Investigating facet joint osteoarthritis in the context of adolescent idiopathic scoliosis

Daniel Bisson

Experimental Surgery

Faculty of Medicine

McGill University, Montréal

October 2022

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree

of Doctor of Philosophy

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Abstract

Adolescent idiopathic scoliosis (AIS) is the most common musculoskeletal disease affecting youth aged between 10 and 16 years old worldwide. Detrimental outcomes for the patient's health-related quality of life such as decreased pulmonary function and back pain are associated with spinal curve severity in scoliotic patients. Current treatment options are aimed at preventing curve progression but are limited by the idiopathic nature of AIS evading our current comprehension of causal factors leading to the disease. Spinal instability during growth has been hypothesized as a driving force for AIS progression. However, the structural deformation of the vertebrae and other spinal features remain poorly understood.

This thesis hypothesized that scoliotic spines would bear anatomical evidence of spinal instability in the form of facet joint osteoarthritis because they are the spinal features most engaged in keeping rotational stability. The three following aims were formulated for this approach:

1. Characterize facet joint cartilage samples from AIS patients compared to non-scoliotic organ donors to evaluate the presence of osteoarthritis.
2. Investigate the pattern of facet joint degeneration with the 3D deformation of scoliotic spines.
3. Investigate Toll-like receptors (TLRs) as disease-modifying therapeutic targets to treat facet joint osteoarthritis

We found an increased prevalence of facet joint degeneration in scoliotic samples compared to non-scoliotic controls. The loss of proteoglycans, fibrillation of the cartilage surface, chondrocyte proliferation and expression of pro-inflammatory cytokines and proteases were confirmed as hallmarks of osteoarthritis in scoliotic facet joints. (Manuscript I) However, the pattern of degeneration in facet joints across the thoracolumbar spine did not match the coronal

deviation using pre-operative frontal radiographs. This discrepancy led us to believe the variation in osteoarthritic severity in samples collected from AIS patients would be associated with the 3D deformity in each respective intervertebral segment. Using the EOS system for spinal reconstructions, we determined that facet joint bone and cartilage degeneration were associated with axial intervertebral deformation in scoliotic spines. Furthermore, the spinal levels most vulnerable to asymmetrical and bilateral facet joint osteoarthritis were in kyphotic intervertebral segments subjected to axial rotation. This vulnerable back region also correlated with increased perceived back pain in AIS patients. (Manuscript II) These results suggest that facet joint osteoarthritis could be both a cause and consequence of spinal rotational instability at the core of AIS pathogenesis and pain. Due to the lack of disease-modifying treatment options available for facet joint osteoarthritis, we next shifted our focus to finding molecular targets to investigate therapeutic options to treat the underlying condition of the degenerating joints. Notably, we found an increased abundance of *alarmins* in scoliotic cartilage which are a class of endogenous agonists activating the constitutively expressed Toll-like receptors (TLR). TLR activation promoted the expression of pro-inflammatory cytokines and proteases which exacerbated proteoglycan loss in *ex vivo* scoliotic facet joint cartilage. Finally, the treatment of activated chondrocytes with TLR antagonists successfully reduced the expression of the catabolic mediators found in scoliotic cartilage. (Manuscript III)

The work in this thesis revealed the prevalence of facet joint osteoarthritis in scoliotic patients induced by axial rotation. These results support the leading hypotheses that AIS is mainly a decompensation of the spine due to axial rotational instability. Taken together, this work suggests that a novel therapeutic approach using TLR inhibition could aim to restore facet joint integrity and in consequence prevent the progression of the spinal deformity.

Résumé

La scoliose idiopathique de l'adolescent (SIA) est la maladie musculo-squelettique la plus courante chez les jeunes âgés de 10 à 16 ans mondialement. Des résultats néfastes pour la qualité de vie liée à la santé du patient, tels qu'une diminution de la fonction pulmonaire et des maux de dos, sont associés à la sévérité de la courbure vertébrale chez les patients scoliotiques. Les options de traitement actuelles visent à prévenir la progression de la courbe, mais sont limitées par la nature idiopathique qui élude notre compréhension actuelle des facteurs causaux menant à la maladie. L'instabilité vertébrale pendant la croissance semble être un facteur important pour la progression de la SIA. Cependant, la déformation structurelle des vertèbres et d'autres caractéristiques de la colonne vertébrale restent mal comprises.

Cette thèse a émis l'hypothèse que les colonnes vertébrales scoliotiques auraient des signes anatomiques d'instabilité vertébrale sous la forme d'arthrose facettaire, car les articulations facettaires demeurent les plus engagées dans le maintien de la stabilité en rotation. Les trois objectifs suivants ont été formulés pour cette approche :

1. Caractériser les facettes articulaires des patients SIA par rapport aux donneurs d'organes non scoliotiques pour évaluer la présence d'arthrose.
2. Étudier l’association entre la dégénérescence des facettes articulaires avec la déformation 3D des colonnes vertébrales scoliotiques.
3. Étudier les récepteurs Toll-like (TLR) en tant que cibles thérapeutiques modificatrices de la maladie pour traiter l'arthrose des facettes articulaires.

La perte de protéoglycans, la fibrillation à la surface du cartilage, la prolifération des chondrocytes et l'expression de cytokines et de protéases pro-inflammatoires ont été confirmées comme des caractéristiques de l'arthrose dans les articulations facettaires scoliotiques. (Manuscrit

I) Cependant, la dégénérescence des facettes articulaires ne correspondait pas à la déviation coronale de la colonne vertébrale du patient. En utilisant le système EOS pour les reconstructions vertébrales, nous avons déterminé que la dégénérescence de l'os et du cartilage des facettes articulaires était associée à une déformation intervertébrale axiale dans les colonnes vertébrales scoliotiques. De plus, les niveaux rachidiens les plus vulnérables à l'arthrose facettaire asymétrique et bilatérale se situaient dans les segments intervertébraux cyphotiques soumis à une rotation axiale. (Manuscrit II) Ces résultats suggèrent que l'arthrose des facettes articulaires pourrait être à la fois une cause et une conséquence de l'instabilité rotationnelle vertébrale au cœur de la pathogenèse de la SIA. En raison du manque d'options de traitement modificateurs de la maladie disponibles pour l'arthrose des facettes articulaires, nous nous sommes ensuite concentrés sur la recherche de cibles moléculaires pour étudier les options thérapeutiques pour traiter l'état sous-jacent des articulations en dégénérescence. Notamment, nous avons trouvé une abondance accrue d'*alarmins* dans le cartilage scoliotique qui sont une classe d'agonistes endogènes activant les TLR exprimés de manière constitutive. L'activation des TLRs a favorisé l'expression de cytokines pro-inflammatoires et de protéases qui ont exacerbé la perte de protéoglycans dans le cartilage articulaire des facettes scoliotiques *ex vivo*. Enfin, le traitement d'antagonistes de TLR a réussi à réduire l'expression des médiateurs cataboliques présents dans le cartilage scoliotique suite a une activation avec des *alarmins*. (Manuscrit III)

Ces résultats soutiennent l'hypothèse selon laquelle la SIA est principalement une décompensation de la colonne vertébrale due à une instabilité de rotation axiale. Pris ensemble, ces travaux suggèrent qu'une nouvelle approche thérapeutique pourrait viser à restaurer l'intégrité de l'articulation facettaire et, par conséquent, à prévenir la progression de la déformation vertébrale.

Acknowledgements

First and foremost, I must thank my mentor Lisbet Haglund who not only believed in me but also made me believe in myself. Although extremely corny, this last statement is true and has meant a lot. We had a disagreement on the hypothesis at the start of this study, and she suggested we should make a bet. I still owe her a beer. Derek Rosenzweig also provided extremely helpful mentoring in experimental work and writing. I had a blast jamming with him at conferences and still believe Take me out by Franz Ferdinand is our best cover attempted to date. For the guidance and fun times at conferences in San Diego and New Orleans, I thank Emerson Krock. As with every body of work, omitting the contribution of colleagues would be folly. I'm especially grateful towards Kai and Semsi who without them the analysis of surgical samples would have been torturous. Additionally, I thank Jean Ouellet, Neil Saran, Michael Weber, McGill spine fellows and the McGill Scoliosis and Spine group for their immense contribution to sample collection for this work. At one point, the wife of Leonardo, a particularly involved spine fellow, accused him of infidelity because of our frequent phone calls in the middle of the night for spine harvests. All of my colleagues and I are very grateful for their voluntary sacrifice of sleep and potential marital troubles. I must thank Julien aka *Captain Contamination* for being the initial bridge between me and the Haglund lab. Julien shares a membership with Gogs, Nic, Ced, Chris and Vinny to an elite club of childhood friends called *Che town boys.* They know I wouldn't be who I am without them. It is evident I wouldn't have finished this endeavour without the constant support of my family. I credit my mom with initiating my love for science when she took me to see Mission to Mir in an Imax theater. My dad certainly accelerated this spark by building my first *laboratory* in a large cardboard box in my room. My sister was the unfortunate and relunctant victim of my early concoctions consisting of Gatorade powder and food colouring. Finally, I reserve my highest praise

for Alexe and Maggie, who support me daily with love and give me strength. I owe them everything.

Contribution to original knowledge (and authors)

This manuscript-based thesis is composed of three multi-authored articles. Each study brought novel contributions to knowledge which will be cohesively explained in this thesis. I (D.G. Bisson) have conducted most experiments, data analysis and writing for each manuscript. This work would not be possible without the contribution of my co-authors for human tissue collection, study design, conducting experiments and reviewing draft manuscripts. L. Haglund and D.H. Rosenzweig both contributed to my training in experimental work, data analysis and scientific writing. The categorical contribution of each co-author for the three manuscripts is listed below.

Manuscript I: Facet joint degeneration in Adolescent Idiopathic Scoliosis (Published in the Journal of Orthopedic Research Spine, 2018)

Study design: D.G. Bisson, L. Haglund, J.A. Ouellet, D.H. Rosenzweig

Conducted experiments and analyzed data: D.G. Bisson, P. Lama, D.H. Rosenzweig

Human tissue collection: J.A. Ouellet, N. Saran, F. Abduljabbar

Writing the manuscript: D.G. Bisson, P. Lama, L. Haglund

Funding: L. Haglund, J.A. Ouellet, N. Saran

Manuscript II : Axial rotation and pain are associated with facet joint osteoarthritis in Adolescent Idiopathic Scoliosis (In review)

Study design: D.G. Bisson, L. Haglund, J.A. Ouellet

Conducted experiments and analyzed data: D.G. Bisson, K. Sheng, S. Kocabas, D.D. Ocay

Human tissue collection: J.A. Ouellet, N. Saran

Writing the manuscript: D.G. Bisson, L. Haglund

Funding: L. Haglund, J.A. Ouellet, N. Saran, C.E. Ferland, D.G. Bisson

Manuscript III: Toll-like receptor involvement in adolescent scoliotic facet joint degeneration (Published in the Journal of Cellular and Molecular Medicine, 2020)

Study design: D.G. Bisson, L. Haglund, J.A. Ouellet

Conducted experiments and analyzed data: D.G. Bisson, S. Kocabas, K. Sheng, E. Krock

Human tissue collection: J.A. Ouellet, N. Saran, A. Teles

Writing the manuscript: D.G. Bisson, L. Haglund

Funding: L. Haglund, J.A. Ouellet, N. Saran, D.G. Bisson

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List of Abbreviations

ADAMTS - A Disintegrin And Metalloprotease with Thrombospondin motif

AIS – Adolescent Idiopathic Scoliosis

BNC2 – Basonuclin 2

DAMP – Danger Associated Molecular Pattern

DMEM – Dulbecco's Modified Eagle Medium

ECM – Extracellular Matrix

GAG – Glycosaminoglycan

GPR126 – G-protein coupled receptor 126

GWAS - Genome-wide association study

HMGB1 – High Mobility Growth Box 1

HRQOL - health-related quality of life

HSP – Heat Shock Protein

IFN – Interferon

IL – Interleukin

ITS – Insulin-Transferrin-Selenium

IVD – Intervertebral Disc -

LBX1 – Ladybird Homeobox 1

LPS - Lipopolysaccharide

MAPK – Mitogen-Activated Protein Kinase

MMP – Matrix Metalloprotease

MyD88 – Myeloid differentiation primary response 88

NF-κB – Nuclear Factor-Light-chain-enhancer of activated B cells

NGF – Nerve Growth Factor

PAX1 – Paired box 1

PBS -Phosphate Buffered Saline solution

SLRP – Small Leucine-Rich Repeat Protein

SPARC – Secreted Protein Acidic and Rich in Cysteine

TIR – Toll/IL-Like receptor domain

TLR – Toll-like receptor

TRAF6 – TNF Receptor Associated Factor 6

TRAM – Translocating chain-associated Membrane protein

TRIF – TIR-domain-containing adapter-inducing Interferon-ß

Chapter 1: Introduction

1.1 The current problem of Adolescent Idiopathic Scoliosis

Adolescent idiopathic scoliosis (AIS) is a 3-dimensional deformation of the spine and the most prevalent musculoskeletal disease affecting children aged 10-16 years old. The disease generally appears in early puberty and affects 1-4% of adolescents worldwide, primarily girls. [1] Many medical consequences can arise if AIS is left untreated. Some studies have reported increased mortality and disability due to pulmonary and cardiovascular issues in untreated patients. [2] Additionally, Sato et al. found that scoliotic patients have a two-fold increase in odds of developing back pain compared to a healthy population. [3] Thankfully, the advancement of treatment options has dramatically improved outcomes for scoliotic adolescents. Current treatment options aim to prevent curve progression and avoid adverse effects such as decreased pulmonary function associated with curve severity. [4] After an initial diagnosis, the curve is monitored by coronal radiography until it reaches a cobb angle of 20°. [5] Afterwards, bracing is recommended to support the spine. Bracing remains the only effective treatment, as demonstrated by a 'dose-dependent' prevention of curve progression according to the total time the brace was worn. [6] In cases where the progression is uncontrolled, spinal fusion surgery is recommended when the cobb angle reaches >50°. [7] The main goal of this procedure is to completely stop the curvature by correcting and realigning the spine with metal rods or tethers. Surgery is considered the final option because of the risks associated with the procedure, adverse psychosocial effects, and back pain. [8][9] The consequences above are thought to contribute to the decreased health-related quality of life (HRQOL) scores still reported by AIS patients.

At the time of this thesis, the main obstacle to the advancement of AIS treatments is our incomplete comprehension of initiating and progressive causes that lead to AIS. Developing

disease-modifying treatment options remains difficult without understanding the underlying mechanisms at the core of the structural spinal deformation. Such therapeutic modalities could help prevent progression and reduce the need for surgical intervention in more severe AIS curves. Recent literature reviews have shown various causes and tissues involved in AIS development. [1] However, the characterization of the scoliotic spine is incomplete. Research aiming to study vertebral dysfunctions in AIS could expand our understanding of the disease etiopathogenesis. In turn, the goal is to help optimize the predictive strength of current monitoring strategies and offer new targets for disease-modifying therapeutic modalities to prevent progression.

1.2 Comprehensive literature review and background

1.2.1 Adolescent Idiopathic Scoliosis

Adolescent idiopathic scoliosis (AIS) is a 3-dimensional structural deformation of the spine first reported by Nicoladoni in 1904. [10] The large diversity of spinal deformities observed in AIS patients can be crudely simplified to combinations of coronal curves along the thoracolumbar spine. Clinicians use the Lenke and King classification systems to divide all AIS spines into 6 or 5 groups. [11] The groups represent combinations of primary 'structural' curves at different spinal levels flanked by 'compensatory' secondary curves above or below. The secondary curves are less severe, more flexible, and their concavity is mirrored in the coronal plane compared to the primary curve. Secondary curves are compensatory because they develop after the primary deformity, which is considered the initiating feature. Diagnosis occurs when the angle between the vertebral endplates at the extremities of the primary curve (Cobb angle) is above 10° in the coronal plane. (Figure 1)

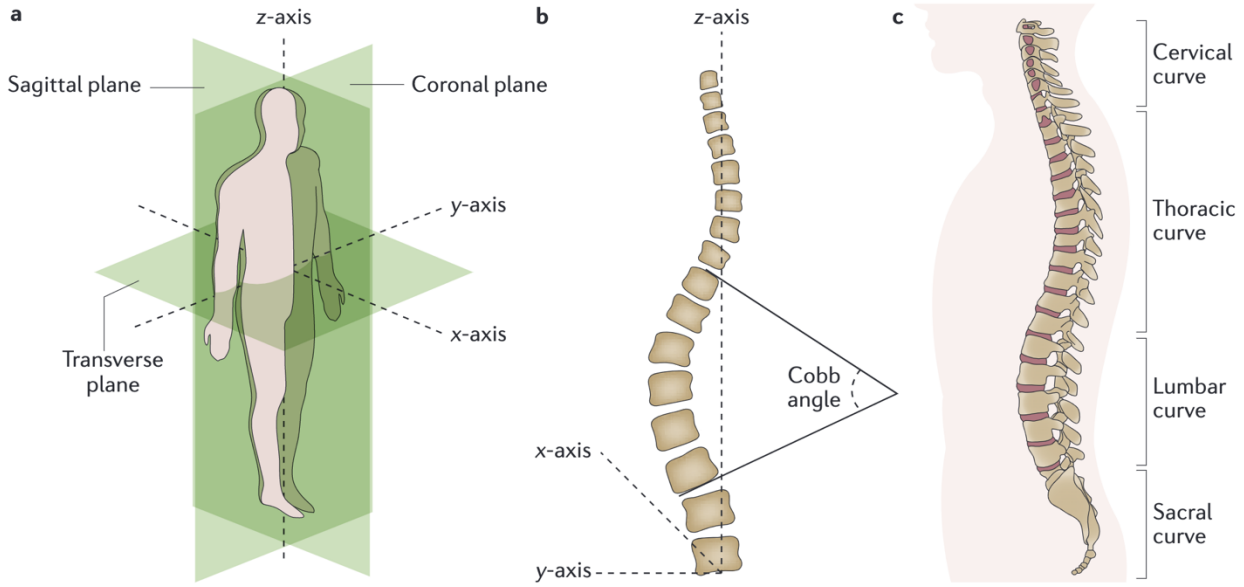

Figure 1 ***Measuring the cobb angle.*** *A) The axes and planes of the human body. B) Scoliosis curves are measured from the y-axis (coronal plane), where the cobb angle is commonly measured by drawing a line that is parallel with the angle of the top of the top vertebra involved in the spine curve and the bottom of the bottom vertebra involved in the curve. The cobb angle is measured where these lines intersect. In addition, the sagittal plane Cobb angle can be measured from the z-axis. The spine can be divided into regions comprising the cervical, thoracic, lumbar and sacral vertebrae. This image was used with permission from the publisher.*

Traditional classification systems are commonly used for clinical practice although studies suggest they could be significantly improved to help guide treatment options. [12] Most importantly, simple coronal and sagittal radiographs for diagnosing AIS prohibits the proper characterization of the 3D deformity, especially in the transverse or axial plane. Roaf et al. demonstrated the implication of axial rotation in vertebrae of AIS patients with CT imagery. Subsequent studies have confirmed maximal axial deviance from the curve apex's midline pointing towards the convexity. [13-17] Significant correlations were also reported between the Cobb angle and axial rotation in primary and secondary curves. [18] Therefore, the coronal and axial deformities appear intrinsically linked and should be evaluated equally. Congenital, neuromuscular, posttraumatic, and adult degenerative scoliosis all bear similar patterns of coronal

and axial deformity. {} However, AIS is the only scoliotic subtype that develops spontaneously in generally healthy individuals.

1.2.1.1 Etiopathology

The term idiopathic describes the lack of consensus on causal factors that lead to AIS in otherwise healthy adolescents. Nonetheless, our current understanding of AIS etiopathology is guided by an accumulation of necessary research published on the subject. A unified model of scoliosis found in figure 2 represents a simplified review of this research at the time of this thesis.

Members in AIS familial cohorts reported very different spinal deformities and variable progression rates, including identical twins. [19][20] To better explain this dichotomy, Cheng et al. proposed a biphasic model of scoliosis where the initiation and progression of the spinal deformity are considered distinct phases of the disease. [21] Notably, AIS initiation is more dependent on genetic factors than progression. Accordingly, a study performed on the Swedish Twin Registry estimated that approximately 38% of the risk of developing AIS was attributed to heredity, whereas the remaining 62% was responsible to environmental factors. [19] The study also showed that monozygotic twins have, on average, twice the rate of idiopathic scoliosis concordance compared to dizygotic twins. [19][22] Also, higher rates of scoliotic diagnosis among relatives than the general population also demonstrate disease heritability. [23][24] Therefore, the importance of genetics in the development of AIS is evident. However, linkage analyses in familial cohorts revealed multiple susceptibility loci with autosomal dominant or X-linked inheritance mechanisms. [25][26] Furthermore, these domestic cases account for less than 10% of total AIS patients, and their loci of interest do not overlap between families. [27] With the availability of genome-wide association studies (GWAS) to evaluate genetic associations in much larger populations, a few genes were linked with the risk of developing AIS across diverse

ethnographic populations such as LBX1, BNC2, PAX1 and GPR126. [28-31] Many other susceptibility loci were discovered but failed to be seen in different populations. The susceptible genes have established roles in many cellular functions of spinal tissues such as bone, cartilage and paraspinal musculature development and homeostasis. This provides the most substantial evidence that AIS is a complex polygenic disease caused in part by inheritable risk factors. Associative studies have provided hypotheses on the precise phenotypical risk factors these SNPs provide, but much more research is needed to elucidate their role in the scoliotic outcome. [32]

Once the deformity is initiated, the structural asymmetry creates abnormal biomechanics along the thoracolumbar spine. This mechanism is believed to drive progression in all different types of scoliotic variants which share similar curve patterns. In short, scoliosis progresses when abnormal biomechanical loading is subjected to a normal spine (neuromuscular scoliosis) or normal loading is applied to an asymmetrical or deformed spine (congenital, adult degenerative, posttraumatic). Asymmetrical loading of the spinal tissues is especially detrimental to balanced growth for its ability to differentially modulate bone metabolism according to the Hueter-Volkmann and Wolff's law. [33] Once deformed, the vertebrae are unable to transmit biomechanical forces evenly across the spine. This feedback loop is best represented as the *vicious cycle* hypothesis, first proposed by Aebi et al. and is depicted in Figure 2. [34]

In this model, the risk factors leading to AIS can be categorized by their involvement in asymmetrical loading or spinal tissue dysfunction leading to vertebral deformity.

Asymmetrical loading of the spine can arise in many circumstances. For example, it is a consequence of an imbalance in paraspinal musculature or impaired somatosensory neurons affecting paraspinal muscle activation unevenly. Developmental issues to the vestibular and central nervous systems also increase the risk of AIS, seemingly through similar asymmetrical

activation and impaired somatosensory control of the paraspinal muscles. [35] The problematic source of asymmetrical loading remains to be fully elucidated, however.

In contrast, any risk factor affecting spinal tissue response to abnormal loads will also promote vertebral deformation and AIS progression. The risk factors observed in AIS cohorts include having osteopenic bone, altered cartilage extracellular matrix and asymmetrical paraspinal muscle thickness. [36] [37] Osteopenia, cartilage dysfunction and uneven musculature can impair the stabilization needed to contain the spinal movements and adapt to increasing abnormal forces. Extensive evidence of these impairments has been found in AIS, including low bone mineral density and volume in 67% of patients and increased bone turnover rates in 76% of patients. [38] Peak curve progression coincides with a growth spurt when the patient is still skeletally immature. [39] The remaining growth is predicted by the Risser score of ossification or the Sanders Maturity score, therefore reliable predictors of AIS curve progression. [36] However, the association between the SNPs found in populations at risk of AIS and their effect on spinal stability needs further research.

In conclusion, it is theorized that AIS is a polygenic disease manifested through an accumulation of genetic and environmental risk factors that hamper spinal stability during growth. This hypothesis must be fully validated with further anatomical evidence and computer models simulating disease progression.

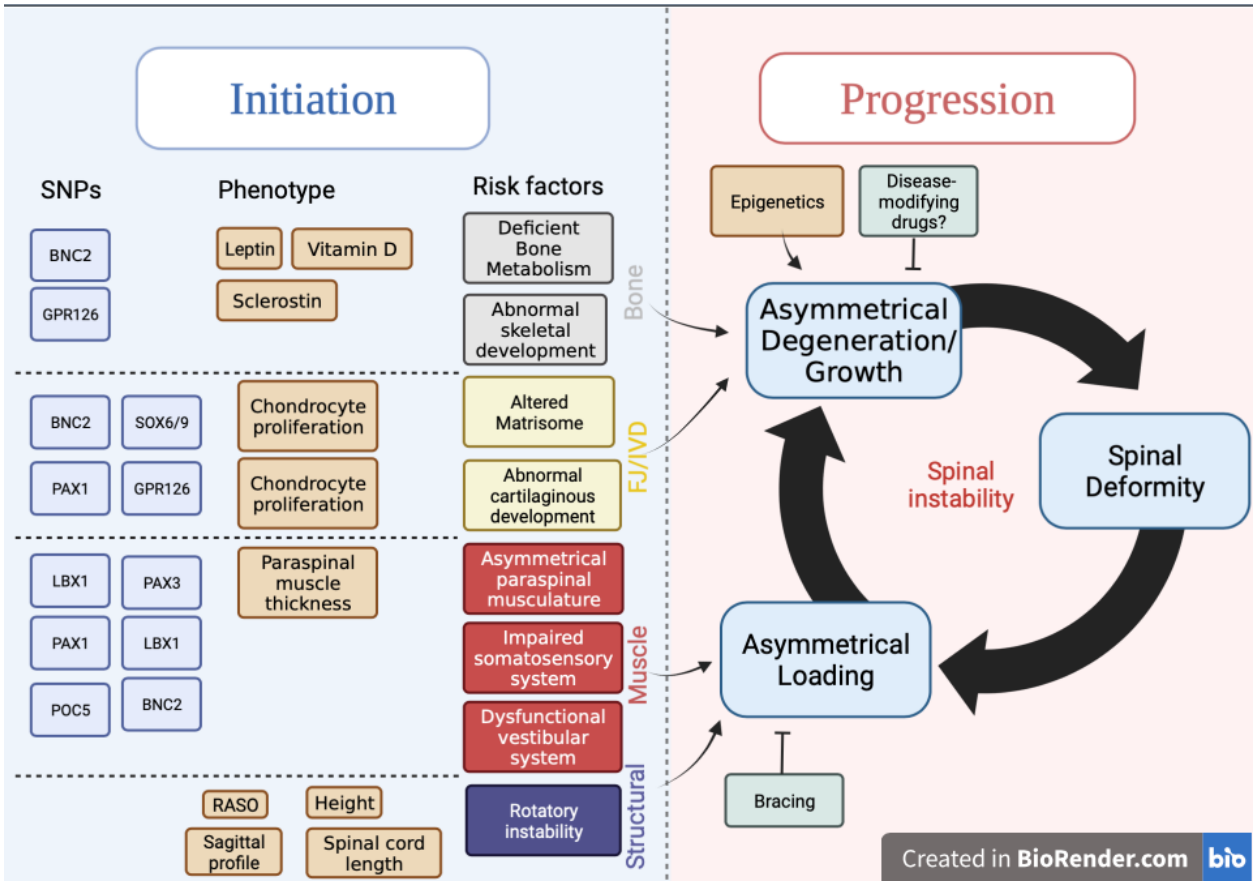

Figure 2 ***Modern model of adolescent idiopathic scoliosis etiopathogenesis.*** *- Illustration by D.G. Bisson.*

1.2.2 Spine

1.2.2.1 Anatomy

The spine is the main characteristic of the vertebrates, an animal taxon comprising all mammals, birds, reptiles, amphibians, and fish. The human vertebral column is comprised of 24 vertebral bodies divided into three parts: cervical (C1-C7), thoracic (T1-T12) and lumbar (L1-L5). Between each adjacent vertebra are located the intervertebral discs (IVD) and a pair of posterior cartilaginous facet (zygapophyseal) joints (FJs). Two transverse processes flank the supporting articular processes in the frontal plane and one spinous process in the sagittal plane joined by the lamina. (Figure 1) The shape and size of all vertebral parts vary gradually across the spinal column

to adapt to biomechanical requirements and form the three sagittal curvatures of our upright spines. The cervical and lumbar regions are convex anteriorly and described as lordotic. Inversely, the thoracic part is posteriorly convex and kyphotic. The length of the pedicles and facet joint orientation help form these sagittal curves. A slight axial rotation and frontal convexity are generally observed close to the heart in the general population. [40][41] Otherwise, the spine is straight in the frontal and axial planes to maintain symmetrical balance.

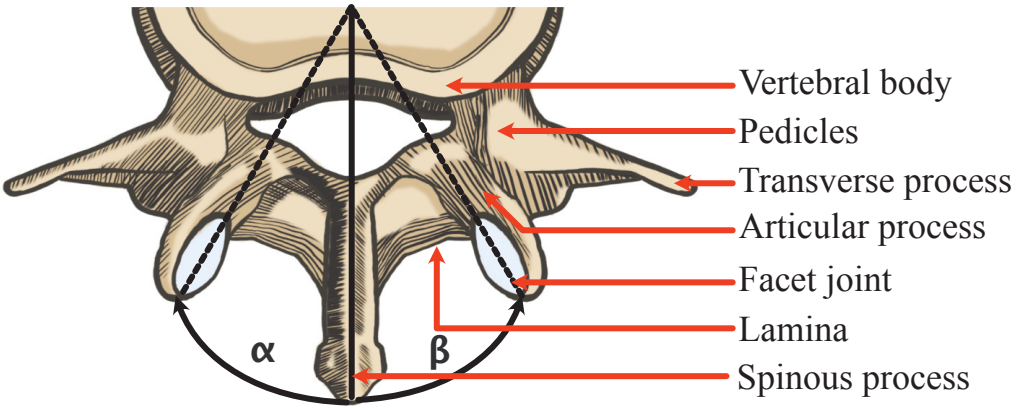

Figure 3 ***Schematic of vertebral features in the axial plane.*** *The angles α, β represent the bilateral facet joint orientation. Facet joint tropism is diagnosed when α and β vary by more than 8° —illustrated by D.G. Bisson.*

1.2.2.2 Function

The spine's complex anatomy is explained by its three functions: 1) protecting the spinal cord and nerve roots, 2) supporting the body 3) allowing for guided trunk mobility. The different components of the spine work together to ensure these three functions are maintained. The pedicles and laminae form a rigid enclosure around the spinal cord to protect the spinal cord and nerve roots. Most support for the upright human body comes from vertebral bodies and IVDs helped by articular processes and FJs. Yang and King estimated that the IVDs support 75-97% of the compressive spinal load whereas the remaining 3-25% is covered by the facet joints. [42] The kyphotic and lordotic curves in the sagittal plane help distribute the biomechanical loads evenly

across the upright spine. Finally, trunk movement is performed through the spinal muscles connected to the transverse and spinous processes, which act as lever arms. The movement allowed in every intervertebral segment is guided by ligaments and the orientation of their facet joint pair. [43] Flexion, extension, lateral flexion and rotation are all permitted by the flexibility of the intervertebral segments and constrained by the recruitment of specific spinal features. Failure to maintain regular physiological forces during trunk mobility is described by Panjabi et al. as loss of spinal stability. [44]

1.2.3 Facet joints

Facet joints are the synovial planar joints covered in hyaline cartilage intersecting the vertebra's superior and inferior articular processes. For each intervertebral segment, a pair of joints are located symmetrically in the mid-sagittal plane, posterior to the spine. (Figure 1) Facet joints assist the IVDs and vertebral bodies transfer biomechanical loads, especially in extension and rotation. [45][42] However, their most important feature is to restrain the intervertebral motion to certain directions. This feature prevents movement between adjacent vertebra that could injure IVDs, the spinal cord and nerve roots. The joint comprises various tissues that enable and limit these complex spinal movements.

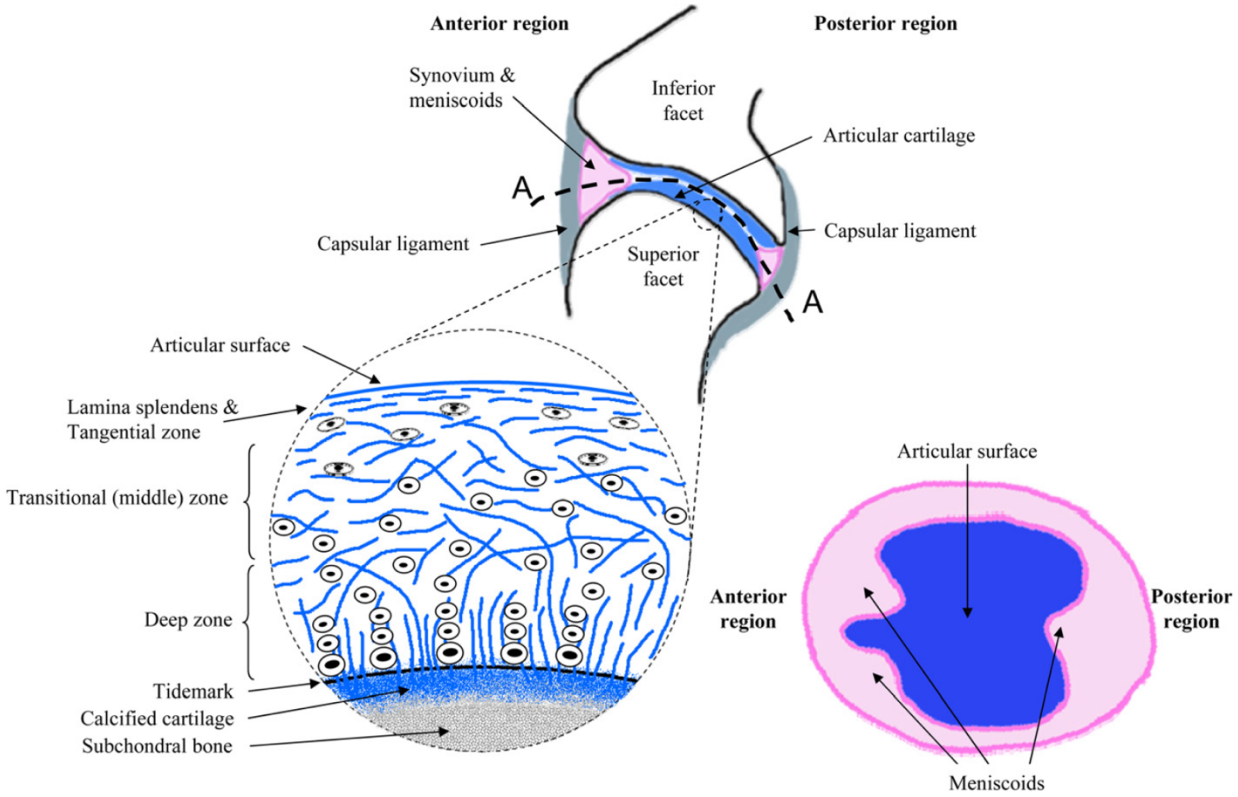

Figure 4 ***Schematic drawings of the facet joint*** *and the primary tissues that compose it, as well as the cartilage and menisci of the facet articulation. The blow up illustrates the different zones of the articular cartilage layer with the collagen fibers and chondrocytes orientations through its depth. A cut through of the facet joint (A-A) is also drawn to show the elliptically-shaped inter-articular surfaces with the cartilage surface on the inferior facet,the synovium, and meniscoids. This image was used in permission with the publisher.*

1.2.3.1 Cartilage

Most importantly, the cartilage surface and synovium permit frictionless motion and compressive loads between the articular processes. Cartilage thickness is maximal in the center of the joint and gradually decreases towards the edges. [46] The cartilage extracellular matrix (ECM) composition is largely responsible for load-bearing capability. The residing chondrocytes are the only cell type found in cartilage and produce the collagen fibers, glycosaminoglycans (GAGs) and proteoglycans in the ECM. The organization of molecules and chondrocytes shift between the superficial, middle, and deep zones between the articular surface and the subchondral bone,

respectively. In the superficial zone, the collagen fibers are oriented parallel to the articular surface and populated by relatively few flattened chondrocytes. The fiber alignment is essential to enable proper resistance to shear and tensile strains when the cartilaginous surfaces slide against each other during movement. Through the middle and deep zones, the collagen fibres transition to a vertical alignment and chondrocytes become more present and organized in columns parallel to the fibers. Proteoglycan concentration follows chondrocyte density and increases incompressibility through the middle and deep zones by trapping water in their negatively charged chains. This gradual change in elastic modulus allows an even transfer of loads from the surface to the subchondral bone. [47] In AIS, genetic studies have shown that alterations of extracellular matrix proteins may be a risk factor for developing the disease. [48] Abnormal chondrocyte metabolism and proliferation were also observed in AIS facet joints. [49] However, facet joint cartilage in the context of AIS was understudied at the start of this doctoral study.

1.2.3.2 Capsule and Synovium

The synovium is a thin membrane of connective tissue between the two articular cartilage surfaces that secretes the synovial fluid to lubricate the joint for frictionless motion. This environment is maintained by a ligamentous capsule surrounding the whole joint between the superior and inferior processes. The capsule consists of compact collagen fiber bundles, proteoglycans, and elastin fibers and is lined with fibroblasts. Its varying thickness provides tensile resistance to the joint, especially in rotation and contains the synovium and surfaces aligned. The capsule is densely populated by neurons providing mechanoception, proprioception and nociception to the CNS. [47] The signals transmitted to the CNS allow proper activation of paraspinal muscles to counterbalance the spine in movement. In the context of scoliosis,

generalized joint laxity was found in multiple AIS cohorts. [50-52] It remains unclear how facet joint capsules behave in AIS under abnormal loading conditions.

1.2.3.3 Bone

Finally, compressive loads are transferred to the vertebral body through the bony articular processes supported by the cartilage. The arrangement of hard cortical bone on the periphery and column-like trabecular bone inside is optimized to provide the most strength with the least bone volume. [53] Articular process orientation varies throughout the spine, potentially as a requirement for the sagittal curves (lordosis and kyphosis) of an upright spine. [54] Lumbar facet joints are oriented 82-86° sagitally and 15-70° in the axial plane to limit axial torsion of the lower back while providing more range of motion in flexion. Thoracic facets oriented between 55-80° sagitally and 85-120° axially permit a bigger range of motion in rotation and flexion for the trunk. Facet joint tropism is diagnosed when the relative bilateral orientation of the facets surpasses 8° (Figure 1) and is associated with curve progression in AIS. [55][56] Many dysfunctional mechanisms in bone remodelling and metabolism are also prevalent in scoliosis. Most importantly, studies suggest that the delicate balance between bone formation by osteoblasts and bone resorption by osteoclasts is disturbed in scoliotic patients. [57] The unbalanced bone remodelling in AIS could be due to impaired osteogenic differentiation of mesenchymal stem cells to osteoblasts. [58] Additionally, an association between ghrelin resistance in osteoblasts from AIS patients was found and could explain the prediction power of increased plasma ghrelin levels and curve progression [59][60] Thus, the generalized osteopenic phenotype of AIS patients, which displays high bone turnover rates, seems to be caused by osteoblast dysfunction.

1.2.4 Facet joint degeneration

Degeneration of the facet joints is a progressive condition that affects all tissues of the articulation (articular processes, cartilage, ligament, synovium). It leads to detrimental changes in the composition and mechanical properties of the tissues involved. [47] Osteoarthritic facet joint cartilage is recognized morphologically by surface fibrillation, fissures, erosion and eventually exposure to subchondral bone in severe conditions. [61] The composition of the extracellular matrix in such conditions Impaired chondrocyte metabolism and increased apoptosis hamper the repair process resulting in a disruption of the collagen network and loss of proteoglycan content. Degenerative changes to the ligament capsule were observed by Boszczyk et al. in osteoarthritic lumbar facet joints in the form of ligament hypertrophy, aberrant fibrous scar tissue and fibroblast proliferation. [62] In osteoarthritic bone, osteophyte formation and thickening of the subchondral trabecula were observed in age-related degeneration. [63][64] However, it remains unclear if facet joints degenerate in the abnormal environment of the scoliotic spine.

1.2.4.1 Consequences

The primary consequence of facet joint degeneration is the mechanical failure of the joint. Fujiwara et al. published a study detailing the increased axial rotation in intervertebral segments bearing degenerate facet joints. [65] Similarly, joint laxity caused by damages to the facet joint capsule was found by Ivancic et al. [66]. Similar studies have proven the relationship between segmental hypermobility and facet joint osteoarthritis. Hypermobility of the facet joint can have cascading effects on the surrounding intervertebral segments by way of improper load distribution. [67][68] A secondary outcome of facet joint degeneration is back pain. [69][70] Manchikanti et al. report that 40% of patients with chronic low back pain suffer from facet joint osteoarthritis.

[71] Mechanistically, it is believed that the noxious stimuli come from inflammatory mediators released by the degenerating facet joints irritating nociceptors in the dorsal root ganglia or capsule. [72] [73] In support, animal models of facet joint degeneration saw their painful behaviour increase in conjunction with the overexpression of inflammatory cytokines. [74-76]

1.2.4.2 Cause

Studies have found a high prevalence of facet joint osteoarthritis increasing with age in the general population. [77] Additionally, sports or motor vehicle accidents are common causes forof injurious facet joint degeneration. [78][79] Mechanotransduction is the process by which cells in bone, cartilage and ligament sense the mechanical stimuli of the loaded joint and respond with an appropriate response to repair or maintain the tissue. The amplitude, frequency and rate of biomechanical loading are the three factors to which cells respond. The cells develop aberrant responses if the biomechanical values extend beyond a physiological window. [80] Generally, inflammation is a primary component of the unusual cell response and is a common occurrence in osteoarthritis. [81][82] For example, an increase in inflammatory cytokines such as IL-6 and IL-1ß was reported in osteoarthritic facet joints by Igarashi et al. [83] Cytokines have an autocrine/paracrine effect on surrounding cells to promote tissue catabolism through the expression of proteolytic enzymes. [81] Xu et al. have shown that overexpression of the collagenase MMP-1 is directly linked to IL-1ß stimulation in osteoarthritic facet joints. [84] Once the collagen network is disrupted, the resulting increase in forces applied to the cells amplifies their aberrant inflammatory response even more.

1.2.5 Disease Modifying drugs for osteoarthritis

The interest in developing disease-modifying drugs for osteoarthritis has steadily grown over the past years. Such treatment options represent the ideal therapeutic scenario by targeting the underlying condition to change the course of the disease instead of treating only the symptoms. In the context of degenerating facet joints, as mentioned above, controlling inflammation should be an optimal target to prevent joint tissue damage. [85] Inappropriate activation of NF-κB has been characterized as a critical mediator of the chronic inflammation observed in osteoarthritis. [86][87] Despite being a promising target for therapeutic intervention, inhibiting NF-κB directly has several downsides to its importance in countless other cellular pathways. [88] For this reason, we have focused our search on a molecular target upstream of NF-κB activation in OA.

1.2.5.1 Toll-like receptors

Toll-like receptors (TLR) are a class of extracellular sensors that bind a wide range of endogenous and exogenous ligands to induce intracellular signalling through MAP kinases and, ultimately transcription factors including NF-κB, AP-1 and IRF-3. (Figure 4) Their molecular pattern recognition and downstream effects are complex due to the combinatory nature of two TLR subunits and ligands to start the molecular cascade. The TLR family is comprised of 10 members in humans. TLR1, 2, 4, 5, 6 and 10 are located mainly on the cell surface, while TLR3, 7, 8 and 9 reside in endosomal vesicles. TLR2/6, TLR1/2 heterodimers and TLR4 homodimers are the dimer pairs most described in the literature. [89] TLR activation is induced in 3 steps [90][91]:

1st step: Two TLR subunits dimerize while trapping the ligand in their leucine-rich repeat ectodomains. Cytoplasmic Toll/IL-receptor (TIR) domains also dimerize due to the proximity between two subunits.

2nd step: Membrane-recruited TRAM or TIRAP organize molecular complexes around MyD88 or TRIF. All TLR dimer pairs activate the MyD88-dependant pathway. However, TLR4 homodimers can also signal through TRIF to induce interferon transcription.

3rd step: TRAF6 signal through MAPK to ultimately translocate NF-κB and AP-1 towards the nucleus to activate transcription of pro-inflammatory genes, amongst others.

1.2.5.1.1 Toll-like receptors in OA

TLR pathways are heavily involved in inflammatory processes required in immune responses and tissue repair. [89] However, recent research suggests that TLRs are also involved in the inflammatory state of OA joints. First, a growing body of literature reports an increase in the abundance of alarmins in degenerating cartilage. [92-95] These include intracellular proteins that bind to TLRs once released from the cytoplasm, such as high mobility group box protein 1 (HMGB1), S100A8/9 and heat shock proteins (HSP). [96] Such alarmins are actively secreted by stressed cells or passively released by dying chondrocytes in damaged joints. Additionally, proteolytic fragments of extracellular matrix (ECM) proteins, including fibronectin, biglycan, fibromodulin and hyaluronic acid, also have the potential to activate TLRs. [97][98] ECM alarmins are prominent in the catabolic state of the OA joint due to the increase of catabolic enzymes such as matrix metalloproteases (MMPs) and a disintegrin and metalloproteases with thrombospondin motifs (ADAMTS). Furthermore, the expression of TLR1-9 in chondrocytes increases with OA grade. [99][100] Activation of TLRs in chondrocytes induce the expression of pro-inflammatory cytokines, and catabolic proteases and reduces ECM synthesis. [101-103][95] For example, gene expression of MMP-1, -3 and -13 by chondrocytes were significantly upregulated after treatment with LPS and peptidoglycan signalling through TLR2/6 and TLR4 dimers. [99] Additional positive

feedback loops reinforce the inflammatory signal by upregulating TLR expression and promoting the creation of more alarmins in the joint space after initial TLR activation. The self-amplifying nature of inflammatory processes directed by TLRs is essential for a robust immune system and efficient responses to injury, but they are at risk of uncontrolled chronic inflammation in the case of OA. Contrary to knee and other long bone joint OA, the involvement of TLRs in facet joint degeneration is comparatively understudied. Jiang et al. demonstrated an upregulation of TRAF6 in OA lumbar facet joint cartilage. A knockdown of this mediator of the TLR pathway successfully inhibited chondrocyte apoptosis and inflammation in LPS-treated chondrocytes. [104] The complete role of TLRs in facet joint osteoarthritis remains to be discovered.

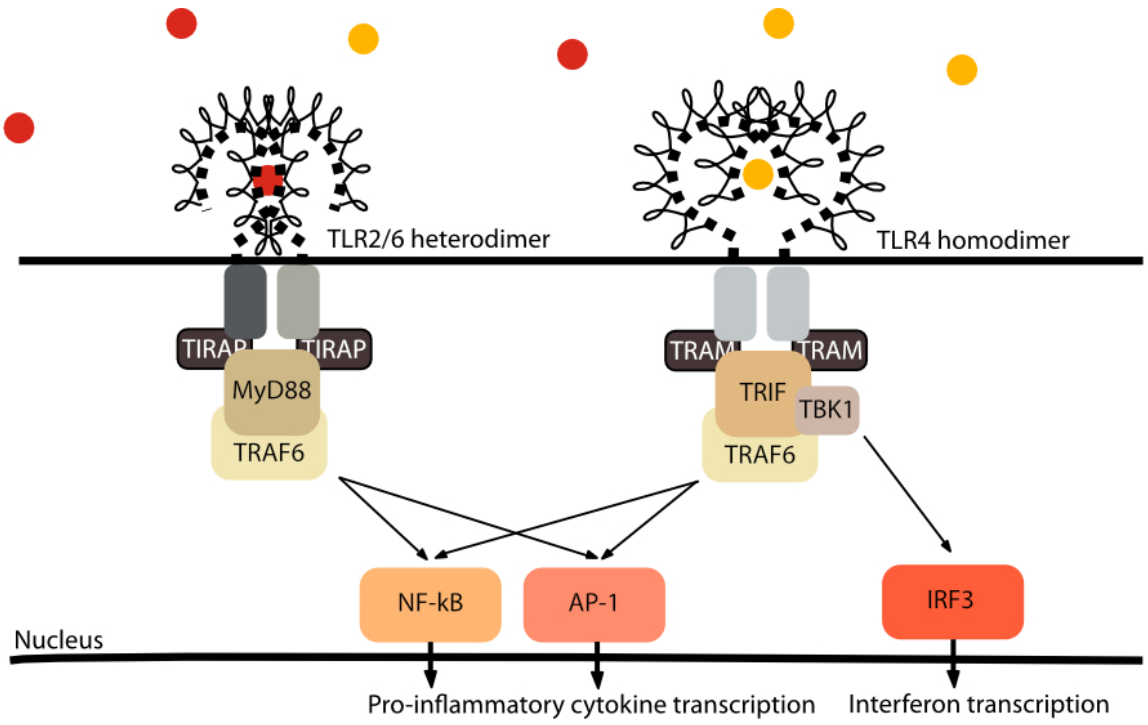

Figure 1 Schematic representation of TLR activation by TLR2/4 agonist and TLR4 agonist and downstream signalling pathways. [105] This image was used with permission from the publisher.

1.3 Rationale

Adolescent idiopathic scoliosis is a prevalent and debilitating disease that remains poorly understood but seems to originate from spinal instability during pubertal growth. Any progress in developing treatment modalities is hampered by the lack of knowledge regarding spinal tissue deformation and mechanics driving the progression of the deformity. Facet joints are crucial for their involvement in keeping rotational stability in healthy spines. The relationship between facet joint state and AIS was unexplored at the start of this thesis work.

1.4 Hypothesis

Facet joint osteoarthritis contributes to spinal instability at the core of Adolescent Idiopathic Scoliosis etiopathogenesis. Toll-like receptors could be the target of disease-modifying drugs to treat facet joint osteoarthritis to contribute to the prevention of AIS progression.

1.5 Specific Aims

1. Characterize facet joint degeneration in Adolescent Idiopathic Scoliosis.
2. Investigate how facet joint degeneration relates to spine deformation and pain in AIS.
3. Explore Toll-like receptors as disease-modifying drug targets to treat facet joint osteoarthritis.

Chapter 2: Facet joint degeneration in AIS

Preface

The incomplete comprehension of AIS etiopathogenesis limits the advancement of treatment options to prevent disease progression. As discussed above, our current understanding of AIS development involves a cascade of asymmetrical loading along the spine, inducing vertebral deformities that exacerbate unbalanced biomechanics. However, the nature of both aberrant forces and their modulatory effects on scoliotic vertebrae remains understudied. One hypothesis postulate that asymmetrical forces can arise from the rotational instability of an upright human spine. The bilateral pair of facet joints are the vertebral features most engaged in keeping the rotational balance of the spine. Facet joint osteoarthritis is characterized by the degeneration of the cartilage, loss of subchondral bone and a generalized weakening of mechanical properties necessary for joint function. Therefore, spinal instability and pain are frequent consequences of facet joint osteoarthritis in the average population. However, a complete characterization of facet joint cartilage in the context of AIS has never been established. Due to their importance in transmitting biomechanical loads symmetrically and limiting vertebral torsion, the characterization of facet joints in scoliotic patients is essential for understanding AIS pathogenesis. Furthermore, the discovery of facet joint osteoarthritic asymmetry would reveal the extent of rotational vulnerabilities and help decipher how asymmetrical loading modulates vertebral growth during disease progression.

In this study, we hypothesized that osteoarthritic hallmarks would be detected in facet joint cartilage from AIS patients. We characterized and graded facet common cartilage osteoarthritis

from scoliotic patients undergoing surgery compared to samples from non-scoliotic organ donors using histological and immunoblotting techniques.

Manuscript I: Facet joint degeneration in Adolescent Idiopathic Scoliosis

Daniel Bisson[1,2], Polly Lama[1,2], Fahad Abduljabbar[1], Derek H Rosenzweig[2], Neil Saran[1], Jean A Ouellet[1,2], Lisbet Haglund[1,2]

[1]Orthopaedic Research Laboratory, Shriners Hospital for Children, Montreal, Canada

[2]Department of Orthopedic Surgery, McGill University, Montreal, Canada

Corresponding Author:

Dr. Lisbet Haglund, Ph. D
Associate Professor,
The Orthopaedic Research Laboratory
Montreal General Hospital,
McGill University,
1650 Cedar Avenue, Room C10.148.2
Montreal, Qc., H3G 1A4
Canada
Telephone: (514) 934-1934 ext. 35380
or
Shriners Hospital for Children
1003, boulevard Décarie
Montréal, Québec H4A 0A9
E-mail : lisbet.haglund@mcgill.ca

Keywords: Scoliosis, facet joint, osteoarthritis, metalloproteases, cytokines, cell proliferation, cartilage, proteoglycans, extracellular matrix

Adolescent idiopathic scoliosis (AIS) is a poorly understood deformity of the thoracolumbar spine which affects the intervertebral discs (IVDs) and the articular facet joints. The knowledge concerning facet joints in this context is very limited, although facet joint degeneration is a known contributor of back pain. In this study, a comprehensive investigation was performed to characterize the facet joint chondrocytes and extracellular matrix within the scoliotic spine.

Surgically removed articular facet joint tissues were collected from patients undergoing spinal corrective surgery for AIS deformities, while non-scoliotic articular facet joint tissues were obtained from cadaveric organ donors. Alterations in cartilage tissue structure were evaluated histologically with safranin-O fast green and a modified OARSI grading scale. Pro-inflammatory cytokines, matrix-degrading proteases, and fragmented matrix molecules associated with cartilage degradation were analyzed by immunohistochemistry and western blotting.

Safranin-O fast green staining revealed that young scoliotic facet joints show clear signs of degeneration with substantial proteoglycan loss, similar to osteoarthritis (OA). The proteoglycan levels were significantly lower than in healthy asymptomatic non-scoliotic control individuals. In comparison to controls, scoliotic articular facets showed increased cell density, increased expression of the proliferation marker Ki-67, immunopositivity to Ki-67, and higher expression of MMP-3, MMP-13 and IL-1β. Expression and fragmentation of the small leucine rich proteins (SLRPs) chondroadherin, decorin, biglycan, lumican and fibromodulin were analysed with western blot. Chondroadherin and decorin were fragmented in cartilage from patients with a curve greater than 70°, whereas biglycan and fibromodulin did not show curve-related fragmentation.

AIS facet joint cartilage shows hallmarks of OA including proteoglycan loss, overexpression of proinflammatory mediators, increased synthesis of matrix-degrading proteases

and fragmentation of SLRPs. As with patients with age-related OA, the premature joint degeneration seen in scoliotic patients is likely to contribute to the pain perceived in some individuals.

Introduction

Adolescent idiopathic scoliosis (AIS) is a structural deformity which manifests as a gradually increasing curvature and rotation of the spine [1]. Patients with AIS can experience mild discomfort which later progresses as impairment in spinal function and impaired breathing due to reduced area for lungs to expand [2]. There is a worldwide prevalence of 3% of children aged 10-16 years who suffer from AIS [3], and currently there are two treatment options: 1) bracing if the curve is below a 50-degree Cobb angle (major curve angle) 2) spinal fusion with instrumentation when the curvature is greater than a 50-degree Cobb angle [2]. Although the treatment options are highly beneficial for managing progression, the long-term effects on patient's quality of life are still unclear [4].

The clinical system used to classify the wide range of curve types in AIS is called the Lenke system, developed by Lawrence Lenke and colleagues [5]. It provides reliable two-dimensional classification using upright coronal and sagittal radiographs to determine 1) Curve type (1-6) 2) Lumbar spine modifier (A, B, C) and 3) Sagittal thoracic modifier (-, N, +). This system allows for an objective classification of every possible curve pattern in scoliosis and helps clinicians choose the best treatment accordingly.

In non-scoliotic individuals, the symmetry of the spine allows its load-bearing to be balanced between the intervertebral discs (IVD) and the corresponding facet (zygapophyseal) joints [6]. Due to the midline location of IVDs and their large size, 70-80% of the load is transmitted through the IVDs and vertebral bodies [7]. The facet joints compensate for the

remaining 20-30% of load distribution. The bilateral position of facet joints give them the role of limiting flexion and torsion of the spine [8]. In patients with AIS, this equilibrium is shifted and consequently the facet joints become subjected to increased and unbalanced loads because of the abnormal torsion and rotation of the spinal column. It is well known that load magnitude affects bone and cartilage development and growth as well as proteoglycan production by chondrocytes [9]. Thus, inadequate or excessive loading has detrimental effects on cartilage matrix metabolism [10]. Apart from traumatic injury, which is the most obvious form of abnormal biomechanics, slight shifts in ambulatory mechanics [11], abnormal tension, shear forces and malalignment of articular joints [12] are known to contribute towards cartilage degeneration. In fact, load-induced changes are one of the triads of primary osteoarthritis, along with genetic influences and age-related changes [10]. In consequence of abnormal spinal curvatures and loading, osteoarthritis (OA) (defined as destruction of articular cartilage and subchondral bone) could be present in the facet joints of younger AIS patients. Osteoarthritis is also known to be prevalent in 15-45% of the facet joints in patients with chronic low back pain cases [13].

Cell proliferation and formation of cell clusters can often be observed in OA. The chondrocytes overexpress pro-inflammatory cytokines such as IL-6, IL-1β, and matrix-degrading enzymes such as MMP-3 and MMP-13 [14]. Increases in these factors shifts matrix homeostasis toward catabolism, thereby promoting tissue destruction. Consequentially, the cartilage loses proteoglycan content early along with its collagen network later in the disease process [15]. The small leucine-rich proteins (SLRPs) which include biglycan, decorin, chondroadherin, fibromodulin and lumican [15], have also been reported to be proteolytically fragmented in OA [16]. SLRPs are found in the pericellular or territorial matrix [17], and their fragments may

potentially act as alarmins [18] by activating toll-like receptors TLR 2 and 4 in articular cartilage. TLR activation triggers the secretion of pro-inflammatory mediators [19].

The aim of this study is to investigate the effects of scoliotic curvature on the articular facet joint homeostasis in patients with AIS. Our underlying hypothesis is that abnormal curvature of the scoliotic spine results in degenerative changes and increased secretion of cytokines and proteases in facet joints, as reported in osteoarthritis.

Materials and Methods

Sample Collection: Multiple superior articular facet joints were collected from AIS patients undergoing spinal fusion corrective surgery after consent. The facet joints collected span multiple spinal levels, with the levels collected depending on the surgical requirement. The orthopedic surgeons classified the patient's spinal curves according to their major curve types using the Lenke system, which categorizes all patients into 6 major curve types with subclassifications for lumbar and sagittal thoracic modifiers using bi-planar radiographs (Table 1) [5]. Articular facet cartilage samples of 20 scoliotic patients aged 11-19 years, with a mean age of 15.23 ± 2.36, were collected. 75% were from female patients (Table 2). The institutional review board approved this study. McGill University (IRB # A00-M113-13B) Montreal, Canada.

Through a collaboration with Transplant Quebec, six asymptomatic non-scoliotic articular facet joint pairs from the thoracolumbar region, age range 17-60 years, with a mean age of 34.33± 13.31 were obtained from cadaveric organ donors who had no history of back pain or other spinal deformities (Table 2).

Tissue processing for explant culture: Articular cartilage was separated from the underlying subchondral bone using a scalpel. Cartilage explants were washed with Phosphate Buffered Saline

(PBS) containing 50µg/ml gentamycin (Life Technologies), and 0.5µg/ml amphotericin B (Life Technologies) for 5 minutes. Cartilage explants were kept in Dulbecco's modified eagle medium (DMEM) with 4.5g/L glucose (Sigma), 25µg/ml gentamycin (Life technologies), 2mM Glutamax (Life Technologies), and 10% fetal bovine serum for 24 hours following dissection. The facets were cultured for 96 hours in serum-free DMEM, 25µg/ml Gentamycin (Life Technologies),1x Glutamax (Life Technologies), Insulin-transferrin-selenium (ITS, Life Technologies), 50µg/ml ascorbic acid on volume per weight basis (10x v/w) at 37º C, 5% CO_2.

Histology: Dissected facet cartilage was fixed in 4% paraformaldehyde overnight, followed by sequential immersion in 10%, 20% and 30% sucrose for 12 hours each before OCT embedding. Cryosectioning was performed on a CryoStar NX70 cryostat (ThermoFisher Scientific) and 12um thick sections retrieved were arranged on charged super frost plus slides (VWR). Prior to staining, slides were heated at 60°C for 15 minutes and rehydrated in PBS-T (0.05% Triton-x) for 5 minutes. Safranin-O (0.025%) (Sigma Aldrich, Canada) and fast green stain (0.01%)(Sigma Aldrich, Canada) was applied to the sections for 5 mins each as mentioned in published protocols [20] and a semi-quantitative grading was performed on the tissue sections.

Immunohistochemistry: 12um thick sections were immunostained for proliferative and pro-degenerative factors. The primary antibody to the proliferative marker Ki-67 (10ng/ml) (NB110-89717) (NovusBio) was applied at 1:100 dilution, while antibodies to pro-inflammatory markers IL1ß (ab9722) (2.5µg/ml), IL6 (5µg/ml) (ab9324) and matrix degrading enzymes MMP3 (2µg/ml) (ab52915), MMP13 (5µg/ml) (ab39012), (Abcam) were used at a 1:200 dilution for 1 hour at room temperature. Followed by PBS washes, DAB chromogen (Abcam) staining kit was used to identify the immunopositive cells. These cells were counted from images captured under 20x and 40x

objectives with Olympus DP70 digital camera (Olympus) pre-fixed to a Leica microscope (Leica DMRB) under visible light.

Histology staining & quantification: A modified OARSI grading scheme (Grade 0-4) was used and grading of the safranin-O fast green stained sections was performed blinded by 3 independent evaluators [21]. Healthy cartilage is given a grade 0 and OA starts at grade 1, where mild abrasion and proliferation can occur. At grade 2, there is discontinuity in the cartilage surface that can transform into fissures at grade 3. At grades 4 and 4.5, erosion and excavation takes place respectively. OARSI grades 5 and 6 (characterized by denudation, deformation and bone remodelling) were excluded since the cartilage was removed from the bone before analysis. For cell counts, DAPI (Vectashield) and Meyer's hematoxylin (Sigma) stained cell nuclei were counted and normalized to cartilage area using ImageJ software (NIH). All slides were examined using the 5X objective. A MatLab script was developed with background normalization, and the area selection tool was used to assess the proteoglycan content through average red pixel intensity in a semi-quantitative manner [22]. In the MATLab script, the red staining intensity of the entire section was quantified by normalizing to the white background and extracting the RGB (red-green-blue) intensities of each pixel in the region of interest, which was drawn over the cartilage and then isolating the red channel only which was finally averaged over the entire region of interest.

Protein extraction and digestion: Articular cartilage was finely minced, and proteins were extracted at 4°C under continuous agitation for 72 h using 15 volumes per gram of tissue of 4 M guanidine hydrochloride (Sigma Aldrich), 50mM sodium acetate and 10mM EDTA (Sigma Aldrich, Canada), and COMPLETE® protease inhibitors (Roche), pH 5.8. The extract was separated from the tissue by centrifugation for 30 minutes at 13,000 × g, and aliquots of 8µl of

were prepared for SDS page. Samples were treated with keratanase (Amsbio LLC) and chondroitinase ABC (Ambsio LLC) prior to SDS-PAGE and western blotting. [16]

Western Blotting: Proteins were fractionated on 10% SDS-PAGE gels and were transferred to a nitrocellulose membrane. The membrane was blocked with 3% skim milk powder in 0.01M Tris-HCL, 0.15 M NaCl, 0.1% Tween20 (TBS-T), pH7.6. Proteins were detected by chemiluminescence (GE Healthcare) and in-house generated antibodies to biglycan, lumican, chondroadherin, decorin, fibromodulin.

Statistics: Unpaired and paired parametric student T-test were performed in GraphPad (Prism) to assess significance valued at $P<0.05$. 95% confidence intervals were used to plot error bars.

Results

Histology and OARSI score: Articular facet joint cartilage from both scoliotic and non-scoliotic groups was stained with safranin-O fast green to assess proteoglycan levels and overall cartilage tissue condition. Hallmarks of osteoarthritis in the form of cartilage fibrillation and erosion ***(Figure 1D)*** were observed in a few samples, while proteoglycan loss was evident mainly from the superficial zone of the scoliotic tissue sections. To assess proteoglycan content semi-quantitatively, a MatLab script ***(Figure 1A, B)*** was used to calculate the mean red pixel intensity of the safranin-O stain of the entire section. The scoliotic facet joint cartilage had significantly ($P<0.0001$) lower pixel intensity compared to the non-scoliotic group. The scoliotic facet cartilage had an average OARSI grade of 2.14 ± 0.81 and the non-scoliotic had an average OARSI grade of 1.04 ± 0.51. The difference was significant ($P<0.05$), with the scoliotic cartilage showing a full grade point higher average score ***(Figure 1C)***. Safranin-O fast green staining intensity revealed

variable proteoglycan content between the two sides in a facet joint pair in scoliotic spines ***(Figure 2),*** whereas no difference was detected in the non-scoliotic group ***(Figure 1A).***

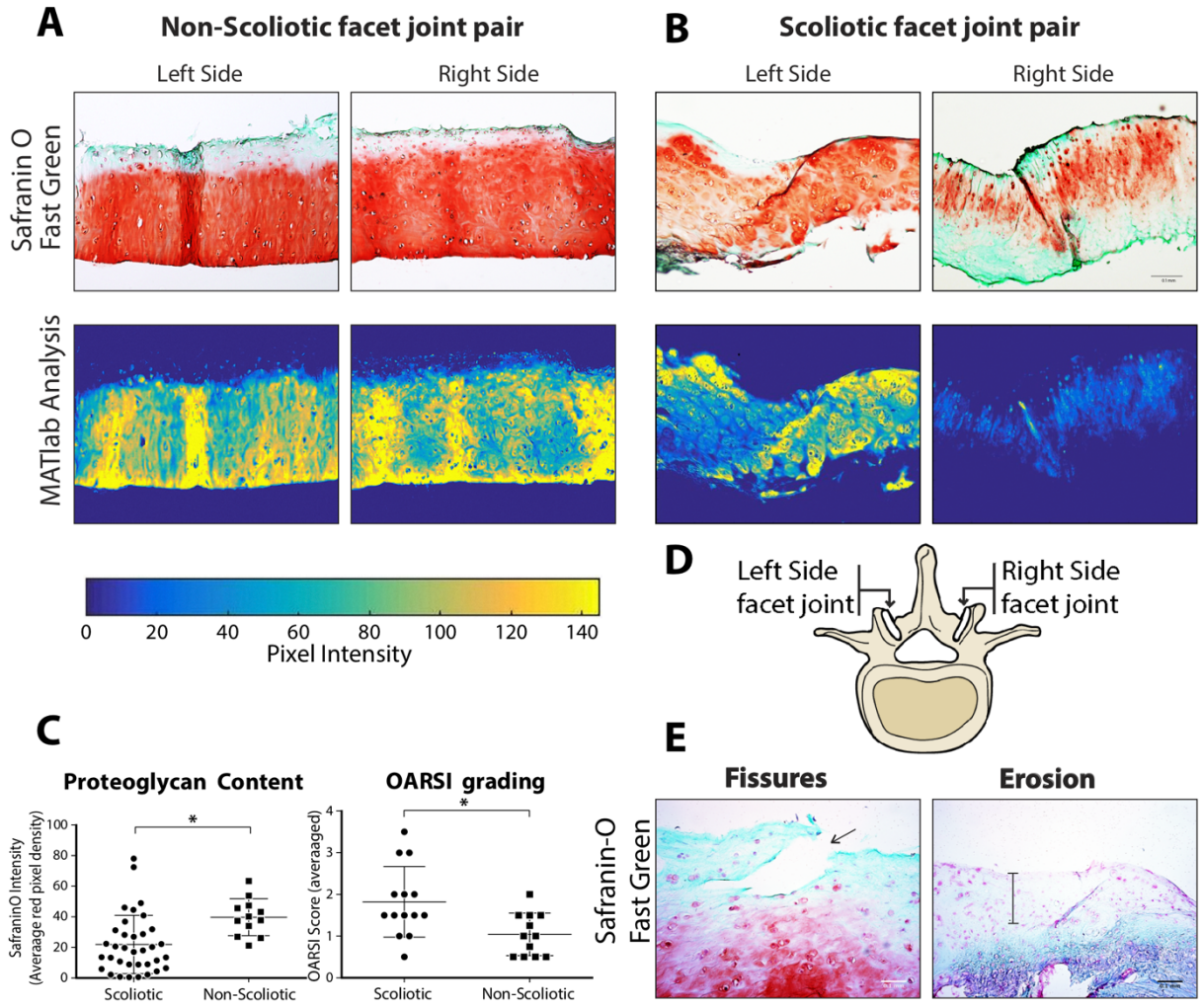

Figure 1 *Safranin-O fast green staining of bilateral facet joint cartilage from* ***A)*** *scoliotic and* ***B)*** *non-scoliotic donors with red staining quantification using MATlab.* ***C)*** *Overall safranin-O intensity and OARSI grading comparison between Scoliotic and non-Scoliotic donors.* ***D)*** *Schematic representing a facet joint pair from the same vertebrae.* ***E)*** *Osteoarthritic markers found in scoliotic cartilage and highlighted by arrow (fissure) and thinning (erosion). Error bars shown are 95% CI. Unpaired parametric student's t-test (**** = p<0.0001, * = p<0.05)*

Cell density and proliferation*:* The number of chondrocytes in the cartilage was determined by labeling cell nuclei with hematoxylin. Scoliotic cartilage had a significantly (P<0.0001) higher numbers of cells, averaging 1.5-fold more compared to non-scoliotic cartilage ***(Figure 3C).*** Some

scoliotic cartilage samples also showed more cell clustering, although this was not seen in all samples. Cell numbers were very similar between the two facet joints from the same spine level in every non-scoliotic donor. Side-to-side differences in cell number were often observed in the scoliotic cartilage at the same spine level. To determine if the cause for higher cell count was due to proliferation, as reported in osteoarthritis [23], the proliferative marker Ki-67 was used for immunohistochemical analysis. Ki-67 positive cell numbers were significantly ($P<0.0001$) greater in all scoliotic samples compared to non-scoliotic and always correlating with higher cell density when compared side-to-side ***(Figure 3C),*** with a high number of proliferating cells in the clusters

Figure 2: Safranin-O fast green staining of articular cartilage from 3 facet joint pairs (left and right side for 3 spinal levels for 6 samples overall) for each of 6 Lenke types.

(Fig 3F).

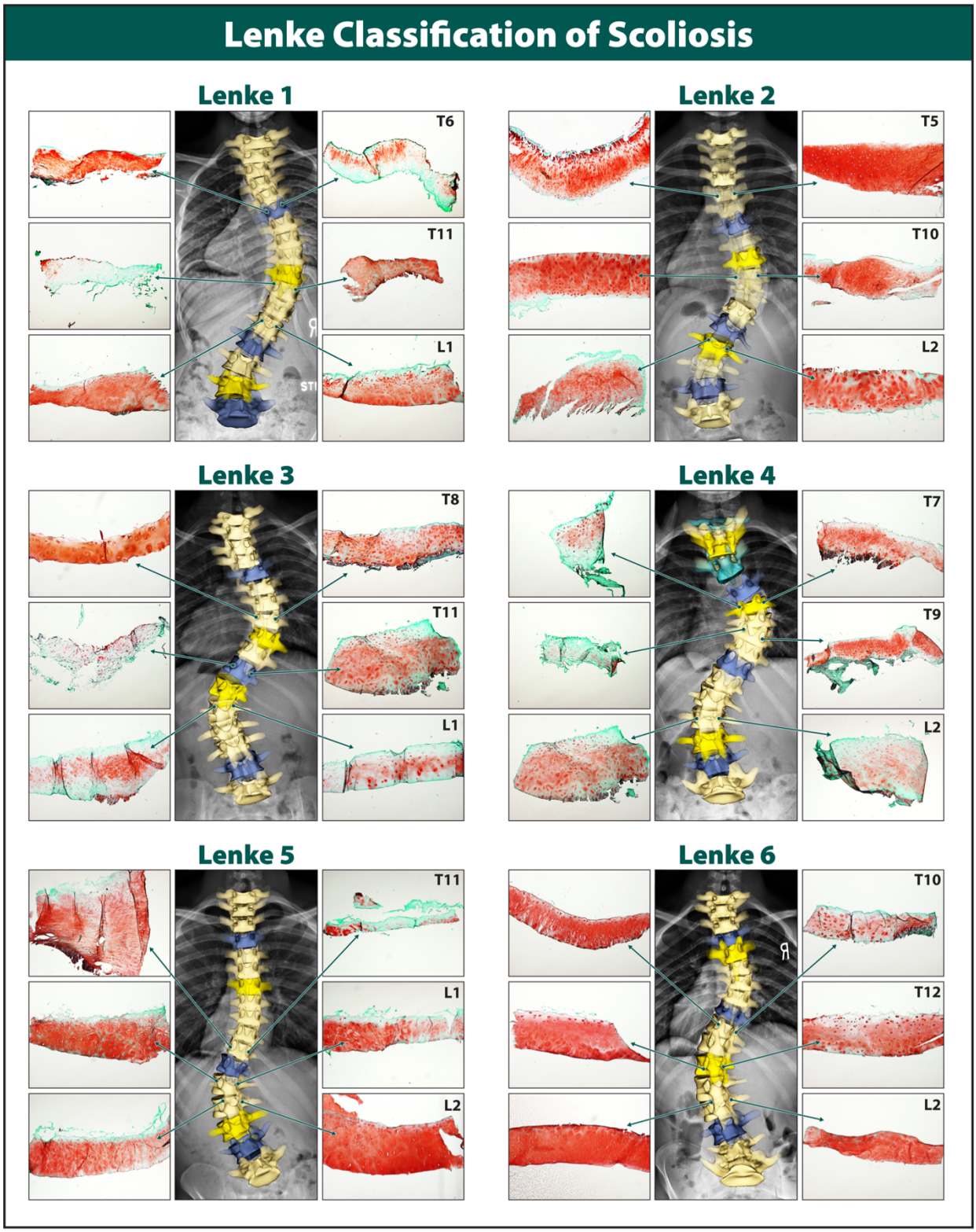

Figure 2: *Safranin-O fast green staining of articular cartilage from 3 facet joint pairs (left and right side for 3 spinal levels for 6 samples overall) for each of 6 Lenke types.*

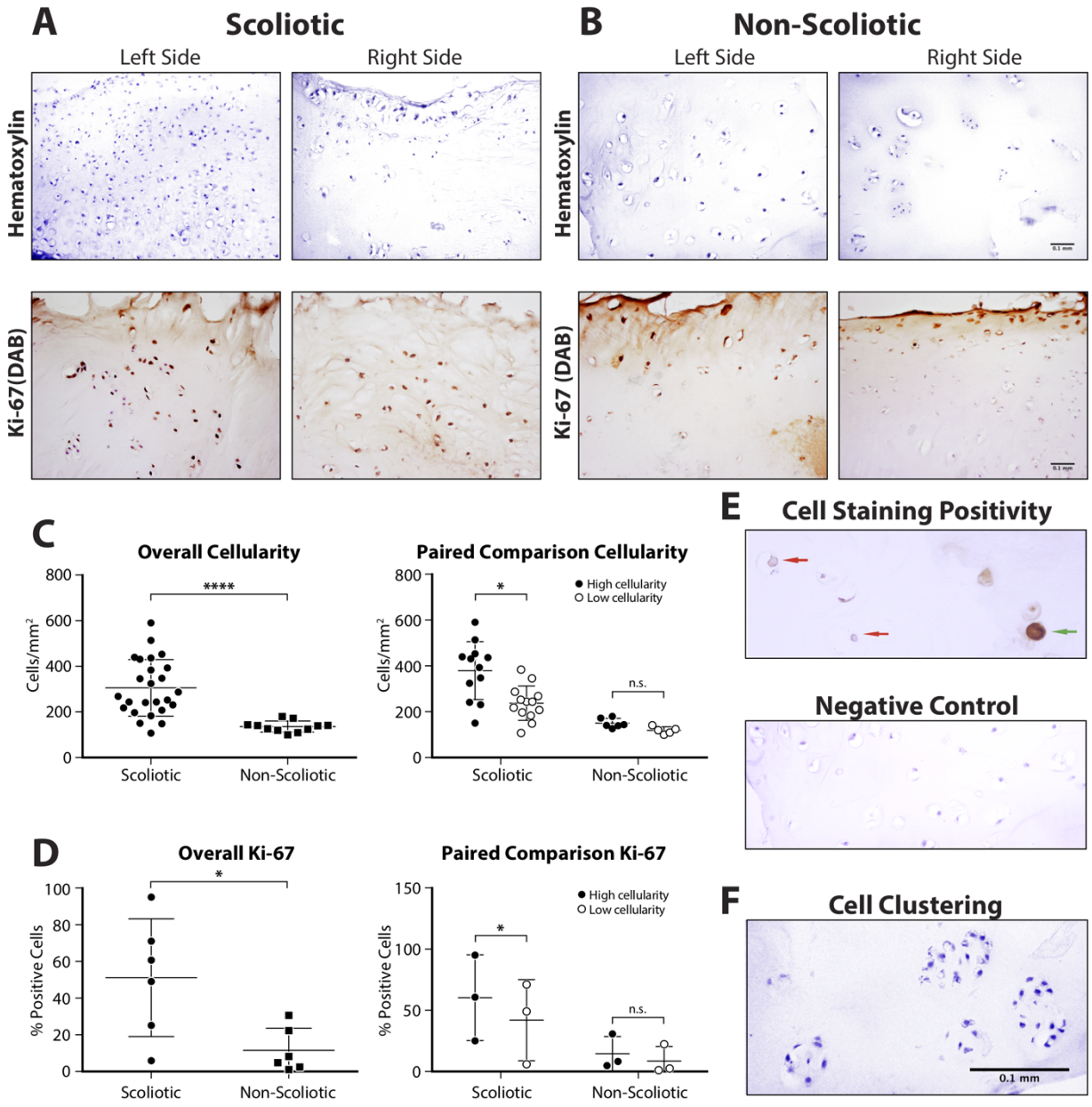

Figure 3: *Cell density and proliferative marker Ki-67 IHC within facet joint cartilage from* ***A)*** *AIS and* ***B)*** *non-Scoliotic groups revealed by hematoxylin and DAB staining respectively.* ***C)*** *Cell density quantification between scoliotic and non-scoliotic groups as well as paired comparison in high cellularity and low cellularity subgroups within the same donors.* ***D)*** *Ki-67 immunopositivity quantification and comparison between scoliotic groups and the same cellularity subgroups as C).* ***E)*** *High magnification Ki-67 IHC to show positive cells (green arrow) and negative cells (red arrow) and negative control (secondary antibody only)* ***F)*** *Image of scoliotic facet joint cartilage showing cell cluster, as revealed by hematoxylin. Error bars shown are 95% CI. Unpaired t-test for overall comparisons and paired t-test for paired comparisons. (**** = p<0.0001, * = p<0.05)*

Degenerative factor expression: Immunohistochemical staining showed a significant (P<0.001) increase in positive staining for MMP-3, -13 and IL-1ß in the scoliotic tissue **(Figure 4).** Positive cells were defined by strong intracellular and pericellular brown stain, which was normalized to all cells counterstained with hematoxylin. IL-6 showed the weakest immune reactivity with no difference between control and scoliotic groups.

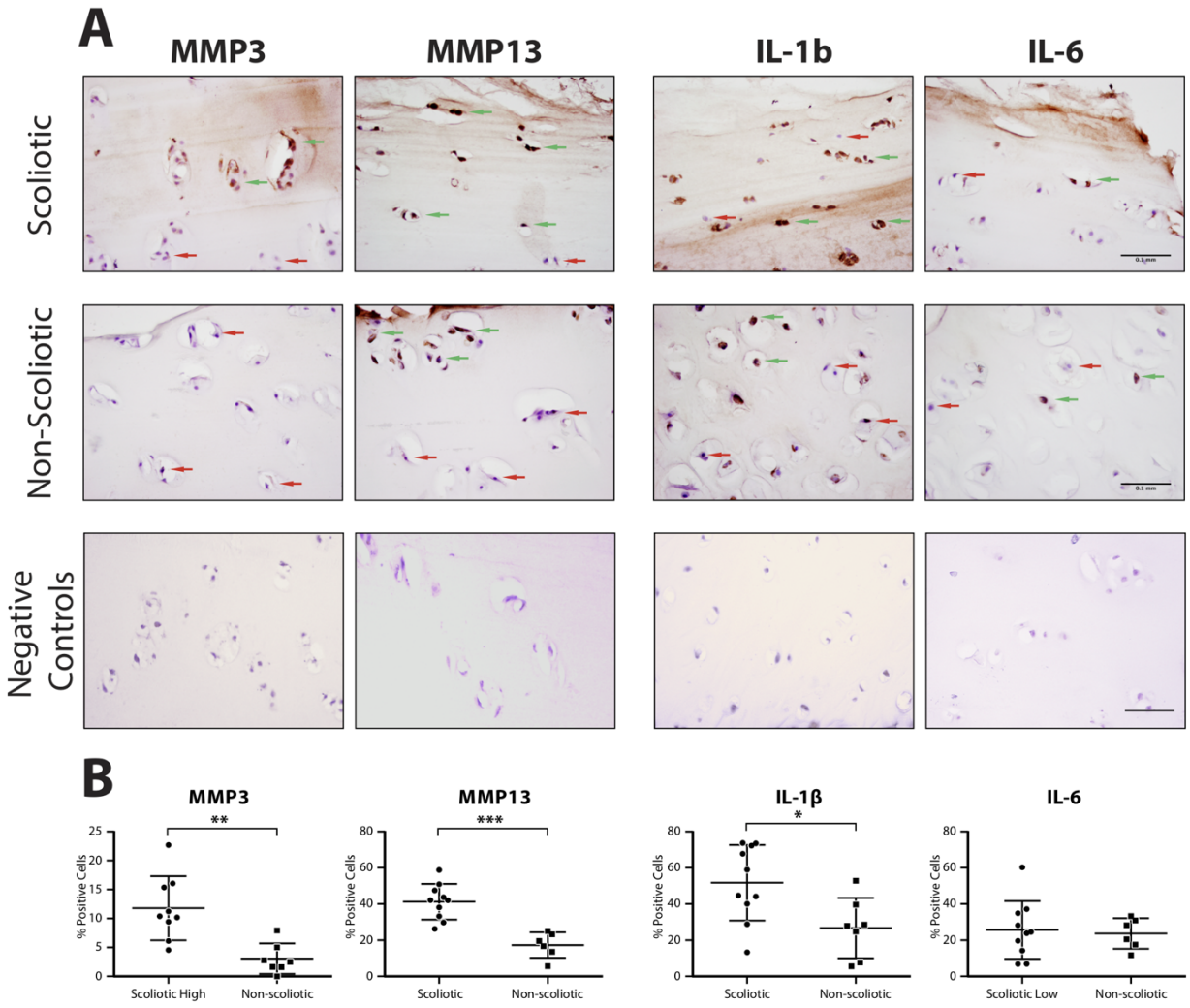

Figure 4: *Scoliotic and non-scoliotic cartilage immunohistochemistry of proteases MMP-3, MMP-13 and pro-inflammatory cytokines IL-1β and IL-6 with cell-positivity quantification. Positive cells are shown by green arrows and negative cells by red arrows. Error bars shown are 95% CI. Unpaired T-test (*** = p<0.001, ** = p<0.01, * = p<0.05)*

Fragmentation of SLRP's*:* Protein extracts of facet joint pairs (left and right facets) at the apex of the curve were pooled for 10 patients. The 10 scoliotic and 1 aged-matched non-scoliotic patient samples were analysed on a weight per volume basis by western blotting using antibodies for chondroadherin, decorin, biglycan and lumican. ***(Figure 5).***

Decorin and chondroadherin: Fragmentation of decorin and chondroadherin appeared as curve severity progressed ***(Figure 5)***. Chondroadherin core protein appeared at 38 kDa with three or four shorter fragments prominent between 22 to 35 kDa. Fragments appeared when the scoliotic curve progressed above 70 degrees of Cobb angle ***(Figure 5A).*** Similarly, decorin core protein appeared at 45 kDa, with two or three prominent fragments between 22 to 36 kDa, ***(Figure 5B).***

Biglycan, Lumican, and Fibromodulin: Biglycan fragmentation was present in all samples irrespective of curvature severity. For all samples, biglycan core protein appeared as a prominent band at around 45 kDa, with fragments evident at 36 kDa in all samples. A second 22 kDa fragment appeared mainly in curvatures above 70 degrees ***(Figure 5C).*** Fibromodulin core protein was apparent as a single or double band around 65 kDa ***(Figure 5D)***. Lumican appeared as a single band at 55 kDa with a faint lower molecular size fragment in a few samples ***(Figure 5E)***.

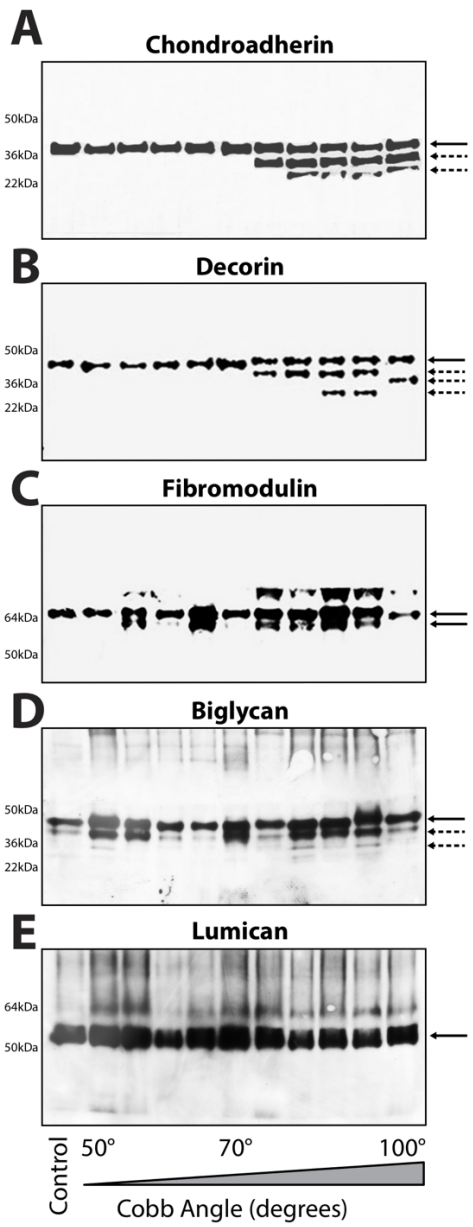

Figure 5: *Western Blot analysis of SLRP's on a 10 % gel. Gels were loaded based on ascending grade of curvatures. The curves ranged from 50 to 100 degrees, the first lane is occupied by non-scoliotic, non-degenerated age-matched control sample* ***5A)*** *Chondroadherin core protein at 38 Kda & its fragments below the core protein are indicated by the arrow.* ***5B)*** *Decorin core protein at 45 Kda with fragments. Note fragments for Decorin & Chondroadherin appear as the spinal curves magnitudes reach 70° of Cobb angle.* ***5C)*** *Doublet bands of Biglycan at 45 kDa along with its fragmented species.* ***5D)*** *Double and single bands of fibromodulin appearing around 65 kDa.* ***5E)*** *Non-fragmented Lumican appears as single band around 50 kDa.*

Discussion

Articular facet joints in AIS patients demonstrated multiple signs of tissue deterioration which occurs in the form of proteoglycan loss, increased cellular proliferation, overexpression of MMP-3, -13 and IL-1ß. The tissue also presents a distinct fragmentation of SLRPs, especially chondroadherin and decorin, with curves characterized by a bigger Cobb angle. The results from this study thus suggest that young facet joint cartilage tissues in AIS patients undergo changes comparable to aged individuals with osteoarthritis.

As the articular cartilage matures after epiphyseal closure around 20 years of age, its proteoglycan content decreases and the tissue slowly degrades with aging, a process that is strongly enhanced in OA [24]. In young scoliotic patients who were less than 20 years of age, loss of proteoglycan from the superficial zone was evident and distinctive fragmentation of SLRPs was apparent confirming an early onset of facet degradation in AIS. This is somewhat similar to IVD matrix disruption as we have previously reported for chondroadherin fragmentation in AIS IVDs [25, 26] and the recently reported aggrecan fragmentation in AIS IVDs [27].

In articular cartilage, the histological progression of OA is evidenced by surface discontinuity, fibrillation and erosion ***(Figure 1E).*** These characteristic changes were evaluated by a modified OARSI grading [20] for the analysis of the AIS and non-scoliotic facet articular cartilage. This grading system shows scoliotic tissues had the strongest OA-related phenotype with significantly higher OARSI grade compared to the control group. Furthermore, proteoglycan loss in facet joints of AIS patients were comparable to affected joints in osteoarthritic patients. In fact, the average age of the scoliotic donors was 15 and that of cadaveric organ donors was 34 years, which further reinforces the finding that known age-related OA associated changes were more pronounced in younger AIS facets. Interestingly, the only age-matched non-scoliotic donor (17

years old) displayed similar amounts of proteoglycan content and OA related changes to the older non-scoliotic donors, suggesting that the difference seen between control and scoliotic groups are not solely age related.

There are many previous studies describing results in the context of a simple 2-dimensional concave or convex side of the spine. However, a simple 2-dimensional analysis by x-ray of spinal concavity and convexity cannot be used to assess differential biomechanical loads across facet joints for two reasons: 1) It does not account for spinal segment rotation which affects the perceived load on each side of the vertebrae 2) Contrary to IVDs where the concave side gets wedged during scoliosis, the facet joints are not oriented perpendicularly to the spine, which alters the load bearing as well. For example, the Lenke 1 facet joints showed advanced deterioration on the concave side (**Figure 2**). However, for more complex curves like the Lenke 3, a different pattern is observed (**Figure 2**). These observations are in accordance with recent studies that found the higher loading on facet joints in degenerative lumbar scoliosis is dependent on the curve intensity, the position of the apex and spinal movements, which do not always correlate with the concavity of the curvature [28]. For these reasons, the scoliotic facet joints in this study were all considered as abnormally loaded, and they were compared to the cadaveric non-scoliotic facet joints which we deemed being exposed to physiological and balanced loading. An in depth 3-dimmensional analysis is needed to decipher the link between biomechanical forces and tissue deterioration.

Although osteoarthritis is an age-related disease [29], there exist other characteristic features that separate osteoarthritis from normal age-related changes. These include cellular proliferation, overexpression of matrix-degrading enzymes and increased pro-inflammatory factors [30]. We found a significantly higher ($p<0.0001$) cellularity in scoliotic cartilage, which

was expected because of the age difference between groups and because cell density in articular cartilage decreases with age. However, since the cell density decrease is not linear during development and the bulk of the cell loss is seen before adolescence [31], we believe the high cell density in scoliotic cartilage is abnormal. To support this argument, the only truly aged matched non-scoliotic donor (17 years old) included in our study had lower cell density than 79% of the scoliotic samples. (**Fig. 3A**) Interestingly, when single donor scoliotic facet joint pairs from one vertebrae are separated and compared one side to the other, there is a constant difference in cell density. This difference is significant ($p<0.05$) when all scoliotic facet joint pairs are separated into "high cellularity" and "low cellularity" subgroups. This separation showed a 1.6-fold difference in cell numbers between the two subgroups. However, this difference is not seen in non-scoliotic cartilage, which has very similar cellularity on both sides of a facet joint pair. We believe this is due to the differential load applied the facet joint pairs when the spine is curved in scoliosis. This hypothesis also needs biomechanical analysis to be validated. To determine the cause of a high cell density and potentially uncover an osteoarthritic marker in cell proliferation, we performed immunohistochemistry of the proliferative marker Ki-67. Scoliotic cartilage had significantly ($p< 0.05$) more proliferating cells, suggesting that the higher cell count in these samples is in fact due to proliferation. Again, the one aged-matched non-scoliotic donor (17 years old) had 20% proliferating cells compared to the scoliotic donors' 80%, which can be used to exclude the age difference as an explanation. Clusters of cells resulting from proliferation are a histological hallmark of osteoarthritis and were of common occurrence ***(Figure 3C)*** in scoliotic facet joints. However, cell clusters were not always present [30, 32], suggesting the possibility that mechanisms other than proliferation, such as cell migration, could have been associated with increased cell density. The chondrons in abnormally loaded cartilage can become distended as the

matrix is shifting under forces. This triggers mechanotransductive signals to the chondrocytes which are driven to proliferate and fill up the larger chondrons, which could be a potential cause seen in the scoliotic samples we studied (Figure 3C) [33].

Tissue homeostasis is critical in maintaining healthy cartilage. If the balance shifts towards matrix catabolism, the cartilage will quickly lose proteoglycans, water content and consequentially its load-bearing function. This is seen in osteoarthritis when hypertrophic chondrocytes start overproducing matrix degrading enzymes and pro-inflammatory factors in response to an inflammatory event. Here, we focused on 4 predominant degenerative factors that are overexpressed in cartilage degradation: proteases MMP3, MMP13 and inflammatory cytokines IL-1ß and IL-6 [34, 35]. Immunohistochemistry revealed an upregulation of the two proteases of interest, MMP3 and MMP13 compared to the non-scoliotic control group. The biggest difference was seen in MMP13, which had on average 2-fold more positive cell staining. These results follow the previous findings in which MMP-13 was found to be the most upregulated protease in OA [36]. In accordance to the previous findings, the pro-inflammatory cytokine IL-1ß was upregulated which is known to induce MMP secretion in articular cartilage [37]. IL-6, however, remained unchanged between the two groups, but its role in OA is also less understood.

Matrix-degrading enzymes MMP -1,2,3,5,7,10,13 and HTRA1 effectively cleave most SLRPs, fibronectin, collagen, and proteoglycans [16, 26, 38]. Overloaded cartilage loses its water content as a consequence of proteoglycan loss and synthesizes complex catabolic and degradative molecules which leads to increased fragmentation of its matrix molecules [39]. In this study, we report an extensive proteolysis of the SLRPs chondroadherin and decorin, which specifically appeared only when the scoliotic curves progressed towards higher Cobb angles with greater complexities. The appearances of fragmented chondroadherin and decorin were not detected in

tissue sample from an age-matched control individual. Extensive fragments of biglycan appeared in all the tissues examined and did not associate with scoliotic curve severities. This suggests that biglycan may be more sensitive to tissue loading in comparisons to the others SLRPs [40]. The molecular mass of the chondroadherin, decorin, biglycan, and fibromodulin fragments were between 22 to 35 kDa and based on previous reports it can be suggested that proteolytic cleavage and abnormal loading in the scoliotic spine may have jointly contributed to the generation of these fragments [26, 40, 41]. Lumican, on the contrary, was not fragmented in any sample.

As very little tissue-specific details exist on the spinal curves in AIS patients, limitations are associated with this study: first, all the scoliotic facets examined in this study were subjected to distinct abnormal loading with variable patterns, which might explain the variable results in the scoliotic group. To assess this, biomechanical analysis would be required to better visualize and correlate the 3-dimensional deformity to actual loading and cartilage degeneration. Second, the scarcity of young cadaveric donors influenced the age difference between the scoliotic and non-scoliotic groups. However, this can be used to reinforce our conclusion by considering that cartilage degenerates with age, and the scoliotic tissues were already more degenerate than the older control group.

In conclusion, this study shows the degenerative effects of chronic abnormal loading on the articular facet joints of patients with AIS. In these tissues, we observed hallmarks of OA such as proteoglycan loss, overexpression of proinflammatory mediators, increased synthesis of matrix-degrading proteases and fragmentation of the SLPRs. As with patients with age-related OA, the premature joint degeneration seen in scoliotic patients is likely to contribute to the pain perceived in some of these individuals.

Abbreviations

OA: Osteoarthritis, AIS: Adolescent idiopathic scoliosis, MMP: Matrix metalloproteases, SLRPs: Small leucine-rich proteins, IVD: Intervertebral discs

Ethics approval and consent to participate: This study was ethically approved by McGill University and Transplant Quebec (IRB # A00-M113-13B). Consent for tissue donation was acquired from the AIS patients and parents as well as next of kin for organ donors.

Funding: Dr. Lisbet Haglund and Dr. Jean Ouellet were jointly awarded a grant from the Shriners Hospitals for Children, Montreal, Canada to perform the studies. Dr Polly Lama received a postdoctoral salary support from the Oral and Bone health network (RSBO), Montreal, Canada.

Author Contributions: Dr. Lisbet Haglund, Dr. Jean A. Ouellet and Dr. Derek Rosenzweig conceived, designed and coordinated the study. Daniel Bisson, Dr Polly Lama and Dr. Derek Rosenzweig collected the experimental data presented in the paper. Daniel Bisson and Dr. Polly Lama drafted the manuscript. Dr. Jean A Ouellet, Dr. Neil Saran and Dr. Fahad Abduljabbar performed all surgeries and provided clinical input to the study design and data interpretation. All authors read and approved the final manuscript.

Acknowledgments: We gratefully acknowledge Professor Peter Roughley for helpful discussions with the western blot analysis and for providing antibodies to SLRPs decorin, biglycan, lumican, and fibromodulin. We would also like to thank Mark Lepik for technical assistance with the preparation of the figures included in this paper.

Conflict of interests: The authors d eclare they have no competing interests.

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Table 1 Lenke classification system for scoliositic spine curvatures.

Lenke Type	Curve Type	Description
1	**Main Thoracic (MT)**	**Thoracic Side-bending cobb >25°**
2	**Double Thoracic (DT)**	**MT + T2-T5 Kyphosis >120°**
3	**Double Major (DM)**	**MT + T10-L2 Kyphosis >+20°**
4	**Triple Major (TM)**	**MT + DT + DM**
5	**Thoracolumbar/Lumbar (TL/L)**	**Lumbar Side-**

		bending cobb >25° + T10-L2 Kyphosis >20°
6	**Thoracolumbar/Lumbar – Structural MT**	**MT (minor) + TL/L**

Table 2 Scoliotic and non-scoliotic donors used with age, sex, Lenke type (scoliotic only), major curve Cobb angle (scoliotic only) and cause of death (non-scoliotic only).

Group	Age	Sex	Lenke Type	Major Curve Cobb Angle (°)	Cause of Death
Scoliotic	18	**F**	**1**	**56**	
Scoliotic	**19**	**M**	**6**	**53**	
Scoliotic	**18**	**M**	**5**	**55**	
Scoliotic	**15**	**F**	**1**	**60**	
Scoliotic	**16**	**M**	**4**	**80**	
Scoliotic	**18**	**F**	**5**	**50**	
Scoliotic	**12**	**F**	**4**	**100**	

Scoliotic	**16**	**M**	**1**	**58**	
Scoliotic	**12**	**F**	**3**	**60**	
Scoliotic	**16**	**F**	**1**	**48**	
Scoliotic	**17**	**F**	**5**	**60**	
Scoliotic	**17**	**M**	**2**	**85**	
Scoliotic	**12**	**F**	**4**	**75**	
Scoliotic	**14**	**F**	**5**	**50**	
Scoliotic	**13**	**F**	**2**	**70**	
Scoliotic	**12**	**F**	**1**	**48**	
Scoliotic	**13**	**F**	**6**	**67**	
Scoliotic	**17**	**F**	**1**	**45**	
Scoliotic	**14**	**F**	**1**	**50**	
Scoliotic	**17**	**F**	**1**	**55**	
Non-Scoliotic	**50**	**M**			**Anoxia**
Non-Scoliotic	**50**	**M**			**Stroke**
Non-Scoliotic	**28**	**M**			**Anoxia**
Non-Scoliotic	**17**	**M**			**Anoxia**
Non-Scoliotic	**34**	**F**			**Stroke**

Non-Scoliotic	**27**	**M**	**Trauma**

Chapter 3: Scoliotic spine and facet joint degeneration

Preface

Facet joint osteoarthritis is associated with spinal instability and back pain. [47] In our previous study, we characterized osteoarthritis of varying severity in facet joints from scoliotic adolescents using histological and immunoblotting techniques. However, we failed to observe a distinct pattern of facet joint degeneration along the coronal spinal deformity seen in the frontal radiographs of patients in our cohort. A likely explanation for this is omitting the axial and sagittal planes of the spinal deformity in our initial analysis. Many studies have confirmed the complex 3D nature of the scoliotic deformity; therefore, all deformation planes should be considered. Based on our findings, other studies classifying facet joints and other spinal features by their convex or concave location relative to the coronal curve have significant limitations. To overcome this problem, we quantified the 3D deformity of each intervertebral segment harbouring the facet joint samples we received from consenting patients undergoing corrective surgery using the EOS imaging system. Since osteoarthritis is a disease affecting all tissues of cartilaginous joints, we extended the characterization of AIS facet joints to include subchondral bone using μCT analysis. To better understand the fate of facet joint cartilage in the context of AIS, we evaluated associations between osteoarthritic severity of cartilage, subchondral bone and 3D deformity parameters. Exploring the details of facet joint osteoarthritis severity along the scoliotic spine helps understand rotational vulnerabilities of the scoliotic spine and provides further evidence to corroborate hypotheses regarding the source of asymmetrical loading in AIS progression. Finally, self-reported localized back pain measurements from the same scoliotic patients were compiled in 3 back regions and compared to facet joint osteoarthritis severity in these regions. Further knowledge of sources of

painful stimuli will help guide treatment modalities for scoliotic adolescents with increased pain and disability.

Manuscript II: Axial rotation and pain are associated with facet joint osteoarthritis in Adolescent Idiopathic Scoliosis

Daniel G Bisson[1,2], Kai Sheng[1,2], Semsi Kocabas[1,2], Don Daniel Ocay [2], Catherine E Ferland[3,4], Neil Saran[1,2], Jean A Ouellet[1,2 §], Lisbet Haglund[1,2 §]
[1]Orthopaedic Research Laboratory, Shriners Hospital for Children, Montreal, Canada
[2]Department of Surgery, McGill University, Montreal, Canada
[3]Department of Clinical Research, Shriner's Hospital for Children, Montreal, Canada
[4]Department of Anesthesia, McGill University, Montreal, Canada
[§]Both authors contributed equally to this work.

Corresponding Author:
Dr. Lisbet Haglund, Ph. D, Professor,
The Orthopaedic Research Laboratory
Montreal General Hospital,
McGill University,
1650 Cedar Avenue, Room C10.148.2
Montreal, Qc., H3G 1A4
Canada
Telephone: (514) 934-1934 ext. 35380
or
Shriners Hospital for Children
1003, boulevard Décarie
Montréal, Québec H4A 0A9
E-mail : lisbet.haglund@mcgill.ca

Abstract

Objective: Facet joints are crucial for spinal stability but develop premature osteoarthritis in patients with adolescent idiopathic scoliosis (AIS). Here, we evaluated the association between facet joint cartilage and subchondral bone homeostasis, perceived back pain and 3-dimensional spinal deformity to better understand the role of facet joint degeneration in AIS progression and pain.

Design: Scoliotic patients were recruited to donate facet joint samples resected during spinal fusion surgery to correct the deformity. Cartilage and bone osteoarthritic states were characterized using histological OARSI scoring, visual morphological grading and μCT analysis, respectively. Back pain was self-reported relative to the location on the back of the patient and graded using a numerical rating scale. The scoliotic curves from our patient cohort were digitally reconstructed using biplanar radiographs and the eOS system (EOS imaging). The deformity was then reduced to 3 intervertebral angles (coronal, sagittal and axial) for each pair of bilateral facet joints. Statistical associations between the intervertebral angles, osteoarthritis parameters and pain intensity were performed using the Spearman method and one-way ANOVA.

Results: Facet joint cartilage degeneration was associated to decreased subchondral bone volume and quality. Most importantly, asymmetrical, and overall degeneration of facet joints is strongly correlated to intervertebral axial rotation. Additionally, kyphotic intervertebral segments in the sagittal plane were good predictors of increased facet joint degeneration and back pain.

Conclusions: Facet joint degeneration is associated with axial deformity, kyphotic intervertebral angle and back pain intensity in AIS. These results suggest facet joints are important features to consider for rotational instability in AIS spines and related disease progression and perceived back pain.

Keywords

Scoliosis, Facet joints, osteoarthritis, rotation, pain

Introduction

Adolescent idiopathic scoliosis (AIS) remains the most prevalent musculoskeletal disease affecting between 1-3% of adolescents worldwide. [1,2] A progressive coronal deviation of the thoracolumbar spine is the main descriptive feature of AIS, but the deformity has a complex 3-dimensional (3D) nature and can lead to back pain and decreased quality of life. [3,4,5] Strikingly, bipeds are the only vertebrates who spontaneously develop scoliosis. [6] Studies have shown the increased risk of rotational instability in upright human spines, which could be a factor driving AIS progression. [7] Similarly, hypokyphosis is most associated with rotational instability and was found highly correlated to the severity of AIS. [8,9] Castelein et al. have reported a pre-existing right thoracic curve in most non-scoliotic spines which could be an initiating factor for the rotatory deformity. [10] Theoretically, the resulting asymmetrical forces applied along the intervertebral segments are at risk of developing deformities according to Hueter-Volkmann and Wolff laws for bone growth and modulation if the paraspinal musculature cannot compensate. [11,12] Thus, axial rotation through spinal instability seems to be an important causal factor leading to development and progression of AIS. [13,14]

Axial rotation is limited by the pair of bilateral facet joints and to a lesser extent the intervertebral discs. These cartilaginous surfaces are oriented to limit the axial component of movements differentially along the spine. Degeneration of the cartilage, bone and capsule during facet joint osteoarthritis can negatively influence spinal stability through increased joint laxity and weakening of vertebral mechanical properties. Additionally, degenerating facet joints can be the source of nociceptive stimuli contributing to chronic back pain. [15] Interestingly, we have previously demonstrated that facet joint cartilage degeneration is prominent in AIS patients. [16] However, we could not find any association between facet joint degeneration and its location on the scoliotic deformity in the coronal plane. [16] Understanding the pattern of facet joint degeneration in the scoliotic spine could improve our comprehension of progressive and pain-generating mechanisms in AIS and reveal new targets for treatment modalities. This study aimed to investigate facet joint degeneration in relation to the 3D spinal deformity and localized back pain in AIS patients.

Methodology

Facet joint sample collection and grading

A total of 526 inferior facet joint samples were collected from 45 consenting AIS patients undergoing spinal fusion surgery at the Shriner's Hospital for Children. Average age was 15 years old and female patients represent 68% of the cohort. High-resolution pictures of the facet joint cartilage surface were taken with a Canon EOS 5D Mark II (Canon) and a Macro lens. Morphological scoring was made by three independent observers using a facet joint scoring system described in Li et al. [17] This study was approved by the McGill University institutional review board in Montreal, Canada. (IRB # A08-M22-17B).

Histology and OARSI scoring

Facet joint cartilage was dissected from the subchondral bone, fixed and cryosectioned at 5uM thickness longitudinally. Safranin-O/Fast green staining on the sections was performed according to standard protocols. The stained specimens were photographed at 20x using a Zeiss Axioscan 7 (Zeiss) microscope slide scanner. To evaluate the severity of osteoarthritis in facet joint cartilage, a modified Osteoarthritis Research Society International (OARSI) scoring system was used as previously described. [16] The median score value of three blinded evaluators was taken for each facet joint. Cartilage thickness and fibrillation index (Surface/width) were additionally measured from the histological images. (Figure 1)

Micro-computed tomography (μCT)

Fixed facet joint samples in plastic tubes were imaged using a Skyscan 1272 (Bruker). Acquisition parameters were set at 70kV, 142uA at 2000msec with an 8uM pixel size and a 0.5mm aluminum filter. Images were captured at a size of 2452x1640. 3D volumes were reconstructed using NRecon (Bruker) and analyzed using CTVox (Bruker). Cortical and trabecular volumes of interest were drawn by hand. Bone volume (BV/TV), trabecular thickness (Trab.Th), trabecular separation (Trab.S), trabecular number (Trab.N) and cortical thickness were measured from the volumes of interest.

Spinal reconstructions

The same 45 consenting patients had their spinal deformity analysed using 3D modeling. This modeling was generated with pre-operative biplanar low-dose radiographs. With the EOS software (EOS imaging) and the patient images, the thoracolumbar spine was modelled in 3-dimensions

starting with the pelvic parameters. Next, the thoracic and lumbar vertebras are simulated by the software and each vertebra was correctly rotated, positioned and deformed by the examiner to match the biplanar radiographs as closely as possible. Two reconstructions were created for each AIS patient and intervertebral angles for each segment were averaged to account for variability. Average angles in the coronal, sagittal and axial planes were matched to every facet joint pair collected in this study. (Figure 2 B, C,D)

Self-reported pain measurement

Pre-operative localized pain assessment was conducted by a research assistant. At the time of x-ray measurement, patients identified the painful location on a schematic back diagram dividing the upper (T1-T5), middle (T6-T11) and lower (T12-L5) back regions and marked the pain intensity using a numerical rating scale ranging from 0-10, where 0 represents no pain and 10 the worst pain imaginable. Patients with right thoracic curves and pain scores in any back region of moderate intensity and above (equal or higher than 4) were included in this analysis.

Correlation and statistical analysis

Spearman correlation analysis was used to evaluate the relationship between the cartilage, bone, intervertebral angles, and pain scores. The non-parametric method was chosen because of the limiting ordinal nature of the OARSI, morphology and pain scores used to evaluate cartilage degeneration. Friedman test was used as a non-parametric one-way ANOVA to assess statistical differences between the pain intensity and facet joint morphology across 3 back regions. A detailed view of sample numbers for each analysis is featured in supplementary figure 1. Significance was attained at $p<0.05$. Correlative and statistical analyses were performed using Prism 9 (Graphpad).

Results

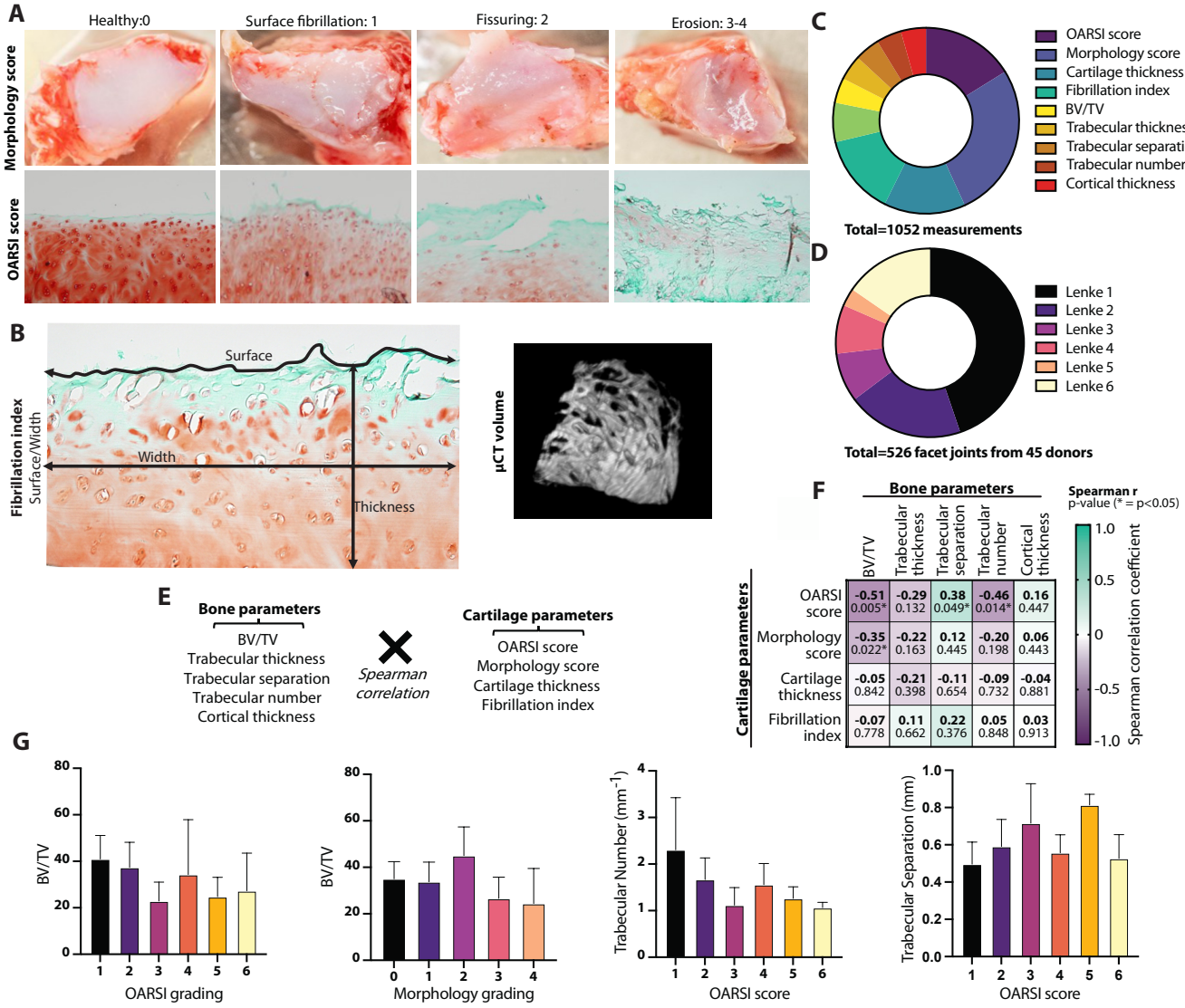


Figure 2 **Bone density is inversely correlated to cartilage degeneration.** A) Facet joint cartilage photographs and Safranin-O Fast Green histology with increasing morphology and OARSI scoring. B) Schematic showing measurements to calculate fibrillation index and cartilage thickness. µCT facet joint reconstruction used to obtain bone parameters. C)D) Frequency distribution of measurements obtained and Lenke types of patient curves used in the study. E)F) Bone and cartilage parameters were cross-analyzed using Spearman correlations and shown in the heatmap. (Spearman r in **bold**, p-value below) (* =p<0.05,**=p<0.01) G) Histograms of

significant correlations between bone and cartilage parameters. Error bars represent standard deviation of the mean. BVTV = Bone volume/total volume

Bone density is inversely correlated to cartilage degeneration in scoliotic facet joints

We evaluated cartilage degeneration using a morphology score, an OARSI score, cartilage thickness and fibrillation index. Subchondral bone volume (BV/TV), cortical thickness and trabecular thickness, separation and number were measured using μCT analysis. Spearman correlation coefficients were calculated for cartilage and bone parameters (Figure 1A,B and C) of each facet joint (n=526 individual facet joints from 45 donors) (Figure 1E). Four significant correlations were found between degenerative cartilage scores and bone parameters. Bone volume (BV/TV) correlated inversely with OARSI score ($r=-0.51$, $p<0.01$) and morphology score ($r=-0.35$, $p=0.02$). OARSI score had significant a correlation with trabecular separation ($r=0.38$, $p=0.049$) and trabecular number ($r=-0.46$, $p=0.01$). (Figure 1F,G) Overall a strong pattern suggests that cartilage degeneration correlates with a lower bone volume (BV/TV), trabecular thickness, trabecular number and a higher trabecular separation. There was no significant relationship found between cartilage parameters and cortical thickness. (Figure 1F) Similarly, cartilage thickness and fibrillation index did not present significant correlations with bone parameters but showed similar trends to cartilage scores in correlations above $r=0.20$. Intergroup correlations revealed a high level of concordance between cartilage parameters and bone parameters. (Supplementary figure 2)

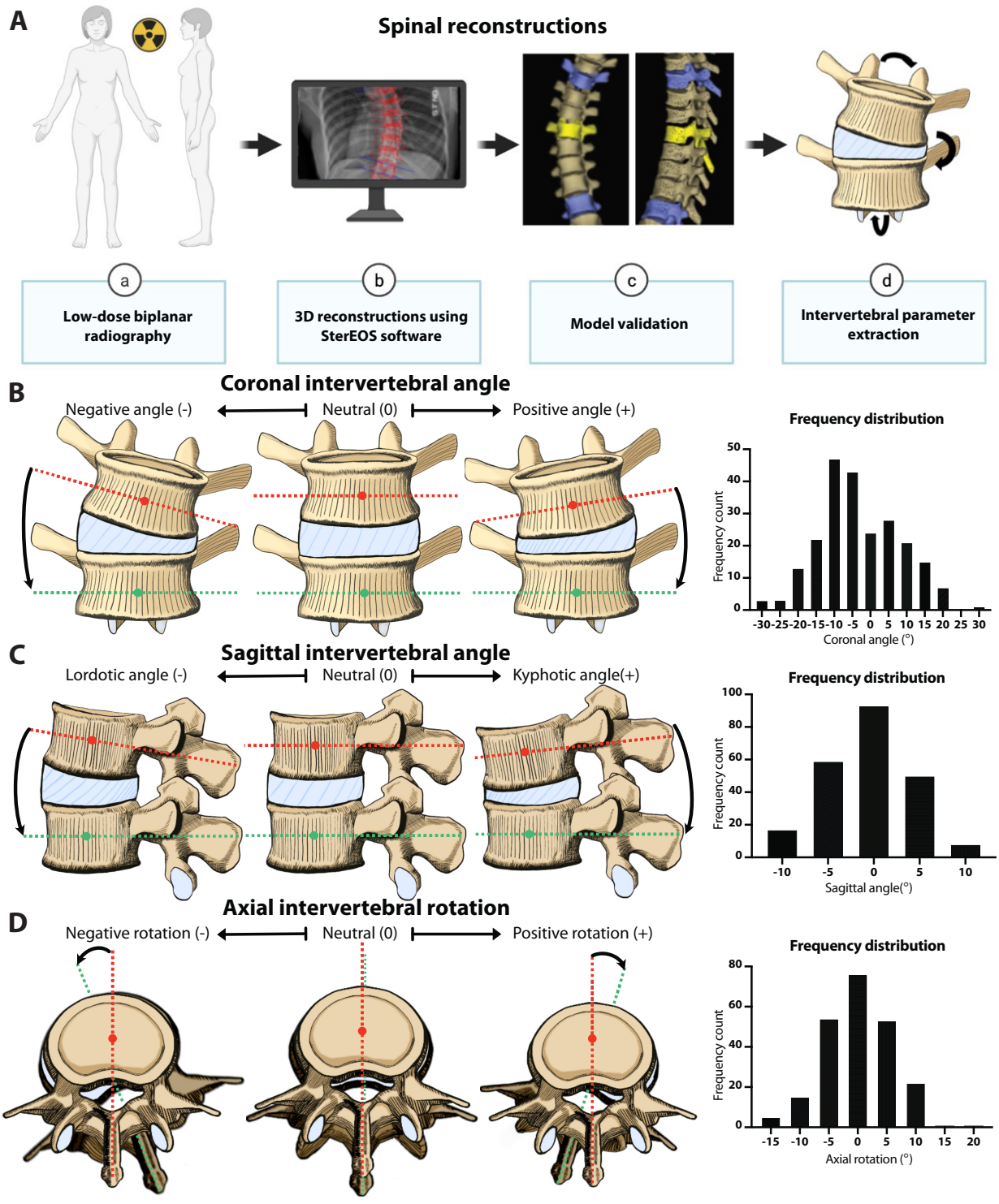

A
Spinal reconstructions
a
Low-dose biplanar radiography
b
3D reconstructions using SterEOS software
c
Model validation
d
Intervertebral parameter extraction
B
Coronal intervertebral angle
Negative angle (-)
Neutral (0)
Positive angle (+)
Frequency distribution
Frequency count
Coronal angle (°)
C
Sagittal intervertebral angle
Lordotic angle (-)
Neutral (0)
Kyphotic angle(+)
Frequency distribution
Frequency count
Sagittal angle(°)
D
Axial intervertebral rotation
Negative rotation (-)
Neutral (0)
Positive rotation (+)
Frequency distribution
Frequency count
Axial rotation (°)

Figure 2 **Spinal reconstructions and derived intervertebral angles**. A) Intervertebral angles were measured using SterEOS spinal reconstructions. Coronal (B), sagittal (C) and axial intervertebral angles (D) of all intervertebral segments used in this study.

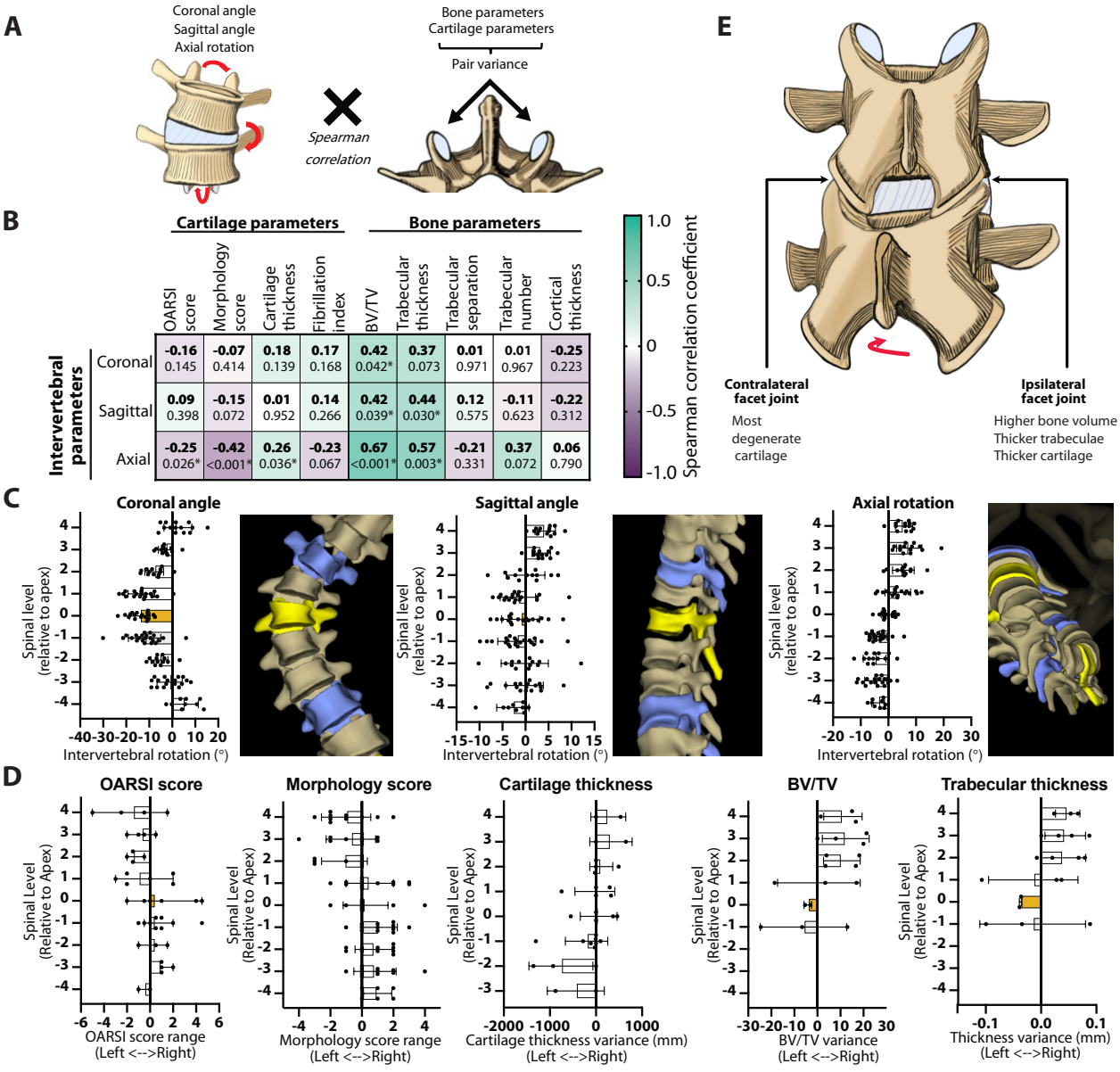


Figure 3 **Bone and cartilage parameter variance in facet joint pairs correlate with axial rotation.** A)B) Intervertebral angles were cross-analyzed with bone and cartilage parameter

variance within a facet joint pair and shown in the heatmap (Spearman r in **bold**, p-value below) (* =p<0.05). C)D) Intervertebral angles and tissue parameters in single right thoracic curve patients were normalized to a spinal level relative to curve apex. (0, yellow) All histograms are representing averaged parameter data and error bars show standard deviation. E) Illustration of the relationship between the axial rotation and tissue parameter variance. Sample sizes are available in supplemental figure 2.

Bone and cartilage variance in facet joint pairs correlate with axial rotation

Pre-operative biplanar radiographs and the sterEOS system (EOS, Switzerland) were used to create and validate 3D models of the spines. (Figure 2A) Coronal intervertebral angles (Figure 2B), sagittal intervertebral angles (Figure 2C), and axial intervertebral rotations (Figure 2D) were extracted from the model and matched to the corresponding samples. The variance in cartilage and bone parameters was calculated within facet joint pairs and was cross-analyzed with the intervertebral angles. (Figure 3A) The sample size for each comparison is illustrated in supplemental figure 2. Moderate to strong correlations were found between axial rotation and most tissue parameters. (Figure 3B) Significant inverted correlations were found between axial rotation, OARSI score (r=-0.25, p=0.03) and morphology score (-0.42, p<0.01) whereas cartilage thickness (r=0.26, p=0.04), bone volume (r=0.67, p<0.01) and trabecular thickness (r=0.57, p<0.01) displayed significant positive correlations. Bone volume correlated significantly with kyphotic sagittal intervertebral angle (r=0.42, p=0.04) and coronal intervertebral angle (r=0.42, p=0.04). A similar relationship was found with the trabecular thickness (r=0.44, p=0.03). To observe these findings in a population of similar curve types, we narrowed our cohort

to right thoracic curves (Lenke 1 and 2) and normalized each segment relative to the apex of the curve. A high level of concordance in intervertebral angles was found with the apex of the curve (in yellow) consistently displaying maximal coronal wedging and minimal axial rotation. Most strikingly, the similarity between the distribution of axial rotation and significant parameter variance in right thoracic curves was evident. (Figure 3 C,D) Taken together, these findings show that the facet joints are less degenerate and have a higher bone density on the side towards the axial rotation (ipsilateral facet joint) compared to the other (contralateral facet joint). (Figure 3E)

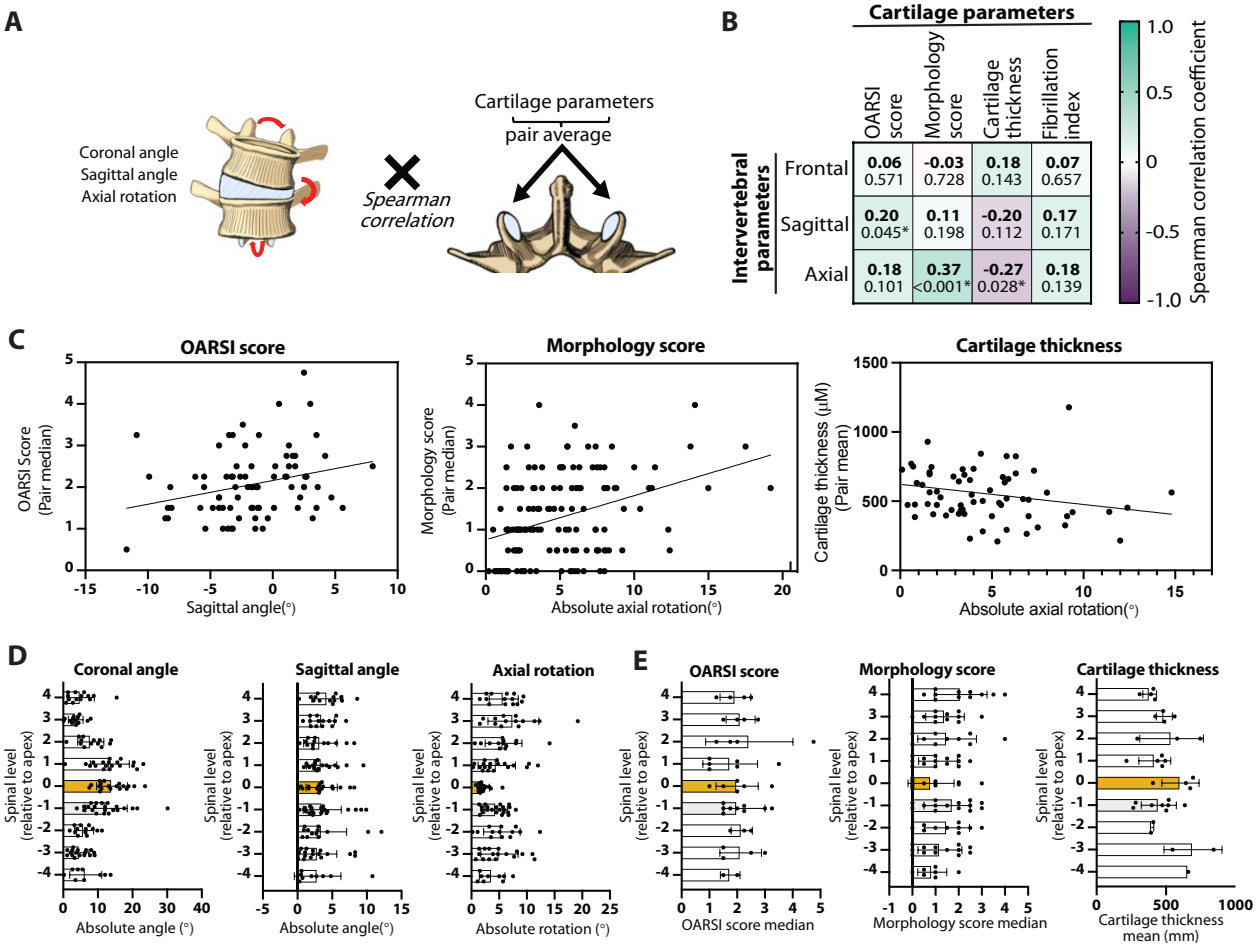


Figure 4 **Degeneration of** facet joint pairs **correlate with axial rotation and kyphotic angle.** A)B) Absolute Intervertebral angles were cross-analyzed with cartilage parameter averages within a facet joint pair and shown in the heatmap. (Spearman r in **bold**, p-value below) (* =$p<0.05$) C) Scatterplots showing significant correlations found in B). D) Absolute Intervertebral angles and tissue parameter averages in single right thoracic curve patients were normalized to a spinal level relative to the curve apex (0, yellow). All histograms are representing averaged parameter data and error bars show standard deviation. Sample sizes are available in supplemental figure 2.

Paired facet joint degeneration correlates with axial rotation and kyphotic angle

To reveal the intervertebral angle most impacting tissue degeneration, we investigated how averaged tissue parameters within a facet joint pair correlated with absolute intervertebral angles. (Figure 4A) The absolute values for intervertebral angles were used to account for the lack of left-right orientation when compared to averaged facet joint tissue data. Sample sizes for each comparison are available in supplementary figure 1. Three significant Spearman correlations were found between the absolute angles and averaged tissue parameters (Figure B) The axial rotation correlated with morphology score ($r=0.37$, $p<0.01$) and cartilage thickness ($r=-0.27$, $p=0.03$) and the sagittal angle with the OARSI score ($r=0.20$, $p=0.045$). (Figure 4E) When accounting all other correlations, we observed a pattern of thinner, more degenerate facet joint cartilage correlating with increasing axial rotation and kyphotic intervertebral angle. No significant correlations were found between paired cartilage parameters and coronal intervertebral angle. The parameters were then evaluated separately in the right thoracic curves where the apical level (yellow) showed thicker, less degenerate cartilage and minimal axial rotation (Figure 4D).

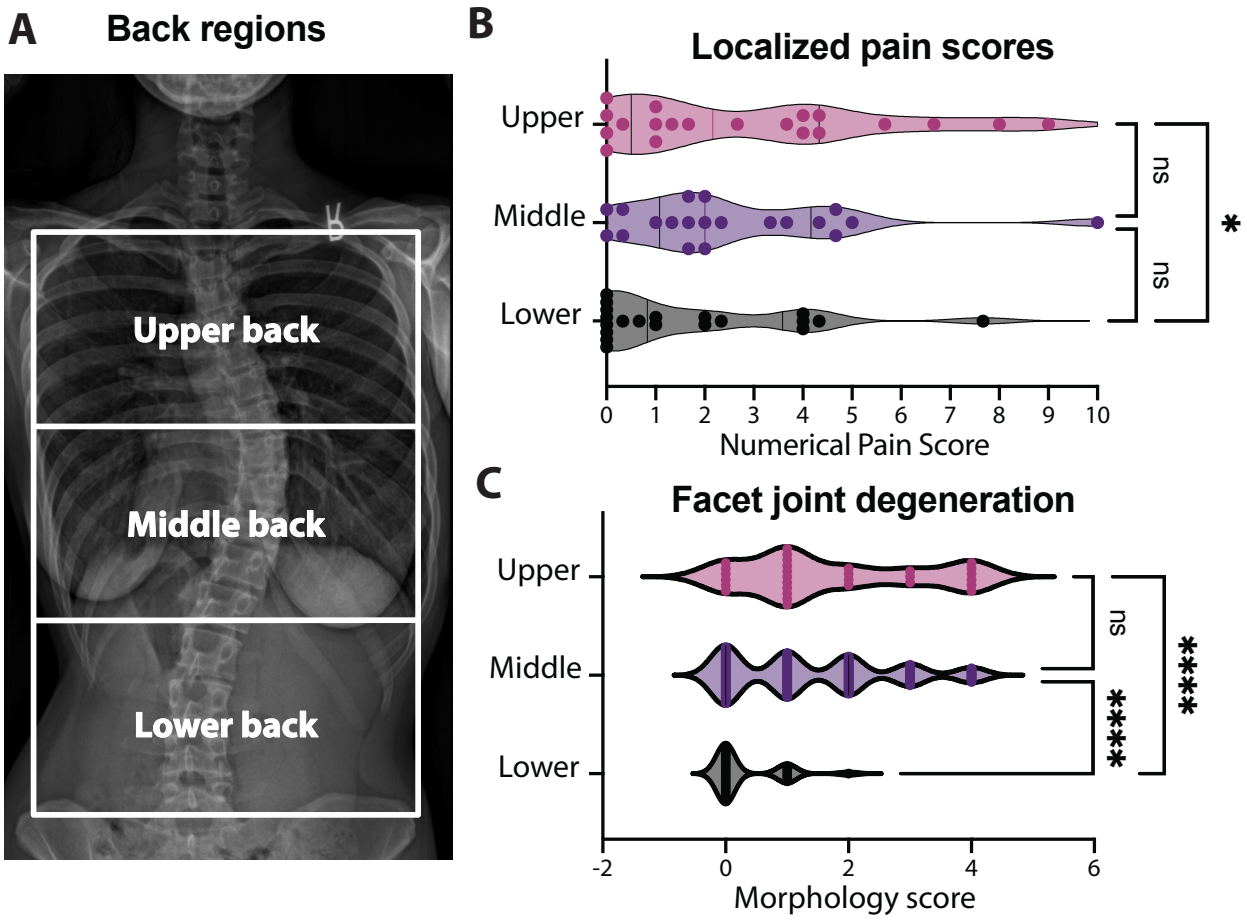


Figure 5 **Back pain location is associated with facet joint degeneration in single right thoracic scoliotic curves.** A) Diagram representing the upper, middle, and lower back regions where pain scores were reported. B) Averaged pain scores and C) facet joint cartilage morphology scores across the back regions of AIS patients with single right thoracic curves. Violin plots represent median values with C.I. of 95% as error bars. Significant differences were analyzed by Friedman test. (*=$p<0.05$, ****=$p<0.001$)

Back pain location and intensity correlate with facet joint degeneration

Finally, we investigated facet joint degeneration in relation to localized back pain in AIS patients with a single right thoracic curve. Self-reported pain scores were pre-operatively assessed and averaged for each region of the back in the single right thoracic curve cohort. (Figure 5A, B)

Facet joint cartilage morphology scores were grouped according to their position across back regions. (Figure 5C) A concordant pattern of increasing pain and facet joint degeneration towards the upper back was found in this cohort of right thoracic curves. Notably, the most significant differences in the parameters were between the lower lumbar region and the middle/upper back regions. These results suggest that back pain is associated with facet joint tissue degeneration in the upper thoracic region of the right thoracic curves.

Discussion

Spinal instability and axial rotation of vertebrae are integral to the pathogenesis of AIS. [13,14] We have previously described the prevalence of cartilage degeneration in scoliotic facet joints but found no correlation between osteoarthritic severity and frontal radiographs. [16] Here, we show that facet joint degeneration is most influenced by axial intervertebral rotation in the context of AIS. Additionally, maximal pain intensity corresponds to the same back region as the most osteoarthritic facet joints. We propose this could be due to the rotational instability and subsequent asymmetrical forces across these joints specifically in patients with AIS.

Osteoarthritis is a disease that affects all the tissues of the cartilaginous joint. [18] In the context of AIS, it was unclear whether facet joint cartilage degeneration is related to subchondral bone sclerosis. Here, we cross-analyzed bone and cartilage parameters measured from AIS facet joints to evaluate this relationship. Parameters quantifying cartilage degeneration significantly and negatively correlated with bone volume, trabecular number and decreased trabecular separation which is corroborated by studies showing trabecular rod loss in adult facet joint osteoarthritis. [19,18,20] There was no association found with the thickness of the subchondral cortical plate. This agrees with Netzer et al. who reported a lack of association between cortical thickness and other

bone parameters in both healthy and osteoarthritic facet joints. [20] These results indicate that facet joint cartilage degeneration is associated with elevated bone resorption.

3D models of the spines were generated, and intervertebral angles were extracted and matched to the corresponding facet joint samples. Frequency distributions of the coronal, sagittal and axial intervertebral angles for each facet joint pair in this study showed normal distributions centered around 0°. However, the distribution range showed that vertebrae were most deformed in the coronal plane, followed by axial and sagittal. Moreover, the coronal angle distribution is asymmetrical and is skewed towards negative values. We speculated that this is due to the high prevalence of right thoracic curves in our patient population, which results in negative coronal intervertebral angles in segments surrounding the apex. This could be verified when all right thoracic curves were grouped and normalized to their apical level. Notably, it is interesting that the apex (level 0) is the segment most wedged in the coronal plane and is the least axially deformed. Importantly, this is not to be confused with the fact that the apical vertebra is most axially rotated because of the summation of intervertebral axial rotations classically found above and below the apex. Jozsef et al. published a study confirming that 95% of scoliotic spines have their most axially rotated segments at the curve apex. [21,22] Our spinal reconstructions corroborate the finding that all scoliotic deformities have common axial deviation patterns associated with the coronal deformity.

Asymmetrical degeneration of vertebral segments is at the core of AIS etiopathology. [2] We evaluated asymmetrical degeneration of facet joints by calculating the range or variance of cartilage and bone parameters between two joints of a pair. When cross-analyzed with

intervertebral angles, we found significant correlations between parameter variance and axial rotation. Spearman correlations showed a significant increase in degenerative changes in the cartilage and lowered bone quality at the contralateral side (opposite from the rotation) In a population of right thoracic curves where each segment was normalized relative to the apex, we could best appreciate the concordance of "S" shapes between parameter variance and axial rotation. Additionally, the upper thoracic segments above the apex displayed the greatest variance between left and right facet joints. This could be due to increased rotational instability in segments with intervertebral kyphotic angles as described by Kouwenhoven et al. [7] Unfortunately, we could not evaluate the biomechanical loads from the static reconstructions. However, we can deduce certain loading properties using the Hueter-Volkmann and Wolff's law along with additional corroborating evidence. In vertebrae, growth is retarded by compressive forces while osteogenic metabolism is activated by intermittent stresses of increasing intensity. [12] As such, we believe that increased compressive forces are applied to the ipsilateral side where bone quality and volume are superior. According to cadaveric studies, the ipsilateral side is subjected to increased loading in axial rotation.[23] Additionally, pedicle asymmetry was reported in many AIS morphometry studies. Interestingly, pedicle width asymmetry throughout a right thoracic curve corresponded perfectly with bone parameters in our study and located the largest discrepancy in facet joint pairs at the levels above the apex, where increased axial rotation is maximal. [24,25,26]

The fate of the three features of an intervertebral segment (2 facet joints and 1 disc) are highly interdependent. Studies have reported a high concordance of degenerative changes in all three tissues in osteoarthritic segments. [18] As such, facet joint cartilage parameters were averaged for each pair and compared with absolute intervertebral angles to reveal which conditions produce the

most degenerate intervertebral segment. Bone parameters were not included in this analysis because bone density has been shown to change relative to spinal level [27] and are not normalized to each other as with parameter variance. Nevertheless, averaged cartilage degeneration in facet joint pairs correlated with increasing intervertebral axial rotation. The significance of this relationship is best visualized in right thoracic curves, where a high concordance between the distribution of averaged parameters and absolute axial rotation is evident. An additional significant association was found between the OARSI score and positive sagittal intervertebral angles. We believe this is due to increased rotational instability and shear loads in upper thoracic segments that display an intervertebral kyphotic angle based on research by Kouwenhoven et al. [7] Taken together, we conclude that facet joints are more likely to degenerate in segments along the kyphotic scoliotic spine where increased axial rotation is present.

Facet joint osteoarthritis has been extensively described as a source of painful stimuli in cases of chronic back pain. [28,15] Here, we investigated the association between the location and intensity of back pain in AIS patients with patterns of degenerative changes in facet joint cartilage. The cohort of right thoracic curves was used for this analysis because previous research from our group shows different pain location patterns depending on Lenke type. [3] Both pain intensity and cartilage morphology scores show a high concordance of intensity across the back regions. The middle and upper back regions display the most degenerative changes in facet joint cartilage and the highest pain scores. These results are in concordance with previous findings in this study where facet joint degeneration is most prominent in upper thoracic segments. Notably, this region is also at risk of maximal ligamentous capsule distention which has been observed to produce nociceptive stimuli in response to axial intervertebral rotation. [29,30] Furthermore, Teles and al. demonstrated

the same pattern of localized back pain in right thoracic curves (Lenke 1 and 2 curve types). [3] These results suggest that facet joint osteoarthritis and rotational instability could be generating pain in scoliotic spines.

There are some limitations encountered in this study. First, the tissue variables are imperfect tools to measure facet joint osteoarthritis. Both grading scores have limited resolution and analytic power because of their ordinal nature and non-linearity. Nevertheless, the analytic power of this study relies on a total of 10 parameters to account for this problem and to widen the conditions surveyed to assess facet joint osteoarthritis. Second, spinal models created here are not *true* reconstructions and are dependent on the observer and the quality of the biplanar radiographs. The fidelity and reproducibility were therefore amplified by averaging two reconstructions of the same biplanar radiographs for each donor. Additionally, we must consider the relative difficulty of recreating the axial rotations compared to the coronal and sagittal angles because of the lack of a transverse plane view in the biplanar radiographs. However, CT analysis and other EOS studies of scoliotic spines corroborates the axial rotation pattern found in this paper. [31, 32]

Spinal instability and aberrant axial deformation are increasingly reported in studies of AIS. The spinal features most important to limit axial rotation are the biplanar facet joints and we have previously shown the prevalence of degeneration of these joints in the context of AIS. Here, we conclude that facet joint cartilage and bone degeneration in AIS are part of the structural spinal deformity and are strongly associated with axial intervertebral rotation. The association between the deformity, pain and facet joint degeneration should be investigated further to improve comprehension and treatment outcomes for AIS.

Acknowledgements

The authors kindly acknowledge the help from staff and surgeons at the Shriner's Hospital for Children.

Contributions

DB, LH and JAO were responsible for the conception and design of the study. DB, KS, SK acquired the facet joint data. DB reconstructed spinal models for the intervertebral angle data. DDO acquired the self-reported pain data. DB performed the analysis and interpretation of the data. All authors participated in the drafting, revising and submission of the final manuscript.

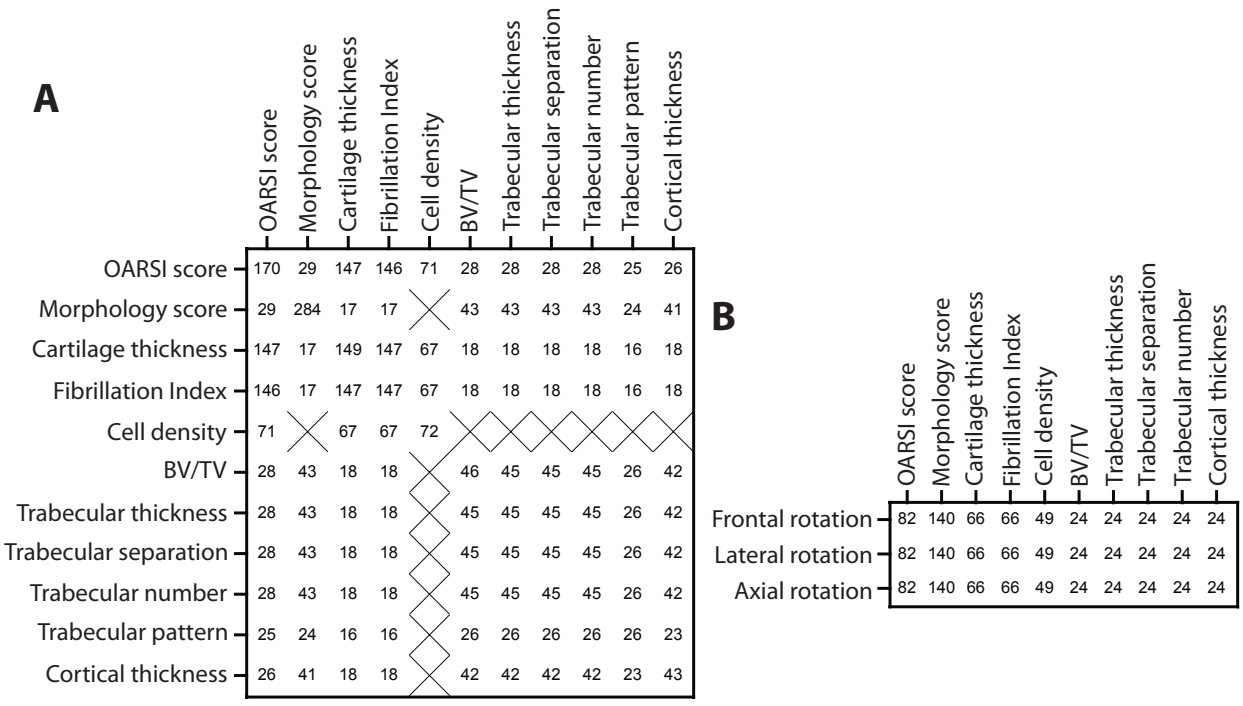
Conflict of interest

The authors report no conflicts of interest.

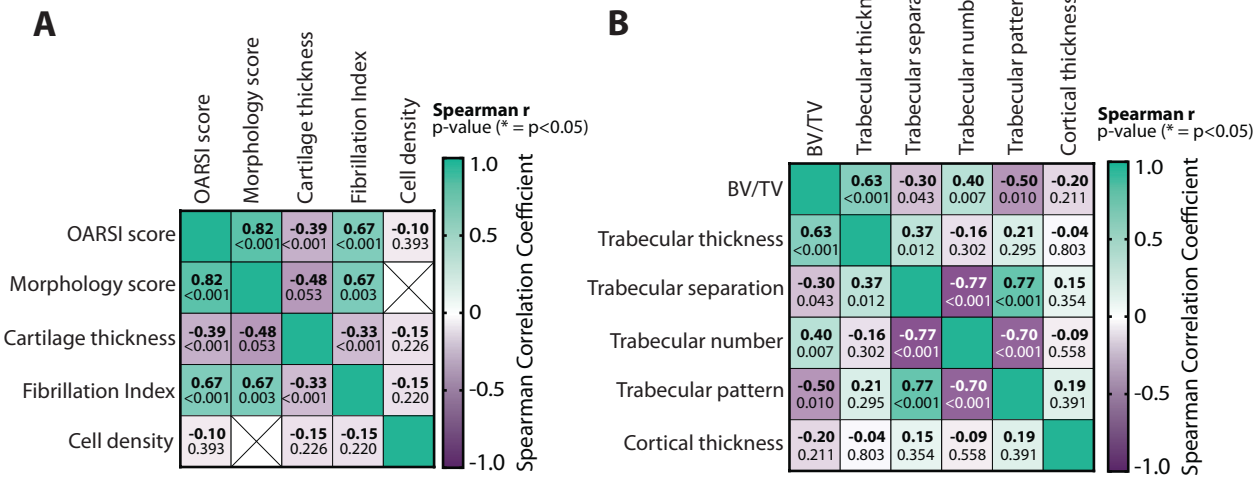
Funding sources

This study was funded by the Shriner's Hospital for Children Basic Research award (85110-CAN-16). DB received a studentship from RSBO.

Supplementary data



Supplementary data Supplemental Figure 1: Sample size (n) for A) cartilage and bone parameter comparisons (n=single facet joints) and B) tissue parameters and intervertebral angles (n=intervertebral segments).



Supplemental Figure 2: Intergroup Spearman correlations between A) cartilage and B) bone parameters. (Spearman r in **bold**, p-value below) (* =p<0.05)

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Chapter 4 : Toll-like receptor involvement in AIS facet joint degeneration

Preface

The first two manuscripts revealed the extent of facet joint osteoarthritis in AIS patients. As discussed, this phenomenon likely contributes to spinal instability leading to curve progression and pain, which remain the most critical hurdles in improving standard treatment procedures. Unfortunately, therapeutic options for osteoarthritis of all joints are limited by the current lack of disease-modifying drugs. In the case of scoliotic cartilage, we found increased expression of pro-inflammatory cytokines and proteases, resulting in extracellular matrix catabolism and loss of proteoglycan content. In osteoarthritis of other cartilaginous joints, similar pathological changes have been associated with Toll-like receptor activation and constitutive downstream translocation of the transcription factor NF-κB. This central pathway is ubiquitous for most inflammatory processes in immunity and tissue repair but remains unclear in the context of facet joint osteoarthritis. Toll-like receptors can recognize and bind alarmins in the extracellular environment, which comprises intracellular and fragmented extracellular molecules that act as danger signals. In the first manuscript, we discovered increased fragmentation of biglycan in AIS samples which has been shown to activate TLRs. The investigation of the involvement of TLRs in AIS facet joints could yield a novel therapeutic target with the potential to combat the underlying cause of osteoarthritis.ß

Based on the literature and previous studies, we hypothesized that Toll-like receptors were involved in facet joints' inflammatory and catabolic state in AIS patients. We compared scoliotic and non-scoliotic chondrocyte gene expression and alarmin presence to reveal the contribution of TLRs in facet joint osteoarthritis. We treated *ex vivo* cartilage explants from scoliotic and non-scoliotic samples with TLR agonists (synthetic and alarmins) and evaluated pro-inflammatory

cytokines, proteases, and proteoglycan loss to determine the effects on cartilage health. Finally, we tested two natural compounds for their potential to prevent pro-inflammatory and catabolic signalling by inhibiting TLR activation in the presence of alarmins.

Manuscript III: Toll-like receptor involvement in adolescent scoliotic facet joint degeneration

Daniel G. Bisson[1,2], Kai Sheng[1,2] Semsi Kocabas[1,2], Emerson Krock[2], Alisson Teles[1], Neil Saran[1], Jean A Ouellet[1,2], Lisbet Haglund[1,2]

[1]Orthopaedic Research Laboratory, Shriners Hospital for Children, Montreal, Canada

[2]Department of Orthopedic Surgery, McGill University, Montreal, Canada

Corresponding Author:
Dr. Lisbet Haglund, Ph. D
Associate Professor,
The Orthopaedic Research Laboratory
Montreal General Hospital,
McGill University,
1650 Cedar Avenue, Room C10.148.2
Montreal, Qc., H3G 1A4
Canada
Telephone: (514) 934-1934 ext. 35380
or
Shriners Hospital for Children
1003, boulevard Décarie
Montréal, Québec H4A 0A9
E-mail : lisbet.haglund@mcgill.ca

Abstract

Facet joint osteoarthritis is prevalent in young patients with adolescent idiopathic scoliosis (AIS) and might contribute to back pain. Toll-like receptors (TLR) have been linked to cartilaginous tissue degeneration but their involvement in facet joint osteoarthritis in AIS patients is still unknown. We compared baseline gene expression levels of TLRs -1, -2, -4, and -6 in scoliotic and non-scoliotic chondrocytes and found higher expression levels in scoliotic chondrocytes with significantly higher TLR2 levels. Furthermore, TLR expression correlated strongly and significantly with inflammatory and catabolic markers in scoliotic but not in non-scoliotic chondrocytes. TLR activation with a synthetic TLR2/6 agonist resulted in a robust induction and release of pro-inflammatory and catabolic factors which exacerbated proteoglycan loss in scoliotic but not in non-scoliotic cartilage. We also detected a higher abundance of alarmins including S100A8/9 and biglycan in scoliotic cartilage. Finally, the small-molecule antagonists Sparstolonin B and o-Vanillin reduced catabolism following induction with naturally occurring alarmins and the synthetic TLR2/6 agonist. The high baseline expression, robust responsiveness and strong and significant correlation with proteases and pro-inflammatory cytokines suggests that TLRs are key regulators of facet joint degeneration in AIS. Blocking their activity could therefore potentially modify disease progression.

Keywords: Adolescent Idiopathic Scoliosis, osteoarthritis, facet joint, cartilage, metalloproteases, cytokines, extracellular matrix

Introduction

Zygapophyseal also known as facet joints are synovial joints located on each side of the posterior spinal column of each motion segment. They play a primary role in spinal stabilization and load bearing. Studies show facet joints can carry up to 25% of the axial compression and 40-65% of torsional and shearing forces in a healthy spine [1-3]. Balanced load dispersion between the intervertebral discs (IVD) and both facet joints is important to prevent degeneration of these cartilaginous tissues. Many studies have confirmed the detrimental effects of non-physiological loading on both facet joints and IVDs, as well as segmental degeneration following injurious loading. One such example is the presence of IVD and facet joint degeneration in Adolescent Idiopathic Scoliosis (AIS) [4-6]. AIS is the most prevalent orthopaedic condition (up to 4%) in young individuals aged from 10 to 16 worldwide that involves a progressive curvature of the spine [7]. The imbalanced load results in wedging that causes disc degeneration, which is thought to contribute to coronal deformation [8]. Similarly, we have recently reported facet joint osteoarthritis (OA) in AIS patients, although, how the altered biomechanics affect the facet joints is still unclear. Histologically, we found that scoliotic and non-scoliotic facet joint OA share many characteristics such as fibrillation of the cartilage surface, erosion, fissuring, osteophyte formation, increased cell density and elevated expression of pro-catabolic and pro-inflammatory factors which suggests that they are closely related [4,9,10].

The underlying pathology of facet joint OA is a catabolic and inflammatory environment that leads to degradation, loss of function and potentially pain. Pro-inflammatory cytokines are secreted by chondrocytes and synovial fibroblasts, and cause catabolism by triggering the production of proteases degrading the extracellular matrix (ECM) [11-13]. Interestingly, a growing body of literature links Toll-Like Receptors (TLR) to cartilaginous tissue degeneration [14-16]. TLRs are a family of pattern recognition receptors with 10 members in human cells. They are

activated by a variety of molecules derived from pathogens, endogenous danger related proteins released by stressed cells or fragmented ECM components. These endogenous proteins such as HSP60, HSP70, S100A8/9, biglycan, HMGB1, fibronectin and aggrecan-fragment belong to a group called *alarmins* and have been measured in higher abundance in degenerating cartilage [17-23]. TLR ligands selectively bind specific TLR hetero- or homo- dimers and generate an inflammatory downstream response [24]. Although TLR1-9 protein expression was found to correlate with degenerative changes in adult OA chondrocytes, most of the alarmins bind to either TLR2 which dimerizes with TLR1 and 6, or to TLR4-heterodimers [25]. TLR homo- or hetero -dimerization generally results in recruitment and activation of MyD88 through the Toll/IL-1R homology domain. This leads to the activation of the transcription factor NF-κB and downstream expression of pro-inflammatory, catabolic and pro-nociceptive factors [16,24,26-28]. There has been a recent interest in using small-molecule inhibitors to block TLR activation. Two such molecules are o-Vanillin and Sparstolonin B which interfere with MyD88 recruitment preventing downstream signalling [29-31]

This study is focused on evaluating a potential involvement of the TLRs in AIS facet joint OA and evaluating the potential of inhibiting TLRs using small-molecule inhibitors as potential disease-modifying therapeutics.

Materials and Methods

Sample collection and processing

Facet joints were resected with consent from AIS patients undergoing corrective surgery. Non-scoliotic thoracolumbar facet joints were from organ donors without known spinal pathology and were obtained with consent through a collaboration with Transplant Quebec. A total of 18 scoliotic

patients and 12 non-scoliotic organ donors were included in this study, with a mean age of 15.0 ± 1.12 and 41.5 ± 9.66 respectively. From each donor, one left and right upper facet joint from the same spinal level were collected and processed (Table 1). The facet joint cartilage was removed from the subchondral bone and was either digested with Collagenase Type II (Gibco) to release chondrocytes [32] or cultured as explants [33] in complete chondrocyte media (Dulbecco's DMEM with 4.5g/L glucose (Sigma Aldrich), 10% FBS, 25µg/ml gentamycin (Life Technologies), 2mM Glutamax (Life Technologies)). Collagenase digestion generated on average $1x10^6$ chondrocytes per 100mg of tissue. The study was approved by the McGill University institutional review board in Montreal, Canada (IRB # A10-113-13B and #A08-M71-14B).

Gene expression analysis

Freshly isolated scoliotic (n=18 from 9 donors) and non-scoliotic (n=12 from 6 donors) chondrocytes were lysed with TRIzol (Life technologies). RNA was extracted according to the manufacturer's instructions and re-suspended in DEPC water (Life technologies). 1µg of RNA was reverse transcribed with the Reverse-transcriptase kit (Life technologies). RT-qPCR was performed on a QuantStudio 7 (Life Technologies) using PowerUp Sybr Green master mix (Life Technologies) and primers listed in Table 2. The results were analyzed by the comparative CT method using ß-actin as the reference gene. When compared to untreated or alarmin-treated controls, the fold change was calculated using the 2^{-ddCT} method and normalized to ß-actin.

Ex vivo culture and TLR activation

Cartilage explants of scoliotic (n=15 donors) and non-scoliotic (n = 7 donors) facet joints were generated with a 8mm biopsy punch. The biopsy was divided in two equal pieces and cultured

separately in complete chondrocyte media for 48h after isolation. The weight of the explants ranged between 16 and 20mg. One half was then subjected to 100ng/ml Pam2CSK4 a TLR2/6 agonist (Invitrogen) in explant media (Dulbecco's DMEM with 4.5g/L glucose (Sigma Aldrich), Insulin-transferrin-selenium (Invitrogen), 2mM Glutamax (Life Technologies), 25µg/ml gentamycin (Life Technologies)) on a 20 volume (ml) per weight (g) of tissue (20x (v/w)) basis for 4 days while the other half served as untreated control in 20x (v/w) explant media. After the treatment period, media was collected, and samples taken for histology as described below.

Secreted Protein analysis

Secreted S100A8/9 was measured in explant culture media using a Quantikine ELISA (R&D Systems). Secreted MMP3, MMP13, IL-6 and IL-8 was measured in explant culture media from tissue with or without TLR2 activation (Raybiotech). Measures were obtained using a VICTOR Nivo microplate reader (Perkin Elmer).

Histology and proteoglycan content analysis

Facet joint cartilage explants were fixed in 4% paraformaldehyde and cryoprotected in 10, 20, and 30% sucrose for OCT embedding and cryosectioning on a CryoStar NX70 cryostat (ThermoFisher Scientific). 12µm sections perpendicular to the joint were placed on SuperFrost plus slides (VWR). Slides were heated at 60˚C for 30 minutes and rehydrated in PBS-T (0.05% Tween 20 (Sigma Aldrich)) for 5 minutes. The slides were subsequently stained in 0.025% Safranin-O (Sigma Aldrich) for 5 minutes, 1% glacial acetic acid for 15 seconds and finally in 0.01% fast green (Sigma Aldrich) for 5 minutes. Images of the sections were taken at 20x on a Leica DMRB microscope (Leica) with a DP70 digital camera (Olympus). The red safranin-O staining was quantified using

a MATlab script which normalizes background and measures the red pixel intensity in a delimited cartilage region of interest. Proteoglycan content released into the media was quantified using the Dimethylmethylene Blue (DMMB) assay [34].

Mass spectrometry analysis

Conditioned media samples from the explant-cultures were reduced with DTT and alkylated with iodoacetic acid. Following trypsinization and re-solubilization in 0.1% aqueous formic acid and 2% acetonitrile, peptides were separated on a Thermo Acclaim Pepmap (Thermo, 75 uM ID × 2 cm C18 3 uM beads) precolumn followed by an Acclaim Pepmap Easyspray column (Thermo, 75 uM × 15 cm with 2 uM C18 beads) using a Dionex Ultimate 3000 uHPLC. Analysis of all peptides with a charge of 2+ or greater were dectected with the Orbitrap Fusion mass spectrometer with HCD. The data was analyzed and compared to known human proteins sequences (Swissprot) using Mascot 2.3. Results were compiled in Scaffold and illustrated using Graphpad prism.

In vitro TLR antagonist evaluation

Monolayer chondrocytes were used at passage 2. 150 000 cells were seeded in 6 well plates and were cultured in complete chondrocyte media to confluent. At confluence, cells were pre-treated with 1/1000 dilution of Sparstolonin B, o-Vanillin or PBS in serum-free chondrocyte media for 1h before adding a final concentration of 1µg/ml S100A8/9 (R&D Systems) or 2.5ug/ml biglycan.(R&D Systems). The synthetic agonist Pam2CSK4 (Invitrogen) was also used here as a positive control at a lower concentration of 1ng/ml. The lower dose was chosen for physiological relevance to simulate the low-grade inflammation found in OA. For gene expression analysis, cells were lysed in TRIzol 16h following alarmin treatment. Culture media was collected after 48h.

Statistical methods

For each comparison between scoliotic and non-scoliotic groups or control and treated groups, a parametric student's t-test was used. For correlations between TLRs and degenerative factors, the Pearson method was used to assess the degree of relation and significance of $p<0.05$. Each statistical method and correlation were calculated using GraphPad Prism.

Results

Baseline gene expression of TLRs and degenerative factors

The proteinases MMP3 and MMP13 and the cytokines IL-1ß, IL6 and IL8 are often linked to OA in adults[35]. We have demonstrated that young patients with AIS show evidence of facet joint OA but the expression of cytokines and proteases has not been well established. Here we found the transcripts to be more abundant in scoliotic chondrocytes compared to non-scoliotic (Figure 1A). MMP3 had a modest 1.28-fold increase while MMP13 showed a 2.37-fold higher expression in scoliotic chondrocytes. The pro-inflammatory cytokine IL-1ß had elevated mRNA levels in scoliotic chondrocytes, with a 2.97-fold higher expression compared to non-scoliotic samples (Figure 1B). A 1.98-fold higher IL-6 expression was seen scoliotic tissues and IL-8 showed no difference between groups.

TLR 1,-2,-4, -6 are the main members involved in alarmin and danger signal recognition and the subsequent induction of pro-catabolic and inflammatory mediators can contribute to tissue degeneration [36,37]. To evaluate the presence of this pathogenic mechanism in degenerating scoliotic cartilage, baseline mRNA expression of TLR receptors was evaluated from isolated chondrocytes (Figure 1C). Interestingly, all four TLRs evaluated were more abundant in scoliotic

cartilage compared to non-scoliotic, with TLR2 having the largest difference with a significant 2.84-fold (p=0.03) higher expression. In the scoliotic samples, TLR6 expression was 1.71-fold higher, TLR1 expression was 1.49-fold higher, and TLR4 expression was 1.15-fold higher compared to non-scoliotic samples. This data reinforces the knowledge of a catabolic and pro-inflammatory state of degenerating facet joint chondrocytes with the added insight of an elevated TLR expression.

Gene expression correlation of TLRs and degenerative factors in scoliotic chondrocytes

A Pearson correlation analysis was performed to assess the relationship between TLR expression and factors linked to cartilage degeneration. The correlation analysis was performed between TLR1, -2, -4, -6, MMP3, -13, and IL-1ß, -6, -8 gene expression levels, of scoliotic and non-scoliotic samples separately (Figure 1D). Two parameters were used to evaluate the correlation of each gene pair: the Pearson coefficient details the correlation strength on a scale where 0 equates no correlation, 0.6 a moderate correlation and 1 being the strongest possible. A positive coefficient represents a proportional correlation whereas a negative coefficient an inversely proportional correlation. An overview of association results from scoliotic samples shows that all pairs of TLR (-1,-2,-4,-6) and degenerative factors (MMP3, MMP13, IL-ß, IL-6, IL-8) correlated positively and significantly with each other (Figure 1D,E). Moreover, all correlations are strong (Pearson>0.6) except the pair TLR1-IL-6 (Pearson=0.547) which showed a moderate correlation. In stark contrast, gene expression in healthy non-scoliotic cartilage were mostly independent from each other as only 8 out of 36 possible correlations were significant (Figure 1E). In order to better understand the relation in expression between individual TLRs, we also evaluated Pearson correlations between each TLR levels in scoliotic and non-scoliotic chondrocytes. In

scoliotic tissues, expression of the four TLRs were strongly (Pearson>0.6) and significantly (P<0.05) correlated. The strongest correlations were between TLR2-TLR4 (Pearson = 0.899) followed by TLR4-TLR1 (Pearson = 0.819) and TLR4-TLR6 (Pearson = 0.780). For the non-scoliotic samples, only 3 out of 6 pairs correlated significantly (TLR1-TLR4, TLR1-TLR6 and TLR4-TLR6). Notably, TLR2 only had weak correlations with TLR1,-4 and -6, in non-scoliotic cells. The strongest correlation in non-scoliotic cells was between TLR4-TLR6 (Pearson=0.864, P=0.0002) Together, the data indicates a strong relationship between TLR expression with inflammation and catabolism in degenerating facet joints.

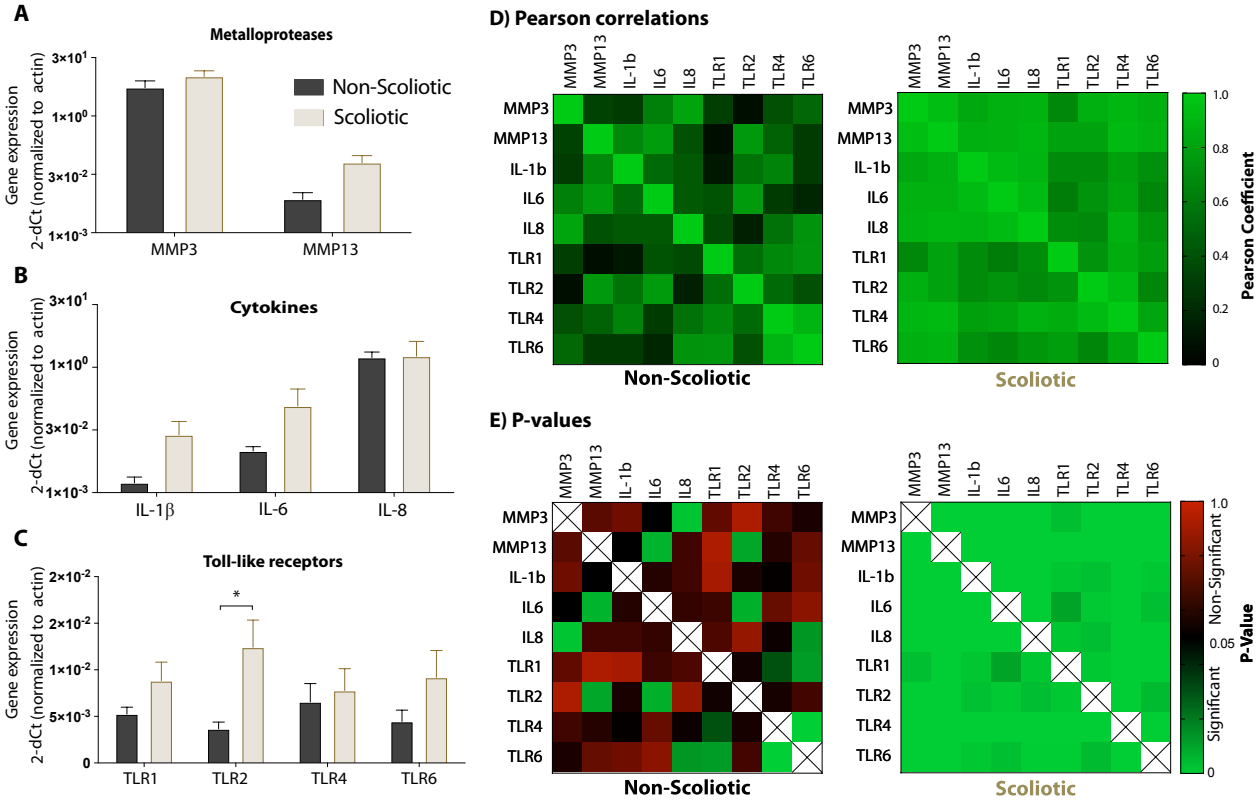

Figure 1 A, B, C) *Baseline MMP3, MMP13, IL-1ß, IL-6 and IL-8 and Toll-like receptor 1,2,4 and 6 gene expression in scoliotic (n=18) and non-scoliotic (n=12) facet joint chondrocytes.* ***D)*** *Pearson correlations of gene expression (cycle numbers) normalized to ß-actin between TLR1, -2, -4, -6 and degenerative factors MMP3, MMP13, IL-1ß, IL-6 and IL-8)* ***E)*** *P-values for each of the Pearson correlations in D). Significance is evaluated at p<0.05.*

TLR activation induced production of catabolic and pro-inflammatory factors

To evaluate the effect of TLR activation we subjected facet joint chondrocytes to the potent synthetic TLR2/6 agonist Pam2CSK4. Gene expression analysis revealed a significant increase of IL-6, IL-8, MMP3 and MMP13 in both scoliotic and non-scoliotic chondrocytes (Figure 2A). Interestingly, scoliotic chondrocytes responded stronger than non-scoliotic cells for all genes tested. IL-6 was increased by 196-fold ($p=0.0003$) and IL-8 by 91-fold ($p=0.0001$). The proteases MMP3 was increased 39-fold ($p=0.0008$) and MMP13 22-fold ($p=0.0007$) in scoliotic cells. Non-scoliotic chondrocytes also responded significantly to the TLR2/6 agonist although to a lesser extent with a 117-fold ($p=0.0009$) and 46-fold ($p=0.0008$) increase in IL-6 and IL-8 respectively. MMP3 expression was increased by 13-fold ($p=0.04$) and MMP13 by 6-fold ($p=0.03$). To verify these finding in intact tissue, a cartilage explant experiment was conducted and protein secretion into culture media assessed by ELISA assays (Figure 2B). TLR2/6 activation significantly increased MMP3 secretion 5.7-fold ($p=0.02$) and 3.43-fold ($p=0.006$) from scoliotic and non-scoliotic explants respectively. For MMP13, the scoliotic samples produced more than the non-scoliotic without activation. TLR2/6 activation significantly increased MMP13 expression with 5.39-fold ($p=0.02$) in scoliotic and by 4.63-fold ($p=0.004$) in non-scoliotic explants. Since IL-6 production in untreated samples was under the detectable threshold of the assay, we could not determine a fold difference in production after TLR2/6 activation. However, both groups had detectable amounts after treatment demonstrating that IL-6 production is also influenced by TLR2/6 activation. Finally, TLR2/6 activation significantly ($p=1.91\times10^{-6}$) ($p=5.73\times10^{-5}$)) increased IL-8 protein expression in scoliotic and non-scoliotic samples with 168-fold and 289-fold difference respectively. Notably, scoliotic explants displayed the strongest induction of all 4 factors analyzed after TLR2/6 activation. Scoliotic explants produced higher levels than non-scoliotic, disregarding the initial amount measured in untreated controls.

TLR activation induced cartilage ECM degradation

Proteoglycan loss is a key feature of OA [38]. To evaluate the effect of TLR2/6 activation on proteoglycan content, safranin-O fast green staining was performed on *ex vivo* treated scoliotic and non-scoliotic cartilage explants (Figure 2C). Proteoglycan content was measured semi-quantitatively (Figure 2D). In scoliotic tissues, the cartilage had a lower baseline proteoglycan content compared to the non-scoliotic cartilage and lost significantly more ($p=0.003$) following TLR2/6 activation. In contrast, TLR2/6 activation did not cause an as robust proteoglycan loss in non-scoliotic samples. Proteoglycan release in the culture media was measured with the DMMB assay and confirmed histological findings with increased release from scoliotic cartilage after TLR2/6 activation (Figure 2E). There was no significant difference in proteoglycan release in non-scoliotic cartilage after TLR2/6 activation. Together, These findings support a role for TLR2/6 in the degenerative cycle of scoliotic facet joint cartilage.

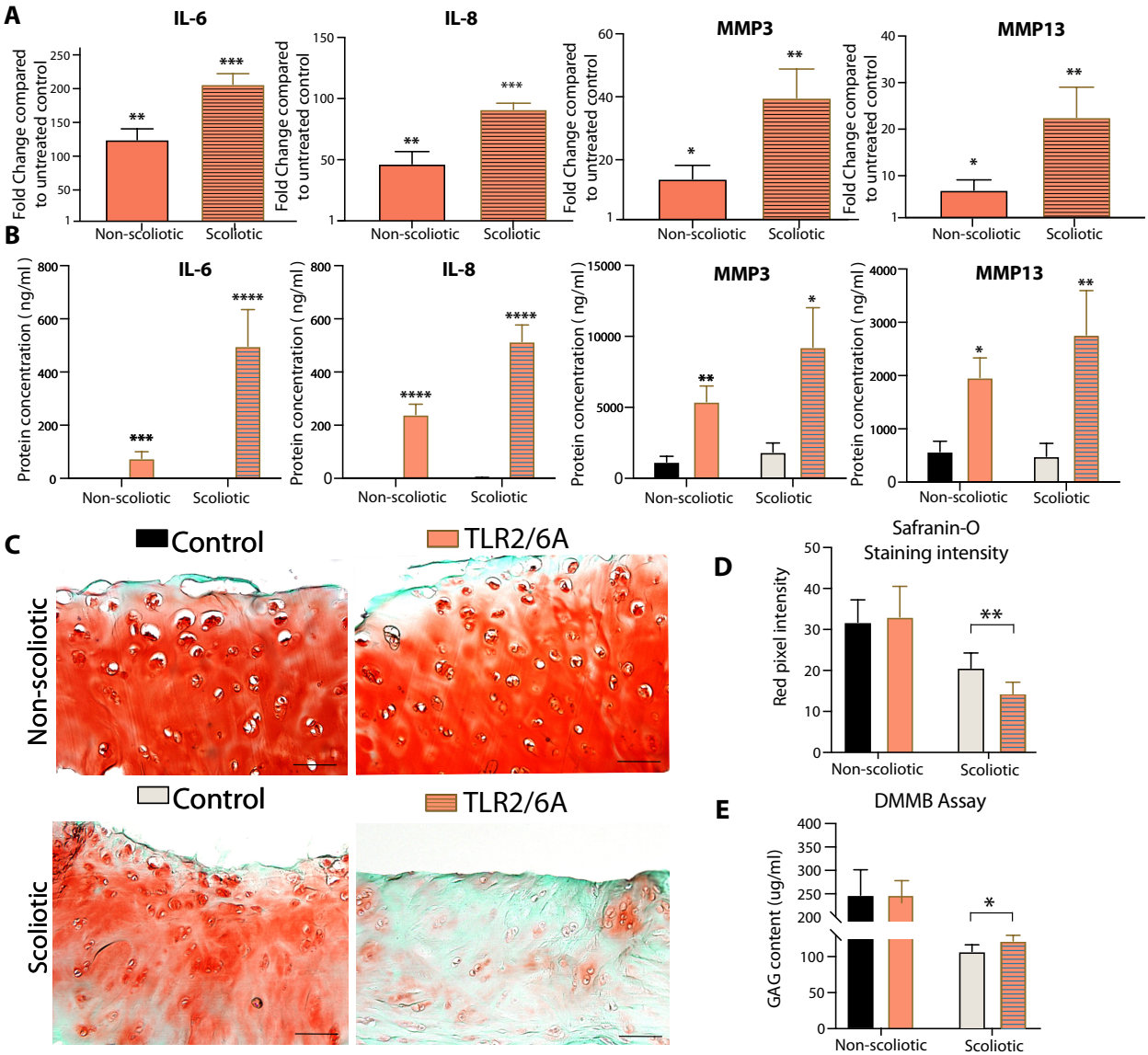

Figure 2 A) *Gene expression analysis of Pam2CSK4 treated scoliotic and non-scoliotic chondrocytes compare to non-treated controls (n=5)* ***B)*** *MMP3, MMP13, IL-6 and IL-8 secretion analysis of ex vivo facet joint cartilage cultured media from scoliotic (n=10) and non-scoliotic groups (n=10) after 4 days of TLR2 activation with Pam2csk4.* ***B)*** *Safranin-O fast green histology of scoliotic (n=30) and non-scoliotic (n=13) facet joint cartilage before (Control) and after (Pam2csk4) TLR2 activation for 4 days.* ***D)*** *Red staining quantification for proteoglycan content using a MATlab script.* ***E)*** *GAG content analysis in the cultured media before and after Pam2CSK4 treatment. Paired student t-test were performed to assess significance defined by *=P<0.05, ** = P<0.01, ****=P<0.001.*

Alarmins in scoliotic cartilage

An increase in abundance of alarmins activating TLRs has been described in joints affected by OA in adults [38]. Mass spectrometry and ELISA assays were used to reveal the alarmin secretion profile of scoliotic and non-scoliotic cartilage (Figure 3A). The z-score heat map shows an increased level of alarmins released from scoliotic cartilage. In contrast protein levels were frequently below the detection threshold non-scoliotic cartilage. Notably, cytoplasmic alarmins such as heat shock proteins (HSP) were found in all scoliotic samples but were mostly undetected in the non-scoliotic group. S100A8/A9 has been strongly linked to adult OA. [39] Although, not detected with mass spectrometry analysis we set out to quantitively measure the concentration. Scoliotic cartilage released 2.9-fold ($p=0.03$) more S100A8/A9 than non-scoliotic. The concentration was 10.33ng/ml in scoliotic and 3.57ng/ml in non-scoliotic cartilage (Figure 3B). These results indicate an increased release of alarmins from degenerating scoliotic cartilage that could activate TLRs.

Alarmins induced pro-inflammatory and catabolic factor production

Alarmins and TLR activation are believed to contribute to the chronic low-grade inflammatory state present in adult OA [40]. To evaluate the potential of naturally occurring alarmins to participate in facet joint cartilage degeneration, isolated scoliotic chondrocytes were subjected to S100A8/9 and biglycan, which are both released by scoliotic cartilage. A lowered concentration of the TLR2/6 agonist was used in these experiments to more accurately compare to the alarmin induced responses. As expected, the agonists caused an increase in IL-6 an IL-8 gene expression (Figure 3C). S100A8/A9 had the strongest effect on IL-6 with a significant 5.14-fold ($p=0.0009$) increase. MMP3 and MMP13 gene expression was only affected by S100A8/A9, with

a 2.34-fold increase. Biglycan treatment increased TLR2 expression with a modest 1.829-fold increase. As expected, the low-dose TLR2/6 agonist increased gene expression of the 4 genes to a similar level as found with S100A8/9. These results suggest a role for alarmins in scoliotic facet joint degeneration.

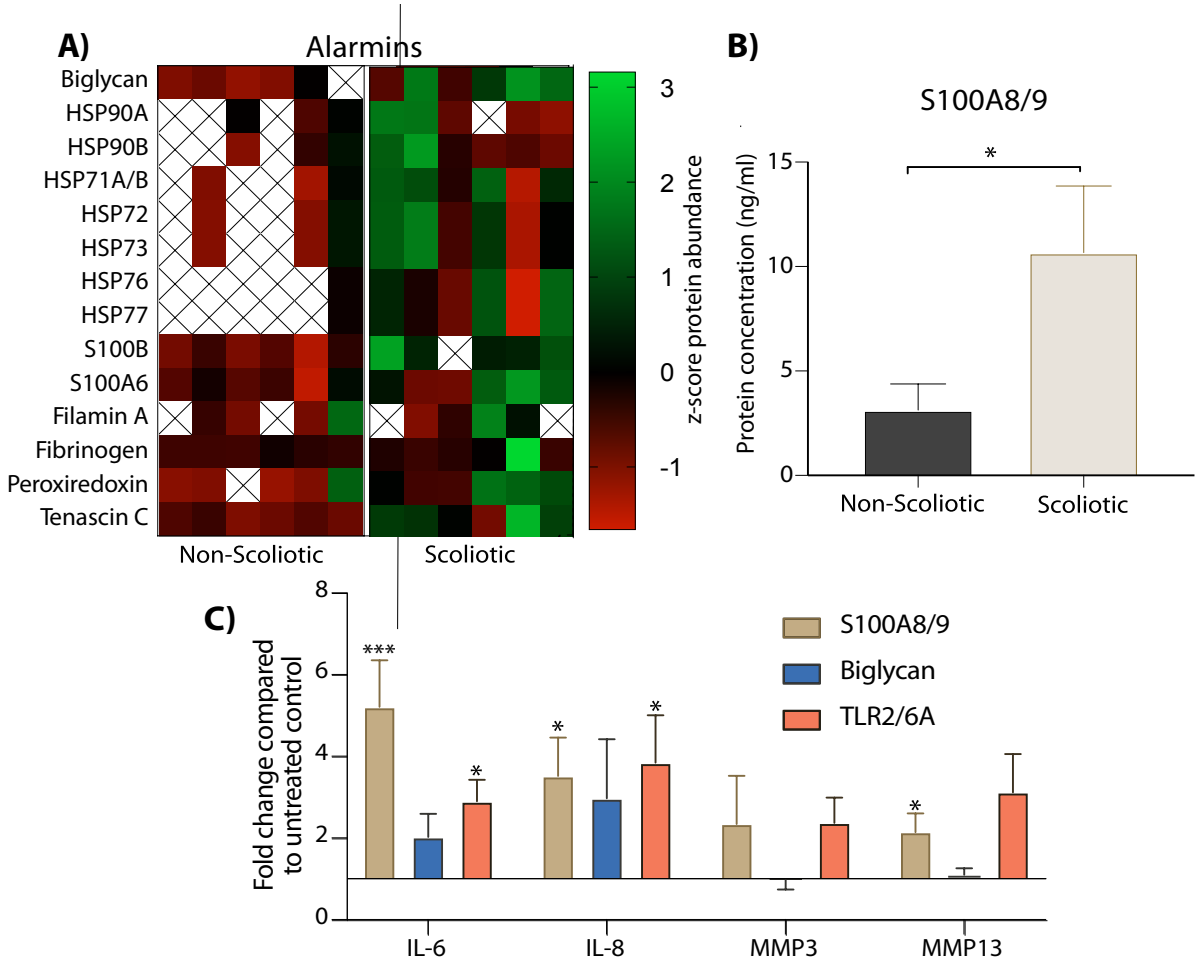

***Figure 3**: **A, B)** Mass spectrometry and ELISA of alarmin and S100A8/9 abundance in scoliotic and non-scoliotic cartilage explant conditioned media (n=6). **C)** Gene expression analysis of IL-6, IL-8, MMP3, MMP13 after stimulation by alarmins S100A8/9 and Biglycan and low dose Pam2CSK4 in scoliotic facet joint chondrocytes (n=4). Significance was evaluated by student t-tests. (* = p<0.05, *** = p<0.001)*

Sparstolonin B and o -Vanillin reduced alarmin-induced TLR activation

As TLR expression is elevated and the chondrocytes are strongly responsive to alarmins in scoliotic cartilage blocking their activity could potentially modify disease progression. To assess the effect of blocking TLR activation, two naturally-derived compounds that prevent the recruitment of MyD88 to the TIR domain of TLRs, and thus TLR signaling, were used [31,41]. The antagonists did not significantly modulate gene expression in scoliotic chondrocytes challenged with biglycan (Figure 4A, B). However, Sparstolonin B and o-Vanillin significantly suppressed S100A8/9-induced IL-6 gene expression by 6.12-fold (p=0.0009) and 7.43-fold (p=0.0007) respectively. IL-8 expression decreased with both antagonists but significance was only reached with o-Vanillin (3.98-fold). In contrast, MMP3 expression was reduced by 2.14-fold (p=0.0008) with Sparstolonin B treatment, and MMP13 gene expression was unchanged by both antagonists, although a small decreasing trend after o-vanillin treatment was found. This indicates that TLR inhibition overall reduces the alarmin-induced pro-inflammatory response.

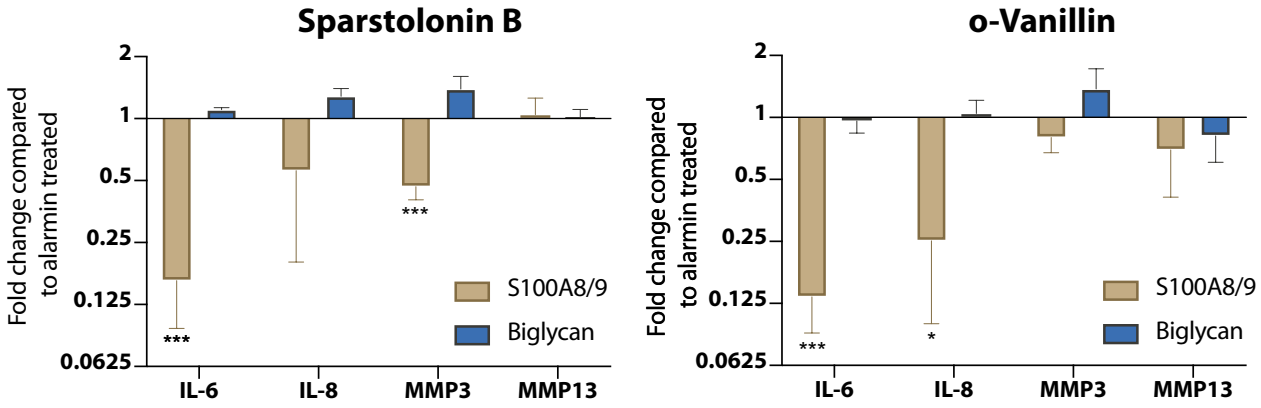

Figure 4 *: Gene expression analysis of degenerative factors under stimulation of alarmins S100A8/9 and biglycan in conjunction with antagonists Sparstolonin B and o-Vanillin. Significance between control and treatment was evaluated at ***=P<0.001. Significance between alarmin and alarmin + antagonist was assessed at ++= P<0.001 and += P<0.05.*

Alarmins increased TLR gene expression

TLR activation has been linked to an increased expression of TLRs . This mechanism can drive a vicious cycle in the presence of abundant alarmins such as in degenerating cartilage [42]. We therefore evaluated TLR gene expression in response to activation in scoliotic cartilage. TLR4 gene expression was unchanged following biglycan, S100A8/9 and TLR2/6 agonist treatment. (Figure 5A). S100A8/9 had the strongest effect and induced TLR1, -2 and -6 expression by 6.242-fold ($p=0.04$), 2.799-fold ($p=0.02$) and 3.271-fold respectively compared to untreated controls.

Treatment with Sparstolonin B and o-Vanillin significantly reduced the levels of TLR1 and TLR2 expression induced by S100A8/9 (Figure 5B). TLR1 and TLR2 gene expression also decreased by 2.04-fold ($p=0.007$) and 1.81-fold (p-0.03) following Sparstolonin B treatment. TLR1 and TLR2 decreased with a similar magnitude, 1.9-fold ($p=0.04$) and 1.87-fold ($p=0.03$) following o-Vanillin treatment. TLR4 and TLR6 expression however, was not affected by either of the antagonists. These results show the potential of using MyD88-targeting small molecule inhibitors to block TLR signaling to potentially restore a homeostatic balance in degenerating facet joints in AIS patients.

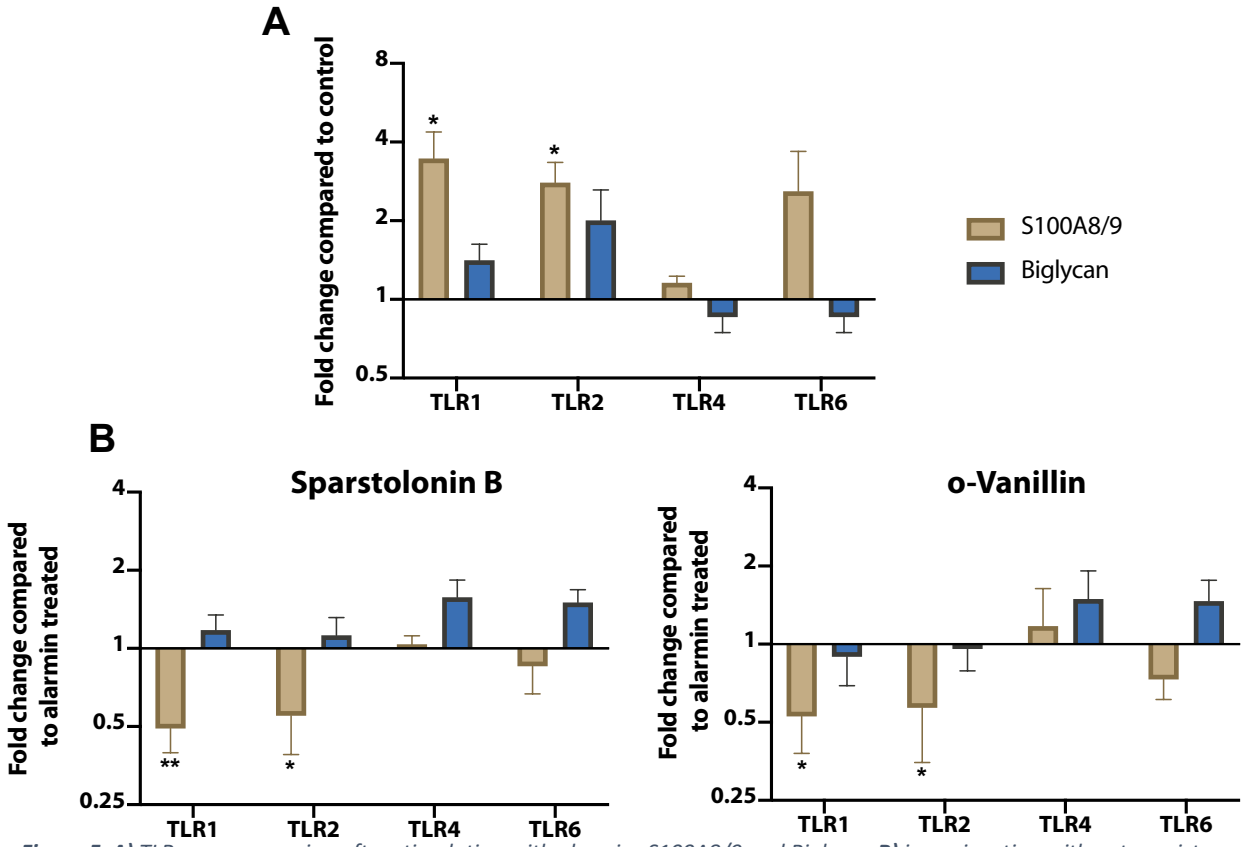

Figure 5 A) *TLR gene expression after stimulation with alarmins S100A8/9 and Biglycan* ***B)*** *in conjunction with antagonists Sparstolonin B and o-Vanillin. Significance between control and treatment was evaluated at * = p<0.05, **=P<0.01.*

Discussion

Our findings suggest that TLR activation is contributing to scoliotic facet joint degeneration. We found higher TLR gene expression and strong correlations with degenerative factors in scoliotic cartilage. TLR activation by synthetic agonists and naturally occurring alarmins induced the expression of pro-inflammatory cytokines and proteases, which exacerbated cartilage breakdown in scoliotic facet joint cartilage. A potential beneficial effect of blocking TLR signalling was suggested from the effects of the MyD88 inhibitors Sparstolonin B and o-Vanillin Both reduced alarmin-induced expression of proteases, pro-inflammatory cytokines and TLR receptors.

A current hypothesis of the catabolic shift and chronic inflammation in osteoarthritic cartilage implicates the formation of alarmins through tissue degradation with a subsequent TLR activation [43]. We confirmed that this mechanism is active in facet joints of young patients with scoliosis.

First, baseline gene expression for key catabolic factors and TLRs were measured. The increase in baseline gene expression of proteases MMP3 and MMP13 match extensive previous research showing a robust increase of these proteases in OA cartilage. Furthermore, it corroborates with immunohistochemistry findings in our previous study on scoliotic facet joint degeneration [4]. Gene expression analysis of TLRs revealed that chondrocytes from degenerating scoliotic cartilage had an elevated baseline expression of TLRs compared to the non-degenerate non-scoliotic cells. These findings also align with other studies showing that TLR expression is upregulated in articular cartilage with increasing OA severity in adults [25]. Notably, TLR2 had the largest and most significant difference between the two groups which is in accordance with data from osteoarthritic knee chondrocytes in adults [26]. The amount of highly significant and strong correlations between TLRs (1,2,4,6) and degenerative factors (MMP3, MMP13, IL-1ß, IL-6, IL-8) in scoliotic chondrocytes suggests that there is a strong link between TLR expression and degeneration in scoliotic cartilage. The fact that healthy non-scoliotic cartilage lacked this strong correlation further support this. We could only detect a significant correlation between TLR2 - MMP13 and TLR2-IL-6 in cells from non-scoliotic tissue. One explanation could be that TLR2 homodimers are more prevalent in healthy tissues whereas TLR2/6 and TLR2/1 heterodimers are more prominent in scoliotic cartilage, but this would need to be confirmed. The fact that all TLRs were found to be upregulated in adult OA cartilage could further support why they correlate strongly with each other only in the scoliotic samples [25].

The responsivity of facet joint chondrocytes to TLR2/6 activation was strong, as evidenced by large fold-changes when compared to untreated controls. As expected, cytokines were the most increased after activation in both groups. The stronger response in cells from scoliotic tissue might be a reflection of elevated expression levels of TLRs detected. The *ex vivo* cartilage explant experiment supports the gene expression analysis of isolated cells confirming elevated levels of protease and cytokine release into the media after TLR2/6 activation. The robust induction of proteases and cytokines in cells from scoliotic samples following TLR activation indicates that they are more susceptible to TLR-induced joint catabolism. *Ex vivo* cartilage explants subjected to TLR2/6 activation revealed a pronounced proteoglycan loss in scoliotic samples, which supports the susceptibility to TLR activation in scoliotic cartilage. Gene expression analysis of aggrecan suggests that the proteoglycan loss is not due to a change in synthesis since gene expression levels did not change after TLR activation (Data not shown). The induced expression of proteases (MMP3, MMP13) and pro-inflammatory cytokines (IL-6, IL-8) likely contributed to this drastic catabolism. Proteoglycan loss from the tissue and release to culture media was not affected in non-scoliotic explants and might reflect on three possibilities. First, the semi-quantitative measurement of proteoglycan staining might not be sensitive enough to detect the loss from healthy tissue with a much higher initial content than what is present in scoliotic cartilage. However there was also no change proteoglycan released to the culture media. Secondly, as non-scoliotic cells have a lower baseline expression of TLRs, the short exposure of 4 days to the agonist might not have been enough to allow for receptor upregulation and subsequent release of degenerative factors

. Thirdly, the agonist might not readily penetrate healthy cartilage to induce degenerative factors. However, isolated non-scoliotic cells also responded less to the agonist. Other studies have shown proteoglycan loss following TLR activation in non-degenerate cartilage and a longer

treatment period would likely have resulted in the loss seen with scoliotic samples. The literature also supports a role for MMP13 in OA, where overexpression of MMP13 was shown to be sufficient to cause osteoarthritis in mice [44]. Another plausible explanation is a modulation of TLR signalling by a yet unknown mechanism in scoliotic facet joint OA, which should be investigated further.

To better assess the potential of TLR activation in degenerating scoliotic facet joints, we quantified the alarmin profile. The observable trend of increased abundance of alarmins in scoliotic samples is corroborated by studies showing increased alarmins in degenerating cartilage of adults [38]. The alarmins detected has been described in the literature to activate TLR1,-2,-4, or -6. For example, the extracellular matrix molecule biglycan interact with TLR2 and TLR4 and induces pro-inflammatory molecules in macrophages [20]. S100A8/9 which was released at a significantly higher level from scoliotic cartilage, has been described to induce pro-inflammatory responses in macrophages [19]. Here we evaluated the effect of biglycan and S100A8/9 in scoliotic chondrocytes where treatment increased gene expression of IL-6, IL-8, MMP3 and MMP13, which is similar to experiments done OA chondrocytes [39]. The alarmins had a similar response to a low concentration of the synthetic TLR2/6 agonist, mimicking a chronic low-grade inflammation found in OA. The difference in gene expression induction between high and low concentration of the synthetic TLR2/6 agonist reflect an increase proportional to concentration. Further studies are needed to fully elucidate the detrimental role of TLR activation following exposure to alarmins, since every alarmin has a different binding potential that could result in varying downstream effects.

Although this study suggests the involvement of TLRs in scoliotic facet joint OA, the clinical relevance of blocking TLRs to slow disease progression still needs to be evaluated. In

support of our suggestion, a joint-saving outcome of blocking TLRs has been described in an experimental osteoarthritis models [45]. The successful reduction of alarmin-induced protease and pro-inflammatory cytokine production by Sparstolonin B and o-Vanillin opens the possibility of using small molecule inhibitors to suppress chronic inflammation and catabolism in scoliotic facet joints. Therefore, blocking TLR signalling should be studied further to explore a potential disease modifying effect of such inhibitors.

A limitation in this study was the age differences between scoliotic and non-scoliotic subjects. The scoliotic cohort had a lower and homogeneous age range whereas non-scoliotic organ donors had a wider age range and a higher average age. This is explained by the window at which patients with AIS undergo corrective surgery and the variable age of organ donors. However, there was a variability in curve severity and degree of OA in AIS patients whereas all non-scoliotic organ donors had healthy spines with no signs of OA and no prior spine deformities. Therefore, we believe that the heterogeneity in age is counterbalanced by the clear differences in cartilage health between the groups.

Conclusions

In conclusion, our results suggest that TLRs are integral to cartilage degeneration in scoliotic facet joints. The higher baseline expression of TLRs in scoliotic cartilage and the strong and significant correlation with proteases and pro-inflammatory cytokines suggests that they are key regulators of tissue degradation. Taken together, these findings provide an insight into a potential target for future molecular therapies aiming at restoring tissue homeostasis and prevent tissue degradation and loss of function.

Author Contributions

Dr. Lisbet Haglund, Dr. Jean A. Ouellet and Daniel Bisson conceived and designed the study. Daniel Bisson, Semsi Kocabas, Kai Sheng and Dr. Emerson Krock collected the experimental data presented in the paper. Daniel Bisson and Lisbet Haglund drafted the manuscript. Dr. Jean A Ouellet, Dr. Neil Saran and Dr. Alisson Teles performed all surgeries and provided clinical input to the study design and data interpretation. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to acknowledge Dr. Derek Rosenzweig for reading the manuscript and providing comments on the experimental procedure.

Conflicts of interest

The authors have no conflict of interest to disclose. Dr. Lisbet Haglund and Dr. Jean Ouellet were jointly awarded a grant from the Shriners Hospitals for Children (Montreal, Canada) to perform the studies.

Data statement

The data generated in this study are available from the corresponding author on reasonable request.

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Table 3 Tissue donor information for both Scoliotic and Non-scoliotic groups.

Group	AGE	SEX	MAJOR CURVE COBB ANGLE (°)	CAUSE OF DEATH
scoliotic	14	Female	68	
scoliotic	13	Female	67	
scoliotic	17	Female	45	
scoliotic	14	Female	56	
scoliotic	15	Female	50	
scoliotic	17	Female	55	
scoliotic	19	Male	50	
scoliotic	13	Male	78	
scoliotic	18	Male	62	
scoliotic	14	Female	50	
scoliotic	17	Male	50	
scoliotic	13	Female	70	
scoliotic	13	Female	64	
scoliotic	14	Female	50	
scoliotic	15	Female	60	
scoliotic	13	Female	55	
scoliotic	19	Male	55	
scoliotic	12	Female	48	

Non-scoliotic	60	Female	Stroke
Non-scoliotic	34	Male	Stroke
Non-scoliotic	49	Female	Hemorrhage
Non-scoliotic	53	Male	Hemorrhage
Non-scoliotic	17	Male	Anoxia
Non-scoliotic	22	Male	Anoxia
Non-scoliotic	53	Female	Anoxia
Non-scoliotic	28	Male	Anoxia
Non-scoliotic	27	Male	Trauma
Non-scoliotic	42	Male	Stroke
Non-scoliotic	57	Male	Hemorrhage
Non-scoliotic	56	Male	Anoxia

Table 2 Human oligonucleotides used in gene expression analysis.

Gene of interest	*Forward Primer*	*Reverse Primer*
TLR1	cagtgtctggtacacgcatggt	tttcaaaaaccgtgtctgttaagaga
TLR2	ggccagcaaattacctgtgtg	Aggcggacatcctgaacct
TLR4	cagagtttcctgcaatggatca	gcttatctgaaggtgttgcacat
TLR6	gaagaagaacaaccctttaggatagc	aggcaaacaaaatggaagctt
MMP3	aatggcattcagtccctctatg	Gacaggttccgtgggtac
MMP13	gatgacgatgtacaagggatcc	Agggtcacatttgtctggc
IL-1ß	aagcttggtgatgtctggtc	acaaaggacatggagaacacc
IL-6	tgaaccttccaaagatggctg	caaactccaaaagaccagtgatg
IL-8	tcctgatttctgcagctctg	gtctttatgcactgacatctaagttc
Actin	gtcttcccctccatcgtgg	Aatccttctgacccatgcc

Chapter 5: Conclusions

Summary

The progressive nature of AIS follows a *vicious cycle* of asymmetrical loading and differential modulation of vertebral growth to drive the deformity in a rotationally unstable spine. However, the fate of facet joints which prevent non-physiological rotation remains unclear in the context of a scoliotic spine. We hypothesized that facet joint osteoarthritis is dependent on the spinal deformity and could contribute to rotational instability and pain. Our research aimed to deepen the knowledge of structural scoliotic deformities to provide new targets for disease-modifying treatment options and prevent AIS progression and pain.

Our first study sought to characterize facet joint cartilage in AIS patients and evaluate the severity of tissue degeneration by comparing it with facet joint samples from non-scoliotic organ donors. We found many hallmarks of osteoarthritis in scoliotic facet joints, including proteoglycan loss, surface fibrillation and cartilage erosion. Furthermore, the expression of pro-inflammatory and catabolic mediators such as IL-1β, MMP-3 and MMP-13 was increased in AIS cartilage. Secondary outcomes of facet joint degeneration were also found in AIS facet joints, such as increased chondrocyte density, proliferation, and fragmentation of extracellular matrix components. The prominence of these hallmarks in AIS facet joints confirms the premature degeneration of facet joints in AIS.

In our first study, the pattern of degenerative changes in facet joints could not be matched to the scoliotic curve using only coronal radiographs. [106] Following the consensus that scoliotic spines deform in all three planes, we investigated the association between facet joint degeneration and the spinal deformity reduced to intervertebral angles in the sagittal, axial and coronal planes. We found a strong association between axial intervertebral rotation, facet joint cartilage

degeneration and subchondral bone loss. Areas of peak facet joint degeneration on scoliotic spines correlated with increasing intervertebral kyphosis, axial rotation and patient-reported localized pain scores. These findings suggest that facet joint degeneration is driven by asymmetrical forces from rotational instability and could be a pain-generating source in AIS patients.

With the apparent implication of facet joint degeneration in AIS disease progression and pain, we started investigating targets for disease-modifying treatment options for facet joint osteoarthritis. [107] Several previous studies have implicated Toll-like receptors (TLRs) in osteoarthritis and intervertebral disc degeneration, including our research. [108-111] In scoliotic facet joints, we found an increase in TLR2 expression, which correlated with elevated levels of pro-inflammatory and catabolic mediators. Alarmins, which signal through TLRs to induce an inflammatory response, were elevated in AIS facet joint cartilage. Strong induction of the same pro-inflammatory and catabolic mediators was achieved by a synthetic TLR2/6 agonist and exacerbated proteoglycan loss in *ex vivo* scoliotic cartilage. However, the inflammatory response to alarmins and synthetic agonists in AIS chondrocytes was dampened by small-molecule antagonists Sparstolonin B and o-Vanillin. [105] These results identify a potential pathway to target the underlying inflammation driving facet joint osteoarthritis and pain.

Discussion

Using human tissues to study AIS

The main strength of this study is the use of human tissues with the excellent cooperation of Transplant Quebec and Shriner's Hospital for Children. Translational research relies on basic science to understand clinical concepts and is especially important in the complex case of AIS being a polygenic and multifactorial disease. The lack of bipedal animals with similar spinal

structures further limits the development of clinically relevant models to research AIS etiopathology. Our analysis of facet joints in AIS patients presents a unique perspective on vertebral deformities with exceptional translatability to a clinical setting. The research was utterly non-intrusive as the tissue samples and radiographs were by-products of standard treatment for evaluating and correcting spinal deformity. The bi-planar radiographs taken with the EOS system during evaluations are designed to enable 3D reconstructions while remaining low-dose to avoid adverse outcomes from repeated radiation. The facet joints usually are resected and discarded during spinal fusion to anchor pedicle screws in the vertebra and were instead collected with consent. Including cartilage and subchondral bone in the samples received allowed the evaluation of the two most important features of a diarthrodial joint. Unfortunately, our analysis was not designed to include other spinal features such as the capsular ligament, synovium and intervertebral discs. Additional research is needed to expand our characterization of facet joint osteoarthritis in AIS.

Rotational instability and AIS

An essential contribution of this thesis can be attributed to finding anatomical evidence of rotational instability in AIS. Spinal instability is the loss of ability to maintain the spine in a physiological threshold relative to a neutral zone. [44] Unbalanced forces exacerbated by an unstable scoliotic spine are theorized to form a *vicious cycle* of progressive deformity by modulating the growth of vertebras asymmetrically. [13][15][112-114] However, the uneven development of the spine in response to altered biomechanics remains unclear. We first described prominent degeneration of facet joint cartilage and loss of subchondral bone quality in AIS surgical samples. The presence of osteoarthritic hallmarks such as fibrillation and erosion with severe

proteoglycan loss likely reflects changes in the elastic modulus and compressibility of the cartilage. [47] Additionally, we found strong associations between cartilage degradation and lowered subchondral bone volume. These findings suggest facet joints are undergoing similar changes as other diarthrodial joints in early osteoarthritis, which have been characterized extensively. [47] As such, the deterioration of cartilage and subchondral bone suggests a weakening of facet joint mechanical properties. Fujiwara et al. reported an increase in rotational instability in patients with facet joint osteoarthritis. [65] Other studies confirm the loss of mechanical stability due to the presence of facet joint osteoarthritis. [115-117] Taken together, the integrity of facet joints is crucial to maintain spinal stability and therefore suggests their degeneration in AIS represents a failure in keeping axial alignment due to spinal instability.

Rotational instability is also manifested by asymmetrical degeneration of the facet joints. [118][117] In AIS samples, we found high variability in the osteoarthritic state between the two facet joints in a bilateral pair. This discrepancy was not observed in non-scoliotic samples. [106][119] We speculate that the segments presenting the most considerable variability are subjected to the most unbalanced biomechanical loading. In thoracic curves, we confirm an increase of asymmetrical degeneration at junctional segments more vulnerable to rotational instability. Additionally, facet joint tropism is a condition most often observed with asymmetrical degeneration. [120] The diagnosis is made by calculating an asymmetry in the facet joint angles from an axial view. (Figure 1) Cadaveric studies reported a shift in stress distribution due to tropism, and the resulting asymmetrical forces are believed to accelerate the degeneration of facet joints. [117][121][120] Facet joint osteoarthritis and tropism were notably reported in facet joints of degenerative scoliosis and spondylolisthesis patients. [122-124] Due to the lack of CT imaging,

we could not evaluate this parameter in our patient cohort. However, we speculate that facet joint tropism is likely present in AIS as observed, as in degenerative scoliosis. [56][122]

A major advantage of this study is the ability to compare facet joint degeneration to the 3D spinal deformity using the EOS system. Our analysis of facet joint degeneration patterns across scoliotic spines could contribute to a better understanding of common vulnerabilities in differing scoliotic shapes. Most importantly, we found significant correlations between axial rotation and facet joint degeneration. Due to the difficulty of assessing axial rotation, the rotational deformity has been relatively understudied. Nonetheless, vertebral rotation has been theorized as a dominant factor driving scoliotic progression. [112-114] Clinically, maximal rotation has been consistently observed at the apex of all idiopathic and non-idiopathic scoliotic curves. [125-127] Our third study confirmed this finding and demonstrated a high concordance in the distribution of axial intervertebral rotations in similar scoliotic spines. [119] Although the apical segment is the most rotated, we found the maximal intervertebral rotation at the junction points a few spinal levels above and below the apex. [119] Notably, the junctional segments above the apex for right thoracic curves are on average the most degenerate facet joint pairs among the samples we collected. We speculate that these junctional segments are most vulnerable to rotational instability.

In the sagittal plane, the most degenerate facet joints correlate with thoracic curves' largest kyphotic intervertebral angles. Furthermore, a significant linear association between kyphotic angles and facet joint degeneration suggests that osteoarthritis develops primarily in axially deformed and kyphotic segments. These findings are compatible with the hypothesis that dorsal shear loads applied to kyphotic vertebras are the main driver of instability in upright spines. [114] Due to decreased contact forces, facet joints risk losing the ability to restrict axial forces in kyphotic segments. If vulnerabilities arise from kyphotic segments, the risk would be determined

by the initial sagittal curves of the patient. Indeed, several studies have shown the risk of familial AIS associated with the inheritance of sagittal spinal profiles. Generally, slender and tall spines with hypokyphosis are more likely to develop AIS, according to population studies. The relative lack of kyphosis would subject more vertebras to increased dorsal shear loads and could drive rotational instability. Assuming the location of vulnerable segments differs depending on the sagittal profile, we could expect the center of rotations to develop as consequence of the initial deformity of the vulnerable segment. This could explain why initial sagittal profiles of patients with lumbar curves were distinct from those with thoracic curves and can help differentiate between progressive and nonprogressive cases. [128][129] Additionally, Lee and al. found an association between the location of the apical level and Lenke curve types. [130] In our analysis, the improved state of facet joints at the apex compared to those at the junctional segments support the hypothesis that rotatory decompensation is at the core of AIS etiopathology. Assuming this theory is correct, the vulnerable levels unable to control exacerbated rotation could represent the initiating deformity which progresses by 'cascading' unbalanced biomechanics down the spine. The location of the vulnerable segments in an average spine coincides with the level of pre-existing spinal curves in most humans. [131] Taken together, we speculate that vulnerable segments dictated by the sagittal curves could be an initiating factor that drives axial decompensation into the 3D deformity during growth.

Our main limitation in the search to understand the fate of facet joints in AIS is the lack of biomechanical analysis. This was due to the complexity of developing finite element models of the spine without CT imaging of scoliotic patients. Nonetheless, we can speculate on the nature of asymmetrical forces leading to facet joint degeneration in AIS. A logical explanation for asymmetrical facet joint degeneration would involve unbalanced biomechanical loading between

the two joints. According to anatomical studies using pressure sensors, axial rotation during torsion is the only movement that subjects significant asymmetrical loading between bilateral facet joints. The contralateral facet joint (away from the rotation) bears most of the stress, up to twice as much as the ipsilateral side (towards the rotation). [132] The high bone density we found in overloaded contralateral facet joints is aligned with Hueter-Volkmann and Wolff's laws for bone modulation. Interestingly, the pattern of facet joint degeneration we observed in AIS indicates a vulnerability of thoracic torsion from one side. Since the spinal region above the apex on the left side displayed the most extensive degeneration in the right thoracic curves, we could assume that this cohort has a torsional weakness towards the right. Finite element models of degenerative lumbar scoliosis cases found differential facet joint stress distributions between left and right torsion movements. Right degenerative curves reported the most extensive asymmetrical distribution at the junctional segment above the apex. Notably, the contralateral facet joint received peak loading while the ipsilateral side was minimally engaged. [133] In AIS spines, biomechanical analysis confirms that torsional forces are most potent at the same junctional segments where we described the most degenerative changes in facet joints. [134] Taken together, these studies corroborate our hypothesis that asymmetrical loading modulates facet joint state.

From our assessment of rotational vulnerabilities in scoliosis, we believe expanded research is needed to understand the associated risk factors related to disease progression. For example, studies have shown that certain physical activities may increase the risk of developing scoliosis. Both adult and adolescent ballet dancers are more likely to develop spinal deformities than the general population. [135] Additionally, children who participated in indoor swimming before age one have significantly increased odds of AIS. [136] These activities could be an initial source of

torsion, leading to AIS; however, additional research is needed to understand the risk of torsion on the development of the spine.

Disease-modifying treatment exploration

The relationship we described between rotational instability and facet joint degeneration emphasizes the need to develop pharmaceutical treatment options to prevent AIS progression. Based on our findings, we propose a novel approach based on a localized administration of a therapeutic compound to improve the state of specific facet joints in the most vulnerable segments. By improving the condition of the cartilage and bone, we could regain equilibrium in the capacity to bear the load and grow symmetrically, especially between facet joints of rotationally unstable pairs. Unfortunately, no disease-modifying compounds are currently available to treat osteoarthritis in articular joints. At the core of the joint dysfunction, we identified an underlying inflammatory and catabolic state produced by the residing chondrocytes, which are hallmarks of osteoarthritis. Several studies have implicated the transcription factor NF-κB for the expression of multiple pro-inflammatory cytokines and proteases, among other factors contributing to tissue catabolism. [137] In the review entitled *For whom the disc tolls: intervertebral disc degeneration, back pain and toll-like receptors* we implicated Toll-like receptors (TLR) in the priming of the NF-κB pathway in degenerative conditions of both cartilaginous joints and intervertebral discs. Increasing evidence supports the nefarious role of TLRs on chondrocyte homeostasis, apoptosis, inflammation, and catabolism in osteoarthritis. [138-141] The production of endogenous TLR agonists or *alarmins* released from hypertrophic chondrocytes or the degrading extracellular matrix is at risk of propagating an inflammatory signal beyond homeostatic limits. The increased production of alarmins we found in degenerating AIS facet joints could be explained by the

cleaving of matrix proteins by catabolic enzymes such as MMP3 and MMP13. A synergistic effect on the inflammatory response was observed when chondrocytes were exposed to pro-inflammatory cytokines and different types of alarmins. [142] We confirmed the presence of multiple alarmin sources in AIS facet joints with the fragmentation of extracellular matrix proteins such as fibronectin and biglycan, as well as the release of intracellular S100 and heat-shock proteins (HSPs). TLR2 was essential in the inflammatory process, as demonstrated by elevated gene expression and associated pro-inflammatory cytokine production. The abundance of alarmins and TLR2 expression in chondrocytes suggests that the TLR pathway likely contributes to the production of pro-inflammatory and catabolic factors at the core of facet joint osteoarthritis. [105] In support, the knockdown of TRAF6 downstream of TLR activation blunted the release of inflammatory and catabolic mediators in lumbar facet joint osteoarthritic chondrocytes. [104]

Additionally, studies indicate TLR activation favours bone resorption. [143] In *ex vivo* facet joint studies, the activation of TLR4 using LPS induced the expression of MCP-1, which attracted monocytes to promote osteoclastogenesis. [144] The subchondral bone loss associated with osteoarthritic cartilage in AIS facet joints raises the question of crosstalk between chondrocytes and bone cells mediated by TLR activation. Evidence of this molecular transfer was found in the modulated osteoblast activity after culturing with osteoarthritic chondrocytes. [145] We have initiated research on this phenomenon in AIS facet joints by monitoring the secretome of AIS primary chondrocytes and osteoblasts after TLR activation. Preliminary results indicate a role for TLR in the bone resorption observed in degenerate scoliotic facet joints. Taken together, we believe inhibiting the TLR pathway could become an effective target to prevent cartilage and bone degeneration in AIS facet joints.

Despite frequent cases of back pain in AIS, the source of the nociceptive stimuli remains unknown. [146] Using a localized numerical pain scale, we found a correlation between maximal facet joint degeneration and the highest pain scores in the upper back of patients with right thoracic curves. Additionally, Cavanaugh et al. demonstrated that the activation of nociceptors in the ligamentous capsule is caused in response to excessive stretch. [147] Because torsional movement produces most of the facet joint capsular stretch, this could explain the association between pain and rotational instability in the upper thoracic region of our cohort. Teles et al. reported differing pain locations in relation to the Lenke curve type, suggesting that the painful intervertebral segments follow the most rotationally unstable spinal level. [148] However, this does not prove that the painful stimuli originate from facet joints. Studies have shown that non-physiological loading of facet tissues produces pain and neuronal hyperexcitability. [149] The nociceptive stimuli are thought to originate partly from neuro-inflammation due to the 'leaking' of pro-inflammatory mediators from the osteoarthritic cartilage to the nearby dorsal root ganglion. NGF, a potent growth factor, is linked to the development of nociceptors in painful and degenerating facet joints. [150] Because TLR and NF-κB pathways are heavily involved in producing NGF and inflammatory cytokines in chondrocytes, these targets could also alleviate back pain in AIS. In support, Krock et al. successfully ameliorated the painful behaviour of SPARC-null mice using a TLR4 antagonist (TAK242). [151] In a clinical setting, using small-molecule inhibitors would be most compatible with the concept of localized facet joint injection for a novel treatment approach targeting TLRs. The ubiquitous nature of TLRs in countless cell programs and their importance for innate immunity presents an argument against systemic treatment. Using two widely available compounds targeting the TLR pathway (o-Vanillin and Sparstolonin B), we demonstrated the ability to blunt the production of inflammatory and proteolytic mediators from chondrocytes in the

presence of alarmins. [105] Nonetheless, many hurdles are left to prove the effectiveness of TLR inhibition as a disease-modifying target to prevent tissue degradation and pain in AIS.

Conclusion

In conclusion, this study has revealed the presence of facet joint osteoarthritis in the context of adolescent idiopathic scoliosis. More specifically, the asymmetrical degeneration of facet joints is related to axial intervertebral deformity and is highest in kyphotic segments. These results suggest the scoliotic spine is a victim of rotational instability and therefore could explain the asymmetrical forces at the core of AIS progression. TLR inhibition seems like a promising target for a disease-modifying treatment approach to treat facet joint osteoarthritis in the optic of regaining spinal stability to prevent scoliotic curve progression.

Future Directions

Listed below are questions remaining to be answered stemming from the work in this thesis.

1. *Which biomechanical forces are contributing most to facet joint degeneration in AIS?*

   To fully answer this question, a finite element model using CT imaging should be constructed with representative mechanical properties of the tissues in the AIS spine. By inputting the patient weight and gravity, the facet forces could be computed in all trunk movements to understand which movement and attributed facet forces drive the degeneration of the joint.

2. *Could facet joint degeneration become a marker to evaluate the risk of progression?*

   Since our analysis is restricted to patients who need surgical intervention to correct the deformity, we could not evaluate facet joint degeneration in patients whose spinal curves did not progress as much. However, CT imaging could present a non-invasive research avenue to

explore the state of facet joints in non-progressive cases. Furthermore, a longitudinal study of spinal deformity and biomechanics on progressive and non-progressive curves could reveal initial markers to predict AIS progression better.

3. *Investigate the role of TLRs on bone and cartilage cross-talk.*

TLRs are notoriously promiscuous in both ligand preference and downstream targets. Therefore, the complex message transferred between TLR-activated chondrocytes and bone cells regulating turnover needs to be better understood. These findings could help decipher the different states of bone remodelling during osteoarthritis.

4. *Can blocking TLRs prevent facet joint degeneration?*

The next step in answering this question would be to test TLR inhibitors on animal models of facet joint degeneration. For example, facet joint osteoarthritis in rats can be induced with monosodium iodoacetate or by surgical restriction of an intervertebral segment. Then, the state of the facet joints could be monitored after localized injection of TLR inhibitors or vehicle controls.

5. *Can preventing facet joint degeneration slow down AIS progression?*

An animal model, such as the pinealized chicken with an upright spine developing spontaneous scoliosis due to osteopenia, could be administered with TLR antagonists to study this effect.

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Appendices

Related Works

During my Ph.D. study, I contributed to 5 manuscripts in the field of Intervertebral disc degeneration and pain as coauthor:

Rosenzweig D, Gravel JT, **Bisson D**, Ouellet J, Weber M, et al. Comparative analysis in continuous expansion of bovine and human primary nucleus pulposus cells for tissue repair applications. European Cells Mater. 2017;33:240–51.

Krock E, Rosenzweig DH, Currie JB, **Bisson DG**, Ouellet JA, Haglund L. Toll-like Receptor Activation Induces Degeneration of Human Intervertebral Discs. Sci Rep-UK. 2017;7(1):17184.

Cherif H, **Bisson DG**, Jarzem P, Weber M, Ouellet JA, Haglund L. Curcumin and o-Vanillin Exhibit Evidence of Senolytic Activity in Human IVD Cells In Vitro. J Clin Medicine [Internet]. 2019;8(4):433. Available from: https://www.ncbi.nlm.nih.gov/pubmed/30934902

Cherif H, **Bisson DG**, Mannarino M, Rabau O, Ouellet JA, Haglund L. Senotherapeutic drugs for human intervertebral disc degeneration and low back pain. Elife [Internet]. 2020;9:e54693. Available from: https://www.ncbi.nlm.nih.gov/pubmed/32821059

Beaulieu-Laroche L, Christin M, Donoghue A, Agosti F, Yousefpour N, Petitjean H, …, **DG Bisson** et al. TACAN Is an Ion Channel Involved in Sensing Mechanical Pain. Cell. 2020;180(5):956-967.e17.

Furthermore, I authored a review on Toll-like receptors and intervertebral disc degeneration:

Bisson DG, Mannarino M, Racine R, Haglund L. For whom the disc tolls: intervertebral disc degeneration, back pain and toll-like receptors. European Cells Mater. 2021;41:355–69.

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Date	01/01/1977
Language	English
Country	United States of America
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