleus [A,B,C]. For females, only the trends in collagen content and extent of Dpd in the anulus were statistically significant ($p < 0.05$). Degenerate male nuclear samples had significantly higher collagen content compared to degenerate female samples ($p < 0.05$) and vice versa for the anulus. Error bars represent one standard deviation.

* = ANCOVA tests, taking age as a covariate, where $p < 0.05$.

** = $t$-test comparing means between degenerate (II and III) male and females samples, where $p < 0.05$. 

**THORACIC DISC REGIONS**
5.4.4 DISCUSSION

5.4.4.1 Disc regional influences

Significant regional differences in the biochemical matrix were noted between the anulus and nucleus of normal and moderately degenerated samples. However with severe degeneration, no disc regional differences in the matrix were observed. The finding that the nuclei of normal and moderately degenerated discs had lower collagen content and higher extents of Pyd and Dpd compared to the anulus, was consistent with that reported by Eyre (1995), and Duance et al (1998). However, this trend contradicted the findings of Pokharna and Phillips (1998), whose anuli and nuclei data were not significantly different. Their data were however consistent with the present findings for severely or grade III degenerated thoracic anuli and nuclei. The significance of these disc regional findings provides evidence that the collagen content and extent of collagen crosslinks varies in different stages of disc degeneration. Therefore data from a variety of normal and moderately degenerated anuli and nuclei, should not be pooled for statistical analysis, instead should be analysed separately.

Studies on lumbar discs had reported that the boundary between the anulus and nucleus in severely degenerated discs eventually appeared indistinct morphologically (Thompson et al 1990, Nerlich et al 1997). In the present study, similar macroscopic changes were seen in severely degenerated thoracic discs. This loss of distinction between the anulus and nucleus in grade III discs was also reflected in the biochemistry of the disc matrix. This finding was not surprising, as the nucleus had been reported to change its biochemical composition with age (Adams et al 1977) and degenerate at a faster rate than the anulus (Buckwalter 1995, Nerlich et al 1997). As the nuclear matrix aged, it became more fibrocartilaginous, with an increase in collagen fibers, hence its degenerated matrix closely resembled that of the anular matrix (Hutton et al 1998).

5.4.4.2 Age influences

Previous studies had reported degeneration changes in lumbar spinal discs as early as the second decade of life, and that these changes usually first observed in the nucleus (Buckwalter 1995, Nerlich et al 1997). The present study found similar grade III macroscopic degeneration changes in the nucleus of thoracic discs, from as early as 18 years of age. In the anulus however, severe degeneration changes were only noted in the Old age group (>60 years old). Reasons for the early nuclear degeneration could be due to several factors. The nucleus might be susceptible to nutritional deficiency and oxidative stress, being in the centre of the disc and dependent on diffusion of nutrient from the end plates (Urban and Maroudas 1980, Nerlich et al 1997, Horner and Urban 2001). Inadequate nutrition reduces the number
of viable cells, hence decreased matrix synthesis (Horner and Urban 2001). Sustained or increased compression forces on the disc during spinal loading might add to the nutrient deprivation in the nucleus, hence inhibiting cellular synthesis, and increasing the susceptibility to degeneration (Oshima et al 1993, Hutton et al 1998). The rapid loss of water and proteoglycan in the nucleus was also associated with ageing, especially for the nucleus (Eyre et al 1989, Pearce et al 1991, Buckwalter 1995). The propensity for early degeneration in the nucleus may be due to these three factors or a combination of the factors.

Results from this study and those in Study 5.3 showed that the normal aged disc samples had lower collagen content and extent of Pyd. This finding suggests an imbalance in the turnover of these collagenous constituents in the disc extracellular matrix favouring a net loss of collagen and Pyd over the life span. Therefore the ageing disc matrix would have an impaired matrix tensile strength, making the disc more susceptible to injury and degeneration (Pearce et al 1987, Pearce et al 1991, Buckwalter 1995, Duance et al 1998, Hutton et al 1998). When aged and degenerated samples were analysed, the combination of these two factors showed a higher collagen content in both the anular and nuclear samples, although only statistically significant in the nucleus (Figure 5.14). The extent of Dpd was also significantly higher with degeneration, suggesting no contrasting or compounding effect between age and degeneration.

The extent of Pyd was generally lower with degeneration and age, however was only statistically significantly related to age changes. Duance et al (1998) and Pokharna and Phillips (1998) also found decreasing trends for the extent of Pyd in degenerate lumbar discs, which were not statistically significant. A possible reason for the non-significant findings for the changes in Pyd might be due the small number of degenerated anular and nuclear samples analysed (76 of 303 samples for the present study). Grade III anular samples were only found in the Old Age group, making it difficult to determine degeneration effects without the influence of age. Another compounding factor was that mature crosslinks usually increase with age up to 25 years (Herbert et al 1975, Kivirikko and Myllyla 1982, Eyre et al 1988), therefore mature crosslinks such as Pyd, would be expected to decrease only after the Mid age group in this study.

5.4.4.3 Degeneration influences
The finding that the collagen content was higher with degeneration, especially in the nucleus, was not new, and was consistent with most studies for lumbar discs (Pearce et al 1987, Pearce et al 1991, Olczyk 1992). The high collagen content seen in degenerate nuclear samples in the
Young and Old age groups, in contrast to a decreasing trend with increasing age in normal samples, might be due to the increased formation of granulation tissues due to inflammatory responses to injury (Pearce et al 1987, Crean et al 1997, Hutton et al 1998). The change in the collagenous matrix might also be influenced by a change in the other disc biochemical components, such as water, proteoglycans and non-collagenous proteins (Urban and Maroudas 1980, Pearce et al 1987), which were not measured in this study.

It was interesting to note that the high collagen content in grade III nuclear samples, especially in the Young and Old age groups, was not accompanied by a high extent of Pyd. In fact the extent of Pyd was lower in grade III nuclear samples in these 2 groups. This provided support for the assumption that the crosslinking ability of newly formed collagen fibers in the degenerated nucleus might not be the same as that of the existing or normal collagen fibers (Pokharna and Phillips 1998). According to Eyre (1988), the collagen in aged discs had less capability to form crosslinks, hence the increased collagen content did not necessary translate to an increase in extent of Pyd. However there was a higher extent of Dpd.

The non-degenerate nucleus usually had more than 85% of Type II collagen, which has double the crosslinking potential of Type I collagen (Eyre 1988). From this study the extent of Pyd decreased from a mean of 2 mol/mol collagen in the normal nucleus to 1.7 mol/mol collagen in the severely degenerate nucleus. It was possible that the new collagen formed in grade III nuclear tissues might consist primarily of Type I collagen, common in granulation tissue (Ng et al 1986), which had a reduced cross linking ability compared to collagen Type II fibers, to form Pyd (Eyre et al 1984b).

The inconsistent degeneration trends noted in grade II and III anular and nuclear samples in each of the age groups (Figure 5.14), might be a reflection of the dynamic state of the tissues as it turned-over due to the processes of injury, inflammation and repair (Kang et al 1996, Gruber and Hanley 1998). Disc tissues may be exposed to repeated episodes of high compression and torsional stresses, hence injury may occur in the matrix. The frequent injury and repair processes may result in a matrix that is continually turning-over due to increased matrix synthesis and tissue remodelling (Pearce et al 1987, Crean et al 1997, Hutton et al 1998), hence the variation in the collagenous disc matrix in different degeneration state. The present cross-sectional study provided a static view of the status of the matrix, therefore it was limited in tracing the biochemical changes due to degeneration and ageing. In addition, there was often a time-lag between morphological degenerative and biochemical matrix changes (Olsewski et al 1996, Hutton et al 1998), which was dependent on the matrix synthesis rate and the time lapse after injury (Bayliss et al 1988). The disc matrix even in
aged discs, was therefore able to change its biochemical content, inspite of its avascularity (Walmsley 1953, Urban et al 1977).

5.4.4.4 Spinal region influences
Degenerated thoracic discs did not have significantly different collagenous disc matrix in the different thoracic levels, except in the anterior anulus of the lower thoracic region (Figure 5.13). Generally, degenerated anterior anular samples in the lower thoracic region had a higher collagen content but lower extent of Pyd and Dpd. In the non-degenerate thoracic discs, the lower thoracic region also had a lower extent of Pyd and Dpd, but a lower collagen content. The main difference between the degenerate and non-degenerate thoracic discs in the anterior anulus of the lower thoracic region is the higher collagen content. The biochemical changes in the other disc regions were similar to degeneration trends, with a higher collagen content and extent of Dpd and a lower extent of Pyd. The lower thoracic region is also the site for a higher prevalence of thoracic disc degenerative changes (Singer 2000), which may be associated with a higher collagen content and extent of Dpd.

5.4.4.5 Gender influences
The degeneration trends in the distribution of collagen and the extent of collagen crosslink were different between male and female samples. Taking age as a covariate, the biochemical matrix in males tended to change significantly with increasing degeneration compared to females, in particular, with a higher collagen content in the nucleus. The higher collagen content in the degenerated matrix of males might be due to the increased granulation tissue in the disc matrix as a result of inflammatory processes leading to disc degeneration (Crean et al 1997, Hutton et al 1998). Males also had a higher propensity for disc degeneration compared to females, due possibly to an increased exposure to spinal stresses from gender-biased occupation and recreational activities (Swärd et al 1991, Videman et al 1995b, 1997). There is currently very limited gender data in the literature with which to compare, hence these findings provide original information for future comparisons.

5.4.5 CONCLUSION
The nucleus was found to have a predominance of severe macroscopic degeneration changes at an earlier age interval compared with the anulus. In severely degenerated thoracic discs, the loss of distinction in the morphological boundary between the anulus and nucleus seen macroscopically was also accompanied by non-significant differences in the collagen content.
and extent of Pyd and Dpd in the anular and nuclear matrices. The collagen content and extent of Pyd decreased significantly, and the extent of Dpd was significantly higher with increased age in both the anulus and the nucleus. However, after accounting for age effects, increasing degeneration, was only significantly associated with a higher collagen content in the nucleus, and a higher extent of Dpd in the anulus. The extent of Pyd increased significantly with moderate degeneration, however it was not significantly different between severely degenerated and normal discs.

Significant decreasing craniocaudal trends were noted in degenerate anterior anular tissues for the extent of Pyd and Dpd. Degenerate male nuclear samples had significantly higher collagen content compared to degenerate female nuclear samples. The thoracic disc matrix was observed to have significantly different collagenous content, which varied with different degeneration status, thoracic level, disc region, age and gender. Therefore the biochemical analysis of spinal disc matrices should take all these factors into consideration, as they may affect the results.
ELASTIC fibers are commonly reported in the matrices of ligament connective tissues, for example ligamentum flavum, ligamentum nuchae and articular ligaments, as well as in vascular connective tissues and lung tissues (Thomas et al 1963, Yong-Hing et al 1976, Starcher 1977). More recently, studies have also reported the presence of elastic fibers in spinal discs (Buckwalter et al 1976, Hickey and Hukins 1981, Johnson et al 1982, Johnson et al 1985, Mikawa et al 1986, Yu et al 2002).

Elastic fibers contain a number of amino acid groups which are involved in the formation of elastin crosslinkages, two of which are Des and Isodes (Partridge et al 1963, Thomas et al 1963). Although Des and Isodes crosslinks comprise only 0.5 to 2% of the total amino acid content of human elastin, however these crosslinking amino acid groups are found uniquely in elastin proteins (Starcher and Galione 1976, Rosenbloom 1987). It was hypothesised that elastin and collagen crosslinks had a complementary function in the disc matrix, to resist tension and to enable the annular lamellae to return to resting position after mechanical deformation (Buckwalter et al 1976, Ghosh et al 1977, Hickey and Hukins 1981). Few studies had used biochemical analyses to provide quantitative data on the collagen and elastin content in human intervertebral discs (Mikawa et al 1986, Olczyk 1994b). Olczyk (1994b) reported that there was more elastin in lumbar anulus compared to the nucleus and that the elastin content decreased with increasing age. Currently there are no data reported in the literature on the elastin (Des and Isodes) crosslink content in human spinal disc tissues.

The objective of the present study was to determine the collagen and elastin content in human intervertebral discs and LF, in terms of the Des and Isodes crosslinks content, the extent of collagen crosslinks; Pyd and Dpd, using reversed phase HPLC analyses. Variations of these biochemical components in the disc and LF due to age, gender and spinal regional influences were also investigated.

5.5.1 MATERIAL AND METHOD

5.5.1.1 Tissue collection
A total of 26 cadaver spines (24 formalin-fixed and 2 fresh) were used for harvesting the LF and intervertebral disc tissue samples (Table 5.11). The LF (n = 364) from all spinal levels, cervical to lumbar, was removed from 18 formalin-fixed and 2 fresh spines, after routine post-
mortem procedures. The mean age of the spines was 56 ± 25 years, with 13 males and 7 females. Spinal intervertebral discs (n = 77) were also removed from various spinal regions from 7 formalin-fixed and 2 fresh cadavers, after routine post mortem procedures. The mean age of the spines was 66 ± 25 years, with 5 males and 4 females. Spines with disc pathology from fractures, scoliosis, previous surgery or tumour were excluded from the study.

5.5.1.2 Harvesting LF and Intervertebral Discs
The spinous process and laminae were detached from the vertebral bodies by sectioning through the pedicles. The LF was excised between adjacent laminae from all cervical, thoracic and lumbar levels. Where possible the ligament was removed intact, unless calcified. Care was taken to avoid ligamentous tissues with calcification [Figure 5.17 (A)]. Each harvested ligamentum sample was approximately 1.0 to 2.0 mm thick. Table 5.9 shows the total number of LF and intervertebral discs harvested. In one spine the LF was completely calcified at thoracic levels T6, T7, T8 and T10, therefore these were not used for biochemical analysis. In another spine L5 had fused with S1, therefore the ligament was unavailable for harvesting. In total, ligament flava tissues were harvested from the cervical to the lumbar regions from only 10 spines. For the remaining 10 spines, either the cervical or both the cervical and lumbar spines were removed for neurological examination of the spinal cord and not available for this study.

Mid plane sections of intervertebral discs were removed axially from the cervical (n = 6), thoracic (n = 57) and lumbar (n = 14) regions [Figures. 5.17 (B) and (C)]. Each slice was approximately 1.5 to 2 mm thick, was then separated into anterior and posterior anulus, and nucleus, from which 3 to 4 mm³ pieces were prepared for biochemical analysis. In total there were 150 anular and 77 nuclear tissues analysed. Only four samples of anular tissue (three anterior and one posterior) were not analysed due to advanced calcifications or contamination during tissue preparation.
Table 5.11 The number of discs and ligamentum flava (LF) removed from fresh and formalin-fixed cadaver spines \((n = 26)\), with age, gender, age groups, spinal levels and the cause of death.

<table>
<thead>
<tr>
<th>Code</th>
<th>Age</th>
<th>Gender</th>
<th>Age group</th>
<th>Spine level</th>
<th>No of LF</th>
<th>Spine level</th>
<th>No of discs</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>1</td>
<td>F</td>
<td>Young</td>
<td>Nil</td>
<td>Nil</td>
<td>C2 - C4, L1 - L5</td>
<td>8</td>
<td>Cause of death unknown, no history of trauma</td>
</tr>
<tr>
<td>A2</td>
<td>7</td>
<td>M</td>
<td>Young</td>
<td>C2 - L5</td>
<td>23</td>
<td>Nil</td>
<td>Nil</td>
<td>Intracranial haemorrhage</td>
</tr>
<tr>
<td>A3</td>
<td>18</td>
<td>M</td>
<td>Young</td>
<td>C2 - L5</td>
<td>23</td>
<td>Nil</td>
<td>Nil</td>
<td>Spinal cord with autolysis</td>
</tr>
<tr>
<td>A4</td>
<td>29</td>
<td>M</td>
<td>Young</td>
<td>C2 - L5</td>
<td>23</td>
<td>Nil</td>
<td>Nil</td>
<td>Cerebral trauma</td>
</tr>
<tr>
<td>A5</td>
<td>32</td>
<td>F</td>
<td>Young</td>
<td>C2 - L5</td>
<td>23</td>
<td>Nil</td>
<td>Nil</td>
<td>Head injuries</td>
</tr>
<tr>
<td>A6</td>
<td>33</td>
<td>M</td>
<td>Young</td>
<td>T1 - T12</td>
<td>12</td>
<td>Nil</td>
<td>Nil</td>
<td>Drug overdose</td>
</tr>
<tr>
<td>A7</td>
<td>37</td>
<td>M</td>
<td>Mid</td>
<td>C2 - L5</td>
<td>23</td>
<td>Nil</td>
<td>Nil</td>
<td>Hypoxic encephalopathy</td>
</tr>
<tr>
<td>A8</td>
<td>40</td>
<td>F</td>
<td>Mid</td>
<td>C2 - T5, T9, T11 - L4</td>
<td>18</td>
<td>Nil</td>
<td>Nil</td>
<td>Cerebral swelling &amp; pulmonary congestion</td>
</tr>
<tr>
<td>A9</td>
<td>42</td>
<td>F</td>
<td>Mid</td>
<td>C2 - L5</td>
<td>23</td>
<td>Nil</td>
<td>Nil</td>
<td>Head injury</td>
</tr>
<tr>
<td>A10</td>
<td>48</td>
<td>M</td>
<td>Mid</td>
<td>C2 - L5</td>
<td>23</td>
<td>Nil</td>
<td>Nil</td>
<td>Cerebral swelling</td>
</tr>
<tr>
<td>A11</td>
<td>52</td>
<td>M</td>
<td>Mid</td>
<td>T1 - L5</td>
<td>17</td>
<td>Nil</td>
<td>Nil</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>A12</td>
<td>63</td>
<td>F</td>
<td>Old</td>
<td>T1 - T12</td>
<td>12</td>
<td>Nil</td>
<td>Nil</td>
<td>Pulmonary congestion</td>
</tr>
<tr>
<td>A13</td>
<td>67</td>
<td>F</td>
<td>Old</td>
<td>Nil</td>
<td>Nil</td>
<td>L1 - L4</td>
<td>4</td>
<td>Lung Cancer</td>
</tr>
<tr>
<td>A14</td>
<td>71</td>
<td>M</td>
<td>Old</td>
<td>Nil</td>
<td>Nil</td>
<td>T10 - T12</td>
<td>3</td>
<td>Cause of death unknown</td>
</tr>
<tr>
<td>A15*</td>
<td>72</td>
<td>M</td>
<td>Old</td>
<td>T1 - L4</td>
<td>16</td>
<td>L1 - L4</td>
<td>4</td>
<td>Pneumonia</td>
</tr>
<tr>
<td>A16</td>
<td>72</td>
<td>M</td>
<td>Old</td>
<td>Nil</td>
<td>Nil</td>
<td>C5 - C7, T1,</td>
<td>12</td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>A17</td>
<td>73</td>
<td>M</td>
<td>Old</td>
<td>Nil</td>
<td>Nil</td>
<td>T1 - T9</td>
<td>9</td>
<td>Cause of death unknown</td>
</tr>
<tr>
<td>A18*</td>
<td>73</td>
<td>F</td>
<td>Old</td>
<td>T1 - T12</td>
<td>12</td>
<td>T1 - T12</td>
<td>12</td>
<td>Motor Neuron Disease</td>
</tr>
<tr>
<td>A19</td>
<td>75</td>
<td>M</td>
<td>Old</td>
<td>C2 - L5</td>
<td>23</td>
<td>Nil</td>
<td>Nil</td>
<td>Myocardial infarct</td>
</tr>
<tr>
<td>A20</td>
<td>77</td>
<td>M</td>
<td>Old</td>
<td>C2 - L5</td>
<td>23</td>
<td>Nil</td>
<td>Nil</td>
<td>Pituitary tumour</td>
</tr>
<tr>
<td>A21</td>
<td>77</td>
<td>F</td>
<td>Old</td>
<td>T1 - T12</td>
<td>12</td>
<td>Nil</td>
<td>Nil</td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>A22</td>
<td>79</td>
<td>F</td>
<td>Old</td>
<td>T1 - T12</td>
<td>12</td>
<td>Nil</td>
<td>Nil</td>
<td>Bronchopneumonia,</td>
</tr>
<tr>
<td>A23</td>
<td>83</td>
<td>F</td>
<td>Old</td>
<td>T1 - T12</td>
<td>12</td>
<td>T1 - T12</td>
<td>12</td>
<td>Cause of death unknown</td>
</tr>
<tr>
<td>A24*</td>
<td>85</td>
<td>M</td>
<td>Old</td>
<td>T2 - T12</td>
<td>11</td>
<td>T1 - T12, L4</td>
<td>13</td>
<td>Aspiration Pneumonia</td>
</tr>
<tr>
<td>A25</td>
<td>90</td>
<td>M</td>
<td>Old</td>
<td>T1 - T12</td>
<td>12</td>
<td>Nil</td>
<td>Nil</td>
<td>Myocardial infarct</td>
</tr>
<tr>
<td>A26</td>
<td>90</td>
<td>M</td>
<td>Old</td>
<td>C2 - L5</td>
<td>23</td>
<td>Nil</td>
<td>Nil</td>
<td>Myocardial infarct</td>
</tr>
</tbody>
</table>

| 57.15 | 16 M | Total | 364 | Total | 77 |

* fresh spines  **IVD samples that yielded results  C = Cervical  T = Thoracic  L = Lumbar
Figure 5.17 Photomicrograph of a 100 μm horizontal histological section at T11-12, to highlight ossification within the LF [curved arrow] (A). The ligament was bounded by attachments to the superior articular process [SAP] and laminae [L] as it helped form the dorsal wall of the vertebral canal between the paired zygapophyseal joints laterally and adjacent laminae. Mammillary process [MP]. This ligament was excised en bloc for biochemistry assays (avoiding areas of ossification). Disc samples were taken axially from the mid-plane [arrow] (B), and small samples excised from both the annular and nuclear regions (C). Black arrows depict the location of ossicles between T10-T11, which project into the LF (B).
5.5.1.3 Collagen and Elastin biochemical analysis
Disc and ligament tissues were weighed, dried and hydrolysed as described in Study 5.1. The cooled sample hydrolysate was analysed for Pyd, Dpd, Des and Isodes, using the HPLC (I) procedure described in Study 5.1. The collagen content was measured in terms of the hydroxyproline content using the method described in Study 5.1.

5.5.2 DATA REDUCTION AND STATISTICAL ANALYSIS
Data for the LF samples were divided into 3 age groups for statistical analyses. The age range for each age cohort were "Young" < 35 years, "Mid" = 36 to 60 years and "Old" > 61 years. ANOVA was used to determine changes due to increasing age and spinal levels. The relationship between age and the biochemical parameters were also examined using regression analysis. Unpaired t-tests were used to determine gender differences. All statistical tests were performed using Statview 4.1 statistical software package (Abacus Concept Inc., USA). A probability level of $p < 0.05$ was adopted for all statistical analyses in determining meaningful differences. Where multiple t-tests were performed, Bonferroni correction was applied, therefore for age group evaluations, $p < 0.0167$ was accepted as statistically significant. For the purposes of statistical analysis, each disc and ligament was treated as a separate case. Raw data is recorded in Appendix F.

5.5.3 RESULTS
The elastin content for the LF and disc was derived from the sum of the Des and Isodes crosslink content in the tissues. The collagen content was calculated from the hydroxyproline content and the extent of Pyd and Dpd was calculated number of mole of crosslinks per mole of collagen.

5.5.3.1 Intervertebral discs
Isodesmosine and Des crosslinks were only detected in lumbar anular tissues ($n = 12$, wet weight range = 423 to 725 mg, mean 572 mg) and lumbar nuclear tissues ($n = 4$, wet weight range = 515 to 704 mg, mean 631 mg) from the 72-year old male spine (Table 5.12). The minimum content of Isodes and Des detected in the disc tissues were 0.02 nmol/mg dry wt. There were no elastin crosslinks detected in the remaining cervical, thoracic and lumbar anular and nuclear tissues ($n = 211$, wet weight range = 9 to 854 mg, mean 171 mg). The 10 lumbar disc tissues that did not yield elastin results were samples with a lesser wet weight, that is less than 500 mg (range = 10 to 129 mg, mean = 51 mg). All 364 LF tissues tested
yielded results for collagen and elastin crosslinks (Table 5.12). The mean wet weight of LF tissues was 109.1 mg (range 11 to 423 mg).

**Table 5.12** The mean and standard deviation of the collagen content, extent of Pyd and Dpd, Des and Isodes crosslink content in anulus \( (n = 12) \), nucleus \( (n = 4) \) and LF \( (n = 22) \).

<table>
<thead>
<tr>
<th></th>
<th>Anulus</th>
<th>Nucleus</th>
<th>Ligamentum Flavum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet weight (mg)</td>
<td>572 [119]</td>
<td>631 [86]</td>
<td>109 [45]</td>
</tr>
<tr>
<td>Dry weight (mg)</td>
<td>189 [50]</td>
<td>205 [21]</td>
<td>41 [18]</td>
</tr>
<tr>
<td>Collagen (nmol/mg dry wt)</td>
<td>1.07 [0.23]</td>
<td>0.56 [0.08]</td>
<td>1.13 [0.25]</td>
</tr>
<tr>
<td>Pyd (mol/mol collagen)</td>
<td>1.14 [0.14]</td>
<td>1.58 [0.24]</td>
<td>0.71 [0.15]</td>
</tr>
<tr>
<td>Dpd (mol/mol collagen)</td>
<td>0.18 [0.0035]</td>
<td>0.035 [0.0062]</td>
<td>0.14 [0.034]</td>
</tr>
<tr>
<td>Pyd/Dpd</td>
<td>65.6 [10.5]</td>
<td>45.0 [4.8]</td>
<td>5.5 [1.3]</td>
</tr>
<tr>
<td>Isodes (nmol/mg dry wt)</td>
<td>0.021 [0.0033]</td>
<td>0.025 [0.0049]</td>
<td>8.4 [2.5]</td>
</tr>
<tr>
<td>Des (nmol/mg dry wt)</td>
<td>0.019 [0.0034]</td>
<td>0.017 [0.0040]</td>
<td>11.5 [3.6]</td>
</tr>
</tbody>
</table>

A comparison of the biochemical composition of the LF and intervertebral disc revealed that the collagen content in the ligament was similar to that in the anulus and higher to that in the nucleus (Figure 5.18). The ligament had a significantly lower extent of Pyd compared to the anulus and the nucleus \( (p < 0.001) \), but had a significantly higher extent of Dpd \( (p < 0.001) \). The LF also had a significantly higher Isodes and Des crosslink content \( (p < 0.001) \), more than 400 times more Isodes and 650 times more Des compared to the anulus and the nucleus (Figure 5.18). The mean Pyd to Dpd ratio for the LF was 6:1 compared to 66:1 for the anulus and 45:1 for the nucleus.
5.5.3.2 Ligamentum Flavum

5.5.3.2.1 Age and Gender influences
The collagen content and the extent of Pyd and Dpd increased significantly with age for LF ($p < 0.05$). The Des and Isodes content did not change significantly with increasing age (Figure 5.19). The LF tissues had significantly higher collagen ($p < 0.05$), Isodes ($p < 0.01$) and Des ($p < 0.01$) content in females compared to males, especially in the old age groups. The extent of Pyd and Dpd were significantly higher in males compared to females, in the young and mid age groups ($p < 0.01$).

5.5.3.2.2 Spinal level and Gender influences
The cervical and lumbar regions had significantly higher collagen content compared to the thoracic region ($p < 0.01$). The lumbar region also had significantly higher extents of Pyd and Dpd and Des and Isodes content compared to the cervical and thoracic regions ($p < 0.05$, Figure 5.20). The trends for collagen and elastin crosslink content were similar for both males and females. In the thoracic region, females had significantly higher collagen, Des and Isodes content compared to males ($p < 0.05$). The cervical and lumbar regions did not show gender variations for all biochemical components, except for a significantly higher Pyd crosslink extent in females in the cervical region ($p < 0.05$), and significantly higher Isodes crosslink content in females in the cervical and lumbar regions ($p < 0.05$).

5.5.3.2.3 Spinal level and Age influences
The collagen content in LF increased in all spinal regions with increasing age and was significant in the thoracic ($p < 0.01$) and lumbar regions ($p < 0.05$, Figure 5.21). The extent of Pyd increased significantly with increasing age ($p < 0.05$), and was generally highest in the Mid age group but this was statistically significant only for the thoracic region ($p < 0.01$). Deoxypyridinoline extent was significantly higher in the Old age group but this association was only significant in the cervical and lumbar regions ($p < 0.05$). Elastin crosslinks decreased significantly with increasing age, mainly in the lumbar region ($p < 0.05$). The Des content was significantly lower in the Mid age group ($p < 0.05$).
5.5 Collagen and elastin crosslinks in spinal discs and LF

Figure 5.18 The collagen content and extent of Pyd, Dpd, and the Des and Isodes content in the anulus, nucleus and ligamentum flavum (LF) are depicted. The LF had a significantly lower extent of Pyd and the highest extent of Dpd, Des and Isodes crosslinks compared to the anulus and the nucleus ($p < 0.001$). Error bars represent one standard deviation.

* = Significant $t$-tests between AF and LF, where $p < 0.001$.

** = Significant $t$ tests between NP and AF, where $p < 0.001$.

*** = Significant $t$-tests between NP and LF, where $p < 0.001$. 
**Figure 5.19** The collagen content and extent of Pyd, Dpd and Des and Isodes content in Young (< 35 years), Mid (36 to 60 years) and Old (> 60 years) age groups, according to male and female groups in the LF. Des and Isodes content in the LF did not change significantly with age, however the collagen content and extent of Pyd and Dpd increased significantly with age ($p < 0.05$). The LF in females had significantly higher collagen and elastin content compared to males in the Old age groups ($p < 0.01$). Error bars represent one standard deviation.

* = significant ANOVA test comparing the means in the three age groups, $p < 0.05$.

** = significant $t$-test between male and female groups, where $p < 0.01$. 
Figure 5.20 The collagen content and extent of Pyd, Dpd, and the Des and Isodes content in the LF pooled for the three spinal regions, and separated into male and female data for each spinal region. All biochemical parameters were significantly higher in the lumbar region compared to the cervical and thoracic regions ($p < 0.05$), except for the collagen content, which was not significantly higher than the cervical region. These regional trends were broadly similar for males and females. Error bars represent one standard deviation. C = cervical, T = thoracic, L = lumbar.

*= significant ANOVA tests comparing the means in the three spinal region, $p < 0.05$.

**= significant $t$-tests between male and female in each spinal region, where $p < 0.05$. 
Figure 5.21 The collagen content and extent of Pyd, Dpd and the Des and Isodes content in Young (Y), Mid (M) and Old (O) age groups, according to spinal regions in the LF. The ligaments in the lumbar region had significantly higher collagen and lower elastin content with increasing age ($p < 0.05$). The collagen content and extent of Pyd was significantly higher in the thoracic region with increasing age ($p < 0.01$). Error bars represent one standard deviation.

* = Significant ANOVA tests comparing the means in the three age groups, where $p < 0.05$. 
5.5.4 DISCUSSION

Although the presence and percentage of elastic fibers in spinal intervertebral discs had been examined histologically (Buckwalter et al. 1976, Johnson et al. 1985, Mikawa et al. 1986), few had quantified the elastin content using biochemical analyses (Olczyk 1994b). The current study found only trace amounts of Des and Isodes crosslinks in spinal discs and these were detected only in lumbar disc samples that weighed more than 420 mg wet weight. No elastin crosslinks could be detected in lumbar tissues that weighed less than 129 mg wet weight or none at all in cervical or thoracic discs. The positive elastin findings in adult lumbar discs was consistent with that reported by Johnson et al. (1985), who found elastic fibers throughout the lumbar anular matrix and none in the inner layers of the cervical and thoracic disc.

Previously Johnson et al. (1982) had used light microscope examination to report the presence of elastic fibers in the superficial layers of the anulus and nucleus in cervical and thoracic discs, in the region of attachment to the hyaline end-plate and vertebral bone. No elastic fibers were observed in the middle layers of the cervical and thoracic anuli or nuclei. In contrast, Buckwalter et al. (1976) and Mikawa et al. (1986) reported the presence of elastic fibers in spinal discs from various spinal regions. However they did not state where the elastic fibers were located. However, the recent study by Yu et al. (2002) reported finding elastic fibers throughout the anular and the nuclear matrices in bovine lumbar discs. All anular and nuclear tissues in this study were taken from the mid-section of the spinal discs, and this sampling procedure may have contributed to the negative elastin findings for the thoracic and cervical discs. Further investigations using cervical or thoracic anular and nuclear tissues adjacent to the end-plates and vertebral attachment, and larger tissue sizes, may be required to confirm the findings observed by Johnson et al. (1982) and Yu et al. (2002).

The mean Pyd:Dpd ratio of 55:1 for the disc was consistent with other studies (Eyre 1995, Tan et al. 2000a) which have reported ratios of greater than 50:1. Eyre (1995) reported a ratio of 3.5:1 for bone, 6:1 for muscle and > 20:1 for joint capsule. The ratio of 6:1 for the LF, suggested a matrix where the Pyd:Dpd ratio was close to that for muscle tissue. In view of the mechanical function of LF and the higher proportion of Type I and III collagen in LF tissues (Specchia et al. 2001), the Pyd:Dpd ratio was probably more likely between bone and muscle tissues.

The LF had significantly higher elastin content (> 400 times) compared to the lumbar discs, which is consistent with other studies (Johnson et al. 1982, Mikawa et al. 1986). The higher elastin content in the LF, confers considerable elasticity in the ligament, helping to restore the ligament’s original shape and resting length following spinal flexion (Yong-Hing et al. 1976,
Johnson et al (1982). The elasticity in the LF also prevents buckling of the ligament hence reduces compression of the spinal cord due to the LF during spinal extension (Nachemson and Evans 1968). Ponseti (1995) suggested that the large elastin content in the LF enables the ligament to assist in vertebral motion and provides intrinsic stability to the spine, therefore is most abundant in those mammals requiring greater spinal mobility compared to those with relatively less spinal motion.

The disc in contrast to LF, had a marked collagen content and a high extent of Pyd but low Dpd. The collagen and collagen crosslinks provided the tensile strength to the disc during weight bearing, and movement (Hukins 1988). The low extent of Dpd in the disc matrix compared with LF, suggested a lesser role compared with Pyd, however this hypothesis remains to be confirmed. In contrast, the LF had a high collagen content accompanied by a surprisingly high extent of Dpd instead of Pyd. The function and role of these collagenous crosslinks in the matrix were still relatively unknown. It was difficult to speculate on how collagen and elastin crosslinkages interact to provide tensile and elastic functions in the LF and the disc, apart from affording a balance in tensile strength and elastic recoil, which together, serve spinal level and total spinal mechanics.

The small amount of elastin detected in the disc matrix may suggest that it did not have a major role in the elastic function of the disc (Buckwalter et al 1976). However, studies have reported that even small amounts of elastin in the skin (0.6% of the total dry weight) (Uitto 1979), were considered physiologically functional (Mikawa et al 1986, Rosenbloom 1987). Other studies suggested that the function of elastin in the disc is different from that of ligament tissues, because it had a composition that was different from that of the ligament, and the elastic fiber was also smaller in diameter (Mikawa et al 1986, Nakagawa et al 1994). It had been postulated that elastic fibers in the anulus might be important in supporting collagen fiber recovery after deformation (Ghosh et al 1977, Johnson et al 1982). Since elastic fibers were found at the disc periphery (Johnson et al 1982), their role might be to provide an elastic component to the collagen fibers as they attached the anular lamellae into the vertebral bone.

The elastin content in the LF did not change significantly with age. However when data was grouped according to spinal regions, the Des and Isodes content decreased most significantly with age in the lumbar region (Figure 5.21). In contrast, other studies had reported that elastic fibers and the elastin content in LF did not change significantly with age, although there was a decreasing age trend (Yong-Hing et al 1976, Mikawa et al 1986, Chen et al 2000). These studies however, did not differentiate the spinal regions from which the LF tissues were
tested. Interestingly, studies on other connective tissues have found a decrease in the elastin content with age for example lung tissues (Starcher 1977), skin (Pearce and Grimmer 1972) and lumbar anular tissue (Johnson et al 1985, Olczyk 1994b). In contrast, the collagen content and extent of Pyd and Dpd in LF increased significantly with age (Figure 5.19). This finding was similar to that reported by Chen et al (2000), although they found the increase with age was not statistically significant. The impact of a decrease in elastin and an increase in collagen content in these ligaments with age probably contributes to spinal stiffness along with other age-related degenerative changes.

Although the differences between spinal regions were not always statistically significant, this study found that the LF in the lumbar region had significantly higher collagen and elastin content and extent of Pyd and Dpd compared to the other spinal regions (Figure 5.19). The reason for the high collagen and elastin content in the lumbar region may be related to the function of these ligaments in this region. The lumbar region is usually exposed to greater spinal loading compared to the cervical and thoracic regions (Pal and Routal 1987), and larger amplitudes of spinal motion compared to the thoracic region (White and Panjabi 1990b). Consequently the LF in this region might be prone to matrix degradation and higher turnover, especially with increasing age, as seen in the significant decrease in elastin content and increased collagen content with age. Nachemson and Evans (1968) also reported significant decrease in the modulus of elasticity of lumbar LF with increasing age, from 1.000 kg/cm² in the young to 200 kg/cm² in the aged. A degradation of collagen fibers and loss of elastin crosslinks in the matrix is suggested to influence negatively on the ability of the connective tissue to withstand tensile forces (Minns et al 1973, Olczyk 1994b), possibly resulting in accelerated degeneration.

Female ligamentum tissues had significantly higher elastin and collagen content compared to males, except for the extents of Pyd and Dpd (Figure 5.20). This gender trend was similar for all age groups. There are few studies that have examined gender differences, however Mikawa et al (1986) reported no gender difference in the elastin content of male and female ligamentum flava. When grouped for spinal regions, similar significantly higher collagen and elastin trends in females compared to males were noted for the thoracic region. Reasons for this gender difference are not clear, although generally females might be more supple (Brandner 1970, Nachemson et al 1979) and had greater flexibility and range compared to males (Van Herp et al 2000).
5.5.5 CONCLUSION

This study provided preliminary data on collagen and elastin crosslink (Des and Isodes) content in human lumbar disc tissues. It also provided a comprehensive report on the distribution of collagen and elastin crosslinks in human LF with increasing age, gender and spinal regions. Collagen and elastin crosslinks were detected in all LF tissues, however only limited traces of Des and Isodes crosslinks were detected in the largest samples of lumbar disc tissue. Ligament tissues had more than 650 times more Des and 400 times more Isodes crosslinks compared to lumbar disc tissues. Elastin crosslinks, Des and Isodes content, in LF did not change significantly with age; were significantly higher in females compared to males ($p < 0.01$); and were significantly higher in the lumbar region, ($p < 0.05$). The collagen content and extent of Pyd and Dpd in the LF increased significantly with age ($p < 0.05$). The collagen content was also significantly higher in females compared to males ($p < 0.01$) and was higher in the lumbar region compared to the thoracic region ($p < 0.05$). The LF in the lumbar region also had significantly higher extent of Pyd ($p < 0.05$) compared to the cervical and thoracic regions.
STUDY 5.6 BIOCHEMICAL AND MRI EVALUATION OF AGED HUMAN THORACIC INTERVETERbral DISCS AND LIGAMENTUM FLAVA: TWO CASE STUDIES

MRI studies from Chapter 4 have provided findings on the prevalence of vertebral body and disc degenerative changes in the thoracic spine. Studies 5.2 to 5.5 had determined the collagenous and elastic constituents and distribution within the thoracic discs and spinal LF. Thompson et al (1988) and Tertti et al (1991) reported that changes in the T2-weighted MRI signal intensity of the human lumbar intervertebral discs were closely associated with the biochemical changes in the disc matrix, which was difficult to measure by histological or morphological examination. Studies by Pearce et al (1983) and Olczyk (1994c) had shown that macroscopic disc degenerative changes were often associated with high collagen and elastin content and low proteoglycan content. Consequently, there is a need to determine the correlation between what was observed on MRI with the biochemical constituent in the disc matrix, in particular for human thoracic discs, where there is currently no similar study in the literature. In addition, there is no study reporting on the association of collagen and elastin crosslinks in the disc matrix and the corresponding MRI signal intensity or macroscopic disc changes, even for lumbar discs.

Calcifications of spinal LF has been reported in the literature (Yong-Hing et al 1976, van Oostenbrugge et al 1999), however, the influence of calcification on the distribution of proteoglycan, collagen and elastin crosslinks in the ligamentous matrix has not been reported in the literature. Therefore the objective of this final thesis study, was to investigate the association of MRI and macroscopic grading of thoracic discs and LF with the matrix components of collagen, proteoglycan, elastin (Des and Isodes) and collagen (Pyd and Dpd) crosslinks. For thoracic discs, changes in both the MRI and macroscopic evaluations were investigated, however for the thoracic LF, only the macroscopic evaluation of the degree of calcification of the LF was assessed.

5.6.1 MATERIALS AND METHOD
5.6.1.1 Tissue collection and preparation
As formalin fixation affects the release of proteoglycans for biochemical analysis (Boskey et al 1982, Toledo et al 1996), only non-fixed disc and LF samples were used for the study. Thoracic spinal columns (T1 to T12) from two unfixed cases: Case A (female aged 73 years) and Case B (male, aged 85 years) were examined, following routine post-mortem procedures. The fresh spinal columns were sectioned cranially throughout the disc space at C7-T1
juncture, and at the L5-S1 junction caudally, then wrapped and stored at -20°C until required for investigation.

5.6.1.2 Radiographic and MRI investigations
The spines were thawed by immersing in room temperature water for a duration of at least 12 hours. Lateral and anterior-posterior plain film radiographs were taken of each spine, followed by sagittal T2-weighted MRI (Figure 5.22). Screening of the spines was performed using radiographs to exclude vertebral fractures, severe vertebral deformity or tumours. From the radiographs, it was noted that Case B was sectioned at the T1-T2 disc level instead of C7-T1, therefore it was not possible to view the T1-T2 disc using MRI or macroscopic examinations (Figure 5.23). The corresponding LF at that segment, was also not available for macroscopic examination or biochemical investigation.

A Picker Vista 1.0 Tesla MRI unit (Cleveland, Ohio, USA) was used for spinal MR imaging. The MRI parameters were T2-weighted fast spin-echo sequences, with repetition time = 3000 msec, and echo time = 80 msec. The slice thickness was 4mm, with a field of view of 44–50 mm and an acquisition matrix range of 192 x 256.

![Figure 5.22](image)

**Figure 5.22.** Sequence of investigations using non-fixed human thoracic cadavers. X-ray was used to exclude bony fractures, deformities or tumours. Sagittal MRI was used to grade the discs, followed by macroscopic disc and LF examinations. Finally tissues were assayed biochemically to determine the PG, collagen and elastin crosslink content.

Degenerative changes in the nucleus, anulus, and the presence of osteophytes observed on MRI, were graded using a modified 3-point scale as described in Study 4.1. All sagittal T2-weighted thoracic sequences were examined for disc degenerative changes at all available thoracic levels (T1 to T12). The T2-T3 disc from Case A MR image was obscured due to the specimen holder, therefore the disc grade at that level was not recorded (Figure 5.23).
Figure 5.23  Mid sagittal T2-weighted MR images of Case A (T1 to T12) and Case B (T2 to T12). For Case A, grade II changes were noted in the nucleus and annulus, and grade III osteophytes were noted at T11-T12 disc (arrow). In Case B, grade III changes were noted in the nucleus and annulus and osteophytes at T6-T7 disc (arrow).

5.6.1.3 Macroscopic investigation

After MRI, the spines were sectioned at the junction of the pedicle and vertebral body to separate the laminae and spinous processes from the vertebral bodies. Thoracic intervertebral discs ($n = 23$) and LF ($n = 23$) were removed from all available thoracic levels of both spines. The disc was removed axially at the mid plane, with the annulus and the nucleus intact. Each axial slice was approximately 1.5 to 2 mm thick, and graded macroscopically using the same descriptors from the modified 3-point sagittal grading scale described in Study 5.3 (see Figure 5.24).

The LF was removed from each thoracic level and graded macroscopically for the presence and the amount of calcification, in proportion to the whole ligament tissue. Grade I indicated the absence of calcification; grade II for moderate (< 50% of the tissues were calcified); and grade III for severe calcification (> 50% calcification of the ligament tissues). The thickness of the LF increased craniocaudally and tissues removed varied between 1 and 2 mm.
thickness, however where the LF was calcified, only limited amount of tissues could be harvested. The total number of discs and LF tissues graded was 23.

5.6.1.4 Collagen and Elastin biochemical analysis
Disc and ligament tissues were weighed, dried and hydrolysed as described in Study 5.1. The cooled sample hydrolysate was analysed for collagen and elastin crosslinks using the HPLC (I) procedure, and collagen content using the hydroxyproline assay described in Study 5.1.

5.6.1.5 DMB Assay for Chondroitin Sulphate
The DMB assay for CS in fresh spinal disc and ligament tissues was developed using a modification of the methods by Farndale et al, (1982), Sabiston et al (1985), Chandrasekhar et al, (1986) and Carroll (1987), described in detail in Appendix B3. In an earlier pilot investigation, papain was unable to digest the formalin-fixed spinal tissues completely, and the resulting supernatant when tested, did not yield any positive results for CS, therefore the assay was used only for fresh spinal ligament and disc tissues.

The DMB assay for sulphated glycosaminoglycans is rapid, simple, quantitative and more importantly, reliable (Chandrasekhar et al 1986, Carroll 1987). It was reported by Carroll (1987) to be significantly correlated to the glycosaminoglycan assay using radioimmunoassay (correlation coefficient of 0.6, \( p < 0.001 \)), and has a sensitivity of not less than 1 \( \mu g \) (4 \( \mu g/ml \)) of CS (Farndale et al 1982).
Figure 5.24 Macroscopic examination of mid axial (left) and mid sagittal (middle) sections of thoracic discs, showing Grade I, Grade II, and Grade III changes, using descriptors from the Thompson et al (1990) grading scale for discs in the sagittal plane (right).
A range of 20 to 30 mg of disc and ligamentum tissues were diced finely for papain digestion. Disc samples were digested with 1.5 ml of papain solution containing 5 units/ml papain, 5 mmol/L cysteine, 5 mmol/L Sodium EDTA in 0.1 mol/L sodium acetate buffer (pH 5.5). Tubes were sealed and incubated at 65°C overnight for not less than 12 hours. The supernatant, consisting of varying amounts of chondroitin sulphate, Tris HCl, and NaCl, were added into the microtiter plate for CS analysis, using DMB assay. The plates were read using a Flow Spectrophotometer plate reader (Titertek Multiskan MC, Eflab Oy, Helsinki, Finland), at a wavelength of 540 nm, immediately after adding DMB reagent. The concentration of CS in the samples was calculated against the standard curve by GraphPad Prism™ (Hallogram Publishing, Aurora CO, USA), and reported as µg/mg wet weight.

### 5.6.2 DATA REDUCTION AND STATISTICAL ANALYSIS

A comparison between MRI and macroscopic grades was performed using crosstabs to determine correlation coefficient or kappa statistic (SPSS, version 6.1, SPSS Inc, Illinois, USA). For the purposes of statistical evaluations, each disc and ligament was treated as separate cases, an assumption that may limit the generalisability of these data. The total possible number of disc and LF samples removed from both spines was 23 (12 from Case A and 11 from Case B).

### 5.6.3 RESULTS

Due to grade III calcification and limited tissue availability, two LF tissue samples from Case A (T3-T4 and T4-T5) and one from Case B (T4-T5) were not available for CS assay (Table 5.13). There was only sufficient ligamentum tissue for collagen and elastin essays, and insufficient tissue for further CS assay especially for severely calcified LF samples. Des and Isodes crosslinks were only detected in the LF tissues. There were no elastin crosslinks detected for the anulus or the nucleus despite using large amounts of disc tissues (mean = 204 mg wet weight, maximum wet weight = 410 mg).

### 5.6.3.1 MRI and macroscopic examinations

The kappa correlation coefficient of the MRI grading with the macroscopic grading scale for the anulus \( k = 0.3 \), nucleus \( k = 0.4 \) and the presence of osteophytes \( k = 0.3 \) were fair (Table 5.13). Generally, MRI grading identified a higher percentage of grade II and III discs (54%) compared with macroscopic grading (36%). Macroscopically osteophytes were mainly
located laterally, or anteriorly, especially in the upper and mid thoracic levels (T3 to T6). No MRI comparisons were made for the LF as these tissues were only graded macroscopically.

5.6.3.2 Biochemical matrix and grade I tissues
Comparing anular and nuclear MRI grade I discs, the collagen content was higher in the anulus (Table 5.14 and Figure 5.25), while the CS content and extent of Pyd and Dpd were higher in the nucleus compared to the anulus. The biochemical differences between the anulus and the nucleus for macroscopic grade I discs were also similar to the findings using MRI grades (Figure 5.26).

The biochemical data for the LF could only be compared to the anular and nuclear data using the macroscopic grades for degeneration and calcification (Figure 5.26). Grade I normal LF tissues had higher extent of Dpd compared to the anulus and the nucleus (Table 5.14). The ligamentum tissues had lower collagen content compared to the anulus, however was higher than the nucleus. The extent of Pyd was lower in the ligament compared to both the anulus and the nucleus.

5.6.3.3 Biochemical matrix and grades II and III tissues
Generally, increasing MRI and macroscopic grades were associated with a higher collagen content in the nucleus and the LF, and a lower collagen content in the anulus (Figures 5.25 and 5.26). Collagen content was higher in degenerated nucleus graded using MRI (Figure 5.25, Table 5.14). In contrast, a lower collagen content was observed in degenerated anulus when graded macroscopically (Figure 5.26).

For LF tissues, Des and Isodes content and collagen and CS were higher with increasing calcification grades. Generally a decrease in the CS content was noted in the grade II and III nuclear matrix, using macroscopic and MRI grading. In contrast, an increase in CS content was observed for grade III anuli (more obvious using macroscopic compared to MRI grading scale) and grade II ligamentum tissues.
**Table 5.13** The MRI and macroscopic (MAC) grading of the nucleus (NP), anulus (AF), presence of osteophytes (OS) and calcifications in the LF for all thoracic levels in the 2 cases, ($n = 23$ discs and 23 ligamentum flava), female 73 years old (A) and male 85 years old (B).

<table>
<thead>
<tr>
<th>Spine level</th>
<th>Case A</th>
<th></th>
<th>Case B</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MRI</td>
<td>MAC</td>
<td>MRI</td>
<td>MAC</td>
<td>MRI</td>
</tr>
<tr>
<td>T1-T2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>T2-T3</td>
<td>0</td>
<td>I</td>
<td>0</td>
<td>I</td>
<td>II</td>
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<td>T3-T4</td>
<td>II</td>
<td>I</td>
<td>I</td>
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</tr>
<tr>
<td>T5-T6</td>
<td>II</td>
<td>II</td>
<td>I</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>T6-T7</td>
<td>II</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>T7-T8</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>I</td>
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<tr>
<td>T8-T9</td>
<td>I</td>
<td>I</td>
<td>I</td>
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</tr>
<tr>
<td>T9-T10</td>
<td>II</td>
<td>II</td>
<td>I</td>
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<td>T10-T11</td>
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<tr>
<td>T12-L1</td>
<td>II</td>
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</table>

0 = missing data
Table 5.14 The mean (± SD) for collagen, CS, Des and Isodes content, and extent of Pyd and Dpd in thoracic anulus (AF), nucleus (NP) and LF of grade I, II and III MRI and macroscopic (MAC) grades (n = 23 disc and 23 ligamentum tissues).

<table>
<thead>
<tr>
<th>Grade</th>
<th>Collagen</th>
<th>Pyd</th>
<th>Dpd</th>
<th>CS</th>
<th>Isodes</th>
<th>Des</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol/mg dry wt)</td>
<td>(mol/mol collagen)</td>
<td>(mol/mol collagen)</td>
<td>(µg/mg wet wt)</td>
<td>(nmol/mg dry wt)</td>
<td>(nmol/mg dry wt)</td>
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<tr>
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<td>MRI</td>
<td>MAC</td>
<td>MRI</td>
<td>MAC</td>
<td>MRI</td>
</tr>
<tr>
<td>I AF</td>
<td>1.50 ± 0.13</td>
<td>1.53 ± 0.14</td>
<td>1.23 ± 0.12</td>
<td>1.18 ± 0.13</td>
<td>0.017 ± 0.003</td>
<td>0.016 ± 0.006</td>
</tr>
<tr>
<td>NP</td>
<td>0.54 ± 0.08</td>
<td>0.61 ± 0.13</td>
<td>1.51 ± 0.15</td>
<td>1.39 ± 0.18</td>
<td>0.016 ± 0.006</td>
<td>0.016 ± 0.006</td>
</tr>
<tr>
<td>II AF</td>
<td>1.44 ± 0.17</td>
<td>1.31 ± 0.06</td>
<td>1.16 ± 0.09</td>
<td>1.18 ± 0.03</td>
<td>0.020 ± 0.002</td>
<td>0.021 ± 0.0001</td>
</tr>
<tr>
<td>NP</td>
<td>0.68 ± 0.11</td>
<td>0.64 ± 0.09</td>
<td>1.14 ± 0.22</td>
<td>1.55 ± 0.17</td>
<td>0.015 ± 0.004</td>
<td>0.015 ± 0.0002</td>
</tr>
<tr>
<td>III AF</td>
<td>1.47 ± 0.29</td>
<td>1.26 ± 0.20</td>
<td>1.12 ± 0.11</td>
<td>1.22 ± 0.05</td>
<td>0.018 ± 0.0002</td>
<td>0.018 ± 0.001</td>
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<tr>
<td>NP</td>
<td>0.65 ± 0.10</td>
<td>0.63 ± 0.12</td>
<td>1.56 ± 0.12</td>
<td>1.54 ± 0.11</td>
<td>0.018 ± 0.004</td>
<td>0.021 ± 0.004</td>
</tr>
<tr>
<td>I LF</td>
<td>1.06 ± 0.19</td>
<td>0.84 ± 0.13</td>
<td>0.12 ± 0.022</td>
<td>8.4 ± 5.32</td>
<td>5.78 ± 1.35</td>
<td>8.83 ± 1.95</td>
</tr>
<tr>
<td>II LF</td>
<td>1.27 ± 0.38</td>
<td>0.83 ± 0.19</td>
<td>0.12 ± 0.019</td>
<td>16.1 ± 11.1</td>
<td>8.32 ± 2.23</td>
<td>19.35 ± 6.47</td>
</tr>
<tr>
<td>III LF</td>
<td>1.48 ± 0.31</td>
<td>0.71 ± 0.02</td>
<td>0.11 ± 0.020</td>
<td>7.91 ± 2.68</td>
<td>17.49 ± 9.03</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.25 The collagen content, extent of Pyd and Dpd, and CS content in grade I, II and III anular and nuclear tissues, using the 3-point MRI grading scale. The collagen content in the nucleus was higher with increased grades, however the CS was lower instead. In the anulus, only the extent of Pyd showed a decreasing trend. Error bars represent one standard deviation.
Figure 5.26 The collagen content, extent of Pyd and Dpd, and CS content in grade I, II and III anular (AF) and nuclear (NP) and LF tissues, using the 3-point macroscopic examination. Des and Isodes content in grade I, II and III LF were also reported. The collagen content was lower in the anulus with increasing grade. The elastin crosslinks, Des and Isodes, collagen and CS content in the LF were higher with increasing calcification. Error bars represent one standard deviation.
5.6.4 DISCUSSION

This study provided a preliminary comparison of the biochemical composition of the disc and LF according to degenerative changes observed using MRI and macroscopic examinations. The agreement between the two grading scales for the disc components was fair. One could argue that they should have been very good. A larger sample size, consisting of different spine cases, would improve the power for statistical analysis. Other reasons may be due to the different axes that the discs were examined. For MRI examination of the discs, all T2-weighted sagittal sequences were used, whereas only the mid transverse plane slices were examined macroscopically. In addition, the macroscopic grading criteria used to grade the axial slices were adapted from the 3-point grading scale, originally described by Thompson et al (1988) for examination of lumbar discs in the sagittal plane. Therefore, the ‘good’ correlation reported by Thompson et al (1988) between the MRI and macroscopic grading scales, may not be applicable for macroscopic grading of discs using axial disc slices and for lumbar discs. The correlation between macroscopic and MRI grading of axial disc slices has not been examined for thoracic discs and is warranted in future research.

When using MR images for assessment, the degeneration grades for thoracic anulus and nucleus, were consistently higher compared to macroscopic grading. These findings were similar to those reported by Thompson et al (1988) for lumbar discs. They attributed this trend to a higher sensitivity of MRI to detect early matrix changes compared with macroscopic examination. This increased sensitivity led Tertti et al (1991) to caution that the biochemical changes in the matrix associated with early MRI or grade II changes, might not be representative of true pathologic processes, instead, might be indicative of normal ageing in the matrix. However, findings from the present study revealed that grade II changes on MRI were associated with higher collagen content in the nucleus. The association of an increase in collagen content in the nucleus with increasing degeneration found in the present study, was consistent with reports from lumbar studies (Eyre 1979, Pearce et al 1987, Olczyk 1992) and earlier findings in Study 5.4. In addition, the relationship of collagen to MRI signal intensity changes has also been reported by Pearce et al (1991) and Aguila (1985), who noted that the dark intranuclear line assessed as grade II for nuclear tissues on MRI, was associated with a higher collagen content. Macroscopic examinations were instead, associated with lower collagen in the anular tissues.

The decreasing CS trend in the nucleus was similar to that reported in other lumbar disc studies (Thompson et al 1988, Pearce et al 1991, Tertti et al 1991, Olczyk 1994a). Thompson et al (1988) and Pearce et al (1991) suggested that the low proteoglycan content in the nucleus
was closely associated with low MRI signal intensity, and was an early sign of ageing and degeneration of the nuclear matrix.

It was interesting to note that the CS content in grade II and III anular samples was higher compared to grade I samples in the present study. In the anulus, only the collagen content was significantly lower with increasing degeneration grades, evident on MRI and macroscopic examination. This increasing CS trend in the anulus was in contrast to results from other lumbar studies, which reported significant decreases in large proteoglycans (Inkinen et al 1998), and CS content in degenerate lumbar anulus (Thompson et al 1988). However Pearce and Grimmer (1983) did report no change in chondroitin 6-sulphate and a decrease in keratan sulphonate in degenerated lumbar discs.

The controversial CS finding in degenerated anular samples might suggest a heterogeneous anular matrix due to various degeneration and repair processes in the tissue matrix or a change in other disc matrix components, such as water and non-collagenous proteins. The increase in CS in the anulus might also be due to an increase in small proteoglycans, decorin and biglycan, found in degenerate lumbar anulus (Inkinen et al 1998). Decorin and biglycan consist of CS and dermatan sulphate chains, but in a smaller proportion compared to the large proteoglycans (Heinegård and Sommarin 1987, Inkinen et al 1998). These small proteoglycans were not usually found in normal anular tissues and, when present in degenerate tissues, were proposed to be responsible for binding to collagen fibrils and inhibiting fibrillogenesis of collagen fibers (Vogel et al 1984). Although the higher CS content in degenerate anular samples might not have a negative influence on the biomechanical function of the tissue, the loss of collagen in the anulus would impair the matrix’s ability to withstand loading and twisting, due to a loss of tensile strength (Adams and Muir 1976, Heinegård and Sommarin 1987, Inkinen et al 1998).

Comparison of the biochemical content of calcified and non-calcified LF tissues had not been reported in the literature. A higher collagen, CS, Des and Isodes content were observed in calcified ligamentum tissues. Further study to examine for the collagen and proteoglycan changes with larger numbers of severely calcified ligamentum tissues (grade III) would be needed to confirm these trends. Roberts et al (1994) had reported similar increases in the PG content in calcified sheep and human intervertebral discs. They found that calcified disc tissues were associated with an increase in chondroitin-6-sulphate, native CS and chondroitin-4-sulphate, compared to non-calcified tissues. They suggested that the deposition of calcium salts was associated with these proteoglycan components, however the mechanism was not
known. This phenomenon might also explain the increase in CS content in the anulus if the degenerated tissue had some calcification present.

Other spinal studies have found no significant changes in the elastin content with age both in the LF (Chen et al 2000) and in lumbar discs (Mikawa et al 1986, Olczyk 1994b) or spinal spondylosis (Yong-Hing et al 1976). The present study could only compare changes in the Des and Isodes between normal and calcified LF tissues. The finding that Des and Isodes crosslinks was higher in calcified LF tissues compared to non-calcified tissues was unexpected. According to Eyre (1979) and Bailey (1998), calcified tissues have been reported to have a higher Dpd content, such as bone, however there has not been any report of elastin in calcified tissues. The present study did present with a higher extent of Dpd in grade II calcified LF, however the sample size was too small to be tested for statistical significance.

The lack of elastin crosslinks in the thoracic anulus and nucleus found in this study might be due to similar reasons suggested in 5.5.4 of Study 5.5. The higher collagen content in thoracic grade I anulus compared to the LF samples, was consistent with other lumbar disc comparisons (Mikawa et al 1986). Although the extent of Pyd was lower in the LF, it had a higher extent of Dpd compared to disc tissues. The LF also had lower CS content compared to the anulus and the nucleus. Comparison of these collagenous crosslink data in the spinal LF have not been reported in the literature, and provides preliminary information upon which to base further investigations.

5.6.5 CONCLUSION

Disc and LF tissues from the two non-fixed case studies provided a preliminary comparison of the radiological, macroscopic and biochemical markers of degeneration of human thoracic discs and LF. The kappa correlation coefficient between MRI and macroscopic grading for the discs were fair, ranging from 0.3 to 0.4. Generally MRI grading tended to be higher than the corresponding macroscopic grading for the discs.

Using MRI, higher nuclear grades were associated with higher collagen content. Macroscopically, the collagen content in the anulus was lower with increased anular grade. The CS content was lower in the degenerate nucleus and higher in degenerate anulus. For the LF, the collagen, Dpd, CS, Des and Isodes content was higher in calcified ligaments.
OVERALL CONCLUSIONS FOR CHAPTER 5

In Study 5.1 formalin-fixed spinal disc and ligament tissues were investigated to determine their feasibility for analysing collagen, Des and Isodes crosslink content and extent of Pyd and Dpd.

1. Human spinal discs and LF fixed in 10% buffered formalin from 1 to 25 weeks did not have significantly different collagen, Des and Isodes content, or extent of Pyd and Dpd, compared with fresh unfixed control samples.
2. There were also no significant differences in these biochemical components of collagen, Pyd, Dpd, Des and Isodes, in the disc and ligament tissues over the 25 weeks of fixation.

In Studies 5.2 and 5.3 the distribution of collagen content and the extent of Pyd and Dpd in the extracellular matrix of 209 non-degenerate thoracic disc over a wide age range (1-90 yrs) was analysed. Data were also regrouped to analyse for age, gender, disc and spinal regional influences on the collagenous constituent of the thoracic disc matrix.

3. Regionally, the nucleus was found to have a significantly lower collagen content but significantly higher extent of collagen crosslinks compared to the anulus (p < 0.05).
4. The anterior anulus had significantly lower extent of Pyd compared to the posterior anulus (p < 0.05).
5. The anular tissues in the mid thoracic region had the highest variation of collagen, Pyd and Dpd in different regions of the disc, compared to the upper and lower anular tissues.
6. With increasing age, the collagen content and extent of Pyd were significantly lower in all disc regions (p < 0.001), in contrast, the extent of Dpd was higher in all regions, but was only statistically significant in the nucleus (p < 0.001).
7. Younger male discs had a significantly higher extent of Pyd compared to females (p < 0.01); however with age this gender difference was reversed. The collagen content in the anulus was also significantly higher in male samples compared to females (p < 0.05).
8. The main spinal regional trends noted were a lower extent of Dpd and Pyd in the anulus and nucleus, respectively, in the lower thoracic region. In the mid thoracic region, the collagen content of the anterior anulus was significantly lower (p < 0.05), however, the extent of Pyd was significantly higher, compared to the other thoracic regions (p < 0.05).
In Study 5.4, the influence of disc degenerative changes on the distribution of collagen content and extent of collagen crosslinks in 303 thoracic discs were evaluated. In addition, the changes in the distribution due to age, gender and thoracic regions were also determined.

9. The nucleus had a predominance of severe macroscopic degeneration changes at an earlier age interval compared with the anulus. In severely degenerated thoracic discs, there was no difference between the anulus and the nucleus, in terms of the collagen and crosslink measurements.

10. After accounting for age effects, degenerate nuclear tissues had significantly higher collagen content ($p < 0.05$), compared with aged non-degenerate thoracic discs.

11. Although present in small amounts, the extent of Dpd increased significantly with increased age and degeneration ($p < 0.001$).

Study 5.5 provided comprehensive data on collagen and elastin crosslink content in 364 human spinal LF and 77 disc tissues. The association of these biochemical components in the LF with increasing age, gender and spinal regions was also examined.

12. Collagen and elastin crosslinks were detected in all LF tissues, however only small amounts of Des and Isodes crosslinks were detected in a limited number of lumbar disc tissues.

13. Ligamentous tissues had over 650 times more Des and 400 times more Isodes crosslinks, compared to lumbar disc tissues.

14. Des and Isodes crosslinks in LF did not change significantly with age, but were significantly higher in females compared to males ($p < 0.05$), and were higher in the lumbar region (but only statistically significant for Isodes crosslinks).

15. The collagen content and extent of Dpd in the LF was significantly higher with age ($p < 0.05$); while the extent of Pyd remained unchanged with age.

16. The collagen content and Pyd extent in the LF was significantly higher in females ($p < 0.05$), but there was no gender difference for Dpd.

The preliminary trends in Study 5.6 for two unfixed spine cases was used to compare MRI, macroscopic and biochemical data from human thoracic discs and LF. Inferences from this study are limited due to the small sample.

17. The kappa correlation coefficients between MRI and macroscopic grading scales for the discs were fair, ranging from 0.3 to 0.4. Generally MRI grading tended to be higher than the respective macroscopic grading for the discs.
18. With increased MRI and macroscopic grades, the collagen content was found to be higher in the nucleus and lower in the anulus.

19. With increasing degeneration grades, the CS content showed a decreasing trend in the nucleus but a higher content in the degenerated anulus instead.

20. In calcified LF, the Des and Isodes crosslink, extent of Dpd and collagen content were higher compared to normal tissues.

21. Grade I LF samples had almost similar collagen content to the anulus, which was higher to that in the nucleus; and a lower extent of Pyd; a higher extent of Dpd; and a lower CS content compared to the anulus and nucleus.
CHAPTER 6 DISCUSSION

6.1 OVERVIEW

The spinal intervertebral disc is a unique connective tissue that is structured to contribute to spinal load bearing and flexibility. Although much is known about the spinal discs in the lumbar and cervical region, information on thoracic discs is more limited, especially in terms of the pathogenesis of degeneration. Disc degeneration is a common phenomenon in the spine especially in lumbar and cervical discs, often leading to disc herniation and spinal pain due to compression of nerve roots (Skubic and Kostuik 1991, Buckwalter 1995, Kang et al 1996). Extrapolation of degeneration trends from the lumbar to thoracic spinal discs cannot be assumed, given that the latter are subject to different biomechanical stresses, due to the position of the thoracic spine between the lumbar and cervical regions; different shape and height of the thoracic discs (Pooni et al 1986); attachment of thoracic vertebrae to the ribs and sternum (Veleanu et al 1972); and the kyphotic curvature (Schmorl and Junghanns 1971).

Studies have demonstrated that the thoracic region also has a high prevalence of disc degeneration, in terms of Schmorl’s nodes (Hilton et al 1976), osteophytes (Nathan 1962) and anular and nuclear degeneration (Wood et al 1995, Singer 2000). In addition, this region is frequently involved in severe kyphotic and scoliotic spinal deformities (Lambrinudi 1934, Schmorl and Junghanns 1971, Leong et al 1999). The thoracic region might have a high prevalence of disc degeneration, however compared to the cervical and lumbar regions, surgically operated disc herniations are not as common (Otani et al 1988, Maiman and Pintar 1990, Skubic and Kostuik 1991). Wood et al (1995) reported a high percentage (37%) of thoracic herniated discs apparent on MRI, which are asymptomatic. In addition, thoracic herniations have been reported to mimic lumbar (Ito et al 1999, Lyu et al 1999) and cervical (Chok and Wong 2000, Arana et al 2004) symptoms, therefore thoracic disc herniations, may be missed during routine clinical examinations in the lumbar or cervical regions.

Thoracic disc degenerative studies using cadaver (Nathan 1962, Singer 1997), histological (Singer 1997) and radiographical (Lawrence 1977, Singer 1997) data also revealed that age, thoracic segmental levels and gender-related occupational and recreational activities also have an influence on the pathogenesis of disc degeneration in the thoracic region. The prevalence of thoracic disc degeneration using MR imaging is less frequently reported compared to lumbar and cervical discs (Goh et al 2000b).
Studies have also sought to understand the pathogenesis of disc degenerative changes from a biochemical perspective, in particular changes in the proteoglycan and collagen content (Galante 1967, Akeson et al 1977, Boos et al 1997a), as these were the two major structural components of the disc extracellular matrix. Collagen fibers interacting with proteoglycan and elastin fibers, form a composite fiber that provide for the main tensile strength (Minns et al 1973) in the disc anular matrix. More importantly, Eyre et al (1989) have suggested that, fundamentally, it is the mature intermolecular collagen crosslinks that provide the major contribution to the tensile strength of the collagenous network in the extracellular matrix. Information on the distribution of these pyridinium (Duance et al 1998, Pokharna and Phillips 1998) and elastin crosslinks (Olczyk 1994b) are limited not just for thoracic discs, but also for lumbar and cervical discs.

The amount of elastic fibers found in spinal discs was reported by many studies to be a small percentage (< 10% disc dry weight) (Hickey and Hukins 1982, Johnson et al 1985) (Buckwalter et al 1976, Yu 2002). In contrast, elastic fibers were found more abundantly (47% dry weight) in spinal ligament tissues (Mikawa et al 1986, Kashiwagi 1993), and studies on spinal ligament tissues provided more information on the distribution of elastic fibers, especially elastin crosslinks Des and Isodes (Chen et al 2000).

It is apparent from the literature that there is limited information on the prevalence of disc degeneration using MRI, particularly in the thoracic region, and even fewer studies reporting on the biochemical aspect of spinal disc matrix. These gaps of information in the literature provided the motivation for investigations in this thesis, which sought to add to the information on the prevalence of thoracic disc degenerative changes over the life span using MRI. In particular, the association of degeneration and other factors, such as age, gender and spinal regions, and their influence on the biochemical (collagenous and elastin) matrix of the disc and spinal LF (Figure 2.19) were also investigated.

### 6.2 USE OF MRI TO EVALUATE DISC DEGENERATIVE CHANGES IN THE THORACIC DISCS

Numerous studies have utilised T2-weighted MRI films to subjectively grade lumbar discs for degenerative changes (Eyre et al 1989, Yu et al 1989, Boden et al 1990a), and results from Study 4.1 supported the use of a modified 3-point grading scale for assessing thoracic discs using MR images. The subjective intra-rater assessment of thoracic discs using a 3-point modified scale from the original Thompson 5-point scale (unpublished information) reported
by Eyre et al (1989) for T2-weighted MRI films, was found to be “good” to “excellent” ($k = 0.71$ to $0.87$). The inter-rater reliability for the 3-point scale was also high ($k = 0.64$ to $0.88$), and better than the inter-rater reliability for the 5-point scale ($k = 0.54$ to $0.83$).

The 3-point scale also had a higher intra-rater kappa coefficient compared to the 5-point scale of Thompson, which was developed for the lumbar region (Eyre et al 1989). This finding supports the use of MRI to reliably grade disc degeneration in the thoracic region, where the discs are reportedly smaller and more difficult to assess compared to the lumbar discs (Raininko et al 1995). Reliability of the Thompson schema (Eyre et al 1989) for MRI disc grading scale, has not been reported for the thoracic region. In addition, there is no MRI reliability study reported for discs in the upper thoracic region to date.

Consistency of MRI grading was generally highest in the upper thoracic region, followed by the mid and lower thoracic regions, using both the 3- and 5-point scales. The lower intra-rater reliability for discs in the mid thoracic compared to the upper thoracic region may be due to the smaller disc height in this region (Pooni et al 1986, De Smet et al 1988, Goh et al 1999), therefore it may be more difficult to examine the discs in the mid thoracic region (Raininko et al 1995).

The 3-point grading criteria used for each of the four disc components had the highest kappa correlation coefficient for soft tissue changes, especially in the nucleus, and the lowest for bony changes, such as osteophytes. These findings were similar for studies reporting for reliability of reading lumbar disc and osteophyte changes (Raininko et al 1995). According to Martin et al (1992) and Maravilla and Cohen (1991), osteophytic changes were better assessed using T1-weighted MR images instead of T2-weighted MRI, which is more suited for bony tissues. Hence assessment of thoracic vertebral body changes in Study 4.3 utilised T1-weighted MR images, which had a correspondingly high intraclass correlation coefficient of 0.99.

Data comparing the assessment of disc degenerative changes using MRI and macroscopic examination was performed on fresh spine cadavers in Study 5.6. The correlation between MRI and macroscopic grading was fair, however the mean collagen content, collagen crosslinks and CS content was almost similar for each grade in the two grading schema. Results from this preliminary comparative study, showed that degenerative changes noted in MR images and macroscopically, especially in the nucleus, were associated with a higher collagen but a lower CS content. In contrast, degenerative anular changes on MRI and macroscopic inspection, were associated with a lower collagen but a higher CS content. The
nuclear findings were consistent with lumbar disc studies which have reported a decrease in CS in the degenerated lumbar nucleus (Thompson et al 1988, Pearce et al 1991, Tertti et al 1991, Olczyk 1994a). Biochemical studies on lumbar discs have proposed that the low PG content in the degenerate nucleus, which was closely associated with low MRI signal intensity, was an early feature of ageing and degeneration of the nuclear matrix (Thompson et al 1988, Pearce et al 1991).

Based on the above findings in Study 4.1 and 5.6, the use of MRI, with its enhanced sensitivity to provide a greater appreciation of the spectrum of spinal disc abnormalities, and to evaluate soft tissue disc and vertebral body degenerative changes was found to be useful for the thoracic region. Results from Studies 4.2 and 4.3, using archived MRI films from various hospitals have their limitations, as it is not representative of an asymptomatic population. These patients selected for the survey, did not have a diagnosis or indication of thoracic vertebral body fractures, tumours, surgery or deformity. However the degenerative trends observed, provides an illustration of the spectrum of disc and vertebral body changes in the thoracic spine from a large population of patients referred for MRI. Therefore in clinical practice, the relevance of these disc and vertebral changes observed on routine thoracic MRI need to be interpreted according to the age, gender and regional influences. More importantly, these MRI changes should also be related to presenting clinical symptoms for better diagnostic accuracy. Even for lumbar discs, Borenstein et al (2001) reported that the predictive value of MR disc degenerative features for the development of spinal pain is still low.

6.3 USE OF FORMALIN-FIXED SPINAL TISSUES FOR BIOCHEMICAL ANALYSIS

Formaldehyde is commonly used to preserve tissues, and it forms crosslinks with the protein molecules, which can render tissues subsequently unsuitable for biochemical analysis. Findings from Study 5.1 were important in determining if formalin-fixed cadaver tissues might be used for collagen and elastin crosslink detection and analysis. Formalin fixation did not significantly influence the collagen and elastin content or the extent of insoluble collagen crosslinks, Pyd and Dpd, in spinal disc and LF tissues employed in this thesis.

Study 5.1 was completed in 1999 and published in 2002. During this period there were no similar studies to compare results with. However a recent publication by Abe et al (2003) has provided interesting results for comparison. Using HPLC to identify and quantify Pyd,
pentosidine, Des and Isodes in frozen and formalin-fixed spinal LF and hip joint cartilage tissues, they found that formalin fixation did not affect the concentration of collagen, Pyd and pentosidine in both tissues, but significantly lowered the concentrations of Des and Isodes instead. The findings for collagen and Pyd by Abe et al (2003) support the results reported in this thesis, however the different findings in Des and Isodes are interesting and worth discussing.

One reason may be due to the different preparation of the tissues for HPLC analysis. Abe et al (2003) stored the fresh tissues at −80°C, compared to the −20°C used in this study, and the eluate after hydrolysis was separated from the other constituents by vacuum evaporation, instead of the ion-pairing method used in Study 5.1. Storing at such extremely low temperatures may cause further protein denaturation in the tissues (Arakawa et al 2001, Cao et al 2003), which the formalin tissues were not exposed to. Another reason for the different findings may be due to the sampling of tissues in the two studies. Abe et al (2003) used tissues removed during surgery, which may not have a uniform matrix, as degenerative changes in the tissues have been found to have a lower distribution of elastic fibers (Kashiwagi 1993). In Study 5.1, care was taken to ensure that tissues were divided in the midline and samples taken were matched to identical regions on each half, without the obvious presence of degenerative changes. Findings from Study 5.6 also revealed that the degree of calcification of spinal LF tissues will affect the amount of Des and Isodes in the ligament matrix. Abe et al (2003), did not specify the degeneration status of the tissues and whether they were matched samples.

Abe et al (2003) were also not able to explain reasons for the significantly lower amounts of Des and Isodes in formalin-fixed tissues, except to mention that formalin was not able to completely fix the elastin crosslinks in spinal LF and hip joint cartilage tissues, hence were destroyed or altered during biochemical analysis. However histological studies on collagen and elastic fibers have shown that the number of collagen and elastic fibers were preserved in mummified tissues (Montes et al 1985) and, biomechanically, formalin fixation did not affect the elasticity of fixed ligament tissues, therefore the elastin tissues were not altered by fixation (Fung and Sobin 1981). Whether acid hydrolysis is able to release these crosslinks in formalin-fixed tissues is not reported. In Study 5.1, formalin fixation did not appear to significantly hinder the release of elastin crosslinks from spinal soft tissues for biochemical analysis.

The findings for Pyd and Dpd in this thesis and by Abe et al (2003) is not surprising as they are mature, end-point insoluble crosslinks, hence should not be altered by formalin induced
crosslinks. Acid hydrolysis of both fixed and unfixed tissues was able to ensure the release of peptide forms into free ions for ion-pairing and identification using HPLC. Des and Isodes are also insoluble crosslinks, therefore it is speculative if they can be fixed or altered by formalin induced crosslinks. The loss of elastin crosslinks may be due to other methodological process used by Abe et al (2003) besides being masked or altered by formalin induced crosslinks.

The variable elastin findings from Abe et al (2003) notwithstanding, the non-significant results for collagen crosslinks reported in their study and in Study 5.1 does provide evidence that formalin crosslinks may not significantly alter the collagenous matrix of spinal disc and LF matrix constituent. For the purpose of this thesis, elastin crosslinks were found to be not significantly affected after 6 months of formalin fixation. However, in view of the results of Abe et al (2003), further investigations of these crosslinks in fresh and formalin-fixed tissues is warranted to confirm if findings in this thesis are maintained. Formalin-fixed archived tissues may therefore be appropriately utilised for biochemical analysis of collagen and mature collagen crosslinks in human spinal and LF tissues. The opportunity to access information from archival tissues are preferred, as it is usually safer, more accessible compared to fresh human tissues and tissues are less fragile during handling (Cavanaugh and King 1990). In addition, such information can enhance the understanding of the effect of formalin fixation on the biochemical proteins in human spinal intervertebral disc and ligament tissue matrices.

6.4 PATHOGENESIS OF THORACIC DISC DEGENERATION

Results from Studies 4.2 and 4.3 presented in Chapter 4, provided further evidence that age, gender and thoracic spinal and disc regions have a significant influence on the pathogenesis of disc degeneration in the thoracic region. Results from the MRI survey on degenerative changes in the thoracic spine were consistent with existing radiological (Lawrence 1977, Singer 1997), MRI (Videman et al 1995a, Wood et al 1995) and cadaver (Nathan 1962, Singer 1997) studies. More importantly, it provided original data on the prevalence of thoracic disc degenerative and vertebral morphology changes in terms of gender and spinal level using MRI. Studies 5.3 to 5.6, in Chapter 5, provided evidence from a biochemical perspective of changes in the collagen and elastin crosslink content, and the extent of pyridinium crosslinks in degenerated thoracic discs. This discussion will review the association of disc degenerative trends due to age, gender and spinal level influences, with the biochemical disc matrix distribution in the thoracic spine.
6.4.1 Disc and spinal regional influences on thoracic disc degeneration

6.4.1.1 Disc regional influences – anulus and nucleus

From the literature, lumbar disc studies reported that the biochemical matrix in the anulus was different from the nucleus. The layers of collagen in the anulus increases its tensile strength (Nachemson 1965b, Markolf and Morris 1974), and the high proteoglycan content in the gel-like nucleus being more suited for absorption of water into the disc, to withstand spinal compression loading (Nachemson 1965a, Hukins 1988). Findings for the non-degenerate nucleus had a higher extent of Pyd and Dpd and a lower collagen content, compared to the anulus, which is consistent with data from lumbar discs studies (Table 6.1). Eyre (1988) proposed that the higher extent of Pyd and Dpd is probably due to the higher proportion of Type II collagen in the nucleus, which has twice the capacity to form mature collagen crosslinks, compared to Type I collagen in the anulus. The Des and Isodes content in the thoracic anulus were not significantly different from the nucleus (Table 6.1). This finding is similar to the Des and Isodes results of 0.4 and 0.05 amino acid residues/1000 amino acid residues respectively, reported by Mikawa et al (1986) for lumbar anulus and nucleus.

Johnson et al (1985) and Olczyk (1994b) however reported a higher elastic fiber and elastin content respectively, in lumbar anulus compared to nucleus. The latter studies did not report specifically on Des and Isodes crosslinks.

The MRI survey revealed a higher prevalence of degeneration in the nucleus (86% of 216 cases) compared to anulus (75%), with the lowest degeneration rate in the end-plates (63%). This finding is consistent with cadaver studies for the lumbar spine (Miller et al 1988, Lebkowski 2002). The nucleus is more prone to degeneration for a number of reasons, the anulus has a higher tensile strength, hence is more resistant to injury from mechanical forces (Galante 1967); the poor circulation to the disc results in limited oxygen and nutrition to the nucleus (Urban et al 1977), therefore the nuclear matrix is more prone to degeneration (Maroudas 1988, Horner and Urban 2001) compared to the anulus.

From a biochemical perspective, the different collagenous disc matrices between the normal thoracic anulus and nucleus observed in Study 5.3, was not evident in Study 5.4 for grade III or severely degenerated thoracic discs. It had been commonly reported in lumbar disc studies that morphologically, the nucleus loses its distinctive soft gel-like appearance and resembles the more fibrous anulus in degenerated discs (Thompson et al 1990, Nerlich et al 1997, Hutton et al 1998). Hence the loss of the distinctive morphological boundary between the degenerated thoracic anulus and the nucleus is also accompanied by changes in the biochemical nuclear matrix, with the latter having a higher collagen content, which is equal to that in degenerated anulus (Study 5.4). There was also a reduction of the extent of Pyd and an
increase in Dpd with increased degeneration status, such that there were no significant difference in these crosslinks between the degenerated anulus and nucleus.

**Table 6.1** Comparison of the mean collagen content (nmol/mg dry wt), extent of Pyd and Dpd (mol/mol collagen) and Des and Isodes content (nmol/mg dry wt) from various lumbar spine studies and current thoracic disc data from Study 5.3 (Thesis 2004) for the nucleus and anulus.

<table>
<thead>
<tr>
<th>Studies</th>
<th>Tissues</th>
<th>Nucleus</th>
<th>Anulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olczyk 1992,1994</td>
<td>Collagen</td>
<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Elastin (mg/g)</td>
<td>16</td>
<td>22</td>
</tr>
<tr>
<td>Scott et al 1994</td>
<td>Collagen</td>
<td>0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Eyre et al 1995</td>
<td>Pyd</td>
<td>2.0 to 2.8</td>
<td>1.6 to 1.9</td>
</tr>
<tr>
<td></td>
<td>Dpd</td>
<td>&lt; 0.03</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td></td>
<td>Pyd:Dpd</td>
<td>&gt;50:1</td>
<td>&gt;50:1</td>
</tr>
<tr>
<td>Thesis 2004 (non-degenerate)</td>
<td>Collagen</td>
<td>0.80</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>Pyd</td>
<td>2.00</td>
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</tr>
<tr>
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<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Pyd:Dpd</td>
<td>50:1</td>
<td>65:1</td>
</tr>
<tr>
<td></td>
<td>Des (nmol/mg)</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Isodes (nmol/mg)</td>
<td>0.03</td>
<td>0.02</td>
</tr>
</tbody>
</table>

6.4.1.2 Disc regional influences – anterior and posterior anulus

It was also interesting to note that within different regions of the non-degenerate thoracic anulus, the biochemical matrix was found to be different, particularly between the anterior and posterior anulus. Findings in Studies 5.2 and 5.3 revealed that generally the extent of Pyd was significantly lower in the anterior anulus compared with the posterior anulus. The extent of Dpd was also lower in the anterior anulus, however the collagen content was similar (Study 5.2). The non-degenerate posterior anulus did not show any significant biochemical trends in the different thoracic regions.

These regional disc differences in the non-degenerate collagenous content may be explained by the different mechanical forces experienced in the anterior and posterior anulus, especially in the mid thoracic region. Studies on scoliotic spines have also reported that the biochemical constituents of the anular matrix within the same disc was different depending on whether it
was on the concave or convex side (Bushell et al 1979, Brickley-Parsons and Glimcher 1984, Crean et al 1997, Duance et al 1998). Duance et al (1998) reported a higher extent of Pyd on the convex side of scoliotic spines, compared to the concave side. This finding is similar to the lower extent of Pyd in the anterior thoracic anulus, which is on the concave side of the kyphosis. Bushell et al (1979) and Brickley-Parsons and Glimcher (1984) also reported a higher amount of collagen Type I fiber on the concave side of scoliotic discs. Collagen Type I fibers, as mentioned above, have less crosslinking ability compared to Type II fibers, hence the lower extent of Pyd on the concave anterior anulus, and may also explain the lower extent of Pyd and Dpd in the anterior anulus compared to the posterior anulus.

Longitudinal animal spine studies also provide further evidence to support that compression forces or altered spinal biomechanical forces result in changes to the disc biochemical matrix (Hutton et al 1998, 1999, Edwards et al 2001). Therefore, the exposure of the anterior anulus in the mid thoracic region to sustained compression loading, which is also the apex of the thoracic kyphosis (Schmorl and Junghanns 1971, White and Panjabi 1990a, Singer 1997), might be responsible for the different biochemical matrices compared to the posterior anulus, even in the absence of degenerative changes. In degenerate thoracic discs, these collagenous matrix differences were not observed between the anterior and posterior anulus (Study 5.4). Biochemical analysis of the collagenous matrix in spinal discs should therefore take into consideration the particular disc region examined, especially in non-degenerate thoracic discs.

### 6.4.1.3 Spinal regional influences – regional degeneration trends

Using MRI archives reported in Studies 4.2 and 4.3, the degenerative pattern for thoracic anular, nuclear and end-plate lesions revealed significant increasing craniocaudal trends with a higher prevalence from the mid to lower thoracic regions, in particular, from T6 to T12 (Table 6.2). In addition, the mid and lower thoracic regions were found to be highly susceptible to vertebral morphological changes of anterior wedging and vertebral compression, evidenced by a low Ha/Hp and Hp/D index respectively, especially with increased age (Study 4.3). The mid and lower thoracic trends for lower Hp/D values appeared to mirror distribution patterns of vertebral compression fracture or deformity in the thoracic spine, where an increased prevalence is commonly reported in these regions (De Smet et al 1988, Hedlund et al 1989, Melton et al 1993, Edmondston et al 1997, Ismail et al 1999).

The prevalence of osteophyte formation did not have an increasing craniocaudal trend, peaking instead at the mid thoracic region, from T5 to T8 (Studies 4.2 and 4.3). These findings for osteophyte formation although consistent with the cadaver study by Nathan
(1962) and the radiological study by Singer et al (1997), were however different from reports from recent radiographic (O’Neill et al 1999) and MRI (Videman et al 1995a) studies, which found a higher prevalence in the lower thoracic region instead. Possible explanations for the difference between Study 4.2 and the MRI study by Videman et al (1995a), may be due the use of T1-weighted films to grade the osteophytes, and the predominantly male twin subjects used in their study. Subjects were also much younger (35 to 69 yrs) compared to the cohort in this thesis (1 to 90 yrs). O’Neill et al’s (1999) study used X-ray films, which may not be comparable with the T2-weighted MRI film analysis used in Study 4.2.

Literature on spinal biomechanics support this predominance of vertebral body deformations and disc degenerative changes in the mid and lower thoracic regions, which represent thoracic regions that are biomechanically at risk to trauma and injury. The mid thoracic region is exposed to both higher rates of rotational strain (Gregerson and Lucas 1967) which particularly increases the potential for anular injury (Farfan et al 1970); as well as the cumulative effect of accentuated axial loads on the apex of the kyphosis (Schmorl and Junghanns 1971, Singer et al 1990). Compression loads in particular also effect gradual shape adaptations of the vertebral body (Schmorl and Junghanns 1971, Goh et al 1999). Similarly in the lower thoracic region, the combination of excessive torsional and compression forces due to the less restricted T11 and T12 thoracic segments with no sternal-rib attachment (Malmivaara et al 1987, Singer et al 1989); and greater axial load (Pal and Routal 1987), increases its potential for disc injury and degeneration, hence the higher prevalence seen in these two thoracic regions.

In the non-degenerate thoracic discs, the upper thoracic region generally had a higher collagen content and extent of Pyd and Dpd compared to the other thoracic regions, except for the mid thoracic region, where the extent of Pyd was highest in the anterior anulus (Table 6.2). There were no significant biochemical trends in the different thoracic regions, except for the lower collagen and higher extent of Pyd noted in the mid thoracic region of the anterior anulus and the lower extent of Dpd in the lower thoracic region for all disc regions, that is anterior and posterior anulus and nucleus. These findings suggest that differences in the distribution of the collagen crosslinks may be related to the normal biomechanical loading experienced due to posture and physical activities over the life span.
Table 6.2 Summary data from Studies 5.3 and 5.4 to show the association of thoracic regional degenerative trends for disc and vertebral body changes and the distribution of collagen and extent of Pyd and Dpd in thoracic nucleus and anulus (nucleus/anulus).

<table>
<thead>
<tr>
<th>Thoracic regions</th>
<th>Upper</th>
<th>Mid</th>
<th>Lower</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRI degenerative features</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteophytes</td>
<td></td>
<td></td>
<td>AF and NP degeneration</td>
</tr>
<tr>
<td>AF and NP degeneration</td>
<td></td>
<td></td>
<td>EP</td>
</tr>
<tr>
<td>EP</td>
<td></td>
<td></td>
<td>Increased bi-concavity</td>
</tr>
<tr>
<td>Increased compression index</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen nmol/mg</td>
<td>1.1/1.7</td>
<td>1.0/1.5</td>
<td>0.8/1.6</td>
</tr>
<tr>
<td>Non-degenerate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyd mol/mol collagen</td>
<td>2.1/1.7</td>
<td>1.9/1.8</td>
<td>1.8/1.7</td>
</tr>
<tr>
<td>Dpd mol/mol collagen</td>
<td>0.07/0.04</td>
<td>0.05/0.03</td>
<td>0.04/0.03</td>
</tr>
<tr>
<td>Pyd:Dpd</td>
<td>30/43</td>
<td>38/60</td>
<td>45/57</td>
</tr>
<tr>
<td>Degenerate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen nmol/mg</td>
<td>0.8/1.3</td>
<td>1.0/1.4</td>
<td>1.0/1.6</td>
</tr>
<tr>
<td>Pyd mol/mol collagen</td>
<td>2.0/1.7</td>
<td>1.75/1.5</td>
<td>1.70/1.40</td>
</tr>
<tr>
<td>Dpd mol/mol collagen</td>
<td>0.08/0.07</td>
<td>0.08/0.07</td>
<td>0.09/0.06</td>
</tr>
<tr>
<td>Pyd:Dpd</td>
<td>25/25</td>
<td>22/21</td>
<td>19/23</td>
</tr>
</tbody>
</table>

6.4.1.4 Spinal regional influences – distribution of elastin crosslinks
Analysis of human spinal discs from the cervical, thoracic and lumbar regions in Study 5.5, confirmed the presence of very small amounts of Des (0.02 nmol/mg dry wt) and Isodes (0.02 nmol/mg dry wt) crosslinks, particularly in lumbar discs (Table 6.1). The present study did not detect any elastin crosslinks in the cervical and thoracic discs, which according to the literature may be due to two limiting factors: the location of the tissue sampled and the amount of tissues used for biochemical analysis.

Johnson et al (1982), using light microscopy to examine for the presence of elastic fibers in cervical and thoracic discs, found these fibers only in the superficial layers of the anulus and nucleus which were attached or connected to the bony vertebral body or hyaline end-plate. No elastic fiber was observed in the body or “bulk” of the anulus and nucleus. However Yu et al (2002) reported the presence of elastic fibers between and within each anular lamellae in bovine spinal discs. Therefore elastic fibers should be found in the bulk of the anulus as well. Mikawa et al (1986) and Buckwalter et al (1976) did not define specific locations for the
elastic fibers when staining lumbar discs, instead they advocated that large quantities of disc tissues (800 mg wet weight) was necessary for analysis and detection of elastin.

These controversial findings on the location of elastic fibers in spinal discs will need further studies to confirm the specificity of location in determining elastic fibers. The anular and nuclear tissues in Study 5.5 were taken from the mid height, transverse sections of the spinal discs, which may have contributed to the negative results for the cervical and thoracic discs. On further analysis of the weight of the tissues sampled, it was also possible that the latter reason proposed by Mikawa et al (1986) and Buckwalter et al (1976) may be the crucial limiting factor, as only elastin crosslinks were detected in lumbar disc samples that had a mean wet weight of more than 500 mg. The cervical and thoracic tissues sampled in this thesis, had a mean wet weight of 25 mg and 254 mg respectively. Even lumbar disc samples that were less than 500 mg wet weight did not yield any elastin crosslinks. The importance of the amount of tissue sampled for elastin crosslinks is an acceptable assumption, as lumbar disc studies have observed that although elastic fibers are present, the amount is however very small, hence difficult to locate, unless there is ample spinal tissue for analysis. Further biochemical investigations on the presence of elastin crosslinks in cervical and thoracic disc tissues should take into consideration the specific disc locations, such as the attachment between the anulus and the hyaline end-plates or vertebral body (1982), as well as the amount of tissues sampled for biochemical analysis and detection (Mikawa et al 1986).

It is difficult to compare data with other lumbar disc studies, as studies by Olczyk (1994b) reported only elastin content and not the amount of Des and Isodes. Most of these studies usually report the concentration of elastic fibers in the tissue (Buckwalter et al 1976, Yu 2002). The small amount of Des and Isodes crosslinks found in Study 5.5 were comparable to the small amounts of Des and Isodes 0.4 and 0.05 amino acid residues per1000 total amino acid residue reported for lumbar disc matrices by Mikawa et al (1986). The highest amino acid present in elastin is proline at 279 amino acid residues per 1000 total amino acid residues. The elastin (Des and Isodes content) to collagen ratio for the lumbar anulus (1:0.04) and nucleus (1:0.07) in this thesis was also similar to that reported by Olczyk (1994b), with a range of 1:0.02 to 1:0.07. From their sample of 182 spines, Olczyk (1994b) found that the amount of elastin was distinctly higher in the anulus compared to the nucleus. The present study (Study 5.5) did not find any difference in the Des and Isodes content between the lumbar anular and nuclear samples, which is also reported by Mikawa et al (1986) for lumbar discs, although Mikawa et al (1986) revealed a higher percentage of Des compared to Isodes in lumbar disc tissues. Current findings are supported by Anwar (1965) who reported that Des and Isodes crosslinks are usually found equally in aorta, cartilage and ligament tissues.
Findings in this thesis, however support reports for lumbar discs, that the elastin content is very small (< 5% dry wt) (Johnson et al 1985, Mikawa et al 1986, Yu 2002).

The small proportion of elastic fibers had in the past eluded various researchers to suggest that it had an insignificant role in the function of the disc (Buckwalter et al 1976, Eyre 1987). However Yu et al (2002) in a recent histological study on bovine discs proposed that these elastic fibers, however few, had an important function to support the collagen fibers in the anular and nuclear matrices, and were specifically arranged within and between the anular lamellae, as well as radially in the nucleus. Various studies have suggested that elastic fibers usually exist with collagen fibers (Minns et al 1973), and are important to enable the latter to recover after deformation (Buckwalter et al 1976, Olczyk 1994b).

Mikawa et al (1986) also proposed that the amino acid composition of the elastic fibers in spinal discs were different from that of other ligament tissues in the body, having varying amounts of amino acid residues except for glycine, isoleucine and phenylalanine. The elastic fibers were also smaller in diameter and more sparsely distributed in the disc compared to the ligamentum flavum, hence may play a different mechanical role. This assumption was challenged by the recent morphological study by Yu et al (2002) who felt that the bovine spinal disc cells were unlikely to synthesise different types of elastin protein, instead the mechanical function of the elastic fibers was determined by the orientation and location in spinal discs. Unlike the random and sparse distribution reported by Mikawa et al (1986), Yu et al (2002) found that elastic fibers were highly organised and oriented radially in the nucleus, and parallel to the collagen fibers in the anulus of bovine spinal discs. They proposed that the radial orientation of the elastic fibers in the nucleus enabled transmission of load to the surrounding anulus, while the criss-crossed elastic fibers between and within the anular lamellae, enable the recovery of the anular lamellae to its original structure after deformation. The limited elastin crosslink data reported in this thesis however is not able to add further to current information on the function of elastic fibers in the disc.

### 6.4.2 Age influences on the mechanism of thoracic disc degeneration

Age as a contributing factor in lumbar disc degeneration (Miller et al 1988, Buckwalter 1995), was also associated with the morphological (Studies 4.2 and 4.3) and biochemical changes (Studies 5.3 and 5.4) observed in thoracic disc degeneration in this thesis. The cumulative effects and adaptations of the thoracic spine to biomechanical demands imposed on it over time, was evidenced by the increased vertebral body bi-concavity and anterior vertebral wedging, typical of a kyphotic posture found in aged female subjects (Study 4.3);
coupled with a higher prevalence of degenerative anular, nuclear changes and osteophytes (Study 4.2) especially in the mid thoracic region. These age-related degenerative changes were also associated with significant biochemical changes in the thoracic disc matrix, depending on whether the discs also had degenerative changes as well. In non-degenerated aged thoracic discs, the collagen content was lower in the nucleus (Study 5.3), but was higher in aged and degenerated tissues, especially in the nucleus (Study 5.4, Table 6.3).

Present findings for non-degenerate aged thoracic discs in Study 5.3 were similar to data reported by most lumbar disc studies (Olczyk 1992, Scott et al 1994, Crean et al 1997). Olczyk (1992) and Scott et al (1994) however reported an increase in collagen content in lumbar disc matrices before maturity, which started to decline with age, especially after the 4th decade. The lower collagen content and extent of Pyd, reported in Study 5.3, in non-degenerate thoracic discs across the life span, provides new normative information for human thoracic discs. Pokharna and Phillips (1998) reported a lower extent of Pyd in aged and degenerated lumbar discs, which were not statistically significant. More importantly, the implications of these findings is that the natural ageing process resulted in a disc matrix with less tensile strength, hence is more susceptible to injury and degeneration (Pearce et al 1987, Pearce et al 1991, Buckwalter 1995, Duance et al 1998, Hutton et al 1998). The tensile function of the disc matrix is determined by the number of collagen crosslinks formed and not the number of collagen fibers (Eyre 1980, Kivirikko and Myllyla 1982).

The net loss of collagen and collagen crosslink Pyd, with increased age in the absence of disc injury or pathology is unexpected, as the half life of collagen is reported to be very long, ranging from 100 to 400 years (Bank et al 1998, Verzijl et al 2000). The low collagen turnover rate in spinal disc matrix is suggested to be due to the poor blood supply to the disc, especially in the nucleus (Eyre 1979, Liu et al 1991, Bartels et al 1998), however it is also this same reduced blood supply which makes the nuclear tissue susceptible to matrix degradation with increased age. In the absence of pathology, a lack of oxygen and nutrients increases the acidity in the tissues resulting in cell apoptosis (Urban et al 1977, Stairmand et al 1991, Horner and Urban 2001). Such an acidic condition will also reduce matrix synthesis (Horner and Urban 2001), decrease cellular metabolism and increase the action of degradative enzymes such as cathepsins (Maroudas 1988). Eyre et al (1988) also proposed that the collagen formed in aged disc matrices are not able to form crosslinks as the activity of lysyl oxidase is decreased with age. It is therefore interesting to note that the extent of Dpd is increased instead. Reasons for this trend will be discussed below in Section 6.4.5. Therefore in the absence of degeneration and a lower collagen content in the matrix, the lower extent of
Pyd is probably due to a higher collagen turnover and a lesser ability of existing collagen fibers to form post-translational crosslinks.

**Table 6.3** Comparison of the mean collagen content (nmol/mg dry wt), extent of Pyd and Dpd (mol/mol collagen) between normal and degenerated thoracic discs, and due to age and gender influences from Studies 5.3 and 5.4.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Nucleus</th>
<th>Anulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>0.80</td>
<td>1.50</td>
</tr>
<tr>
<td>Pyd</td>
<td>2.00</td>
<td>1.70</td>
</tr>
<tr>
<td>Dpd</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>Pyd:Dpd</td>
<td>50:1</td>
<td>65:1</td>
</tr>
</tbody>
</table>

**Non-degenerate (mean for all ages)**

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Nucleus</th>
<th>Anulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td><strong>1.00</strong></td>
<td>*1.30</td>
</tr>
<tr>
<td>Pyd</td>
<td>*1.70</td>
<td>*1.50</td>
</tr>
<tr>
<td>Dpd</td>
<td><strong>0.08</strong></td>
<td><strong>0.07</strong></td>
</tr>
<tr>
<td>Pyd:Dpd</td>
<td>*40:1</td>
<td>*40:1</td>
</tr>
</tbody>
</table>

**Degeneration**

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Nucleus</th>
<th>Anulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>*0.50</td>
<td>1.50</td>
</tr>
<tr>
<td>Pyd</td>
<td>*1.70</td>
<td>*1.50</td>
</tr>
<tr>
<td>Dpd</td>
<td><strong>0.10</strong></td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td>Pyd:Dpd</td>
<td>50:1</td>
<td>*60:1</td>
</tr>
</tbody>
</table>

**Aged discs (> 60yrs) (non-degenerate)**

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Nucleus</th>
<th>Anulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td><strong>1.00</strong>/0.80</td>
<td>*<em>1.80</em>/1.50</td>
</tr>
<tr>
<td>Pyd</td>
<td><em><em>2.20</em>/1.80</em>*</td>
<td><em><em>2.00</em>/1.60</em>*</td>
</tr>
<tr>
<td>Dpd</td>
<td>0.05/<strong>0.07</strong></td>
<td><strong>0.04/0.03</strong></td>
</tr>
<tr>
<td>Pyd:Dpd</td>
<td><em>44:1</em>/26:1</td>
<td><em>50:1</em>/53:1</td>
</tr>
</tbody>
</table>

* = lower value compared to non-degenerate values
** = higher value compared to non-degenerate values

### 6.4.3 Gender influences on the thoracic disc degeneration

From the literature, the influence of gender-related factors on the prevalence of thoracic disc degeneration and vertebral morphometric changes is limited. The present MRI surveys in Studies 4.2 and 4.3, revealed a predisposition towards a higher rate of disc degenerative changes in males, and a higher rate of vertebral body morphology changes in females, especially in the mid thoracic region of the aged female spine. This gender difference in thoracic disc degenerative trends was first observed and proposed by Schmorl and Junghanns (1971), and later supported by more recent MRI studies (Wood et al 1995), population surveys (O’Neill et al 1999) and radiological studies (Singer 2000). A similar predominance
of disc degeneration in males had also been reported for the lumbar and cervical discs (Lawrence 1977, Miller et al 1988), however Lawrence (1977) reported a higher prevalence of thoracic disc degenerative changes in the female population instead.

A possible reason for the contrasting findings between current MRI data and Lawrence’s (1977) data may be due the use of X-rays instead of MRI to examine for disc degenerative changes. The ability of X-rays to detect soft tissue changes is not as accurate as MRI, and is better suited for assessing vertebral bony changes instead (Modic et al 1984, Kaiser and Ramos 1990, Rosenbloom 1991). Using MRI in Study 4.3, the prevalence for vertebral body deformities was higher in aged female subjects, and may have biased the radiographic findings by Lawrence’s (1977) population study, which unfortunately did not provide data on thoracic disc degeneration to compare the age and gender trends.

According to Schmorl and Junghanns (1971), while cumulative compressive and torsional stresses over time induces degeneration of the anterior anular fibers, particularly in males, in contrast, the effect on the female spine is usually a deformation of the vertebral body. This phenomenon was evidenced by the higher prevalence of anterior vertebral wedging and osteophyte formation in the mid thoracic region of the female spines noted in Study 4.3. Hormonal changes leading to gender-related bone loss particularly in females may be the key reason for the higher prevalence of vertebral morphologic changes in the thoracic spine (Schmorl and Junghanns 1971, De Smet et al 1988, Kanis and McCloskey 1992, Goh et al 2000b). In addition, the vertebral body in females tended to be more slender and smaller, therefore may be more prone to vertebral body deformity (Brandner 1970).

Thoracic vertebral body and discs are generally larger in males compared to females (Brandner 1970, Singer and Goh 2000), which is also similar for lumbar discs (Miller et al 1988). It is possible that gender-related degeneration might be related to the larger disc size in males compared to females. The large disc size increased the diffusion distance for oxygen and nutrition through the disc matrix, especially to the nucleus, hence reduces disc nutrition and increased the susceptibility to degeneration (Urban et al 1977, Stairmand et al 1991, Horner and Urban 2001). Another reason for the propensity for disc degeneration in males may be due to the more frequent exposure to vigorous occupational and recreational activities, imposing cumulative compressive and torsional stresses on the discs over time (Miller et al 1988, Swärd et al 1991, Videman et al 1995b, 1997, Riihimaki et al 1998).

Biochemically, the main gender difference in non-degenerate thoracic disc matrices was a higher collagen content and extent of Pyd in young males, but a lower Pyd/Dpd ratio
compared to females (Table 6.3). The literature is limited on gender-related biochemical data from which to compare. Zanze et al (1997) in their study of healthy infants and children (8.5 to 27.5 months) suggested that boys generally had an overall higher extent of collagen crosslinks in the body, compared with girls. They speculated that young girls (2 years old) had a higher rate of collagen degradation and a decreased rate of collagen formation. Using data for older subjects, it was observed that the early gender bias in childhood was reversed with age and degeneration, such that males had lower extent of Pyd and a lower collagen content (Study 5.4), hence higher turnover rate. Only the distribution of extent of Dpd in non-degenerate and degenerate discs did not show any gender trends. Cahoon et al (1996) investigating gender differences in bone turnover rates in adult rhesus monkeys also reported no gender differences in the turnover of Pyd and Dpd in bone degradation.

In summary, the series of MRI and biochemical investigations in Studies 4.2, 4.3, 5.3 and 5.4, supports gender-related MRI trends in thoracic disc degenerative and vertebral shape changes, such that although the younger male disc matrix may have a higher collagen and extent of Pyd and Dpd compared to females, however with age, this trend is reversed. Whether this gender and age trend in the biochemical matrix increases the propensity to disc degenerative changes in males, noted in Study 4.2, is speculative at this stage. Similar decreasing trends were noted also for older females, however the loss of collagen and collagen crosslinks were not as significant as in males. Therefore the increased vertebral body changes in older female subjects is not accompanied by significant changes in the disc collagenous matrix, especially if there is no degeneration in the discs. Although the mechanism for these gender biases was not investigated in this thesis, drawing from biomechanical spine studies, it is possible that the greater frequency of exposure to and accumulation of, repetitive and sustained torsional and compression forces in males, and the tendency to accelerated spinal bone loss, due to hormonal and physiologic systemic changes in females, may be some of the gender-related causes for thoracic disc and vertebral body degeneration trends observed in this thesis. The effect of hereditary and sex-linked genetic pathologies may also be other reasons for the gender-related degeneration trends (Cassinelli et al 2001). However these issues were beyond the scope of this thesis.

6.4.4 Comparison of radiological, macroscopic and biochemical changes in thoracic disc – pathomechanism of disc degeneration

The series of MRI and biochemical studies on thoracic discs reported in Studies 4.2 and 4.3 and Studies 5.3 and 5.4, respectively, provided survey data which were not directly correlated with each other. However, the case studies reported in Study 5.6 provided a preliminary
comparison of the biochemical composition of the thoracic intervertebral disc with the degenerative changes observed using MR images and macroscopic examinations. Additionally these two unfixed cadavers enabled an investigation on the distribution of proteoglycans, which was not possible with formalin-fixed tissues (Chapman et al 1990, Toledo et al 1996). This difficulty to analyse PG in fixed tissues was evident when a pilot study was conducted on formalin-fixed tissues, which showed that PG in these tissues could not be digested with papain and yielded negative results for CS. The association of MR and macroscopic findings with matrix biochemical constituents in thoracic discs has not been previously reported in the literature.

Results from the preliminary comparative study (Study 5.6), showed that degenerative changes noted in MR images as well as macroscopic changes in the nucleus, were associated with a higher collagen but a lower CS content, which is similar to biochemical findings reported in Study 5.4 and other lumbar disc studies (Table 6.4). The nuclear findings were consistent with lumbar disc studies which also reported a decrease in CS in the degenerated lumbar nucleus (Thompson et al 1988, Pearce et al 1991, Tertti et al 1991, Olczyk 1994a). In contrast, degenerative anular changes on MRI and macroscopic inspection, were associated with a lower collagen but a higher CS content (Study 5.6). The finding for the higher CS content in degenerated thoracic anular tissue has not been reported, although generally lumbar disc studies suggest a lower CS content (Thompson et al 1988, Inkinen et al 1998). Olczyk (1994a) found a decrease in the CS of lumbar anular tissues with age, but did not investigate changes in the anular matrix with degeneration. Pearce and Grimmer (1983) also reported no change in chondroitin 6-sulphate but a decrease in keratan sulphate in degenerated lumbar discs.

From the summary data in Tables 6.2 and 6.3, it is apparent that age, gender and spinal regional differences are associated with changes in the distribution of collagen and collagen crosslinks, Pyd and Dpd in non-degenerate thoracic anulus and nuclear matrices. Whether degenerative processes would expedite or alter the normal age changes in the disc collagenous matrix was investigated in Study 5.4. The high prevalence of osteophytes, end-plate lesions and disc degeneration in the mid and lower thoracic regions observed on MRI, could be associated with the higher collagen content and extent of Dpd, but lower extent of Pyd, especially in the degenerated thoracic anulus (Study 5.4). When examining degenerated tissues, the differences in collagen and collagen crosslinks in the thoracic matrix, due to gender and thoracic regional influences were not so obvious. The main difference in the biochemical matrix between normal aged and degenerated thoracic disc matrices is a higher collagen content in degenerated thoracic discs.
Table 6.4 Summary data for the collagen content (nmol/mg dry wt), extent of Pyd and Dpd (mol/mol collagen) and Des and Isodes content (nmol/mg dry wt) from various lumbar spine studies, compared with thoracic data from the Study 5.4 (Thesis 2004) for degenerated nuclear and anular tissues.

<table>
<thead>
<tr>
<th>Biochemical matrix</th>
<th>Nucleus</th>
<th>Anulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pearce et al 1991</td>
<td>Collagen 0.8</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>PG (μmol/g) 73</td>
<td>NR</td>
</tr>
<tr>
<td>Olczyk 1992, 1994</td>
<td>Collagen 1.0</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Elastin (mg/g) 18</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>PG (μg/mg) 150</td>
<td>70</td>
</tr>
<tr>
<td>Duance et al 1998</td>
<td>Pyd 1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Pokharna &amp; Phillips 1998</td>
<td>Pyd 3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Thesis 2004</td>
<td>Collagen 1.00</td>
<td>1.30</td>
</tr>
<tr>
<td></td>
<td>Pyd 1.70</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>Dpd 0.08</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Pyd:Dpd &gt;50:1</td>
<td>&gt;65:1</td>
</tr>
<tr>
<td></td>
<td>CS (μg/mg) 67</td>
<td>103</td>
</tr>
</tbody>
</table>

NR = not reported

The degenerated thoracic disc was also associated with a higher extent of Dpd, but no significant change in the extent of Pyd, although the latter was lower in the degenerated disc matrix (Table 6.3). The extent of Pyd was not significantly affected by degeneration status as much as ageing, where there was a significantly lower extent of Pyd in non-degenerate aged thoracic discs. There is also a need to consider that an increase in collagen content in the degenerated nucleus could be a result of a decrease in other biochemical components, such as water, proteoglycans and other non-collagenous disc proteins (Urban and Maroudas 1980, Pearce et al 1991, Duance et al 1998), which were not measured in the present study. Little is known about the relationship between collagen crosslinks and the interaction of these crosslinks with the other biochemical components of the extracellular matrix. These findings are similar to other lumbar disc studies, which have reported an increase in collagen with degeneration (Pearce et al 1991, Olczyk 1992, Scott et al 1994), and no significant changes in the extent of Pyd (Duance et al 1998, Pokharna and Phillips 1998, Kaapa et al 2000). The degenerated thoracic disc matrices had an increased collagen content, which was associated with a lower extent of Pyd and a higher extent of Dpd (Study 5.4). A possible reason for this observation may be due to a change in the collagen type, as degenerate discs then to have
more collagen Type I compared to Type II fibers, which may affect the Pyd crosslink density in the matrix.

The lack of a consistent degeneration trend in the various age groups in Study 5.4 also suggests that degeneration in the disc matrix is a dynamic process where tissue breakdown and repair occurred simultaneously (Kang et al 1996, Crean et al 1997). According to Roberts (2002), the normally avascular disc is observed to have an increase in blood vessels and innervation with degeneration and disease. Without the proliferation of vascular tissues, Schmorl and Junghann (1971) proposed that it was not possible to have regeneration of tissue. The increase in reducible collagen crosslinks in degenerated and aged nuclear tissues (Olczyk 1992) provides evidence that there is matrix repair and regeneration in progress, as these crosslinks are not usually present in normal aged discs, being replaced by the matured insoluble crosslinks instead (Eyre et al 1984b). The tissue inflammatory process might stimulate and encourage new collagen synthesis in the disc matrix while other matrix metalloproteinases resulted in tissue breakdown instead (Pearce et al 1987, Kang et al 1996, Crean et al 1997, Hutton et al 1998, Pokharna and Phillips 1998), such that at any given point of tissue sampling, no two degenerate disc tissues were in a similar biochemical state of synthesis and degradation even though they may appear morphologically similar. In addition, longitudinal spinal studies in animals have shown that morphological degenerative changes usually preceded biochemical changes in the disc matrix, as the latter might take more than eight months to occur (Olsewski et al 1996, Hutton et al 1998, Hutton et al 1999, Lotz and Chin 2000). This time lag required for changes in the biochemical disc matrix to occur further supports the varied biochemical changes noted in the degenerated thoracic disc matrices in the different age groups in Study 5.4, which is difficult to authenticate using cross-sectional surveys.

The finding that the extent of Pyd decreases with age and did not increase with degeneration in thoracic disc matrices is puzzling, as the degenerated matrix usually has a higher collagen content (Olczyk 1992, Kaapa et al 2000) and a higher amount of reducible collagen crosslinks (Olczyk 1992), which are the precursors to the mature collagen crosslinks (Eyre 1980). A possible reason for the non-significant Pyd changes in the present thoracic disc study might be due to the small number of degenerated anular and nuclear samples analysed (76 degenerated discs of 303 samples in Study 5.4). In addition, the majority of Grade III anular samples were only found in the Old age group, making it difficult to determine degeneration effects without the influence of age. However, when data were analysed using samples only in the Old age group (> 60 years old), similar non-significant results were noted for Pyd with
increasing degeneration grade, thus confirming the findings for Pyd in degenerated thoracic disc matrices.

Other possible reasons for the lack of change in the extent of Pyd may be due to a higher collagen Type I content compared to Type II in the degenerated disc matrix or a preferential increase in the extent of Dpd instead, which is noted in Study 5.4. Collagen Type I has less crosslinking ability compared to Type II. Kaapa et al (2000) however found no change in both Pyd and Dpd in degenerated lumbar anular tissues. Besides the data from Eyre et al (1984a, 1995) there is very limited information on the distribution of Dpd in spinal discs to date. Reasons for the preferential formation of Dpd instead of Pyd will be discussed below.

6.4.5 Changes in Deoxypyridinoline – function in the disc

Deoxypyridinoline or lysylpyridinoline, is usually analysed as a bone marker with very few studies reporting its presence in spinal discs, probably because its content in spinal discs is very low (Table 6.1). An earlier study by Duance et al (1998) did not detect any Dpd in lumbar discs. Typically, Dpd was identified as a marker of collagen turnover in bone studies (Eyre et al 1984a, Randall et al 1996) and found more commonly in calcified tissues (Bailey et al 1998). However Eyre et al (1984a, 1995) has reported data on Dpd in spinal discs which are comparable with current thesis findings. The mean extent of Dpd in non-degenerate thoracic anular tissues is 0.04 mol/mol of collagen and 0.06 mol/mol collagen for the nucleus (Study 5.3). The average ratio of Pyd to Dpd was 50:1 in non-degenerate thoracic discs, which was comparable with the >50:1 ratio reported by Eyre (1995) for spinal discs, and similar to the ratio for cartilage tissues (40:1).

While the extent of Pyd was lower in aged thoracic disc tissues, the extent of Dpd showed a contrasting trend, and was significantly higher with both age and degeneration. The findings in Studies 5.3 and 5.4 for the extent of Dpd in non-degenerate and degenerated spinal thoracic discs provided new data in the literature (Table 6.4). Takahashi et al (1995) however reported no significant changes in the extent of Dpd in other human tissues, such as bone, cartilage, ligament, tendon, meniscus and muscle tissues with increasing age. The reason for the contrasting trends in the two collagenous crosslinks, Pyd and Dpd, is not known and the specific role of Dpd in the collagen network of the matrix has not been reported, apart from the fact that it is one of the pyridinium crosslinks present (Eyre 1987). The higher extent of Dpd with age and degeneration observed in thoracic discs in this thesis does suggest that it was preferentially formed or accumulated in disc tissues, especially in thoracic anular tissues. However what facilitates the formation or destruction of the pyridinium crosslinks remains
unknown (Last et al 1990). According to Last et al (1990), the higher extent of Dpd in
degenerated anular tissues suggested a bias or a competition for the same hydroxylysine
residues, which favoured the Dpd pathways.

During collagen synthesis, the type of collagen crosslink to be formed is committed
intracellularly during the hydroxylation stage (Eyre et al 1984b, Bailey et al 1998). Both the
enzymes prolyl-4-hydroxylase and lysyl hydroxylase are important as they influence
hydroxylation of lysine molecules intracellularly, hence providing sites for carbohydrate
attachment, preparing the collagen molecule for crosslinking extracellularly (Kivirikko and
Myllyla 1982, Robins 1982, Eyre et al 1984b); and for hydroxylation of proline residues,
which provide thermal stability to the collagen triple helix structure at body temperature
(Prockop et al 1979, Eyre 1980). Pyridinoline is formed from three hydroxylysine residues
whereas Dpd is formed from one lysine and two hydroxylysine residues (Eyre et al 1984b).
Therefore more hydroxylysine residues are required to form Pyd compared with Dpd.

According to Eyre (1979), collagen Type II has a higher number of hydroxylysine residues,
hence it has twice the crosslinking ability compared to Type I fibers. In addition, Type I
collagen lacks a lysine residue at the aldehyde site in its C-terminal telopeptide, which may
reduce the number of crosslinking sites available (Eyre 1988). In the degenerated disc matrix,
the number of collagen Type I fibers synthesised during matrix repair is higher than Type II,
hence the number of hydroxylysine residues available for crosslinking is reduced (Burgeson
1982). Collagen Type I fibers have been reported to increase in tissues under sustained
compression loading, such as disc tissues on concave side of scoliotic spines (Bushell et al
1979, Brickley-Parsons and Glimcher 1984); and during induced compression in animal
spines (Hutton et al 1998, Lotz and Chin 2000). In addition, these studies reported a lower
proportion of collagen Type II fibers in the degenerated disc matrix (Hutton et al 1998, Lotz
and Chin 2000). Type II fibers were instead increased on the convex side of scoliotic spines
(Bushell et al 1979, Brickley-Parsons and Glimcher 1984). It may be speculated that this
reduced hydroxylysine residue in the extracellular matrix, due to the higher collagen Type I
fibers in degenerated matrices, may reduce the formation of Pyd. It is proposed that the
increased in Dpd instead is probably due to the lack of lysyl hydroxylase activity.

The differential formation of Pyd and Dpd is influenced by the enzyme, lysyl hydroxylase in
the nascent α1 chains, during hydroxylation intracellularly (Prockop et al 1979, Eyre et al
1984b), with a lack of the enzyme favouring the formation of Dpd (Bailey et al 1998). Kaapa
et al (2000) only reported an increase in prolyl 4 hydroxylase in degenerated disc matrices,
however the other enzyme responsible for hydroxylation of lysine residues is lysyl
hydroxylase, which was not measured in their study. According to Kaapa et al (2000), the increase in prolyl 4-hydroxylase activity in degenerated lumbar disc tissues, did not seem to increase the extent of Dpd, hence it could be that lysyl hydroxylase is the key enzyme determining the type of collagen crosslinks formed extracellularly. Lysyl hydroxylase is usually present in younger nuclear matrices hence the higher Pyd compared to Dpd in these matrices. However in the aged disc matrix, the crosslink pathways are reversed with a preference for Dpd pathways instead (Eyre et al 1984b). According to Eyre et al (1984b), the action of lysyl hydroxylase is dependent on the type of tissue and not as much on the collagen type. Similar results have been reported in clinical disease syndromes, such as Ehlers Danlos syndrome VI, where lysyl hydroxylase enzyme deficiency was associated with a higher extent of Dpd in the tissues (Eyre et al 1984b, Bailey et al 1998). Therefore the higher extent of Dpd instead of Pyd in aged and degenerated discs is probably due to the decrease in lysyl hydroxylase in the matrix, hence reduced hydroxylation of lysine residues intracellularly; coupled with a lower number of hydroxylysine residues available to form Pyd crosslinks extracellularly due to the higher collagen Type I fibers instead of Type II fibers.

Another factor to consider is the degree of calcification in the tissues. According to Bailey et al (1998), Dpd was also more commonly found in calcified tissues. Therefore calcification in the degenerated disc matrix may encourage the formation of Dpd instead of Pyd. However Hoshino et al (1995) reported a lower extent of Dpd in calcified aortic tissues compared to Pyd, which was higher instead. They also proposed that calcification is able to influence collagen crosslinking at the intracellular level, such that lysyl hydroxylase prevented the formation of the deoxy form of collagen crosslinks. Current findings for calcified spinal LF tissues in Study 5.6 concur with Hoshino et al (1995), where the extent of Dpd and Pyd were observed to be lower in calcified tissues. According to Eyre et al (1984b), typically mineralisation immobilises the collagen molecules, and hinders crosslinking at the collagen telopeptide sites, however which crosslink is hindered is speculative at this stage. Current findings in spinal LF and the literature show a trend of reduced Dpd in calcified soft tissues, which is different for bony tissues. It is possible that mineralisation effects between bone and soft tissues are different and reasons for this contrasting finding is beyond the scope of this thesis. The contrasting age and degeneration trend between Pyd and Dpd in thoracic discs were unexpected findings and may suggest that Dpd can be identified as a biochemical marker of ageing and degeneration in thoracic or spinal disc matrices.

Various authors have suggested that the major function of Pyd is to provide tensile support to the disc matrix (Eyre et al 1989, Bailey et al 1998, Duance et al 1998), therefore it is important to maintain these collagenous crosslinks in the disc matrix. In Studies 5.3 and 5.4,
the extent of Pyd and Dpd increased initially from the Child to the Young age groups. Subsequently, the only collagen crosslink that was noted to increase in the older age groups and with degeneration was Dpd. Despite the significant increase in the extent of Dpd with age and degeneration, the ability of Dpd to provide support to the collagen network and compensate for the lower extent of Pyd is questionable. This is because the amount of Dpd (0.04 mol/mol collagen) present is 40 to 50 times less than Pyd (2 mol/mol collagen), which is the major collagenous crosslink in the disc matrix (Eyre 1995). Deoxypyridinoline probably has more influence on the matrix of bony tissues, where the Pyd:Dpd ratio is lower 3.5:1 (Eyre 1995). In degenerated discs, especially in the anulus, the Pyd:Dpd ratio decreased, dropping to 25:1 in both severely degenerated and aged anuli and nuclear samples, which is almost similar to that observed in articular cartilage tissues (Eyre 1995). The function of Dpd in the disc is not conclusive, however its small amount notwithstanding, it may still play an important role in sustaining the aged and degenerated disc matrix, similar to the small amount of elastic fibers in the disc, mentioned previously.

6.5 BIOCHEMICAL CHANGES IN SPINAL LIGAMENTUM FLAVUM

6.5.1 Comparison between ligament and spinal disc tissues

The literature has limited information on the biochemical constituent of spinal LF tissues, especially in terms of elastin and collagen crosslinks. Current findings in Study 5.5 provide original data on collagen and elastin crosslinks in spinal LF tissues from the cervical, thoracic and lumbar regions (Table 6.5). In contrast to the findings for the disc, Des and Isodes crosslinks were easily detected in the extracellular matrix of the LF in Study 5.5. The spinal LF had a significantly higher amount of elastin crosslinks, more than 400 times more, compared to the lumbar discs. This finding is consistent with results using staining of elastic fibers in spinal disc and ligament tissues by Mikawa et al (1986). The LF tissues also had higher extents of Dpd compared to the lumbar discs, but had lower collagen content and extent of Pyd (Study 5.5). The higher collagen content in the anulus compared to the ligament was consistent with observations on collagen fibers by Mikawa et al (1986). The LF also had significantly lower CS content compared to the anulus and the nucleus (Study 5.6). This finding is not surprising as PG is mainly required in tissues that bear large compression forces, such as in articular cartilage and the nucleus pulposus (Eyre 1979, Culav et al 1999).

The Pyd to Dpd ratio of the spinal LF averaged at 7:1, which is closest to that reported for muscle tissues (6:1) (Eyre 1995), and may suggest a tissue function which is similar to such tissues, which is to withstand large contractile and tensile forces during movement. The spinal LF is responsible in restoring the spine to the erect position after spinal flexion and extension.
(Yong-Hing et al 1976); to prevent buckling or compression of the spinal cord in the spinal canal (Nachemson and Evans 1968) and to provide spinal stability during movement (Ponseti 1995). It also pre-stresses the intervertebral disc to assist in maintaining the disc height (Nachemson and Evans 1968). In comparison, the Pyd:Dpd ratio is much higher in spinal disc tissue (> 50:1), whose function is to resist compression and tensile forces; but lower in bone tissues (3.5:1) (Eyre 1995), where compression forces are predominantly supported by the osteochondrocytes and calcium deposits. Comparison of the collagen and elastin data between spinal disc and LF provided preliminary data, which had not been reported for human LF tissues in the literature previously.

Table 6.5 Summary data for collagen content, extent of Pyd and Dpd in various human tissues from the literature and Studies 5.5 and 5.6 (Thesis 2004).

<table>
<thead>
<tr>
<th>Studies</th>
<th>Tissues</th>
<th>Nucleus Des</th>
<th>Anulus Des</th>
<th>Spinal LF Des</th>
<th>Articular cartilage</th>
<th>Muscle Des</th>
<th>Cortical bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eyre et al</td>
<td>Pyd</td>
<td>2.6 to 2.8</td>
<td>1.6 to 1.9</td>
<td>*0.41</td>
<td>1.5 to 2.3</td>
<td>ND</td>
<td>0.26</td>
</tr>
<tr>
<td>1984,1995</td>
<td>Dpd</td>
<td>&lt; 0.03</td>
<td>&lt;0.03</td>
<td>ND</td>
<td>&lt;0.03</td>
<td>6:1</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Pyd:Dpd</td>
<td>&gt;50.1</td>
<td>&gt;50:1</td>
<td>*14:1</td>
<td>&gt;40:1</td>
<td>3.5:1</td>
<td></td>
</tr>
<tr>
<td>Takahashi et</td>
<td>Pyd</td>
<td>*0.70</td>
<td>0.9</td>
<td>0.22</td>
<td>0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>al 1995</td>
<td>Dpd</td>
<td>*0.05</td>
<td>0.08</td>
<td>0.06</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chen et al</td>
<td>Pyd</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1999</td>
<td>Dpd</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pyd:Dpd</td>
<td>4:1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Des</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Isodes</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(mmol/mol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thesis</td>
<td>Collagen</td>
<td>0.80</td>
<td>1.50</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2004</td>
<td>Pyd</td>
<td>2.00</td>
<td>1.70</td>
<td>0.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dpd</td>
<td>0.05</td>
<td>0.03</td>
<td>0.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pyd:Dpd</td>
<td>50:1</td>
<td>65:1</td>
<td>7:1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Des</td>
<td>0.02</td>
<td>0.02</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(nmol/mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Isodes</td>
<td>0.03</td>
<td>0.02</td>
<td>12</td>
<td></td>
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<tr>
<td></td>
<td>(nmol/mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CS (µg/mg)</td>
<td>67</td>
<td>103</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
From the literature, the ratio of Pyd: Dpd in the ligament matrix is variable ranging from 4:1 reported by Chen et al (2000), to 14:1 by Takahashi et al (1995), with Study 5.5 reporting a ratio of 7:1 (Table 6.5). Reasons for the different results may be due the different ligament tissues and standards used, as Takahashi et al (1995) and Eyre et al (1984a) did not specify what type of ligament tissue or standards were used for analysis. Another reason for the varied results may be due to the different method of analysis for the collagen crosslinks, as early studies by Eyre et al (1984a) did not detect any Dpd in the tissues, which due to the small amount present, required more sensitive methods of identification and analysis.

6.5.2 Spinal regional influences on ligamentum flavum tissues

From Study 5.5, it was observed that the LF in the lumbar region had the highest elastin crosslink content compared to the cervical and thoracic regions. According to Ponseti (1995), the large elastin content of the LF enables it to assist in vertebral motion and provide intrinsic stability to the spine, therefore elastin was most abundant in mammals requiring greater spinal mobility compared to those with relatively less spinal motion. White and Panjabi (1990a) also found that the LF in the lower thoracic region had the greatest tensile strength in the spine. Similar high amounts of elastin content were also reported in the lower thoracic and lumbar regions of the posterior longitudinal ligaments by Nakagawa et al (1994). Therefore the presence of a high amount of elastin and collagen crosslinks in the lumbar spinal LF supports the above assumptions, as the lower thoracic and lumbar regions are exposed to increased mechanical stresses due to the greater spinal flexion and extension range of motion (White and Panjabi 1978). These regions also bear large compression loads due to the weight of the upper body (Pal and Routal 1987, Edmondston et al 1997).

Although the cervical region is also very flexible, with a similar range as the lumbar region (White and Panjabi 1978), the amount of compression load that it bears is lower than in the lumbar region, hence the lower amount of elastin crosslinks observed in the cervical ligaments. Study 5.5 also provided new data on the collagen and elastin crosslink content for cervical and thoracic spinal LF, and the influence on age, gender and calcification on the LF matrices, which has not been previously reported.

6.5.3 Age influences in ligamentum flavum

The increase in age was also accompanied by an increase in collagenous content but no significant change in the elastin crosslinks, although there was a decreasing trend (Study 5.5). This age-related finding for elastin crosslinks in this thesis was consistent with other studies.
(Yong-Hing et al 1976, Mikawa et al 1986, Kashiwagi 1993, Chen et al 2000), which also reported a decreasing trend that was not statistically significant. However only the elastin crosslinks in the lumbar region was statistically lower with increased age in Study 5.5. This latter finding is consistent with studies involving other connective tissues, such lung tissues (Starcher 1977), skin (Pearce and Grimmer 1972) and lumbar anular tissue (Johnson et al 1985, Olczyk 1994b), which reported significantly lower elastin crosslinks with increased age.

Various studies also report an increase in the collagen content, in particular collagen Type II content in aged (Kashiwagi 1993) and calcified (Yoshida et al 1992, Specchia et al 2001) LF tissues. There is generally an increase in the stiffness or a loss of elastic resilience in the ligament (Nachemson and Evans 1968: Starcher, 1977 #314) and disc tissues (Olczyk 1994b) with increased age. Nachemson and Evans (1968) reported a significant decrease in the Modulus of Elasticity for lumbar LF with increasing age, from 1,000 kg/cm2 in the young to 200 kg/cm2 in the aged. Minns et al (1973) also reported a higher tensile strength in tissues with a higher collagen compared to elastin content. Therefore the change in elasticity in the ligament tissue may be due to the increase in the collagen content accompanied by a decrease in elastin crosslinks, which was observed in this thesis. The significant age-related finding in the lumbar region suggests that changes in the distribution of elastin content is affected by a combination of mechanical (spinal flexibility) and age-related biochemical degeneration of tissues, compared to the cervical and thoracic regions.

### 6.5.4 Gender influences on biochemical components in ligamentum flavum

Significant gender-related trends were noted in the distribution of elastin crosslinks in the spinal LF tissues. Female ligamentum tissues had significantly higher elastin and collagen content compared to males. When regrouped for spinal regions, male LF generally had higher collagen and collagen crosslinks compared to females in the lumbar region, but lower elastin crosslinks. This finding contrasts with Mikawa et al (1986), who found no gender differences in the elastin content of spinal LF. Reasons for this gender trend, which was similar for all age groups, has not been previously described and there is also very limited information on gender-related differences for the elastin content in spinal LF tissues.

From the literature males generally are more likely to be exposed to greater spinal mechanical stresses during occupational and recreational activities (Swärd et al 1991, Riihimaki et al 1998, Videman and Battié 1999, Hartvigsen et al 2001), therefore it is puzzling that the elastin and collagen content in the male spinal LF was lower than that found in female LF
tissues in Study 5.5. This finding may suggest that flexibility rather than compressive strength is the main determinant for elastin content in the tissues. Ponseti (1995) as mentioned earlier, also proposed that tissues with great flexibility are usually accompanied by a high elastic content in the matrix. It is possible that the more slender female spine (Brandner 1970), with its greater spinal flexibility (Van Herp et al 2000) compared with males might be the determining factor influencing this gender-related difference in the spinal LF, hence the higher elastin content noted in the females and the lumbar region compared to males.

### 6.5.5 Comparison of calcification and biochemical changes in the ligamentum flavum

In Study 5.6, although the LF tissues were not graded by MR images, however macroscopically the tissue was examined for signs of degeneration in terms of the amount of calcification present. In Study 5.5, the Des and Isodes content did not change with increasing age, however when there was calcification present in the LF tissues, noted in Study 5.6, there was a higher elastin crosslink content in the LF matrices. There was also an associated increase in the collagen content but no change in the Pyd and Dpd content in calcified spinal LF tissues. The higher elastin content in calcified LF has not been reported in the literature, although studies in other connective tissues have suggested that calcification inhibits spontaneous crosslinking formations (Bailey and Peach 1971, Walters and Eyre 1983, Hoshino et al 1995, Bailey et al 1998).

Reasons for the increased elastin crosslink is not known, as generally Des and Isodes have been reported to decrease with age (Chen et al 2000). Normal LF tissues consisted of collagen Type I fibers and did not have collagen Type II fibers (Yoshida et al 1992, Specchia et al 2001), which was found to predominate in aged and calcified LF tissues (Kashiwagi 1993, Specchia et al 2001). According to Specchia et al (2001) and Kashiwagi (1993), the presence of collagen Type II cells in calcified LF tissue matrix provided evidence that there was higher chondrocyte and osteoblast cellular activity, probably resulting in ossification of the LF tissues. The increase in elastin crosslinks in calcified LF tissues may be due to the hypertrophic chondrocytic activity resulting in an increase in matrix components, such as collagen Type II and elastin crosslinks. The increase in collagen Type II fibers may account for the higher collagen content in calcified tissues, however this was not accompanied by an increase extent of Pyd or Dpd in Study 5.6.

It is also possible that the increase in elastin crosslinks in calcified ligament tissues may be due to a decrease in other matrix biochemical components, such as water and other glycoproteins. Collagen and CS were noted to be increased in calcified LF. Olczyk (1994b)
also reported a higher elastin content in degenerated nuclear lumbar tissues. Confirmation of these biochemical findings is recommended with more calcified samples, especially of grade III severity. In the present study, only 3 of the 23 LF tissues were categorised as severely calcified.

6.6 SUMMARY

Spinal disc degeneration is a complex phenomenon. From the literature, it was identified that the main contributors to spinal disc degeneration were age, spinal level, gender and altered or abnormal mechanical loading history or trauma. The contribution of these factors in altering the disc matrix and precipitating degenerative changes in the disc was outlined in Figure 2.19 in Chapter 2. The series of MR and cadaver investigations on human thoracic discs provided new data, which further support the observed patterns of thoracic disc degenerative and vertebral morphologic changes.

Findings from the cross-sectional MRI studies in Chapter 4 support the use of MR imaging to reliably examine for disc degenerative changes as well as the pattern of vertebral body shape changes in the thoracic spine. In particular, gender-related vertebral body changes in the mid thoracic region provide evidence that the female thoracic spine is highly susceptible to gender-linked accelerated bone loss and compressive loads acting on the apex of the thoracic kyphosis over the life span. In addition, the predominance of thoracic disc degenerative changes, especially osteophytes, in the mid and lower thoracic regions may reflect the greater and sustained exposure to biomechanical stresses acting on the thoracic column, especially from vigorous, repetitive and cumulative compression and torsional forces. These stresses in the mid and lower thoracic regions may be created by the typically kyphotic posture, and from daily occupational and recreational activities over the life span (Swärd et al 1991, Videman et al 1995b, 1997, Riihimaki et al 1998), respectively. End-plate lesions did not show any age-related trends, and showed equal prevalence in all age groups, especially after 20 years of age. These trends were also reported by other cadaver (Nathan 1962), radiographic (Singer 1997) and MRI (Wood et al 1995) studies on the thoracic spine.

Cross-sectional studies on the macroscopic degeneration status, and collagen and elastin crosslink content of thoracic discs, were examined in Chapter 5. Results from Study 5.1 support the use of formalin-fixed tissues to analyse the collagen content and extent of Pyd in spinal tissues. However, results for the elastin crosslinks content in formalin tissues were different from that reported by Abe et al (2003), and will require future studies to confirm these findings.
Data from the series of studies in Chapter 5 also provided new information mainly for the collagen and collagen crosslinks Pyd and Dpd, in human thoracic discs. The summary of biochemical constituents observed in non-degenerate and degenerate thoracic disc matrices in this thesis is shown in Figure 6.1. The collagen, CS content and extent of Pyd in the thoracic annular and nuclear matrices are similar to that reported in the literature for lumbar discs (Eyre 1995). The elastin content was only identified in the larger lumbar discs, with none of the thoracic discs yielding any result, probably due to the small tissue size. A novel finding from this series of biochemical studies is the influence of age, gender, degeneration and spinal region on the extent of Dpd in thoracic discs (Studies 5.2 to 5.6). Information on the extent of Dpd extends the data provided by Eyre (1995) for spinal disc tissues.

![Figure 6.1 Summary of the biochemical constituents of the normal or non-degenerate and degenerate annulus and the nucleus using data from this thesis.](image)

Data from the cadaver series of non-degenerate thoracic discs in Study 5.3 demonstrated significant changes in the disc collagenous matrix, probably in response to biomechanical loading, which may be further aggravated by gender-related hormonal changes and physical activities over the life span. Generally the thoracic discs in younger males had a higher collagen content and extent of Pyd compared to females. Within the thoracic spine, the biochemical matrix in each thoracic region were almost similar, except in the anterior annulus of the mid thoracic region, where collagen content was higher and the extent of Pyd was lower. The age-related trend in normal thoracic discs with less collagen content and extent of Pyd, was suggestive of a disc matrix with less strength, rendering it more susceptible to degeneration from normal postural and activity-related biomechanical forces.

While it was difficult to differentiate age-related from degenerative changes using morphological findings, however results from Studies 5.3 and 5.4 revealed interesting
differences in the collagenous content between degenerate and non-degenerate aged disc matrices. Normal aged disc matrices revealed significantly low collagen content and extent of Pyd, however degenerative changes *per se* were associated with a higher collagen content, but no change in the extent of Pyd. The extent of Dpd in thoracic disc matrices was found to be higher with both age and degeneration. There is currently no study in the literature for comparison of Dpd data in spinal discs, even for lumbar discs.

Data from this series of biochemical studies in Studies 5.3 and 5.4 on the thoracic discs may be used as markers for age and degeneration processes. The collagen content may be a useful marker for the presence of degeneration, especially in the younger thoracic disc matrix, whereas the extent of Dpd reflects the presence of age and degeneration processes instead. In addition, the decreased extent of Pyd may serve as a marker for ageing in non-degenerate disc matrices, as changes in degenerated disc matrices were not statistically different, similar to findings for lumbar disc matrices (Duance et al 1998, Pokhrana and Phillips 1998). There is currently limited information on the extent of Pyd in non-degenerate spinal tissues.

The current investigations in Study 5.6, also demonstrate that MRI and macroscopic examination of thoracic disc degenerative changes could be related to the biochemical content of the disc, especially the collagen content in the nucleus. Decreased MR signal intensity in the nucleus was significantly associated with a higher collagen content. Grading of disc degeneration using MR images produced a fair agreement with macroscopic findings, although MRI observations were generally higher hence were better able to reveal early disc degenerative changes.

Elastin crosslinks were found in significantly larger amounts (more than 400 times more) in spinal LF tissues. The present findings in Study 5.5, added to the knowledge on the biochemical distribution of collagen and elastin crosslinks in spinal LF in the different spinal regions and over the life span. In particular, the elastin crosslink content in spinal LF was higher in the lumbar region, and for females. These data support the assumptions that the elastin content in the matrix is higher in tissues that have greater flexibility (Ponseti 1995), such as in the lumbar region, and in the more slender and flexible female spine.

The degree of calcification in the spinal LF tissue was also observed to influence the biochemical matrix constituent. The elastin crosslinks and collagen content were significantly increased in LF tissues especially if there was calcification. However with increased age, only the collagen content was increased, with the elastin crosslinks showing a decreasing trend instead. Such data add to the information on spinal LF tissues in the literature. Reasons for the
increased elastin crosslinks in mineralised tissue remains to be investigated and may be related to the increase in collagen Type II fibers or increased chondrocytic and osteoblast activity in calcified LF tissues (Kashiwagi 1993, Specchia et al 2001).

The series of MRI and biochemical investigations in this thesis provided insights and further evidence to support the influence of age, gender-related and spinal regional influences on the prevalence of thoracic disc morphological degenerative changes and the distribution of the collagenous thoracic disc matrix (Figure 6.2). In particular there is evidence to support the different biochemical matrix changes due to the natural ageing process, which is different from changes due to degenerative processes. More importantly, the associations between MRI macroscopic degenerative changes and the biochemical disc matrix changes have provided some preliminary insights into the pathogenesis of degeneration and ageing in human thoracic disc tissues.

Figure 6.2 Schematic diagram to show the interactions and influences of age, gender, spinal level and trauma, on the mechanical loading, disc nutrition and matrix metabolism, leading to disc degeneration. Dashed lines represent responses that accentuate degenerative causative factors. Dashed blocks indicate features that were investigated in the thesis. M = Male bias and F = Female bias.
CHAPTER 7  CONCLUSIONS

The main conclusions arising from these series of investigations on the thoracic intervertebral discs, spinal discs and ligamentum flavum, using MRI and macroscopic examinations, and biochemical analyses are summarised as follows:

Study 4.1 examined the inter- and intra-rater reliability of the modified 3-point and 5-point scales to examine T2-weighted thoracic MR images.

7.1 The intra- and inter-rater reliability was highest for soft tissue changes especially in the nucleus (0.87, 0.88 respectively) compared to osteophytes (0.78, 0.64 respectively) using the 3-point scale.

7.2 The inter-rater reliability was higher using the 3-point ($k$ range = 0.64 to 0.88) scale compared to the 5-point scale ($k$ range = 0.54 to 0.83) except for the rating on end-plates, which was slightly higher for the 5-point scale of Thompson in Eyre et al (1989).

7.3 The intra-rater $k$ coefficient was highest for the upper thoracic region and lower for the mid and lower thoracic regions for both scales. There were however, a few exceptions. Using the 5 point-scale, the $k$ coefficient for end-plate lesions was highest in the lower thoracic (0.76). For the 3-point scale, the only exception was the $k$ coefficient which was highest for anular grading in the mid thoracic (0.96).

Study 4.2 provided prevalence data on thoracic disc degeneration in relation to age, gender and thoracic region, from an audit sample of 216 thoracic T2 weighted MR images. In addition, Study 4.3 examined the association of these degenerative trends with the changes in vertebral morphology, using 169 corresponding T1 weighted MR images.

7.4 The prevalence of thoracic disc degenerative changes and osteophytes was significantly associated with increased age ($p < 0.05$). In contrast, end-plate lesions did not show an age-related trend.

7.5 There was a trend for a higher prevalence of thoracic disc degenerative changes from the mid to lower thoracic regions. A significant increasing craniocaudal trend from T1 to T12 was noted for degenerative changes in the nucleus and anulus, and for end-plate lesions ($p < 0.05$). Osteophyte location however tended to peak in the mid thoracic region.
7.6 There was also a significant linear age-associated decrease in the antero-posterior and mid-posterior vertebral height ratios, reflecting an increase in anterior wedging, associated with an increase in the bi-concavity configuration of the vertebral body ($p < 0.05$). Age-related changes in anterior wedge deformation were particularly prevalent in the mid-thoracic region of older female cases.

7.7 Vertebral deformity and osteophyte formation were predominant in the older female cases, however, disc degenerative changes were more commonly found in males compared to females.

In Study 5.1 formalin-fixed spinal disc and ligament tissues were investigated to determine their feasibility for analysing collagen, Des and Isodes crosslink content and extent of Pyd and Dpd.

7.8 Human spinal discs and LF fixed in 10% buffered formalin from 1 to 25 weeks did not have significantly different collagen, Des and Isodes content, or extent of Pyd and Dpd, compared with fresh unfixed control samples.

7.9 There were also no significant differences in these biochemical components of collagen, Pyd, Dpd, Des and Isodes, in the disc and ligament tissues over the 25 weeks of fixation.

In Studies 5.2 and 5.3 the distribution of collagen content and the extent of Pyd and Dpd in the extracellular matrix of 209 non-degenerate thoracic disc over a wide age range (1-90 yrs) was analysed. Data were also regrouped to analyse for age, gender, disc and spinal regional influences on the collagenous constituent of the thoracic disc matrix.

7.10 Regionally, the nucleus was found to have a significantly lower collagen content but significantly higher extent of collagen crosslinks compared to the anulus ($p < 0.05$).

7.11 The anterior anulus had significantly lower extent of Pyd compared to the posterior anulus ($p < 0.05$).

7.12 The anular tissues in the mid thoracic region had the highest variation of collagen, Pyd and Dpd in different regions of the disc, compared to the upper and lower anular tissues.

7.13 With increasing age, the collagen content and extent of Pyd were significantly lower in all disc regions ($p < 0.001$), in contrast, the extent of Dpd was higher in all regions, but was only statistically significant in the nucleus ($p < 0.001$).
Chapter 7

Conclusions

7.14 Younger male discs had a significantly higher extent of Pyd compared to females ($p < 0.01$); however with age this gender difference was reversed. The collagen content in the anulus was also significantly higher in male samples compared to females ($p < 0.05$).

7.15 The main spinal regional trends noted were a lower extent of Dpd and Pyd in the anulus and nucleus, respectively, in the lower thoracic region. In the mid thoracic region, the collagen content of the anterior anulus was significantly lower ($p < 0.05$), however, the extent of Pyd was significantly higher, compared to the other thoracic regions ($p < 0.05$).

In Study 5.4, the influence of disc degenerative changes on the distribution of collagen content and extent of collagen crosslinks in 303 thoracic discs were evaluated. In addition, the changes in the distribution due to age, gender and thoracic regions were also determined.

7.16 The nucleus had a predominance of severe macroscopic degeneration changes at an earlier age interval compared with the anulus. In severely degenerated thoracic discs, there was no difference between the anulus and the nucleus, in terms of the collagen and crosslink measurements.

7.17 After accounting for age effects, degenerate nuclear tissues had significantly higher collagen content ($p < 0.05$), compared with aged non-degenerate thoracic discs.

7.18 Although present in small amounts, the extent of Dpd increased significantly with increased age and degeneration ($p < 0.001$).

Study 5.5 provided comprehensive data on collagen and elastin crosslink content in 364 human spinal LF and 77 disc tissues. The association of these biochemical components in the LF with increasing age, gender and spinal regions was also examined.

7.19 Collagen and elastin crosslinks were detected in all LF tissues, however only small amounts of Des and Isodes crosslinks were detected in a limited number of lumbar disc tissues.

7.20 Ligamentous tissues had over 650 times more Des and 400 times more Isodes crosslinks, compared to lumbar disc tissues.

7.21 Des and Isodes crosslinks in LF did not change significantly with age, but were significantly higher in females compared to males ($p < 0.05$), and were higher in the lumbar region (but only statistically significant for Isodes crosslinks).
7.22  The collagen content and extent of Dpd in the LF was significantly higher with age ($p < 0.05$); while the extent of Pyd remained unchanged with age.

7.23  The collagen content and Pyd extent in the LF was significantly higher in females ($p < 0.05$), but there was no gender difference for Dpd.

The preliminary trends in Study 5.6 for two unfixed spine cases was used to compare MRI, macroscopic and biochemical data from human thoracic discs and LF. Inferences from this study are limited due to the small sample.

7.24  The kappa correlation coefficients between MRI and macroscopic grading scales for the discs were fair, ranging from 0.3 to 0.4. Generally MRI grading tended to be higher than the respective macroscopic grading for the discs.

7.25  With increased MRI and macroscopic grades, the collagen content was found to be higher in the nucleus and lower in the anulus.

7.26  With increasing degeneration grades, the CS content showed a decreasing trend in the nucleus but a higher content in the degenerated anulus instead.

7.27  In calcified LF, the Des and Isodes crosslink, extent of Dpd and collagen content were higher compared to normal tissues.

7.28  Grade I LF samples had almost similar collagen content to the anulus, which was higher to that in the nucleus; and a lower extent of Pyd; a higher extent of Dpd; and a lower CS content compared to the anulus and nucleus.
CHAPTER 8 DIRECTIONS FOR FUTURE RESEARCH

The current series of investigations has provided original data on the biochemical composition of human thoracic discs, in particular the collagen content and the extent of Pyd and Dpd. In addition, the data on the influence of degeneration, age, gender and spinal regions on these biochemical components in the disc and LF have increased the existing body of information on spinal discs and ligament. More importantly, the pattern of degenerative changes in the thoracic discs and vertebrae and its associated influence on the biochemical disc matrix were also examined. In the course of these investigations, a number of issues were raised that deserve further consideration.

8.1 Correlative studies investigating radiological, biomechanical and biochemical aspects of spinal discs.

Future correlative studies are needed to further our understanding of the mechanisms involved in spinal disc degeneration. Reviewing the various factors that were investigated in this thesis in Figure 6.1 (Chapter 6), it is apparent that correlative studies are needed to determine the impact of biomechanical forces on the spinal disc matrix over time. Figure 8.1 presents further gaps of information in understanding the sequelae of disc degeneration. Biomechanical studies on lumbar discs have been reported by McNally et al (1995) on disc stress profilometry, to predict potential disc degeneration (Figure 8.2). Such data on the pressure profile of thoracic discs have not been reported and would warrant an investigation to examine the pressure profiles during loading, particularly given the different loading conditions experienced in the thoracic regions due to the physiological kyphosis.

Such a correlative cross-sectional study was attempted in an earlier pilot study using the methodology outlined in Figure 8.3, attempting to link the radiological, macroscopic, biomechanical and biochemical data for spinal discs. This methodology is an extension of the procedures described in Study 5.6 on the two non formalin-fixed thoracic spines. Investigations for this thesis, were unfortunately suspended as the pressure transducer was damaged during experimentation. More detail on this methodology is provided in Appendix G.
Figure 8.1 Schematic diagram to show the interactions and influences of age, gender, spinal level and trauma, on the mechanical loading, disc nutrition and matrix metabolism, leading to disc degeneration. Items in dashed boxes were investigated in this thesis. Items in shaded boxes are recommended for future investigations to advance the body of knowledge for the mechanism of disc degeneration in the thoracic region.

Figure 8.2 Example of the stress profile for a degenerated lumbar disc taken from McNally and Adams (1992).
Figure 8.3 Sequence of investigations using human spinal discs. X-ray to be used to exclude bony fractures, tumours and deformities, MRI, to be used to grade the discs and vertebral morphology, followed by macroscopic examination of the discs for degeneration changes. Before the samples were removed from the disc for biochemical analysis, a stress profile of the disc would be performed using a pressure transducer drawn horizontally, through the mid section of the disc, as described by McNally and Adams (1992).

The investigation of the distribution of biochemical components in the disc matrix due to various age, gender, spinal regional factors has enabled a better understanding of the pathogenesis of spinal tissue degeneration and what factors are involved in this process. However such cross-sectional studies are limited to providing correlative information, because there is a time lag from the onset of mechanical changes and the observation of biochemical matrix responses. Therefore longitudinal studies are necessary to evaluate the biochemical responses (Ziran et al 1994, Hutton et al 1998, Hutton et al 1999). Such longitudinal studies have usually been performed on animal lumbar and caudal discs, which have provided useful information of the biomechanical effect on the disc biochemical matrix over time.

8.2 Biochemical studies to investigate the presence and distribution of advanced glycation end-products (AGE) and other matrix degradation products

Of particular interest in the biochemical constituent of the disc was the presence of AGE or glycated collagen crosslink, pentosidine (Sell and Monnier 1989). Pentosidine had been identified as a biochemical marker for age-related and degenerative changes in connective tissues and lumbar discs (Sell and Monnier 1989, Pokharna and Phillips 1998). Pentosidine is the end-product of glycosylation of glyco-proteins, and is formed from long-lived collagen molecules (lysine residues) forming covalent bonds with glucose residues in the extracellular matrix, followed by Amadori or ketoamine rearrangements (Monnier et al 1984, Sell and Monnier 1989) (Figure 8.4). The slow matrix turnover of collagen in spinal disc tissues predisposes the tissues to accumulation of AGE from non-enzymic glycosylation (Eyre et al 1989, Hormel and Eyre 1991, Verzijl et al 2000). Pentosidine had been found to be present in aged tissues, particularly in diabetic patients and in rheumatoid patients for example.
(Takahashi et al 1998). Future investigations on age and degeneration changes in the disc matrix should include an analysis of these AGE and their usefulness as pathologic and senescent biochemical markers. The presence of pentosidine and other pathological biochemical markers in thoracic discs and spinal LF over the life span had not been investigated. Details of the methodology for investigating pentosidine is outlined in Appendix H.

Figure 8.4 Formation of pentosidine from oxidative degradation of pentose and reactions with lysine and arginine side chains. Figure taken from Paul and Bailey (1996).

Besides pentosidine there are other disc matrix degeneration markers that may be investigated. Nerlich et al (1997) had reported the presence of N-carboxymethyllysine (CML) as well as the phenotypic changes in collagen types in degenerated lumbar discs. The distribution and changes in collagen types in normal and degenerated disc matrices have also been reported by Boos et al (1991) who found the presence of collagen Type X in aged and degenerated lumbar disc matrices, which is usually reported in hypertrophic tissues, such as growth plates. Recent studies by Kaapa et al (2000), Pattison et al (2001) Cassinelli et al (2001) and Horner et al (2002) provide a more indepth and comprehensive information on collagen synthesis during disc degeneration, investigating the changes in the enzymic activities of hydroxylases (Kaapa et al 2000), the expression and breakdown of the disc matrix by MMPs (Pattison et al 2001), the use of gene mapping and transfer therapy to modify these changes in the matrix (Cassinelli et al 2001), and the role of disc cell populations to maintain the distinctive matrix composition in different parts of the disc.
(Horner et al 2002). Such information is important in explaining and elucidating the changes in collagen type and the distribution of collagen and collagen crosslinks in the disc matrix with increased age and degeneration.

8.3 Further studies using MRI
Some advantages of using MRI as an assessment tool for research studies, in terms of accessibility and high inter- and intra-rater reliability when evaluating T1- and T2-weighted MR images for investigating disc degenerative and vertebral morphology changes, have been demonstrated in Studies 4.1, 4.2 and 4.3. Further evaluation of the degenerative changes using MRI within specific symptomatic cohorts, for example scoliotic or patients with referred nerve root symptoms, preferably in a prospective longitudinal study (over 1 or 2 years), would provide more information on the significance and predictive value of MR investigations for diagnosis and treatment of thoracic disc and vertebral degenerative changes (Figure 8.1).

In addition, recent MRI studies on lumbar studies have attempted to objectively measure the signal intensity, in order to determine MRI parameters that would distinguish symptomatic from asymptomatic features (Boos et al 2000, Borenstein et al 2001, Vaithianathar et al 2003). Given the high prevalence of asymptomatic thoracic degenerative changes, correlation of MRI parameters with clinical findings may advance the use of MRI as a diagnostic and measurement tool.

8.4 Elastin and deoxypyridinoline in spinal discs
This thesis provided baseline information on the collagen crosslinks for thoracic discs. With the establishment of the appropriateness of formalin-fixed spinal disc and ligament tissue for evaluation of collagen and elastin crosslink evaluations, there is potential for further studies to gather similar biochemical data, especially for Des and Isodes crosslinks, for discs in the cervical, thoracic and lumbar regions. Such studies should take into consideration using larger disc tissue samples to examine the extent of Dpd, Des and Isodes crosslinks in spinal discs. According to Johnson et al (1985) and Yu et al (2002) elastic fibers in spinal discs are located in specific sites, especially near the attachment to bony tissues and between the lamellae. Future investigations for these biochemical components should attempt to map the location of such fibers and determine a density profile for specific disc regions through histological staining methods, as well as to correlate these profiles with the biochemical analyses for elastin crosslink content.
CHAPTER 9 REFERENCES


