Tomographic and Histological Analyses of Ectopic Calcification Associated With Diffuse Idiopathic Skeletal Hyperostosis (DISH)

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Abstract

Diffuse idiopathic skeletal hyperostosis (DISH) is a non-inflammatory spondyloarthropathy characterised by the formation of ectopic calcifications of the ligaments/enthuses along the anterolateral aspect of the vertebral column. Despite affecting 15-25% of North Americans over the age of 50, the aetiology of DISH is poorly understood and there are no disease modifying treatments. Our discovery-based approach implemented a transdisciplinary research team to study the basic biology, imaging science, clinical anatomy and significance of DISH in humans. Our novel characterisation defined distinct morphological presentations of ectopic calcifications associated with DISH in cadaveric specimens using micro-computed tomography imaging. Subsequent histopathological analyses demonstrated that flowing bridges characteristic of DISH are composed of both ectopic ossifications and discrete regions of amorphous calcifications within fibrocartilage structures. These findings provide newfound insight into the pathogenesis of DISH and that the current diagnostic criteria captures a heterogenous population of presentations, or distinct pathologies that must be further defined.

Keywords

Diffuse idiopathic skeletal hyperostosis (DISH), spine[vertebral column], calcification[ossification], micro-computed tomography, histology, human[cadaver].
Co-Authorship statement

Chapter 2 was drafted by Dale E. Fournier and co-authored by Chris JD. Norley, Steven I. Pollmann, Dr. Christopher S. Bailey, Dr. Fahad Al Helal, Dr. Katherine E. Willmore, Dr. David W. Holdsworth, Dr. S. Jeffrey Dixon, and Dr. Cheryle A. Séguin (senior author). This study was prepared for submission to Journal of Bone and Mineral Research. Chris JD. Norley assisted with µCT processing which included outlining the workflow to stitch and rescale full volumes. Steven I. Pollmann outlined the computational workflow to generate digitally reconstructed radiographs. Drs. Christopher S. Bailey and Fahad Al Helal shared their clinical expertise and diagnosed the specimens. Drs. Katherine E. Willmore, David W. Holdsworth, S. Jeffrey Dixon and Cheryle A. Séguin provided insight into the study design and the interpretation of results. All other experiments and data analyses were performed by Dale E. Fournier in the laboratory of Dr. Cheryle A. Séguin. The manuscript was written by Dale E. Fournier with suggestions from Drs. Katherine E. Willmore, David W. Holdsworth, S. Jeffrey Dixon, and Cheryle A. Séguin. All authors were involved in the critical revision of the manuscript content and approved the final version to be submitted.

Chapter 3 was drafted by Dale E. Fournier and co-authored by Dr. Patti K. Kiser, Ryan J. Beach, Dr. Katherine E. Willmore, Dr. David W. Holdsworth, Dr. S. Jeffrey Dixon, and Dr. Cheryle A. Séguin (senior author). This study was prepared for submission to Annals of the Rheumatic Diseases. Dr. Patti K. Kiser provided a pathologist’s report of the histopathological features of ectopic calcifications/ossifications associated with DISH. Ryan J. Beach assisted in the troubleshooting of tissue techniques and operated the diffractometer during the XRD analysis. Drs. Patti K. Kiser, S. Jeffrey Dixon, and Cheryle A. Séguin provided insight into the study design and the interpretation of results. All other experiments
and data analyses were performed by Dale E. Fournier in the laboratory of Dr. Cheryle A. Séguin. The manuscript was written by Dale E. Fournier with suggestions from Dr. Cheryle A. Séguin and Katherine E. Willmore. All authors were involved in the critical revision of the manuscript content and approved the final version to be submitted.
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Third, I wish to acknowledge the past and current lab members of the Séguin lab, whom I have been fortunate to connect, learn, and grow with; and, I am proud to call friends.

And lastly, without doubt, my father, mother, and brother.

I am hopeful my gratitude has been previously expressed and feel a few written words will not do justice, so I have selected to list those associated with the present thesis:

<table>
<thead>
<tr>
<th>Séguin lab members</th>
<th>Committee members</th>
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<tbody>
<tr>
<td>Courtney Brooks</td>
<td>Dr. S. Jeffrey Dixon</td>
<td>Dr. Marjorie Johnson</td>
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<td>Geoffrey Kerr</td>
<td>Dr. David W. Holdsworth</td>
<td>Haley Linklater</td>
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<td>Mark Kim</td>
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<td>Matthew Veras</td>
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Dr. Fahad Al-Helal, Dr. Christopher Bailey, Ryan Beach, Dr. Stephen Ferrier, Dr. Roberta Flemming, Dr. Yara Hosein, Linda Jackson, Dr. Patti Kiser, Chris Norley, Caroline O’Neil, Dr. Michael Pest, Steven Pollmann, Dr. Amin Rizkalla, Alex Rupert, Dr. Todd Simpson, Dr. Joseph Umoh, and the Department of Pathology at University Hospital, London Health Sciences Centre.
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<tr>
<td>µCT</td>
<td>micro-computed tomography</td>
</tr>
<tr>
<td>BMP2</td>
<td>bone morphogenic protein</td>
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<td>DISH</td>
<td>diffuse idiopathic skeletal hyperostosis</td>
</tr>
<tr>
<td>DRR</td>
<td>digitally reconstructed radiograph</td>
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<tr>
<td>ENT1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>equilibrative nucleoside transporter 1 null</td>
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<tr>
<td>EDX</td>
<td>energy dispersive x-ray spectroscopy</td>
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<tr>
<td>HU</td>
<td>Hounsfield Units</td>
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<tr>
<td>IVD</td>
<td>intervertebral disc</td>
</tr>
<tr>
<td>MIP</td>
<td>maximum intensity projection</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of active B cells</td>
</tr>
<tr>
<td>OPLL</td>
<td>ossification of the posterior longitudinal ligament</td>
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<tr>
<td>PGI2</td>
<td>prostaglandin I2</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>C or T#</td>
<td>cervical or thoracic #</td>
</tr>
<tr>
<td>XRD</td>
<td>x-ray diffraction</td>
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Chapter 1 General introduction

The present chapter is an introduction to the anatomy of the human vertebral column and spinal pathologies associated with ectopic calcification and/or ossification. Inherent preference is given to studies related to humans as opposed to animal models.

1.1 Anatomy of the human vertebral column

The vertebral column composes the majority of the adult human axial skeleton via the interaction of 24 presacral vertebrae (Figure 1.1A) which are interspersed by 23 intervertebral discs (IVDs), divided into three anatomical regions: cervical (7 vertebrae and 6 IVDs), thoracic (12 vertebrae and IVDs), and lumbar (5 vertebrae and IVDs)\(^\text{(1)}\).

The basic functional unit of the vertebral column is a motion segment, defined as a single isolated IVD, the adjacent superior and inferior vertebral bodies, and the associated connective tissues connecting the vertebrae (e.g. muscles and ligaments - Figure 1.1A).

1.1.1 Osseous features of the vertebral column

The osseous features of the vertebrae are generally preserved across the different anatomical regions – spinous and transverse processes, pedicles, laminas, superior and inferior articulating facets, vertebral bodies, and numerous foramen for nerves (Figure 1A)\(^\text{(1)}\). The morphology of the osseous features differs slightly in each anatomical region, including differences in overall size/mass (lumbar > thoracic > cervical), orientation of the articulating facets (to facilitate diverse spinal ranges of motion), and the projection angle of the spinous process (lumbar = parallel to the transverse plane, thoracic = oblique superior-inferior angle)\(^\text{(1)}\). These differences contribute to the biomechanical stability of the vertebral column and facilitate the multi-directional movements associated with its
Figure 1.1. Anatomical rendering of the vertebral column.

(A) Superior view of an individual vertebrae with the significant bony landmarks labelled. Note: the counter “inferior articular process” is not visible in this schematic. (B) Median sagittal view of a thoracic region, labelled are the relevant connective tissues. Further identified are the specific tissues of the intervertebral disc. Orientation is presented for each Panel via axis legend.
1.1.2 Connective tissues of the vertebral column

Connective tissues (e.g. ligaments and general connective tissues) and muscles connect each individual motion segment in the vertebral column via numerous different structures. The posterior aspect of the vertebral column is heavily supported by muscolotendinous and ligamentous attachments (Figure 1.1B). The major role of the musculature is to facilitate the large, gross movements of the torso while also supporting the axial skeleton during peripheral movements\(^2\). The ligamentous structures connect the adjacent osseous features (e.g. anterior longitudinal ligament interfaces with the anterior waist of each superior and inferior vertebral body), while also restricting injurious extremes in range of spinal motion. Along the anterior and posterior aspect of the vertebral bodies are broad ligaments that cover the outer surface of the vertebral bodies and IVDs termed the anterior and posterior longitudinal ligaments, respectively (Figure 1B\(^1\)). The anterior longitudinal ligaments extend from the first cervical vertebrae to the sacrum, becoming more thick and narrow within the thoracic region and more wide spanning in the cervical and lumbar regions\(^3\). These ligaments are anchored to the mid-waist of the vertebral body and adhere to the outer layers of the annulus fibrosus as they cross the IVD\(^3\). The anterior longitudinal ligaments are the only ligamentous structure along the anterior aspect of the vertebral column and they prevent extremes in spinal hyperextension, while the posterior longitudinal ligaments limit the amount of vertebral column hyperflexion\(^1\). The posterior longitudinal ligaments also extend from the first cervical vertebrae to the sacrum, but reside within the spinal canal\(^3\). The posterior longitudinal ligaments attach to the margins of the vertebral bodies as well as the
posterior aspect of the IVD\(^{(3)}\). In the cervical and superior thoracic regions, the posterior longitudinal ligaments are broad and uniform in width; while, in the inferior thoracic and lumbar regions they become narrow over the vertebral bodies and wide over the IVD\(^{(3)}\).

1.1.3 Intervertebral discs

IVDs are complex fibrocartilaginous joint structures that are interspersed between each vertebrae. Biomechanically, IVDs serve to stabilize the vertebral column, provide flexibility, and bear load during axial compression. The IVDs are composed of three distinct, but interacting tissue types: the annulus fibrosus, nucleus pulposus, and cartilage endplates (Figure 1.1B).

The annulus fibrosus forms the periphery of the IVD and is identified by the organisation of concentric bundles of type 1 collagen in consecutive lamellae\(^{(4)}\). In humans, the annulus fibrosus are arranged into roughly 20-25 concentric alternating rings that are oriented at oblique angles from one another to form an angle-ply structure\(^{(5,6)}\). Biomechanically, this arrangement serves to resist tensile strain experienced during bending, twisting, as well as during compressive loading of the IVD\(^{(5,6)}\). The AF interacts with the adjacent vertebrae via elastic and Sharpey’s fibers that anchor the IVD to the bone\(^{(7,8)}\). The AF is populated by elongated cells that morphologically resemble fibroblasts\(^{(9)}\), formed by the somatic mesenchyme during development. The AF is a fibrocartilaginous tissue with significant variations in its extracellular matrix composition depending on the location (i.e. inner versus outer layers)\(^{(10)}\). In a healthy IVD, the inner AF serves as a transition from the outer fibrous AF to the gelatinous NP, through a decreased abundance of type 1 collagen and a greater type II collagen and proteoglycan content, the latter localized to the interlamellar matrix\(^{(10)}\).
The AF surrounds the inner gelatinous nucleus pulposus, which is composed primarily of proteoglycans and water within an irregular network of type II collagen and elastin fibres\(^7\). The biomechanical function of the NP is to resist compressive loads placed on the IVD. The NP is an avascular tissue containing a heterogeneous population of cells formed by large vacuolated notochordal cells, that are gradually replaced by rounded chondrocyte-like cells over time\(^9\).

Lastly, the cartilage endplates are thin layers of hyaline cartilage that attach the IVD to the superior and inferior vertebral bodies. The cell type of the CEP are chondrocytes, secreting a type II collagen and proteoglycan rich extracellular matrix\(^7,9\). The CEPs serve an essential function in nutrient exchange for the IVD, allowing transport of nutrients from the vascularized bone to the avascular NP via passive diffusion\(^11\).

### 1.2 Pathologies of ectopic spinal calcification

With increased age, it is common for the connective tissues of the vertebral column to calcify and stiffen; although, the underlying mechanisms are not well known.

#### 1.2.1 Axial spondyloarthopathies

Axial spondyloarthritis is an umbrella term that captures a collection of spinal pathologies including psoriatic and reactive arthritis, ankylosing spondylitis (AS), and undifferentiated spondyloarthritis (i.e. non-radiographic and/or early stage AS)\(^12\). AS is an inflammatory auto-immune disorder involving the axial skeleton, characterised by sclerosis and/or fusion of the sacroiliac and facets joints\(^13\). Changes to the IVD occur at the discovertebral junctions (i.e. attachment of annulus fibrosus to vertebral body) and are detected radiographically as “Romanus lesions,” which are sclerotic regions confined to
the anterior edges (both superior and inferior) of the vertebral bodies\(^{13}\). The formation of sclerotic regions ultimately leads to a reactive pathological healing response that results in the ossification of the periphery of the annulus fibrosus\(^{13}\). Symptoms associated with AS include inflammatory back pain and/or stiffness that occurs during late adolescence and/or early adulthood\(^{13}\). AS has been strongly associated with human leukocyte antigen and familial heredity of the disease is common\(^{14,15}\).

1.2.2 Vertebral spondylosis

Spondyloses is an umbrella term referring to degeneration of vertebral column structures\(^{16}\). It is the result of natural ageing and deterioration of the connective tissues supporting the vertebral column. The pathological mechanism resulting in osteophyte formation is described as follows: Phase I, micro-trauma or dysfunction of the annulus fibrosus leading to mechanical instability; Phase II, morphological changes to the IVD as a result of the biomechanical changes; and Phase III, as IVD space narrows osteophytes forming from the vertebral bone unite and bridge\(^{16}\). Typically, these osteoarthritic changes to the vertebral column often occur in the elderly population (but in cases of trauma can occur in younger aged groups) and is characterised by facet joint space narrowing, vertebral body subchondral sclerosis, and osteophyte formation. Generally, the lumbar spine is the initial site of vertebral spondylosis and symptoms of lower back pain are prominent due to IVD space narrowing\(^{16}\).

1.2.3 Ossification of the posterior longitudinal ligament

Ossification of the posterior longitudinal ligament (OPLL) is defined by pathological bone formation occurring within the posterior longitudinal ligament. OPLL is more common within Japanese populations and preferentially affects the cervical region of the
spine\(^{(17,18)}\). A major complication associated with ectopic bone formation in OPLL is the compression of the spinal cord and/or nerve roots travelling through the vertebral foramen\(^{(19,20)}\). Genetic and environmental factors have been associated with increased incidences of OPLL, including familial inheritance of mutations in the genes encoding \textit{BMP4}, \textit{BMP9}, and \textit{COL6A1}\(^{(21,22)}\). A genome wide association study identified six loci to be more frequent in OPLL patients compared to controls: \textit{HAO1}, \textit{RSPO2}, and \textit{CCDC91} which may promote endochondral ossification, as well as \textit{RSPH9} and \textit{STK38L} which may promote intramembranous ossification\(^{(23,24)}\).

1.2.4 Diffuse idiopathic skeletal hyperostosis

Diffuse idiopathic skeletal hyperostosis (DISH) is a non-inflammatory spondyloarthropathy characterised by the formation of ectopic calcifications along the anterolateral aspect of the vertebral column (\textbf{Figure 1.2}). Ectopic calcifications in DISH are thought to occur due to ossification of the anterior longitudinal ligament, but the underlying aetiology of the disease is unknown\(^{(25)}\). Although the calcifications associated with DISH appear analogous to those seen in OPLL, the genetic relationships identified with OPLL have not been found with DISH\(^{(26)}\). DISH often co-exists with other spinal and systemic pathologies but is recognized as a distinct clinical entity\(^{(27)}\).

1.3 Overview of diffuse idiopathic skeletal hyperostosis

The first systematic description of DISH was published in 1950 by Dr. Jacques Forestier, the report outlined clinical, radiological and pathological criteria of the pathology termed “senile ankylosing hyperostosis of the spine” (later renamed Forestier’s disease and subsequently DISH)\(^{(28)}\). The fundamental characteristics of the disease were detailed
Figure 1.2. Stylised comparison of a healthy vertebral column compared to DISH.

Thoracic regions from segments 8 through 12 are presented from (A) a 73-year-old male without ectopic calcifications associated with DISH; and (B) an 87-year-old male identified with ectopic calcification associated with DISH (further displayed via the red colouring). Note the extremely large outgrowths and their implications with surrounding anatomy, the contiguous flowing connection with adjacent vertebral bodies, and the classically described “right sidedness” (i.e. opposing the aorta that travels along the left side of the vertebral column – in most cases).
as bony outgrowths along the anterior aspect of the vertebral column, projecting upwards from inferior to superior vertebral segments (resembling the appearance of a “candle-flame”) and affecting the elderly population\textsuperscript{(28)}. The bony structures were characterized as having a dense cortex, often co-existing with osteophytes but having entirely different anatomical features\textsuperscript{(28)}.

Although first described in detail in 1950, ectopic calcifications of the vertebral column characteristic of DISH have been documented throughout the historical record via paleo-archeological studies\textsuperscript{(29,30)}. It should be noted that in conjunction with the changes to the axial skeleton, the nomenclature of DISH includes the presence of extraspinal calcifications within the appendicular skeleton, most commonly at the Achilles tendon attachment to the calcaneus, patellar ligaments, elbows, and shoulder joints\textsuperscript{(31–33)}. Close to 70 years following the seminal description of this disorder\textsuperscript{(28)}, our understanding of DISH remains limited and DISH is often underdiagnosed or misdiagnosed due to unfamiliarity in clinical practice\textsuperscript{(34,35)}.

1.3.1 Prevalence, clinical presentation, and treatment

The estimated prevalence of DISH in North America is 15\% of females and 25\% of males over the age of 50\textsuperscript{(36)}. DISH has been reported globally, with prevalence ranging from 0.8\% to 42.0\% of the population depending on the geographic region (Table 1.1). The likelihood of DISH increases in all populations per decade of age beyond 50 years\textsuperscript{(37–39)}, with only few reports diagnosing DISH in patients under 40 years of age\textsuperscript{(40–43)}. The prevalence of DISH is greater for males compared to females (~2:1) as well as for individuals with metabolic disorders such as obesity, diabetes mellitus, high waist circumference, hypertension, hyperinsulinemia, dyslipidemia, and hyperuricemia\textsuperscript{(38,44–48)}. 
Table 1.1. Summary of reports on the prevalence of DISH.

Tabulated overview of the literature on the prevalence of DISH from 1971 to 2017. The authors and year of publication are presented with the geographical region and patient populations which the studies were conducted. The total number of individuals in the respective studies are presented along with the prevalence of DISH, expressed as a percentage and segmented by sex.

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<td>Outpatient population</td>
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<td>100</td>
</tr>
<tr>
<td>Hofst et al. (2011)</td>
<td>United States</td>
<td>MiOS (osteoporosis)</td>
<td>573</td>
<td>1074</td>
</tr>
<tr>
<td>Kogotani et al. (2014)</td>
<td>Japan</td>
<td>ROAD (prospective OA)</td>
<td>599</td>
<td>529</td>
</tr>
<tr>
<td>Nardo et al. (2014)</td>
<td>United States</td>
<td>General populations</td>
<td>524</td>
<td>1038</td>
</tr>
<tr>
<td>Yoshimura et al. (2014)</td>
<td>Japan</td>
<td>OPLL patients</td>
<td>888</td>
<td>612</td>
</tr>
<tr>
<td>Fujimori et al. (2016)</td>
<td>Japan</td>
<td>Screening survey (cancer)</td>
<td>300</td>
<td>258</td>
</tr>
<tr>
<td>Katsumoto et al. (2017)</td>
<td>New Zealand</td>
<td>Hospital patients (X-ray and CT)</td>
<td>212</td>
<td>247</td>
</tr>
<tr>
<td>Katsman et al. (2017a)</td>
<td>California, USA</td>
<td>Rancho Bernardo (CVD risk)</td>
<td>630</td>
<td>961</td>
</tr>
<tr>
<td>Katsman et al. (2017b)</td>
<td>United States</td>
<td>Osteoporotic groups</td>
<td>1500</td>
<td>1265</td>
</tr>
<tr>
<td>Mori et al. (2017)</td>
<td>Japan</td>
<td>Screening survey (lung diseases)</td>
<td>1752</td>
<td>1261</td>
</tr>
<tr>
<td>Pariante-Rodrigo et al. (2017)</td>
<td>Cantabria, Spain</td>
<td>General population</td>
<td>987</td>
<td>987</td>
</tr>
<tr>
<td>Toyoda et al. (2017)</td>
<td>Japan</td>
<td>Spinal disorders</td>
<td>163</td>
<td>118</td>
</tr>
</tbody>
</table>
The symptomatic profile of DISH is highly variable and usually considered to be mild at disease onset and evolve slowly with disease progression. Symptoms include increased spine stiffness (particularly in the morning, during rainy weather, or after long periods of inactivity) and decreased spinal range of motion with or without back pain, radiculopathy, polyarticular pain, and/or monoarticular synovitis\(^\text{(33,49,50)}\). In advanced stages, ectopic calcifications can interfere with surrounding anatomical structures causing severe complications, such as sleep apnea or airway obstruction\(^\text{(51–54)}\), dysphagia\(^\text{(55–57)}\), dysphonia\(^\text{(58–60)}\), and compression of spinal nerve roots and/or cord\(^\text{(61)}\). DISH is also associated with a greater risk of spinal fracture\(^\text{(62,63)}\) and post-surgical heterotopic ossifications\(^\text{(64–66)}\). Due to our limited understanding of the pathobiology of DISH there are no disease-modifying treatments to delay, stop, or reverse the progression of ectopic calcification\(^\text{(67)}\). The development of pharmaceutical treatments for DISH have been hampered by the lack of suitable mechanistic studies or pre-clinical models to study the disease pathobiology\(^\text{(25,68)}\). Instead, disease management is restricted to conservative approaches centred on symptom relief through physiotherapy, anti-inflammatory medications, muscle relaxants, and control of associated metabolic disorders\(^\text{(67)}\). Surgical interventions are reserved as a last resort for patients with severe complications. Surgical resection of ectopic calcifications is achieved through invasive high-speed burring and piecemeal removal protocols\(^\text{(55,69)}\). Although initially effective, the rates of post-surgical recurrence of ectopic calcifications are extremely high (with or without symptoms) in patients\(^\text{(70)}\).
1.3.2 Diagnostic criteria

The diagnosis of DISH is based exclusively on the detection of ectopic calcifications involving the axial skeleton, using radiographic criteria first developed by Resnick and Niwayama in 1976, as follows: (i) flowing calcifications and/or ossifications along the anterolateral aspect of four contiguous vertebral segments, with or without osteophytes; (ii) preservation of IVD height in involved areas of ectopic calcification (to differentiate from degenerative disc disease pathologies); and (iii) the absence of bony ankylosis of facet joints, sacro-iliac erosion, sclerosis or fusion (to specifically differentiate from ankylosing spondylitis)\(^{(71)}\). It is acknowledged that the current diagnostic criteria are limited to an advanced disease state, and may possess ambiguity in the evaluation of morphological features associated with calcifications, as determined through radiographs\(^{(68,72)}\).

Given limitations associated with assessing the stage of disease, several attempts have been made to expand the diagnostic criteria for DISH. Table 1.2 highlights a subset of specific modifications to the diagnostic criteria. These modifications generally propose an enhanced emphasis on the extent and involvement of extraspinal manifestations\(^{(33)}\), reduced number of contiguous motion segments involved\(^{(33,38,73)}\), and classifying the specific features of bridging, such as the angle of the calcification bridging relative to the vertebrae\(^{(74–76)}\). Moreover, the variability the diagnosis of DISH based on the specific criteria used was demonstrated in a study that evaluated a sample of 253 human remains using four of the different proposed criteria\(^{(77)}\). Depending on the criteria used, the prevalence in this sample population ranged from 5.5% to 17\(^{(77)}\). Despite the proposed modifications, the rudimentary criteria proposed by Resnick and Niwayama in 1976
Table 1.2. Summary of proposed modifications to the diagnostic criteria.

Summary table highlighting a few of the proposed modifications to diagnostic criteria of ectopic calcifications associated with DISH. Resnick and Niwayama’s seminal criteria that remains the gold-standard in clinical practice. Common modifications to the criteria include emphasising the number of motion segments involved in the vertebral column, the health of the associated intervertebral disc at levels of ectopic calcification, the presence and extent of extraspinal manifestations (e.g. Achilles tendon, patellar ligament, olecranon ligaments), and developing criteria to characterise the morphological appearance of ectopic calcifications associated with the vertebral column.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Year</th>
<th>Number of segments</th>
<th>IVD characteristics</th>
<th>Peripheral characteristics</th>
<th>Bridging characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resnick and Niwayama(^{[71]})</td>
<td>1976</td>
<td>4+ contiguous vertebral bodies</td>
<td>Relative preservation of IVD height in involved areas</td>
<td>*not included in criteria</td>
<td>&quot;Flowing&quot; calcification and ossification along anterolateral aspect</td>
</tr>
</tbody>
</table>
| Utsinger\(^{[33]}\)             | 1985 | 2-4 contiguous vertebral bodies | Exclusion when abnormal disc space height in the involved areas | Symmetrical enthesopathy (e.g. posterior heel, patella, or olecranon) | Continuous ossification
  • fine ribbon-like wave
  • broad, bumpy, buttress-like |
| Rogers and Waldron\(^{[73]}\)   | 2001 | 3+ vertebral bodies   | Should not be used for exclusion of DISH       | Evidence of extra-spinal calcification or ossification | Changes to right side of thoracic vertebrae                   |
| Oudkerk et al.\(^{[74]}\)      | 2017 | 3 IVD spaces or 4 vertebral bodies | Exclusion when severe IVD degeneration         | *not included in criteria | Bridging angle to differentiate flowing ossification vs. degenerative osteophyte |
remains the gold-standard in the clinical diagnosis of DISH\textsuperscript{(68,72,78)}. To improve clarity in
the interpretation and reliability of Resnick’s criteria, a study was conducted that further
defined each criterion as: (i) the number of segments involved as bridging across four
vertebral bodies (or three IVD levels); (ii) bridging angles greater than 90° relative to the
vertebrae (<90° defined as an osteophyte); and (iii) inclusion of mild or moderate disc
degeneration (but severe disc degeneration remained an exclusion criteria in the diagnosis
of DISH)\textsuperscript{(74)}. A qualitative systematic review of the proposed diagnostic criteria clearly
demonstrated the need for a standardized criterion that reflects the progression of the
disease as opposed to the current dichotomous outcome associated with the detection of
an advanced disease state (i.e. DISH vs. no-DISH)\textsuperscript{(72)}.

1.3.3 Classification of spinal calcifications associated with DISH

The hallmark radiographic finding of DISH is the presence of ectopic calcifications along
the anterolateral aspect of the vertebral column\textsuperscript{(71)}. Since DISH is considered to develop
slowly over time, care needs to be taken to differentiate potential stages or classifications
of the disease.

Forestier’s follow-up work proposed three stages in the formation of ectopic
calcifications associated with DISH based on radiographic appearance: (i) thickenings
along the anterior aspect of the vertebral bodies; (ii) accentuated vertebral thickenings
which lengthen into a thick spur, resembling the flame of a candle; and (iii) fusion of
bony spurs creating a flow of calcification\textsuperscript{(79)}. A subsequent report using fine detail
radiography investigated potential early stages in the progression of ectopic calcification
associated with DISH\textsuperscript{(80)}. Three distinct stages were proposed: (i) new bone at the
entheses of the anterior longitudinal ligaments (the site of attachment to the vertebral
body) but independent from the IVD at two separate vertebral bodies; (ii) continuous entheseal new bone spanning across two vertebral bodies; and (iii) entheseal new bone that had completely fused with the adjacent vertebral body and spanned across two or more vertebral bodies\(^{(80)}\). Up until this point, initial predictions into the pathogenesis of DISH proposed that ligamentous changes progressed into the characteristic bridging seen in DISH, but excluded were the extraspinal changes associated with DISH\(^{(81)}\). In 1985, Utsinger et al. were first to incorporate the presence of extraspinal manifestations into a criterion to classify patients for epidemiological evaluation of DISH\(^{(33)}\). Patients with “definite DISH” had continuous ossification along the anterolateral aspect of at least four contiguous vertebral bodies\(^{(33)}\), while patients with “probable or possible DISH” had continuous ossification along the anterolateral aspect of at least two contiguous vertebral bodies and/or symmetrical and peripheral enthesopathy\(^{(33)}\). The only other study to consider the involvement of extraspinal manifestations in the classification of DISH included the presence or absence of enthesophytes (i.e. bony projections at the attachment of tendons or ligaments) at principal locations (e.g., shoulders, elbows, hip, knees, ankles)\(^{(75)}\). Importantly, this study evaluated each motion segment independently and classified each as either: (i) no ossification present; (ii) ossification present without bridging; (iii) ossification present with incomplete bridging of the disc space; or (iv) complete bridging of the disc space by ossification\(^{(75)}\).

More recent classifications for DISH have adopted computed-tomography (CT) imaging. In 2014, Yaniv et al. evaluated 26 patients diagnosed with DISH that had undergone consecutive CT examinations at two or more time points over a period of 10 years \(^{(82)}\). The study introduced a semi-quantitative scoring system that assessed bridging
osteophyte formation at individual motion segments using a six point scale ranging from no osteophytes, to anterior longitudinal ligament calcification, to ligament calcification and osteophyte formation\(^{(82)}\). Interestingly, two distinct bridging patterns were observed concomitantly in more than half of the patients assessed, those being: osteophyte fusion associated with calcification of the ALL (identified in 66% of motion segments examined), and osteophyte fusion without ALL calcification (identified in 33% of motion segments examined)\(^{(82)}\). The authors postulated that these patterns may indicate an underlying inflammatory rather than a degenerative pathogenesis\(^{(82)}\). Most recently, in 2018 Kuperus \textit{et. al.} retrospectively examined consecutive chest CTs from the same patients and identified 55 patients without DISH at the first timepoint but with DISH on a subsequent scan, as well as 90 patients with DISH at both timepoints\(^{(83)}\). They focused their scoring on the presence and completeness of a bony bridge spanning the IVD (0=no bridge to 3=completely fused bridge), the fluency or flow of DISH (1=sharp angle to 3=flowing wide angle), and the circumferential extent of DISH formation (via an analog clock overlay in the axial view)\(^{(83)}\). A bone-forming progression from incomplete, pointy bone bridges to more flowing complete bridges was proposed\(^{(83)}\). Collectively, the proposed classification schemes for DISH have focused on the changes associated with the vertebral column but are limited to a qualitative interpretation of morphological differences in calcified structures detectable by imaging. It is evident that ectopic calcifications associated with DISH are slow developing and include intermediate stages that need to be characterized using quantifiable methodologies that can be implemented across disciplines and researchers.
1.3.4 Bone mineral density

The current literature remains ambiguous as to whether vertebral and/or overall bone mineral density increases\(^{(84,85)}\) or decreases\(^{(86–88)}\) in association with DISH. As such, evaluation of patients using bone mineral density scanning may complement the radiographic screening for ectopic spinal calcifications. A recent longitudinal evaluation of bone mineral density using consecutive CT analysis in a cohort of DISH patients over a minimum of 5 years showed that measurements of vertebral body bone mineral density did not differ between individuals with DISH and unaffected controls, while the newly formed ectopic calcifications did increase in density over time\(^{(89)}\).

1.3.5 Spatial distribution of calcification associated with DISH

The distribution of ectopic calcifications associated with DISH varies along the axial skeleton with a predominance in the thoracic, inferior cervical, and superior lumbar spine segments\(^{(17,83,90–92)}\). A unique characteristic associated with DISH is the presence of ectopic calcifications preferentially along the right anterolateral aspect of the vertebral column, opposing the descending thoracic aorta\(^{(83,93)}\). It has been proposed that the pulsating action of the aorta may act to prevent and/or shape the formation of ectopic calcification\(^{(93)}\). Supporting this hypothesis are case reports of individuals with *situs inversus* (i.e. reversed organization of internal anatomy resulting in the aorta descending along the right side of the vertebral column) presenting with ectopic calcifications on the left anterolateral aspect of the vertebral column (i.e. opposing the aorta)\(^{(94–97)}\).

1.3.6 Histological features of DISH

The ectopic calcifications associated with DISH are thought to arise from the anterior longitudinal ligaments, annulus fibrosus, and surrounding fibrocartilaginous structures
such as the enthuses (Figure 1B). Early studies by Resnick et. al. described two types of DISH that were differentiated by the affected tissue(s): Type I was defined by ligamentous changes characterised by islands of metaplastic cartilage deposited within the fibres of the anterior longitudinal ligament; and Type II was associated with alterations to the IVD, particularly extensions of the annulus fibrosus, leading to traction changes and periosteal new bone formation\textsuperscript{(81)}. Similarly, another study identified differently sized “syndesmophytes” (i.e. bony outgrowth inside a ligament) with features of bone thickening and extensions of annulus fibrosus material between the encroaching bridging segments\textsuperscript{(98)}. Also noted were vascular invasions of the lateral edge of the annulus fibrosus, discrete islands of bone within the lateral fibrocartilage extensions, and a normal IVD thickness\textsuperscript{(98)}. An early histopathological evaluation of ectopic calcifications associated with DISH was conducted on fragments of tissue retrieved during surgery to relieve complaints of dysphagia and dysphonia in a patient with DISH\textsuperscript{(99)}. This analysis demonstrated that the DISH-tissue contained a thin cortex structure with osteons haphazardly arranged and intramedullary islands of metaplastic hyaline cartilage\textsuperscript{(99)}. However, a limitation of this report is that tissue retrieved from surgery is extensively fragmented, preventing accurate anatomical orientation. Thus, a recent pilot study was conducted that involved the histological evaluation of 10 cadaveric spines identified with DISH, and scored: (i) the presence of a bone bridge (complete, partial, none); (ii) the type of bone (>75% cancellous, mixed cancellous and cortical bone, >75% cortical bone); (iii) the presence of woven bone (yes or no); and (iv) the shape of the IVD (regular, tapered, spatulate, irregular)\textsuperscript{(100)}. Based on the morphological retention of IVD height in motion segments from DISH specimens and unaffected controls, it was proposed that the IVD
serves a limited (or non-existent) role in the pathogenesis of ectopic calcifications associated with DISH\(^\text{100}\). Finally, a recent study used cryomacrotome sectioning of four cadaveric specimens with DISH to examine changes to the anterior longitudinal ligament\(^\text{101}\). From their analysis, the authors concluded that the anterior longitudinal ligaments are displaced by the formation of ectopic calcification rather than itself being ossified, suggesting the pathogenesis of DISH is not related to the ossification of the anterior longitudinal ligament\(^\text{101}\).

Collectively, initial insight into the pathobiology of DISH can be summarised by two potential mechanisms: (i) the involvement of the anterior longitudinal ligament in the formation of ectopic calcifications; and/or (ii) the role of the IVD (particularly the annulus fibrosus) in the development of ectopic calcifications. Clearly, further investigation is warranted to elucidate the tissue-specific changes associated with ectopic calcification of the vertebral column and to identify potential mechanistic targets for disease-modifying therapeutics.

1.4 Overview of thesis

1.4.1 Rationale for current studies

A comprehensive review of the literature reveals a high prevalence of DISH in the general population (15-25% of North Americans over the age of 50) but a remarkably limited knowledge of its aetiology, progression, and pathobiology; which, has lead to a lack of disease-modifying treatments. The limitations associated with the current radiographic criteria for the diagnosis of DISH suggests an inability to detect or classify early disease. Therefore, the goal of the present thesis is to provide a novel
characterization of ectopic calcification associated with DISH using advanced medical imaging and histological analyses of cadaveric tissues.

1.4.2 Aims and hypotheses
The proposed research is discovery driven and was designed to better understand and characterise the pathological features associated with ectopic calcification of the vertebral column in humans. The overall goal is to evaluate the morphological and histopathological features of ectopic calcification associated with DISH in a human cadaveric population.

Aim 1
The radiographic criteria developed by Resnick in 1976 remains the most clinically accepted diagnostic tool for DISH. However, many limitations have been highlighted with respects to this criteria, including: diagnosis restricted to late-stage disease, the inability to classify, stage, or detect early disease, and the ambiguity in the interpretation of each criterion\(^{(72)}\). Given that these criteria were developed using plain film radiographs, modern imaging modalities should provide newfound insight into unique characteristics of the disease. Thereby the first aim of the present thesis is to re-evaluate the current radiographic criteria using advanced micro-computed tomography imaging to assess unique features of ectopic calcification associated with DISH. We hypothesize that a detailed investigation of specimens using \(\mu\)CT will reveal distinct subsets of pathological presentations associated with DISH, differentiated by the morphology and density of pathological ectopic bridges, as well as the anatomical structures affected.

Aim 2
To date, few studies have furthered the understanding of the pathobiology underlying the formation of ectopic calcification of the human vertebral column. These limitations are primarily associated with tissue availability, since DISH is often diagnosed secondarily or remains undetected throughout adult life and is not revealed until autopsy. Moreover, tissues resected from surgery eliminate anatomical orientation and/or preservation of adjacent tissue structure that may have relevance to understanding pathogenesis. Therefore, the second aim of the present thesis is to conduct a thorough histological and structural analyses of human cadaveric samples to characterize the tissue-specific changes associated with ectopic calcification of the vertebral column. We hypothesize that ectopic calcifications meeting the diagnostic criteria for DISH will be localized to the fibrocartilaginous tissues of the vertebral column, including the annulus fibrosus and enthuses.
1.5 References


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Chapter 2  Ectopic spinal calcification associated with diffuse idiopathic skeletal hyperostosis (DISH): A quantitative µCT analysis

In the present chapter, a novel application of µCT was implemented with intact human cadaveric vertebral columns. This chapter has been written for submission to the peer-reviewed Journal of Bone and Mineral Research.

2.1 Abstract

Diffuse idiopathic skeletal hyperostosis (DISH) is a non-inflammatory spondyloarthropathy identified radiographically by calcifications and/or ossifications along the anterolateral aspect of the vertebral column. The aetiology and pathogenesis of ectopic calcifications are unknown, and the diagnosis of DISH is based solely on radiographic criteria associated with advanced disease. To further characterise the features of ectopic calcification associated with DISH, the present study used micro-computed tomography imaging to quantitatively evaluate a cohort of 19 human cadaveric vertebral columns. Fifty-three percent of the cohort examined (n=10; 3 females, 7 males, mean age of death = 81 years, range 67-94) met the current radiographic criteria for DISH, with ectopic calcification of four or more contiguous vertebral segments. In almost all cases, the lower thoracic region (T8-12) were affected by calcifications/ossifications, primarily characterised as large, horizontal outgrowths of bony material. In contrast, ectopic calcifications localised to the upper thoracic regions demonstrated variability in their presentation, categorised as either “continuous vertical bands” or “discontinuous-patchy” lesions. In addition to the variable morphology of the ectopic calcifications, our analysis demonstrated remarkable heterogeneity of the properties of ectopic calcification, which displayed tissue densities resembling cortical
bone as well as hyper-dense material. These findings suggest that the current radiographic criteria for DISH capture heterogeneous presentations of ectopic calcifications that can be differentiated based on the morphology and density of ectopic calcifications. These findings may indicate a naturally heterogenous disease, potential stage(s) in the natural progression of DISH, or distinct pathologies of ectopic calcification.

**Keywords** (5): diffuse idiopathic skeletal hyperostosis, micro-computed tomography, intervertebral disc, spine, ectopic calcification

### 2.2 Introduction

Diffuse idiopathic skeletal hyperostosis (DISH) is a non-inflammatory spondyloarthropathy primarily characterized by ectopic calcification and/or ossification along the anterolateral aspects of the vertebral column\(^1\). DISH also involves ectopic calcification in the entheses of extra-spinal tendons and ligaments (e.g. olecranon ligaments, patellar ligament, and Achilles tendon attachment to the calcaneus)\(^2,3\). DISH often manifests as back pain and stiffness\(^4-6\); however, surrounding anatomical structures can be affected leading to dysphagia\(^7\), airway obstruction\(^8\), or compression of the spinal cord and nerve roots\(^9,10\). The ectopic calcifications associated with DISH are also associated with an increased risk of spinal fractures\(^11\) as well as postsurgical heterotopic ossifications\(^12\).

The pathology of DISH is relatively unknown and, as such, the aetiology of the disease is unknown. Consequently, there are no disease-modifying treatments for DISH so clinical interventions are largely focused on symptom relief\(^13\) with severe cases managed
through surgical resection of problematic tissues\cite{7}. Systemic metabolic changes related to obesity, diabetes mellitus, high waist circumference, hypertension, hyperinsulinemia, dyslipidemia, and hyperuricemia have been associated with DISH\cite{14-18}. The reported prevalence of DISH is 15-25\% of North Americans over the age of 50, with a greater prevalence per decade of age beyond 50 years\cite{19} and for males compared to females (~2:1 ratio)\cite{20}. It has been postulated that the reported prevalence of DISH is likely an under-representation due to the variable and vague symptoms, the rise in suspected risk factors (e.g. advanced age and metabolic disorders), and the lack of diagnostic criteria enabling early disease detection\cite{21,22}.

Currently, DISH is diagnosed through radiographic detection of calcified outgrowths along the vertebral column, based on criteria proposed by Resnick and Niwayama in 1976: (i) flowing calcifications and/or ossifications along the anterolateral aspect of at least 4 contiguous vertebral bodies, with or without osteophytes; (ii) preservation of intervertebral disc (IVD) height in the affected areas (vs degenerative disc disease); and (iii) absence of bony ankylosis of facet joints, sacroiliac erosion, sclerosis or fusion (vs ankylosing spondylitis)\cite{23}. These criteria differentiate DISH from other vertebral column pathologies but are limited to the detection of DISH at advanced stages and do not classify progression of the disease\cite{22}. To further classify DISH, modifications to the radiographic criteria have been proposed that consider the extent of extra-spinal involvement\cite{5}, the number of contiguous vertebral segments affected\cite{4,5}, defining characteristics of ectopic calcification bridging angle\cite{24-26}, and schemes for staging or scoring of disease progression\cite{5,25,27-29}. Nonetheless, the defining features of flowing calcifications/ossifications remain the basis for clinical diagnosis of DISH\cite{22,30}.
Current knowledge of the pathogenesis, cell biology and clinical manifestations of DISH is limited due to access to human tissues. As such, previous work has detailed the radiography and computed tomography features of ectopic calcifications/ossifications associated with DISH, but DISH is considered to be under-diagnosed and/or misdiagnosed\(^{(31)}\). Although the radiographic hallmarks of DISH are defined, we postulate that the current radiographic criteria capture a heterogeneous population that can be further classified based on the specific spinal tissues affected and the properties of ectopic calcifications. The aim of this study was to provide a detailed characterization of ectopic calcification in human vertebral columns associated with DISH, using advanced micro-computed tomography imaging techniques.

2.3 Materials and methods

2.3.1 Specimens and dissection

This study included a random cohort of 19 embalmed human cadavers (6 females, 13 males; mean age at death = 81 years, range 65-94) from the Haase Education in Anatomy & Research Technologies Lab at The University of Western Ontario (London, Canada). All data were obtained in accordance with the Anatomy Act of Ontario and Western’s Committee for Cadaveric Use in Research (REF# 22062016 - Appendix A). Where possible, intact vertebral columns (cervical to thoracic) were dissected by sagittal cuts along the ribs (3-5 cm lateral to the vertebrae) and complete resection inferior to the ribcage (Figure 2.1A). A subset within this cohort were previously dissected and therefore did not have segments of the cervical/upper thoracic region \(n=4\) or had laminectomies performed \(n=5\) as part of our institution’s medical curriculum.
Figure 2.1. Sample cohort and characteristics.

(A) Thick maximum intensity projections (MIPs) corresponding to 10.8 ± 0.54 mm mid-sagittal sections (representing the internal third of the vertebral body) of the 19 cadaveric vertebral columns included in the study cohort, grouped by sex (left panel - males, n=13; and right panel - females, n = 6). (B) Characteristic data and diagnostic results of cohort determined by blinded assessment of the micro-computed tomography and digitally reconstructed radiograph images using Resnick diagnostic criteria, denoted by the white asterisks. Scale bar represents 5 cm.
Specimens with signs of previous spinal surgery (e.g. cervical fusion) were excluded from our study since donor medical history was not available.

2.3.2 Micro-computed tomography (µCT)
Following dissection, specimens were scanned using a GE Locus eXplore Ultra X-ray conebeam µCT imaging system (GE Medical Systems, London, Canada) at a peak volatage of 80 kVp and tube current of 50 mA. Image acquisition consisted of 1000 X-ray projections, 16 ms in duration, and acquired over a full 360-degree rotation of the gantry around the specimen. To capture the entire length of specimens, the scanning bed was moved in 86 mm increments between scans to accommodate the scanner’s available axial field of view. The individual scan acquisitions were corrected for beam hardening and back projected using a Feldkamp filtered-back projection algorithm\(^{(32)}\). The reconstructed volumes were then cropped and stitched into a single 3-dimensional volume with an isotropic voxel spacing of 154 µm\(^3\). Each specimen was scanned with an internal calibrator consisting of water, air, and a bone-equivalent 450-SB3 phantom with a known density of 1073 mg/cm\(^3\) (58% volume x 1.8247 g/cm\(^3\); Gammex Inc. Middleton, WI, USA)\(^{(33)}\). The final volumes were scaled in Hounsfield Units (HU) using the average grayscale values of air and water.

Prior to analyses, the image volumes were oriented into anatomical position, interpolated for smooth image quality, and set to a window and level value of 3000 and 900, respectively (MicroView 2.5.0; GE Healthcare Biosciences, Parallax Innovations, 2017). A variety of imaging data were generated, including: maximum intensity projections (MIPs), in-plane sections, and 3-dimensional isosurface renderings. For comparison to computed tomography used in clinical practice, µCT image volumes were rebinned to
non-isotropic voxel sizes (616 µm in-plane, 1077 µm axially) and resized for volume equivalence using a trilinear extrapolation algorithm. Digitally reconstructed radiographs (DRR) were generated using custom software\(^{(34)}\) that simulated clinical parameters (e.g. tube-to-film distance, perspective view, central ray at mid-thoracic)\(^{(35-37)}\). All figure images were exported from MicroView (2.2: GE Healthcare and 2.5.0: Parallax Innovations Inc.) or VG Studiomax (Volume Graphics, GmbH) and formatted in Adobe Photoshop (CC 2017.1.1).

2.3.3 Diagnosis evaluation based on radiographic criteria for DISH

Two blinded clinician-evaluators assessed the µCT and DRR images according to Resnick’s radiographic criteria to diagnose DISH\(^{(23)}\). The two image modalities were assessed independently on separate days, with the order of images randomized to address any potential priming bias. A diagnosis of DISH was determined via either imaging modality and in the event of a discrepancy, a common diagnosis was determined among the two evaluators. The µCT data were presented as a representative sagittal section along with anterior and lateral MIPs. The DRRs were displayed as left-lateral, anterior-posterior, and right-lateral projections.

2.3.4 Histomorphometric analyses

Specimens that met the radiographic criteria for DISH were assessed for density and volume of ectopic calcifications. For each motion segment of the vertebral column (i.e. single IVD and two adjacent vertebral bodies), a region of interest (ROI) was manually contoured to capture the content of ectopic calcification for each specimen based on predetermined criteria: (i) external to the IVD, based on the boundary created by the adjacent superior-inferior vertebral bodies (i.e. to include material outside the borders of
the IVD); and (ii) bounded in axial height by the subchondral cortical bone of adjacent vertebral bodies (i.e. to include material associated with IVD height) (Appendix B). The resultant 3D ROIs provided volumetric measurements (in mm$^3$) and were adjusted by the average intervertebral disc height of the associated segment for comparison across specimens and anatomical regions. The sidedness of ectopic calcification/ossification was evaluated by the percent ROI volume to either side of a midline plane (bisecting spinous process, and center of the vertebral foramen and body). Custom software was used to determine the mineral density of the total ROI and to analyse the internal properties based on the minimum (0.182 g/cm$^3$) and maximum (1.250 g/cm$^3$) densities of normal cortical bone, averaged from sites of normal bone across the specimens, using a protocol from Beaucage et al.$^{(38)}$. Material below the minimum density of normal cortical bone (<0.182 g/cm$^3$) was defined as non-calcified tissue. Material exceeding the maximum density of normal cortical bone (>1.250 g/cm$^3$) was defined as hyper-dense tissue.

2.3.5 Data analysis and statistics

Numerical data were imported into GraphPad Prism (Version 6.01, 2012) for statistical analyses. Normality was assessed using the Shapiro-Wilk test. All parametric data were evaluated using one-way ANOVA, with Bonferroni’s multiple comparisons test. Non-parametric data was analysed using the Kruskal-Wallis test, with Dunn’s multiple comparisons. All data are presented as scatterplots with mean ± 95% confidence intervals. Differences were accepted as statistically significant at p <0.05.
2.4 Results

2.4.1 Ectopic calcification/ossification associated with DISH

Of the 19 specimens examined in the study, 15 were intact vertebral columns, while the remaining four had intact thoracic segments but were missing portions of the cervical region (Figure 2.1A). Fifty-three percent of the cohort (n=10; 3 females, 7 males, mean age of death = 81 years, range 67-94) met Resnick’s radiographic criteria for DISH based on the collective viewing of µCT and DRR images, with ectopic calcification of four or more contiguous vertebral segments (Figure 2.1B). A representative specimen with DISH is presented in Figure 2.2. Mid-sagittal (Figure 2.2A) and transverse views (Figure 2.2B) demonstrate the extent of ectopic calcification detected along the anterior aspect of the vertebral column between T7 and T11. Consistent with the radiographic criteria, the µCT imaging demonstrated the maintenance of IVD height at levels associated with ectopic calcification, compared to adjacent unaffected levels. Three-dimensional isosurface renderings of the same specimen revealed the flowing, wax-like appearance of ectopic calcification that is characteristic of DISH (Figure 2.2C).

We next determined if the imaging modality used to assess each vertebral column influenced the ability to detect areas of ectopic calcification/ossification meeting the diagnostic criteria for DISH. The µCT data were used to generate images with degraded resolution, comparable to computed tomography parameters used in clinical practice as well as DRRs, which mimic plain film radiographs. A comparison of image modalities for the same representative motion segments are shown in Figure 2.3. As anticipated, the resolution of the imaging modality affected the clinical assessment of the spinal segments.
Figure 2.2. Example of micro-computed tomography imaging of ectopic calcifications associated with an 85-year-old male identified with DISH.

(A) Single 154µm slice in the sagittal view of thoracic region (cut C6 to full T12) displaying the extent of ectopic calcification along the anterior aspect of the vertebral column (white arrowheads) and preservation of the intervertebral disc space at each affected level. (B) Transverse section showing the superior view of T9 (corresponding to the red dotted line in panel A) demonstrating the size, density, and sidedness of the ectopic calcification (black arrowheads) adjacent to the vertebral bone. (C) Three-dimensional isosurface rendering of the vertebral column shown in panel A (cut T4 to cut T12) demonstrating the 3-dimenisonal presentation of ectopic calcification associated with DISH. Note the flowing wax-like appearance of ectopic calcification characteristic of DISH (yellow arrowheads).
Figure 2.3. Comparison composite of µCT, synthesised CT, and DRR.

Representative specimens from (A) 78-year-old male diagnosed with DISH in the mid-thoracic region through µCT; (B) 72-year-old male diagnosed with DISH in the lower-thoracic region based on radiographs; and (C) 87-year-old male diagnosed as DISH positive in the mid-thoracic region irrespective of imaging modality. CT, computed tomography; DRR, digitally reconstructed radiograph.
Specifically, 2 of 10 specimens were identified as meeting the radiographic criteria for DISH exclusively through µCT (Figure 2.3A), and 2 of 10 specimens were identified as meeting the radiographic criteria for DISH exclusively through DRR images (Figure 2.3B). However, the majority of specimens (6 of 10) met the radiographic criteria for DISH independent of the imaging modality used in the current study (Figure 2.3C).

2.4.2 Morphological characterisation of ectopic calcification associated with DISH

Using the 10 vertebral columns identified as meeting the radiographic criteria for DISH, we conducted a detailed analysis of the thoracic vertebral column since this region was present in all specimens and was consistently affected by ectopic calcification. We examined the presence of ectopic calcification corresponding to each thoracic motion segment. At sites with ectopic calcification, ROIs were manually contoured to examine each motion segment, external to the IVD and limited to the IVD height (Appendix B). Individual motion segments were excluded from this analysis when no IVD space was detectable (due to degenerative changes, n = 2 motion segments), or when there was evidence of post-mortem tissue damage resulting in displacement of contour boundaries (n = 2 motion segments).

In total, 79% of thoracic motion segments (76/96 analysed) showed evidence of ectopic calcification within the borders of the IVD height. In half of the vertebral columns assessed (5 out of 10), the ectopic calcification was detected as a single contiguous band, extending along five or more motion segments (range 5-10). In the remaining five vertebral columns, multiple areas of ectopic calcification/ossification were detected: in each, one band of ectopic calcification was associated with at least four contiguous
motion segments, and additional areas of ectopic calcification were detected in the thoracic region separated by one or two unaffected motion segments.

The general appearance of ectopic calcifications/ossifications was similar between specimens as an outer, dense cortical band of calcified tissue associated with the anterolateral aspect of the IVD. The resolution afforded by μCT imaging highlighted characteristics of ossification (i.e. dense trabecular network) as well as discrete regions of amorphous material. Ectopic calcifications associated with DISH were categorised as vertical bands when resultant bridging angle was greater than 90° (21%: n=16/76; Figure 2.4A), or horizontal outgrowths when resultant bridging angle was less than 90° (58%: n=44/76; Figure 2.4C, D)(74). In addition, a third presentation was categorised as discontinuous-patchy areas of ectopic calcification (21%: n=16/76; Figure 2.4B). It was common for these presentations to be detected at separate motion segments within the same vertebral column, or even within the same individual motion segment (Figure 2.4C, D).

2.4.3 Histomorphometric analyses of ectopic calcifications in the thoracic vertebral column

We next assessed the location and prevalence of each presentation of ectopic calcification throughout the thoracic vertebral column of the 10 specimens that met the radiographic criteria for DISH. Ectopic calcifications were identified at all levels of the thoracic vertebral column but with differing prevalence across the cohort (Figure 2.5A), with the highest prevalence detected in the inferior regions of T8-12 (97%; 36/37 of available segments). Ectopic calcifications in these regions were predominantly horizontal outgrowths (86%; n=32/37 motion segments). In contrast, motion segments in the upper
Figure 2.4. Isosurface renderings demonstrating the 3-dimensional presentation of ectopic calcifications associated with DISH.

(A) Continuous vertical bands depicting the candle-wax description of DISH, T3-4, 85-year-old male; (B) discontinuous-patchy presentation associated with DISH vertebral columns (not necessarily critical to DISH diagnosis), T8-9, 72-year-old male; (C) large horizontal outgrowths with a dense interior of trabecular bone paired with dense vertical bands underneath, T7-8, 78-year-old male; and (D) large horizontal outgrowths with an interior occupied by IVD tissue paired with the previously described discontinuous-patchy presentation, T10-11, 86-year-old male. Representative sagittal images adjacent to the renderings retrieved from the location of the dotted red line, all sections oriented with anterior to the right and superior to the top of the figure. Scale bar represents 10 mm.
Figure 2.5. Quantitative analyses of ectopic calcification from vertebral columns identified with DISH.

(A) The anatomical distribution of the three described presentations expressed as a percent of the total cohort. (B) The volume of ectopic calcification (mm$^3$), expressed as a corrected value relative to IVD height (mm), at each motion segment level and shown per specimen (different symbols). Red line indicates mean and bars are 95% confidence intervals. Significance of $p < 0.05$ denoted by the matching lettering (a = difference from T9-10, b = difference from T10-11, and c = difference from T11-12). $ns$, not significant.
thoracic regions (T1-T7) were predominantly the discontinuous-patchy and/or vertical presentations of ectopic calcifications (**Figure 2.5A**). The volume of ectopic calcifications were greatest in the lower thoracic regions (T9-T12) compared to those in the upper thoracic regions (**Figure 2.5B**). Moreover, the volume of ectopic calcification associated with individual motion segments was largest for horizontal outgrowths, compared to both vertical and discontinuous-patchy presentations (**Appendix C**).

A right-sided dominance of greater than 75% volume of ectopic calcification was observed in the motion segments at the level of and below the T3-4 IVD (**Appendix D**). The horizontal outgrowths presented the greatest right-sided dominance (Right = 87%, Left = 13% of volume to the midline plane of the vertebrae). Both the discontinuous-patchy and vertical presentations were largely right-sided, but also extended more towards the left side of the vertebral column (both discontinuous-patchy and vertical – Right = 67%, Left = 33% of volume).

In addition to their anatomical location along the vertebral column, the various presentations were differentiated by the properties of ectopic calcification (**Figure 2.6**). In all ectopic calcification associated with the IVD, our analysis demonstrated a heterogeneous distribution of densities marked by discrete regions of hyper-dense material (i.e. exceeding the density of normal cortical bone) (**Figure 2.6A- C**). There was a significantly greater average volume of calcified material (within the density range of normal cortical bone) between motion segments associated with the horizontal presentation compared to either the discontinuous-patchy or the vertical presentations (**Figure 2.6D**). No significant difference in the average volume of calcified material was noted between the discontinuous-patchy and vertical presentations. The volume of hyper-
**Figure 2.6. Analysis of the properties of ectopic calcifications associated with DISH.**

(A-C) Representative images of 154 µm transverse sections (superior-to-inferior perspective) at the level of the IVD illustrating the characteristics of (A) patchy (T6-7 from a 72-year-old male), (B) vertical (T6-7 from an 85-year-old male), and (C) horizontal (T7-8 from an 86-year-old female) presentations of ectopic calcification demonstrating the heterogeneity of densities as colour maps. Purple represents non-calcified material (<0.182 g/cm$^3$) while red indicates material above the density of normal cortical tissue (>1.250 g/cm$^3$). (D) The volume (mm$^3$) of calcified material (grey bars) and hyper-dense material (white bars) associated with individual motion segments, corrected for the average disc height (mm) of the motion segment for each of the presentations. (E) The average bone mineral density retrieved from each ROI of ectopic calcification, per presentation. Each data point corresponds to measurements from an individual motion segment. **Note for (D), the break in y-axis scale bar and difference in scales. Significance denoted via asterisks. Scale bar of 10 mm.
dense material associated with each motion segment displayed a similar trend, yet a statistical difference was only found between the horizontal outgrowths and the discontinuous-patchy presentations. The analysis of total ROI mineral density revealed a significant difference between the presentations of ectopic calcification; the vertical lesions demonstrated a significant increase in bone mineral density compared to the horizontal and discontinuous-patchy presentations of ectopic calcifications (Figure 2.6E).

2.5 Discussion

The radiographic criteria used in the diagnosis of DISH is based on the presence of flowing calcifications and/or ossifications along the anterolateral aspects of the vertebral column but is limited to detection of an advanced disease state by its dependence on the number of contiguous motion segments affected. This study applied µCT imaging of human cadaveric vertebral columns and conducted a detailed analysis of ectopic calcifications to investigate the characteristics associated with DISH. We demonstrate that the current radiographic criteria used to assess DISH captures a heterogeneous population that can be further classified based on quantifiable properties including the morphology, volume, and density of ectopic calcifications. We postulate that further investigation of these presentations may inform on disease pathogenesis or characteristics relevant to detection of early stage disease.

In the current study, 53% of the cohort examined met the radiographic criteria for DISH. This prevalence is well above previous reports conducted in European (17%: 22.7% males, 12.1% females) and North American (20%: 25% males, 15% females) populations (19, 20). The high prevalence in the present cohort is most likely attributed to the
advanced age of the population examined (mean age = 81 years, range 67-94), since the prevalence of DISH is known to increase with each decade of age beyond 50 years\(^{(4,19,20)}\). In addition, our ability to detect ectopic calcifications across spinal tissues was facilitated by the high resolution of the imaging modality used, as there is a marked increased in sensitivity to detect structural changes with computed tomography compared to radiographs. Moreover, the application of µCT results in images at a remarkably higher resolution, which allowed for the analyses of features not possible with clinical computed tomography.

The detailed analyses of morphological characteristics of ectopic calcifications carried out in the current study further described three distinct presentations at the level of the individual motion segment: (i) discontinuous-patchy calcifications, (ii) continuous, vertical bands resembling cortical bone, and (iii) large, horizontal outgrowths containing cortical and trabecular bone. Within the cohort examined we noted remarkable heterogeneity within specimens based largely on anatomical location: motion segments in the upper thoracic regions (T1-T7) were predominantly associated with discontinuous-patchy and/or vertical presentations; while, motion segments in the lower thoracic regions were predominantly associated with horizontal outgrowths. Moreover, the presentations observed were not exclusive rather occurred simultaneously at individual motion segments within the same vertebral column (all three presentations = 5/10; two of three presentations = 4/10; only one presentation = 1/10), and even within the same motion segment (n=15/76). Interestingly, the analysis of all three presentations demonstrated focal regions of amorphous, hyper-dense material either within the adjacent annulus fibrosus of the intervertebral disc, or within the larger horizontal ectopic calcifications
(Figure 2.6A-C). This may be an artifact of the pathobiology of DISH or may highlight a pathological process distinct from DISH. Histological analyses of these tissues are necessary to elucidate their relevance.

These findings are in keeping with previous reports that have identified variability in the presentation of DISH. The nature of bridging calcifications have been categorized as flowing (i.e., vertical presentation) when resultant bridging angles of the new calcified formation are $\leq 45^\circ$ (26) or when related to the vertebral bodies the resultant bridge is $>90^\circ$ (24,39). Alternatively, the presentations not fulfilling these classifications have been described as sharp horizontal outgrowths, resembling osteophytes (i.e., horizontal presentation). Moreover, the discontinuous-patchy presentation outlined in the current study has been noted in previous reports as “unknown floating particles” requiring further characterization (28,39). Interestingly, the most prominent presentation of large, horizontal outgrowths detected in the present study (58%: n=44/76 segments) resembles what has been described as “late, advanced, or terminal-stage” of DISH (22,28,39,40). Of note, the internal properties of ectopic calcifications identified as horizontal outgrowths were also heterogeneous, with some demonstrating an internal trabecular network resembling normal bone, others contained regions of non-calcified tissue that appeared to guide the calcification anteriorly, and few had amorphous hyper-dense material within. Based on our current analysis, it is unclear if the radiographic criteria applied to identify DISH captures a naturally heterogenous disease morphology, different stages in the progression of ectopic calcification, or numerous but distinct pathological processes associated with ectopic calcification of the vertebral column. Furthermore, since medical histories are not available for the donors studied, it is unknown if the symptomatic profiles for patients
differ between the presentations we detected. Further studies assessing the progression of ectopic calcification paired with clinical evaluation in a longitudinal study design would be extremely valuable to investigate these potential implications.

In addition to the characterization of calcifications within motion segments meeting the radiographic criteria for DISH, we extended our analysis to adjacent motion segments that showed evidence of discontinuous-patchy ectopic calcifications. Similar to previous studies, the prevalence of incomplete bridging in these adjacent “non-DISH” regions suggests they may be an indicator of disease progression. If this holds true, it may be that ectopic calcification originate as hyper-dense areas of incomplete bridging that progress to form the vertical and/or horizontal presentations associated with advanced DISH. Furthermore, if large horizontal outgrowths are representative of late-stage disease, their predominance in the lower thoracic regions (T8-12) may suggest a common site for the origination of ectopic calcification. Importantly, our analysis affirmed that the current radiographic criteria for the diagnosis of DISH do not capture potential indicators of early disease initiation and/or indicators of disease progression that may be important for clinical interventions.

Although the imaging findings from this study cannot be correlated to the symptomatic profile of the individuals within our cohort, this study has highlighted intriguing findings with clinical relevance to understanding the aetiology of DISH. First, the predominance of ectopic calcification in the lower thoracic regions suggests this region may be a prime target for efforts aimed at developing early detection strategies of DISH or disorders of ectopic calcification. Second, the detection of increased bone mineral density seen in the vertical presentation of DISH may provide insight into its detection through a non-
invasive imaging modalities, since a recent report has shown bone mineral density measurements may have diagnostic value\textsuperscript{(41)}. Identifying image-based characteristics of ectopic calcification associated with the early stages of DISH will be critical to conduct longitudinal assessments and evaluate the associated symptoms.

In summary, in the current study µCT imaging enabled the identification and quantification of features of ectopic calcification associated with DISH. Although the presentations described herein are in keeping with the descriptions reported previously for DISH, the enhanced quantification of features we report revealed specific differences in the volume, anatomical location, and internal properties of ectopic calcifications. These findings underscore the conclusion that the current radiographic criteria for the diagnosis of DISH captures a population of presentations associated with ectopic calcification that may include a naturally heterogenous disease, potential stage(s) in the natural progression of DISH, or multiple distinct pathologies of ectopic calcification. It is anticipated that findings from this study will aid in the classification of ectopic calcification associated with DISH, and ultimately in the development of early diagnostic criteria that will enable longitudinal studies to better understand the disease progression and clinical implications of DISH.

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2.7 Author roles

Study design: DEF, KEW, SJD, DWH, CAS; Study conduct: DEF, CSB, FAH; Data collection: DEF; Data analysis: DEF, CJDN, SIP; Data interpretation: DEF, KEW, SJD, DWH, CAS; Drafting manuscript: DEF, KEW, SJD, DWH, CAS; Revising manuscript content: DEF, KEW, SJD, DWH, CAS; Approving final version of manuscript: All authors
2.8 References


Chapter 3  Pathological changes associated with diffuse idiopathic skeletal hyperostosis involve heterotopic calcification in spinal fibrocartilage tissues as well as ossification

Through analyses of human cadaveric vertebral columns for DISH with μCT, we demonstrated heterogeneity in the morphological appearance of what is captured with the current radiographic criteria for DISH. In the present chapter, we investigate the tissue-specific changes of ectopic calcifications associated with DISH. This chapter has been written for submission to the peer-reviewed *Annals of the Rheumatic Diseases*.

3.1  Abstract

**Objectives.** To evaluate the histopathological features and composition of ectopic calcification associated with diffuse idiopathic skeletal hyperostosis (DISH) in human tissues.

**Methods.** Thoracic spines from six cadaveric specimens (five males, one female; mean age 81 years) meeting the diagnostic criteria for DISH were evaluated using radiographic, histological, and elemental analyses. Surface analytic techniques (SEM, EDX, XRD) allowed for the identification of elemental composition and crystalline diffraction patterns of the ectopic calcifications associated with DISH.

**Results.** The histological features of ectopic calcification within intact motion segments were heterogeneous: regions of mature bone, primary woven bone, and multifocal areas of fibrosus were identified within the anterior aspects of individual motion segments. In addition to findings of ectopic ossification, our analyses revealed regions of amorphous, calcified material within the anterior longitudinal ligament, annulus fibrosus and/or fibrocartilage extensions of the IVD associated with DISH. These regions were further
characterised as having a disorganised “chalky” appearance by SEM, with a high content of calcium and phosphorus, a crystalline diffraction pattern matching hydroxyapatite, and density values equivalent to or exceeding those of normal (unaffected) cortical bone.

**Conclusions.** Our findings indicate that the formation of pathological bony bridges associated with DISH are formed by both ectopic ossification and amorphous calcification in fibrocartilage tissues. The current radiographic criteria for the diagnosis of DISH capture a heterogeneous group of morphological presentations, which may include a distinct subset of pathologies related to ectopic calcification.

**Keywords:** diffuse idiopathic skeletal hyperostosis (DISH); histology; spine; ectopic calcification, ossification.

3.2 **Introduction**

Diffuse idiopathic skeletal hyperostosis (DISH) is a non-inflammatory spondyloarthropathy characterised by ectopic calcifications and/or ossifications along the anterolateral aspect of the vertebral column, particularly in the thoracic spine\(^1\). DISH is diagnosed based on the radiographic detection of flowing calcifications and/or ossifications across four contiguous vertebral segments, the preservation of intervertebral disc (IVD) height in affected areas, and the absence of bony ankylose of the facet joints and/or sacrum\(^2\). Though not included in the diagnostic criterion, DISH is often associated with the presence of extraspinal calcifications, commonly in the knee, ankle, hip, shoulder, and elbow joints\(^3\). Symptoms associated with DISH are variable, ranging from spine stiffness and decreased spinal range of motion (with or without back pain) to severe cases of dysphagia, dysphonia, or spinal cord/nerve root compression\(^4-6\). Notably,
both the clinical symptoms and radiographic diagnosis of DISH are limited to an advanced disease state\(^7\).

The prevalence of DISH in North America and Europe are estimated to be 15-25% and 17% of the population over the age of 50, respectively\(^8,9\). Risk factors for DISH include ethnicity (e.g. Caucasians)\(^8\), sex (e.g. males > females)\(^8,9\), advanced age (e.g. per decade of age beyond 50 years)\(^8,10\), and co-morbidities (e.g. obesity, diabetes mellitus and metabolic syndrome)\(^11\). Together, the lack of early-stage disease detection of DISH paired with the rise in potential risk factors suggests that the prevalence of DISH is greater than reported and is expected to increase.

To date, the aetiology underlying the formation of ectopic calcifications associated with DISH is unknown. Systemic metabolic changes related to obesity, diabetes mellitus, high waist circumference, hypertension, hyperinsulinemia, dyslipidemia, and hyperuricemia have been associated with DISH\(^4,11–14\). At the cellular level, changes to the Wnt-\(\beta\)-catenin, NF\(\kappa\)B, BMP2, PG\(\iota\)2, and endothelin-1 pathways have been associated with the presence of ectopic calcifications, although the association between pathway deregulation and the induction of disease pathogenesis has not been established\(^4,15\). Familial cases of DISH\(^16,17\), although rare, paired with the characterisation of ectopic calcifications in animal models\(^18–20\) strongly suggests the contribution of genetic factors in the aetiology of DISH. A related disorder ossification of the posterior longitudinal ligament (OPLL), has been reported in patients with DISH and is postulated to share a similar pathogenesis\(^21\). OPLL has a greater prevalence in the Japanese population and has been associated in genome wide association studies with a single nucleotide polymorphism in the \textit{COL6A1} gene, which encodes the \(\alpha\)1 chain of type VI collagen\(^22–24\). Mechanical
factors have also been suggested to influence the formation of calcification associated with DISH, particularly due to the characteristic right sidedness of formation, which is thought to be a consequence of aortic pulsation\(^{(25)}\). Ultimately, the limited understanding of the pathobiology of DISH has resulted in the lack of disease modifying treatments and insufficient prognostic indicators for clinicians and patients\(^{(26)}\).

The hallmark finding of flowing ectopic calcifications and/or ossifications associated with DISH has been described through radiographic and tomographic studies. The histopathological characterisation of DISH is less thorough; initial reports described cellular features of endochondral ossification of the anterior longitudinal ligament\(^{(27)}\) as well as changes in the morphometry of the IVD\(^{(28,29)}\). Recent studies in cadaveric tissues demonstrated that, in contrast to previous reports, the anterior longitudinal ligament does not incorporate into the mature calcifications but is instead displaced by it\(^{(30)}\). Taken together, these findings underscore the need to identify and characterise the tissue types and cellular changes associated with the induction and progression of pathological changes leading to DISH, the composition and/or source of the ectopic calcification, and if any of these features change with disease progression. The current study was designed to investigate these basic questions of disease pathobiology by combining radiographic, histological, and elemental analysis of human spinal segments affected by DISH.

3.3 Methods

3.3.1 Cohort characteristics and dissection

Human cadaveric spine tissues were obtained from the medical education program at the University of Western Ontario, in accordance with the Anatomy Act of Ontario and
Western’s Committee for Cadaveric Use in Research (REF#22062016 – Appendix A).
Removal of the head and neck musculature exposed the first cervical vertebrae, and complete resection was performed inferior to the twelfth thoracic vertebrae. The ribs were dissected 3-5 cm lateral to the maintained costovertebral joints, the connective tissues were preserved, and the descending thoracic aortas were removed. The thoracic spine was further dissected to allow for subsequent analysis.

3.3.2 Micro-computed tomography imaging (μCT)
Intact vertebral columns were scanned by μCT as previously described (Chapter 2) using a GE Locus eXplore Ultra X-ray conebeam imaging system at a peak voltage of 80 kVp and tube current of 50 mA. The 1000 X-ray projections were reconstructed into a single 3-dimensional volume with isotropic voxel spacing of 154 μm$^3$ and rescaled into Hounsfield units using an internal calibrator of SB3 (Gammex Inc. Middleton, WI, USA), air, and water. The μCT data were used to generate a series of images for each specimen (in-plane sagittal sections, anterior and lateral maximum intensity projections, and digitally reconstructed radiographs representing left-lateral, anterior-posterior, and right-lateral projections) that were assessed by two clinician-observers to diagnose DISH using Resnick and Niwayama’s radiographic criteria$^{(2)}$. Three-dimensional isosurface renderings and pseudo-coloured μCT images were exported from MicroView 2.2: GE Healthcare, and 2.5.0: Parallax Innovations Inc.

3.3.3 Histological evaluation of tissues
Six thoracic spines (Figure 3.1) meeting the diagnostic criteria for DISH (five males, and one female; mean age of 81 years, range 72-89) were dissected into individual motion segments by transverse cuts across the waist of the superior and inferior vertebrae to
Figure 3.1. Micro-computed tomography (μCT) based three-dimensional isosurface renderings of the specimens included in the study.

Isosurface renderings showing the 3-dimensional involvement of ectopic calcifications from (A) 85-year-old male, thoracic spine segments 4-11; (B) 77-year-old male, thoracic spine segments 3-7; (C) 87-year-old male, thoracic spine segments 7-9; (D) 78-year-old male, thoracic spine segments 4-6; (E) 86-year-old female, thoracic spine 4-7; and (F) 72-year-old male, thoracic spine segments 3-9. Arrows indicate the disc level of the motion segments included in the study; yellow correspond to segments evaluated for histology, and green correspond to motion segments analysed by SEM/EDX/XRD. Scale bar represents 10 mm. SEM, scanning electron microscopy; EDX, energy dispersive X-ray spectroscopy; XRD, X-ray diffraction.
preserve the intervertebral disc and connective tissues; while, oblique cuts removed the posterior osseous features from the vertebral body (Appendix E). From the six specimens, multiple motion segments from each were selected for analysis based on the appearance of previously described presentations of ectopic calcification (e.g. patchy, vertical, and horizontal) (Chapter 2). A total of 15 motion segments were evaluated.

To isolate tissues of interest, a sagittal slice was made through the central regions of the ectopic calcification associated with each motion segment using a rotating diamond bladed saw (Appendix E). In cases of extreme right-sidedness, oblique cuts were made through the center of the ectopic calcification (Appendix E). From each motion segment, a single one-millimetre slice of tissue was dissected in the associated sagittal/oblique plane, and half of the tissue was imaged using a digital x-ray system (Planmeca ProX, Helsinki, Finland) prior to decalcification with Shandon TBD-2 (14-21 days; Thermo Scientific). The other half of the tissue from each motion segment was used for subsequent analysis of tissue composition. Following decalcification, the tissues were processed, embedded, and sectioned at 5 µm thickness using a Leica RM2255 microtome and collected on 50 x 75 mm slides (Brain Research Laboratories). Serial sections were stained with Haematoxylin and Eosin as well as Masson’s Trichrome. Low magnification micrographs were captured using a Gen5 CellPlate reader, and high magnification images (including under polarised light) were captured using an Olympus BX41 optical microscope equipped with a digital camera (Olympus U-TVO.5XC-3) and Infinity Analyze software (Version 6.5.5, Lumenera Co.). All images were imported into Adobe Photoshop CC 2018 (Version 19.1.5) for figure construction.
3.3.4 Scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDX), and X-ray diffraction (XRD)

The one-millimetre slices of tissue (n = 4) taken from the central region of the ectopic calcification of each motion segment were desiccated with Drierite (W.A. Hammond Drierite Co., Ohio, USA) for 30 days, imaged with a Canon (EOS 40D digital single-lens reflex camera), rinsed in 100% chloroform for 1 h and subsequently re-dried for 1-2 days before being coated with a 10 nm coating of osmium using previously established protocols\(^\text{(31)}\). SEM imaging was performed using a Zeiss 1540XB FIB/SEM instrument (Carl Zeiss, Oberkochen, Germany) at the University of Western Ontario Nanofabrication facility. EDX analyses were performed using an Oxford Instruments X-max50 analysis system and ICNA software. Multiple regions of interest within the ectopic calcifications (10-16 per motion segment) were analysed, along with internal “control” regions of normal bone along the unaffected posterior vertebral bone. All EDX data were expressed as a percentage of atomic element weight. Data were imported into GraphPad Prism (Version 6.01, 2012) to assess statistical significance. Data were examined for normality using the Shapiro-Wilks test, and no outliers were identified using GraphPad’s ROUT method with Q = 1%. Parametric data was assessed using a one-way ANOVA with Bonferroni’s multiple comparisons test, and statistical significance was accepted at P < 0.05.

The same desiccated tissues were assessed for crystalline diffraction pattern using a Bruker D8 Discover diffractometer with a 60 mm Co Gobel mirror and a 300 µm snout (Department of Earth Sciences, University of Western Ontario). The nominal beam diameter for each measurement was 300 µm. The samples were manipulated using a
remote-controlled XYZ stage-laser system, and 5-7 targets within regions of ectopic calcification as well as regions of normal cortical bone from the posterior aspect of the vertebrae were selected for each motion segment using a microscope equipped with a CCD camera and a laser targeting system. Data were collected by a 2-D General Area Detector System, and analysed with Diffrac Suite EVA software for comparison with patterns from the International Centre for Diffraction Data database, as previously described\(^{(32)}\).

3.4 Results

3.4.1 Samples and inclusion criteria

Based on our previous studies which characterised the morphometric properties of ectopic calcification associated with DISH in cadaveric human spines using \(\mu\)CT imaging (Chapter 2), we carried out a detailed analysis of a subset of six specimens (five males, one female; mean age of 81 years, range 72-89), for a total of 15 individual motion segments (Figure 3.1). Using \(\mu\)CT-based analysis, these specimens were selected based on i) having met the diagnostic criteria for DISH (with at least four contiguous segments affected in the thoracic spine), and ii) demonstrating heterogeneity in the morphological appearance of ectopic calcifications based on the presentations we outlined in our previous studies (e.g. each contained at least two of the three morphological presentations described in Chapter 2 - patchy, vertical, or horizontal).

3.4.2 Histological features of spinal tissues associated with DISH

To localise areas of ectopic calcification within the anatomical structures of individual motion segments affected by DISH and assess their morphological appearance, we
carried out histological evaluation of decalcified tissue sections (Figures 3.2-4). In order to correlate histological features with areas of tissue calcification, digital radiographs were taken prior to tissue decalcification. **Figure 3.2** is representative of a motion segment with the morphological features of “horizontal” and “patchy” calcifications; **Figure 3.3** is representative of a motion segment with a characteristic right sided “horizontal” calcification; and **Figure 3.4** is representative of a motion segment with a “vertical” calcification.

Histopathological analysis of intact motion segments (arrows in **Figure 3.1**) demonstrated consistent histological features within the intervertebral discs examined (Figures 3.2-4). In general, the nucleus pulposus was marked by granular changes indicative of mild to moderate degeneration; although, radiographic IVD height was maintained in keeping with Resnick’s criteria\(^{(2)}\). In all specimens examined, varying degrees of IVD degeneration were observed marked by the loss of a distinct transition between the nucleus pulposus and surrounding annulus fibrosus, as well as moderate to severe disorganisation of the lamellar layers of the annulus fibrosus most often detected along the anterior aspect. The cartilage endplates showed variable irregularities, ranging from loss of organisation of the cartilaginous matrix to small clefts and disruption of the subchondral bone (i.e. Schmorl’s nodes, detected in 3/15 motions segments from 3/6 specimens; **Figures 3.2,3.3**). The degenerative changes detected in the IVD tissues were not unexpected given the advanced age of specimens (range 72-89 years). The overlaying
Figure 3.2. Histological appearance of spinal tissues with horizontal and patchy presentations associated with DISH.

Images correspond to specimen shown in Figure 3.1B, T6-7. (A) A digital radiograph of the tissue prior to decalcification which displays the ectopic calcification with a trabecular network and the presence of calcified material within the IVD. (B) Representative serial section stained with H&E at 1.25 x magnification demonstrating the appearance of the intact tissue, scale bar represents 10 mm. Panels C-E correspond to the yellow box in Panel B, highlighting features of mature bone (arrowheads) along the anterior portion of the outgrowth, including compartmentalised bone marrow cavities (black asterisk). Panels F-H correspond to the white box in Panel B, showing a region of primary woven bone (arrows) and features of endochondral ossification (white asterisk). Panels C, F are stained with H&E; D, F are stained with Masson’s Trichrome; and E, H are micrographs of C, F under polarised light. Scales bars for Panels C-H represent 100 µm. Orientation is indicated in Panel A. H&E, Haematoxylin and Eosin.
Figure 3.3. Histological appearance of spinal tissues with the horizontal presentation associated with DISH and morphological changes to the IVD.

Images correspond to specimen shown in Figure 3.1C, T8-9. (A) A digital radiograph of the tissue prior to decalcification which displays the outermost shell of ectopic calcification and the presence of non-calcified material within the core. (B) Representative serial section stained with H&E at 1.25 x magnification demonstrating the appearance of the intact tissue, scale bar represents 10 mm. Panels C, F, I correspond to the yellow box in Panel B, highlighting the anterior-most portion of the fibrocartilage extension (asterisk). Panels D, G, J correspond to the white box in Panel B, revealing unique transitions zone marked by the presence of amorphous granular material (black
arrowhead) and multifocal areas of fibrosis (white arrowheads). Panels E, H, K correspond to the black box in Panel B, displaying the internal-most aspect of the fibrocartilage disc extension (asterisk) and a localised region of ossification (black arrow). Panels C-E are stained with H&E; F-H are stained with Masson’s Trichrome; and I-K are micrographs of C-E under polarized light. Scales bars for Panels C-K represent 100 µm. Orientation is indicated in Panel A. H&E, Haematoxylin and Eosin.
Figure 3.4. Histological appearance of spinal tissues with the vertical presentation associated with DISH.

Images correspond to specimen shown in Figure 3.1A, T4-5. (A) A digital radiograph of the tissue prior to decalcification which displays the outermost ectopic calcification and ambiguous internal composition. (B) Representative serial section stained with H&E at 1.25 x magnification demonstrating the appearance of the intact tissue, scale bar represents 10 mm. Panels C-E correspond to the yellow box in Panel B, highlighting an amorphous region of calcified material within the annulus fibrosus (asterisk) and characterised by a granular appearance and poor staining. Panels E-H correspond to the white box in Panel B, displaying a region of mature bone features, marked by organized
osteons (black arrowheads). Panels I-K correspond to the black box in Panel B, revealing amorphous material within the anterior longitudinal ligament (black arrows). Panels C, F, I are stained with H&E; Panels D, G, J are stained with Masson’s Trichrome; and E, H are micrographs of C-E under polarized light. Note: Panel K is a micrograph of Panel J under polarized light due to tissue folding. Scales bars for Panels C-K represent 100 μm. Orientation is presented in Panel A. H&E, Haematoxylin and Eosin.
anterior longitudinal ligaments (when in-section) were either well-preserved or contained discrete regions of calcification.

3.4.3 Histological features of ectopic calcifications/ossifications associated with DISH

In all motion segments examined, the primary characteristic of DISH detected was the formation of an osseous shell adjoining the superior-inferior vertebrae across an IVD segment (Figures 3.2-4). Consistent with our previous work with μCT imaging (Chapter 2), there was tremendous heterogeneity in the histological appearance of ectopic calcifications associated with DISH.

First, the characteristic bridging ectopic calcifications associated with DISH varied in maturity, thickness, length and volume. In all presentations, the bony bridges showed features of developed, mature, and distinct lamellar bone with organised osteons and an internal network of bone marrow (Figure 3.2C-E, Figure 3.4F-H). Focal areas of fibrosis and primary woven bone were often noted adjacent to or interrupting areas of mature ossification (Figure 3.2F-H).

Second, areas of fibrocartilage were often detected extending from the IVD and localised between the calcified outgrowths from the superior and inferior vertebrae (Figure 3.3C-K). Within these regions, the fibrocartilage was interrupted by multifocal areas of granular degeneration, fibrosis, and ossification (Figure 3.3C-K). At these sites, the transition from fibrocartilage to nests of chondrocytes within cartilage to sites of woven bone is consistent with the process of endochondral ossification.
Lastly, our analysis highlighted the presence of isolated regions of amorphous calcified material in all morphological presentations of DISH. These structures were located within the annulus fibrosus or the fibrocartilage extensions (Figure 3.2F-H, Figure 3.4C-E) or within the anterior longitudinal ligament (Figure 3.4I-K). The islands of amorphous material were often inconsistently stained and granular in appearance, which may indicate a mixture of calcified material, degenerated fibrocartilage and gelatinous extracellular matrix. The origin of this material could not be determined. Systemic indicators of inflammation were absent; although, small areas of “clean-up” inflammatory cells and neovascularisations within the ossified tissues were noted.

3.4.4 Elemental composition and crystalline diffraction patterns of ectopic calcifications/ossifications associated with DISH

Given that areas of ectopic calcification and/or ossification associated with DISH contained both mature bone and amorphous calcified material, we sought to determine the composition of these structures. Regions of interest within 4 individual motion segments from 2 vertebral columns identified with DISH (Figure 3.1A&F) were analysed by EDX to determine the elemental composition in areas of mature ectopic ossification (n=5-10 ROIs/motion segment) and of amorphous calcification (n=3-8 ROIs/motion segment) (Figure 3.5). The content of calcium was significantly greater in all sites of ectopic calcification and/or ossification associated with DISH compared to normal (unaffected) vertebral cortical bone (indicated as internal control). Sites of amorphous calcification were also associated with a greater phosphorus content compared to normal (unaffected) vertebral cortical bone (Figure 3.5A). Moreover, both
Figure 3.5. EDX and XRD analyses of ectopic calcifications and/or ossifications associated with DISH.

(A) Tabulated results from the elemental analysis expressed as percent of atomic weight and Ca/P ratio, bolded are the average mean values and italicised are the standard deviations. Multiple regions of interest (ROI) were examined within sites of amorphous calcification, ectopic ossification, and internal controls of unaffected posterior vertebral bone in four different motion segments. * indicates statistical significance (p < 0.05) compared to internal controls of unaffected posterior vertebral bone; † indicates statistical significance (p < 0.05) between the amorphous calcification and the ectopic ossification.

(B) Representative diffraction patterns from individual regions of interest within sites of amorphous calcification, ectopic ossification, and internal controls of unaffected posterior vertebral bone are displayed in a stack plot and matched to hydroxyapatite (PDF 86-1201), as indicated by the bar pattern.
the calcium and phosphorus content within sites of amorphous calcification were higher than those detected within regions of ectopic ossification associated with DISH (Figure 3.5A). The calcium/phosphorus ratios were greater at all sites of ectopic calcification and/or ossification compared to normal (unaffected) vertebral cortical bone (Figure 3.5A).

To assess the associated crystalline diffraction patterns within these regions, tissues were subsequently analysed by XRD. Regions within sites of amorphous calcification and ectopic ossification associated with DISH, as well as sites of normal (unaffacted) vertebral cortical bone showed a diffraction pattern matching hydroxyapatite (Figure 3.5B).

3.4.5 Surface topography and density analysis of ectopic calcifications and/or ossifications associated with DISH

The areas of ectopic calcification and/or ossification associated with DISH were further characterised in individual motion segments by SEM and μCT (Figure 3.6). Gross examination of mineralized foci by SEM revealed distinct areas of disordered “chalky” material associated with sites of amorphous calcification, distinct in appearance from the adjacent mature woven bone. Lastly, μCT data were used to generate pseudo-coloured maps of densities within the same regions assessed by SEM, EDX and XRD. This analysis demonstrated remarkable heterogeneity in the range of densities detected in motion segments associated with DISH (Figure 3.6). Within the motion segments examined, the highest density detected was localised within sites of amorphous calcifications (the density within these areas exceeded that of the “normal” vertebral cortical bone).
Figure 3.6. Characterisation of ectopic ossifications and amorphous calcifications associated with DISH.

Composite figure demonstrating the gross morphological appearance of the one mm thick desiccated tissues and corresponding SEM and μCT analysis of A) Figure 3.1B, T9-10 (ectopic ossification); B) Figure 3.1F, T3-4 (ectopic ossification and amorphous calcification); and C) Fig 3.1A, T3-4 (ectopic ossification and amorphous calcification). Topographical analysis of tissues by SEM (middle panels and areas denoted by dotted yellow boxes in left panels) highlight the “chalky” disorganised appearance of amorphous calcifications within the intervertebral disc that is distinguished from adjacent tissues. These corresponding areas were assessed via pseudo-colored density maps from μCT data. Densities equivalent to that of normal bone are range from blue-to-green while densities exceeding normal cortical bone are presented as yellow-to-red. The black and white coloured scale bars represent 10 mm and the yellow coloured scale bars represent 1 mm. Orientation is presented in Panel A with “s” representing superior and “a” representing anterior, the reciprocal inferior and posterior axis are not shown or labelled.
3.5 Discussion

The features of ectopic calcification and/or ossification that serve as the hallmark of DISH have been extensively studied using medical imaging, whereas few reports have examined the histopathological features within intact human spinal tissues. The present investigation combined radiographic, histological and elemental analysis to characterise the pathological features of ectopic calcifications and/or ossifications associated with DISH in human cadaveric tissues. To our knowledge, this is the first study to show that ectopic material associated with the diagnosis of DISH is formed through a mixture of amorphous calcifications and ectopic ossification associated with spinal tissues in humans. Based on these, and previous studies from our group, we postulate that the current radiographic criteria for DISH captures a heterogeneous subset of presentations that require further differentiation or separation into distinct pathologies in order to understand the aetiology of disease pathogenesis.

The hallmark radiographic finding of DISH is the presence of flowing calcifications and/or ossifications along the anterolateral aspect of the vertebral column\(^\text{(1)}\), but previous reports by our group identified heterogeneity in the morphological appearance of ectopic calcification associated with DISH (Chapter 2). This heterogeneity was likewise evident in the histological evaluation of these tissues, as seen in the variability in structure, organisation, thickness, and maturity of features associated with the ectopic bridges formed along the anterior aspect of motion segments. For example, despite demonstrating a common radiographic appearance, some of the motion segments examined demonstrated features of mature woven bone, including an internal network of trabecular bone and bone marrow, whiles others demonstrated areas of encapsulated fibrocartilage...
material with hallmarks of endochondral ossification. While it is tempting to speculate that these features may be related to the stage of disease or the pattern of disease progression at individual motion segments, further studies are required to determine if the specific histopathological features relate with the spatio-temporal pattern of disease progression.

Important to the diagnosis of DISH is the exclusion from other vertebral pathologies, such as degenerative disc disease via the maintenance of IVD height based on radiographic evaluation. A pilot study conducted using spinal tissues from ten cadaveric spines suggested a lack of involvement of the IVD in the pathogenesis of DISH due to the preservation of IVD height between DISH specimens and controls. Our histopathological evaluation demonstrates that characteristics of mild-to-moderate IVD degeneration were detected in all specimens examined but did not affect the radiographic appearance of disc height (hence, did not influence the diagnosis of DISH). These findings are in keeping with recent studies which demonstrate degenerative IVD changes in patients with DISH, and proclaim to de-emphasise mild-to-moderate changes as exclusion criterion for DISH, as co-morbidities are thought to be common with DISH\(^{33,34}\). As such, changes associated with the onset of IVD degeneration should not be excluded as potential contributors to the pathogenesis of DISH.

Despite the radiographic preservation of IVD height (with or without mild-moderate degeneration), the current study demonstrates that morphological changes to the outer annulus fibrosus appear frequently in DISH. Our findings agree with previous studies that identified fibrocartilage extensions of the IVD localised between ectopic calcifications and/or ossifications extending from both superior and inferior directions, lying beneath
the anterior longitudinal ligament\textsuperscript{(29,35)}. In fact, Kuperus \textit{et al.} proposed a scoring system specific based on the based on qualitative histological characteristics of ectopic briding which included characterisation of the shape of these fibrocartilage extensions from the IVD (defined as regular, tapered, spatulate, or irregular, many of which were noted in the current investigation)\textsuperscript{(29)}. Within these regions, histopathological evaluation in this study noted indicators of endochondral ossification, including nests of chondrocytes, primary woven bone, and areas of fibrosis. Unique to our investigation was the identification of discrete regions of amorphous calcified material within the fibrocartilage extensions, the outer layers of the annulus fibrosus, and the anterior longitudinal ligament (amorphous calcifications identified in 9 of 15 motion segments examined), regardless of the morphological presentation of the ectopic bridge. These amorphous regions did not possess typical histological features or cell types characteristic of bone and also demonstrated greater calcium and phosphorus contents than normal (unaffected) cortical bone, with a crystalline pattern matching hydroxyapatite; consequently, we proposed they should be termed “calcifications”. Also, \(\mu\)CT analysis of these amorphous regions demonstrated a density exceeding that of normal cortical bone. These novel findings in human tissues closely resemble the amorphous pathological calcifications reported in the \textit{ENT1}\textsuperscript{\textminus\textminus} mouse model of DISH\textsuperscript{(20)}, underscoring the potential of the \textit{ENT1}\textsuperscript{\textminus\textminus} mouse as a pre-clinical model of DISH for future mechanistic and/or pharmaceutical studies.

The involvement of the anterior longitudinal ligament in the pathogenesis of DISH has been controversial. Some authors report the preservation of the anterior longitudinal ligament associated with pathological findings of DISH\textsuperscript{(1,28,30)}; while, other propose its mineralization is involved in disease pathogenesis\textsuperscript{(27,28,36)}. When present in the
histological sections examined in the current study, the structure of the anterior longitudinal ligaments were typically preserved; however, in a selected subset of specimens we detected focal regions of amorphous granular material within the anterior longitudinal ligament. Further studies are required to specifically examine if changes associated with calcification of the anterior longitudinal ligaments are associated with DISH or if these pathological findings should be considered as a distinct pathology, such as “calcification of the anterior longitudinal ligament.”

The overt morphological changes to the vertebral column resulting from the formation of large horizontal outgrowths of ectopic calcification and/or ossification external to the IVD has led to the hypothesis that DISH is associated with osteophyte formation, that is more common in vertebral spondyloses, and osteoarthritis\(^{(4)}\). Since the prevalence of DISH increases with age, it may frequently coexist with these pathologies, making it difficult to distinguish from other vertebral pathologies, particularly in early stages. In contrast, the formation of amorphic calcifications with the annulus fibrosus may be a unique feature specific to DISH and distinct from other pathologies. Although the origin of this material could not be determined in the current study, a theory for its existence could be due to local micro-trauma or instability within the outer concentric layers of the annulus fibrosus associated with early degenerative changes. Perhaps in a subset of individuals with contributing metabolic factors, a local reactive immune response is initiated in the fibrocartilaginous tissue of the annulus fibrosus, leading to the accumulation of discrete region(s) of amorphous calcified material. The presence of the heterotopic calcification triggers ectopic ossification from the surrounding tissue(s) to further stabilise the IVD structure, ultimately leading to the characteristic flowing
calcifications and/or ossifications across the IVD associated with DISH. Further studies are required to assess tissues at the early stages of disease pathogenesis, including adjacent segments with signs of early calcification and/or ossification, to test the assumptions underlying this proposed model.

Taken together, the findings from the current study provide further evidence that the current radiographic criteria for DISH need to be re-evaluated as it currently captures a heterogeneous population of pathological features related to ectopic calcification and/or ossification of the vertebral column. The current investigation infers two distinct types of ectopic calcification and/or ossification based on the tissues affected, the morphological appearance of changes, the presence of amorphous material, and/or the density of the calcified material. We propose the use of the term “calcification of the anterior longitudinal ligament” in instances where there is direct evidence of anterior longitudinal ligament involvement. The radiographic presentation of large, horizontal outgrowths (with varying degrees of maturity) may become a distinct feature in the classification of DISH. Future work is required in order to validate the association of these radiographic and histological features in a larger cohort of specimens, to correlate histological features with disease symptoms and/or co-morbidities, to identify biomarkers to differentiate these presentations in clinical studies, as well as to associate clinical symptoms and disease features with progression of the disease.

3.6 Acknowledgements

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3.7 Author roles

Study design: DEF, KEW, SJD, DWH, CAS; Study conduct: DEF, RJB; Data collection: DEF; Data analysis: DEF; Data interpretation: DEF, PKK, KEW, SJD, DWH, CAS; Drafting manuscript: DEF, SJD, CAS; Revising manuscript content: All authors; and Approving final version of manuscript: All authors
3.8 References


Chapter 4  General discussion and conclusions

The studies in the current thesis aimed to enhance the understanding of the pathogenesis of ectopic calcification associated with DISH through analyses of human cadaveric tissues. The overall goal was to evaluate the morphological and histopathological features of ectopic calcification associated with DISH in a human cadaveric population. We explored this discovery driven research through two specific objectives:

(1) Re-evaluate the current radiographic diagnostic criteria to facilitate early disease detection and classify unique features of pathological spinal calcification; and

(2) Characterise the tissue-specific histopathological features associated with ectopic calcifications in human specimens.

4.1 Overall conclusions

By adopting unique and novel technologies and methodologies, we have contributed new information to the field of DISH- and spine-research. Chapter 2 detailed the application of µCT imaging to quantify the distribution and morphology of ectopic calcifications associated with DISH. We demonstrated:

- remarkable heterogeneity in the morphological appearance of ectopic calcifications that can be classified into three categorisations: (i) large, horizontal outgrowths associated with the IVD; (ii) continuous vertical bands of tissue resembling cortical bone; and (iii) discontinuous-patchy calcifications that do not form a complete bridge across the IVD; and
• heterogeneity in the imaging properties of the ectopic calcifications, notably the presence of hyper-dense material that exceeded the density of normal (unaffected) cortical vertebral bone.

Chapter 3 applied a combination of radiographic, histological, topographical and physical analyses to identify and characterise the tissue types, cellular features, and composition of ectopic calcification and/or ossification associated with DISH. We demonstrated:

• motion segments from specimens meeting the radiographic criteria for DISH showed histopathological features consistent with areas characteristic of ectopic ossification as well as areas of amorphous calcification within the annulus fibrosus, fibrocartilage extensions from the IVD, and anterior longitudinal ligament; and

• areas of ectopic ossification showed evidence of endochondral ossification; and

• areas of amorphous calcifications had increased calcium and phosphorus content compared to normal (unaffected) cortical bone, crystalline diffraction pattern matching hydroxyapatite, and densities that often exceeded that of normal cortical bone with µCT imaging.

4.2 Imaging characteristics related to DISH

The radiographic findings of flowing calcifications and/or ossifications along the anterolateral aspect of the vertebral column remains the hallmark characteristic of
DISH\(^{(1)}\). However, numerous studies have acknowledged that the current diagnostic criteria are limited to the detection of DISH in an advanced disease state\(^{(2,3)}\). Moreover, there are currently no indicators or prognostic criteria enabling the detection of DISH at an early disease stage. In Chapter 2, we used µCT imaging to characterise the morphological features and characteristics of ectopic calcifications associated with DISH. We outlined three distinct classifications of ectopic calcification associated with DISH based on µCT: i) large, horizontal outgrowths associated with the IVD; ii) continuous vertical bands of tissue resembling cortical bone; and iii) discontinuous-patchy calcifications that do not form a complete bridge across the IVD. The progression of ectopic calcification associated with DISH is thought to occur over a lengthy period of time, although the underlying mechanism remains unknown\(^{(4,5)}\). Based on the findings from Chapter 2, we speculate that the discontinuous-patchy presentations detected at sites within vertebral columns meeting the diagnostic criteria for DISH are indicative of an early disease state. The initiation of these focal sites of ectopic calcification may then develop into either the continuous vertical or large horizontal outgrowth presentations we described. Together, these findings infer two major classifications of DISH that can be distinguished by standard clinical imaging modalities (e.g. computed-tomography and plain-film radiography).

4.3 Knowledge transfer related to DISH
DISH is commonly under-diagnosed or misdiagnosed in the clinical setting due to medical professionals being unfamiliar with the pathology and/or its implications\(^{(6)}\). For example, a series of case reports have described unforeseen complications following clinical intubation of patients, which was later revealed to be due to the presence of large
ectopic calcifications and/or ossifications located in the cervical spine\(^{(7-12)}\). Moreover, surgeons routinely tasked with spinal procedures need to be aware of potential complications associated with DISH, that being the displacement or obstruction of typical anatomy due to the growth of ectopic calcifications and/or ossifications\(^{(13)}\). Overall, a greater awareness of DISH paired with an improved knowledge of its associated symptoms and common co-morbidities would aid health care providers (e.g. radiologists, primary care providers, and physiotherapists) in the recognition of DISH. The development of early stage diagnosis of DISH would benefit the understanding of the disease through the implementation of large cohort, longitudinal studies to monitor its progression and associated clinical presentation and/or symptoms in humans. Information from these studying can be used to identify at-risk patients (in early stages) and the creation of conservative treatments plans (e.g. physical therapy) aimed to prevent the disease from reaching a more advanced state.

4.4 Potential mechanisms underlying DISH pathogenesis

A poor understanding of the disease aetiology and pathological mechanisms underlying the formation of ectopic calcifications and/or ossifications associated with DISH has resulted in the lack of disease-modifying treatments and prognostic information for clinicians and patients\(^{(14)}\). Understanding the histological features of ectopic bridges associated with DISH will contribute to better understanding the pathogenic mechanism(s). A detailed investigation in cadaveric human tissues was carried out in Chapter 3, which revealed remarkable heterogeneity in the histological features of ectopic calcifications associated with DISH. Primarily localised to the outermost region of ectopic bridges (i.e. anterior) were ectopic ossification spanning across the intact
motion segment, with histological features of varying degrees of maturity ranging from primary woven bone to osteon formation and the development of bone marrow compartments. We propose a pathological mechanism involving osteophyte formation from the superior and inferior vertebral bone in response to the natural process of aging and intervertebral disc degeneration. Perhaps genetic factors or changes to the tissue microenvironment from systemic metabolic deregulation associated with co-morbidities make certain populations more vulnerable to excessive ectopic calcifications and/or ossifications, resulting in the large horizontal outgrowths associated with DISH.

A novel observation from the study detailed in Chapter 3 was the characterisation of discrete regions of amorphous calcified material localised to fibrocartilaginous structures within the ectopic calcifications and/or ossifications. These regions appear to be composed of hydroxyapatite based on XRD analysis and were found to be equivalent or exceed the density of normal cortical bone based on µCT. A proposed pathological mechanism could be that these areas of ectopic calcification are the result of micro-trauma to the outer concentric lamellae of the annulus fibrosus, leading a local inflammatory response which triggers the secretion of amorphous calcified material by fibrocartilage cells to maintain the biomechanical stability of the IVD and vertebral column. The resulting alterations in the IVD biomechanics induce structural bone formation from adjacent cartilage and bone tissues to maintain the long-term structural integrity of the vertebral column. In this scenario, the amorphous calcified material may represent an early indicator of DISH pathogenesis.
4.5 Limitations and future work

In the present thesis, we provide a descriptive characterisation of ectopic calcification and/or ossification associated with DISH in humans using µCT, histological, topographical, and physical analyses. Our findings have provided new insight into the pathobiology of DISH; however, inherent limitations must be acknowledged related to our study design. The cadaveric tissues used in this work correspond to individuals of advanced age with associated co-morbidities (e.g. joint replacements, chronic cardiovascular disease, terminal cancers), and therefore does not reflect the general population (e.g. sex, ethnicity). As a result, our findings are limited to specimens with an advanced disease state of ectopic calcification and/or ossification. Future work should focus on examining motion segments adjacent to those affected by DISH, in addition to the cervical and lumbar spine, as these may serve as indicators of an early disease state and/or stage(s) of progression. The embalming of tissues for preservation also created many challenges in the application of traditional methodologies for tissue characterisation (e.g. decalcified histology) and limited the type of analysis that could be conducted. Moreover, the array of modalities used in the current thesis are not feasible in the clinical setting where access to human spinal tissues is sparse. Despite these limitations, the studies described in the current thesis have provided valuable knowledge, allowing for the development of a variety of future studies related to the radiographic assessment and basic biology that were not possible before. For example, development of a finite element model based on the µCT images of intact human vertebral columns would allow for the computational evaluation of the influence ectopic calcifications and/or ossifications have on spinal mobility or fracture risk, at specific anatomical locations (e.g. upper vs. lower
thoracic spine) or during different stages of disease progression (e.g. early vs. late).

Quantifiable metrics from such studies may be particularly valuable to better understand the correlation between clinical presentations and/or symptoms and the radiographic findings. It is clear that early stage detection of DISH is crucial for the initiation of large cohort longitudinal studies with living populations. Moreover, an improved understanding of the patient-population’s specific needs is necessary to guide future care and treatments. Collectively, the goal of future work in the field of DISH research should be aimed at correlating the patient needs, clinical presentation and/or symptoms, and histories to the findings observed through traditional medical imaging, such as computed-tomography, to better understand the onset and progression of DISH.

Currently, the scientific literature associated with DISH consists primarily of individual or small case reports from clinicians, with little emphasis or research focused on enhancing the understanding of the underlying disease mechanism. Moreover, many studies have proposed modifications to the diagnostic criteria of DISH to facilitate disease detection, yet the initial criteria proposed in 1976 remains the gold-standard in the diagnosis of DISH. Given our limited access to human tissues affected by DISH, particularly at the early stages of disease, pre-clinical animal models are necessary for investigations of disease pathobiology.

4.5.1 Animal models of DISH

Ectopic calcifications and/or ossifications associated with DISH have been described throughout the archeological record in humans, as well as in animals (e.g. dinosaurs, saber-toothed cat, Rhesus monkeys). Moreover, selected breeds of dogs (e.g. Boxer) have been reported to present with a naturally high prevalence of ectopic calcifications in
the vertebral column resembling DISH in humans\textsuperscript{(25–27)}. In addition, our group has previously characterised a mouse model (lacking expression of the gene encoding the nucleoside transporter ENT1) that displays ectopic calcification of the paraspinal ligaments, rib enthuses, and IVDs with remarkable resemblance to DISH\textsuperscript{(28,29)}. Characterisation of the cell-type specific changes associated with the onset and progression of ectopic calcification using these models may provide insight into the physiology of the disease and allow for the development of disease-modifying interventions to slow, stop, or reverse the formation of ectopic calcifications and/or ossifications associated with DISH.
4.6 References


Diffuse idiopathic skeletal hyperostosis (DISH) and spondylosis deformans in purebred dogs: a retrospective radiographic study. Vet J. 2011;190(2):e84-90.


Appendices
Appendix A. Use of Cadaveric Materials – Ethics Approval Notice.

Letter of permission to use and photograph cadaveric materials for the purposes of research from the cohort of 19 vertebral columns obtained during the summer of 2016.
Appendix B. Isosurface 3-dimensional renderings highlighting the regions of interest used for quantification.

(A) right-lateral; (B) anterior; (C) right-lateral; and (D) posterior views displayed in 90° rotations about the vertical axis. Note the posterior view has the posterior bony landmarks removed for visual clarity. This individual motion segments shows T9-10 from an 85 year-old male identified as DISH according to Resnick’s radiographic criteria: i) contiguous ectopic calcification along the antero-lateral aspect of the vertebral column; and ii) preservation of intervertebral disc height. Regions of interest (highlighted in red) corresponding to the ectopic calcification were manually contoured based on predetermined criteria: i) anterior to the intervertebral disc, and ii) bound superiorly-inferiorly by the subchondral cortical bone of the adjacent vertebral bodies. These regions were then used for subsequent quantitative analyses. Scale bar represents 10 mm.
Appendix C. Volumetric analysis of ectopic calcifications from each individual motion segment.

Each data point is normalised by the corresponding disc height, from spines associated with DISH (n=10/19, M=7, F=3). A total of 96 motion segments were available for analyses, from which 76 presented with ectopic calcification. These regions were divided into three broad categories based on their gross morphological features: i) discontinues-patchy (21%: n=16/76 motion segments); ii) continuous vertical bands (21%: n=16/76 motion segments); and iii) large horizontal outgrowths (58%: n=44/76 motion segments). Dotted red line indicates the mean with 95% confidence intervals. Statistical significance of P < 0.05 denoted by the asterisk.
Appendix D. The sidedness of ectopic calcifications.

The sidedness of ectopic calcification expressed as the percent of ROI volume displaced to the left and right of a midline plane (bisected through the spinous process and vertebral foramen and body), per individual motion segment. (A) All presentations of ectopic calcification displayed across anatomical location; (B) discontinuous-patchy presentation only; (C) continuous vertical bands presentation only; and (D) large horizontal outgrowth presentation only. Dotted red line indicates the mean percent volume of ectopic calcification per each side of the midline.
Appendix E. Schematic demonstrating the dissection approach.

All images are superior views of a sample vertebrae. (A) Laminectomies corresponding to the white dotted lines were first performed to open up the posterior aspect of the vertebral foramen (i.e. spinal canal). Second, oblique cuts corresponding to the red dotted lines were performed medial to the costovertebral joints (as to preserve them) for the removal of the transverse processes. The isolated vertebral body was then sliced (B) sagittally through the center of the ectopic calcification/ossification; or (C) in cases of extreme right sidedness, oblique cuts through the center of the ectopic calcification/ossification was performed, represented by the blue dotted lines. For subsequent analyses, a one mm slice was then carefully dissected from either one of the two halves before one was decalcified and the other stored.
Curriculum vitae

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Graduate Teaching Assistant
The University of Western Ontario
2016-2017, 2017
