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Traumatic Heterotopic Ossification: Pathophysiological Mechanisms, Epidemiological Characteristics, and Risk Factors

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Pathophysiological Mechanisms, Epidemiological Characteristics, and Risk Factors

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Declaration of Authorship

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

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Abstract

Introduction

Traumatic heterotopic ossification (tHO) refers to the complication of pathological formation of ectopic bone in soft tissues, which follows tissue insult secondary to burn, neurological and orthopaedic injury [2]. Common to these diagnoses is the involvement of central and or peripheral nervous system trauma. Although known risk factors are associated with developing tHO, the underlying cellular and molecular mechanisms are not fully understood. Research to date, primarily in single injury cohorts, has provided minimal translatable results [2]. Thus, clinical guidelines for early screening and prophylactic drug therapy are lacking, and treatment options are limited. To move the body of knowledge forward, machine learning approaches, such as IBM Watson for Drug Discovery (WDD), may offer a solution to enhance basic science discovery by providing important insights into the biological processes and genetic influences implicated in tHO. Investigating the key genes and signalling pathways that are dysregulated in and related to the development of tHO through analysis of human biospecimens may lead to the identification of putative targets that could be used to elucidate the underlying mechanisms of pathological ectopic bone formation. Fibroblasts, which can acquire bone-forming characteristics under dysregulated neuroinflammatory conditions induced by injury, make them an attractive candidate progenitor cell that may contribute to tHO pathogenesis.

The challenges to enhancing clinical tHO management are highlighted in the burn patient context, as despite four decades of research, the lack of translatable evidence is stark, and difficulties in conducting multi-centre trials due to low event rate and patient care heterogeneity remain [2]. Thus, injury complicated by tHO significantly impacts patient quality of life, hampering physical and psychosocial functioning and impeding rehabilitation [3]. As such, it is hypothesised that a diagnosis of tHO in trauma patients prolongs hospital length of stay (LOS) and increases healthcare costs.

Furthermore, at the commencement of this program of research, there were no published epidemiological data available for Western Australian (WA) tertiary hospital trauma patients to help

understand tHO prevalence, LOS outcomes and risk profile or data evaluating the accuracy of medical diagnostic coding and specificity of clinical documentation for tHO diagnoses. Purposeful multi-centre data pooling of at-risk trauma populations was therefore warranted and instigated as part of this research program to address several aforementioned research, understanding and clinical practice gaps. Specifically, to comprehensively explore and identify novel tHO risk factors and to quantify the impact of tHO on the health care system. By combining information from burn patients and individuals with a higher prevalence of tHO, this program aimed to unveil underlying causes that may not be apparent due to the relative rarity of tHO following burns in the WA cohort. This research program aimed to explore and seek a novel understanding of the pathophysiology, epidemiology, clinical characteristics, and risk factors of traumatic heterotopic ossification in adult trauma patients. To confirm novel and applicable results, the methods were designed to build a detailed risk profile and define the mechanisms that perpetuate the tHO process to provide new targets for detection, prevention, and intervention.

Methods

Building on previous research conducted by the candidate, new literature reviews were undertaken on (i) epidemiology, risk factors, diagnostic tools, and treatment for tHO in trauma populations and (ii) known pathophysiological mechanisms, disease biomarkers and emerging therapeutic targets in diseases of ectopic bone formation. Unlike traditional literature reviews designed on the principle of scarcity to facilitate human inference, IBM Watson for Drug Discovery, arguably the first machine learning adjunct of its kind, was used to synthesise a global corpus of evidence on pathophysiological mechanisms and disease biomarkers in tHO and identify plausible new genes and proteins that may be involved in tHO. To contextualise the literature findings, the retrospective audit was undertaken to determine the prevalence of tHO and, in doing so, evaluate the accuracy of medical diagnostic coding and clinical documentation for tHO diagnoses within the WA tertiary hospital network. The multi-centre, matched cohort study of pooled trauma populations was conducted first to explore the association of inpatient tHO diagnoses on hospital length of stay (LOS) and second to analyse clinical variables known at and during hospital admission to identify common risk factors for tHO development, seeking markers that could facilitate preventive practices. Finally, the research program was extended further through a national, multi-centre study, which comprised a series of basic science studies utilising biospecimens collected from burn-

injured adults who did (tHO+) and did not develop tHO (tHO-) after injury. The objectives of the study, therefore, were (i) to identify candidate genes and signalling pathways that differ in fibroblasts from tHO+ and tHO- patients after burn injury and (ii) to determine whether fibroblasts from burn-injured tHO+ patients are more susceptible to osteoblastic differentiation compared to control fibroblasts.

Results and Discussion

The use of IBM Watson for Drug Discovery proved successful in identifying a novel set of plausible candidate gene targets that may participate in the pathogenesis of tHO. Interrogation of the literature highlighted that, of the top 25 ranked genes, six genes (MMRN1, MSC/MyoR, ITGAM/CD11b, PDGFD, GREM1 and NELL1) were identified to have plausible evidence of likely association with the mechanisms of tHO development. Further validation of their biologic relevance in tHO pathobiology is required, presenting opportunities as the focus of future investigations to develop surveillance biomarkers or treatments based on these findings.

Evaluation of medical diagnostic coding accuracy for tHO diagnoses identified that HO-specific ICD-10-AM codes failed to identify more than 1/3rd of true tHO cases, with a high prevalence of non-specific HO codes (19.4%) and cases identified incidentally via manual chart review (25.3%). The reported sensitivity of M61 codes for correctly diagnosing tHO after burn injury was only 50%, indicating that using M61 diagnostic codes is a less than acceptable method to classify tHO cases accurately. Marked inconsistencies and variation in clinical documentation for tHO diagnoses was detected across facilities. These results provide a new benchmark for current practice for clinicians and medical coders and may have implications for future retrospective research and patient care.

The contribution of a tHO diagnosis during hospitalisation as a co-morbid complication was determined to be the most significant predictor of an increased hospital LOS for patients following burns, spinal cord injury and traumatic brain injury. Trauma patients diagnosed with tHO during hospitalisation stayed 56% longer than trauma patients of the same age, gender, and injury severity who did not develop tHO. These findings provide a new and valuable understanding of the effect of tHO on trauma patient outcomes, which has significant implications for clinical practice, as early recognition and treatment of tHO could potentially reduce LOS and health resource utilisation.

Considerations must be made around resource allocation and early tailoring of care for trauma patient populations to reduce prolonged hospital stays.

Multivariate regression analysis revealed that total hospital LOS and concomitant injury to the hip region and thigh independently increased the risk of developing tHO during hospitalisation. Further, novel predictors of tHO relating to local and systemic infection sources included infectious agents *Staphylococcus* species, *Acinetobacter calcoaceticus-baumannii* complex and *Enterobacter cloacae* complex.

The laboratory studies provided evidence through RNA sequencing analysis, which identified 136 genes significantly differentially expressed in tHO+ dermal fibroblast samples, of which 16 were bone-related genes and included osteogenesis signalling pathways, namely Wnt signalling. Except for *CD26,* validation of RNAseq data by qRT-PCR confirmed consistent gene expression changes in genes associated with osteogenic processes; *CADM1*, *NFATC2*, *STEAP4* and *WNT4.* Alkaline phosphatase activity staining discovered the osteogenic differentiation potential of dermal fibroblasts. Mineralisation was observed in tHO+ fibroblast cultures by alizarin red staining; however, the quantification of calcium deposition did not differ significantly when compared with control fibroblasts. tHO+ fibroblasts expressed baseline levels of osteogenic markers *RUNX2*, *ALPL, IBSP* and *PHEX*. QRT-PCR verified osteogenic pathway activation in fibroblast cultures under BMP-2 stimulation. With the exclusion of *PHEX*, temporal expression changes over 21 days were as expected for all osteogenic gene markers studied. Collectively, results indicate that dermal fibroblasts derived from tHO patients are primed towards early, pre-osteoblastic lineage in response to BMP-2 and may participate in pathological ectopic bone formation after burn injury.

Conclusions and Clinical Implications

- Machine-learning methods extend the human capacity to assimilate previous research, discover plausible gene targets, and further understand pathophysiology in complex disease states such as tHO.
- Healthcare professionals should strive to improve the standardised recording of clinical data for suspected or confirmed tHO diagnoses and employ standardised clinical terminology from

the point of care to enhance the specificity of medical diagnostic coding for injury-specific classifications of rare events like tHO.

- Clinicians and wider hospital administration staff must be aware of the risk a tHO diagnosis has on prolonging inpatient hospital stay for trauma patients. Trauma patients who develop tHO should be considered immediately as an at-risk population who may benefit from the increased allocation of resources and complex discharge planning.
- Examination of the tHO risk profile in pooled trauma patients confirmed known and identified novel, independent risk factors common across the burn and neurological trauma populations. Clinicians should be alert to common risk factors for tHO development and develop strategies for early targeted surveillance of high-risk trauma patients.
- Laboratory investigations have provided valuable clues for highlighting the characteristics of tHO after burn injury and support that multiple genes and cell signalling pathways in tHO+ tissues are dysregulated in subjects that develop tHO. Thus, putative targets that could be used to elucidate the molecular mechanisms underlying tHO were identified. Future investigations linking these biomarkers to potential new therapies are proposed as a pathway to improve patient outcomes.
- Finally, these findings add to the body of knowledge on the underlying mechanism of tHO and, particularly, dermal fibroblast plasticity. The *in vitro* research identified a potentially novel fibroblast subtype that may possess the ability to phenotypically switch towards the osteogenic lineage in response to burn injury and contribute to the genesis of tHO. Therefore, dermal fibroblasts may be a prime cell type for targeted therapeutics in burn-induced tHO.

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List of Abbreviations

AA: L-Ascorbic acid
ACVR1: Activin A Receptor Type 1
AD: Alzheimer's Disease
ALP/ALPL: Alkaline Phosphatase
ANK: Ankylosis Protein
ARDS: Acute Respiratory Distress Syndrome
ASIA: American Spinal Injury Association
AUC: Area under the curve
B-ALP: Bone-specific Alkaline Phosphatase
BBB: Blood Brain Barrier
BMP: Bone Morphogenetic Protein
BMP-2: Bone morphogenic protein 2
BMP-3: Bone Morphogenic Protein 3
BMP-4: Bone Morphogenic Protein 4
BMP-6: Bone Morphogenic Protein 6
BMP-7: Bone Morphogenic Protein 7
BMP-9: Bone Morphogenic Protein 9
BMPR2: Bone Morphogenetic Protein Receptor Type 2
BNB: Blood Nerve Barrier
BSP: Bone Sialoprotein
CADM1: Cell Adhesion Molecule 1
CD4+: Cluster of Differentiation 4
CD8+: Cluster of Differentiation 8
CD11b: Cluster of Differentiation 11b
CDH5: Cadherin 5
CGRP: Calcitonin gene-related peptide
CI: Confidence Interval
CK2: Casein Kinase 2
CNS: Central Nervous System
COL2A1: Collagen Type II Alpha 1
COL6A6: Collagen Type VI Alpha 6 Chain
COVID-19: Coronavirus Disease
COX: Cyclooxygenase
CT: Computed tomography
CTX: Cardiotoxin
DCN: Decorin

Dlx5: Distal-less homeobox 5
DMEM: Dulbecco's Modified Eagle Medium
DNA: Deoxyribonucleic acid
DPO: Dendriform Pulmonary Ossification
DPP4: Dipeptidyl Peptidase 4
DVT: Deep Vein Thrombosis
ECM: Extracellular matrix
EHDP: Etidronate Disodium
EMR: Electronic medical records
ENPP1: Ectonucleotide Pyrophosphate/Phosphodiesterase 1
ERK: Extracellular Signal-Regulated Kinase
FBS: Fetal bovine serum
FGF-2: Fibroblast Growth Factor 2
FGF-23: Fibroblast Growth Factor 23
FGFR3: Fibroblast Growth Factor Receptor 3
FOP: Fibrodysplasia Ossificans Progressiva
GCS: Glasgow Coma Scale
GDF2: Growth Differentiation Factor 2
GREM1: Gremlin-1
H&E: Hematoxylin and eosin
HAUSP: Herpesvirus-Associated Ubiquitin-Specific Protease
HD: Huntington's Disease
HO: Heterotopic Ossification
Hh: Hedgehog
IBSP: Integrin-Binding Sialoprotein
ICD-10-AM: International Statistical Classification of Diseases and Related Health Problems, 10th Revision, Australian Modification
ICU: Intensive Care Unit
IL-1α: Interleukin-1 alpha
IL-1β: Interleukin-1 beta
IL-22: Interleukin-22
IL-6: Interleukin-6
IQR: Interquartile Range
ITGAM: Integrin Subunit Alpha M
ITGAM: Integrin Alpha M
JNK: c-Jun N-terminal Kinase
KEGG: Kyoto Encyclopedia of Genes and Genomes
KRT18: Keratin 18
LEC: Lymphatic Endothelial Cell
LOS: Length of Stay
MAPK: Mitogen-Activated Protein Kinase
MCP-1: Monocyte Chemoattractant Protein-1

MEDLINE: Medical Literature Analysis and Retrieval System Online
MEM α: Minimum essential medium alpha
MITF: Melanocyte Inducing Transcription Factor
MMP11: Matrix Metallopeptidase 11
MMP13: Matrix Metallopeptidase 13
MMP27: Matrix Metallopeptidase 27
MMP9: Matrix Metallopeptidase 9
MMPs: Matrix Metalloproteinases
MMRN1: Multimerin-1
MO: Myositis Ossificans
MRI: Magnetic Resonance Imaging
MRSA: Methicillin-resistant Staphylococcus aureus
MSC: Mesenchymal Stem Cell
MYOR: Myogenic Repressor
NCAM1: Neural Cell Adhesion Molecule 1
NF-kβ: Nuclear Factor kappa B
NF-κB p65: Nuclear Factor kappa B subunit p65
NFATC2: Nuclear factor of activated T cells 2
NFATc1: Nuclear Factor of Activated T Cells 1
NGF: Nerve growth factor
NHO: Neurogenic HO
NK1r: Neurokinin 1 receptor
NPP: Nucleotide Pyrophosphatase/Phosphodiesterase
NSAID: Non-Steroidal Anti-Inflammatory Drug
NT-3: Neurotrophin-3
OPLL: Ossification of the Posterior Longitudinal Ligament
OPN: Osteopontin
OR: Odds Ratio
OSX: Osterix
P38 MAPK: p38 Mitogen-Activated Protein Kinase
PBS: Phosphate-buffered saline
PC-1: Phosphocholine Cytidylyltransferase 1
PD: Parkinson's Disease
PDGFR: Platelet-derived growth factor receptor
PHEX: Phosphate Regulating Endopeptidase Homolog X-Linked
PI3K: Phosphoinositide 3-Kinase
PNS: Peripheral Nervous System
PPAR-y: Peroxisome Proliferator-Activated Receptor gamma
PPi: Pyrophosphate
PTA: Post-Traumatic Amnesia
PTLH: Parathyroid Hormone-Like Hormone
qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction

RLT: RNeasy lysis buffer
RNA: Ribonucleic acid
RNA-seq: RNA Sequencing
ROC: Receiver operating characteristic
ROM: Range of Motion
RUNX2: Runt-related Transcription Factor 2
S1: Proximal Site 1
S2: Distal Site 2
SCI: Spinal Cord Injury
sP: Substance P
SP7: Osterix
STEAP4: Six-Transmembrane Epithelial Antigen of Prostate 4
SUSI: Spectroscopic ultrasound
Smad: Small Mothers Against Decapentaplegic
TBI: Traumatic Brain Injury
TBSA: Total Body Surface Area
TDS: Three Times Daily
TGF-β1: Transforming Growth Factor Beta 1
TGF-β3: Transforming Growth Factor Beta 3
THA: Total Hip Arthroplasty
tHO: Traumatic Heterotopic Ossification
THO+: Patients who do develop traumatic heterotopic ossification
THO-: Patients who do not develop traumatic heterotopic ossification
THR: Total Hip Replacement
TIMP: Tissue Inhibitors of Matrix Metalloproteinases
TLR-2: Toll-like Receptor 2
TNAP: Tissue-Non-Specific Alkaline Phosphatase
TNF: Tumour Necrosis Factor
TNF-α: Tumour Necrosis Factor Alpha
TRAP: Tartrate-resistant acid phosphatase
TrKA: Tropomyosin receptor kinase A
US: Ultrasonography
USP7: Ubiquitin-Specific Protease 7
VEGF: Vascular Endothelial Growth Factor
VEGF-A: Vascular Endothelial Growth Factor A
WA: Western Australia
WDD: Watson for Drug Discovery
WNT: Wingless-related Integration site
WNT4: Wingless-related integration site 4
β-catenin: Beta-Catenin

Chapter 1.

Introduction

Overview

This chapter will outline the background, rationale and research questions for the studies undertaken as part of this research programme. The overall aim of this research programme was to explore and seek a novel understanding of the epidemiology, pathophysiology, clinical characteristics, and risk factors of traumatic heterotopic ossification (tHO) in adult trauma patients. **Chapter 2** summarises a review of the published literature on the epidemiology, risk factors, diagnostic tools, and treatment for tHO in trauma populations. Reported in **Chapter 3** is an investigation using an AI-enhanced search tool, IBM Watson for Drug Discovery (WDD), to identify novel gene and protein candidates that may be implicated in tHO pathogenesis. A supporting literature review on known pathophysiological mechanisms, clinical biomarkers and emerging therapeutic targets in tHO is provided in the supplementary material. Where there was a dearth in the literature on tHO populations, data collected in other disease states characterised by ectopic bone formation were reviewed. **Chapter 4** reports an evaluation of the accuracy of medical diagnostic coding and clinical documentation for tHO diagnoses within the West Australian (WA) Tertiary Hospital network, the results of which provide a benchmark of current practice for clinicians and medical coders. **Chapter 5 (Part 1)** presents a matched case-control study undertaken to explore the association of inpatient tHO diagnoses on hospital length of stay. **Chapter 5 (Part 2)** was a retrospective analysis of clinical variables known at and during hospital admission and carried out to identify common risk factors for tHO development in pooled trauma populations. Finally, the findings of the laboratory investigations using human clinical

biospecimens to characterise the molecular and cellular contributions of tHO after burn injury are reported in **Chapter 6**.

1.1 Rationale for Thesis

Traumatic Heterotopic Ossification (tHO) refers to the formation of mature lamellar bone outside of the skeletal structure and is a severely debilitating complication of burn, neurological and orthopaedic trauma [2]. Contemporary understanding of the neuroinflammatory regulation of bone remodelling suggests that injury to the central or peripheral nervous system may provide the common neurotrauma mechanism propagating tHO formation in trauma populations. However, there remains an incomplete understanding of the pathophysiological mechanisms involved [2].

Due to the lack of enlightenment available from burn patient tHO research, alternative approaches to interrogating large clinical data sets were considered as they may be beneficial, particularly to assist in the discovery of relevant genes and proteins of interest. Machine learning methodologies, like IBM Watson for Drug Discovery, can serve as valuable tools for enhancing the efficiency of fundamental scientific exploration by providing novel insights into the biological processes and genetic influences implicated in complex pathologies such as tHO [1, 4, 5]. Furthermore, analysing human biospecimens with contemporary techniques to study dysregulated genes and signalling pathways implicated in tHO may unveil potential targets, thus facilitating a comprehensive understanding of the underlying mechanisms. The *in vivo* evidence suggests that fibroblasts have the potential to transdifferentiate into ossifying cells in response to inflammatory reactions after injury and participate in pathological ectopic bone formation in soft tissues. However, the interaction between dermal fibroblasts and traumatic heterotopic bone formation is yet to be elucidated.

The lack of translatable evidence and challenges in conducting multi-centre trials, mainly due to low event rate and patient care heterogeneity, hinders progress in clinical practice and understanding of tHO management. Despite the existence of several identified risk factors associated with tHO development, there are no established clinical guidelines to facilitate early, routine screening of high-risk trauma patients; thus, a clinical diagnosis does not allow for prophylactic drug therapy, and therapeutic options remain limited. As a result, it is established that the presence of tHO profoundly affects the quality of life for trauma patients, impeding both

physical and psychosocial functioning and complicating the rehabilitation trajectory [3]. Further, it is hypothesised that a diagnosis of tHO contributes to a prolonged hospital length of stay (LOS) and increased healthcare costs.

The systematic multi-centre data pooling of high-risk trauma populations was proposed to comprehensively investigate and ascertain novel risk factors associated with tHO and quantify the impact of tHO on the health care system. By integrating data from burn patients with those from populations exhibiting a comparatively high prevalence of tHO, we endeavoured to uncover underlying causes that may not be apparent due to the relative rarity of tHO following burns injury in the WA cohort. Building a comprehensive risk profile will encourage the development of novel diagnostic technologies and prophylactic interventions and facilitate mechanistic research to prevent and or ameliorate tHO. Ultimately, the principal goal is to achieve prevention and early detection, thus, reducing the development of tHO where feasible and, consequently, the burden of illness on the patient and the health care system. New insights gathered through such investigations may provide for the further development of surveillance and treatment guidelines for best practices across trauma populations.

1.2 Aims

The aims of this thesis were:

1. To investigate the pathophysiological mechanism(s), diagnosis, and treatment in diseases of ectopic bone formation
2. To identify and describe the epidemiological characteristics of tHO in WA Tertiary hospitals
3. To investigate the length of stay outcomes in trauma patients diagnosed with tHO
4. To identify risk factors associated with tHO
5. To analyse the key genes and signalling pathways that are implicated in tHO after burn injury
6. To decipher the interaction among dermal fibroblasts and traumatic heterotopic bone formation

1.3 Objectives

The objectives of this thesis were:

1. To examine a broader corpus of novel literature using the IBM Watson search engine
    1.1. To conduct a review of the published literature on the pathophysiological mechanisms, clinical biomarkers, and emerging therapeutic targets in diseases of ectopic bone formation
    1.2. To investigate novel gene and protein candidates in tHO
2. To conduct a retrospective audit of the West Australian trauma database to:
    2.1. Establish the prevalence of tHO in trauma patients in West Australian tertiary hospitals
    2.2. Evaluate the accuracy of ICD-10-AM diagnostic coding and clinical documentation for tHO diagnoses in West Australian tertiary hospitals
    2.3. To examine the relationship between an inpatient tHO diagnosis and hospital length of stay
    2.4. To explore and identify common risk factors associated with tHO development in pooled trauma patients
3. To investigate mechanistic data from the collection of human biospecimens to:
    3.1. Identify the key genes and signalling pathways that are different in dermal fibroblasts from tHO+ and tHO- patients after burn injury
    3.2. Determine whether dermal fibroblasts from burn-injured tHO+ patients are more susceptible to differentiating to an osteoblastic phenotype than control fibroblasts.

1.4 Guiding Questions

This research program set out to answer the following research questions:

1. What is the current state of the science regarding pathomechanisms, diagnosis and treatment of diseases associated with ectopic bone formation?
2. What are the epidemiological characteristics of tHO diagnoses in West Australian hospitals over a 14-year period?
3. Is tHO associated with increased inpatient hospital length of stay?
4. What common risk factors are associated with developing tHO in adult trauma patients?

5. What are the key genes and signalling pathways that differ in dermal fibroblasts from patients who do and do not develop tHO after burn injury?
6. Do burn patients that develop tHO after injury have a dermal fibroblast phenotype that is more susceptible to osteogenic differentiation?

Chapter 2.

Traumatic Heterotopic Ossification (tHO)

Overview

This chapter will introduce Traumatic Heterotopic Ossification (tHO), including a summary of the epidemiological characteristics and risk factors for tHO development. The diagnostic tools and treatment will also be reviewed.

2.1 Introduction

First described in the early 1800s, heterotopic ossification (HO) is a pathological process characterised by the production of mature, lamellar bone within non-skeletal tissue [6-8]. The etymological roots of the term HO derive from the Greek “Heteros topos”, meaning different place, and the Latin “ossificatio", meaning bone formation. Therefore, the literal translation is ‘bone formation in another place’ [9]. While in the general population, genetic causes of HO, such as Fibrodysplasia Ossificans Progressive (FOP), are exceedingly rare (1.36 per million people) [10], the incidence of traumatic HO (tHO) remains clinically significant [11, 12]. No genetic mutation has been identified as responsible for driving heterotopic bone formation in the trauma context.

2.2 Epidemiology

Cases of traumatic HO are broadly classified into three aetiological categories: tHO following burns [2] and high-velocity blast injury [9], neurogenic HO (NHO) resulting from traumatic brain injury (TBI) and spinal cord injury (SCI) [13-15]; and orthopaedic HO developed after fracture, dislocation

and soft tissue trauma [16, 17]. Post-traumatic HO [18], traumatic HO [19] or acquired HO [20] are used interchangeably in today's literature. As the term heterotopic ossification refers only to the environment in which the bone process occurs, the more specific term myositis ossificans (MO) is commonly used to classify aberrant bone formation that occurs within skeletal muscle due to direct trauma or repeated micro-injuries [21, 22].

2.2.1 Burns

As the survival rates for patients with major burns have increased, so has the incidence of tHO. Based on three large burn centre reports, Hu et al. [23] recently summarised the incidence rate of tHO after burn injury to be 3.5-5.6% [24-26], with prior estimates ranging from 0.15 to 1.2% [27-31].

2.2.2 Neurological (SCI and TBI)

Following central nervous system (CNS) injury, a reported tHO rate of 10-53% in SCI patients versus 11-20% in TBI patients has been described [15, 32]. However, it must be noted that within the current literature, reports of tHO incidence and prevalence following CNS injury generally do not distinguish between traumatic cases and non-traumatic cases. Thus, these estimates must be interpreted cautiously as they may not accurately represent the post-traumatic populations [33]. Reznik et al. [13] described a prevalence of tHO in TBI patients as one-third of that found in traumatic SCI patients, ~4% and 11%, respectively. Rawat and colleagues [34] conducted a 17-year retrospective analysis of 303 patients who received inpatient treatment for traumatic SCI. The overall incidence of tHO reported by these authors was 6.3%, consistent with previous work reporting cases with a history of traumatic injury.

2.2.3 Orthopaedic Injury

The rates of orthopaedic tHO following operative fixation of upper limb fractures range from 5.5-18.8% and increase significantly with associated CNS injuries [35]. In a WA tertiary hospital, Bochat et al. [36] carried out a retrospective review of a theatre database for procedures undertaken for elbow trauma over a five-year period and identified an overall tHO incidence of 22% (34 of 153 patients), which was similar across high and low energy injuries (23% vs. 22%). However, post-operative tHO following fracture is often asymptomatic and incidentally detected during routine post-operative radiographs [17]. The reported incidence of asymptomatic tHO was 18.8% in a cohort of patients with elbow fractures requiring surgical fixation [37]. Traumatic HO in the lower limbs has been reported in 28.8% [38] and 38% [39] of patients following acetabular fractures and forms in ~44% of patients undergoing total hip arthroplasty [16], where a great variability in incidence depends on the surgical approach [40]. In combat-injured patients, tHO occurs as a sequela in >60% of blast injuries, rising to 90% in trauma-related amputations [41, 42].

2.3 Risk factors

Several clinical studies have attempted to identify risk factors associated with tHO to stratify predisposed patients. However, most investigations have focused mainly on single-centre outcomes making results challenging to interpret.

2.3.1 Burns

More commonly, tHO develops in joints with overlying burns and in patients with extensive full-thickness injuries (TBSA >20%) [24, 43, 44]. However, exceptions to the high-risk categories have been reported; for instance, tHO formation has been described in joints without overlying burn injury [3, 45] and Chen et al. [27] confirmed the development of tHO in patients with an 8% TBSA. The most strongly implicated risk factors for tHO after burn injury are shown in **Table 1**. However, it should be noted that many of these findings are from smaller, single-institution reports.

Table 1. *Risk factors for developing traumatic heterotopic ossification after burn injury identified from multivariable analysis techniques*

First author, year of publication	Study design	Risk factors
Thefenne et al., 2017 [26]	Single centre tHO+, *n*=32 tHO-, *n*=96	Use of air-fluidised bed (OR 39.6) Duration of use of air-fluidised bed (OR 1.1) Curare use (OR 24.1) Pulmonary infection (OR 21.5) Cutaneous infection (OR 7.5) Length of stay in ICU (OR 1.1) Mean total burn area (OR 1.1) Mean depth of burns (OR 1.1)
Schneider et al., 2017 [43]	Multi-centre tHO+, *n*=98 tHO-, *n*=2,660	1% increase in TBSA up to 30% (OR 1.14, p=0.004) 1% increase in TBSA above 30% (OR 1.04, p=0.000) Arm burn requiring graft (OR 5.06, p=0.005) Head/neck burn requiring graft (OR 2.72, p=0.000) Trunk burn requiring graft (OR 2.40, p=0.005)
Orchard et al., 2015 [25]	Single centre tHO+, *n*=19 tHO-, *n*=19	Longer time to active movement (OR 1.48)
Levi et al., 2015 [24]	Multi-centre tHO+, *n*=98 tHO-, *n*=2,979	Arm burns requiring skin graft (OR 96.4, p=0.04) TBSA >30% (OR 11.5, p<0.001) Number of trips to operating theatre (OR 1.32, p<0.001) Number of days on mechanical ventilation (OR 1.035, p<0.001)
Klein et al., 2007 [28]	Single centre tHO+, *n*=45 tHO-, *n*=45	Time to elbow wound closure (OR 1.08, 95% CI 1.4-1.12)

n = no. of subjects. tHO+: subjects with tHO. tHO-: control subjects without tHO. tHO: traumatic heterotopic ossification, ICU: intensive care unit; TBSA: total burn surface area.

2.3.2 Neurological (SCI and TBI)

In a systematic review and meta-analysis of the SCI population, males, smokers, and patients with complete injury, pneumonia, spasticity, and pressure ulcers had significantly higher odds of developing tHO [32]. In SCI, tHO appears to occur below the neurological level of the injury and is more common in cervical and thoracic injuries than lumbar injuries. However, completeness of the spinal cord lesion is suggested to be more predictive of the development of tHO than the level of injury [46]. Risk factors for tHO after TBI include the severity of the injury, surgically treated concomitant fractures, autonomic dysregulation, duration of immobilisation, spasticity and presence of pressure ulcers and systemic infection [13, 47-49]. Most studies, however, have been conflicting in their outcomes, shown limited association and or are limited by design.

THO in the neurological population typically occurs more often in spastic than flaccid limbs. However, it is unclear if tHO precedes spasticity or vice versa [46]. Reznik et al. [13] found that the risk factors associated with tHO following TBI and SCI were almost entirely distinct, with deep vein thrombosis (DVT) remaining the only common risk factor in both cohorts. Thus proposing, the mode of injury contributes to tHO formation, as the causes of upper motor neuron lesions differed in TBI and SCI patients [13]. Location of injury (cervical vs thoracic) and DVT were not found to be associated with tHO development after SCI [32]. Combined CNS and peripheral musculoskeletal injuries (i.e., muscle injuries and bone fractures) are associated with an increased risk of developing tHO [15, 50]. Patients with bone fracture combined with severe TBI are at increased risk of tHO development [51]. TBI patients show a faster rate of fracture healing accompanied by hypertrophic callus formation or heterotopic ossifications [52]. However, the development of tHO occurring in the presence of peripheral musculoskeletal injuries after burn injury remains unexplored.

2.3.3 Orthopaedics

Traumatic HO has been linked to tissue injury from total hip arthroplasty (THA) [53, 54]. The occurrence of tHO is thought to depend on the THA surgical technique [55]. Patients who undergo a lateral or anterolateral approach are at a higher risk of HO than those undergoing a posterior approach [17, 53, 56]. Although, HO has been noted in 10-40% of patients having direct anterior or anterior-based muscle-sparing approaches. Wilke et al. [55] concluded that the direct anterior approach had the lowest overall odds for HO, and the anterolateral approach (OR 1.85, p=0.033) demonstrated an increased association with the post-operative development of severe HO (Brooker

Grade 3, 4). This concurs with studies assessing the role of surgical approach in the risk of tHO after fixation of acetabular fracture [57]. The higher degree of surgical trauma associated with lateral approaches compared to an anterior and posterior approach may reflect an increased level of soft-tissue injury, which is implicated in the pathogenesis of tHO [58].

2.3.4 Common Trauma Risk Factors

2.3.4.1 Critical Care Factors

In trauma aetiologies, the extent and progression of bone formation are proportional to the severity of injury incurred [54]. Prolonged immobilisation due to coma and sedation, the requirement for ventilatory support and early contracture positioning and splinting results in prolonged pressure on high tHO risk areas [2]. Huang et al. [52] suggested that inadequate local blood and oxygen supply to tissues may contribute to a favourable environment for tHO development. Further supporting mechanical compression contributing to tHO risk following burn injury, is using the elbow for leverage when exiting bedrest and the constant pressure at the elbow in immobilised patients, often positioned in upper limb extension with the forearm supinated [2]. Prolonged mechanical ventilation may change the body's electrolyte and acid balance, thereby increasing fluid pH, promoting calcium deposition, and accelerating fracture healing and callus formation [27].

Inflammatory conditions, vascular diseases and bone-forming diseases such as ankylosing spondylitis appear to affect the risk of tHO [59]. Interpretation of serum calcium, phosphorus, and alkaline phosphatase (ALP) levels to identify those at risk shows conflicting results [24, 60, 61], potentially due to the disruption related to post-trauma hypermetabolism, electrolyte imbalance or sepsis [2, 24, 27, 45]. Serum ALP is an indicator of bone or liver disease, and bone specific ALP (B-ALP), an isoform of ALP, is considered a marker of bone formation [62]. Elevated ALP expression correlates with early pathological ectopic ossification *in vivo* [63-65]. Differentiating early tHO formation from the aetiologies of elevated ALP in trauma patients, such as healing fractures, vitamin D insufficiency and thyroid or parathyroid disease, is a challenge due to the reliability of raised serum ALP or B-ALP as a sensitive indicator [14, 27, 61].

2.3.4.2 Local and Systemic Infection

Acute or chronic infection, either systemic or local, are common risk factors associated with trauma-induced HO [26, 66]. Pavey et al. [67], using a rat model of high-energy combat injury, showed a

significantly higher volume of ectopic bone at the amputation site with the early colonisation of Methicillin-resistant *Staphylococcus aureus* (MRSA), the predominant pathogen in severe burn wound infections [66]. Human clinical studies are required to validate these experimental findings. After a neurological injury, patients who developed tHO were more frequently infected with gram-negative bacilli, particularly *Pseudomonas* aeruginosa. However, this data must be interpreted cautiously due to the small sample size (n=6). Orchard et al. [25] illustrated a univariate association between sepsis and tHO after burn injury; however, after adjustment for injury severity, sepsis was no longer significantly associated with the risk of tHO. Thus, to the best of the author's knowledge, no prior investigations in burn and neurological trauma cohorts have specified the infectious pathogen associated with tHO risk using multivariate analysis.

2.3.4.3 Concomitant CNS/PNS Injury

An elevated risk of tHO has been established with mechanical injury to peripheral nerves [53]. Adaptive changes to the compartments of peripheral nerves caused by stretch, compression or transection lead to the opening of the BNB [68]. The choice of surgical technique performed at the hip may lead to stretching of the adjacent sciatic nerve, significantly disrupting the tight junction regulation, a hallmark of the BNB [53]. In the residual limbs of amputees without concomitant injury, tHO is often associated with neuroma and schwannoma formation, postulated to be due to the severing of the nerves that innervated the limb [8, 69, 70]. Elbow injuries have a significantly higher risk for tHO, with the incidence up to 37% following fracture or dislocation of the elbow [8, 53, 54]. Levi et al. [24] proposes that the anatomy of the elbow joint, with the superficial ulna nerve, predisposes it to mechanical compression or stretching. Fracturing or dislocating the bone is almost impossible without injuring the nerve [8]. However, this link has not been documented after burn injury despite the association of injury to the central or peripheral nervous system with tHO.

2.3.4.4 Future Directions

Thus, although there are known risk factors that are associated with tHO, research to date has provided minimal translatable results to enhance clinical management. Given the dearth of high-level evidence, particularly in the burn patient context, more purposeful data pooling may be a solution to overcome the research challenges due to the low event rate of tHO in individual trauma populations.

2.4 Onset and Differential Symptomology

Pain, decreased ROM, intrinsic muscle weakness, and joint contracture remain the principal clinical signs of HO [2]. In the burn population, Foster et al. [3] highlighted the unique symptomology of tHO and listening to the patient's voice in the early stages before formal diagnosis of tHO. Participants described a unique and severe pain experience of distinctive nature and location, distinguishable from pain related to their burn (wounds and, or scars). Patients reported pain on palpation over the triceps tendon and medial epicondyle. The location of pain experienced was consistent with previous findings that, at the elbow, tHO typically develops posteriorly, deep to the triceps extending from the epicondyles to the olecranon process [54, 71]. Patients noted a 'locking sensation' at extension-flexion limits of the elbow, not associated with scar contracture alone. Many stated the aggressive and rapid loss of movement at the joints affected. Foster et al. [3] results suggest movement restrictions present at 4-5 weeks post-burn injury may indicate tHO.

While the hip is the dominant site in both SCI (up to 97% of cases) and TBI patients (up to two-thirds of cases) [72], it is more common for TBI patients to present with tHO in other joints, such as the elbow and shoulder and multisite involvement is more common than in SCI patients [13, 17] Traumatic HO is commonly found at two months post neurological injury, however this can range from two weeks to twelve months [14, 33]. At the hip, tHO most commonly forms in the flexor (anterior) or adductor (medial) anatomical compartments and is associated with warmth, swelling, erythema and soft tissue breakdown. These symptoms are similar to other inflammatory conditions, including osteomyelitis, cellulitis, and DVT, challenging definitive tHO diagnosis. Localised pain can occur from the prominence of the tHO under the skin and or from compression of neurovascular structures [3].

In ~20% of neurogenic HO patients, bone formation progresses to cause peripheral nerve impingement/entrapment. Compression of the sciatic nerve at the hip can lead to neuropathic pain, numbness, and lower limb weakness [54]. The resultant restrictions in ROM and severe pain experienced can be extremely debilitating [73]. Acute ulnar nerve entrapment is present in ~12% of cohorts with tHO. The symptoms of neuropathic pain and marked motor and sensory deficits of the upper limb are diagnostic for tHO, although not often the initial signs complained of in neurological cohorts [2].

The extent of HO after hip arthroplasty varies from small islands of bone to complete ankylosis of the hip and, commonly, pain is experienced at the lateral hip region [54, 56]. In an orthopaedic population, Kocic et al. [74] demonstrated that severe HO reduced clinical outcomes, although overall range of motion in tHO affected hips, has no significant effect on reported pain levels. In contrast, localised pain was reported at the site of tHO following intramedullary nailing of femur fractures. Typically, ectopic bone forms in the abductor muscle adjacent to the entry site of the nail [54].

2.5 Detection and Diagnosis

Without an accurate and reliable early diagnosis of tHO, there is little chance for early intervention, and late detection may result in maturation of tHO and joint contractures, nerve entrapment and marked pain [2]. Emerging research focuses on diagnostics with high sensitivity and specificity for early detection of tHO, particularly scanning and tissue imaging [66] dependent on the specific trauma in question, anatomic location of HO, swelling, the extent of soft tissue involvement, and the tHO burden [66].

Clinicians rely on radiography (X-ray) followed by 3-phase bone scintigraphy to confirm the diagnosis and establish the extent and the metabolic activity of HO [7, 66, 75]. Yeh et al. [76] reported that ectopic bone generally does not show on plain film until four to eight weeks after the initial symptoms, suggesting an x-ray has low sensitivity or specificity for early diagnosis of HO—similarly, Lin et al. [75] reported tHO an average of 23 days after symptom development, supporting that X-ray was inappropriate for early diagnosis. Three-phase bone imaging is the gold standard for visualising early tHO [7, 66]. Using this scan, soft tissue swelling is detected after three weeks, and calcification can be identified a week later [66]. However, soft tissue inflammation, as seen in burns and combat-associated tHO, leads to potential difficulties in discriminating tHO from other traumatic processes of the skeleton and false positives [66, 75].

Hybrid SPECT/CT, when applied to 99mTc-MDP bone scintigraphy, assists in more precise localisation and confirmation of abnormal radiotracer activity within soft tissues and improves the diagnosis of tHO lesions [77]. Alternative imaging modalities such as Raman Spectroscopy and Positron emission tomography (PET) combined with CT, either using radiolabelled fluoride (F18) or

radiolabelled glucose (FDG) are novel diagnostic modalities showing promise though lacking robust validation in human contexts [78-80].

Expensive and time-consuming radiographic techniques, including CT and MRI, provide high-resolution visualisation of late-stage tHO, and 3D CT reconstruction can show the exact anatomic location of tHO and is thus helpful for preoperative evaluation [2, 27]. MRI can be used to diagnose mature tHO, since the signal associated with early tHO lesions is heterogeneous [81]. Combining MRI and CT imaging dramatically increases the sensitivity to distinguish early vs mature tHO, infections, and soft tissue inflammation [12].

Chen et al. [27] proposed a cost-effective solution to the early differential diagnosis of HO in a stroke patient [76]. The case report indicated that serial ultrasonography (US) provided an earlier and more specific diagnosis of a hip muscle HO lesion than X-ray or MRI; the latter indicated necrotising fasciitis initially [76]. Ultrasonography depicted clear disease progression, with calcification visible on day 15 after the onset of symptoms [76]. US showed high sensitivity and specificity for early HO diagnosis one week after total hip arthroplasty [82]. Further, evidence of HO was detected by US up to 10–14 days before radiographic evidence appeared [82]. In contrast, Perosky et al. [83] stated that although US can detect HO sooner than conventional radiology, it cannot distinguish new bone formation from less mineralised mature bone. However, due to accessibility, low relative cost and lack of radiation, US is a feasible and safe bedside screening tool for early diagnosis of post-traumatic HO. Although user-specific, US is a convenient and repeatable modality for following up on the disease progression [27, 76]. The efficacy of Spectroscopic ultrasound (SUSI) has been demonstrated by Ranganathan et al. [84] in the identification of developing HO lesions as early as one-week post-injury. Utilising supra-spectral ultrasound frequency, this novel modality penetrates dense tissue structures otherwise impermeable to conventional ultrasound [12, 85].

2.6 Classification

The severity of tHO formation can be characterised by site-specific classification systems most relevant for per-articular tHO [86, 87]. At the elbow, the severity of HO is commonly classified using the Hastings and Graham scale. This scale of 3 classes (I-III) depicts functional limitation of ascending severity, with class III depicting complete ankylosis of the elbow [87]. The degree of tHO

at the hip is characterised by the four-stage ascending severity classification by Brooker [86]. This simple and valid measurement correlates with the clinical picture of hip function. In the orthopaedic population, a higher severity (Brooker grades 3 and 4) of tHO may cause functional limitation and associated reduced hip range of movement (ROM), antalgic gait, reduced walking capacity and increased use of analgesia [88, 89]. Pohl et al. [88] concluded that tHO grades 1 and 2 did not influence the functional status of the hip following THR.

2.7 Prevention and Treatment

The selection of appropriate treatment is complicated by the variability of the inciting injury predisposing to ectopic bone, complex risk stratification and diagnostic limitations. Thus, there is little consensus on optimal prophylactic regimes and treatment options [2, 3, 12].

2.7.1 Pharmacotherapy

Indication for drug therapy varies with the stage of tHO, and studies have included prophylactic measures to prevent the formation of ectopic bone [7, 90]. Non-steroidal anti-inflammatory drugs (NSAIDs) prevent the formation of tHO by inhibiting cyclooxygenase (COX) enzymes through prostaglandin inhibition and suppression of mesenchymal cell differentiation into osteoblastic cells [27, 91]. It is thought that blocking prostaglandin synthesis may also indirectly mediate BMP expression in soft tissues and inhibit heterotopic bone formation [14]. In a randomised clinical trial of SCI patients, prophylactic treatment with slow-release indomethacin (75mg daily for three weeks) from 3 weeks after injury significantly reduced the incidence (25%) of early tHO, compared with placebo (65%; $p<0.001$) [92]. Macfarlane et al. [93] corroborated these findings, achieved with a 7-day minimum course of indomethacin postoperatively (25mg three times daily [TDS] or 50mg twice daily) after THA and 25mg TDS for six weeks following acetabular surgery.

After hip surgery, non-selective (COX-1) NSAIDs did not significantly differ from selective (COX-2) NSAIDs in the prevention of tHO [94]. However, COX-1 NSAIDs cause more gastrointestinal side effects and are more likely to be discontinued than COX-2 NSAIDs or placebo [94]. The benefit of indomethacin and COX-2 NSAIDs in preventing and treating tHO with wounds and burns is unclear as clinical trials are yet to be conducted.

Etidronate disodium (EHDP), a bisphosphonate, inhibits hydroxyapatite crystal formation and osteoclast function and delays matrix mineralisation and may diminish the incidence or severity of tHO [95]. In SCI, TBI, and orthopaedic surgery populations, EDHP was purported to prevent the development and progression of the tHO [96]. Yet, as bisphosphonates do not inhibit bone matrix synthesis, other studies suggest that EHDP is ineffective or that its inhibitory effect is transitionary and tHO returns with discontinuation of the drug [97-101]. Treatment with new generation nitrogen-containing biphosphates, such as alendronate and pamidronate, has fewer adverse effects; however, current data is conflicting [102]. Further research is needed to ascertain the effectiveness of second-generation bisphosphonates in the secondary prevention of tHO after burn and other trauma [25, 103, 104].

In addition to BMP signalling, other dominant signalling pathways in tHO pathogenesis are thought to occur through the mammalian target of rapamycin (mTOR) and retinoic acid receptor pathways [105-107]. Rapamycin has been shown to suppress bone formation in experimental models of tHO through inhibition of mTOR complexes and is currently being studied in a phase II clinical trial [UMIN000028429] for the treatment of genetic HO, fibrodysplasia ossificans progressiva; however, new insights may also extend to the post-traumatic population [105].

Promising reports of effective inhibition of trauma-induced HO via the pharmacological activation of Palovarotene, a retinoic acid receptor γ (RARγ) agonist, are emerging [108-110]. Chondrogenesis requires decreased retinoid signalling with an upregulation of pro-chondrogenic pathways, including BMP signalling. Shimono et al. [108] suggested that RARγ agonists likely act to inhibit the chondrogenic stage of endochondral tHO by maintaining active retinoid signalling whilst dampening BMP signalling. In a rodent model following blast-related amputation, treatment with Palovarotene dampened the systemic inflammatory response, as well as the local inflammatory response and produced a 98% decrease in osteogenic connective tissue progenitor colonies with decreased expression of osteo- and chondrogenic genes [111]. Based on experimental evidence, these results suggest that Palovarotene is a potent inhibitor of traumatic HO. In a HO mouse model, Sinha et al. [110] demonstrated that when combined with corticosteroids, Palovarotene inhibits the recruitment of key tHO instigators, immune and inflammatory cells, at the site of injury. A phase III clinical trial [NCT03312634] is underway exploring Palovarotene for the prevention of new HO

lesions in adults with FOP. However, RAR agonists remain understudied in humans with post-traumatic HO [108].

2.7.2 Surgery

Surgical resection is often associated with complications and a high risk of recurrence [112]. The recurrence rate of clinically significant tHO is 17– 58% [112]. Surgical resection of HO is indicated in patients with limited ROM after non-surgical treatment. To reduce recurrence, a delay of 12–24 months is recommended [113]. However, prolonged dysfunction is potentially compounded by secondary scar, soft tissue contracture, joint fibrosis, joint arthrosis from ligament and joint capsule fibrosis, muscle atrophy, and articular cartilage degeneration [113, 114]. Delay in surgery was associated with up to 30% of patients with permanent loss of ROM (>25% of normal range), [115, 116] and up to 25% postoperative persistent ulnar nerve lesions [117]. Lee et al. [118] reported a high overall complication rate of 22.6% following the excision of tHO around the elbow. These complications include recurrence requiring reoperation, fracture, infection, nerve palsy and wound complications and can result in substantial morbidity.

In contrast, delayed surgery did not correlate with the clinical necessity of tHO resection after neurological trauma [72]. Early excision of HO has been explored. Chen et al. [113] suggested that early excision simplified the operative procedure and reduced the risk of damage to structures important for elbow stability. After SCI, early excision of peri-articular tHO with early mobilisation has effectively allowed patients to reach their functional potential and reduced pain associated with joint ankylosis [119, 120].

2.7.3 Radiation Therapy

Radiation therapy (RT) is suggested to inactivate mesenchymal stem cells and prevent their differentiation into skeletogenic cells [108, 121]. Most studies assessing the effectiveness of prophylactic RT on tHO occurrence and reoccurrence include cohorts undergoing hip surgery. A meta-analysis of twelve randomised controlled trials determined no significant difference between RT administered preoperatively or postoperatively in preventing tHO progression [122, 123].

After SCI, Sautter-Bihl et al. [124] concluded that RT is an effective neoadjuvant and adjuvant treatment option with progressive bone formation prevented in 50 out of 70 hips [124].

Furthermore, a meta-analysis of randomised trials shows that RT administered after major hip procedures is, on average, more effective than NSAIDs in preventing clinically significant tHO (Brooker Grade 3 or 4) and further when RT is administered at higher doses exceeding 6 Gy, there is evidence for the presence of a dose-response relationship with RT becoming increasingly superior to NSAIDs [125]. Maender et al. [126] recommended using peri-operative RT routinely in conjunction with surgery to reduce recurrence rates of tHO after burn injury. However, the literature on the burn's population is limited and remains controversial, with many clinicians expressing concerns about the long-term side effects of local radiation, such as the development of secondary malignancies [45, 126].

2.8 Length of Stay Outcomes

Due to diagnostic limitations, lack of consensus on optimal prophylactic regimes and sparse treatment options, both primary and secondary symptoms accumulate over time, leading to severe functional limitations, which impact the independence and speed of recovery of the patient [3]. Loss of movement is undoubtedly associated with poor functional outcomes, and in many patients, tHO can become the prime factor in the inability to perform activities of daily living efficiently [3]. For example, with a loss of elbow ROM, it is challenging for patients to compensate at adjacent joints, particularly for personal hygiene, requiring near full extension, and other routine activities requiring near full flexion [44, 113]. As such, it is reasonable to postulate that tHO is associated with a prolonged index hospital length of stay (LOS), increased medical care utilisation and associated costs, and a heightened risk of re-hospitalisation.

Previous investigations reporting on LOS outcomes in tHO patients have been conducted in Australian [13] and international trauma centres [25, 47, 127]. Prior studies of a tHO diagnosis and hospital LOS have been limited by design and interpretation, including small sample sizes and failure to account for confounders of a prolonged hospital stay, such as age and injury severity.

2.8.1 Burns

Only one prior study assessed the association of tHO with hospital LOS by multivariate regression analysis that adjusted for burn injury severity. Orchard et al. [25] demonstrated a statistically significant difference in the length of hospital admission between the tHO (n=19) and the non-tHO

(n=19) group (99.8 days vs 15days, p<.001). However, after adjusting for injury severity, binomial logistic regression did not identify LOS as a variable independently associated with tHO development. Other data have allowed only a superficial examination of the factors related with prolonged LOS in tHO cohorts. The direction of effect between these variables has not been confirmed **(Table 3)**.

Table 2. *Length of stay outcomes associated with tHO after burn injury*

	ICU LOS	**Acute LOS**	**Rehabilitation LOS**	**Total LOS**
Yelvington et al., 2019 [128]	-	tHO+, 65 (31) *vs.* tHO-, 31 (37) $p < 0.0001$	tHO+, 30 (32) *vs.* tHO-, 21 (14) $p=0.0465$	-
Levi et al., 2015 [24]	-	-	-	tHO+, 74 (48) *vs.* 20 (21) $p<0.001$
Orchard et al., 2015 [25]	tHO+, 22 (15-34) *vs.* tHO-, 7 (3-12) $p<0.001$	-	-	tHO+, 99.8* *vs.* tHO-, 15 (5-26) $p<0.001$
Medina et al., 2014 [45]	-	-	-	tHO+, 128.8±14.5**
Chen et al., 2009 [27]	tHO+, 82±76 (range 26-240)**	-	-	-
Hunt et al., 2006 [91]	tHO+, 79±56**	-	-	tHO+, range 26-349**

Length of stay outcomes included for index hospital admission only. Presented in days as mean±SD or median (IQR) if not stated otherwise. *IQR not reported. **no comparison group and univariate analysis not conducted. tHO+: burn injured patients diagnosed with tHO, tHO-: burn injured patients without a diagnosis of tHO, ICU: intensive care unit, LOS: length of stay

2.8.2 Neurological (SCI and TBI)

Prior studies assessing the relationship between tHO and a prolonged hospital LOS in neurological trauma populations have shown weak association in univariate analysis but have failed to show significance when subjected to multivariate analysis. In a logistic regression analysis of factors associated with tHO in TBI patients, Reznik et al. [13] did not find total LOS to be independently associated with tHO (OR 1.00, 95% CI 1.00-1.01, p=0.120), and no significant univariate association was determined between LOS and tHO in SCI patients (tHO+, 207±175 vs. tHO-, 122±113, p=0.055). Johns et al. [129] revealed no significant differences in acute care LOS in TBI patients matched (1:1) on age, gender and injury severity however, the tHO group stayed an average of 22 days longer in inpatient rehabilitation than the non-tHO group (tHO+, 50.89 *vs.* tHO-, 28.62, p<0.01). This study was also limited by small sample size (tHO+, n=26) and not able to determine the contribution of secondary factors to differences in the LOS [129].

2.8.3 Orthopaedic Injury

In the orthopaedic literature, studies investigating LOS outcomes and tHO explore the duration of time interval between injury to operative procedure and include mostly critically ill patients [57, 130]. Delayed time to surgery is positively correlated with tHO risk [57, 131, 132]. After THA, average hospital LOS was similar between patients who did and did not develop tHO (tHO+, 3.02± 1.69 *vs.* tHO-, 2.67±2.61, p=0.30) [130]. However, the incidence of acute fracture was only 9.6% and these findings may not be true reflection of a post-traumatic population. Thus, as in the burn and neurological literature, prior studies have largely focused on modelling LOS as a factor in tHO development and thus, by design have not explored the independent influence of a tHO diagnosis on total hospital LOS.

2.8.4 Future Directions

Reducing hospital LOS is a core strategy for reducing health care costs. Further, finding potential solutions to reduce long-term occupation of beds helps trauma units operate efficiently, tailor individual patient care, set realistic patient expectations and thus, improve patient outcomes. However, prior investigations across trauma populations have focused on single centre and single population outcomes, where small sample sizes due to low event rate have limited the power of statistical analysis to identify tHO as an independent factor for predicting LOS. Thus, in order to improve the quality and efficiency of acute and rehabilitation injury care multi-centre studies by

design and by pooling at-risk trauma populations will enable a sufficiently large sample to estimate the magnitude of effect of tHO on LOS through multivariate modelling

Chapter 3.

IBM Watson AI-enhanced Search Tool Identifies Novel Candidate Genes and Provides Insight into Potential Pathomechanisms of Traumatic Heterotopic Ossification

Overview

The first guiding question addressed in this thesis, "*What is the current state of the science regarding pathomechanisms, diagnosis and treatment of diseases associated with ectopic bone formation?*", is addressed in this chapter.

Although systematic reviews are considered the gold standard in knowledge synthesis, they have significant limitations. Typically, execution of the methodology is a time expensive process, purposefully focused on a narrowed clinical question. Rigorous searches and syntheses of refined evidence has yet to provide clear understanding of the pathophysiology of tHO. Thus, alternative methods, such as cognitive computing approaches are needed to interrogate a pooled corpus of research reports and change the outcome expected from synthesising literature in traditional ways.

This Chapter incorporates the novel application of a cognitive computing platform, IBM Watson for Drug Discovery (WDD), to investigate novel gene and protein candidates that may be implicated in tHO. This chapter comprises Study 1 of this programme of research, which was published in *Burns Open* in July 2023 [133] (Appendix 8). The results of this study contributed to the rationale behind the investigation of clinical risk factors in tHO development (Chapter 5, Part B) and the design of basic science studies (Chapter 6).

To support the plausibility of the results presented in Study 1, WDD was used to conduct a literature review of known pathophysiological mechanisms, biomarkers, and emerging therapeutic targets in diseases of ectopic bone formation was carried out. The supporting literature review can be found in Appendix 1I.

3.1 Introduction

Traumatic heterotopic ossification (tHO) is a pathological process characterised by the production of mature, lamellar bone at non-skeletal sites, such as within muscle and connective tissue [134]. Post-traumatic HO [18], traumatic HO [19] or acquired HO [20] are used interchangeably in today's literature. Well known to burn clinicians, tHO is a debilitating sequela of local and systemic inflammatory insult [2]. Other accounts of tHO have been reported following high-velocity blast injury [9, 12]; traumatic brain injury and spinal cord injury [13-15]; and after fracture, dislocation and soft tissue trauma [16, 17]. The rate of disability is variable, with some tHO lesions considered clinically irrelevant. In contrast, others may incur significant patient morbidity, and patient quality of life can be compromised by movement dysfunction and severe pain [3, 135].

Over recent years, a body of research has emerged to elucidate the pathophysiological processes underlying tHO. The contemporary understanding of tHO pathogenesis describes a process induced by central or peripheral nervous system (CNS, PNS) injury [8, 11, 53, 136, 137]. It is thought that tHO arises due to aberrant tissue repair in the presence of persistent neuroinflammatory dysregulation, leading to ectopic bone formation at the peripheral injury site via endochondral or intramembranous ossification [8, 11, 53, 136, 137]. Multipotent cellular contributors of tHO have been identified from a host of tissues; specifically, the multipotency of local mesenchymal stem cell (MSC) populations - identified as chondro-osseous progenitors that appear to arise from different

local soft tissues and, or peripheral nerves [12, 138-140]. However, despite recent efforts to elucidate the pathological processes underlying tHO, the precise mechanisms by which injury initiates tHO formation are poorly understood [141]. No genetic mutation has been found to be causally linked to the development of tHO in trauma cases [19].

New technologies such as cognitive computing offer a means by which the assimilation of novel answers to challenging research questions can be discerned [142]. With the increasing volume and diversity of biomedical literature sources, manual assimilation of the body of knowledge is becoming increasingly challenging for human practitioners [143]. This creates a gap in the ability to design future studies based on the sum of knowledge generated to date and may increasingly hamper future research, particularly in complex and rare conditions such as tHO. Cognitive computing provides a new method to rapidly retrieve pertinent information from a wide corpus of the biomedical literature, making it easier to uncover and extract new insights from existing data [5, 144]. Integrating diverse literature datasets, including clinical trials, patents, and other sources, into the searchable repository can further enhance the utility of cognitive computing approaches to assimilate research reports and assist in the development of novel hypotheses or identification of potential therapeutic targets [5, 142].

The capability of the IBM Watson for Drug Discovery (WDD) platform to facilitate the generation of new hypotheses about the relevance, function, or linkage between genes, drugs, and diseases of interest has been validated through recent scientific discoveries [1, 4, 145]. By combining natural language processing (NLP) with expert-curated data sources found in the literature, the cognitive-based computing platform, embodied by WDD may offer an effective solution to accelerate basic science discovery in traumatic HO [142].

In this study, we utilised WDD to interrogate the literature and identify candidate genes and pathways that may play a role in tHO. To the best of our knowledge, this is the first time this strategy has been applied to develop new hypotheses to investigate the pathological processes underlying the genesis and propagation of traumatic HO.

3.2 Methods

3.2.1. General Methodology and Approach

IBM Watson for Drug Discovery (WDD) is a discovery platform that uses cognitive computing and natural language processing to read large bodies of text and then apply predictive analytics to text to help researchers identify and rank promising genes and protein candidates for further evaluation. WDD's functionality has been described in detail previously [1, 4, 146]. The WDD platform enables two main types of capabilities. First, large volumes of information from unstructured natural language text can be read in a manner with a nuanced understanding of the syntax and meaning of complex biological relationships between genes, diseases, and drugs. WDD can produce interactive visualisations of these relationships (biological relationship network extraction). Semantic relations are discovered using rule-based and machine-learning-based approaches to understand mentions of two distinct entities according to their context, which co-occur in the same sentence of a document [142]. WDD is also disease agnostic in that it will identify common gene and drug relationships across conditions, thereby enabling researchers to further understand a gene or drug's role through publications in completely different therapeutic areas. This capability facilitates generating new hypotheses about the relevance, function, or linkage between genes, drugs, and conditions of interest. By incorporating data from both unstructured and external structured sources, including data presented in figures and tables, WDD can provide a broader domain understanding [142].

The second primary function is that WDD enables the evaluation of a potential list of candidate genes, diseases, or drugs through a sophisticated predictive ranking model (predictive analytics). This function leverages features of text to suggest, for example, similarities between genes whose connection may never have been explicitly identified in the literature to have a role in, or link to, a given disease. Or it may have the same potential role that a group of genes already known to have that linkage based on individual features. The WDD Predictive Analytics engine requires two key inputs: a list of known entities (genes, drugs, disease, or chemicals) and a list of potential candidate entities to map a concept space based on linguistic similarity [142]. Instead of using known biology, the predictive analytics algorithms look for patterns in how entities of interest are described in reports [142].

The general application of WDD to this specific study was:

1. To use WDD's biological relationship network extraction analysis function to search the literature and identify common themes and biological relationships of relevance to tHO with different aetiology.
2. To use the predictive analytics function of WDD to identify new candidate genes that may be implicated in HO.

3.2.2. Biological Relationship Network Extraction

The directed relationships-based biological network analysis function of WDD was utilised first to extract the known semantic relationships between biological concepts from the scientific literature. Here, "Heterotopic ossification" was used as the initial search entity (condition) to identify known biological interaction types and establish explicit, directional relations between entity types, i.e., heterotopic ossification to genes and conditions/diseases. The strength of the relationship between two entities is depicted by value, representing the total number of connections to HO identified by WDD in the literature

Flexible, simple network visualisations of the genes and conditions based on the strength of association to heterotopic ossification were produced, with the ability to select the relationship of interest between HO (target type; condition) and a gene (source type). For example, a gene (source) with a predisposition or a regulation relationship with HO (target). The interactive relationship network allowed the user to select individual links in the network and drill down into the linked literature to understand how and confirm the plausibility of the relationship that connected the two entities was discovered. To validate WDD's predictions of genes that may be associated with tHO, a thorough search and review of the relevant linked literature supporting each association were conducted [146]. In addition to gene relationships, utilising the disease-agnostic capabilities of WDD (Table A1.3, Appendix 1E), literature synthesis and review of associated conditions of interest were also carried out.

3.2.3. Predictive Analytics Process

Having established that IBM Watson methodology is valid and capable of identifying genes and proteins likely to be involved in tHO (Appendix 1I), the predictive analytics application was then used to predict new interactions between genes and heterotopic ossification.

3.2.3.1 Candidate and Known Gene Sets for Predictive Analytics

Two sets of entities were uploaded for predictive analytics analysis, one with known similarities or properties (the Known set) and the other with unknown similarities or properties (the Candidate set). A set of 100 known genes was created based on the genes' previously defined associative roles in ectopic bone formation. This known gene set (Table A1.4, Appendix 1F) was generated through WDD's biological relationship network analysis function as previously described and was used to interrogate the candidate gene list. A candidate gene set containing 233 genes with a potential role in ectopic bone formation, obtained from a previous study, was included (Table A1.5, Appendix 1G) [147].

All entered entities were then queried over known medical literature available to the end of 2019, including 29 million MEDLINE abstracts, all the Open-Source PMC full-text journal articles and over a million licensed medical journal articles, patents, and public government reports to find the most relevant articles containing mentions of these entities in the literature. The WDD platform then calculated a similarity matrix containing a similarity index for each pair of entities, presented as a distance network (Figure A1.2, Appendix 1B) or similarity tree, providing a visual representation of the similarity between the candidate set and known genes entered into the search. Using a graph diffusion algorithm, Watson computed a predictive similarity score based on the similarity matrix, measuring each entity's similarity to all the known entities [147]. Predictive analytics output delineated a ranked list of 233 candidate entities based on their computed similarity score. The top 50 ranked from this list is shown in Table A1.6, Appendix 1H. A final list of the top 25 ranked genes **(Table 3)** most likely to be involved in HO was subsequently produced from the rank product analysis. An in-depth review of the relevant literature was conducted to identify any previous association with tHO before 2020.

3.2.4. Statistical Validation

To validate the predictive power of the model generation by WDD, a custom validation method was performed. The top five ranked entities (BMPR2, ACVR1, PTHLH, GDF2, TGFB3) in our known set comprised the validation set which was subsequently placed in the candidate set. This set was used to verify how similar WDD ranked these known entities compared to others in the candidate set. Watson then supplied a rating to the overall candidate validation based on the yielded p-values (1-

(low)-.05(medium)-0.01-(High)-0) based on two statistical significance tests: the Fisher's Exact Test and the Wilcoxon Rank Sum, as shown in **Table 4**.

3.2.5. An Augmented Literature Search using WDD

An in-depth review of the available literature up to the end of 2019 was carried out to assess (i) the ranked list of 100 genes and associated conditions with HO identified using the biological network analysis function (Appendix 1I), and (ii) the top 25 ranked genes produced from the rank product analysis. Firstly, to validate potentially novel entities (genes) identified by WDD, a search was conducted which included the (gene name) including gene symbol and other known aliases, with the following search terms, used in different combinations; "ossification", "calcification", "ectopic ossification" and "ectopic bone" and "osteogenesis". To identify texts that focused on the pathophysiological mechanisms, additional searches were conducted using the following terms: (gene name) AND "pathophysiology", "physiology", "pathogenesis", and "molecular mechanisms". To capture the depth and breadth of data, searches were not restricted by publication dates.

Articles were eligible for inclusion if they were published in English and full text, peer-reviewed primary research reports investigating each gene function(s) and, or pathophysiological role(s) in various disease states. In the absence of higher-level evidence, case series/reports allow the identification of rare clinical conditions and can provide a thorough review of important topics [148]. Thus, animal studies, case studies and case series have been included. As the pathophysiology of tHO in burns children was likely to differ from that in adults, paediatric studies were excluded. Conference proceedings and editorials were also excluded. The references of selected studies were pursued for articles that may have been missed via the electronic search, and full-text articles were retrieved where possible. Findings from each study were then analysed and developed into key domains to enable the recognition of recurring relationships across data.

3.3 Results

3.3.1. Biological Entity Network Visualisations

WDD automatically extracted relationship networks and illustrated the extracted genes with specific relationships between them. This was achieved by representing the sentence-level gene-condition connections identified earlier as a matrix. One hundred genes associated with

heterotopic ossification were mentioned in at least one document published before the end of 2019. They comprised the relationship network illustrated in **Figure 1** [1]. Watson's ranking of 100 genes based on the strength of association to heterotopic ossification and the depicted value for each associated gene is shown in **Table A1.2** (Appendix 1D). Bone Morphogenic Protein 2 (BMP-2) and Bone Morphogenic Protein 4 (BMP4) were identified as the top candidates by WDD, ranking 1 and 2 respectively and thus, representing the closest connected gene entities to HO as the search entity. Comparatively, Neural Cell Adhesion Molecule 1 (NCAM1) and Collagen Type II Alpha 1 (COL2A1), with ranks 66 and 65, respectively, are presented in farther circles, representing a less significant association with HO.

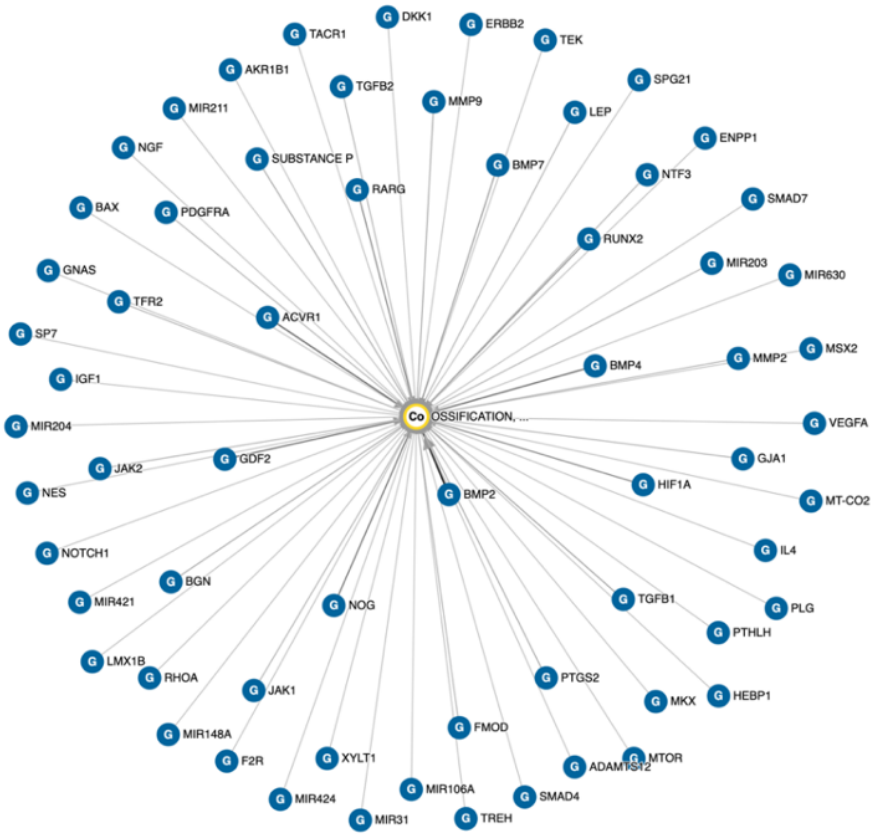


Figure 1. *Biological entity network visualisation of genes associated with heterotopic ossification. The searched entity (heterotopic ossification, condition [Co]) is represented by a white circle and connected entities (genes [G]) by blue circles. Distance from the searched entity (heterotopic ossification) represents the number of documents (value) supporting the connection: nearer circles are connected by relationships in more documents than farther circles [1].*

The semantic relationships between HO and genes identified by WDD and extracted as interaction networks of biological entities are illustrated in **Figure A1.1** (Appendix 1A). Evaluation of each connection in detail reveals sentence-level extractions, which in aggregate create a list of directional relations between two known entities. This relationship can be direct or indirect and is linked through a domain-relevant verb occurring in the same sentence, termed a trigger word. **Table A1.1** (Appendix 1C) provides examples of biologically relevant trigger words curated and used by WDD for semantic relationship extraction between genes (source) and heterotopic ossification (target). For instance, WDD extracted a negative regulation relationship between RUNX2 and HO that contained a direct relation via the trigger word 'inhibit'. The curated evidence supporting the semantic relationships identified by Watson guided an in-depth literature review to validate WDD's predictions of genes associated with HO (Appendix 1I).

The network of conditions that Watson has produced in real-time by using its annotators to extract relationships between HO and other associated conditions is shown in **Table A1.3** (Appendix 1E). The relationship evidence supporting the associations between other conditions and HO was further evaluated by review of the curated literature, as previously described (Appendix 1I). Conditions ranked highly by WDD for an association to HO included age-related or degenerative diseases such as arthritis and osteoporosis, as well as fibrosis and multiple bone-related cancers. Notably, conditions with an increased prevalence of HO previously reported in the literature, including burns [43], spinal cord and brain injury [13] and fractures [149], were all ranked in the top 30 conditions by WDD.

4.2 Results

4.2.1. Predictive Analytics

The predictive analytics application was used to expand research targets in HO and prioritise genes most likely to be of interest, where the corpus of data analysed by Watson was restricted to literature published up to the end of 2019. WDD ranked 233 candidate genes (Table A1.5, Appendix 1G) that may be associated with pathological ectopic ossification using a known set of 100 genes (Table A1.4, Appendix 1F) identified by WDD's biological relationship network analysis function as having previously defined associations with HO.

Watson ranked each candidate gene based on the semantic similarity of the gene to the 100 known genes, producing a final ranked list of candidate genes most likely to be involved in HO (Table A1.6, Appendix 1H). The predictive similarity score generated by WDD for each gene, as shown in **Table A1.6** (Appendix 1H), is a measure of an entity's similarity to all the known entities and ranked highest to lowest. A similarity score of 0.081 was generated for MMRN1 as the highest-ranked entity, indicating it is the most similar of the set. The ranked list of candidate entities was further analysed through visualisations. For example, the distance network in **Figure A1.2** (Appendix 1B) considers the values between all entities in the similarity matrix and represents the distance between any two entities. Candidate entities appear as red circles and when hovered on, the five most similar known entities (green triangles) are shown, enabling visualisation of the closest nodes to a node of interest.

The top 25 results of this ranking are shown in **Table 3**. Nineteen of the top 25 genes predicted by Watson to be associated with tHO, a thorough literature search demonstrated these genes had been previously investigated before 2019 and identified as likely candidates in tHO. Six of the top 25 genes were determined to have no apparent link to tHO before 2019 including Musculin/Myogenic repressor (MSC/MyoR), Integrin Alpha M/Cluster of Differentiation 11b (ITGAM/CD11b), Platelet-Derived Growth Factor D (PDGFD), and Gremlin-1 (GREM1) and NEL-like molecule-1 (NELL1). Further interrogation of the literature highlighted that these candidate genes had previously defined roles in inflammation, aberrant tissue repair and regeneration, extracellular matrix remodelling and mineralisation, endochondral or intramembranous bone formation and injury-associated bone reactions. Additionally, the genes had functional roles in pathways of osteogenic differentiation, including BMP and Wingless-related integration site (WNT) signalling and or defined roles in pathways relevant to HO and highlighted in the known gene list. Thus, these genes represented attractive candidates and were assessed further with an in-depth literature review.

***Table 3**. Combined Rank top 25 gene list*

Gene	Name	Rank	Score (GD)	Evidence of association with tHO prior to 2019
MMRN1 / EMILIN-4	Multimerin 1 / Elastin microfibril interfacer 4	1	0.081	No
IL-1α	Interleukin 1 Alpha	2	0.064	Yes
IL-15	Interleukin 15	3	0.049	Yes
MSC / MyoR	Musculin / Myogenic Repressor	4	0.049	No
ITGAM / CD11b	Integrin Subunit Alpha M / Cluster of Differentiation molecule 11B	5	0.048	No
PDGF-D	Platelet Derived Growth Factor D	6	0.046	No
SOST	Sclerostin	7	0.045	Yes
GREM1	Gremlin 1	8	0.044	No
CD14	Cluster of differentiation 14	9	0.043	Yes
CD8A	CD8a molecule	10	0.042	Yes
SPARC / ON	Secreted protein acidic and rich in cysteine / osteonectin	11	0.042	Yes
MMP2	Matrix metalloproteinase 2	12	0.041	Yes
CDKN1A / p21	Cyclin-dependent kinase inhibitor 1A	13	0.041	Yes
EPO	Erythropoietin	14	0.041	No
NOS2 / iNOS	Nitric Oxide Synthase 2 / Inducible nitric oxide synthase	15	0.04	Yes
IL1RN	Interleukin 1 Receptor Antagonist	16	0.04	Yes
SMAD7	SMAD family member 7	17	0.04	Yes
DKK1	Dickkopf WNT signalling pathway inhibitor 1	18	0.039	Yes
ANG1	Angiopoietin 1	19	0.039	Yes
VWF	Von Willebrand factor	20	0.039	Yes
TIMP1	Tissue inhibitor of metalloproteinases 1	21	0.038	Yes
KITLG / SCF	KIT ligand / Stem cell factor	22	0.038	Yes
IL2RA / CD25	Interleukin 2 receptor subunit alpha	23	0.038	Yes
NFATC1	Nuclear Factor of Activated T cells 1	24	0.037	Yes
NELL1	NEL-like molecule-1	25	0.037	No

4.2.2. Validation of Predictive Analytics Data

The results of the custom validation test showed how highly Watson ranked the validation entities compared with the remaining candidate set, demonstrating that Watson's predictive model for heterotopic ossification was highly accurate. **Table 4** shows WDD's ranking of 5 genes comprising the validation set when removed from the known set and placed into the candidate set. All five genes ranked within the top 15 places out of 233 candidate genes. Based on two statistical significance tests, the Fisher's Exact Test and the Wilcoxon Rank Sum, the yielded p-values were between 0 and .01, and the rating Watson supplied to the overall candidate validation was high.

Table 4. *Results of the custom validation test*

Validation set	Rank
GDF2	3
PTHLH	6
ACVR1	7
BMP2R	10
TGFB3	11
Fishers Exact Test P-value: 0.000151834 Wilcoxon Rank Sum P-value: 0.000116413 Validation rating is HIGH	

4.3 Discussion

This unbiased, evidence-based predictive approach of IBM Watson identified six candidate genes as potential new research targets in tHO, MMRN1, MSC/MyoR, ITGAM/CD11b, PDGFD, GREM1 and MSC. Scientific validation of these entities may contribute to understanding pathological changes identified in tHO.

Although systematic reviews are considered the gold standard in knowledge synthesis, they are, by definition, limited in scope and clinical or research applicability. This is particularly so where a paucity of high-quality studies are not available. There remains a substantial repository of knowledge that is difficult to assimilate in a systematic manner. Typically, the execution of the

methodology is a time-demanding process and is often focused on a narrow clinical question. However, in a clinical condition where rigorous searches and synthesis of refined evidence is yet to elucidate the unique pathophysiologic process, alternative approaches to interrogate large data sets may be beneficial to guide the identification of relevant genes, proteins and, or drugs of interest.

This study identified six new genes of interest in a list of 25 top-ranked genes, including 19 genes with evidence in the literature of a role in tHO. Of the six genes, further literature investigations demonstrated roles for these proteins in physiological and pathological processes that are known to be involved in tHO. Recent literature that has been published since 2019 supports several of the lower-ranked genes in our results [150]. Among the Top 6 genes, MMRN1 (ranked 1), also known as EMILIN-4, is a glycoprotein stored in platelets, endothelial cells (ECs) and megakaryocytes that is deposited into the extracellular matrix (ECM) [151]. MMRN1 is differentially expressed in several cancer cell lines [151, 152], inflammatory diseases [153, 154], and bacterial and viral infections [155, 156]. It may be necessary to maximise platelet adhesion at vascular injury sites by binding to collagen [157]. Recently, upregulation of MMRN1 was observed in serum exosomes from burn patients, a population at risk of HO development [158].

Similarly, *MMRN1* upregulation in the vasculature in response to increased VEGF-A and TGF-β signalling in the tumour microenvironment has been reported, suggesting a possible role in angiogenesis and VEGF signalling, which are involved in tumorigenesis and implied in the pathogenesis of tHO [159-163]. Currently, there are few studies relating MMRN1 to bone remodelling. Liron et al. [164] reported that the MMRN1 gene is expressed in bone tissue, pre-osteoclasts and non-differentiated osteoblasts. The authors observed a 55% lower bone volume in mutant mice attributable to the deletion of *MMRN1*, suggesting a physiological role of *MMRN1* in bone metabolism. Further research is required to elucidate the contribution of other distinct ECM influencers, such as MMRN1 and other members of the multimerin family, in the control of tissue microenvironments and hematopoietic stem cell niche function that can favour osteogenesis leading to pathological ossification. Considering that circulating Mmrn1 levels have been detected in the blood plasma, serum, urine, and saliva of cancer patients, Mmrn1 may serve as a potential biomarker for HO. However, further study is required [165].

Myogenic repressor (MyoR), also known as Musculin, is another candidate gene predicted by WDD to have a potential association with HO. MyoR was initially identified as a transcriptional repressor of muscle differentiation, and its expression is restricted to precursors of the skeletal muscle lineage [166]. MyoR is expressed at high levels in proliferating myoblasts and is downregulated early during myogenesis at the onset of differentiation. It has been speculated whether MyoR may be critical for selectively delaying the expression of certain muscle-specific genes during primary myogenesis or inhibiting myogenesis by inducing cell death or stimulating cell proliferation [167] [167]. Gagan et al. [168] showed that miR-378, a microRNA that is upregulated during differentiation by MyoD, promotes the transition from proliferating myoblasts to differentiating myotubes through targeting MyoR and repressing myogenic differentiation via inhibition of Myogenic differentiation 1 (MyoD) transcriptional activity. Along this line, it is worth noting the finding of Hupkes et al. [169]. This study demonstrated an effect of miR-378 on promoting BMP-2-induced osteogenic differentiation of C2C12 myoblast-like cells [169]. In addition to the critical role(s) of MyoR in skeletal myogenesis, tissue repair, differentiation, and regeneration [170, 171], accumulated evidence has also expanded MyoR function to the regulation of the inflammatory reaction and immune function in the context of trauma and disease [172-175]. Yu et al. [175] suggests that MyoR may act as a novel regulatory factor for maintaining the balance between excessive inflammatory reaction and tissue repair in the intestinal epithelium, whereby MyoR deficiency enhances inflammation via excessive secretion of IL-22, leading to aggravated colonic epithelial injury [175]. A more comprehensive understanding of the mechanistic actions of MyoR and how it affects the differentiation of myogenic and non-myogenic cells during adult tissue regeneration and interactive feedback in the context of acute traumatic injury is therefore warranted.

Ranked five by Watson was CD11b/Integrin subunit alpha M (ITGAM), a receptor originally described on neutrophils and macrophages that is responsible for supporting adhesion and molecular cross-talk of these cells with ECM proteins [176]. CD11b has been implicated in the development of several inflammatory diseases [177, 178] and has an oncogenic role where it is expressed on myeloid lineage osteoclast precursors [179]. However, a direct association between CD11b and pathological ossification has yet to be established. The findings of Park-Min et al. [180] identified CD11b as a negative regulator of the earliest states of osteoclast differentiation. These authors demonstrated that *CD11b*-deficient mice exhibited decreased bone mass associated with increased osteoclast numbers and reduced bone formation. Ehirchiou et al. [176] found that CD11b is expressed in

human chondrocytes and the ECM of articular cartilage from OA patients. The authors established a novel role of CD11b signalling in preventing chondrocyte hypertrophy and chondrocyte mineralisation *in vitro* [176]. More so, primary murine CD11b KO chondrocytes exhibited greater alkaline phosphatase (*Alp*) gene expression levels and production of pro-mineralising cytokine Interleukin-6 and monocyte chemoattractant protein 1 (MCP-1), sustaining the loop between inflammation and calcification [176]. Thus, it is possible that down-regulation of these calcification factors, via cross-talk with CD11b-dependent signalling pathways such as JNK and Nuclear factor-kappa B, maybe a potential mechanism by which CD11b exerts its role as a negative regulator of mineralisation in chondrocytes [176].

Notably, the presence of soluble CD11b has been confirmed in human plasma [177] and in the synovial fluid of OA patients [176], postulating that the primary source could be synovial cells and infiltrating inflammatory cells. These results suggest that CD11b may participate in mineral deposition, and it may be worth exploring the interaction of CD11b integrin signalling in the regulatory mechanisms that govern pathological mineralisation. Modulating CD11b integrin signalling may be an effective strategy to protect traumatised joints from developing inflammation-associated peri-articular ossifications.

Platelet-derived growth factors are potent mitogens and chemoattractants for cells of mesenchymal origins [181] and play an indirect role in facilitating bone formation via induction of MSC migration to the site of bone regeneration and by supporting the expansion of osteoprogenitor cells and promoting angiogenesis [181]. PDFG-D is mainly expressed in endothelial cells, smooth muscle cells and macrophages and plays a role in wound healing [182], fibrotic processes [183] and in disease processes including various cancers [184, 185] and atherosclerosis [186]. However, little is known about the role of PDGF-D in regulating physiological and pathological bone formation [187]. Wang and colleagues [188] showed that the downregulation of PDGF-D led to the inactivation of Notch-1 and NF-kB DNA binding activity, which resulted in the downregulation of target genes such as VEGF and the activity of MMP-9. Huang et al. [184] revealed that Pdgf-d regulates osteoclastic differentiation and promotes intraosseous tumour growth associated with increased osteoblastic bone responses in mice. These authors highlighted a novel function of PDGF-D in early bone remodelling. It was demonstrated that PDGF-D-specific signal transduction upregulated the expression of nuclear factor of activated T cells 1 (NFATc1), a master transcription factor for

osteoclast differentiation [181]. Thus, PDGF-D may be considered an osteoinductive factor that can modulate osteogenic capacity in a proinflammatory microenvironment and contribute to aberrant tissue healing and injury-associated bone reactions.

Gremlin 1 (Grem1) is a secreted glycoprotein and a BMP antagonist that preferentially binds to BMP-2/4/7 ligands in the ECM, opposing BMP effects on osteoblastic differentiation and function *in vitro* and *in vivo* [189, 190]. Thus, it has been proposed that GREM1 may play a key role in regulating endochondral bone formation [191]. Gazzerro et al. [189] demonstrated that deletion of *Grem1* in the bone microenvironment led to sensitisation of BMP and Wnt/β-catenin signalling and activity, enhanced ALP expression and increased bone formation *in vivo.* Moreover, the downregulation of Gremlin in osteoblastic cells increased the BMP-2 stimulatory effect on the Suppressor of Mothers Against Decapentaplegic (SMAD) signalling, ALP activity and enhanced expression of osteogenic markers (osteocalcin and Runx2). As Grem1 can block BMP activity, it likely influences SMAD 1/5/8 signalling and, in turn, the expression of hypertrophic markers. It has been proposed that bone-derived Grem-1 may work in conjunction with BMP-4 to initiate a catabolic and tissue remodelling program in hypertrophic chondrocytes and osteoblasts that favours the pathological remodelling of the osteochondral junction in OA subchondral bone [191]. Aberrant expression of Grem1 has also been associated with rheumatoid arthritis [192], fibrosis of the lung [193, 194] and kidney [195], in various cancers [196, 197] and other skeletal and connective tissue disorders [198, 199]. However, until recently, an association between Grem1 and tHO was not reported. During our WDD investigation, a paper by Yu et al. [200] showed that Grem1 expression is decreased in the early stages of traumatic HO in an Achilles tendon tenotomy rat model. Further investigation into the signalling mechanisms engaged by GREM1 under pathological conditions associated with ectopic ossification is warranted.

NELL-1 (NEL-like molecule-1; NEL [a protein strongly expressed in neural tissue encoding epidermal growth factor-like domain]), ranked highly by WDD as a potential candidate gene in tHO, is reported to control skeletal ossification [201]. NELL-1 is recognised as an osteo-specific growth factor with anti-adipogenic activities, capable of promoting osteochondrogenic cell differentiation and mineralisation *in vitro* [202-205]. The molecular mechanisms underlying NELL1-induced osteogenic differentiation are not fully understood; however, findings suggest that the osteoinductive activity of NELL-1 is particular to the osteochondral lineage [202, 204, 206]. NELL-1 mediates critical

downstream effects of master osteogenic regulator RUNX2 and plays a role in intramembranous and endochondral ossification [202, 207-209]. Aghaloo et al. [204] demonstrated that Nell-1 promoted osteogenesis in MC3T3-E1 osteoblasts and induced *in vivo* bone regeneration equivalent to BMP2. The ability of NELL-1 to direct BMP2-treated cells toward osteogenesis and repress adipogenesis requires intact Wnt signalling, and it has been proposed that Nell1- may also regulate Runx2 via canonical Wnt signalling [209]. In a mouse intramuscular transplantation model, bone marrow stromal cells transduced with *the Nell-1* gene formed mature bone via an endochondral ossification mechanism *in vivo.* Nell-1 was found to act synergistically with BMP to increase the responsiveness of myoblastic C2C12 cells to BMP-2 stimulation and promote bone regeneration. However, Nell-1 could not promote osteoblastic differentiation leading to ectopic bone formation independently of BMP-2 [210]. These findings demonstrate that Nell-1 activates Runx2 via a mechanism independent of the BMP/SMAD pathways and functions selectively on cells in the osteochondrogenic lineage or multipotent cells to undergo osteochondral differentiation and bone formation [202]. In an *in vivo* ectopic bone formation assay, overexpression of Nell-1 significantly enhanced mineralisation and maturity of BMP-9-induced bone formation of MSCs, whilst effectively suppressing BMP-9-induced adipogenesis [205]. Targeting the synergistic bone formatting activity of NELL-1 and osteogenic BMPs (such as BMP-2 and BMP-9) may be a viable therapeutic target in treating undesirable heterotopic bone formation.

In summary, this study has applied a cognitive computing approach to identify genes to be factors influencing trauma-induced heterotopic bone formation. The six most promising genes identified in this study may aid in accelerating focused research on specific candidates for laboratory assessment and validation. To aid this approach, WDD should be supplemented with known manually curated pathway information, e.g., Kyoto Encyclopedia of Genes and Genomes (KEGG), to augment what we extract from the text. Further assessment and laboratory validation of potentially novel targets identified will be necessary to conclusively assess the utility of the WDD [147]. Understanding the critical cellular events and pathways responsible for aberrant cell fate in HO might inform other disease processes such as muscle fibrosis, vascular calcification and other pathological processes involving the aberrant bone formation and allow improved targeted therapies that are amendable for therapeutic interventions of traumatic heterotopic ossification.

3.4.1. Limitations

While the use of WDD in tHO proved successful in identifying a novel set of plausible candidate genes that may participate in the pathogenesis of tHO, our approach has some limitations. Firstly, as Watson uses only sentence-level information to identify associations to a known set of proteins, analysis based on semantic similarity may pose challenges when evaluating particularly complex biological relationships, relying on a deeper understanding of the surrounding context and nuance of individually written sentences identifying the association. To mitigate this limitation, this study employed a manual review of the curated evidence supporting the semantic relationships identified by Watson to validate its predictions further. It is a potential limitation that at the time of this study, we did not have the technology, or scope to complete full paper translations, and papers and abstracts written in languages other than English had to be excluded.

In addition, the use of specific known genes is required to identify candidate genes of interest using the predictive analytics method. In contrast to gene ontologies or standard pathway analyses, given that the current input is likely based on literature evidence, this approach is only valuable for investigating genetic data with a priori hypothesis. It is even more pertinent to consider this when investigating conditions such as tHO with minimal high-level evidence in the current literature of implicated biological processes and genetic influences [4]. These limitations will likely be primarily overcome with advancements in AI platforms, algorithms, and the computing power available.

3.4 Conclusion

This study illustrated the utility of machine learning approaches to support the exploration, identification and prioritisation of promising gene relationships and targets in rare diseases like tHO. The genes identified in this study (MMRN1, MSC/MyoR, ITGAM/CD11b, PDGFD, GREM1 and NELL1) are potential new gene candidates for future studies investigating the pathobiology of tHO. Combining whole genome approaches and more extensive molecular-driven studies with advanced analytics and machine learning approaches can significantly accelerate research, thus streamlining pathways from basic science to clinical translation.

3.5 Key Points

- IBM Watson identified six novel candidate genes potentially involved in tHO
- Application of IBM Watson in tHO provided insight into potential pathobiological mechanisms
- Machine learning may aid target discovery in diseases with unclear pathophysiology.

Chapter 4.

Evaluation of the Accuracy of Diagnostic Coding and Clinical Documentation for Traumatic Heterotopic Ossification Diagnoses in Western Australian Tertiary Hospitals

Overview

The second guiding question in this thesis, "*What are the epidemiological characteristics of tHO diagnoses in West Australian hospitals over a 14-year period*?" was addressed in this chapter. Before this research, there were no published epidemiological data on tHO prevalence or data evaluating the accuracy of medical diagnostic coding and clinical documentation for the diagnosis of tHO. To address this methodological challenge and deficiency in existing data, this chapter presents an audit of the WA trauma database over a 14-year period to first establish the prevalence of tHO in trauma patients in WA tertiary hospitals and, through this process, evaluated the accuracy of ICD-10-AM coding and clinical documentation for tHO diagnoses. This chapter entails the introduction, methodology, results, and discussion for Study 2 of this research program. Study 2 was published in *Injury* in January 2024 [211] (Appendix 9).

4.1 Introduction

Traumatic heterotopic ossification (tHO) refers to the development of extra-skeletal bone in muscle and soft tissues following tissue insult secondary to trauma [2]. Cases of tHO are broadly classified into three aetiological categories: neurogenic HO resulting from traumatic brain injury (TBI) and spinal cord injury (SCI), orthopaedic HO developing after fracture, dislocation, and soft tissue trauma; and HO following burns and high-velocity blast injury [11, 12]. Traumatic HO manifests as a progressive condition, marked by rapid movement loss at affected joints and severe pain [3]. Each aetiology of tHO is associated with a unique clinical picture of prevalence, risk profile, and area of formation, which must be considered by treating clinicians [12].

The International Classification of Diseases (ICD) codes, currently in its tenth revision with Australian modification (ICD-10-AM), is a standard diagnostic coding system used by healthcare facilities to classify health problems and is essential for monitoring disease prevalence [212-215]. Coding accuracy directly impacts the decisions based on codes such as quality, costs, and effectiveness of care and influences funding, clinical and research decisions [212, 213]. However, ICD-10-AM diagnostic classifications typically do not provide enough detail to sufficiently capture patient data at the point of care for meaningful use in clinical and operational improvement and retrospective research [216]. The Systematised Nomenclature of Medicine - Clinical Terms, Australian release (SNOMED CT-AU), is an extensive dictionary of clinical terminology designed to record clinical information that has been adopted by health systems, especially those with electronic medical records (EMR) to facilitate consistent clinical language and documentation at the point of care, and the aggregation and exchange of clinical data between facilities that has a commonly understood meaning [216].

Assessing the accuracy of diagnostic coding is pivotal to ensuring the validity and reliability of administrative diagnostic data [215]. As completeness and accuracy of coding can vary based on coding practices and depends on the institutional culture of clinical documentation, it is essential to assess diagnostic coding in that local context [212]. There are no prior studies evaluating the accuracy of medical diagnostic coding for diagnosing tHO across Western Australia (WA) trauma centres or traumatic injury populations. Thus, the primary aim of this study was to evaluate and compare the clinical documentation and accuracy of ICD-10-AM coding for the diagnosis of tHO across four WA hospitals.

4.2 Methods

4.2.1. Study Participants

A retrospective data audit sought to identify patients with tHO admitted to four WA hospitals in April 2020. Adult patients were included if admitted at age 18 or over and discharged between 1st May 2005 and 1st May 2019 following neurological (TBI and SCI), burn or orthopaedic trauma. Subjects were excluded if death occurred or comfort care/palliation was instigated during their hospital stay.

A flow chart of patient identification, screening protocol and selection is shown in **Figure A2.1** (Appendix 2A). Potential tHO cases were identified in a cascading, three-tier search of the ICD-10-AM coded WA trauma database. Both primary diagnosis codes and secondary conditions were captured and given equal weight. Tier one included patients with HO-specific code M61 (calcification and ossification of muscle) and its subclassifications [217]. Tier two included patients with an additional set of miscellaneous 'non-specific' musculoskeletal codes, including previously defined codes, to capture all patients with a possible diagnosis of tHO [217]. The third tier included searching all trauma admissions with an inpatient hospital length of stay (LOS) ≥ seven days to identify any confirmed cases of tHO+ not identified by medical coders and without an HO-specific or non-specific ICD-10-AM code, i.e., 'no code'. Time to tHO diagnosis has been reported to occur as early as four weeks from injury onset and in patients with greater injury severity and a prolonged hospital LOS [3]. Therefore, the filter criteria of LOS ≥7 days ensured an injury cohort at higher risk of developing tHO was captured.

A screening protocol was conducted on a case-by-case basis to confirm or exclude a tHO diagnosis and traumatic injury mechanism. This included a manual review by a single investigator (NF) of patient EMRs, medical and allied health clinical documentation, discharge summaries and imaging reports. The presence of radiological and clinical evidence of a tHO diagnosis and a primary traumatic injury mechanism classified a confirmed case of tHO+.

4.2.2. Statistical Analysis

The frequency and distribution of HO-specific M61 and non-specific ICD-10-AM codes and tHO diagnostic characteristics were assessed for each trauma population across the overall WA tertiary hospital network. Nominal parameters were evaluated using Pearson's Chi-square test and are shown as frequencies and percentages. In the burns cohort, the predictive performance of the HO-

specific (M61) diagnostic codes in discriminating actual tHO cases from those without tHO was evaluated using receiver operating characteristic (ROC) curve analysis. The discriminative ability of M61 codes was assessed using the area under the curve (AUC) for the conversion outcome, where an AUC ≥ 0.8 would indicate that a test has favourable sensitivity and specificity characteristics [218]. Statistical significance was defined at the conventional 5% level. All computations were performed using IBM SPSS Statistics for Macintosh, version 29.0 (IBM Corp, Inc.).

4.2.3. Ethical Considerations

Ethics approval was granted by the South Metropolitan Health Human Research Ethics Committee (RGS3452) and from The University of Notre Dame, Fremantle (2020-013F). Site Governance approval was granted by South Metropolitan Health Service (Fiona Stanley Hospital), East Metropolitan Health Service (Royal Perth Hospital) and North Metropolitan Health Service (Sir Charles Gairdner Osborne Park Health Care Group).

4.3 Results

4.3.1. Patient Cohort

Eighty-seven patients were identified in the tier one search to have M61 ICD-10-AM codes for tHO. After chart review, only 37 patients were confirmed to have clinical and radiographic evidence of HO with a primary traumatic injury mechanism. The remaining 50 patients were excluded; 11 had no clinical or radiographical evidence of HO and thus were deemed incorrectly coded. Tier two captured a cohort of 1,422 patients with non-specific, miscellaneous musculoskeletal ICD-10-AM codes, of which 13 patients were identified as true tHO+ cases. Therefore, of the 1,509 patients identified from the HO-specific and non-specific ICD-10-AM code search, the true prevalence of tHO was determined to be 3.3% (50 of 1,509). Tier three captured 7,478 patients, including 17 true tHO+ cases with neither HO-specific nor non-specific ICD-10-AM codes among trauma admissions with a LOS ≥ seven days.

4.3.2. Analysis of ICD-10-AM Coding for Traumatic Heterotopic Ossification

The final cohort of 67 patients was confirmed to have clinical and radiographic evidence of tHO+ with a preceding traumatic injury mechanism. An inpatient diagnosis of tHO was most commonly

observed, accounting for 71.6% (p<0.001) of total diagnoses. An outpatient diagnosis was significantly more frequent in the orthopaedic population (n=12, 92.3%) than for burns (n=1, 5.6%), SCI (n=4, 23.5%) and TBI (n=2, 10.5%) patients (Table A2.1, Appendix 2C).

As shown in **Figure 2**, 37 patients (55.2%) were coded with an HO-specific code and 13 with a non-specific code (19.4%). Patients with radiographical and clinical evidence of tHO, missing an HO-specific and non-specific code (n=17), constituted a quarter of total true tHO cases. The frequency and distribution of ICD-10-AM codes for all inpatient and outpatient tHO diagnoses, stratified according to primary injury cohort, is shown in **Table A2.2 to A2.5** (Appendix 2D).

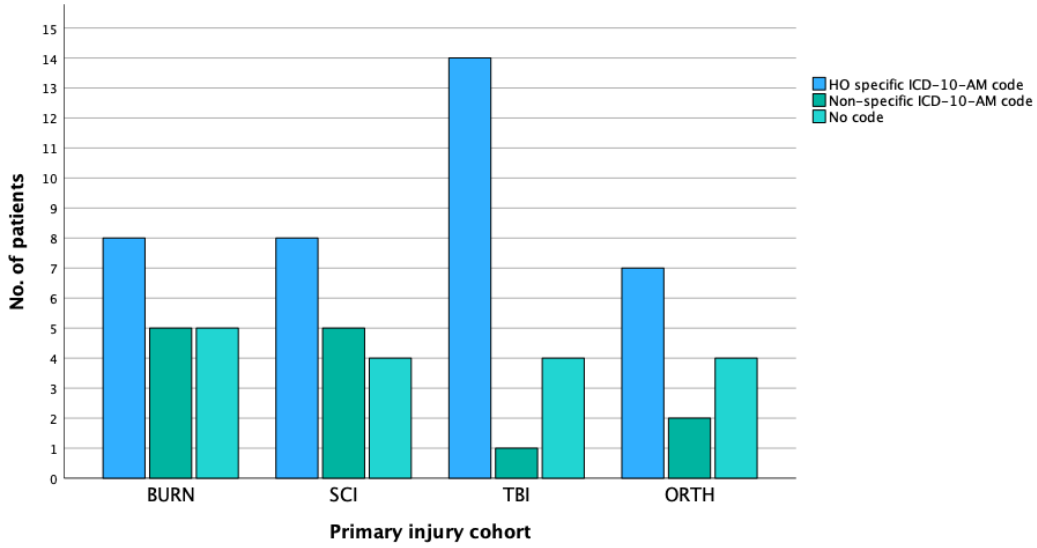


Figure 2. *The frequency and distribution of ICD-10-AM codes for inpatient and outpatient tHO diagnoses (n=67) according to primary injury cohort*

The frequency (code count) of HO-specific (M61) ICD-10-AM codes used for inpatient and outpatient diagnoses of tHO (n=37) is shown in **Figure 3** and **Table A2.4** (Appendix 2F). Only a single SCI patient was identified to be incorrectly coded with the M61.1 code for fibrodysplasia ossificans progressiva (FOP) and, in addition, had an atraumatic MOI and was subsequently excluded from the analysis. Therefore, in the final trauma cohort, zero tHO cases were identified with the M61.1 code, suggesting that the ICD-10-AM coding for post-traumatic HO was used and distinguished correctly from FOP, a genetic form of pathological ectopic bone formation.

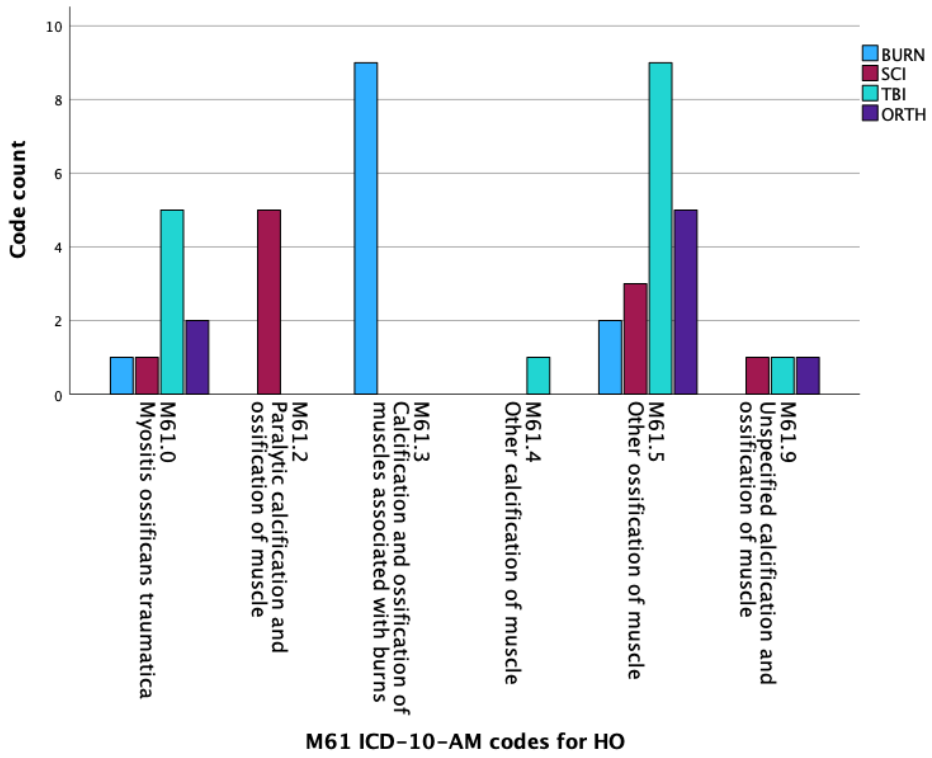


Figure 3. *Bar graph showing frequency (code count) of HO-specific ICD-10-AM codes (N=46) for true inpatient and outpatient tHO diagnoses (n=37 patients), stratified by primary injury cohort.For patients coded with >1 code, the total number of codes per patient was included in the final frequency calculation.*

For patients with an inpatient diagnosis of tHO, only 60.4% of patients were correctly identified with an M61 code **(Table 5)**. Over a third of true tHO cases were identified from non-specific codes only

(n=9) or incidentally through manual chart review, i.e., no code (n=10). A quarter of the burn cohort was not coded with the M61 code attributing tHO to the burn injury M61.3 (Calcification and ossification of muscles associated with burns). Similarly, half of the SCI cohort were coded with 'other' or 'unspecified' M61 codes instead of M61.2 (Paralytic calcification and ossification of muscle).

In a sub-cohort analysis, the reported sensitivity of M61 codes for correctly diagnosing tHO after burn injury was 50%. Thus, for half the true tHO cases, the M61 code was not used when it should have been, returning a high rate of false negatives. Only a single false positive case was identified, equating to a 96.3% specificity rate. The positive and negative predictive values of an M61 code were 88.9% and 76.5%, respectively. ROC analysis showed that M61 ICD-10-AM codes as a predictor of a confirmed positive tHO diagnosis was 0.731 (95% CI=0.561-0.902, *p*=0.012), indicating only fair performance [218].

Table 5. *Description and distribution of coding method used to identify inpatient tHO diagnoses by primary cohort*

		BURN	SCI	TBI	ORTHO	TRAUMA (TOTAL)
HO-specific (M61)	*n*	**8**	**8**	**13**	**0**	**29**
	Primary injury %	47.1%	61.5%	76.5%	0%	**-**
	Total %	27.6%	27.6%	44.8%	0%	**60.4%**
Non-specific	*n*	**5**	**3**	**1**	**0**	**9**
	Primary injury %	29.4%	23.1%	5.9%	0%	**-**
	Total %	55.6%	33.3%	11.1%	0%	**18.75%**
No code	*n*	**4**	**2**	**3**	**1**	**10**
	Primary injury %	23.5%	15.4%	17.6%	100%	**-**
	Total %	40%	20%	30%	10%	**20.8%**
TOTAL		**17 (35.4%)**	**13 (27.1%)**	**17 (35.4%)**	**1 (2.1%)**	**48**

n = no. of patients tHO: traumatic heterotopic ossification, SCI: spinal cord injury, TBI: traumatic brain injury, Ortho: orthopaedics

4.3.3. Evaluation of Clinical Documentation for Traumatic Heterotopic Ossification

A manual review of EMRs identified considerable variation in clinical documentation for 67 tHO cases, revealing 69 different descriptive terms used for tHO across the hospital network **Figure 4**

and Table A2.6 (Appendix 2H). A tHO diagnosis was not documented on the medical discharge summary for almost a third of patients with inpatient tHO diagnoses (n=48), and 38 patients had an inpatient tHO diagnosis stated on the medical discharge summary. However, 43.8% had no evidence of tHO recorded as a 'complication' or 'co-morbidity' under secondary diagnoses. For patients under acute and rehabilitation specialities, 29.2% of cases were not coded under the correct episode of care the initial tHO diagnosis was received.

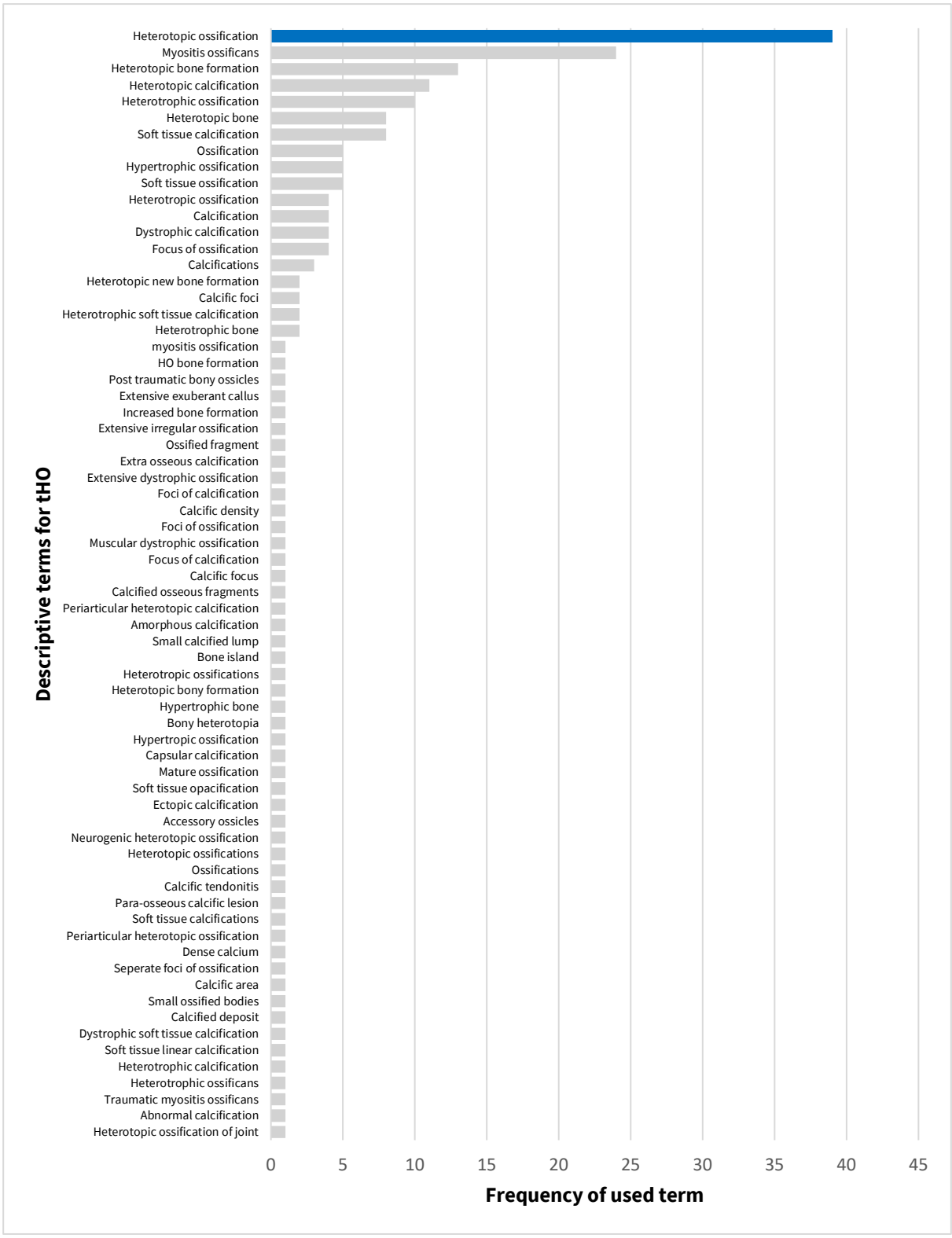


Figure 4. *Descriptive terms for tHO in decreasing order of frequency. Health professionals used 69 individual descriptive terms relating to tHO in clinical documentation for 67 true tHO cases across the network of hospitals.*

4.4 Discussion

This study brings attention to the marked variability and inaccuracies in ICD-10-AM coding and clinical documentation for the diagnosis of tHO after burn, neurological and orthopaedic trauma. Across four major WA hospitals, 1,509 patients were screened from the ICD-10-AM code search, and tHO prevalence was found to be 3.3%; a relatively conservative finding compared to other recently published figures [13, 26, 43]. Notably, the manual chart review revealed 17 tHO cases that were not coded with appropriate HO-specific or non-specific codes, meaning that almost half the tHO cases in the final cohort were incorrectly coded. These data support that the low overall tHO prevalence captured by the ICD-10-AM code search may, in part, be attributable to the integrity of clinical coding and clinical documentation for the complication of tHO [217, 219].

As the term heterotopic ossification refers only to the environment in which the bone process occurs, the term myositis ossificans (MO) is commonly used in the literature to classify aberrant bone formation within skeletal muscle [21]. However, tHO is not entirely specific to muscle and can develop within other soft tissue types such as joint structures, periosteum, ligaments, and tendons [220]. However, the HO-specific M61 ICD-10-AM codes are not attributable to calcification or ossification of other commonly involved soft tissue and peri-articular structures. Therefore, the inclusion of an HO-specific ICD-10-AM code that is distinct from MO of skeletal muscles, e.g., 'Calcification and ossification of the joint region, [site]' may be necessary for distinguishing MO from other sub-forms of tHO with no intramuscular involvement.

Unlike the M61.2 and M61.3 codes for tHO associated with burns and SCI/TBI, there is currently no M61 code that specifies an association with orthopaedic injury or procedures. Including an M61 code that encapsulates HO patients treated under the orthopaedic speciality would be beneficial for a more accurate classification of tHO that is distinguishable between trauma populations. Additionally, there would be further use in the inclusion of an ICD-10-AM code for post-operative HO, which may help distinguish between tHO and HO associated with elective surgical procedures without a traumatic mechanism of injury, e.g. joint replacement – an orthopaedic population observed to have high rates of HO [221-223].

It is known that post-operative tHO following fracture is frequently asymptomatic and incidentally detected during routine post-operative radiographs [17]. A reported incidence of asymptomatic

tHO was 18.78% in a cohort of patients with elbow fractures requiring surgical fixation [37]. In the present study, the higher frequency of outpatient tHO diagnoses after orthopaedic injury could be an explanatory factor contributing to the relatively conservative estimates of orthopaedic tHO. More so, subclinical cases of asymptomatic tHO may not be captured as systematically in the outpatient setting than if they were to be routinely surveilled as an inpatient. These findings highlight the need to implement surveillance guidelines in both inpatient and outpatient settings to achieve cost-effective screening and early diagnosis of symptomatic and asymptomatic tHO cases.

This investigation noted considerable variation in the documentation for tHO by clinicians across the hospital network. Clinical coders rely on the accuracy of discharge summaries completed by treating medical officers as one of the primary sources of clinical documentation for verifying clinical concepts and justifying code assignments for patients' episode(s) of care [212]. As such, using accurate and consistent documentation for tHO between clinicians and across institutions may improve the specificity of coding for injury-specific classifications of infrequent events like traumatic HO.

Another commonly used code set in health is the SNOMED Clinical Terms [224]. SNOMED terminology and ICD-10-AM code classification enable standardisation and semantic interoperability between healthcare systems [216]. Standardised clinical languages and concepts are pivotal to this occurring [223]. However, as evidenced in the present findings, there is a deficiency of a structured and standardised vocabulary of terms and concepts used by health professionals in patient EMRs, for the naming and identification of tHO. This may have ramifications for the accurate sharing of clinical information relating to the diagnoses between healthcare systems, without a loss of detail or change to meaning [216]. Additionally, clinical data analysis relies on consistent data entry through standardised identifiers [225]. To improve standardised data recording for tHO at the point of care, such as for patients' discharge summaries, clinicians should implement SNOMED CT-AU as a reference for consistent clinical terms relating to tHO.

These data lead us to suggest that clinical documentation by health professionals should inform and distinguish between the following categories of clinical information relating to tHO and referenced against SNOMED clinical terms (Figure A2.2, Appendix 2B):

1. Primary aetiology: Traumatic vs. atraumatic vs. genetic HO (FOP)

2. Preceding injury category: neurogenic (paralytic) tHO associated with traumatic spinal cord and brain injury vs. tHO associated with burns vs. tHO related to orthopaedic trauma
3. Primary anatomical site(s): joint region vs. intramuscular site (MO)
4. Anatomical structures involved: skeletal muscle (MO) vs. other soft tissues, i.e., ligament/tendon/extra-capsular/intra and extra-articular
5. A spectrum of pathological calcium deposition: calcification vs. ossification.

For tHO with articular involvement, a measure of HO severity based on existing site-specific classification schemes that correlate with joint function can be used with consistent and distinguishable clinical documentation relating to tHO diagnoses [87, 226, 227].

Overall, the present findings allude to the poor specificity of medical coding for tHO diagnoses across the WA tertiary hospital network, which may have implications for future retrospective research reliant on accurate injury diagnostic coding. In the burn cohort, the reported sensitivity of M61 codes for correctly diagnosing tHO was only 50%, indicating that using M61 diagnostic codes is a less than acceptable method to accurately classify tHO cases after burn injury. The implications of coding inaccuracies may be of even greater significance to rare disease populations such as tHO, where the true event rate and impact of risks and complications of diagnoses may be inappreciable due to poor coding practices, resulting in unreliable data for outcomes-based research [217]. Consequentially, clinician and hospital reimbursement may be impacted, which has potentially detrimental effects on the quality of future care for trauma patients.

4.4.1. Limitations

Although the multi-centre design of this study may improve the generalisability of our findings, this study is of retrospective design, and conclusions must be confirmed in a larger, prospective investigation. Finally, as the determination was not uniform by a classification system and the methods of detecting tHO varied, the different tHO rates between tertiary sites could be due to an interobserver error. For this reason, consensus must be made on the definition of tHO, and a standard method or classification system must be consistently implemented to reduce the heterogeneity among studies and institutions.

Given the large number (n=5,151) of orthopaedic admissions without HO-specific or non-specific ICD-10-AM codes in this study, it was not feasible within the timeframe of this research program to

perform individual retrospective chart review to definitively confirm or rule out a tHO+ diagnosis. In a WA tertiary hospital, Bochat et al. [36] conducted a retrospective study of a theatre database for procedures undertaken for elbow trauma over a five-year period and reported a higher overall tHO incidence (34 of 153 patients; 22%) than found in the present study. In addition, the number of confirmed tHO cases identified from manual chart review of burn and neurological admissions suggests the reported rate of orthopaedic tHO may be underestimated and, thus, a potential limitation of this study.

4.5 Conclusion

This study highlights the inaccuracies in medical diagnostic coding and inconsistencies in clinical documentation for the diagnosis of HO in WA tertiary hospitals, which may have implications for future research and patient care. Clinicians should consistently employ standardised clinical terminology from the point of care to increase the likelihood of accurate medical diagnostic coding for tHO diagnoses. The poor specificity of M61 codes in identifying true tHO cases should be considered in future retrospective studies utilising an ICD-10-AM code search, which should incorporate a broadened search criteria to include non-specific musculoskeletal codes for identifying tHO patients.

4.6 Key Points

- Existing M61 ICD-10-AM codes for traumatic heterotopic ossification (tHO) diagnoses failed to identify more than 1/3rd of true tHO cases.
- A high prevalence of tHO cases was identified from a broadened search of non-specific musculoskeletal codes (19.4%) and individual EMR reviews (25.4%)
- The predictive performance of the M61 diagnostic codes in discriminating true tHO cases after burn injury did not show favourable sensitivity and specificity characteristics.
- A tHO diagnosis was not documented on the medical discharge summary for a near-third of patients diagnosed with tHO during hospital admission.
- Clinicians should consistently employ standardised clinical terminology in clinical documentation from the point of care to increase the likelihood of accurate coding for injury-specific classifications of particularly rare events like tHO.

Chapter 5.

PART 1

Trauma Patient Heterotopic Ossification Diagnosis is Associated with Increased Hospital Length of Stay

Overview

This chapter presents the studies using purposeful multi-centre data pooling to quantify the impact of tHO on hospital LOS (Part 1) and to develop a risk profile of tHO with clinical utility in trauma patients (Part 2). Chapter 5 consists of two parts; the guiding question addressed in **Part 1** was, *"Is tHO associated with increased inpatient hospital length of stay?"* and, in **Part 2**, *"What common risk factors are associated with developing tHO in adult trauma patients?".* **Part 1** of this chapter entails the introduction, methodology, results, and discussion for Study 3 of this research programme, an excerpt of which was published as original research in *Injury* in January 2024 [228] (Appendix 10).

5.1 PART 1

5.1.1 Introduction

Traumatic HO (tHO) is a substantial barrier to rehabilitation for trauma-injured patients [3, 10]. Typically, tHO manifests as a progressive condition marked by rapid movement loss at affected joints and severe pain [3]. Secondary complications such as nerve entrapment and muscle weakness are commonly associated with ectopic bone formation. Longstanding joint dysfunction is often compounded by secondary scar and soft tissue contracture, joint arthrosis, muscle atrophy and articular cartilage degeneration [3]. The myriad of symptoms further impacts the rehabilitation trajectory for these already functionally compromised individuals after trauma, accounting for impaired patient quality of life and significant caregiver burden [3, 10]. Due to the functional impairments associated with tHO, increasing evidence indicates a relationship between tHO and a prolonged hospital length of stay (LOS).

Length of hospital stay is an essential metric for assessing the quality of trauma care, and it is a marker of costs and resource utilisation in trauma centres [229]. Decreased LOS has been associated with a reduced risk of hospital-acquired infections and improved treatment outcomes and patient quality of life [230]. To ensure trauma units operate optimally, reducing long-term occupation of beds is vital [231]. Injury severity, Intensive Care Unit (ICU) admission, surgical interventions, injury complications and comorbidities are known predictors of prolonged hospital stay in trauma patients [229, 232, 233]. Reliable predictions of LOS can enable hospitals to plan and allocate resources efficiently, tailor individual care for patients, and set reasonable patient expectations. Thus, the primary aim of this study was to assess the relationship between tHO and hospital LOS. It was hypothesised that tHO would be associated with an increased index hospital LOS compared to patients of the same injury severity with no tHO diagnosis.

5.1.2 Methods

5.1.2.1 Study Participants

A retrospective data audit was conducted, including patients admitted to three WA tertiary hospitals: Sir Charles Gairdner Hospital (SCGH), Fiona Stanley Hospital (FSH) and Royal Perth Hospital (RPH), and a trauma rehabilitation facility, Osborne Park Hospital (OPH). The inclusion

criteria were adult patients following neurological (TBI and SCI) or burn injury and or tHO/myositis ossificans (MO) diagnosed during inpatient hospital stay; patients aged 18 years or over at the time of admission; discharge was recorded between 1st May 2005 and 1st May 2019. Subjects were excluded if death occurred or comfort care/palliation was instigated during their hospital stay.

5.1.2.1.1 Identification of Traumatic Heterotopic Ossification Cohort

Patients with tHO (tHO+) were identified using the three-tier search of the ICD-10-AM coded WA trauma database, as described in Chapter 5 (Section 5.3.1). For all patients with specific (n=87) and non-specific HO diagnosis codes (n=1,422), individual medical records and imaging were screened by a single reviewer (NF) to confirm an inpatient tHO diagnosis and traumatic injury mechanism. Patients were only included if HO was diagnosed as an inpatient and excluded if HO was confirmed as an outpatient. Sixty-seven patients were confirmed to have clinical and radiographic evidence of tHO after trauma, and 48 were diagnosed as an inpatient. As only a single orthopaedic patient received an inpatient HO diagnosis, the orthopaedic cohort (n=1) was excluded entirely from the statistical analysis. Thus, a final cohort of 47 tHO+ patients met the inclusion criteria and comprised the study group.

5.1.2.1.2 Identification of Comparison Cohort

A search of the WA trauma database was conducted using injury-specific search criteria, diagnosis-related group codes for injury severity and via manual chart review to identify control subjects; patients with evidence of a traumatic injury mechanism and no diagnosis of tHO (tHO-), matched (1:3) by age, gender, and injury severity factors. A total of 33,879 trauma admissions were captured in the initial search. Time to tHO diagnosis has been reported to occur as early as four weeks from injury onset and in patients with greater injury severity and a prolonged hospital LOS [25, 47]. Therefore, the filter criteria of LOS ≥7 days ensured an injury cohort at higher risk of developing tHO was captured. A cohort of 7,478 trauma patients with LOS ≥ seven days was subsequently screened to identify subjects according to the matching criteria, comprising the control group (tHO-, n=141). All patient demographic and clinical variables that comprised the matching criteria per injury cohort were crosschecked via medical record and imaging review.

5.1.2.1.3 Classification and Matching by Injury Severity

Comparison of tHO+ and tHO- groups according to matching criteria is shown in **Table A3.1** (Appendix 3B). In addition to injury-specific severity indices, injury severity was also compared

between groups using the abbreviated injury scale (AIS). The AIS is an anatomically-based injury severity scoring system that classifies each injury by body region on a six-point ordinal scale [234, 235]. The AIS was calculated for index admission for the respective trauma-related injury according to the primary injury category: AIS-burn, AIS-spine and AIS-head. Rather than relying on ICD-10 discharge codes, the medical records and images were analysed to ensure accuracy, and AIS scores were assigned using AIS 2008 scoring paradigms [235, 236].

The severity of burn injury was indicated by Total Body Surface Area (TBSA) at the time of injury, and patients were matched according to their TBSA (%). Burn-injured patients were also scored according to the AIS related to TBSA and depth of burn injury, denoted as AIS-burn [237].

For SCI patients, the level and extent of a neurological deficit are measured by the Neurological Level of Injury (NLI), The International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) and the American Spinal Injury Association (ASIA) impairment scale, scored at the time of hospital admission [238]. These data were supplemented with AIS coding data; the score for the spine body region was retrieved and denoted as AIS-spine. However, as with burn injury, the AIS has limitations as a predictor of mortality after SCI [237, 239]. Consistent with AIS codes, SCI patients were classified as complete or incomplete injuries at the cervical (subclassified: C3 or above, C4 or below), thoracic, or lumbar spine, with or without associated fractures or dislocations at the same level as the traumatic SCI [240].

Traumatic brain injury refers to brain injury acquired through a traumatic event, and the severity of the injury can be categorised as mild, moderate, severe and extremely severe [241]. The severity indices used in this study were the Glasgow coma scale (GCS) and duration of post-traumatic amnesia (PTA), determined using a validated screening tool, [242]. Head AIS, in combination with GCS, has been used to rate the severity of head injury for selecting and stratifying patients for clinical studies of TBI [243-245]. Thus, the severity of TBI was further defined by an AIS-head score (AIS 1 to 2, 3 to 4 and 5-6 indicate mild, moderate and severe TBI, respectively [246].

5.1.2.2 Length of Stay Outcomes

The dependent variable predicted in modelling was the total inpatient LOS, defined as the cumulative number of days between inpatient admission and hospital discharge, including

rehabilitation. Administrative data relating to LOS was confirmed via manual review of clinical records and discharge summaries stipulating date of admission and data of discharge from respective facilities. The association of demographic and clinical variables with tHO and hospital LOS was assessed. Due to the model of care difference between neurological and burn cohorts, acute and rehabilitation LOS outcomes were only comparable between the SCI and TBI groups.

5.1.2.3 Statistical Analysis

Clinical characteristics and demographic data were extracted and recorded from electronic medical records (EMR) relating to inpatient and outpatient activity, including discharge summaries, clinical documentation and radiology and pathology reports. Descriptive statistics summarise and compare age, gender, and injury severity for each injury type by the presence or absence of tHO. Categorical variables were compared using Pearson's Chi-square or Fisher's exact test and presented as frequencies and percentages. Continuous variables were compared using t-tests or a non-parametric Wilcoxon rank-sum test and presented as means and standard deviations, or medians and interquartile ranges (IQRs), respectively.

Hospital LOS in days best fitted a negative binomial distribution, and a negative binomial regression model was used to model the effect of inpatient HO diagnoses on total LOS. Covariates found to have a statistically significant association ($p \leq 0.05$) with total LOS in the univariate analysis were included in the multivariate model and further refined by backward elimination. The association between comorbidities and LOS is presented in terms of the estimated incidence rate ratio (IRR), with its 95% confidence interval (95% CI). The IRRs indicate the rate of increase or decrease in LOS in one group compared to the reference group for categorical covariates or an increase of 1 unit in continuous covariates. The differences in the main effects were observed by evaluating the Estimated Marginal Means. Statistical significance was defined at the conventional 5% level. All computations were performed using Stata version 16.1 (StataCorp. 2021. Stata Statistical Software: Release 16.1).

5.1.2.4 Ethical Considerations

Ethics approval was granted by the South Metropolitan Health Human Research Ethics Committee (RGS3452) and from The University of Notre Dame, Fremantle (2020-013F). Site Governance approval was granted by South Metropolitan Health Service (Fiona Stanley Hospital), East

Metropolitan Health Service (Royal Perth Hospital) and North Metropolitan Health Service (Sir Charles Gairdner Osborne Park Health Care Group).

5.1.3 Results

A total of 47 patients with an inpatient diagnosis of tHO following burns (n=17, 36.2%), SCI (n=13, 27.7%) and TBI (n=17, 36.2%) were identified with 14.9% (n=7) female and 85.1% (n=40) were male; and a mean age of 36±14.1 years. Control subjects (tHO-, n=141) by design had similar age (tHO+, 36±14.1 vs. tHO-, 37±14.7years), gender ratio (tHO-, 15.6%:84.4%) and injury severity. Baseline demographic and injury severity characteristics of the tHO+ and tHO- groups according to the primary injury cohort are compared in **Table A3.1** (Appendix 3B).

Univariate results of tHO diagnostic characteristics according to the episode of care and by primary injury cohort are shown in **Table A3.2** (Appendix 3C). The median time to radiological evidence of tHO was 53 days (IQR 38-81) for all sites and diagnoses. For neurotrauma patients with distinct acute and rehabilitation episodes of care (i.e., patients transferred to a rehabilitation ward during index admission), tHO was most frequently diagnosed in the rehabilitation ward (66.6%, p=0.003) than during the acute care episode. TBI cohort accounted for all cases of acute care diagnoses (TBI, n=10 *vs.* SCI, n=0).

Total inpatient LOS was compared between the tHO+ and tHO- subjects according to primary injury cohort **(Table 6).** The median hospital LOS for all trauma patients was 77 days (IQR, 40-143). Patients who developed tHO during hospitalisation had a significantly higher median LOS (142 days, IQR 74-178) than matched trauma patients who did not develop tHO (61 days, IQR 31.5-113) **(Figure 5a).** In the tHO+ cohort, SCI patients had the highest median LOS (178 days, 95% CI, 144-235) vs. burns (123 days, 95% CI, 67-154) and TBI patients (113 days, 95% CI, 69-196) **(Figure 5b)**.

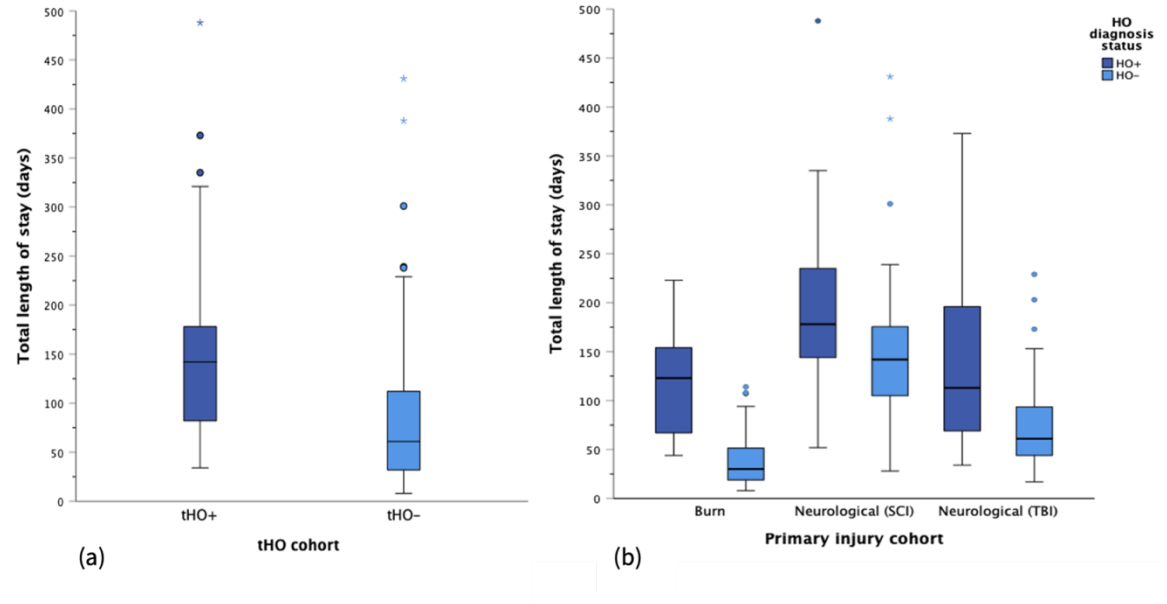


Figure 5. *(a) Box plots of total length of hospital stay (LOS) stratified by HO diagnosis status and (b) by primary injury cohort.*

Table 6. *Univariate analysis of length of stay variables for tHO+ and tHO- subjects*

	Burn			Neurological (SCI)			Neurological (TBI)			TRAUMA (TOTAL)		
	tHO+	**tHO-**	***p***	**tHO+**	**tHO-**	***p***	**tHO+**	**tHO-**	***p***	**tHO+**	**tHO-**	***p***
ICU												
n	17	51		12	39		14	51		43	141	
LOS	21 (17-34)	5 (0-11)	<0.001	9 (2-22)	7 (3-11)	0.482	11 (7-13)	9 (6-12)	0.423	14 (7-24)	7 (3-11)	**<0.001**
Acute care												
n	-	-	-	13	38		17	51		30	140	
LOS	-	-	-	24 (14-52)	19 (16-26)	0.265	35 (30-59)	26 (19-34)	<0.001	32 (24-54)	23 (17-31)	**<0.001**
Rehabilitation												
n	-	-	-	13	39		17	51		30	141	
LOS	-	-	-	126 (86-162)	122 (76-156)	0.684	110 (34-212)	37 (18-64)	0.003	114 (46-206)	64 (29-118)	0.025
Total												
n	17	51		13	39		17	51		47	141	
LOS	123 (67-154)	30 (19-52)	<0.001	178 (144-235)	142 (101-180)	0.108	113 (69-196)	61 (44-97)	<0.001	142 (74-178)	61 (31.5-113)	**<0.001**

Categorical variables are presented as n (%). Continuous variables are presented as median (IQR). Due to model of care differences for burn and neurological trauma patients, LOS outcomes for acute and rehabilitation episodes of care are comparable only in the neurological cohorts. LOS: length of stay, tHO: traumatic heterotopic ossification, SCI: spinal cord injury, TBI: traumatic brain injury, ICU: intensive care unit, *p:* p-value

Univariate analysis of LOS variables by primary injury cohort is shown in **Table 6,** and univariate results are provided in detail in Appendix 3C and Appendix 3D. After backward stepwise elimination, the relationships between total LOS and ICU LOS, long bone fracture, urinary tract infection (UTI) and sepsis were insignificant. Following the elimination process, the final model presents the influential factors on hospital LOS **(Table 7)**. The resulting incidence rate ratios (IRR) indicate that patients who develop tHO during hospitalisation are predicted to stay in hospital 56% longer (IRR 1.56, 95% CI, 1.35-1.79, p<0.000) than trauma patients who do not develop tHO. The Estimated Marginal Means corresponded to an average increase in adjusted total LOS for a tHO+ case of 2.4 days compared to 1.9 days for matched trauma controls (Figure A3.1, Appendix 3A).

Table 7. *The final model for multivariate negative binomial regression examining hospital length of stay predictors*

VARIABLES ASSOCIATED WITH TOTAL LOS	**IRR**	**95% CI**	***p***
tHO diagnosis	1.56	1.35 – 1.79	<0.001
Mechanical ventilation (hours)	1.00	1.0002 – 1.0005	<0.001
Injury to hip region and thigh	1.48	1.24 – 1.76	<0.001
Other ossification disorder	1.33	1.16 – 1.53	<0.001
ICU admission	1.38	1.09 – 1.74	0.007
Pressure injury	1.34	1.15 – 1.57	<0.001
DVT	1.20	1.01 – 1.42	0.035

LOS: length of stay, tHO: Traumatic heterotopic ossification, ICU: intensive care unit; DVT: deep vein thrombosis, IRR: incidence rate ratio, CI: confidence interval, *p:* p-value

5.1.4 Discussion

This is the first study to show that tHO is independently associated with greater in-hospital LOS for patients following a burn, spinal cord, and traumatic brain injury. Patients diagnosed with tHO during hospitalisation stayed 56% longer than matched trauma patients who did not develop tHO. Thus, the study also demonstrated the value of pooling comparative epidemiological data on LOS outcomes from individual tHO populations across four trauma centres. After adjustment for age, gender, injury severity and critical care factors and the presence of multiple co-morbidities, the seven determinants of hospital LOS were: diagnosis of tHO, injury to the hip region and thigh, ICU admission, pressure injury, other ossification disorder, and mechanical ventilation hours. This study presents a novel quantification of the impact of acute tHO on the health care system and is a first step towards identifying the health resource costs associated with the complication of tHO.

Trauma patient LOS is highly variable and is closely governed by factors beyond the diagnoses, injury severity and procedures, including pre-existing co-morbidities, post-operative and injury-related complications, rehabilitative needs and resources or options for discharge disposition [229, 247, 248]. No prior study has robustly assessed the independent contribution of a tHO diagnosis to the LOS trajectory in burn and neurological trauma patients. Previous data have only allowed a superficial examination of such factors that may be associated with a prolonged hospital stay in tHO cohorts and have shown only a weak association or no attempt was made to subject these factors to a multivariate analysis owing to the inclusion of a relatively small sample size. As such, this study differs from others assessing the association between tHO and length of hospital stay.

Previous work has emphasised the contribution of critical care factors such as ICU admission and the requirement for mechanical ventilation to a prolonged LOS in trauma patients. Our results demonstrate similar effects of an ICU admission (IRR 1.37, p=0.007) and mechanical ventilation hours (IRR 1.00, p<0.001) on increasing LOS. However, in contrast to previous work, tHO was determined as a significant predictor of an increased total LOS, independent of the effects of an increasing ICU LOS and mechanical ventilation time [25, 249, 250]. Thus, a more prolonged ICU admission is not necessarily a causal factor in increased total LOS in the presence of a tHO diagnosis.

There are several possible explanations for these findings. Traumatic HO increases the risk of health morbidities, such as acute and chronic pain, pressure ulcers and impaired wound healing [3]. More

so, HO-specific symptomology shares similarities with other acute inflammatory conditions such as DVT, cellulitis and osteomyelitis, posing challenges for early, definitive tHO diagnosis [7]. Without accurate and reliable early diagnosis, there is little chance of early intervention, and late detection fails to limit the unabated progression of tHO. As such, tHO further impedes patients' physical and psychosocial functioning throughout the rehabilitation [3]. Loss of movement is associated with poor functional outcomes, and a lack of normal movement negatively impacts areas such as personal care, participation in social roles and returning to independent daily living [3]. Reduced functional independence increases the reliance on supportive others when transitioning home from the rehabilitation facility, complicating discharge planning and likely delaying time to discharge [3]. These factors support the argument that tHO prolongs hospital LOS and increases medical care utilisation and associated costs.

Factors influencing outcomes have variable influence at different times during hospitalisation, emphasising that different phases of care may have different optimal therapies [251]. Univariate analysis showed that TBI patients with tHO had a significantly longer rehabilitation LOS than controls and other primary injury cohorts. However, time to diagnosis was the earliest in this group (38 days [IQR 33-58], p=0.020), which is similar to previous reports [34, 250, 252]. Differences between injury cohorts may be attributable to different spatiotemporal patterns of disease onset and progression between primary injuries. Early disease onset after TBI may lead to a more established and clinically significant disease progression during rehabilitation, affecting mobility and prolonging LOS in the rehabilitation unit. Or maybe attributable to the consistency and implementation of systematic screening protocols and therapeutic strategies for tHO in respective acute and rehabilitation facilities. However, no established clinical guidelines are available for clinicians in WA hospitals to guide early, routine surveillance of tHO in trauma patients.

It is worth noting that patients who encounter severe burns experience long periods of reduced consciousness and narcotic and sedating medications and, thus, are likely to have difficulties processing and communicating information, such as their experience of tHO symptoms [253]. Similarly, TBI patients experience protracted periods of PTA, often compounded with cognitive and language deficits. These communication barriers are particularly relevant for detecting clinical suspicion of tHO, as the onset of tHO and experience of symptoms appear to occur before evidence on conventional radiographs [25]. This suggests a possible benefit from clinical trials involving the conduction of an early surveillance program comprising early risk stratification by clinicians and

routine screening of high-risk patients utilising diagnostics sensitive for detecting early or potential tHO lesions such as bedside ultrasound or three-phase bone scintigraphy.

Overall, the findings of this study emphasise the importance of addressing tHO diagnoses at specific points in hospitalisation. Identifying critical care junctures can guide interventions for improved quality of care and patient outcomes in at-risk trauma populations. There is a need for standardised patient management protocols for early surveillance and interventions targeting tHO prevention, and it is important to consider tHO patients as a distinct population when allocating resources or planning quality improvement interventions. Early diagnosis through increased clinician awareness, patient education, surveillance, and improved diagnostic algorithms can facilitate prompt treatment and slow disease progression. This approach may effectively improve patients' functional outcomes and reduce the requirement for an extensive rehabilitation stay and the risk of readmission.

5.1.4.1 Limitations

This study has several limitations. The determination of tHO was not uniform within the classification system and the methods of detecting tHO varied. As such, in the investigation of images, the detection sensitivity and determination of tHO may differ among the various institutes. Although purposeful multi-centre and multi-diagnosis study design may improve the generalisability of our findings to other burn and trauma centres, this retrospective-cohort study has limited causative conclusions, and results need to be confirmed in future prospective clinical trials. Finally, despite controlling for many known confounding variables through matching criteria and choice of statistical tests for analysis, it is possible that other variables with an effect on hospital LOS were not included in the results.

5.1.4.2 Future Research

It is understood that patients with a longer LOS have significantly higher odds of readmission, and high readmission rates may reflect different processes of care [254]. Due to the chronicity of primary tHO symptoms and associated secondary morbidities, tHO patients are at higher risk of re-hospitalisation [3]. Further, the magnitude of the financial cost associated with tHO has not been thoroughly investigated in trauma populations. Some insight may be gained from an international survey examining the burden of illness of fibrodysplasia ossificans progressiva. The mean cost of care (~$164,000 U.S. dollars per year) highlights the increasing financial burden of severe FOP-

related mobility restrictions. To fully elucidate the burden of illness associated with tHO and ensure comprehensive measurement of LOS outcomes, tHO-related re-admission rates and associated financial costs should be considered in future investigations.

5.1.5 Conclusion

The findings of this study demonstrated that a diagnosis of traumatic heterotopic ossification during hospitalisation independently prolonged hospital LOS for burn and neurotrauma patients. This investigation presents a novel first step towards understanding the economic impact of acute tHO. It offers valuable insight into healthcare service utilisation and resource costs associated with LOS outcomes in tHO patients.

5.1.6 Key Points

- Traumatic HO is an independent predictor of a prolonged index hospital LOS
- Burn and neurotrauma patients diagnosed with tHO stay in hospital 56% longer than trauma patients of the same injury severity, who do not develop tHO
- The severe functional limitations associated with tHO complicate trauma patients' rehabilitation trajectory and delay discharge
- Early routine surveillance for tHO should be consistently implemented in trauma centres to facilitate early diagnosis and minimise the impact of disease progression on patients' functional outcomes, hospital LOS, medical care utilisation and costs.

Chapter 5.

PART 2

Risk Factors for Heterotopic Ossification Common in Adult Trauma Patients

Overview

The research question answered in **Part 2** of this chapter was, *"What common risk factors are associated with developing tHO in adult trauma patients?"*. Previous investigations have focused on single-centre outcomes in diagnostic trauma cohorts, where small sample sizes limit the tHO event rates and statistical power to identify multiple risk factors. To address these methodological limitations, **Part 2** uses data triangulation across pooled trauma populations to identify novel tHO risk factors. The methodology described in Part 1, Section 5.1.2.1, is relevant to the following chapter. This chapter (Part 2) expands on Part 1 methodology and details the results and discussion for Study 4 of this research programme.

5.2 PART 2

5.2.1 Methods

5.2.1.1 Study Participants

A retrospective data audit of the WA trauma database was carried out to identify the tHO+ cohort and tHO- comparison group, as described in Chapter 5, Part 1, Section 5.1.2.1.

Clinically relevant characteristics and demographic data from all electronic medical records (EMR) relating to inpatient and outpatient activity, including discharge summaries, clinical documentation and radiology and pathology reports, were manually reviewed, and data extracted by a single reviewer (NF). **Table A4.1** (Appendix 4A) and **Table A4.2** (Appendix 4B) list all recorded data fields.

5.2.1.2 Statistical Analysis

Descriptive statistics summarise age, gender, and injury severity for each injury type by the presence or absence of tHO and were tested to confirm the matched group comparability (Table A3.1, Appendix 3B). The dependent variable in modelling was the tHO diagnosis in patients following a burn, spinal cord injury and traumatic brain injury. Univariate association of categorical parameters was evaluated using Pearson's Chi-square test or Fisher's exact test and presented as frequencies and percentages. Continuous variables were compared using t-tests or a non-parametric Wilcoxon rank-sum test and are presented as means and standard deviations, or medians and IQRs, respectively.

Multiple matched tHO negative patients were included to account for interpersonal variation in the statistical analysis. Covariates found to have a statistically significant effect ($p \leq 0.05$) on tHO risk in the univariate analysis were included in the multivariable model. Backward-stepwise logistic regression analysis was performed to model the effects of multiple covariates on the dependent variable, tHO diagnosis. Potential risk factors were eliminated on $p \geq 0.1$ for the final model. The post-estimation linearity test was acceptable, so the linearity assumption was met. The association between comorbidities and tHO is presented using the estimated odds ratio (OR), with its 95% confidence interval (95% CI). The differences in the main effects were observed by evaluating the

Estimated Marginal Means. All computations were performed using Stata version 16.1 (StataCorp. 2021. Stata Statistical Software: Release 16.1) and IBM SPSS Statistics, version 29.0 (SPSS Inc).

5.2.1.3 Ethical Considerations

Ethics approval was granted by the South Metropolitan Health Human Research Ethics Committee (RGS3452) and from The University of Notre Dame, Fremantle (2020-013F). Site Governance approval was granted by South Metropolitan Health Service (Fiona Stanley Hospital), East Metropolitan Health Service (Royal Perth Hospital) and North Metropolitan Health Service (Sir Charles Gairdner Osborne Park Health Care Group).

5.2.2 Results

A total of 47 patients were diagnosed with tHO during an inpatient hospital stay following burn (n=17, 36.2%), SCI (n=13, 27.7%) and TBI (n=17, 36.2%) and frequency of tHO locations are shown in **Figure 6**. A cohort of 141 matched controls comprised the comparison group (tHO-). Baseline demographic and clinical characteristics of tHO+ and tHO- cohorts were comparable in **Table 8** and **Table A4.2** (Appendix 4B). Males accounted for 84.6% of the total cohort. The total mean age at the time of injury was 37.5 years (tHO+, 36±14.1 vs. tHO-, 38±14.8).

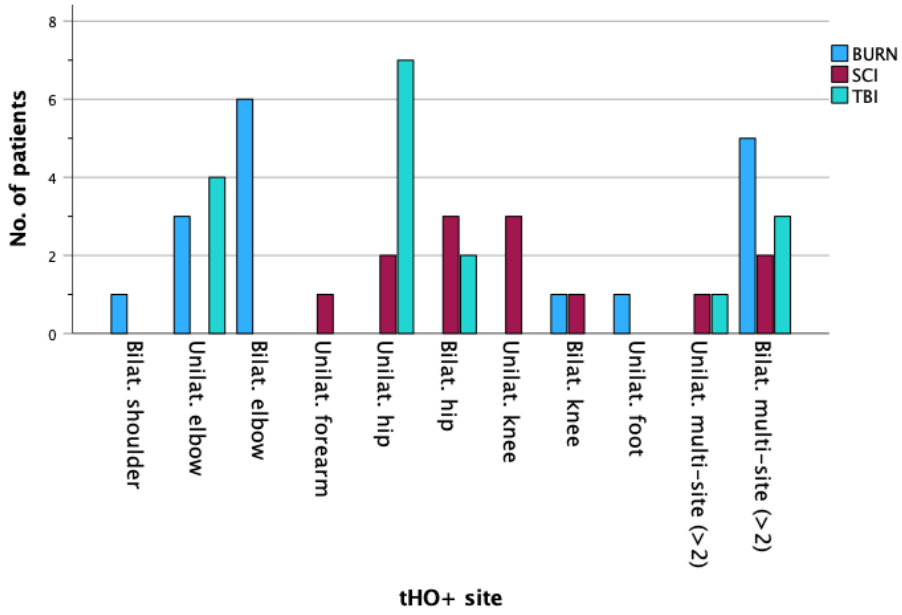


Figure 6. *Bar graph of patient count by site of tHO, stratified by injury*

Table 8. *Baseline demographic and clinical variables for tHO+ and tHO- patients*

	TOTAL (n=188)		**tHO+ (n=47)**		**tHO- (n=141)**		***p***
Variable name	**mean/n**	**sd/%**	**mean/n**	**sd/%**	**mean/n**	**sd/%**	**-**
Injury age	37.5	14.6	36	14.1	38	14.8	0.425
Gender							0.907
Female	29	15.4	7	14.9	22	15.6	
Male	159	84.6	40	85.1	119	84.4	
ICU LOS	11.4	14.2	19.3	21.3	9	10.1	**0.001**
ICU admission	155	84.2	39	90.7	116	82.3	0.192
Total LOS	104.2	102.7	167	149.2	83.3	70.7	**0.000**
Mechanical ventilation (hours)	203.9	356.9	375.3	534.2	154.8	270.1	**0.005**
Smoke	94	50	18	38.3	76	53.9	0.066
Long bone fracture	80	42.6	29	61.7	51	36.2	**0.003**
Injury to hip region and thigh	55	29.3	24	51.1	31	22	**0.000**
Injury to CNS or PNS	40	21.3	18	38.3	22	15.6	**0.001**
UTI	88	48.9	28	62.2	60	44.4	**0.041**
Pressure injury	84	45.9	25	54.3	59	43.1	0.186
DVT	21	11.2	5	10.6	16	11.3	0.894
Sepsis	60	31.9	24	51.1	36	25.5	**0.001**

tHO: traumatic heterotopic ossification, ICU: intensive care unit, LOS: length of stay, UTI: urinary tract infection, DVT: deep vein thrombosis, *p:* p-value

Variables identified as having an association with tHO in the univariate regression analysis are shown in **Table A4.3** (Appendix 4C). The logistic, multivariable model identified factors independently associated with the primary outcome variable, tHO (Table A4.4, Appendix 4D). In the final model, independent predictors for tHO development, identified by backward stepwise logistic regression, are shown in **Table 9**.

Table 9. *Final multivariate model after backward stepwise logistic regression examining significant predictors of tHO*

VARIABLES ASSOCIATED WITH tHO	**OR**	***p***	**95% CI**
LOS total	**1.02**	**0.000**	**1.00-1.03**
Sepsis	2.46	0.095	0.86-7.08
Injury to hip region and thigh	**4.14**	**0.021**	**1.24-13.87**

LOS: length of stay, OR: odds ratio, *p*: p-value, CI: confidence interval

Sepsis was a confounder for the length of stay and tHO and thus was included in the multivariate model. Further univariate and multivariate regression analyses identified the microbiological organisms of interest **(Table 10).**

Table 10. *Backward stepwise logistic regression examining predictors of tHO relating to sources of local and systemic infections*

VARIABLES ASSOCIATED WITH tHO	OR	*p*	95% CI
Other ***Staphylococcus***	2.42	**0.026**	**1.11-5.27**
Acinetobacter calcoaceticus-baumannii	4.43	**0.026**	**1.19-16.38**
Enterobacter cloacae complex	3.81	**0.003**	**1.55-9.35**

OR: odds ratio, *p*: p-value, CI: confidence interval

5.2.3 Discussion

The results of this pooled, matched cohort study confirmed known and novel, independent risk factors for tHO that are common across at-risk trauma populations. Total hospital length of stay and having an injury to the hip region and thigh are significant predictor variables for tHO. Also revealed was the presence of previously unrecognised infectious agents – *Staphylococcus* species, *Acinetobacter calcoaceticus-baumannii* complex and *Enterobacter cloacae* complex – to be independently associated with tHO across diverse trauma populations.

Traumatic HO in multiple joints bilaterally was more commonly observed across trauma patients. This supports the hypothesis that tHO may be propagated by a systemic process in addition to a local pathological response. Symmetrical joint involvement appeared most frequently in the elbows after burn injury, supporting prior reports [3, 25]**.** In line with previous findings in the neurotrauma literature [13], unilateral elbow joint involvement appears almost exclusively associated with the TBI population. Similarly, tHO in the hip(s) was solely associated with CNS-injured patients. However, noting the involvement of other large joints of the shoulder, knee, and smaller joints of the foot should be considered by burn and trauma clinicians when it comes to risk stratification and surveillance.

Plain radiography was the most frequently used diagnostic modality for initial tHO diagnosis. However, plain radiographs are not sensitive for detecting early phases of tHO [6, 255, 256]. Thus, it is likely that utilising a diagnostic modality more sensitive to detecting early or potential tHO lesions, i.e. bedside serial ultrasonography, 3-phase bone scintigraphy or single-photon emission computed tomography (SPECT), may have resulted in an earlier diagnosis of tHO in this cohort, providing a greater window of opportunity for targeted therapeutics to halt disease progression [78]. Notably, the onset of tHO is not always simultaneous in cases of multi-joint involvement, including cases involving symmetrical joints. With the added understanding that tHO signs and symptoms are often present before evidence on plain radiography, it therefore appears justified to recommend, particularly in the presence of confirmed unilateral tHO, that clinicians surveil for multi-joint involvement and consider imaging bilateral symmetrical joints, even in the absence of clinical suspicion of the contralateral joint [3, 25, 85, 257-259].

Length of Hospital Stay

Several studies have explored the length of hospital stay and tHO risk with conflicting outcomes [13, 25, 47, 127, 260]. Extending previous findings, this study presents total hospital LOS to affect tHO risk significantly (OR 1.02, 95% CI 1.00-1.03, p<0.001), independent of the effects of concomitant injuries and secondary complications or comorbidities. Trauma patients who develop tHO during hospitalisation experience severe physical impairments, functional limitations, and an increased reliance on others to complete personal and daily living tasks [3]. Such factors complicate the rehabilitation trajectory and may delay discharge to the community setting. Length of stay represents a multifactorial variable, and although it was not possible to determine the direction of association or infer causality from the present analysis, it is plausible that increasing hospital LOS is a risk factor for developing tHO. These findings are essential for quality improvement and resource utilisation at acute care and rehabilitation hospitals.

Comorbidities

When accounting for demographic and injury severity factors in a large cohort of pooled neurological and burn-injured patients, secondary complications, such as DVT and pressure injuries, were not identified as significant predictors of tHO, negating previous findings [13, 24, 26, 32, 260]. The influence of smoking on bone biology has been described. However, in contrast to multiple prior studies, smoking status was not associated with tHO risk [32]. Conditions affecting bone turnover, such as ankylosing spondylitis, Paget's disease, hypertrophic osteoarthritis, and

comorbid parathyroid and Vitamin D deficiency disorders, have been noted in higher proportions in the tHO orthopaedic cohort [59]. Although Vitamin D deficiency and parathyroid disorders were not included in the conditions analysed in the present study, it was determined that collectively, diseases associated with pathological calcification or ossification did not have an independent association with tHO. Considering prior investigations have proposed potential shared pathobiological mechanisms between tHO and other conditions characterised by pathological ectopic mineralisation, this discovery is somewhat unexpected [261].

Systemic and Local Infection

Acute and chronic infections can further augment the pro-inflammatory response after tissue injury [262, 263]. Secondary infections, either systemically or locally, are among the recently explored risk factors for tHO development after burn and neurotrauma; however, most studies are limited by design and interpretation [25, 264]. In the current report, sepsis increased the odds of developing tHO by over 2-fold; however, this factor did not reach a statistically significant level after multivariable analysis (p=0.095).

Urinary tract infection (UTI) has been associated with tHO, most convincingly in the neurological cohort, with no significant relationship previously described in the burn literature [26, 127]. Although a significant relationship has been observed in this study between the presence of UTI and the formation of tHO (OR 2.05, 95% CI 1.03-4.11, p=0.041), this risk factor was not independently associated with tHO development.

Thefenne et al. [26] identified pulmonary and cutaneous infections as independent predictors of tHO formation complicating burn injury; however, they did not account for injury severity, which is likely an important confounding factor in the analysis. Major burn wounds are often colonised by multi-drug resistant organisms, necessitating serial debridements [262]. The number of trips to the operating theatre for burns requiring grafting and time to elbow wound closure are known independent risk factors for tHO after burns, representing multiple possible avenues related to increased infection risk [28]. An increased rate of critical bacterial colonisation in wounds of tHO patients injured in combat has been reported [265, 266].

A detailed microbiology analysis identified S*taphylococcus* species (excluding *Staphylococcus aureus*), *Acinetobacter calcoaceticus-baumannii* complex, and *Enterobacter cloacae* complex as

specific infectious agents associated with an increased risk of developing tHO independent of age, injury severity or hospital LOS. These organisms are notable for their intrinsic resistance and so are selected through antibiotic exposure and typically identified later in hospital admission. Prolonged hospital LOS increases trauma patients' risk of hospital-acquired infections [267].

These findings suggest that the increased bioburden induced by persistent colonisation of bacteria and chronic infection may contribute to the proinflammatory environment necessary to promote tHO formation. However, future investigations are required to elucidate the specific temporal relationships underpinning infection-related risk factors and tHO. This may be achieved by studying burn and trauma patients longitudinally and modelling the probability of tHO with time-varying covariables such as onset and resolution of infection, depending on time points in which tHO symptoms appear, as well as the anatomical location and method of detection to indicate the presence of a local or systemic infection of the responsible organism.

Concomitant Injuries: Injury to the Central and Peripheral Nervous System

Increasing evidence supports a pathobiological link between the central and peripheral nervous system and tHO development [8, 138, 268-270]**.** Whilst the prevalence of tHO in CNS-injured populations is well documented, the contribution of a concomitant CNS or PNS injury to the development of tHO after burn injury is unknown. Indeed, a significantly more significant proportion of burn patients with tHO presented with concomitant CNS or PNS injuries (tHO+, 40% *vs.* tHO-, 9%, p=0.007). Univariate regression analysis showed that the odds of developing tHO after injury increased by over 3-fold with a concomitant CNS/PNS injury (OR 3.3, 95% CI 1.59-7.06, *p*=0.001).

CNS inflammatory responses have been identified after severe burns (≥20% TBSA) where burn-induced systemic inflammation involving dysregulated cytokine activity can stimulate blood-brain-barrier (BBB) dysfunction [263, 271]. Intriguingly, acute peripheral insults such as sepsis have also been shown to influence CNS function in severe burns patients [42]. Moreover, patients who have sustained burns or CNS injuries, particularly those requiring ventilatory support, often experience prolonged periods of narcotic, muscle relaxant and sedating medication [272]. Although critical care variables such as ICU LOS and time on mechanical ventilation were not identified as independent predictors of tHO in the final model, univariate analysis showed a statistically significant association between mechanical ventilation hours and tHO. Mechanical ventilation

triggers CNS responses through functional connections between the lungs and the brain [273]. Circulating neuroinflammatory mediators, including cytokines released from injured lungs, may trigger neuronal injury and promote a proinflammatory state in the brain by impacting the CNS directly or by influencing blood-brain-barrier (BBB) permeability to brain-toxic metabolites such as administered analgesics and sedatives [274-276]. Thus, in the absence of primary CNS trauma, these findings illustrate a possible mechanism by which burn-injured patients may incur secondary neurological insults that initiates or maintain the dysregulated neuroinflammatory response driving tHO. These data are similar to the recently reported prevalence of HO in the COVID-19 population [277-281].

It is further understood that burn injuries induce widespread peripheral nervous system damage [282, 283]. Even after superficial injury, there is potential for burn-induced neuroinflammatory and or neuronal-mediated mechanisms to induce changes to peripheral nerves [283]. After a burn, evidence of elevated substance P-positive fibres in the skin through to functional changes in cell activation and signal transduction has been documented [284]. Bone morphogenic protein-2 can act directly on sensory neurons by entering the blood-nerve barrier (BNB), in part by the degrading actions of matrix metalloproteinases (MMPs), to stimulate neuroinflammation, resulting in nerve remodelling and the migration and release of osteogenic and other stem cells from the nerve [270]. This finding is particularly relevant owing to the well-documented role of substance P (sP) as a potential early prognostic biomarker of neurological HO [249, 285]. MMPs have also been identified as critical players in developing BBB/BNB dysfunction after severe burn injury [286]. This research suggests that targeting sP and increased BBB/BNB permeability associated with its release after injury may affect tHO, either by acting locally and preventing tHO development at the peripheral injury site or by acting centrally where attenuating TBI outcomes may result in reduced tHO volume [249]. Overall, these findings support the notion that dysregulated neuroinflammation and disrupted neural signalling resulting from damage or alterations in the excitability of neural pathways from CNS/PNS injury after burn injury may play a multifaceted role in tHO pathogenesis.

Concomitant Injuries: Fracture

Investigation of concomitant musculoskeletal injuries showed that long bone fracture was significantly associated with tHO formation after trauma from univariate analysis. However, the correlation of the fracture site was not particularly related to the area of tHO formation. These

findings suggest that the fracture itself may not act as the immediate local stimulus to induce tHO development. Instead, a systemic response to the fracture repair process may provide the sufficient trigger that stimulates an aberrant healing response contributing to tHO pathogenesis. Further clinical evidence supports this theory, whereby tHO has been shown to develop in joints without overlying burn injury and in anatomical locations away from the site of localised trauma [3]. The amount of operative soft tissue damage has been suggested to impact the risk of tHO; however, there is limited conclusive evidence in the burn and neurotrauma literature surrounding the influence of surgically treated fractures on tHO risk [48]. In the current study, a significant association (p=0.010) was determined between tHO and fracture management variables, i.e., surgically treated vs conservatively managed fractures, with the most important proportion of patients managed surgically in the tHO+ cohort (tHO+, 34% *vs.* tHO-, 14.2%). This implies that the local soft tissue trauma inflicted by surgery may contribute as a predisposing factor to tHO.

Concomitant Injuries: Injury to the Hip Region and Thigh

In the present study, a concurrent injury to the hip region and thigh independently predicted tHO (OR 4.14, 95% CI 1.24-18.87, p=0.021) and had the most significant effect size. Further, some evidence at the univariate level suggests an association between the type of injury sustained to the hip and thigh region and tHO development ($p<0.001$).

Together, dislocation with or without fracture and soft tissue injury, including contusion, haematoma and laceration, accounted for over half the cases involving hip and thigh injuries in the tHO+ group, with zero cases of dislocation and only 16% of patients presenting with soft tissue injury in the control group. Trauma close to skeletal structures and soft tissue, such as joint dislocation and laceration or contusion-type injuries, may cause acute physical nerve damage, and an associated elevated risk for tHO development has previously been established for particular injuries and interventions which are linked with a higher likelihood of injury to peripheral nerves by stretch, compression or transection [53]. For example, elbow injuries have a significantly higher risk for tHO, with the incidence reported as high as 37% following fracture or dislocation of the elbow [8, 53, 54]. Levi et al. [24] proposes that certain anatomical aspects of the elbow joint, notably the ulna nerve's superficial location, predispose the nerve to further mechanical compression and stretch injury. This may explain the high prevalence of elbow joint involvement described in the present cohorts of burn and TBI patients.

Pelvic ring injuries and hip dislocation are significant risk variables for developing tHO following operative fixation for acetabular fractures. It is thought that the choice of surgical technique performed for cases requiring fracture fixation may lead to stretching of the adjacent sciatic nerve [53, 58]. Interestingly, there was a significantly higher proportion of the tHO- control cohort with lower limb fracture (tHO-, 77% *vs.* tHO+, 30%). This suggests that the soft tissue trauma with possible peripheral nerve injury, which occurs secondary to dislocations, contusions, and laceration-type injuries, may be a more significant contributor to the pathogenesis of tHO rather than the coexistent fracture itself. In combat-wounded patients, CNS trauma and musculoskeletal polytrauma, i.e., widespread soft tissue trauma secondary to blast injury and amputation, are independent risk factors for developing tHO [42]. Although concomitant amputation only contributed 9% to injuries of the hip region and thigh within the current tHO+ cohort, soft tissue injuries, including amputation, particularly in the presence of CNS or PNS injury, should be taken into consideration as risk factors for tHO by burn and trauma clinicians treating civilian trauma patients.

Although local factors may play a supporting role, the current understanding of tHO pathogenesis suggests that a confluence of factors contributes systemically to its initiation and propagation. Thus, these factors above, in unity, may offer the common neurotrauma mechanism that stimulates tHO formation across trauma populations with different aetiologies. Adequate management of these risk factors could help reduce the overall incidence of tHO and improve trauma patient outcomes.

5.3.1 Limitations

Even though all clinical variables extracted from the administrative databases were confirmed and strengthened by a manual review of patient electronic medical records, the retrospective design of this study limits the inference of causal factors. Rural patients with geographic barriers pose a challenge for discharge and may have higher incidences of extended LOS. However, the effect of geographic location on LOS was not accounted for in the analysis and, thus, may be considered a limitation of this study. A number of the infectious agents studied had too few observations to gain significance or power to be used in multivariable regression analysis. Thus, to increase the strength of future studies, including a larger number of events through a national, multicentre study design would be recommended.

5.10 Conclusion

This study of pooled injury cohorts has revealed novel risk factors for tHO development after burn injury. These findings may contribute towards developing a valid and comprehensive risk profile for early targeted surveillance of trauma patients at high risk of developing tHO.

5.11 Key Points

- tHO most commonly develops bilaterally in trauma patients in >2 anatomical sites.
- Clinicians are encouraged to surveil for multi-joint involvement and consider assessing the symmetrical bilateral joint of patients with confirmed unilateral tHO, regardless of an absence of clinical suspicion.
- Prolonged hospital LOS and having an injury to the hip region or thigh were identified as significant, independent predictors for developing tHO after burn and neurotrauma.
- Infectious agents identified as common independent factors for tHO development include *Staphylococcus* (not including Staph. Aureus), *Acinetobacter calcoaceticus-baumannii* and *Enterobacter cloacae complex.*
- Risk stratification in burn patients should include concomitant injuries - a novel association with tHO development. In addition, CNS/PNS injury and long bone fracture, particularly if requiring surgical management.

Chapter 6.

Investigating the Molecular and Cellular Contributions of Traumatic Heterotopic Ossification after Burn Injury

Overview

The guiding questions addressed in this chapter are "What *are the key genes and signalling pathways that differ in dermal fibroblasts from patients who do and do not develop tHO after burn injury?"* and "Do *burn patients that develop tHO after injury have a dermal fibroblast phenotype that is more susceptible to osteogenic differentiation?*". The use of human biospecimens was intended to represent a more clinically translatable sample and were derived from adult patients who did and did not develop tHO after burn injury. Investigating potential biomarkers implicated in tHO pathogenesis may lead to the development of cell-specific therapeutic strategies to prevent or treat tHO after burn and other trauma, with an aim to benefit patient outcomes. This chapter, provided in a draft manuscript format, presents Study 5 of this research programme and, as such, includes repetition of the earlier thesis text.

6.1 Introduction

Traumatic heterotopic ossification (tHO) is the pathological formation of mature, lamellar bone in non-skeletal sites, such as within muscle and connective tissue [287]. Well known to burn clinicians, tHO is a debilitating sequela of local and systemic inflammatory insult, reported to occur following injury to the skin, nervous system, or direct musculoskeletal trauma [2]. It is thought that tHO occurs via endochondral or intramembranous ossification as a consequence of aberrant tissue repair associated with persistent inflammatory dysregulation [136]. Contemporary understanding of the neuroinflammatory regulation of bone remodelling suggests that injury to the central or peripheral nervous system impacts the regulatory molecules that control skeletal growth and maintenance [35]. However, despite these recent developments, the precise mechanism(s) by which inflammatory insult dysregulates the balance between anabolic factors, cytokines, and osteogenic pathway agonists and antagonists towards the pathological formation of ectopic bone remains largely unknown.

Mesenchymal stem cells (MSCs) are multi-potent cells capable of differentiating osteogenically into mature cells of several mesenchymal tissues, such as bone. Fibroblasts comprise the primary cell type of connective tissue, possessing a spindle-shaped morphology, and function to produce extracellular matrix (ECM) responsible for maintaining the structural integrity of the tissue. Characterisation of mesenchymal fibroblasts in the dermis of those with fibrotic skin disease using single-cell RNA sequencing identified expression of mesenchymal progenitor markers previously defined to be involved in osteoblast differentiation and ossification [288-290]. More so, fibroblasts have shown a programmable potential to transdifferentiate towards pro-fibrotic and pro-regenerative phenotypes, with a multipotent ability to differentiate into osteoblasts. According to the transcript and anatomic position, fibroblasts can be phenotypically moderated in response to different inflammatory milieus, leading to varying outcomes of tissue regeneration, including bone formation. In response to inflammatory reactions after severe injury, PDGFRα+ cells, a typical fibroblast-like cell found throughout most adult tissues, contributed to ectopic bone formation in soft tissue [20, 291]. Hortells et al. [292] illustrated the ability of cardiac fibroblast cells to acquire osteoblastic characteristics and participate in pathological ectopic calcification of injured vasculature. As alluded to by these authors, it is currently unknown as to whether the phenotypic transition of fibroblasts into calcifying or ossifying cells occurs at the transcriptional level of specific genes such as RUNX2 and ALPL or whether there are more global epigenetic changes at play in the

pathogenesis. In this context, bone morphogenic protein (BMP) signalling is essential in regulating osteoblastic lineage commitment. Furthermore, the activation of Wnt signalling has been reported to facilitate osteogenic differentiation. However, a better understanding of the cell populations that respond to osteogenic signals and the relationship of soft tissue trauma and inflammation to the activation of the osteogenic program among the responsible progenitor cells is needed.

Here, to decipher the interaction among dermal fibroblasts and traumatic heterotopic bone formation, we designed experiments around the hypothesis that burn patients who develop tHO after injury exhibit a dermal fibroblast phenotype that is more susceptible to osteogenic differentiation than those who do not develop tHO. The objectives of the study, therefore, were: (i) to identify candidate genes and signalling pathways that differ in fibroblasts from tHO+ and tHO- patients after burn injury and (ii) to determine whether fibroblasts from burn-injured tHO+ patients are more susceptible to osteoblastic differentiation compared to control fibroblasts.

6.2 Methods

6.2.1 Ethical Considerations

Ethics approval was granted for a national multicentre trial by the South Metropolitan Health Human Research Ethics Committee (Project record number: RGS3452) and from The University of Notre Dame, Australia (2020-013F)**.**

6.2.2 Clinical Sample Collection and Preparation with Host Demographics

6.2.2.1. Tissue Biopsies

Skin punch biopsies (3mm diameter) were obtained from 14 patients who had previously sustained burn injuries using a standard procedure (Appendix 5F). Briefly – 3mm pieces of skin were placed dermis side down on the bottom of a T-25 flask and incubated at 37°C for 1 hour. Dulbecco's Modified Eagle Medium (DMEM)-Glutamax™ (Invitrogen Gibco) with 10% fetal bovine serum (FBS) (Invitrogen Gibco), 1% pen/strep (Invitrogen Gibco), 1% kanamycin (Invitrogen Gibco) and 1% amphotericin B (Invitrogen Gibco) was then carefully added, ensuring the skin did not detach from the flasks. These were then cultured until dermal fibroblasts migrated out of the explants, with the media changed every three days. For burn patients with a diagnosis of tHO (tHO+, n=9), biopsies from two anatomical locations were collected; Proximal Site 1 (S1): from the skin over the location of tHO and distal Site 2 (S2): from unscarred skin distal to the tHO site **(Table 12)**. Only a single

biopsy of unscarred skin was collected from control subjects (burn patients without a diagnosis of tHO (tHO-, n=5) **(Table 13)**.

Table 11. *Demographic and injury variables for tHO+ and tHO- cohorts*

		tHO+ (n=9)	**tHO- (n=5)**	**Total**	***p***
Age at injury (years)		38.5±21 35 (56-21)	37.2±13 42 (43-26)	38.1±18	0.900
Gender	**Male**	8 (88%)	4 (80%)	12 (85.7%)	0.649
	Female	1 (11%)	1 (20%)	2 (14.2%)	
TBSA (%)		55.5±27 62 (80-50)	58.8±13 60 (64-55)	56.7±22.2	0.802

Continuous variables are presented as Mean±SD and median (IQR), and categorical variables as frequencies and percentages. tHO: traumatic heterotopic ossification, TBSA: total burn surface area, *p*: p-value

Table 12. *Demographic and injury data including anatomical location of collected skin punch biopsy samples from tHO+ subjects*

tHO+ subjects n=9	Subject No.	Injury age	Gender	TBSA %	Year of burn injury	Time (years) to sample collection	3mm skin punch biopsy samples			
							Site 1	Anatomical location	Site 2	Anatomical location
	P1	54	M	70	2013	6	✓	R elbow lateral epicondyle	✓*	R upper chest
	P2	22	M	80	2013	6	✓	R knee VMO ~5cm superomedial from superior pole of patella	✓	R upper chest
	P3	35	M	50	2019	1	✓*	L forearm volar surface ~2cm from elbow crease	✓	R forearm volar surface ~2cm from elbow crease
	P4	70	M	51	2013	7	✓	L elbow ~1cm distal to medial epicondyle in region of cubital tunnel	✓	R upper chest
	P5	58	F	80	1998	21	✓	L elbow posteromedial aspect	✓	L lower abdomen
	P6	12	M	80	2005	15	✓	R elbow over region of radial head	✓	Posterior neck
	P7	21	M	7	2012	8	✓	R medial groin region	✓	R lower abdomen
	P8	19	M	61.5	2011	9	✓	L elbow	✓	L mid-thigh
	P9	56	M	20	2010	10	✓	R lateral upper arm ~3cm from amputation scar	✓	R lower abdominal region

* Signifies sample collected but not deemed viable during sample processing or analysis. tHO: traumatic heterotopic ossification, TBSA: total burn surface area, R: right, L: left

Table 13. *Demographic and injury data including anatomical location of collected skin punch biopsy samples from tHO- subjects*

tHO– subjects n=5	**Subject No.**	**Injury age**	**Gender**	**TBSA**	**Year of burn injury**	**Time (years) to sample collection**	**3mm skin punch biopsy samples**	
							Site 1	**Anatomical location**
	C1	54	M	55	2013	7	✓	L medial bicep ~4cm from elbow crease
	C2	26	F	60	2012	8	✓*	L medial bicep ~2cm from elbow crease
	C3	42	M	64	2019	1	✓	R Lower abdominal region ~2cm lateral from navel
	C4	43	M	40	2009	11	✓	L lateral arm ~10cm proximal from elbow crease
	C5	21	M	75	2009	11	✓	L lower abdominal region

* Signifies sample collected but not deemed viable during sample processing or analysis. tHO: traumatic heterotopic ossification, TBSA: total burn surface area, R: right, L: left

Table 14. *Demographic and injury data including anatomical location of collected mature heterotopic bone samples from tHO+ subjects*

tHO+ subjects n=3	Subject No.	Injury age	Gender	Primary injury cohort	Year of injury	Time (years) to sample collection	Heterotopic bone samples	
							Site	Anatomical location
	P10	35	M	Burn	2019	2	✓	R distal femur
	P11	42	M	Orthopaedic	2018	2	✓	R medial femur
	P12	47	M	Orthopaedic	2019	1	✓	R elbow

tHO: traumatic heterotopic ossification, R: right

6.2.3 Histology of Excised Human Heterotopic Bone

Samples of mature heterotopic bone were available to be collected from burn (n= 1) and orthopaedic (n= 2) injured patients who underwent an elective surgical procedure for the removal of heterotopic bone **(Table 14)**. The mean time between injury and tHO excision was 21 months. Samples were fixed in 10% neutral buffered formalin for 48 hours, washed in 1x Phosphate-buffered saline (PBS) to remove residual fixative, and then decalcified in 14% EDTA at 37°C for 14 days with EDTA reagent refreshed daily. Samples were then washed in 1x PBS, dehydrated, processed into paraffin wax using a standard overnight protocol on a tissue processor and embedded in paraffin wax blocks. Each sample was sectioned at 5µm on a Leica automatic microtome, with sections collected in consecutive pairs at two intervals 100 µm apart.

Paired sections were stained with Haematoxylin-eosin (H&E) to provide a general overview of tissue and cell morphology. Tartrate-resistant acid phosphatase (TRAP) staining was performed to visualise bone-digesting osteoclasts. Osteoblastic cells were harvested in parallel from the same tissue sample(s) by mechanical disaggregation and served as a positive control sample in functional assays described below. Images of H&E and TRAP staining were taken with a Nikon Eclipse Ts2 microscope, GMP USB 3.0 camera, and Capture V2.2.1.0 software.

6.2.4 RNA-Sequencing

Fibroblasts from the skin biopsies were cultured using a standard explant method [293] within 24-48 hours of collection (Appendix 5F) and cultured to passage 2. RNA was extracted using a Qiagen RNeasy Mini Kit (Cat No./ID: 74104, QIAGEN, Germany) with an on column DNase digestion also carried out. Nineteen samples were analysed, of which fifteen were tHO+ samples, and four were from controls. A NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA) was used to examine the quality and quantity of the extracted RNA. Quality control tests of the samples were also conducted using the Agilent Bioanalyser and LabChip GX software (v4.2.1745.0). Aliquots of 20µg were then sent to the Australian Genome Research Facility (AGRF), where 1µg of total RNA (total RNA <1600ng) was used for RNA sequencing. Illumina NovaSeq Control Software (NCS) (v1.6.0) for image analysis and Real-Time Analysis (RTA) (v3.4.4) for real-time base calling was used, producing a 100 bp single end run. The Illumina bcl2fastq 2.20.0.422 pipeline was used to generate the primary sequence data.

6.2.5 Bioinformatics Analysis of RNA Sequencing

Genome data was sent from AGRF, downloaded on the server, and processed using MATE Terminal (Version 1.8.0) and R studio version (Version 3.6.1). FastQC (v0.11.3) was used to check the quality of the RNA sequencing raw data, and reads of poor quality (the first 15 bp) were trimmed using the fastp software [294]. The RNA sequencing raw reads were then aligned using the STAR read aligner (v2.7) [295] to the Ensembl release 98 Human GRCh38.p13 reference genome (http://asia.ensembl.org/Homo_sapiens/Info/Index)[296]. SAMtools (v1.9) [297] was used to generate BAM files. Exploratory analysis (sample-to-sample distribution and prinPCA) used rlog transformed values. Differential gene expression analysis was run using DESeq2 package (v1.24.0)[298] on R studio (v3.6.1)[299]. A threshold of the adjusted p-value (Padj) <0.05 and an absolute log2(fold change) >1.5 cut-offs were set to identify differentially expressed genes (DEGs). Gene expression levels for each cell were normalised by total expression, multiplied by a scale factor (10,000), and log-transformed.

One hundred thirty-six significantly differentially expressed genes (DEGs) identified were individually analysed within the current literature. Table A5.11 (Appendix 5B) describes each gene based on its function and identifies genes of interest according to their roles; as a major transcription factor, regulatory role(s) in osteogenesis or chondrogenesis, including previous associations with ectopic bone formation, skin/fibrosis including ECM remodelling; other associated functions or diseases/disorders of the nervous system and the musculoskeletal system.

6.2.6 Gene Ontology and Pathway Enrichment Analysis of Differentially Expressed Genes

To explore the genes and biological pathways differentially expressed in tHO+ fibroblasts, Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using ClusterProfiler (Version 3.12.0)[300] with the DEGs list. GO terms under biological process, cellular component, molecular function categories, and KEGG pathways were analysed to determine significantly enriched genes and associated pathways with the enrichment threshold of $p<0.05$.

6.2.6 Functional Assays

Human fibroblasts were cultured from tHO+ and tHO- subjects as previously described. Normal fibroblasts from non-burn-injured subjects were included as a “true control” sample. The tHO+ osteoblasts harvested from excised heterotopic bone were included as a positive control.

Fibroblasts and osteoblasts were seeded into 24 well plates at a density of 2 x 10^4 cells/well for functional assays **(Figure 7)**. All cell types were cultured in a standard medium composed of alpha minimum essential medium (MEM α, no nucleosides; #12561056, Thermo Fisher Scientific) containing 10% FBS and 1% penicillin-streptomycin (Thermo Fisher Scientific). Cells were incubated in a humidified incubator at 37°C in 5% CO2, replacing media every three days. When nearly 100% cell confluency was reached (~day 12) in the well of the 24-well culture plate, the medium was changed to a mineralisation medium supplemented with various combinations of L-Ascorbic acid (AA; #A4544-25G, Sigma), Human bone morphogenic protein-2 (BMP-2; # RDS355BM010CF R&D Systems Recombinant Human/Mouse/Rat BMP-2 Protein, CF, 10 ug), β-Glycerophosphate disodium salt hydrate (β-G; G9422-10G, Sigma) and Dexamethasone (DEX; # D4902-25MG, Sigma). Media and treatments were replenished every 2-3 days for up to 21 days.

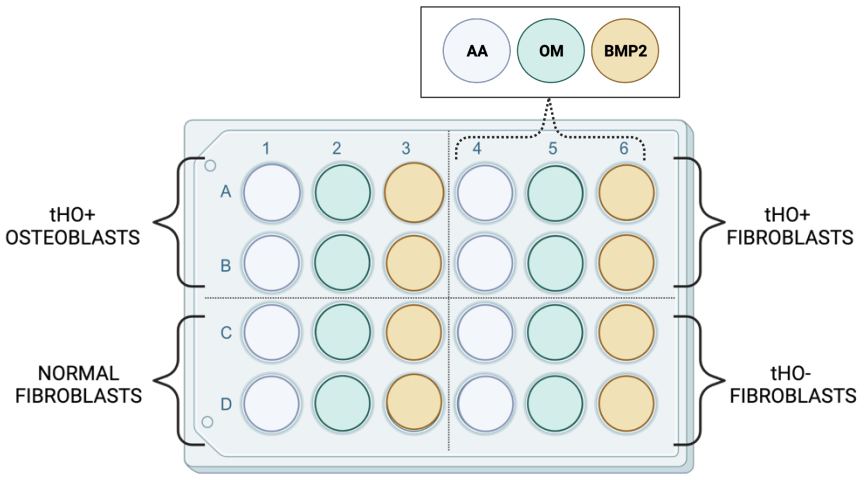


Figure 7. *Plate layout for functional assays*

6.2.6.1 Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) staining was carried out after 17-day osteo-induction. Briefly, cells were washed twice with phosphate-buffered saline (PBS) and fixed with 300µL per well of 4% paraformaldehyde solution (PFA) at room temperature for 15 minutes. Cells were then stained for ALP activity in each well using 5-bromo-4-chloro-3-indolyl phosphate (BCIP®) tablets and nitro blue tetrazolium (NBT) Alkaline Phosphatase Substrate (Sigma-Aldrich) in 10ml distilled H_2O, as per the manufacturer's instructions (Appendix 5I). Finally, micro-structures of ALP staining were observed and photographed using a Nikon Eclipse Ti-S microscope and representative images of each well

were captured in triplicate. Semi-quantitative analysis was performed based on the percentage of area of cells stained by ALP. The area of cells stained by ALP was calculated using Image J v2.0 Software (National Institutes of Health, USA) [301].

6.2.6.2. Osteoblast Mineralisation Assay

The mineralisation-inducing ability of human fibroblasts was investigated using Alizarin Red staining (ARS), a commonly used stain to detect calcium deposition in tissue sections. Fibroblasts were cultured in a mineralisation-inducing medium for up to 21 days under three conditions described above in Section 2.6 and Appendix 5J. The level of mineralised calcium nodule formation was visualised by Alizarin Red S staining at day 21 and imaged using a Nikon Eclipse Ti-S microscope. Additional representative images of wells for each cell line and condition were taken in triplicate.

To quantitatively measure the deposited ARS, 500µL 1% (m/v) cetylpyridinium bromide solution was added into each well of the plate. 100µL solution from each well was transferred into 96-well plates in triplicate, and absorbance was measured at 495nm. Since the cells were confluent in the well of the 24-well culture plate at the time of measurement, we assumed that the number of cells in each well was constant. Wells that had not reached full confluency after 21 days were excluded from the analysis. The 495 nm absorbance of the reaction solution in each well was adopted as the index for alizarin red staining and was spectrophotometrically measured using the Perkin Elmer Wallac 1420 VICTOR2™ microplate reader (PerkinElmer, Inc, USA).

6.2.7 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

6.2.7.1. RNA Extraction, cDNA Synthesis and Analysis

The expression of pro-osteogenic genes was evaluated by qRT-PCR to confirm the osteogenic differentiation potential of dermal fibroblasts under BMP-2 stimulation over a 21-day differentiation period. At indicated time points (Day 0, 14 and 21), fibroblasts were harvested and lysed in 350ul buffer RLT (QIAGEN) and stored at -80°C. All RNA extractions were then carried out using RNeasy Mini Kit (QIAGEN group) per the manufacturer's instructions. Total RNA was subsequently checked for consistent quality and quantified spectroscopically using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Reverse transcriptase polymerase chain reaction (RT- PCR) was used to convert 2µg of RNA to cDNA. mRNA transcripts for four key genes (RUNX2, BSP/IBSP, ALPL

and PHEX) involved in early and late osteogenic differentiation pathways were examined by real-time PCR.

cDNA was synthesised from RNA samples using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) per manufacturers' instructions. mRNA expression for genes of interest was quantified via qRT-PCR with Taqman primer probes. Reactions were prepared according to the manufacturer's instructions, using Taqman™ Universal PCR Master Mix and human probes for CADM1, DPP4, NFATC2, STEAP4, WNT4, RUNX2, BSP/IBSP, ALPL and PHEX (Table A5.3, Appendix 5C). Gene function is documented in Table A5.1 (Appendix 5B). Ct values were normalised by the expression of housekeeping genes 18S and TBP. The amplifications were performed using the QuantStudio 7 Flex Real-time PCR system (Applied Biosystems). Experiments were carried out in triplicate for each biological sample analysed. Relative gene expression for each gene of interest was calculated using the $2^{-\Delta\Delta Ct}$ method (reference), normalised, and compared to the gene expression levels of controls; tHO- fibroblasts and normal, un-injured fibroblasts. Genes found to be significantly deferentially expressed between treatment groups are reported.

7.2.8 Statistical Analysis

All data are expressed as mean ± standard deviation (SD). ALP staining and osteoblast mineralisation assays were analysed using $n = 3$ replicates. qRT-PCR used $n = 2$ replicates. Statistical analysis was performed with the GraphPad Prism software 9.5.1 (GraphPad Software, Inc.), and $p<0.05$ was taken to be statistically significant. For a two-group comparison, Students t-test was used. Multiple comparisons were conducted by the Tukey posthoc test using a one-way analysis of variances (ANOVA).

6.3 Results

6.3.1 Demographic Variables

Table 11 summarises the injury age, gender, and TBSA. For patients who contributed samples for analyses, the anatomical location(s) of the skin punch biopsies and excised heterotopic bone are described in **Tables 12, 13 and 14**, respectively. Males accounted for 88% of the tHO+ cohort and 80% of the control group. There was no significant difference in the mean age (38.5 years vs. 37.2 years, p=0.900) and TBSA (55.5% vs. 58.8%, p=0.802) between the tHO+ and tHO- groups, respectively.

6.3.2 Histological Appearance of Human Heterotopic Bone

6.3.2.1. H&E Staining

To elucidate the histopathological mechanism(s) of tHO, features of mature heterotopic bone were examined on H&E stained sections. A whole-mount tile scan image of a mature tHO case is shown in **Figure 8**. Represented in **Figure 8.1** is the formation of thickened lamellar, mature bone with embedded osteocytes organised into osteons/haversian-like canal structures that resemble native cortical bone. There is evidence of the beginnings of a pseudo-marrow space forming, composed of fibrotic marrow and adipocytes. Thickened bone trabecular surfaces are lined with osteoblasts laying down new bone, indicating active bone synthesis and remodelling. In **Figure 8.2**, bone trabeculae are composed of immature woven bone, and cuboidal bone-lining cells are characteristic of osteoblasts. There is evidence of osteocytes embedded inside the woven bone. Bone is of intramembranous origin in this case, forming directly from MSC/fibroblastic stromal cell condensates (spindle-like cells with elongated nuclei) with prominent bone-lining cells observed around the newly formed bone. Fibrous stroma surrounding new bone trabeculae may serve as sources of MSC/fibroblastic precursors that differentiate into osteoblasts.

6.3.2.2. TRAP Staining

To assess whether the mature tHO specimen was undergoing active bone remodelling, a consecutive section was stained for TRAP, a marker of mature osteoclasts **(Figure 9)**. As shown in **Figure 9.1**, TRAP-positive multinucleated osteoclasts are evident along the exterior bone surface. These cells are actively engaged in bone resorption as evidenced by extended eroded surfaces (bone resorptive pits), indicative of active remodelling and or increased bone erosion, likely reflecting the ectopic nature of the bone mass. Resorbed spaces are infiltrated with fibroblast/stromal cells and scattered TRAP-positive monocytes/macrophages (osteoclast precursors). In addition, TRAP signal was observed within osteocyte lacunae arranged in osteon/haversian canal-like structures, suggestive of osteocytic-osteolysis as occurs in other bone pathologies (PMID: 26796208; PMID: 36331133).

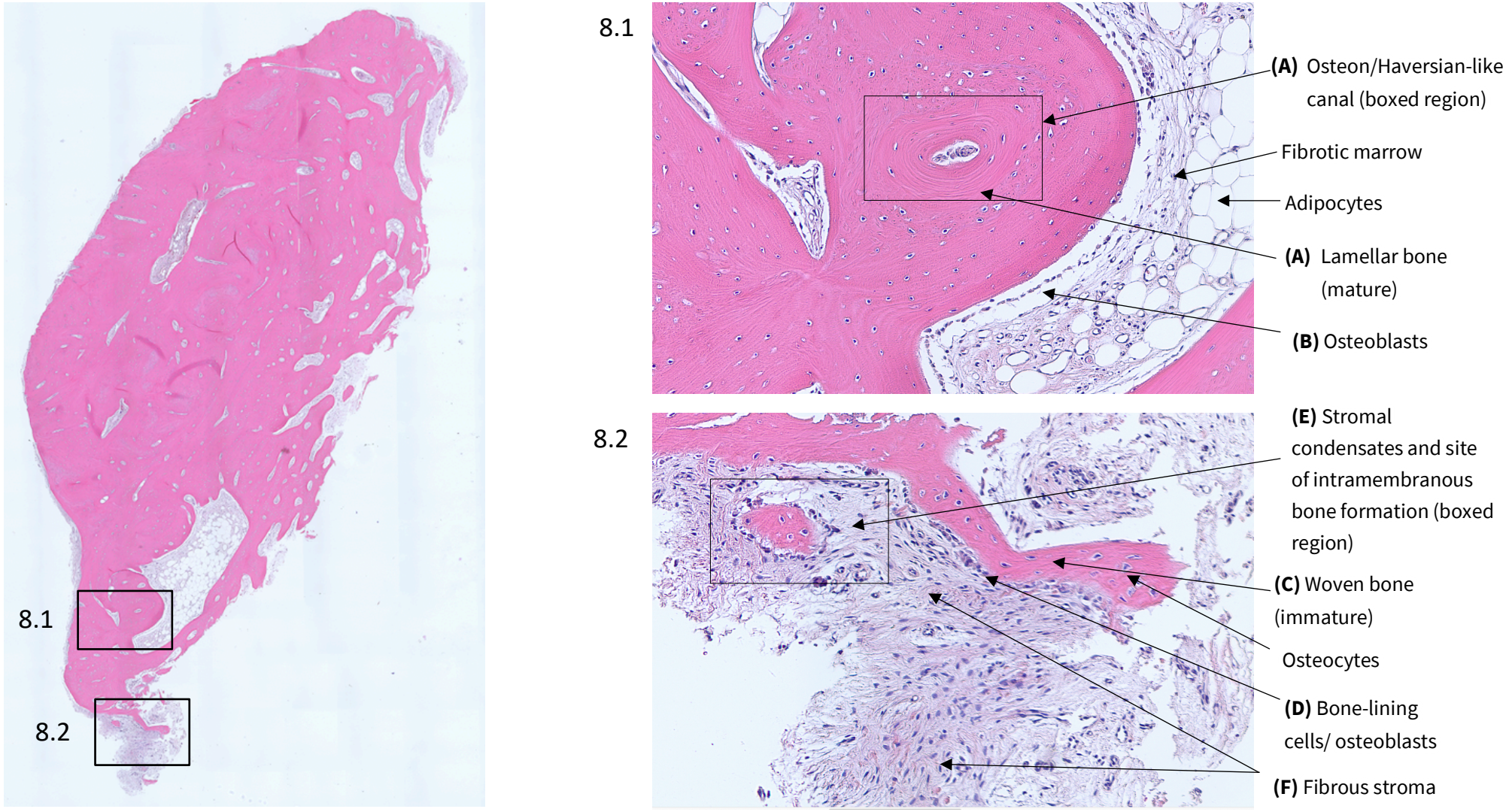


Figure 8*. H&E staining of excised sample of mature tHO+ bone* ***Figure 8.1 (A)*** *formation of thickened lamellar, mature bone that bears resemblance to native cortical bone.* ***(B)*** *Trabecular surfaces are lined with osteoblasts laying down new bone and indicating active bone synthesis and remodelling.* ***Figure 8.2 (C)*** *Bone trabeculae is composed of immature woven bone.* ***(D)*** *Cuboidal bone-lining cells are characteristic of osteoblasts.* ***(E)*** *Bone is of intramembranous origin forming directly from fibroblastic stromal cell condensates with prominent bone-lining cells observed around newly formed bone.* ***(F)*** *Fibrous stroma surrounding new bone trabeculae may serve as sources of fibroblastic precursors that differentiate into osteoblasts.*

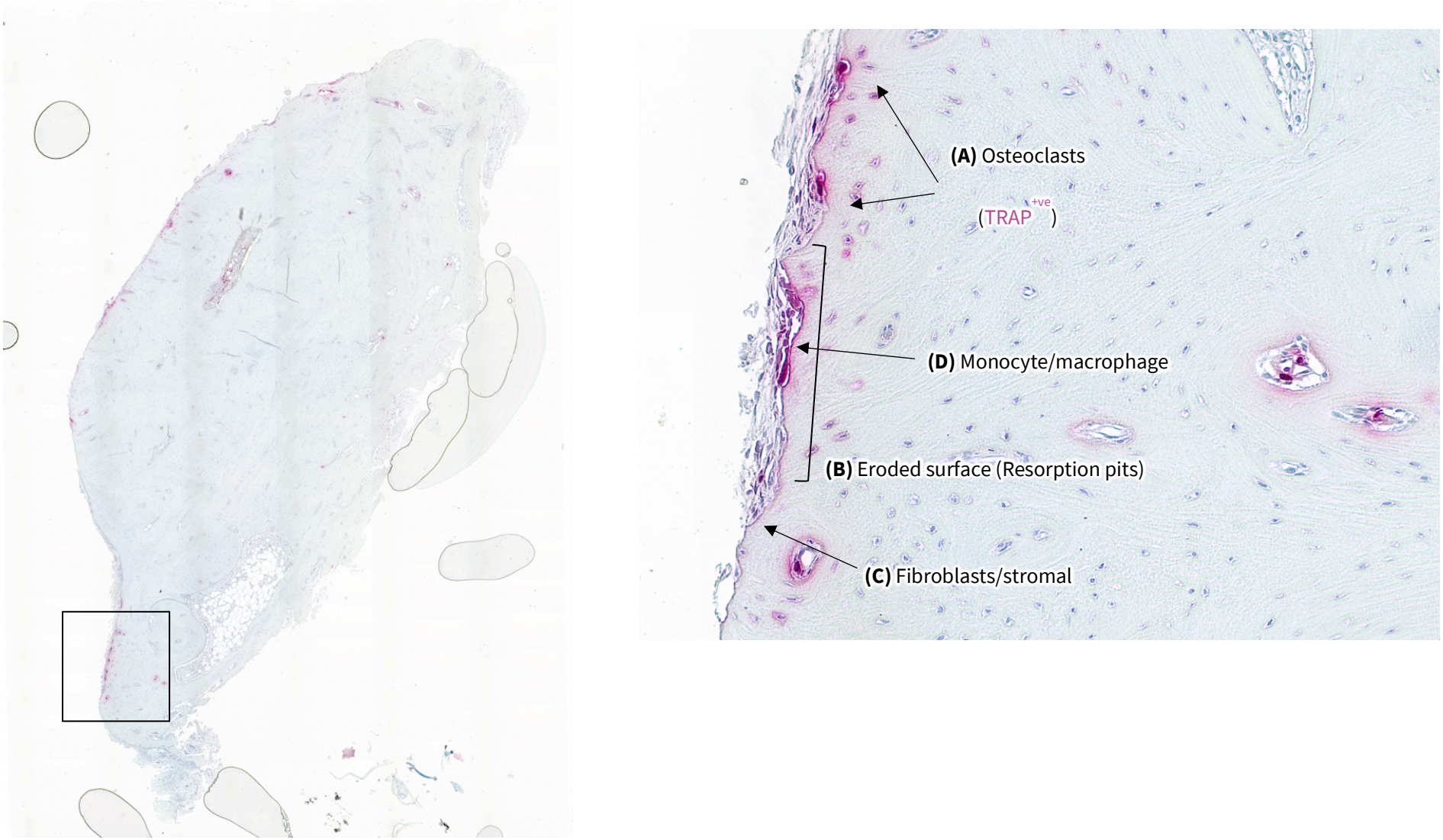


Figure 9. *TRAP staining of excised sample of mature tHO+ bone* ***(A)*** *TRAP positive multinucleated osteoclasts are evident along the exterior bone surface and within osteon/haversian canal-like structures (stained pink).* ***(B)*** *Prominent osteoclast bone-resorptive activity evidenced by extended eroded surfaces (bone resorptive pits) indicative of increased remodelling/increased degradation possibly due to the ectopic nature of the bone mass. Resorbed spaces are infiltrated with* ***(C)*** *fibroblast/stromal cells and* ***(D)*** *scattered TRAP-positive monocytes/macrophages (osteoclast precursors).*

6.3.3 RNAseq of Skin Fibroblasts and Functional Experiments

tHO+ Fibroblasts exhibit a Different Transcriptome Compared to tHO- Fibroblasts

To identify candidate molecules involved in osteoblastogenesis and pathological ectopic bone formation, we compared the transcriptome of tHO+, tHO- and normal fibroblasts with RNAseq analysis. Searching for genes that displayed a log2 fold-change value of >1.5 expression levels in tHO+ site 1 (proximal) fibroblasts and tHO- control (no scar) fibroblasts **(Figure 10A)** and tHO+ site 2 (distal) fibroblasts and tHO- control (no scar) fibroblasts **(Figure 10B)**, we revealed 136 significantly differentially expressed genes in dermal fibroblasts from distal site 2 in tHO+ subjects compared to tHO- subjects, of which 29 were upregulated, and 107 genes were downregulated. Of these, 82 genes were significantly changed between fibroblasts from proximal site 1 compared to tHO- control fibroblasts. We identified many genes with a correlation to osteogenic differentiation and bone formation. More so, we found that genes significantly enriched in tHO+ fibroblasts related to nervous system development and disease, musculoskeletal system development and regeneration, and genes related to fibrotic processes (Table A5.1, Appendix 5B). The heatmap suggests that gene expression significantly differed between the tHO+ fibroblasts from proximal site 1 and tHO- control fibroblasts **(Figure 10A)**. Principal component analysis of datasets demonstrated significant gene expression differences between tHO and control samples **(Figure 11)**.

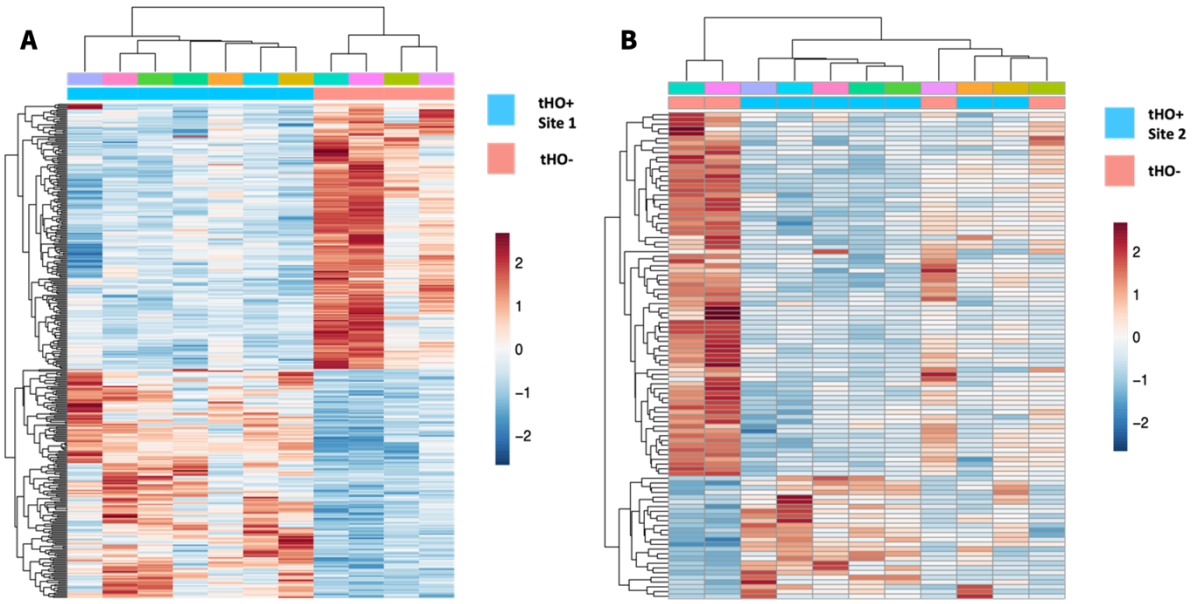


Figure 10. *Heatmap of DEGs with a threshold of Padj<0.05 and absolute log2(fold change) >1.5 differentially expressed in* ***(A)*** *tHO+ site 1 (proximal) fibroblasts and tHO- control (no scar) fibroblasts,* ***(B)*** *tHO+ site 2 (distal) fibroblasts and tHO- control (no scar) fibroblasts. Red refers to up-regulated expression and blue refers to down-regulated expression*

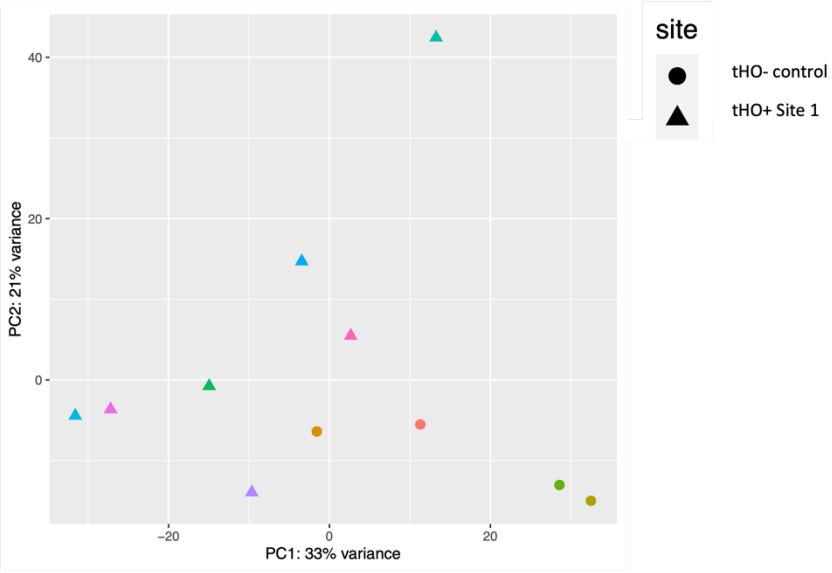


Figure 11. *Principal component analysis of datasets from tHO+ site 1 (proximal) fibroblasts and tHO- control fibroblasts. Coloured circles represent tHO- control samples and coloured triangles represent tHO+ Site 1 (proximal) samples*

To validate the transcriptional analyses of selected genes associated with osteogenic processes, the mRNA expression of CADM1, DPP4, NFATC2, STEAP4 and WNT4 in mature tHO+ osteoblasts and tHO+ fibroblasts against tHO- and uninjured fibroblasts was verified with qRT-PCR **(Figure 11A-D)**. The transcriptional levels of *CADM1, NFATC2 and WNT4* in the tHO+ fibroblasts were upregulated compared to the tHO- group.

CADM1 has been associated with several biological functions, such as heterotopic cell-cell interaction. It has been strongly positively correlated with the ectopic bone-forming capacity of human MSCs *in vitro,* highlighting its role as a regulator of bone mass and skeletal growth [302]. In line with these findings, *CADM1* was principally expressed (20-fold) in tHO+ osteoblasts compared to tHO- fibroblasts. The average increase in *CADM1* expression in tHO+ fibroblasts compared to tHO- fibroblasts was a 9.1-fold enrichment at proximal site 1 and a 7.4-fold enrichment at distal site 2, validating the RNA-seq results. NFATC2 is a gene encoding a member of the nuclear factor of activated T cells (NFAT) family of transcription factors that play a role in osteoclastogenesis. However, the mechanisms whereby Nfatc2 exerts its effects on osteoblastic cells are unclear. We found that *NFATC2* was robustly expressed in tHO+ osteoblasts, and expression of *NFATC2* in tHO+ fibroblasts was up to 4-fold higher than in tHO- fibroblasts. *WNT4* has a role in promoting osteogenic differentiation of MSCs, and a 2.3-fold and a 1.3-fold upregulation was identified in tHO+ fibroblasts at proximal and distal sites, respectively. Six transmembrane epithelial antigen of the prostate 4 (*STEAP4)* expression in human tissue is highest in bone marrow, and its expression is upregulated during osteoclast differentiation. We found that transcriptional levels of STEAP4 were downregulated in tHO+ fibroblasts, as expected. Collectively, upregulated gene expression of *CADM1*, *NFATC2* and *WNT4*, and downregulation of *STEAP4* at the mRNA level was in keeping with transcriptional activity. Conversely, gene expression of fibrotic marker DPP4 (CD26) was inconsistent with the significant downregulation observed by RNAseq.

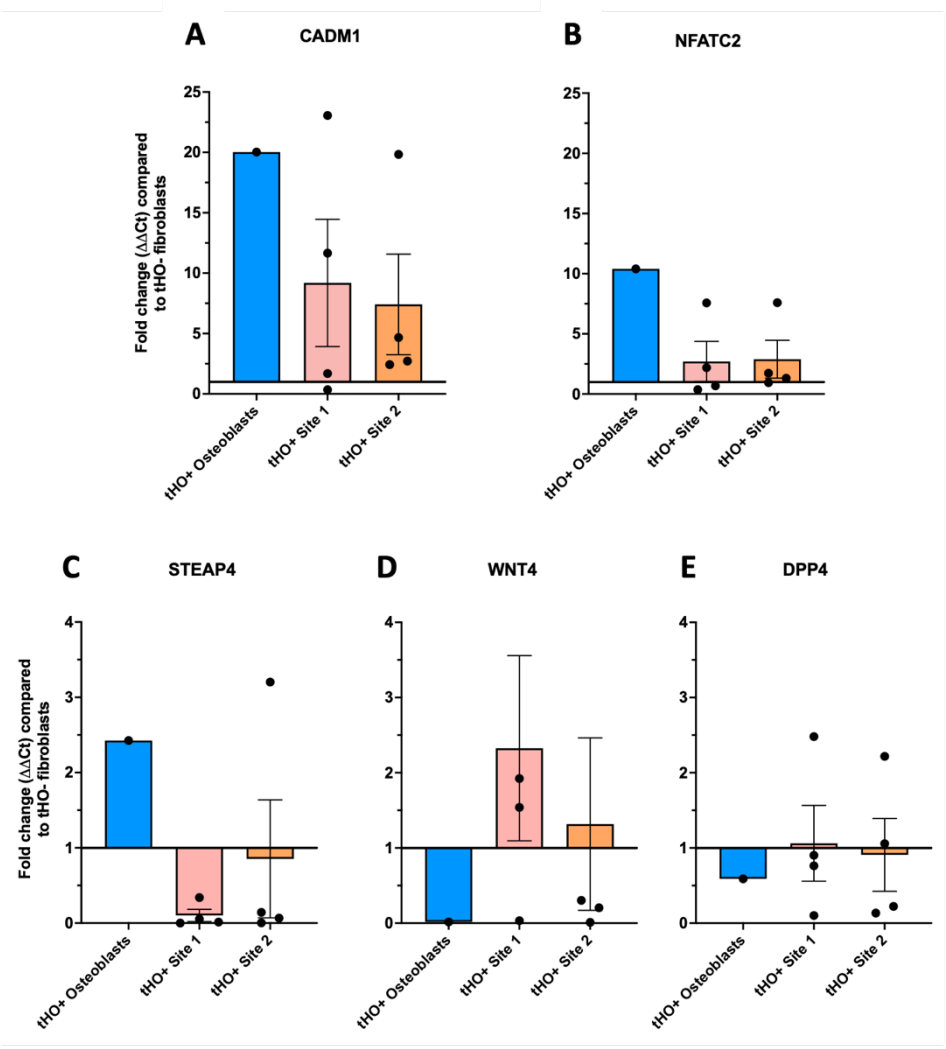


Figure 12. *Validation of individual osteogenic genes evaluated by quantitative RT-PCR.**(A-D)** Relative expression of CADM1, NFATC2, STEAP4, WNT4 and DPP4/CD26 in tHO+ fibroblasts shown as fold change compared to tHO- control fibroblasts. Data represent the mean ± SEM*

Wnt Signalling Appears to be Dysregulated in tHO+ Fibroblasts

There is considerable *in vitro* evidence supporting a role for Wnt/β-catenin (i.e., canonical) signalling in promoting bone formation via stimulating the progression of MSCs from osteoblastic precursor cells into more mature osteoblasts. Higher levels of Wnt signalling, through β-catenin, enhances bone formation with concomitant increases in expression of osteogenic regulators (Runx2, Dlx5 and Osx) whilst suppressing differentiation into adipogenic and chondrogenic lineages [303-306]. Several Wnt proteins, including Wnt4 and Wnt16, have significant roles in osteoblast formation. More so, Wnt and BMP signalling may cooperate and synergise to support osteoblast differentiation

[307]. In our results, several Wnt proteins and Wnt target genes were found to be significantly differentially expressed in tHO+ fibroblasts, including WNT16, CDH5, MMP27, COL6A6, CYP19A1, DPP4/CD26. Further, Gene Ontology and KEGG pathway analysis revealed that dysregulated gene functions of DEGs in tHO+ fibroblasts were significantly enriched in biological processes and primarily associated with pathways, including the Wnt signalling pathway.

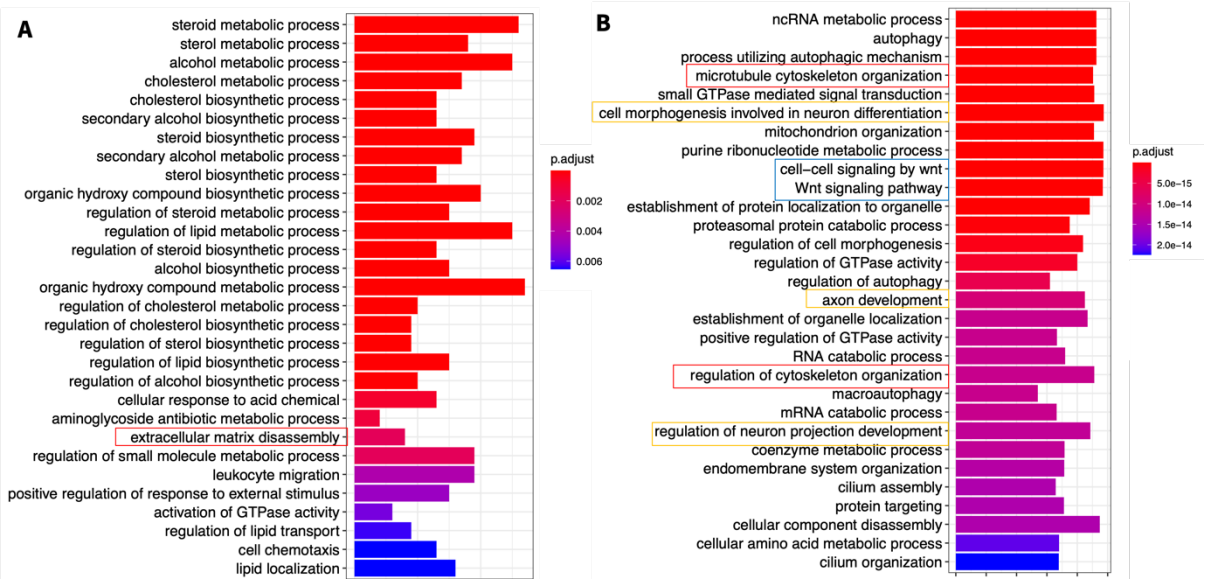


Figure 13. *Gene Ontology of significant DEGs in tHO+ fibroblasts highlighting processes associated with extracellular matrix organisation, the nervous system and Wnt signalling. Bar plot of the top 30 significantly enriched biological processes with a threshold of Padj <0.05 in* ***(A)*** *tHO+ site 1 (proximal) fibroblasts vs. tHO- control (no scar) fibroblasts and* ***(B)*** *tHO+ site 2 (distal) fibroblasts vs. tHO- control (no scar) fibroblasts*

Dermal Fibroblasts from tHO+ Subjects Display a Neural Signature

Results of our transcriptomic profiling revealed the altered expression of various genes and proteins known to have functional roles in neurological system development and the molecular genesis of degenerative neurological diseases (Table A5.1, Appendix 5B). GO biological pathway analysis of DEGs revealed that upregulated genes were significantly enriched in biological processes, including cell morphogenesis involved in neuronal differentiation, axon development and regulation of neuron projection development **(Figure 12B)**. KEGG pathway analysis showed that dysregulated tHO+ genes were highly associated with pathways of neurodegeneration, including Spinocerebellar

ataxia, Amyotrophic lateral sclerosis, Alzheimer's Disease, Huntington's Disease, and Parkinson's Disease.

tHO+ Dermal Fibroblasts are Primed Towards Pre-Osteoblastic Lineage in Response to BMP-2

Like other osteogenic genes, ALP can be transcriptionally regulated by BMP-2, which activates Smad signalling pathways, promotes differentiation of MSCs into osteoblasts *in vitro,* and induces bone formation *in vivo* [308, 309]. It has been documented that one or more cell types can adopt an osteogenic fate and that BMP-2 can induce osteogenic phenotype transitions of skeletal and vascular smooth muscle cells, PDGFRα+ fibro/adipogenic progenitors residing in skeletal tissue, and cardiac fibroblasts favouring calcification or ossification [64, 291]. We examined ALP activity using functional assays to evaluate whether BMP-2 signalling can stimulate osteogenic differentiation from dermal fibroblasts. Representative ALP staining of fibroblast and tHO+ osteoblast cultures treated with BMP-2 for 17 days is shown in **Figure 13A-E**. Our results show that tHO+ osteoblasts exhibited the highest percentage of staining (70%), and a significantly higher intensity of ALP staining was detected in tHO+ fibroblasts from proximal site 1 and distal site 2 compared with tHO- fibroblasts ($p<0.001$) and normal fibroblasts ($p<0.001$) **(Figure 13F)**. Collectively, these results suggest that dermal skin fibroblasts derived from tHO patients are primed towards early, pre-osteoblastic lineage in response to BMP-2 compared to control fibroblasts.

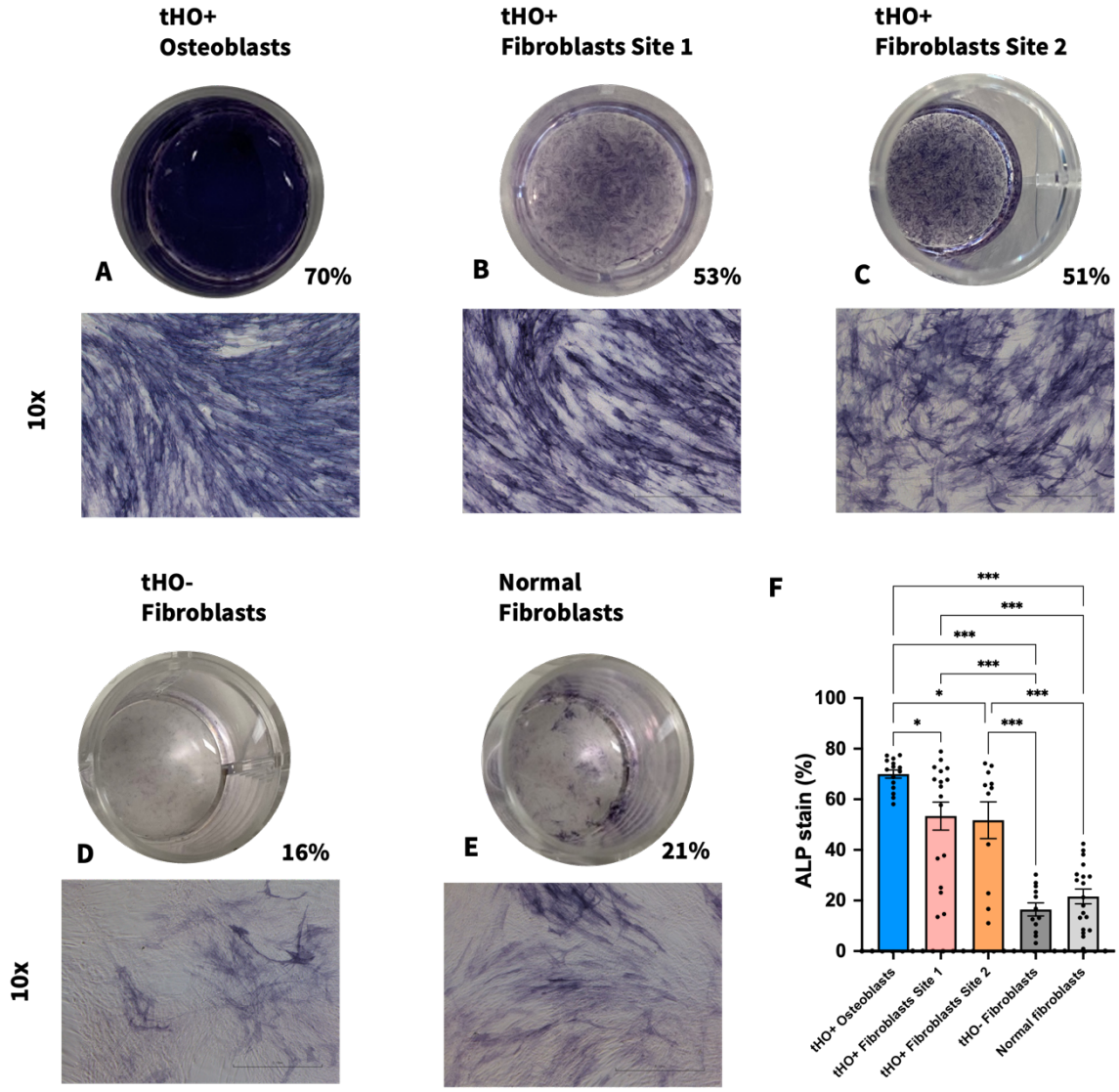


Figure 14. (A-E) *Alkaline phosphatase activity assay after 17-day osteoinduction. Images are representative of n = 5 independent experiments performed in duplicate, showing fibroblasts and tHO+ osteoblasts cultured with BMP-2, using n = 23 biologically independent samples (tHO+ site 1 [n=6], site 2 [n=6], tHO- control [n=4], normal fibroblasts [n=6] and osteoblasts [n=1])* ***(F)*** *Semi-quantitative results of ALP staining are reported as the mean ± SEM. * = p < 0.05, ** = p < 0.001*

Plasma ALP levels collected during index hospital admission for burn-injured patients revealed a significantly higher peak ALP level (u/l) in patients who developed tHO (median 374, IQR 198-398) than burns patients, matched on age, gender and TBSA, who did not develop tHO (median 145, IQR 98-227, p<0.001) **(Figure 14)**. More so, our preliminary investigation found that the peak, or 'spike' in ALP, appears to correlate with the onset of HO-specific symptomology (Figure S1A-G, Appendix 5A). As expected, no radiographical evidence of tHO was evident at this same time point; however, this may still indicate that fibroblasts, or the responsible osteoprogenitor cells, may be differentiating towards an osteoblastic lineage.

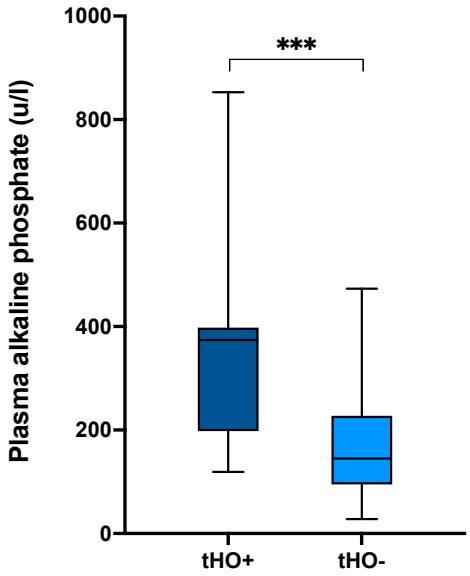
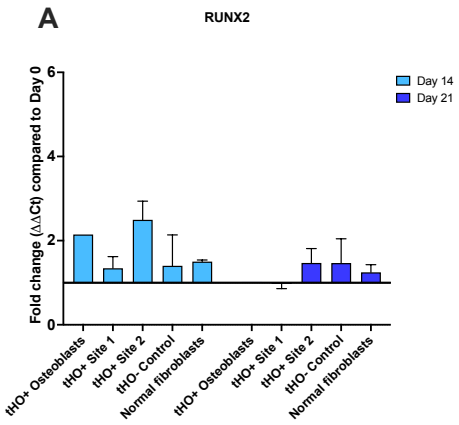
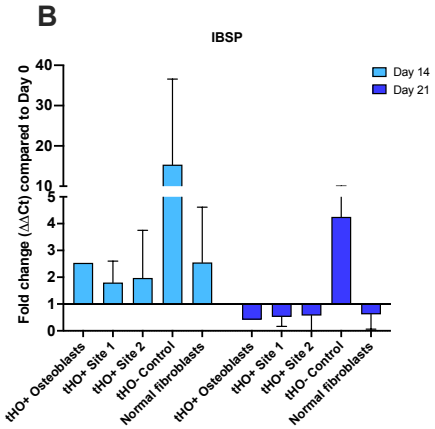
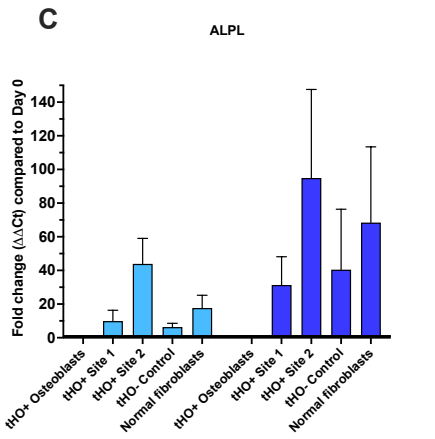


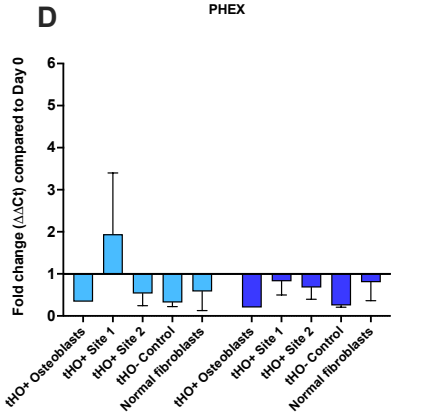
Figure 15. *Comparison of peak levels of plasma alkaline phosphatase (u/l) in tHO+ and tHO- subjects after burn injury. Presented as median (range). Normal range: 30-120 u/l. *** = p < 0.001*

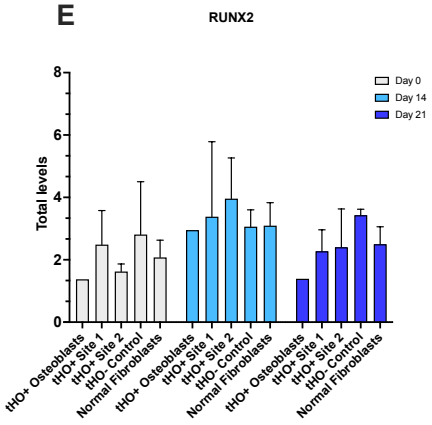
To investigate the mechanisms underlying the effects of BMP-2 on the mineralisation of dermal fibroblasts *in vitro*, the gene expression level of key osteogenic factors in tHO+, tHO-, and normal fibroblasts were obtained by qRT-PCR. Markers of osteogenic differentiation were examined over time (Day 0, 14 and 21) under BMP-2 stimulation and included an early osteoblastic marker; *RUNX2*, for differentiating osteoblasts; *ALPL* and *IBSP* and late-stage mineralising marker; *PHEX*. Data are presented as the fold change in mRNA expression relative to the fold change measured at day 0. Additionally, total gene expression levels over time are presented for comparison. Representative images for individual osteogenic genes are shown in **Figure 15.**

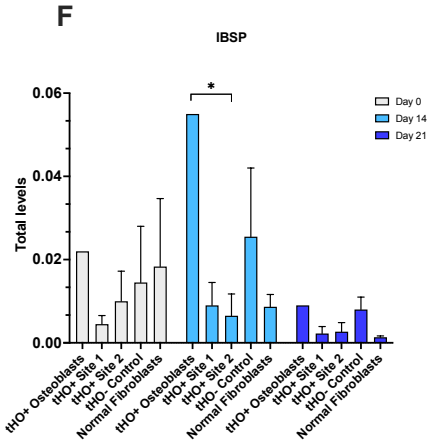

A
RUNX2
Day 14
Day 21
Fold change (ΔΔCt) compared to Day 0
tHO+ Osteoblasts
tHO+ Site 1
tHO+ Site 2
tHO- Control
Normal fibroblasts

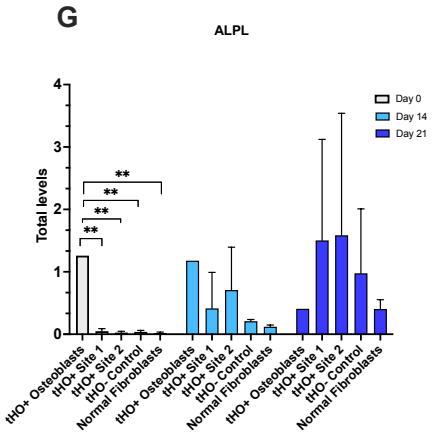

B
IBSP
Day 14
Day 21
Fold change (ΔΔCt) compared to Day 0
tHO+ Osteoblasts
tHO+ Site 1
tHO+ Site 2
tHO- Control
Normal fibroblasts


C
ALPL
Fold change (ΔΔCt) compared to Day 0
tHO+ Osteoblasts
tHO+ Site 1
tHO+ Site 2
tHO- Control
Normal fibroblasts


D
PHEX
Fold change (ΔΔCt) compared to Day 0
tHO+ Osteoblasts
tHO+ Site 1
tHO+ Site 2
tHO- Control
Normal fibroblasts


E
RUNX2
Day 0
Day 14
Day 21
Total levels
tHO+ Osteoblasts
tHO+ Site 1
tHO+ Site 2
tHO- Control
Normal Fibroblasts


F
IBSP
*
Day 0
Day 14
Day 21
Total levels
tHO+ Osteoblasts
tHO+ Site 1
tHO+ Site 2
tHO- Control
Normal Fibroblasts


G
ALPL
**
**
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Day 0
Day 14
Day 21
Total levels
tHO+ Osteoblasts
tHO+ Site 1
tHO+ Site 2
tHO- Control
Normal Fibroblasts

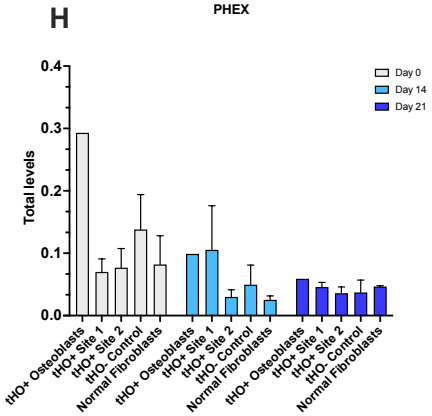

H
PHEX
Day 0
Day 14
Day 21
Total levels
tHO+ Osteoblasts
tHO+ Site 1
tHO+ Site 2
tHO- Control
Normal Fibroblasts

Figure 16. *Gene expression of mature tHO+ osteoblasts (n=1) and tHO+ (n= 8), tHO- (n=2) and normal fibroblasts (n=3) induced by BMP2 stimulation. The relative* ***(A-D)*** *and total* ***(E-H)*** *mRNA levels of RUNX2, ALPL, IBSP and PHEX were evaluated by qRT-PCR after culture in osteogenic medium supplemented with BMP-2 for 14 and 21 days separately. Data represent the mean ± SEM. * = p < 0.05, ** = p < 0.001*

From our transcriptional analyses, differential expression of up to a 2.7-fold upregulation in RUNX2, *ALPL* and *BSP/IBSP* was observed in fibroblasts from tHO+ subjects. Of these, RUNX2 is an established transcription factor responsible for regulating the differentiation of MSCs into pre-osteoblasts. It is needed to form endochondral and intramembranous skeletal elements [65]. As previously demonstrated in mouse HO models and human HO tissues, aberrant expression of RUNX2 appears to contribute to the pathogenesis of ectopic mineralisation [310]. However, the control of *RUNX2* in driving dermal fibroblasts towards an osteoblastic lineage is unknown. Our data demonstrated elevated transcriptional and mRNA levels of *RUNX2* in tHO+ fibroblasts. After 14 days of treatment with BMP-2, relative expression levels of *RUNX2* were higher in tHO+ fibroblasts, most appreciably at distal site 2 compared to tHO- control and normal fibroblasts **(Figure 15A)**. As expected, relative and total expression levels then decreased in tHO+ fibroblast cultures after osteoinduction, with minimal *RUNX2* expression found in mature tHO+ osteoblasts after 21 days **(Figure 15A and E)**.

Another protein favouring ossification is bone sialoprotein (BSP/IBSP), which is involved in the early mineralisation and bone desorption [309]. BSP has previously been identified at sites of ectopic calcification in blood vessels, heart valves, and skeletal muscle [309]. We observed a prominent increase in total *BSP* expression in tHO+ osteoblasts at day 14, which markedly reduced at 21 days (mineralisation stage) **(Figure 15F)**. *BSP* expression was present in fibroblast cultures; however, a similar induction pattern, as demonstrated in tHO+ osteoblast cultures, was not observed in tHO+ fibroblast cells treated with BMP-2. Expression of *BSP* exhibited no significant difference in tHO+ fibroblasts relative to control fibroblasts **(Figure 15B)**.

Alkaline phosphatase has a key role in the propagation of tissue mineralisation. It is among the first functional genes expressed in the mineralisation process by osteoblasts, chondrocytes, and other mineralising cell types such as VSMCs, cardiac fibroblasts and endothelial cells [64, 311]. High enzymatic activity of ALP can reflect enhanced bone turnover and be used as a reliable marker of

the osteoblastic phenotype [312, 313]. Accordingly, analysis of *ALPL* mRNA levels showed that baseline expression levels were significantly higher in mature tHO+ osteoblasts, with low *ALPL* expression consistently evident across all fibroblast cell lines at the same time point (day 0). As expected, tHO+ fibroblasts continued to express *ALPL,* with expression remaining elevated on Day 14 and peaking on Day 21 after osteoinduction **(Figure 15C)**. A downregulation of up to 2.4-fold was identified for an osteogenic marker of last stage mineralising osteoblasts, *PHEX*. Baseline expression levels were highest in tHO+ osteoblasts; however, contrary to expectations, BMP-2 did not promote a relative increase in *PHEX* expression in tHO+ osteoblast or fibroblast cultures after 21 days of osteoinduction **(Figure 15D and H).**

Taken together, qPCR confirmed the induction of early and mature osteogenic gene markers RUNX2, ALPL and BSP in tHO+ fibroblasts. The persistence of osteogenic activity in tHO+ fibroblasts, as demonstrated in our functional assays and verified by qRT-PCR, suggests that BMP-2 can drive osteogenic differentiation of dermal fibroblasts *in vitro.*

Osteoblastic-like Cells from tHO+ Bone Exhibit Characteristics of Mature Osteoblasts

Alizarin red staining was performed to detect the presence of mineralised (calcified) matrix areas in dermal fibroblast cultures. Deposition of calcium was visible in both tHO+ and tHO- dermal fibroblast cultures **(Figure 16A-E)**. By day 21, extensive matrix mineralisation had occurred in mature bone-forming tHO+ osteoblast cultures under BMP-2 stimulation, showing as regions of dark red staining **(Figure 16A)**. Quantification of mineralisation did not determine a significant difference in bone mineral deposition in tHO+ fibroblasts compared to control fibroblasts. **(Figure 16F)**, however, a significant difference in osteoinduction under BMP-2 stimulation was determined between osteoblasts compared to tHO+ fibroblasts and control fibroblasts. This demonstrates that osteoblastic-like cells from tHO+ bone are functionally capable of supporting BMP-2-induced mineralisation *in vitro.* These results align with the histology data, where prominent rims of osteoblasts line the new bone surfaces, whereas fibrous cells remain in a more immature, less differentiated mesenchymal stem cell state with little evidence of bone formation in the surrounding stroma **(Figure 16E and F)**. Thus, evidence suggests that only osteoblastic-like cells arising directly from the HO bone exhibit characteristics of mature osteoblasts.

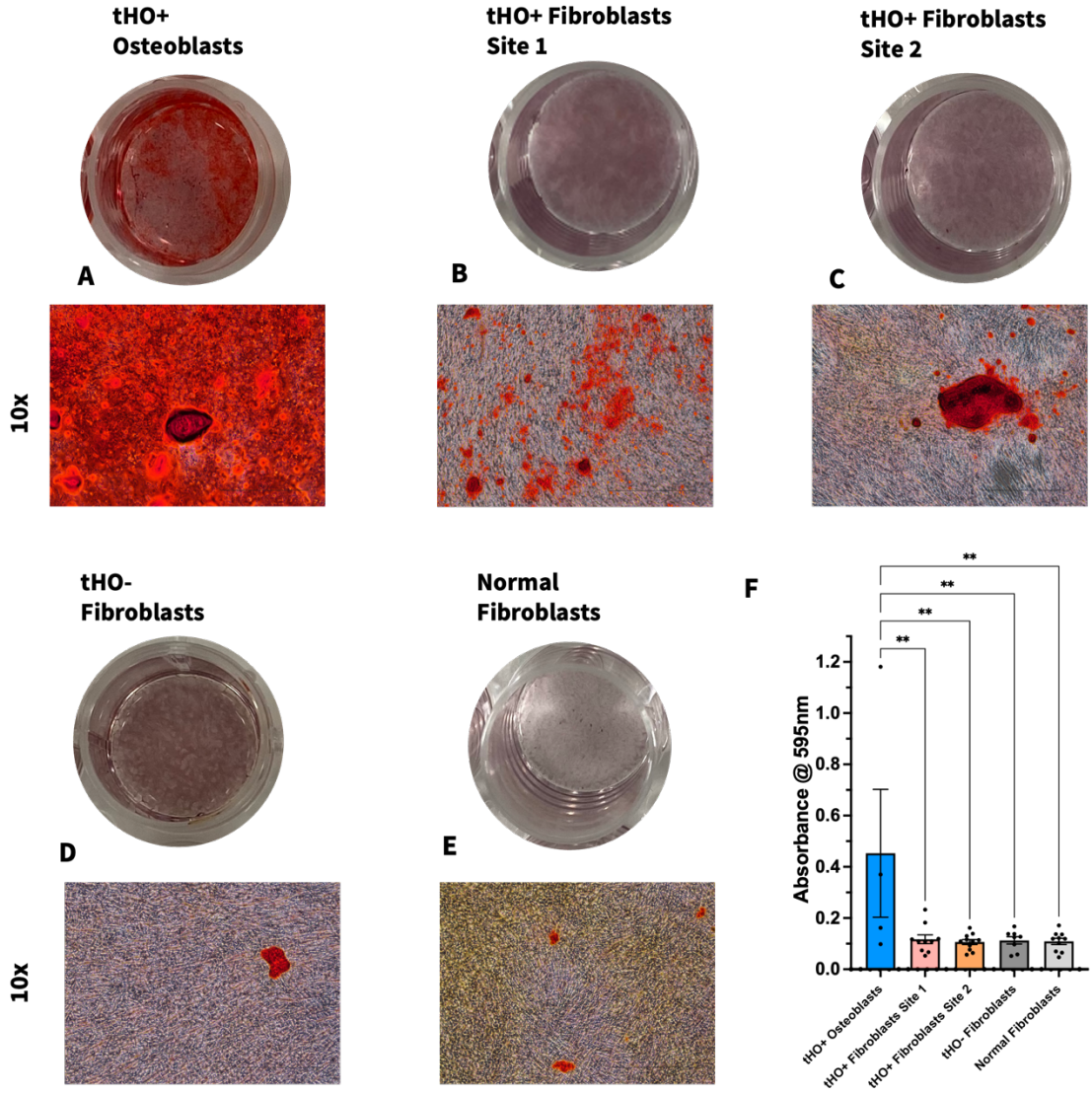


Figure 17. (A-E) *Visualised comparison of osteogenic differentiation potential at day 21 by Alizarin red staining. Images are representative of n=6 independent experiments performed in duplicate, using n=23 separate experimental samples (tHO+ site 1 [n=6], site 2 [n=6], tHO- control [n=4], normal fibroblasts [n=6] and osteoblasts [n=1]).* ***(F)*** *Evaluation of osteogenic potential in mature tHO+ osteoblasts and tHO+ fibroblasts vs. tHO- and normal fibroblasts. Data shown are reported as the mean ± SEM. ** = $p < 0.05$*

6.4 Discussion

The present study combined transcriptomic and bioinformatic methods to identify potential genes, biological processes, and critical signalling pathways involved in traumatic heterotopic ossification after burn injury. We found that tHO+ fibroblasts differ in transcriptome compared to control fibroblasts from patients with and without burn injury. This may imply global transcriptional modifications or dysregulation that led to the aberrant activation of genes participating in the ossification program. The expression profile of osteogenesis-related gene transcripts (CADM1, NFATC2, STEAP4 and WNT4) at the mRNA level, as validated by qRT-PCR, correlated with transcriptional activity. In addition, we provide experimental evidence describing the stimulatory effect of BMP-2 signalling on osteogenic differentiation of tHO-derived mature osteoblasts and dermal fibroblasts from burn-injured human subjects with and without tHO.

Human dermal fibroblasts are derived from mesenchymal stem cells, which are multipotent progenitor cells with the ability to differentiate into multiple tissue types, including bone, cartilage, fat, tendon, and muscle. Abnormal fibroblast function, such as cellular hyperproliferation and excessive extracellular matrix deposition, can directly mediate tissue fibrosis. Various studies have focused on the role of fibroblast cell phenotype switching. In addition to changes in cell function, this phenomenon implies global transcriptional modifications that lead to the aberrant activation of genes involved in the calcification and, or ossification process. *In vitro,* studies demonstrated that cardiac fibroblasts could be induced to calcify under treatment with osteogenic medium [314]. Further, *in vivo,* lineage tracing experiments in a murine line prone to developing myocardial calcification identified that cardiac fibroblasts reside amongst the hydroxyapatite minerals in fibrotic areas and exhibit osteogenic signatures associated with upregulation of *Runx2 [64].*

It is thought that traumatic HO may be an aberrant regenerative process with ectopic bone induction in response to trauma [315]. Like cardiac fibroblasts, dermal fibroblasts exhibit functions to remodel ECM, alter chemical and mechanical signals, participate in angiogenesis, and contribute to fibrosis after injury [316]. Investigations have highlighted an early fibrotic response linked to tHO in traumatised human tissues and have identified an association between tHO and several markers that mediate fibrosis (e.g. Fibronectin 1, SOX9, CCL2/MCP-1, CCN2, CD31) [287, 315, 317-320]. Moreover, it has been suggested that ectopically ossified cells share similar transcriptome characteristics to fibrocartilaginous zone cells [321]. Therefore, besides alterations in the balance

between pro- and anti-calcifying factors, changes in the extracellular matrix may modulate cell phenotype by altering integrin expression, focal adhesions, cytoskeletal organisation, and consequently, intracellular signalling pathways contributing to mineral deposition [309, 322]. In keeping with this, results from our gene ontology analysis identified that significantly DEGs in tHO+ fibroblasts collected from unscarred skin at a distal site to the formation of tHO were significantly enriched in biological processes and involved in pathways of extracellular matrix organisation and structure.

Additionally, several fibrotic markers (*DPP4/CD26*, *COL6A6*, *KRT18*, *STEAP4*, *MMP11*, *MMP27*, *MITF and DCN*) were significantly differentially expressed at the transcriptional level in tHO+ fibroblasts compared to tHO- control fibroblasts. Of these, DPP4/CD26 and STEAP4 gene expression changes were validated by qRT-PCR. These data indicate that the response of ectopic bone formation may follow a similar early fibroproliferative pathway via the expression of similar markers of fibrogenesis and osteogenesis.

Our histology data revealed a surrounding fibrous stroma adjacent to the site of new bone formation, containing fibroblastic precursor cells, which may serve as sources of cells that differentiate into osteoblasts. In addition to the fibrogenic and osteogenic gene signatures identified in tHO+ fibroblasts, this suggests that resident fibroblastic progenitors from the dermis of burn-injured tHO+ subjects may contribute locally to a fibroproliferative response and that fibrosis may be an intermediary step (transient fibrogenic state) contributing to the irregular tissue regeneration process leading to osteogenesis, responsible for pathological ectopic bone formation [315]. Targeting the fibrotic cell phenotype responsible for irregular tissue regeneration, attenuating trans-differentiation of fibroblasts and modulating the intermediary fibroproliferative pathways contributing to ectopic bone formation may provide viable therapeutic advancements in tHO.

It is known that mechanical and inflammatory stresses can initiate MSCs to transition towards cells with mineralising potential, and the upregulation of osteogenic marker genes such as RUNX2, ALPL, BSP/IBSP, type 1 collagen and osteocalcin in pro-calcifying cells is well established [63, 323]. Yet, little is known about the specific regulatory inflammatory signalling pathways that prime a cell to become osteoblast-like and maintain its pro-osteogenic transcriptional and epigenetic changes leading to ectopic ossification. Overactive BMP signalling and its downstream signalling pathways

have been implicated in tHO development. Evidence supports that injury-induced inflammatory changes are sufficient to initiate ectopic bone formation in the presence of increased BMP-2 expression [138, 265, 268-270, 323, 324]. In this study, we established that dermal fibroblasts derived from tHO+ subjects had a greater propensity *in vitro* to transdifferentiate towards an osteoblastic phenotype in response to BMP-2 and exhibit significantly higher ALP activity when compared to cells derived from burn and uninjured control subjects. Further, gene expression analysis confirmed these findings, demonstrating the upregulation of osteogenic marker genes *RUNX2*, *ALP, IBSP, PHEX, NFATC2 and WNT4.* Collectively suggesting that skin fibroblasts derived from both local and distal sites of tHO formation exhibit increased osteogenic potential and may be a candidate progenitor cell that plays a pathoetiogenic role in tHO in traumatised soft tissue.

It is interesting to note that of the significant DEGs associated with osteogenesis, 25 genes have a neuronal signature. Identifying neuronal system pathway enrichment in tHO+ subjects compared to normal fibroblasts from non-burn-injured subjects adds further weight to the findings surrounding burn-induced nervous system morbidity [282]. The contribution of neural inputs in the genesis of ectopic ossification continues to be of intense debate, specifically, how peripheral sensory nerve fibres may modulate abnormal MSC fate decisions after trauma [325]. The possible regulatory role of neural inputs in tHO was further highlighted in our gene ontology and KEGG pathway analysis, which identified the enrichment of biological processes and signalling pathways with known functional roles in nervous system development and neurodegenerative disease. Further, CADM1 protein is involved in a broad, pleiotropic range of conditions and functions, such as neuronal synapse formation, as a tumour suppressor, and the communication between mast cells and smooth muscle cells and in venous thrombosis [302]. An essential role of the synaptic molecule CADM1 has been illustrated in the neuronal regulation of skeletal bone metabolism, where inducing CADM1 expression in excitatory neurons results in increased bone mineral content [326]. Thus, the link between CADM1 and bone formation may shed light on the bone formation processes associated with post-traumatic HO.

Another group of interesting genes are those involved in Wnt signalling. Of the 136 significant DEGs in tHO+ fibroblasts, WNT2 and WNT16 are known Wnt proteins and CDH5, MMP27, COL6A6, CYP19A1 and DPP4/CD26 are identified as Wnt target genes. Further, gene ontology highlighted the enrichment of the Wnt signalling pathway. There is considerable *in vitro* evidence supporting the role of Wnt/β-catenin (i.e., canonical) signalling in promoting bone formation [327-329]. The

activation of the Wnt/β-catenin pathway directs osteogenic lineage allocation by enhancing mesenchymal cell responsiveness to osteogenic factors, such as BMP-2, promoting osteoblast differentiation and activity while indirectly reducing osteoclastogenesis and inhibiting bone resorption [330]. Thus, the Wnt/ß-catenin signalling pathway remains an enticing target for developing drugs to battle skeletal diseases such as tHO (77).

It has been shown that WNT2, WNT4, WNT5a, W11, and WNT16 are detectable in human MSCs [331]. Here, we show that Wnt ligands, WNT2, WNT4 and WNT16, are upregulated at the transcriptional level in dermal fibroblasts from tHO+ subjects. Further, we confirmed elevated mRNA expression of WNT4 and the Wnt target gene, CD26/DPP4. Wnt2 is an extracellular activator of the Wnt/β-catenin signalling pathway and is closely related to osteogenesis [332]. Furthermore, WNT2 activated canonical WNT signalling in primary fibroblasts, which was associated with transdifferentiation towards a pro-migratory and pro-invasive phenotype, and WNT2-mediated fibroblast motility and ECM remodelling [332]. WNT2 activation in MSCs, together with these data, suggests a role of WNT2 as a regulator of mesenchymal stem cell fate [332]. *Wnt16* has been identified as a key molecular regulator of osteogenicity, and overexpression of *Wnt16* induces an increase in trabecular bone mass [333]. Upregulation of WNT16 has been found in articular cartilage after injury, and mechanistic studies have revealed osteoblast lineage cell-derived WNT16 inhibits osteoclast production in mice and humans via direct action on osteoclast progenitor cells and indirectly via upregulating osteoprotegerin expression in osteoblasts [333-335]. Wnt proteins, Wnt2 and 16, may be critical cellular targets that mediate osteogenic transdifferentiation processes associated with tHO formation.

Prior studies have shown that NFAT transcription factors have an essential role in the transcriptional program of osteoblasts [336, 337]. *Nfatc2* is expressed in the osteoblast lineage, and markers of osteoblastic bone formation have been reported to be downregulated in *Nfatc2*$^{-/-}$ mice [337]. In line with these findings, we found that relative expression of NFATC2 was increased 10-fold in tHO+ osteoblasts and, interestingly, a near 4-fold upregulation in tHO+ fibroblasts compared to tHO- fibroblasts, suggesting a potentially novel role of NFATC2 in the pathogenesis of tHO. It has been suggested that NFAT signalling may co-ordinately alter Wnt4, providing a potential mechanism by which NFAT signalling may function at different stages to regulate osteoblast proliferation and function [337, 338]. WNT4 is a promoter of osteogenic differentiation in MSCs, and BMP-induced osteogenic differentiation is enhanced *in vitro* in human and murine MSCs by WNT4

overexpression [339, 340]. Further, WNT4 has been shown to play a role in angiogenesis, suggesting that, *in vivo,* WNT4 may promote bone regeneration by stimulating MSCs to form a conducive microenvironment to generate an entire bone/bone marrow structure [339, 341]. These data and the identification of Wnt4 expression in synovial joint forming regions and non-cartilaginous mesenchymal cells make WNT4 a particularly interesting candidate in the pathogenesis of tHO [342].

Two main pathways regulate the production of bone ALP: Runx2 (mediating the effects of BMP-2, IGF-1, and fibroblast growth factor 23) and b-catenin (mediating the effects of Wnt) [343]. However, the predictive value of plasma alkaline phosphatase as an early marker of tHO formation has yet to be validated [14]. In a previous investigation that followed the temporal association of inflammation and calcification in atherosclerosis, it was found that inflamed areas exhibited high levels of the critical mineralisation enzyme, ALP, before microscopic evidence of calcification [63]. These data align with our results showing significantly raised plasma ALP levels in acute burn-injured patients who developed tHO during hospitalisation. Our preliminary findings indicate a similar temporal association of ALP activity whereby the 'peak' activity correlated approximately with the onset of HO-specific symptoms. However, no radiographic evidence of tHO was evident at this same time point.

It must be noted that ALP can also be falsely elevated with associated long-bone fracture, which is a known common risk factor for tHO development [344]. More so, as there are roughly equal amounts of bone and liver ALP in the circulation comprising more than 90% of the total ALP activity, liver ALP shows approximately 20% cross-reactivity in the assay for bone ALP (BALP) [345, 346]. However, evaluating the longitudinal dynamics of other liver markers, such as the activity of plasma Gamma-glutamyl Transferase (GGT) and alanine transaminase (ALT) and the ratios (ALP: GGT and ALP: ALT), over the same time course, led us to differentiate bone ALP from liver ALP and reasonably postulate that the likely source of elevated plasma ALP in tHO+ patients was largely, from a bone source [347]. Overall, the fundamental role of ALP in vascular calcification and other metabolic bone diseases identifies ALP as a promoter and possible treatment target for disorders of ectopic bone formation [345]. Future studies should consider the prospective evaluation of plasma ALP, or BALP, as a reliable marker for bone metabolism and a potential early diagnostic biomarker for tHO formation in burn and other trauma patients.

Evaluating ALP: ALT and ALP: GGT ratios may be considered by clinicians as an early surveillance tool for differentiating BALP from liver ALP, whereby elevated BALP may indicate increased bone turnover and active tHO formation. Thus, providing a trigger for further investigation with imaging modalities sensitive to detecting early tHO lesions, such as triple-phase bone scintigraphy [348]. Hybrid SPECT/CT, when applied to 99mTc-MDP bone scintigraphy, may allow more precise localisation and confirmation of radiotracer activity in unexpected biodistributions within extraosseous soft tissues and improve the diagnostic interpretation of potential tHO lesions [77].

6.4.1. Limitations and Future Research

Although this research was strengthened through a national, multicentre study design involving several participating burn service sites within Australia, due to limitations of time and expense imposed by this research programme, the results presented only reflect the analysis conducted using biospecimens collected in Western Australia. Thus, the small sample size and datasets included may limit the accuracy of the conclusions. Future research will involve validating the present findings in a larger cohort of burn patients.

The possibility of the condition of tHO itself leading to the local phenotypic changes we have identified in fibroblasts was minimised by including samples from a site proximal to the location of tHO formation, as well as a distal site. Finally, although this study accounted for the possible changes in gene expression and signalling pathways from the burn injury alone by including burn-injured control subjects, the present results reflect a study population that does not include pre-disease samples. Further conclusions regarding genetic predisposition to developing tHO after burn should be made in a prospective investigation and with the inclusion of samples from burn-injured patients pre-tHO development.

Additionally, it is essential to note that the focus of this work relates to tHO secondary to burn injury; thus, it is still being determined whether or not these findings are generalisable to tHO caused by other aetiologies. Future inclusion of samples obtained from neurological and orthopaedic trauma patients will elucidate whether tHO formation shares common pathogenetic mechanisms across different aetiologies and identify possible druggable targets (i.e., single molecules and or signalling pathways) for the effective intervention of tHO across multiple trauma populations.

Although there was no statistically significant difference in the age between the tHO+ and tHO- groups, the tissue samples from subjects in this study represent a wide age range. Age is well known

to negatively affect many biological processes, including healing. Therefore, age-dependent differences in protein expression may be a confounding factor and a source of heterogeneity. The time between sample collection and tHO diagnosis varied between tHO patients, with a significant proportion of patients having mature tHO. For the resected tHO samples, we are limited to those patients who have already developed mature osteoid. Analysis of samples collected in the acute phase of tHO diagnosis, including active osteoblasts derived from immature osteoid, would provide valuable clues for highlighting the phenotypic characteristics of early-stage HO and whether the same key genes, biological processes and pathways identified in the present findings, are implicated across all stages of disease progression. Including normal physiological bone samples in future functional studies may provide an intriguing comparison of the cell proliferation and osteogenic potential between mature tHO+ osteoblasts and normal skeletal osteoblasts. However, as tHO is not a disease of physiological bone formation, considerations must be made around the type and anatomical location of the sample.

To further investigate the correlation of pro-osteogenic gene transcript expression at the mRNA level with functional protein expression and biological relevance, future work will incorporate validating the expression of other critical osteogenic markers by qRT-PCR, including; osterix, which is an essential transcription factor for the osteoblast lineage [349] and osteocalcin and osteopontin, which are involved in mineralisation of the ECM [350].

Finally, few published studies have used proteomics to analyse blood samples in trauma patients who have developed tHO. Concerning clinical translation, there remains potential benefits of such an approach. Although blood plasma samples were collected from tHO+ and tHO- patients over the course of this investigation, they were not analysed due to expenses beyond the scope of this research programme. Thus, future studies will focus on proteomic analysis of blood plasma samples from burn and trauma patients to elucidate potential protein profile discrepancies compared to trauma control subjects. In this way, a putative osteogenic factor may be isolated and readily detectable in the clinical setting.

6.5 Conclusion

The elucidation of pathomechanisms leading to the manifestation of rare disease states such as tHO represents an essential step toward understanding the genesis of this disease. It may provide starting points for the development of new therapeutic intervention concepts. Our findings provide

valuable clues for highlighting the characteristics of tHO after burn injury and support that multiple genes and cell signalling pathways in tHO+ tissues are dysregulated in subjects that develop tHO after burn injury. Thus, we provide potential cellular processes to target. Future investigations linking these biomarkers to potential new therapies are essential for improving patient outcomes. Finally, our findings have broad implications, as they offer additional knowledge on dermal fibroblast plasticity and a potentially novel fibroblast subtype that may possess the ability to phenotypically switch towards the osteogenic lineage in response to burn injury and contribute to the genesis of tHO. Thus, dermal fibroblasts may be a prime cell type for targeted therapeutics in burn-induced tHO.

6.6 Key Points

- tHO+ fibroblasts exhibit a different transcriptomic profile compared to tHO- fibroblasts
- Dermal fibroblasts derived from tHO patients are primed towards early, pre-osteoblastic lineage in response to BMP-2
    - Elevated BALP may be a potential biomarker for early surveillance of tHO
- Only osteoblastic-like cells arising directly from the HO site exhibit characteristics of mature osteoblasts.

Chapter 7.

Thesis Discussion, Clinical Recommendations and Future Directions

Overview

This research program included five studies, and the PhD thesis presents the novel findings, which are assimilated in this, the last of the seven chapters. The overall aim of this research program was to explore and seek a novel understanding of the epidemiology, pathophysiology, clinical characteristics, and risk factors of traumatic heterotopic ossification in adult trauma patients.

This concluding chapter presents the key messages, clinical recommendations, and possible future directions that can be made based on the clinical and basic science studies reported in this thesis.

7.1 Chapter 3

In clinical practice, the diagnosis, prevention, and treatment of tHO are highly variable, partly due to a limited understanding of the pathogenesis. Identifying critical host, injury, clinical intervention, and molecular contributors to the development of tHO remains challenging, limiting the development of effective early diagnostics and treatments. In such cases, machine-learning approaches were considered an effective solution to facilitate target basic science discovery and understanding of pathophysiology in complex disease states where previous efforts focussed, particularly in the burn injury context, have not progressed appreciably in four decades.

As such, an AI-enhanced search tool, IBM Watson for Drug Discovery, was used to synthesise an extensive repository of evidence on pathophysiological mechanisms and disease biomarkers in tHO. To the best of our knowledge, this is the first time this strategy has been applied to develop new hypotheses to investigate the pathological processes underlying the genesis and propagation of tHO [144].

Using a machine-learning approach, this study identified a novel set of plausible candidate gene targets associated with tHO formation. Of the top 25 ranked genes, six genes (MMRN1, MSC/MyoR, ITGAM/CD11b, PDGFD, GREM1 and NELL1) were identified to have evidence of likely association with tHO. Further, interrogation of the literature highlighted that these candidate genes had previously defined roles in inflammation, aberrant tissue repair and regeneration, extracellular matrix remodelling and mineralisation, endochondral or intramembranous bone formation and injury-associated bone reactions. As well as roles in WNT and BMP signalling, important signalling pathways in osteogenic differentiation processes. While the use of WDD in tHO proved successful in identifying promising new gene candidates that may participate in the pathogenesis of tHO, meaningful validation of their biologic relevance in tHO pathobiology is required using basic science studies, which is a limitation of this study and thus, presented opportunities as the focus of future investigations.

7.2 Chapters 4 to 5

Before this study, most epidemiological studies and risk factor investigations in tHO focused on single-centre and single-population outcomes making results challenging to interpret. Utilising the WA trauma database, we could better assess many patients, pooling at-risk injury cohorts across multiple institutions. Considering the data collected during this research program, there are several novel and vital research and clinical findings, promoting new messages and awareness for clinicians and underscoring the need for further investigation.

8.2.1 Chapter 4

This chapter exposed the challenges and provided a novel assessment of the accuracy of medical diagnostic coding and clinical documentation for tHO diagnoses within the WA Tertiary Hospital network. The results provide a new benchmark for current practice for clinicians and medical coders.

The results vividly demonstrate the discrepancies in medical diagnostic coding and the inconsistencies in clinical documentation regarding the diagnosis of tHO within the network of included hospitals.

HO-specific ICD-10-AM codes failed to identify more than 1/3rd of true tHO cases, with a high prevalence of non-specific HO codes (19%) and cases identified incidentally via manual chart review (21%). This is further highlighted in the burn cohort, in which the reported sensitivity of M61 codes for correctly diagnosing tHO was only 50%. Indicating that using M61 diagnostic codes is less than an acceptable method to classify tHO cases after burn injury accurately.

Accurate and consistent documentation for tHO diagnoses between clinicians and across institutions may improve the specificity of medical diagnostic coding for injury-specific classifications of rare events like tHO.

Finally, the higher frequency of outpatient tHO diagnoses after orthopaedic injury could be an explanatory factor contributing to the relatively conservative estimates of orthopaedic tHO identified in the present study. More so, as tHO following operative fracture fixation is often asymptomatic and incidentally detected during routine post-operative radiographs, subclinical cases of asymptomatic tHO may not be captured as systematically in the outpatient setting than if they were to be routinely surveilled as an inpatient. These findings highlight the need to implement surveillance guidelines in both inpatient and outpatient settings to achieve cost-effective screening and early diagnosis of symptomatic and asymptomatic tHO cases.

7.2.1.1. Key Messages and Clinical Recommendations:

1. The inaccuracies in medical diagnostic coding and the wide variability in clinical documentation for the diagnosis of tHO in WA tertiary hospitals may have implications for future retrospective research and patient care.

    a. The inclusion of an HO-specific M61 ICD-10-AM code that is distinct from myositis ossificans (MO) of skeletal muscles, e.g., ‘Calcification and ossification of the joint region, [site]’ may be necessary for distinguishing MO from other sub-forms of tHO with no intramuscular involvement.

b. The addition of an M61 ICD-10-AM code for post-operative HO may help distinguish between tHO and HO associated with elective surgical procedures without a traumatic mechanism of injury, e.g. joint replacement – an orthopaedic population observed to have high rates of HO [221-223].
c. Future retrospective studies utilising an ICD-10-AM code search should consider the poor accuracy of M61 ICD-10-AM codes in identifying true tHO cases and incorporate a broadened search criteria to include non-specific musculoskeletal codes for identifying tHO patients.

2. Healthcare professionals should strive to improve the standardised recording of clinical data for suspected or confirmed tHO diagnoses at the point of care to enhance the specificity of medical diagnostic coding for tHO.

d. Clinicians should implement SNOMED-CT-AU as a reference set for consistency in using clinical terms relating to tHO in conjunction with a measure of tHO severity based on existing site-specific classification schemes that appear to correlate with joint function.
e. As clinical coders rely on the accuracy of discharge summaries completed by treating medical officers, notable documentation of a tHO diagnosis as a 'secondary diagnosis' or 'complication' on patients' medical discharge summaries should be accurately and consistently recorded by the medical officer.

7.2.2 Chapter 5, Part 1

This chapter presents a matched case-control study to explore the association of inpatient tHO diagnoses on hospital length of stay.

To the author's knowledge, this multi-centre study is the first to robustly consider the contribution of a tHO diagnosis during hospitalisation as a co-morbid complication affecting LOS, and it was this factor that was the most significant predictor of an increased hospital LOS for patients following burns, spinal cord injury, and traumatic brain injury. Trauma patients diagnosed with tHO during hospitalisation stayed 56% longer than trauma patients of the same age, gender, and injury severity who did not develop tHO.

The study findings provide a new and valuable understanding of the effect of tHO on trauma patient outcomes, which has significant implications for clinical practice and future patient care, as early recognition and treatment of tHO could potentially reduce LOS and health resource utilisation.

Clinicians and wider hospital administration staff must be aware of the risk a tHO diagnosis has on prolonging inpatient hospital stay for trauma patients. Considerations must be made around resource allocation and early tailoring of care for trauma patient populations to reduce prolonged hospital stays.

This study presents a novel quantification of the impact of acute tHO on the healthcare system. It provides a first step towards identifying the health resource costs associated with the complication. To ensure comprehensive measurement of LOS outcomes, tHO-related re-admission rates should be considered in future investigations. In the context of tHO, early intervention and care coordination in an inpatient hospital setting may significantly influence inpatient LOS. In contrast, the outpatient clinic and patient education may affect the chances of readmission. The latter may be particularly relevant for future evaluation of readmission rates in the orthopaedic tHO, a cohort with significantly higher rates of outpatient tHO diagnoses. Furthermore, burden of illness studies are crucial for improving access to adequate resources, optimising care, and evaluating the benefits of new healthcare interventions [10]. Thus, to fully elucidate the burden of illness associated with tHO, the financial and economic impact of hospitalisations and healthcare resource utilisation associated with tHO requires future evaluation.

Finally, new insights from these data necessitate that accurate clinical documentation and diagnostic coding for tHO is even more paramount to improving evidence-based patient care and LOS outcomes for trauma patients who develop tHO.

7.2.2.1. Key Messages and Clinical Recommendations:

1. Trauma patients with tHO should be considered a distinct patient population when it comes to allocating resources for bed allocation and discharge planning
    a. Due to the severity of primary tHO symptoms and the high risk of developing secondary complications, trauma patients with an in-hospital tHO diagnosis should be flagged in multidisciplinary team meetings to have complex discharge issues and likely longer occupiers of hospital beds

b. Health professionals should engage in early, thorough discharge planning for tHO patients, which is paramount to improving this cohort's LOS outcomes.

7.2.3 Chapter 5, Part 2

This chapter presents a retrospective analysis of clinical variables known at and during hospital admission undertaken to identify common risk factors for tHO development in pooled trauma populations.

Despite the aetiological differences between the trauma induced HO, there are proposed similarities in the underlying neuroinflammatory responses and the structure and characteristics of the ectopic bone formed in all cases. More so, common to all affected anatomical sites is the presence of connective tissue containing progenitor cells which are implicated as having the potential to initiate and or mediate the pathological process of tHO formation. Thus, to enhance understanding, this study was designed purposefully to uncover the similarities across pooled at-risk trauma populations and use the intersection of what is known in each disease state to triangulate data and increase the likelihood of discovering novel causative factors.

This study was strengthened by design; matching on age, gender and injury severity minimised potential confounding factors previously reported to affect the risk of tHO development. Further, the multi-centre study design minimised the impact of low patient numbers and the sample size was found to be sufficiently large to estimate clinically significant effects.

The results of this study confirmed known and identified novel, independent risk factors for tHO that were common across the burn and neurological trauma populations. Increased hospital LOS and concomitant injury to the hip region and thigh significantly increased the risk of developing tHO during hospitalisation. Further, this study reports novel predictors of tHO relating to sources of local or systemic infections.

This investigation adds valuable new information to the burn and broader trauma literature. These findings can be used to assist with the design of forthcoming, larger-scale studies examining the interconnections among these and other variables, with the aims of (i) formulating hypotheses elucidating the fundamental mechanisms underlying tHO development and (ii) establishing a

reliable, time-sensitive risk profile for the implementation of preventative measures in burn and other trauma patients at high risk of development tHO.

7.2.3.1 Key Messages and Clinical Recommendations:

3. Acute care and rehabilitation clinicians should be aware of common risk factors for tHO development and employ early targeted surveillance of high-risk trauma patients
    a. A prolonged hospital length of stay and having an injury to the hip region or thigh are common independent risk factors for developing tHO during hospitalisation
    b. Infectious agents identified as common independent factors for tHO development include *Staphylococcus* (not including Staph. Aureus), *Acinetobacter calcoaceticus-baumannii* and *Enterobacter cloacae complex*
    c. Risk stratification in burn patients should evaluate the presence of concomitant injuries that showed novel association with tHO development after burn injury, injury to the hip region and thigh, long bone fracture (although tHO does not always correlate with the site of tHO formation), and central and or peripheral nervous system injury, e.g., TBI, brachial plexus injuries.
    d. For patients with confirmed unilateral tHO, clinicians should surveil for multi-joint involvement and consider imaging of the symmetrical bilateral joint, even in the absence of clinical suspicion.

4. Patients and health care professionals must be on high alert for early and tHO-specific signs and symptoms, including the 'locking sign' and rapid movement loss (reduced active and passive joint ROM), and specific pain descriptors such as 'deep', 'sharp', and 'stabbing' [3].
    a. Implementing a protocol for educating clinicians, patients, and family members on the early presentation of tHO and its physical and psychological impact as a standard, universal procedure may be beneficial towards improving clinician and patient awareness.

5. Trauma multi-disciplinary team (MDT) members should be aware of common tHO sites and be attentive to the timeframe of tHO symptom onset and diagnosis
    a. In trauma patients, the most commonly develops bilaterally in >2 anatomical sites at a median time of 53 days post-injury

b. Burn patients are more often affected in bilateral elbows; however, clinicians should be on alert for frequently affected other joints, such as the shoulders and knees
c. tHO develops most frequently in bilateral hips in SCI patients and unilaterally at the hip after TBI
d. Radiological evidence of tHO appeared the earliest in the TBI cohort at a median time of 38 days post-injury
e. Traumatic HO was most commonly diagnosed during the rehabilitation episode of care in SCI patients showing radiological evidence of tHO at a median time of 65 days following admission
f. Burns clinicians should be alert of tHO symptom onset, which appears to precede radiological evidence at a median of 35 days, with tHO detectable on plain radiographs at a median of 49 days post-injury

7.3 Chapter 6

The series of basic science studies presented in Chapter 6 (Study 5) was the first to be conducted using clinical biospecimens obtained from adult burns patients with and without tHO. This research was strengthened by design, involving a number of participating burn service sites within Australia. The laboratory investigations have yielded novel experimental evidence highlighting the characteristics of tHO after burn injury, and the results offer several possible future directions.

Specifically, the findings of this study support that multiple genes and cell signalling pathways in tHO+ tissues are dysregulated in subjects that develop tHO. As such, candidate genes, biological processes, and signalling pathways that differ in fibroblasts from tHO+ and tHO- patients after burn injury were identified, providing potential targets that could be used to elucidate the molecular mechanisms underlying tHO. Future investigations linking these biomarkers to novel therapeutics are crucial to improve patient outcomes.

This study was enhanced by accounting for the possible changes in gene expression and signalling pathways from the burn injury alone by including burn-injured control subjects. Further, the possibility of the condition of tHO itself leading to the local phenotypic changes we have identified in fibroblasts was minimised by including samples from a site proximal to the location of tHO formation, as well as a distal site. Further conclusions regarding genetic predisposition to

developing tHO after burn may be a focus of future prospective investigations and with the inclusion of pre-disease samples from trauma-injured patients before tHO development.

The enrichment of biological processes and signalling pathways with known functional roles in nervous system development and neurodegenerative disease underscore the critical regulatory role of neural inputs in the pathogenesis of tHO. Specifically, it highlights the relationship between peripheral inflammation-induced neuroinflammation and tHO development after burn injury. As such, it supports the concept that neurotrauma and associated neuroinflammatory processes are vital factors propagating the development of tHO and a commonality across many conditions predisposing to tHO formation.

In addition, functional experiments were designed and conducted to explore the hypothesis that burns patients who develop tHO after injury exhibit a dermal fibroblast phenotype that is more susceptible to osteogenic differentiation than those who do not develop tHO. This concept is biologically plausible as the contribution of fibroblast cells to ectopic ossification has been illustrated previously. In several disorders, there is clear evidence for their close association.

Analysis of dermal fibroblasts derived from tHO patients revealed the upregulation of key osteogenic marker genes and confirmed dermal fibroblast cells' osteogenic pathway activation capabilities. In addition to the fibrogenic and osteogenic gene signatures identified in tHO+ fibroblasts, our findings also suggest that resident fibroblastic progenitors from the dermis of the burn injured tHO+ subjects may contribute locally to a fibroproliferative response and that fibrosis may be an intermediary step (transient fibrogenic state) contributing to the irregular tissue regeneration process leading to osteogenesis and ectopic bone formation in traumatised soft tissue.

Overall, the study findings support the author's hypothesis and imply that skin fibroblast cells can express bone-forming markers and, thus, could represent a novel, osteoinductive cell type directly involved in developing traumatic HO.

7.3.1. Clinical Recommendations

6. With the knowledge that tHO symptomology presents before radiographic evidence of tHO formation, early diagnostic measures could be implemented to identify the earliest possible

opportunities for the delivery of therapeutic interventions to thwart disease progression and maturation.

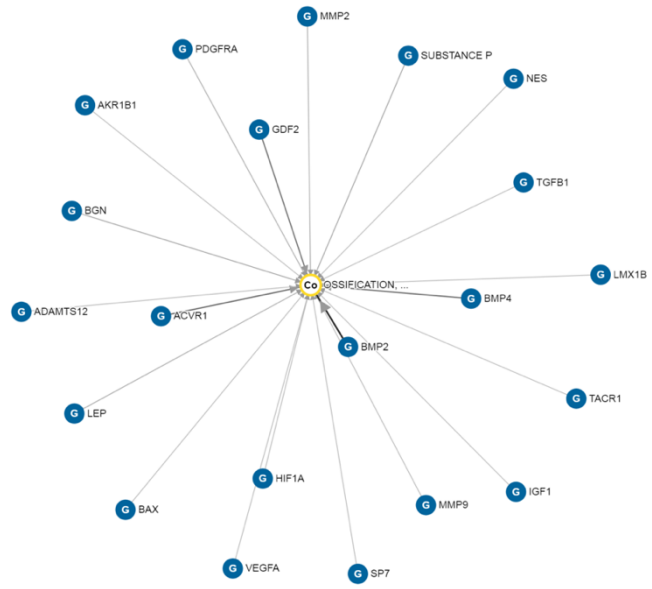
a. The evaluation of plasma BALP (differentiated from liver markers) as part of routine blood work conducted during hospitalisation for trauma patients, particularly in the workup for surgery, may indicate active bone turnover and provide valuable clues of early tHO formation.
b. Elevated BALP may alert clinicians to instigate further investigations utilising sensitive imaging modalities to detect early, active disease states. Employing 3 phase bone scintigraphy for detecting pathological changes in bone metabolism may facilitate early diagnosis of tHO. Specifically, for cases indicating abnormal tracer activity/uptake at commonly affected tHO sites (i.e., large joints of the shoulder, elbow, and hips), additional imaging (i.e., MRI) may be warranted to improve diagnostic differentiation accuracy.
c. Prospective investigations incorporating inexpensive, bedside diagnostic tools with minimal associated risks and side effects, such as ultrasound, may provide additional diagnostic value for the tHO population.

Appendices

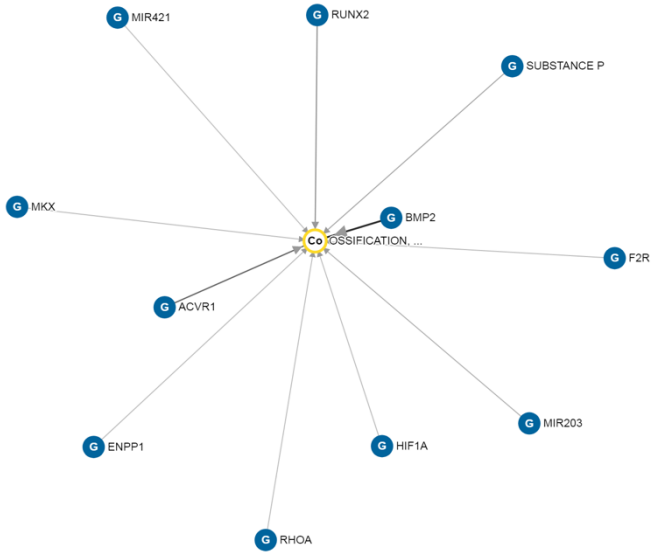
Appendix 1: Supplementary Material (Chapter 3)

Appendix 1A: Visual Representation of Gene Relationships to Heterotopic Ossification

(A)



(B)



(C)

Gene with Predisposition Relationship	Confidence (%)
TGFB1	81
BMP2	79
HIF1A	79
BMP4	77
SP	77
GDF2	76
LEP	76
ACVR1	75
BGN	72
PDGFRA	72
IGF1	69
BAX	66
NES	65
TACR1	63
AKR1B1	63
LMX1B	60
ADAMTS12	59
MMP9	77
SP7	61
VEGFA	69
MMP2	10

Figure A1.1A-C. Genes extracted by Watson that are considered to have a **(A)** predisposition relationship and **(B)** regulation relationship to HO. Relationship types are depicted by Watson so that a user can select and visualise their relationship (E.g. regulation, association, predisposition, modification) to heterotopic ossification without having to read all associated documents supporting the connection. **(C)** confidence (%) represents the level of certainty WDD is of the relationship.

Appendix 1B: Distance Network

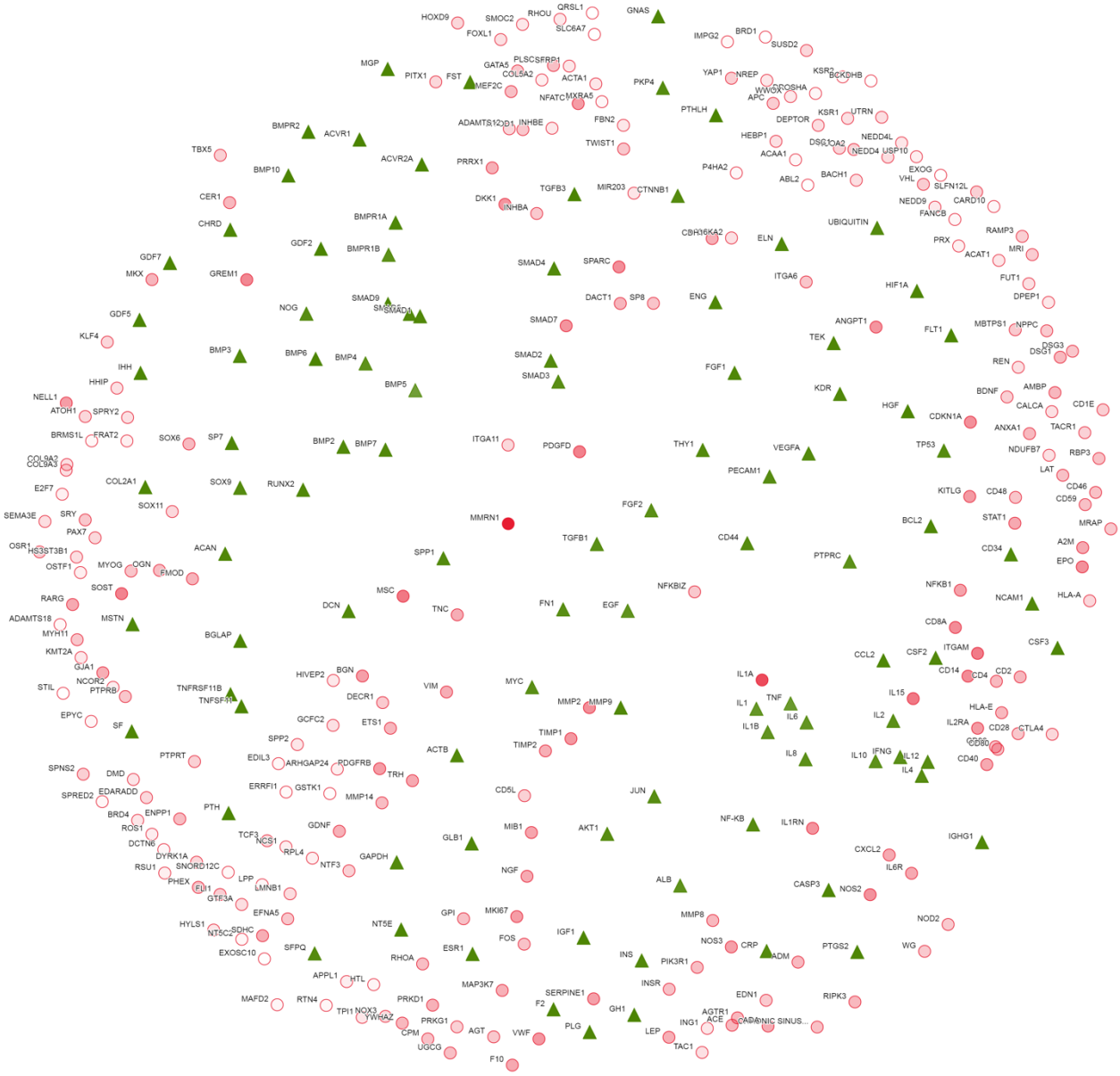


Figure A1.2. The Distance network visualization is optimal when you want to see the closest nodes to a node of interest. Entities that were entered in the first entity group field (the Known set) appear as green triangles, and entities that were entered in the second entity group field (the Candidate set) appear as red circles. In the Watson platform, when hovering on a Candidate entity, the five most similar Known entities are indicated. When hovering on a Known entity, the five most similar Candidate entities are indicated [142].

Appendix 1C: An Excerpt of Associated Literature Supporting Gene Relationships to Heterotopic Ossification

Table A1.1. An excerpt generated by WDD of the supporting literature linked to each associated source (gene) and its relationship to heterotopic ossification.

Document title	Source	Source type	Relationship type	Trigger word (verb)	Target (Text)	Snippet	Field title	Document type	Document publication date	Publication Name	Digital object identifier
Substance P signaling mediates BMP-dependent heterotopic ossification.	SUBSTANCE P	GENE	Regulation	mediates	heterotopic ossification	Substance P signaling mediates BMP-dependent heterotopic ossification.	Title	Medline	Oct 1, 2011	Journal of cellular biochemistry	10.1002/jcb.23259
Synergistic inhibition of endochondral bone formation by silencing Hif1α and Runx2 in trauma-induced heterotopic ossification.	RUNX2	GENE	Regulation Negative	inhibit	HO	The results showed that lacking of Runx2 and Hif1α could inhibit HO formation.	Abstract	Medline	Aug 1, 2011	Molecular therapy : the journal of the American Society of Gene Therapy	10.1038/mt.2011.101
Recombinant human bone morphogenetic protein 2-induced heterotopic ossification of the retroperitoneum, psoas muscle, pelvis and abdominal wall following lumbar spinal fusion.	BMP2	GENE	Regulation Positive	induced	heterotopic ossification	Recombinant human bone morphogenetic protein 2-induced heterotopic ossification of the retroperitoneum, psoas muscle, pelvis and abdominal wall following lumbar spinal fusion.	Title	Medline	May 1, 2010	Skeletal radiology	10.1007/s00256-010-0890-8
Thoracic myelopathy caused by ossification of ligamentum flavum	TGFB1	GENE	Association	played	ectopic ossification	TGF-β1 could have played a role in chondroid metaplasia and ectopic ossification in OLF.	Document Body	Pmcoa	Nov 2, 2006	Journal of Orthopaedic Surgery and Research	10.1186/1749-799X-1-10

of which fluorosis as an etiology factor											
Anaplerotic Accumulation of Tricarboxylic Acid Cycle Intermediates as Well as Changes in Other Key Metabolites During Heterotopic Ossification	MMP9	GENE	Association	linking	heterotopic ossification	Activation of MMP9 is a complex mechanism that requires the presence of plasmin, the active form of plasminogen, thus, linking heterotopic ossification with platelet activation and recruitment to the site of new bone formation.	Document Body	Pmcoa	Dec 31, 2015	Journal of Cellular Biochemistry	10.1002/jcb.25454
Analysis of the mechanism by which nerve growth factor promotes callus formation in mice with tibial fracture	NGF	GENE	Regulation negative	reduced	heterotopic ossification	Local injection of exogenous NGF facilitated the complete healing of the fracture and reduced the formation of heterotopic ossification.	Document Body	Pmcoa	Feb 7, 2017	Experimental and Therapeutic Medicine	10.3892/etm.2017.4108

Appendix 1D: Gene Relationships to Heterotopic Ossification and Associated Values

Table A1.2. A set of 100 genes with associated value extracted by Watson to have a relationship with heterotopic ossification, as shown in the visual representation (Figure 1). The value represents the strength of the relationship i.e., the number of documents supporting the connection.

Rank	Entity name	Value	Rank	Entity name	Value
1	BMP2	595	**51**	AKT1	92
2	BMP4	321	**52**	IL10	92
3	ACVR1	305	**53**	CASP3	90
4	VEGFA	289	**54**	NT5E	90
5	BGLAP	278	**55**	SF	89
6	TNF	278	**56**	CSF2	88
7	RUNX2	263	**57**	GLB1	88
8	BMP7	255	**58**	TEK	88
9	FGF2	228	**59**	PLG	87
10	PTH	218	**60**	BMPR1A	86
11	IL6	213	**61**	IL4	84
12	FN1	204	**62**	BMP3	83
13	GAPDH	193	**63**	BMP5	83
14	TGFB1	190	**64**	PECAM1	83
15	ALB	185	**65**	IGHG1	80
16	INS	183	**66**	SMAD2	78
17	EGF	179	**67**	F2	77
18	PKP4	179	**68**	TGFB3	77
19	SMAD1	174	**69**	BMPR2	76
20	PTGS2	168	**70**	CCL2	76
21	SPP1	166	**71**	MSTN	75
22	IGF1	164	**72**	JUN	73
23	IL1	162	**73**	TNFRSF11B	72

24	BMP6	156	**74**	TP53	72
25	SMAD5	153	**75**	ACVR2A	71
26	SMAD9	140	**76**	CSF3	71
27	CRP	134	**77**	FGF1	70
28	NOG	132	**78**	HGF	70
29	SP7	132	**79**	MYC	70
30	PTPRC	127	**80**	IHH	69
31	CD34	125	**81**	GNAS	67
32	IL1B	125	**82**	IL12	67
33	ACAN	124	**83**	BCL2	66
34	GH1	124	**84**	BMP10	66
35	ACTB	122	**85**	CHRD	66
36	GDF2	119	**86**	FST	66
37	ELN	114	**87**	NCAM1	66
38	SOX9	109	**88**	COL2A1	65
39	NF-KB	108	**89**	SMAD3	65
40	IFNG	107	**90**	SMAD4	65
41	TNFSF11	107	**91**	HIF1A	64
42	THY1	105	**92**	DCN	63
43	IL8	104	**93**	GDF7	63
44	ESR1	103	**94**	FLT1	62
45	GDF5	103	**95**	PTHLH	62
46	IL2	103	**96**	UBIQUITIN	62
47	CTNNB1	100	**97**	BMPR1B	61
48	ENG	96	**98**	KDR	61
49	CD44	94	**99**	MGP	61
50	SFPQ	94	**100**	MMP9	61

Appendix 1E: Conditions Associated with Heterotopic Ossification and Associated Values

Table A1.3. A network of conditions that Watson has produced in real time. Watson uses its annotators to extract relationships between heterotopic ossification and other associated conditions demonstrating its power as a disease agnostic.

Rank	Condition	Value
1	OSSIFICATION, HETEROTOPIC	6156
2	PAIN	1973
3	WOUNDS AND INJURIES	1728
4	ARTHRITIDES, DEGENERATIVE	1117
5	CANCER	1023
6	DISLOCATIONS	936
7	FIBRODYSPLASIA OSSIFICANS PROGRESSIVA	817
8	ARTHRITIS	616
9	AGE RELATED OSTEOPOROSIS	585
10	ASEPTIC NECROSIS OF BONE	492
11	SPINAL CORD INJURIES	484
12	HEMORRHAGE	437
13	RHEUMATOID ARTHRITIS	397
14	BENIGN CHONDROMA OF BONE	382
15	LYTIC BONE LESION	346
16	NECROSIS	346
17	DIABETES MELLITUS	341
18	ANKYLOSES	336
19	FRACTURES, BONE	329
20	BONE RESORPTION	326
21	BONE CANCER	321
22	DEEP THROMBOPHLEBITIS	313
23	FIBROSIS	310
24	BRAIN INJURY	304
25	METASTASES	295

26	POSTOPERATIVE COMPLICATIONS	293
27	BURNS	285
28	HEMATOMA	285
29	INFLAMMATORY RESPONSE	281
30	ABNORMALITY, CONGENITAL	273
31	ANASARCA	257
32	BED SORE	257
33	HYPERTROPHY	254
34	HIP DISLOCATION	252
35	ANKYLOSING SPONDYLARTHRITIDES	250
36	CEREBROVASCULAR ACCIDENT	250
37	CICATRIX	219
38	OBESE	217
39	ANOXIA	212
40	CHRONIC KIDNEY DISEASE	208
41	BONE DISEASE, METABOLIC	207
42	ARTERIOSCLEROSES	205
43	BONE DISEASE	199
44	BREAST CANCER	197
45	CRANIOCEREBRAL TRAUMA	196
46	BONE PAGET DISEASE	195
47	WOUND INFECTION	192
48	BONE MARROW DISEASE	190
49	HYPERTENSIVE VASCULAR DISEASE	186
50	DYSPLASIA	185

Appendix 1F: Known Gene Set for Predictive Analytics

Table A1.4. Known set used for Predictive analytics. A set of 100 genes with previously defined associations with heterotopic ossification was used to interrogate the candidate gene list.

Known set	
BMP2	IL10
BMP4	CASP3
ACVR1	NT5E
VEGFA	SF
BGLAP	CSF2
TNF	GLB1
RUNX2	TEK
BMP7	PLG
FGF2	BMPR1A
PTH	IL4
IL6	BMP3
FN1	BMP5
GAPDH	PECAM1
TGFB1	IGHG1
ALB	SMAD2
INS	F2
EGF	TGFB3
PKP4	BMPR2
SMAD1	CCL2
PTGS2	MSTN
SPP1	JUN
IGF1	TNFRSF11B
IL1	TP53
BMP6	ACVR2A
SMAD5	CSF3
SMAD9	FGF1

CRP	HGF
NOG	MYC
SP7	IHH
PTPRC	GNAS
CD34	IL12
IL1B	BCL2
ACAN	BMP10
GH1	CHRD
ACTB	FST
GDF2	NCAM1
ELN	COL2A1
SOX9	SMAD3
NF-KB	SMAD4
IFNG	HIF1A
TNFSF11	DCN
THY1	GDF7
IL8	FLT1
ESR1	PTHLH
GDF5	UBIQUITIN
IL2	BMPR1B
CTNNB1	KDR
ENG	MGP
CD44	MMP9
SFPQ	IL10
AKT1	

Appendix 1G: Candidate Gene Set for Predictive Analytics

Table A1.5. Candidate gene set used for interrogation using WDD's predictive analytics function. The list contains 233 genes a potential role in ectopic bone formation, extracellular matrix production and fibrosis.

Candidate gene set				
RTN4	PRRX1	A2M	MXRA5	ERRFI1
UGCG	PRX	TIMP1	MIB1	WWOX
BACH1	HLA-A	ACE	EDARADD	SP8
NGF	HYLS1	CD40	SUSD2	FBN2
NCS1	MRAP	CD86	SMOC2	IL6R
BDNF	NDUFB7	DROSHA	SOX6	INSR
GJA1	OGN	PRKG1	UTRN	SEMA3E
GDNF	RPL4	NPPC	CPM	E2F7
MMP2	SPARC	ADM	ANXA1	PIK3R1
BGN	FANCB	IL1A	MYH11	MEF2C
FMOD	EPO	NT5C2	PTPRB	RIPK3
KMT2A	BRD1	RHOA	DSG3	RPS6KA2
CD59	COL9A2	ROS1	MKI67	NOX3
TRH	ACTA1	STIL	EPYC	SFRP1
DPEP1	HLA-E	TCF3	PLSCR4	PRKD1
LEP	WG	TNC	DSC1	CARD10
MIR203	DMD	VWF	INHBA	RSU1
DKK1	IL1RN	AGTR1	YWHAZ	NEDD4L
SOX11	MYOD1	BCKDHB	DSG1	OSR1
SMAD7	NOS2	CD46	LPP	NCOR2
YAP1	MMRN1	CD80	IMPG2	MBTPS1
TAC1	MYOG	CDKN1A	ADAMTS18	DEPTOR
TACR1	APPL1	COL9A3	GCFC2	KSR2
CD2	DECR1	CTLA4	RBP3	NEDD4
RARG	F10	CXCL2	HS3ST3B1	SPNS2
HEBP1	GSTK1	EDN1	PTPRT	USP10

ANGPT1	SERPINE1	EXOSC10	OSTF1	NCOA2
NTF3	NOD2	GPI	NEDD9	RAMP3
ENPP1	SNORD12C	MMP14	KITLG	ING1
PAX7	VIM	MMP8	DCTN6	NFKBIZ
CALCA	ACAA1	PDGFRB	KLF4	IL15
CD48	MAFD2	REN	SPRY2	HIVEP2
FUT1	SDHC	TPI1	FRAT2	LMNB1
CDH1	CD1E	ACAT1	DACT1	MSC
APC	EXOG	AMBP	FOXL1	NFATC1
VHL	ITGAM	CD28	MAP3K7	NFKB1
CD5L	SLC6A7	CD8A	FLI1	GTF3A
ABL2	SOST	ITGA6	QRSL1	AGT
PHEX	CER1	TIMP2	RHOU	FOS
HHIP	GREM1	COL5A2	EFNA5	NELL1
BRD4	HTL	ITGA11	SPRED2	MKX
MRI	CD14	NREP	GATA5	ADAMTS12
LAT	IL2RA	PDGFD	ARHGAP24	DYRK1A
SRY	NOS3	EDIL3	SLFN12L	KSR1
STAT1	TWIST1	P4HA2	ATOH1	HOXD9
TBX5	ADA	SPP2	PITX1	
INHBE	CD4	ETS1	BRMS1L	

Appendix 1H: Ranked Top 50 Gene List and Similarity Scores

Table A1.6. IBM Watson predictive analytics process of top 50 ranked genes. A predictive similarity score is generated by Watson that measures an entities similarity to all known entities. A graph diffusion (GD) score (similarity score) is assigned by Watson to each gene/protein based on semantic similarity of the candidate entity to the positive known set [4]. The higher the number, the more similar an entity is to the set of known entities.

Rank	Gene	Graph diffusion (GD) score
1	MMRN1	0.081
2	IL1A	0.064
3	IL15	0.049
4	MSC	0.049
5	ITGAM	0.048
6	PDGFD	0.046
7	SOST	0.045
8	GREM1	0.044
9	CD14	0.043
10	CD8A	0.042
11	SPARC	0.042
12	MMP2	0.041
13	CDKN1A	0.041
14	EPO	0.041
15	NOS2	0.04
16	IL1RN	0.04
17	SMAD7	0.04
18	DKK1	0.039
19	ANGPT1	0.039
20	VWF	0.039
21	TIMP1	0.038
22	KITLG	0.038

23	IL2RA	0.038
24	NFATC1	0.037
25	NELL1	0.037
26	MKI67	0.037
27	PDGFRB	0.037
28	BGN	0.036
29	SERPINE1	0.036
30	NOS3	0.036
31	NFKB1	0.035
32	MIB1	0.034
33	F10	0.033
34	A2M	0.033
35	RARG	0.033
36	CXCL2	0.033
37	CD40	0.033
38	IL6R	0.033
39	STAT1	0.032
40	TRH	0.032
41	ADA	0.032
42	NGF	0.032
43	TNC	0.031
44	VIM	0.031
45	SDHC	0.031
46	TIMP2	0.031
47	GJA1	0.031
48	CDH1	0.031
49	LEP	0.031
50	PRRX1	0.03

Appendix 1I: Insights into Known Pathophysiological Mechanisms, Biomarkers, and Emerging Therapeutic Targets in Diseases of Ectopic Bone Formation

Overview

This study incorporates the novel application of the IBM Watson for Drug Discovery (WDD) search engine to examine a global corpus of literature of known pathophysiological mechanisms, biomarkers, and emerging therapeutic targets in traumatic HO. Where there was a dearth in the literature on the tHO population, data collected in other disease states characterised by ectopic bone formation were reviewed.

A Summary of the Current State of Understanding of tHO Pathogenesis

Contemporary understanding of tHO pathogenesis describes a process that is induced following a concomitant CNS and or PNS injury to stimulate bone formation at the site of the peripheral injury via endochondral or intramembranous ossification [8, 11, 53, 136]. Neuroinflammation is implicated as the primary driver of tHO formation. It is initiated in the affected tissues through the activation of afferent, sensory fibres of the PNS by noxious mechanical, thermal, or chemical stimuli providing feedback, locally and centrally [8, 137]. The upregulation of neuropeptides characterises the early inflammatory phase; substance P (sP) and calcitonin gene-related peptide [351], and neurotrophic factors; neurotrophin-3 (NT-3), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) [140, 352]. Release of sP initiates expression of cytokines and recruitment of mast cells [8, 11, 53]. Chemokines, released from mast cell degranulation, recruit platelets, neutrophils, and other myeloid cells in a selected innate immune response [136]. Multipotent cellular contributors of tHO have been identified from a host of tissues, including marrow, muscle, adipose, and peripheral nerves; specifically, the multipotency of local mesenchymal stem cell (MSC) populations - identified as chondro-osseous progenitors that appear to arise from different local soft tissues and or peripheral nerves [12, 138-140].

In the late inflammatory to early proliferative phase, secretory macrophages exert autocrine and paracrine effects on local mesenchymal progenitor cells [19]. This occurs via the action of pro-

inflammatory cytokines, chemokines and growth factors, as well as pro-fibrotic factors, including interleukins-3, 4, 6 and 10 (IL-3/4/6/10), C-X-C motif chemokine ligands 1 and 2 (CXCL1/2), and monocyte chemo-attractant protein-1 (MCP-1), which populate the inflammatory microenvironment [353-356]. Local production of growth and pro-inflammatory factors includes fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor- 1 (IGF-1) and Tissue Necrosis Factor-a (TNF-α) [136]. Angiogenesis and osteogenesis are tightly coupled by protein levels of HIF-1α, which rapidly rise to maintain a local hypoxic microenvironment, and vascular endothelial growth factor (VEGF), which stimulates vascularisation [324]. Members of the transforming growth factor-β (TGF-β) superfamily, including TGF-β1 and bone morphogenic protein (BMP) signalling, are additional inductive signals that propagate growth, differentiation, and activation of osteogenic precursor cells [357]. Matrix metalloprotease-9 (MMP9) enzyme activation leads to the degradation of the extracellular matrix (ECM), increasing the permeability of the blood-nerve barrier to BMP-2. BMPs released from bone matrix and macrophage-derived molecular mediators such as oncostatin M may contribute to tHO formation [324, 358, 359].

Due to hypoxia at the peripheral injury site, mesenchymal and osteoprogenitor cells differentiate into chondrocytes and secrete a cartilaginous matrix [11]. Upregulation of osteogenic signalling pathways occurs; IGF-1 coordinates chondrocyte hypertrophy through Wnt/β-catenin signalling leading to the remodelling of the cartilaginous matrix [136]. BMP-SMAD 1,5 and 8 signalling promotes the expression of critical chondrogenic genes: Sox-9, Collagen 10 and 11, and osteogenic genes: BMP-2 and 4, osteopontin, and tissue non-specific alkaline phosphatase [138, 265, 360]. Around the lesion's periphery, bone formation occurs as cartilage is removed and osteoblasts deposit osteoid on the remnants of the cartilaginous template [11]. Other master transcription factors enable the initiation and promotion of MSC differentiation towards an osteogenic fate, specifically runt-related transcription factor 2 (RUNX2) and osterix [270, 361]. RUNX2 controls osteoblast differentiation by regulating bone-specific genes such as osteocalcin, MMP13, bone sialoprotein and tissue inhibitors of matrix metalloproteinases [362]. Over time, mature lamellar bone is remodelled from the initially formed woven bone. The new, mature bone possesses haversian canals, blood vessels and a marrow cavity [141].

Assimilated Literature Review Results

1.1 Introduction

Pathological ectopic bone formation can occur in response to injury or be associated with genetic or acquired diseases, having been described in association with the musculoskeletal, respiratory, cardiovascular, and nervous systems [64, 261, 292, 363-365]. In cases of traumatic HO, lesions are invariably preceded by inflammatory insult, and inflammation is known to play a crucial and multifaceted role in directing cell recruitment, vascularisation, and cartilage remodelling during bone development and repair [12, 136]. Although not fully understood, it is hypothesised that the inflammation-promoting cell subsets, osteogenic and chondrogenic precursor cells, and the interactive cell signalling networks commonly underpin the pathogenic calcification or ossification processes of various body systems [2, 8, 12, 366].

Investigations have yielded fresh perspectives on the mechanistic, bi-directional cross-talk between bone and the central nervous system (CNS), peripheral nervous system (PNS), musculoskeletal system and cardiovascular system [367, 368]. However, the intricate interaction between these systems and bone metabolism, under a normal physiological state and in tHO induced by various forms of injury, remains a subject of intense debate. Investigating the interrelatedness between the pathobiology of tHO and other conditions and diseases associated with pathological calcification and or ossification could expedite the discovery of novel genes, proteins, and signalling pathways responsible for ectopic bone formation in trauma settings and provide viable therapeutic targets.

1.2 Contributory Cell Lineages

Current research has highlighted the likely cellular lineage that contributes to the formation of pathological ectopic tissue [2, 8, 12, 366]. Traumatic HO is thought to result from inappropriate differentiation of progenitor cells and specific cell signalling pathways induced by a pathological imbalance of local and systemic factors and shaped by the homeostatic environment [12]. Controversy exists in the current literature regarding the location and cellular phenotype of the osteoprogenitors of tHO and the mechanism they use to travel to the site of bone formation [138, 268, 369]. Whilst local resident progenitor cells of the mesenchymal lineage are described by Cholok

et al. [12] the contributions of endothelial, neuronal, and epithelial lineage cells to developing tHO lesions have all been reported [268, 370]. Further, the contribution of circulating cellular populations to tHO has been described, suggesting that the initiation and progression of tHO are likely mediated by heterogeneous populations of cells derived from both autochthonous and circulatory reservoirs [12]. This phenomenon is evident clinically whereby bony foci often appear even when the primary trauma occurs anatomically distant from the site of tHO formation [3].

1.3 Neural Inputs in tHO Development

First described in 1968 by Roberts [371], researchers have long debated a relationship between tHO and the nervous system, with some of the most compelling data from statistics in military patients linking tHO to changes in the CNS and PNS [52, 66] [42, 270]. The frequency of tHO after trauma to CNS or PNS following burn, SCI, TBI and orthopaedic injury implicate neuroinflammation as the common trigger required to initiate tHO.

1.3.1. The Role of Neuroinflammation and Neuroinflammatory Priming

Skeletal peripheral nerves include motor and sensory nerves [367]. Nerve regulation of bone metabolism occurs via bi-directional communication between peripheral nerves and the skeleton through neurotransmitters, neuropeptides, neurotrophic and axon guidance factors, and nerve-resident cells [367]. Neuroinflammation in the affected tissues is initiated through the activation of afferent, sensory fibres of the PNS by noxious mechanical, thermal, or chemical stimuli providing feedback, locally and centrally, on pain and temperature [8]. Specifically, a nociceptive ion channel located on sensory nerve endings, called the transient receptor potential cation channel subfamily V member 1 (TRVP1), when activated by factors in the microenvironment, leads to the release of pain mediators substance P (sP) and calcitonin gene-related protein (CGRP) [8, 53].

Release of sP during neuroinflammation initiates expression of immunological chemical messengers or cytokines and recruitment of mast cells to peripheral nerves [8, 11, 53]. In a mouse model of spinal cord injury (SCI), Debaud et al. [372] identified that the acute, systemic release of sP after SCI is positively correlated with neurological HO (NHO) volumes. Similarly, sP was dramatically increased in early lesional tissue in acquired HO patients [373]. The blocking of neuron-specific sP signalling through the neurokinin 1 (NK1r) receptor has been shown to abolish tHO formation, suggesting NK1r inhibition as a potential prophylactic and therapeutic target for tHO [373].

Sensory and sympathetic nerves are crucial in skeletal homeostasis and bone repair [368, 374]. Utilising an in vivo model based on a mild mechanical brain injury combined with cardiotoxin (CTX) induced muscle injury, Tokesi et al. [375] demonstrated that CTX-induced muscle injury without traumatic brain injury (TBI) did not induce calcification in the injured muscle. These results suggest the sympathetic nervous system's role in transmitting the brain injury's stress to the injured tissues. Also found was that adrenaline with concomitant CTX induces calcification in the injured muscle as effectively as the brain trauma. This suggests that adrenaline plays a crucial role in the stress-mediated communication between the CNS and the injured muscle via the sympathetic nervous system [375].

In an SCI mouse model, Debaud and colleagues [372] have helped to explain the effects of SCI on peripheral tissue homeostasis and skeletal muscle regeneration, proposing that the PNS may convey pathological signals from the injured spinal cord to musculoskeletal effectors within denervated injured muscles. It was expected that the interruption of neurotransmitter transport, such as sP, along peripheral nerves by PNS excision would inhibit their intramuscular release; however, an increase in tHO volume in denervated muscles was observed. Worth noting is that burn injuries exert widespread peripheral nervous system damage [282, 283]. There is documented evidence of elevated sP positive fibres in the skin after burn injury, which can lead to functional changes in cell activation and signal transduction [284]. Even after superficial injury, there exists a possibility that burn-induced neuroinflammatory and neuronal-mediated mechanisms could bring about alterations in peripheral nerve function and contribute to tHO formation [283]. These findings suggest a common mechanism by which PNS injury triggers the release of osteoinductive factors and fortifies the pathological propensity for intramuscular progenitor cells to undergo osteogenic differentiation and contribute to pathological ectopic bone formation [376].

1.3.2. Neurotrophic Factors

Nerve growth factor (NGF) is of interest, a neurotrophic factor involved in developing, maintaining and regenerating sensory and sympathetic nerves [377]. NGF accelerated osteogenesis in a mandibular distraction osteogenesis model, unique due to the presence of the inferior alveolar nerve in this bone. Elevated expression of NGF by niche nerves and VEGF by Schwann cells was identified during the bone repair period suggesting their involvement in osteogenic differentiation processes of osteoprogenitor cells leading to new bone formation [378, 379]. Given the similar

progression through endochondral ossification between fracture and post-traumatic HO, Hwang and colleagues [380] hypothesised that neurogenic/neurotrophic signalling via sensory innervation is crucial for pathologic stem cell differentiation and development of tHO.

Using a proven mouse burn/tenotomy model of tHO, these authors identified robust labelling of NGF and regions of colocalisation with infiltrating mesenchymal progenitor cells in tHO tissue at the site of injury [380]. Tropomyosin receptor kinase A (TrKA) is present on innervated bone surfaces and has a high affinity for NGF [19]. Recent work has implicated the critical role of skeletal and sensory nerves in mediating bone formation, and TrKA signalling by sensory nerves is required for bone morphogenesis and repair [325, 374, 381-383]. In an extremity injury model, Lee et al. [325] found that NGF-mediated axon innervation accompanied tHO formation, delayed axonal invasion, and diminished volume of ectopic bone resulting from the selective interruption of NGF-TrkA coupling. Collectively, these data support the proposed role of neurogenic signalling in modulating aberrant wound healing and the mesenchymal cell programming governing tHO [384]. Further, inhibiting neurogenic signalling through NGF-TrkA represents a potential therapeutic target to prevent post-traumatic HO [380].

Neurotrophin-3 (NT-3), a neurotrophic factor in the NGF family of neurotrophins, has been implicated as a neuro-endocrine cytokine that promotes tHO formation through induction of endMT of vascular endothelial cells [140]. These authors further observed the colocalisation of NT-3 and macrophages in injured sites throughout tHO formation. They demonstrated that macrophage-derived NT-3 accelerated osteogenic differentiation and mineralisation of tendon-derived stem cells by activating the extracellular signal-regulated kinase (ERK)1/2 and PI3K/Akt signalling pathways [352]. NT-3 has been shown to enhance BMP-2 and VEGF expression in mineralised cells to mediate bone and blood vessel formation [385]. Additionally, administration of NT-3 *in vitro* led to enhanced expression of osteogenic and chondrogenic gene markers, including Runx2, OSX and OCN, which have previously been implicated in the pathogenesis of tHO [352].

Brain-derived neurotrophic factor (BDNF) produced by macrophages is also responsible for regulating new bone formation via the initiation of osteoblast proliferation and differentiation [386]. BDNF has been shown to enhance fracture healing by promoting osteoblast migration via upregulation of integrin β1 expression and ERK1/2 and protein kinase B (Akt) phosphorylation. As macrophages constitute a central inflammatory mediator in the tHO microenvironment, these

findings suggest that the osteogenic functions of NT-3 and BDNF may promote tHO formation, and targeting this macrophage-mediated paracrine pathway may be effective in attenuating the inducive microenvironment responsible for tHO [355]. Furthermore, due to the previously documented roles of NT-3 in fracture healing and wound repair, further studies are required to investigate whether NT-3, upregulated by different inflammatory phenotypes, may be an attractive injury-induced factor for targeted prevention and treatment of tHO across the orthopaedic and burn injury settings [387, 388].

1.3.3. Neural Regulation via Peripheral Nerve Remodelling

Sympathetic signalling via local serotonin release by mast cells released after tissue injury leads to activating a brown adipocyte-like progenitor within the perineural layer of the nerve, which migrates from nerves to the site of new bone formation [11, 82, 366]. Here, the perineural progenitors differentiate into transient brown adipose-like cells, which create a hypoxic microenvironment favourable for chondrogenesis [82, 269, 366]. Due to hypoxia at the peripheral injury site, mesenchymal and osteoprogenitor cells differentiate into chondrocytes and secrete a cartilaginous matrix [11]. Around the lesion's periphery, bone formation occurs as cartilage is removed and osteoblasts deposit osteoid on the remnants of the cartilaginous template [11]. Over time, mature lamellar bone is remodelled from the initially formed woven bone. The new, mature bone possesses Haversian canals, blood vessels and a marrow cavity [11].

Brown adipose tissue is highly innervated by sympathetic nerves, and there is accumulating evidence suggesting a connection between peripheral nerve remodelling and brown adipogenesis [269, 389]. These brown adipocyte-like cells, derived from the nerve perineurium, express several growth and patterning factors and molecules that regulate oxygen utilisation pathways, depending on their location within the tissue [389]. Data presented by Salisbury et al. [269] showed the presence of a progenitor within peripheral nerves in the mouse that undergo rapid expansion, migration, and differentiation to brown adipocyte-like cells following local intramuscular BMP-2 administration. These findings demonstrate that the perineurium acts as a potential niche for progenitors in adult peripheral nerves, necessary for nerve remodelling associated with tissue regeneration.

Transient brown adipocyte-like cells are defined by their expression of uncoupling protein 1 (UCP1), one of the few proteins to function as a generator of a hypoxic microenvironment [389]. Reducing

oxygen tension in local tissues generates an environment conducive to the pathological formation of ectopic bone. Immunohistochemical analysis has revealed the presence of UCP1+ cells in the perineurium of peripheral nerves and cartilage in human tHO tissue samples, revealing the origin of brown adipocytes in peripheral nerves and possibly, a shared common origin with chondrocytes [389]. Given the presence of nerves embedded within newly formed areas of forming heterotopic bone, these authors propose that the activation of neural progenitors occurs in response to the tissue insult and concomitant injury to peripheral nerves from the initial injury. Of interest, astrocytes within the CNS have been reported to express UCP1 and function to regulate energy metabolism in the brain [389]. In addition, the expression of key neural guidance molecule reelin was identified in a subset of these brown adipocyte-like cells. Astrocytes in the CNS have been observed to express Reelin. It has been proposed that coordination of Reelin and Notch-1 signalling might contribute to regulating neuronal migration and determining neural progenitor cell fate [390, 391]. The importance of Notch signalling for normal human adult brain function is demonstrated by its implication in several diseases with a distinct neurodegenerative component [390]. Furthermore, Notch signalling is essential for MSC differentiation into osteoblasts during skeletal remodelling, and disrupted Notch signalling has been implied in the pathogenesis of tHO [392-394]. Overall, this suggests that transient brown adipose cells may serve an essential function in peripheral nerves, akin to the role played by astrocytes in the brain [389].

In addition to accessory cells migrating from the perineurial layer, Lazard et al. [268] suggests that the induction of tHO leads to the expression of osteoblast-specific transcription factors, SP7 (also known as osterix [Osx]) and distal-less homeobox 5 (DIx5), in cells residing in the endoneurium. These cells exit through the endoneurial vessels and are deposited at the site of new bone formation. It is thought that BMP-2 can directly upregulate DIx5 leading to the expression of SP7. This suggests that progenitor cells in the endoneurium undergo trans- or osteogenic differentiation to osteoblasts in response to BMP-2 signalling [268].

To determine if osteoblasts in tHO are derived from the SP7+ cells within peripheral nerves and if SP7+ cells from the endoneurium are actually exiting the nerve, entering the circulation, and being deposited at the site of new bone formation, Olmsted et al. [138] used a tamoxifen-regulated Wnt1-Cre recombinase lineage-tracing mouse and obtained human tissues encompassing early tHO development induced by traumatic injury. Similar to the finding in mouse tissues, analysis of HO in human tissues indicated a significant presence of phosphoSMAD+ (PS+) cells (PSs 1,5 and 8) and

SP7+ cells in the endoneurium of peripheral nerves near the site of HO formation. Additionally, some PS+ cells in bone tissue were also SP7+ indicating the ongoing expression of BMP proteins during bone formation. The analysis of control tissues and nerves did not demonstrate the presence of PS+ and SP7+ cells. These results confirm the neural origin of osteoblasts and suggest that the endoneurium of peripheral nerves plays a key functional role in trauma-induced HO in both mice and humans.

Lazard et al. [268] found that Osx expression in the nerve was only present on the endoneurial cells for 24 hours and therefore proposed that either expression is downregulated or that these cells exit the nerve immediately via the blood-nerve barrier. Claudin-5 is a neurovascular tight junction protein vital to the physical barrier properties of the BBB, with its loss or disruption acutely involved in neurodegenerative and neuroinflammatory disorders. These endoneurial osteoprogenitors express claudin 5 when in circulation or at the site of new bone formation, even though they do not initially express this marker in the nerve [138, 268]. This suggests a potential role endoneurial cells have through their response to BMP-2, and by upregulating claudin-5 protein, they can exit the nerve through the blood-nerve barrier (BNB) [268]. All of the Osx expression was found to be in the circulating claudin 5+ cells [268]. These results support the notion that both local and circulating osteoprogenitor cells are responsible for tHO formation and have the ability to cross the BNB, enter the circulation, and undergo some level of osteogenic differentiation during transit to the new site of tHO formation.

1.4 Contribution of BMP Signalling

Bone morphogenic proteins have been shown in several nervous system regions and experimental models to interact functionally with neurotrophins to promote neuronal survival and function [355, 388]. Kang et al. [395] demonstrated that in addition to BMP-2, BMP-9, BMP-6, and BMP-7, to a lesser extent, are the most potent osteoinductive BMPs, and stimulated greater alkaline phosphatase (ALP) activity and the most robust and mature ossification at multiple time points. However, unlike BMP-2, BMP-3 exerted no inhibitory effect on BMP-9-induced bone formation, suggesting that BMP-9 may transduce a distinct osteogenic signalling pathway that is significantly different from that of BMP-2, BMP-6 and BMP-7 [395]. LeBlanc et al. [396] found that BMP-9 had an osteoinductive influence on mouse muscle resident stromal cells by increasing their ALP activity and inducing HO in damaged muscle only, whereas BMP-2 promoted HO in skeletal muscle regardless of its state.

Using *in vitro* and *ex vivo* analysis in animal models and patient-derived tissues, Sanchez-Duffhues et al. [397] identified that pro-inflammatory cytokines TNF-α and IL-1β induce EndMT in human primary aortic endothelial cells, sensitising them for BMP-9-induced osteogenic differentiation. BMP-9, found in systemic circulation, regulates vascular homeostasis by controlling proliferation, angiogenesis, permeability, and monocyte recruitment. Downregulation of BMP receptor type II (BMP2R) results in decreased c-JUN N-terminal kinases (JNK) signalling in endothelial cells, enhancing BMP-9 induced mineralisation and contributing to vascular calcification [397]. Understanding the interplay between inflammation and BMP signalling in EndMT-derived cells underlying vascular calcification pathologies may be translatable to other BMP-mediated inflammatory pathologies such as traumatic HO.

1.5. Heterotopic Ossification in Critical Illness and Infection

The increased prevalence of HO in patients with infections or concomitant inflammation after burn and neurological injury, as well as the growing number of HO cases reported after COVID-19 infection, further validates a potential mechanistic link between neuroinflammation as a primary driver in such disease states and its sequential influence on peripheral tissue homeostasis [372, 398].

Cases of periarticular HO have been described in patients with a recent history of COVID-19, indicating the global inflammatory and neuroinvasive potential of COVID-19 may incite HO [281]. Stoira et al. [280] describes the extensive development of HO around the shoulder, elbow and hip in a population with severe COVID-19 requiring prolonged mechanical ventilation, corresponding to a HO prevalence of 19.2%. The prevalence of HO in this population was about 4-fold higher than that reported in patients with ARDS (5%), leading the authors to conclude that prolonged immobilisation as a result of longer sedation and neuromuscular blockade for severe ARDS played a key role for HO in this patient group [280]. In addition, these authors suggest that other factors, such as the deranged calcium metabolism, systemic inflammatory condition and local myositis, possibly due to the SARS-CoV-2 virus, may have contributed to a higher prevalence of HO [280]. In addition to peri-articular ossifications, extensive dendriform pulmonary ossification (DPO) as a sequela of SARS-CoV-2 pneumonia has also been identified [399]. DPO is characterised by diffuse small bone fragments in lung tissue and is frequently associated with fibroblast transformation into

osteoblasts in interstitial lung disease. These data highlight shared characteristics between DPO and tHO and suggest the possible involvement of fibroblasts in other conditions of ectopic bone formation, including HO induced by trauma [400].

The cytokine storm associated with COVID-19 includes upregulation factors previously associated with the formation of HO. The specific genes and signalling pathways shared between the two proposed pathological mechanisms include MMP9, IL-1α, IL-1β, IL-1, IL-6, VEGFA, MCP-1, TLR-2 and NF-κb p65, p38 MAPK and TNF- signalling. The coronavirus spike protein (TNF-a converting enzyme) activates the IL-6/TNF-α axis, causing elevated concentrations of these cytokines [279]. In the initial immune response, pro-inflammatory monocytes contribute to the skewed inflammatory profile, mediated by membrane-bound immune receptors (e.g. Toll-like receptor-2) and downstream signalling pathways (e.g. NF-κB p65 and p38 MAPK) [401]. Followed by cytokine-driven inflammation and infiltration of monocyte-derived macrophages and neutrophils into lung tissue, possibly induced by MCP-1. These mechanisms are concordant with the high expression of inflammatory cytokines and activation of the IL-6/TNF-α axis [279]. Increased expression of transforming growth factor-beta 1 (TGF-β1) and TNF-α may amplify cytokine production and contribute to pulmonary fibrosis by promoting fibroblast proliferation [402]. TGF- β1 gene expression is specific to regenerative macrophages and is a critical regulator of chondrogenic differentiation [162]. Thus, TGF-β1 production in monocytes/macrophages as a cell phenotype marker may be necessary to drive aberrant chondrogenic progenitor cell differentiation and potentiate ectopic bone formation [384]. Similarly, in a mouse model of traumatic HO, increased MCP-1, TNFα, and IL-6 serum levels have been reported [403].

In COVID-19 pathology, the monocyte surface molecule CD14 cooperates with toll-like receptor (TLR)-2 in response to viral infection, activating NF-kB-dependent transcription of genes encoding inflammatory cytokines [401]. It has been shown that activation of macrophages by bacterial ligands leads to the secretion of cytokines stimulating bone formation, suggesting that bacteria-induced inflammatory cues can activate pro-osteogenic pathways and stimulate osteogenesis under appropriate conditions [404]. Osteo-immunomodulatory factors have been strongly associated with several pathological conditions involving extra-skeletal bone formation, such as cochlear ossifications, following Haemophilus *influenzae* meningitis, pulmonary tuberculosis lung ossifications and tHO [67, 405, 406]. Toll-like receptors exist on immune and non-immune cells, including fibroblasts [407]. Lipoteichoic acid (LTA) is a bacterial call-wall derived TLR-2 activator

found on *Staphylococcus aureus* (*S. aureus*) and induces several cytokines found in bone development, such as TNF-α and IL-6. LTA has been identified as an osteo-stimulatory factor and found to enhance bone induction dose-dependently, strengthening an involvement of cell wall associated antigens in the pro-osteogenic response to gi bacteria [408].

COVID-19–associated myositis is also highly interesting because ACE-2, the SARS-CoV receptor, is reportedly expressed in skeletal muscle. If confirmed, COVID-19 may represent the first virus directly capable of seeding infection in muscle fibres [409]. Furthermore, evidence indicates that local inflammation induced by infectious pathogens, *Escherichia coli (E. coli)* or *S. aureus*, in addition to cardiotoxin, may contribute to the development and volume of neurological HO. In an SCI mouse model, local and systemic administration of membrane components from *E.coli* or *S. aureus* significantly increased the volume of NHO, suggesting that inflammation triggered by infectious pathogens may be involved in NHO development [410]. Notably, once bacterial-induced inflammation reached a certain threshold, SCI was not required for NHO development. It has been suggested that using local and, or systemic antimicrobials targeting Gram-positive organisms at the time of injury and early during the treatment course could be a potential adjunct prophylaxis to limit tHO production [67]. However, these experimental findings require future validation in human clinical studies.

Adhesion of inflammatory monocytes and neutrophils to vascular endothelium is selectively enhanced by ICAM-1 expression on endothelial cells, induced by the upregulation of IL-1 and TNF-α signalling. Upregulation of ICAM-1 expression has also been observed in ARDS and cardiovascular pathology, and IL-1 upregulation has been shown to promote neurogenic HO [411]. IL-1RA treatment to dampen IL-1 signalling and subsequent downstream pro-inflammatory has previously proven neuroprotective, affecting both the central and peripheral inflammatory response after CNS injury [412]. Previous studies have reported the ability of IL-1RA to cross the blood-CNS barrier [413]. After a stroke, delayed IL-1RA treatment has been shown to reduce neutrophil infiltration by inhibiting cytokine expression in microglia [412]. Exogenous IL-1RA administration after SCI significantly reduced early neutrophil recruitment and infiltration to the lesion site. These findings suggest that the acute targeting of IL-1 signalling with systemic IL-1RA treatment may be a viable strategy to suppress the early drivers of the dysregulated peripheral inflammatory response after CNS trauma [412] Thereby, reducing immune cell infiltration and attenuating the spread of secondary injury into peripheral structures such as neural tissue [412]. Tseng et al. [411]

demonstrated the inhibitory effect of IL-1RA on NHO-derived FAP mineralisation with an associated reduced RUNX2 expression [411]. These early drivers may promote a heightened inflammatory response contributing to the pathological process driving traumatic heterotopic bone formation.

In an NHO mouse model, gene expression profiling identified that inflammatory responses, IL6-JAK-signal transducer and activator of transcription 3 (STAT3) and TNF-NF-kB signalling gene sets were significantly enriched in injured muscles developing NHO as early as 2 days post-injury, with enhanced expression of oncostatin-M (OSM) and IL-1β [411]. *In vitro,* osteogenic differentiation assays showed that IL-1α and IL-1β, produced by activated human monocytes, stimulated calcium mineralisation and RUNX2 protein expression in human FAPs derived from muscles surrounding NHOs [411]. Increased expression of IL-1β was identified in surrounding fibrotic tissue, and plasma IL-1β concentration was significantly elevated in NHO patients compared with healthy controls. These findings confirm the involvement of IL-1β as an inflammatory promotor of NHO development after SCI and TBI [411]. Interventions aimed at mitigating dysregulated inflammatory signalling and employing inhibitors that target molecular mediators of the maladaptive immune response seen in COVID-19, such as IL-6, TNF-α/Nf-kb, could be explored as potential strategies to target similar immunoinflammatory mechanisms that may play a role in the pathophysiology of tHO.

1.6 Dysregulation of Bone and Soft Tissue Repair Towards Fibrosis and Ectopic Ossification

A hypoxic microenvironment induces the mTOR signalling pathway, and HIF-1α, a downstream intermediate in mTOR signalling, is a key transcriptional regulator of hypoxic cellular responses and promoter of target genes such as VEGF [414, 415]. Cellular communication Network-2 (CCN2), formerly known as Connective Tissue Growth Factor, is a pro-fibrotic factor that is regulated by hypoxia, and HIF-1α has been shown to synergise with TGF-β on driving CCN2 overexpression [416]. Dysregulation of HIF-1α has been strongly implicated in many pathophysiological processes, including tHO [415, 417, 418]. The HIF-1α signalling may act together with other transcription factors that are also upregulated in pathological states and could exert a pro-fibrotic effect in skeletal muscle through the expression of CCN2 in sustained hypoxic conditions [419]. More so, myeloid hypoxic signalling plays a pivotal role in skeletal muscle homeostasis and adequate skeletal muscle regeneration. In a model of soft tissue trauma, it has been shown that mice with a conditional HIF-1α knockout targeted to skeletal muscle or myeloid cells demonstrated delayed

macrophage invasion and fewer myogenic *MyoD* (myogenic differentiation 1) positive cells were recruited to damaged muscles [420]. After BMP-2 injection, *MyoD* expressing satellite cells have been shown to differentiate into fibroblasts in skeletal muscle *in vivo.*

Fibroblasts are highly dynamic cells that play a central role in tissue repair and fibrosis. However, the mechanisms by which they contribute to both physiologic and pathologic states of extracellular matrix deposition and remodelling are just starting to be understood [315, 421]. Under pathological conditions, an aberrant regenerative process of ectopic osteogenesis represented by calcification or ossification and ectopic fibrosis and adipogenesis occurs in traumatised human tissues such as skeletal muscle [422]. Muscle resident multipotent mesenchymal progenitor cells contribute to fibrogenesis and ectopic osteogenesis. During muscle regeneration in response to injury and in chronic disease states, the accumulation and proliferation of fibroadipogenic progenitors (FAPs) into fibroblasts and adipocytes have been observed, suggesting these cells may be involved in the formation of fibrosis and ossification [423]. Numerous arguments incriminate FAPs rather than SCs as the participating progenitor cells in tHO [398, 424].

FAPs are interstitial mesenchymal stromal cells expressing PDGFRα that are emerging as the key effectors of compensatory or maladaptive repair of injured muscles [425]. In response to acute injury, the rapid expansion and accumulation of FAPs precede the presence of inflammatory infiltrate, signifying that FAPs play a key functional role in the coordinated conversion of inflammatory cues into pro-regenerative signals within a precise window of time [425]. In a burn injury/tenotomy mouse model, parabiosis experiments highlighted the involvement of circulating PDGFRα+ FAPs in developing burn-induced tHO in tendons [426]. A combined parabiosis and BMP-2-induced HO mouse model demonstrated an abnormal accumulation of PDGFRα+ FAPs associated with an *in vivo* osteogenic potential, although no circulating FAPs were detected [291]. Tseng et al. [424] showed that after SCI, extensive and uncontrolled FAP survival, proliferation, and differentiation results in the formation of extensive fibrotic areas in which osteogenic differentiation leads to tHO formation rather than muscle repair.

Early inflammatory connective tissue destruction, followed by fibroproliferative cell expansion and HO through an endochondral process, may occur in a dysregulated immune cell response. Different innate and adaptive immune cells' contributions to bone formation, resorption and disease are under continuing investigation. Zhang et al. [427] concluded that CD8 T cells participate in skeletal

muscle regeneration by stimulating the secretion of MCP-1 to recruit macrophages, facilitating myoblast proliferation. In CTX-induced skeletal muscle injury, impaired muscle regeneration, increased matrix deposit and reduced MCP-1 expression were displayed in CD8α-deficient mice compared to wild-type mice. Highlighting a crucial reciprocal interaction between CD8 T cells and MCP-1-producing macrophages in regulating inflammatory microenvironments needed for muscle regeneration after injury.

An accumulation of CD8 T cells has been observed in a dystrophin-deficient (mdx) mouse model of Duchenne muscular dystrophy, a pathology characterised by limited neuronal and skeletal tissue expression of dystrophin with resultant skeletal muscle degeneration and fibrosis [428]. Interestingly, Issac et al. [429] found that dystrophin–utrophin double knockout (dKO) mice have a reduced capacity for bone healing and exhibited spontaneous heterotopic ossification in the hind limb muscles. Similarly, Mu et al. [430] describes extensive HO formation in the skeletal muscle and occurrences of HO in the cardiac muscle of dKO mice, which was localised at sites enriched with damaged myofibres and surrounded by fibrotic and necrotic tissues. Additionally, upregulated gene expression of several inflammatory and pro-fibrotic factors, including BMP-2/4, IL-6, and TNF-α, was identified in the skeletal muscle of mdx mice [430].

Spondylarthritis (SpA) is a heterogeneous group of chronic inflammatory diseases of poorly defined aetiology, hallmarked by the aberrant ossification that occurs in the sacroiliac joints, intervertebral discs and entheses [431]. Entheses are the attachment sites of tendon, ligament, joint capsule, fascia, or muscle to bones, which dissipate mechanical stress and provide optimal myofascial stability. Entheses are hypothesised to be the primary target tissue for inflammation in SpA [431]. Pathological enthesis in SpA is characterised by CD4+ and CD8+ T lymphocyte cell infiltration, inflammation, fibrosis, and new bone formation via endochondral ossification [431]. Park et al. [432] identified significantly elevated TNF-α and IL-6 serum levels in patients with ankylosing spondylitis compared to healthy subjects.

Although limited data exist on the molecular basis controlling the reciprocal interactions between skeletal muscle and bone, both preclinical and clinical data support the concept of muscle-bone crosstalk [433]. The aforementioned results collectively highlight common cellular players involved in perpetuating the exaggerated inflammatory process and immune system dysregulation proposed in tHO and other chronic inflammatory diseases. Thus, expanding the field of potential

therapeutic targets for tHO, which could interfere with the inflammatory process and specific pathways that trigger immune system dysregulation in the context of injury-induced HO.

1.7 Pathways of Osteogenic Cell Signalling

Muscle and bone are subject to stringent regulation by soluble ligands and signalling pathways that function in a coordinated manner to ensure appropriate development and repair [135, 377]. Transcription factors enable the initiation and promotion of MSC differentiation towards an osteogenic fate. Specifically, Runx2 and Osx are the main transcription factors whose activation commits the cells to the osteogenic lineage [434]. Runx2 is a key regulator of bone development requisite for the maturation of hypertrophic chondrocytes and osteoblasts [435]. MMP9 has been identified as a novel target gene of both Runx1 and Runx2 in osteoblasts supporting the concept that RUNX factors are important transcriptional regulators of MMP9 and other MMPs for mediating multiple roles of these enzymes during bone formation [435]. RUNX2 controls osteoblast differentiation from mesodermal cell populations by regulating bone-specific genes such as VEGF, osteocalcin, MMP13, bone sialoprotein (BSP) and tissue inhibitors of matrix metalloproteinases (TIMP) [436].

RUNX2 is also aberrantly expressed in ossified soft tissues. Its role in the pathogenesis of ectopic mineralisation has been demonstrated in both human tHO patients and mouse models of tHO [65, 310, 418]. The literature supports a link between Runx2 with osteolytic diseases and the activities of metastatic cancer cells in the bone microenvironment [436]. Cancer cells metastasising to the bone have higher Runx2 levels than primary tumours and exhibit elevated MMP9 expression and activity [436]. Owing to the systemic nature of the pathological process underlying tHO, whereby the responsible osteoprogenitor cells are capable of entering the blood circulation and migrating to multiple new sites of ectopic bone formation, it is worth considering the similarities between the proposed processes of tHO and metastatic activities in the bone microenvironment.

MMP9 is an essential inducer of events associated with pathological conditions such as rheumatoid arthritis, tumour invasion and metastasis. Along with MMP13, Pratap et al. [436] implicates MMP9 as a downstream target of RUNX2 in bone tissues. These authors demonstrated that a Runx2 knock-down in bone metastatic cancer cells downregulates MMP9 and reduces the invasion properties of these cancer cells. Significant reductions in the mRNA levels of MMP2, MMP13, and VEGF with RUNX2

knockdown were identified compared to the control treatment. This suggests that RUNX2 binding to the MMP9 promoter may lead to a cooperative interaction with other known RUNX2 target genes. For example, VEGF is a primary component of tumour formation and metastasis. Similarly, by targeting the VEGF gene, RUNX2 is a necessary component of a tissue-specific genetic program that regulates VEGF for the progression of intramembranous and endochondral bone formation [65].

By a similar mechanism, in COVID-19 patients with respiratory failure, a significant increase is observed in circulating MMP-9, which strongly correlated with neutrophil count [437]. The co-expression of inflammatory cytokines with regulators of leukocyte recruitment and VEGFα suggests a mechanism by which inflammatory leukocytes may degrade the alveolar-capillary barrier, resulting in increased vascular membrane permeability and capillary leakage and contributing to a permissive microenvironment for vascular calcification [437].

In addition to NF-kβ activation, CD14-positive monocytes in SARS-COV patients show increased phosphorylated mitogen-activated protein kinase (MAPK) p38 [401]. In vascular calcification, it has been proposed that the activation of the p38 MAPK signalling pathway by local stimuli, such as oxidative stress, ECM, mechanical loading and BMP signalling, interfere with other signalling pathways, such as Wnt, which further promotes upregulation of RUNX2 and osteogenic differentiation of VSMC [438]. Immunohistochemical analysis of HO lesions from elbow trauma patients showed increased expression of Wnt/β-catenin as well as Runx2, and, Mir203 targeting of Runx2 inhibits Wnt/β-catenin and effectively inhibited tHO formation [310]. The addition of high phosphate to rat MSCs promoted nuclear translocation of Smad1/5/8 and the activation of canonical Wnt/β-catenin in addition to an increase in BMP-2 expression, ALP activity and osteogenic differentiation into VSMC [439]. Martin et al. [440] found that serum from military and civilian patients with trauma-induced HO had elevated downstream genes associated with the MAPK pathways. MAPK signalling pathway was activated in human adipose-derived stem cells (hASCs) following serum exposure in individuals with tHO [440].

Casein Kinase 2 (CK2) is a highly conserved serine/threonine kinase that serves as a positive regulator of osteoblast differentiation, exerting its specific function on cells of osteoblastic lineage [65, 441]. In contrast to previous *in vitro* findings, Kim et al. [65] demonstrated that CK2 is required for HO formation via a key pathway that stabilises RUNX2, where CK2-induced phosphorylation of RUNX2 leads to the recruitment of herpesvirus-associated ubiquitin-specific protease (HAUSP) [442,

443]. Utilising two established mouse HO models, heterotopic bone was formed via an endochondral pathway, including the formation of cartilage and adipose tissues and recruitment of hematopoietic elements [65]. By contrast, mice with deletion of *Csnk2b* in osteoprogenitors exhibited a notable reduction in HO, while cartilage or adipose tissue still formed at the site of injury [65]. Further, HO was suppressed by *Csnk2b* deletion in osteoblasts or by treatment with either a CK2 or HAUSP inhibitor, revealing a druggable pathway that controls RUNX2 with therapeutic potential for pathological ossification disorders such as tHO [65].

Notably, HAUSP, also known as ubiquitin-specific protease (USP7), controls many substrates mainly identified in the context of cancers, virus-associated host-pathogen interactions and metabolic and neurological pathologies [444, 445]. HAUSP is bound by at least two viral proteins, the ICP0 protein of herpes simplex type 1 and the EBNA1 protein of Epstein-Barr virus, and its interaction with viral proteins suggests that some viruses may influence cellular events by sequestering or altering its activity [444]. A tumour suppressive role was attributed to HAUSP, given its ability to increase the half-life of p53, resulting in growth repression and reactivation of apoptotic pathways [445]. The presence of poly-glutamine repeats in HAUSP indicates its possible link to neurodegenerative disorders, such as Spinocerebellar Ataxia and Huntington's disease [445]. Heterozygous deletion, nonsense mutations or duplications in HAUSP has been reported to lead to an unbalanced neuronal homeostasis [445]. More so, its function in stabilising repressor element 1-silencing transcription factor and positive regulation of Hedgehog (Hh) signalling suggests a role in the maintenance of neural stem/progenitor cells.

HAUSP is the only reported deubiquitinase that positively regulates Hh signalling, a well-known pathway maintaining adult tissue homeostasis [445]. In an animal model, Kan and colleagues [446] provide further evidence to suggest that dysregulated Hh signalling is implicated throughout all stages of endochondral tHO formation, showing that Hh signalling is upregulated in tissue bordering HO lesions, however not within the lesions themselves. Thus, Hh signalling inhibitors have been suggested as a viable treatment for treating tHO [447]. HAUSP dysfunction may also be related to other pathologies, such as metabolic disorders and bone diseases, via interaction with PPAR-y and FOX01. These have previously been shown as critical regulators in other metabolic tissues, such as skeletal muscles and adipose tissues [445]. It has been implied that HAUSP serves as a positive regulator of MSC differentiation; however, the functionality of HAUSP in preventing or buffering excessive bone formation warrants further investigation [12, 448].

1.8 Calcium and Phosphate Homeostasis in Pathological Ectopic Ossification

The control of tissue calcification is dependent on a tight local balance between extracellular levels of inorganic phosphate (P_i) and pyrophosphate (PP_i), regulated by tissue-non-specific alkaline phosphatase (TNAP) [449, 450]. Ectonucleotide pyrophosphate/phosphodiesterase 1 (ENPP1), identified on the surface of chondrocytes and osteoblasts, appears to be necessary for early osteoblast differentiation via generation of extracellular PP_i, a physiologic calcification inhibitor modulated by the membrane channel ankylosis protein (ANK) [451-453]. Increased vascular calcification has been reported in pyrophosphate-deficient Enpp1$^{-/-}$ mice [453], and calcifying vascular smooth muscle cells have been shown to exhibit much higher total NPP activity (generating PP_i) than osteoblasts [311]. In PC-1$^{-/-}$ and *ank/ank* cultured osteoblasts, extracellular PP_i deficiency was associated with reduced osteopontin (OPN, also called bone sialoprotein 1 [BSP-1]), expression and hyper-calcification [453]. Correction of OPN deficiency prevented hyper-calcification signifying the synergistic effects of PP_i and OPN expression on the ability of PC-1 and ANK to mediate calcification [453].

Mice with a truncation mutation (also known as the 'tiptoe walking' mutation) in the ENPP1 gene resulting in loss of function, exhibit symptoms of heterotopic tissue mineralisation [454]. Additionally, several mutations have been identified in the ENPP1 gene associated with ossification of the posterior longitudinal ligament of the spine (OPLL), a pathological condition of the paravertebral ligament that causes ectopic bone formation possibly through the process of endochondral ossification [455-458]. Babij et al. [455] discovered a novel mutation in the ENPP1 (C397S) gene on chromosome 10. In this study, C397S mice presented with striking joint disease and radiography of the skeleton demonstrated a distinct number of joint abnormalities associated with mineralisation of articular cartilage and abnormal calcification of the tendons [455]. Histological evaluation of the vertebrae showed that the joints, specifically and not bone tissue were the origin of the calcifying lesions. These findings also resemble the phenotype of the ENPP1$^{-/-}$ mice that were reported to show fusion of the joints and ectopic ossification of the skeleton [455-457].

Upregulated Hh signalling activity without ENPP1 may be a common mechanism underlying ectopic calcification in distinct tissues [458]. The findings of Jin et al. [458] extends previous studies by

showing that upregulated Hh activity contributes to the joint calcification phenotypes of ENPP1$^{-/-}$ mice. In ENPP1$^{-/-}$ joints, there was an increase in Hh target gene expression associated with chondrocyte hypertrophy and increased expression of osteoblast differentiation markers such as Osx, Col1a1 and Oc (also known as Bglap). This upregulation in Hh activity was detected ectopically before ectopic mineralisation occurred [458]. Another NPP family member, NPP5 (*ENPP5),* is a type 1 transmembrane protein highly expressed in the human brain, respiratory epithelium, and white adipose tissue. Decreased serum insulin levels were identified in Npp5 KO mice Field [446], and gene expression levels were differentially expressed in patients with T2DM and Alzheimer's disease [459]. Thus, as alluded to by Gorelik et al. [451], *ENPP5* may have a potential metabolic role. More so, as membrane glycoproteins of neural cells play crucial roles in axon guidance and neuronal transmission, it has been suggested that NPP5 may participate in neural cell communications [460]. The literature indicates that Pi-PPi metabolism associated with ENPP1 and ENPP5 may play an essential role in regulating ectopic ossification. Targeting the metabolism of local and systemic PP_i and P_i levels may offer an effective strategy to mitigate adverse mineralisation in the pathology of HO [461].

Previous studies have identified that inflammatory cytokines, including TNF-α and IL-1β, suppress endothelial fibroblast growth factor (FGF) signalling with reduced expression and activity of the FGF signalling cascade [462]. Nam et al. [452] showed that FGF-2 induces ENPP1 expression in pre-differentiated but not in differentiated calvarial osteoblasts, and ENPP1 induction depends on Runx2 and MSX2 transcriptional activity. However, research investigating the role of FGF-2 in HO pathogenesis is limited. Zhang et al. [365] found that in specimens from tHO patients, fibroblast growth factor receptor-3 (FGFR3) deletion in LECs decreased lymphatic formation after trauma via upregulated BMPR1α-pSmad1/5 downstream signalling, inhibiting lymphatic drainage and increasing local inflammation potentiating HO progression. More so, excess BMP-2 signalling decreased LEC formation [463]. These findings suggest that the downregulation of FGFR3 in LECs may contribute to HO development, and FGFR3 activation may be a prophylactic and therapeutic strategy for tHO [463].

Fibroblast growth factor 23 (FGF-23) is a bone-derived hormone that plays a fundamental role in PTH secretion and phosphate and Vitamin D metabolism [456]. The principal actions of FGF-23 lower blood phosphate Hyperphosphatemia and increased serum vitamin D levels have been reported in fgf23^{-} mice suggesting that impaired calcium phosphate metabolism may contribute to

FGF-23 mediated vascular calcification [461]. Increased circulating levels of the FGF-23 have been associated with alterations in alterations in bone mineralisation in hypophosphatemic disorders caused by mutations of Enpp1 and Ank, suggesting that bone metabolism is linked to systemic phosphate homeostasis [456]. Enpp1$^{-/-}$ mice disclose an increase in circulating FGF-23 and Fgf-23 mRNA expression, indicating a possible role for Enpp1 in phosphate regulation [456]. More so, decreases in circulating calcium and phosphate levels in ENPP1$^{-/-}$ mice were consistent with excess FGF-23.

Familial tumoral calcinosis (FTC) is a rare and disabling disorder resulting from disturbances in the FGF23-mediated phosphate regulation [464-466]. In FTC, the hyperphosphatemia and high-normal calcium seen with intact FGF-23 deficiency or resistance results in an increased calcium x phosphate product, which likely contributes to the development of ectopic calcifications in the skin, subcutaneous tissues and vascular structures [464, 465]. Ramnitz et al. [464] describes the first case of HO with joint involvement reported in FTC, possibly mediated by chronic systemic inflammation. As in tHO, it is proposed that soft tissue calcifications in FTC often develop in areas of inflammation, tissue hypoxia, or repetitive trauma. However, what exactly precipitates their formation needs to be clarified [464]. Further investigation is warranted into the specific local and systemic mechanisms of fibroblast growth factor signalling in ENPP1 deficiency and disorders affecting phosphate homeostasis resulting in tissue mineralisation and pathological soft tissue calcifications.

Thyroid hormone is a systemic factor that potently regulates skeletal maturation, and thyroid hormone receptor α (TR-α) is essential for regulating the process of endochondral ossification. Wang et al. [467] demonstrated that IGF-1, a stabiliser of β-catenin, and IGF-1 receptor (IGF1R) stimulate Wnt-4 expression and β-catenin activation. Further, regulation of terminal differentiation of growth plate chondrocytes by thyroid hormone is, in part, through the modulation of the Wnt/β-catenin signalling pathway [467]. Parathyroid hormone-like hormone (PTHLH) is an autocrine/paracrine ligand and one of the commonly accepted master regulators of skeletogenesis, including regulating endochondral bone development [446]. Furthermore, Hh signalling promotes chondrocyte differentiation to a hypertrophic state by upregulating the expression of PTHLH, which encodes parathyroid hormone-related protein (PTHrP). In a pathological context, high expression of PTHLH has been identified as playing a role in bone metastasis and osteolysis, whereby RUNX2 and PTHLH have been shown to regulate each other in a reciprocal fashion [468].

Parathyroid hormone (PTH)/Parathyroid hormone-related protein (PTHrP) has been considered an intriguing therapeutic target in the setting of malignancy, particularly in the bone microenvironment owing to their central role in the regulation of serum calcium and phosphate [469]. Regulation of PTHrP expression by Hh signalling is mediated by BMPs, which also initiate chondrocyte differentiation. Most neoplastic tissues that metastasise to bone produce PTHrP, and PTHrP expression correlates with skeletal localisation of tumours [470]. Mak et al. [469] demonstrated that neutralisation of PTHrP induces apoptosis in Giant Cell Tumour of bone (GCT) stromal cells and therefore suggests that PTHrP serves to propagate proliferation in an autocrine manner in GCT stromal cells, contributing to a neoplastic phenotype. The presence of PTHrP in ossifying gastric carcinomas at a relatively high rate indicates that PTHrP may also be related to heterotopic ossification associated with malignancies [471]. More so, immunohistochemical localisation of PTHrP has been found in primary sites of cancer tissue, stroma around heterotopic bone and metastatic sites, suggesting that PTHrP from cancer tissue plays a critical role in its nearby osteogenesis [471].

In previous studies, Nakajima and colleagues [472, 473] demonstrated that PTH and PTHrP regulate endochondral ossification associated with the progression of OPLL. In a genome-wide association study by these authors, the mRNA expression levels of five susceptible genes for human OPLL were analysed in cultured human OPLL and non-OPLL cells subjected to a cyclic tensile strain [474]. This study identified the expression of CCDC91 as one of these five genes and alluded to the location of CCC91 as being adjacent to PTHLH, which encodes a PTH family member. More so, the mRNA expression level for CCDC91 had the largest significant increase due to cyclic tensile stress indicating that CCDC91 may be associated with the progression of ossification caused by mechanical stress [474].

1.9 Neurometabolic Processes and Pathways of Neurodegeneration

The neurovascular unit (NVU) is a functional unit composed of neurons, neuroglia, vascular cells, and the basal lamina matrix [475]. The current understanding of brain injury and neurodegeneration mechanisms highlights an appreciation of NVU's multicellular interactions, including the evolution of blood-brain barrier damage, neuronal cell death or degeneration, glial reaction, and immune cell infiltration. In addition to a mineral constituent regulation, bone homeostasis is controlled by long-range signals such as leptin, glucocorticoids and parathyroid

hormone produced by the adipose tissue, the adrenal glands, and the parathyroid glands, respectively, and by signals originating from the nervous system [398]. Whilst dysregulation of the neuroendocrine system due to lesions of the CNS can result in the abnormal systemic release of numerous mediators that may initiate tHO development, it must be considered whether it is a simultaneous central and peripheral nerve injury that represents endochondral ossification in the context of tHO.

Accumulating evidence shows that TBI triggers several neurobiological processes resembling those operating in neurodegenerative diseases [476]. Prolonged structural and functional abnormalities of the NVU have been associated with many CNS diseases, including TBI [477, 478]. Zhou and colleagues [475] proposed that continued NVU abnormalities following TBI serve as the pathophysiological substrate and trigger yielding chronic neuroinflammation, proteinopathies and oxidative stress, consequently leading to the progression of neurodegenerative diseases such as Alzheimer's Disease (AD) and Parkinson's Disease (PD) [368]. These findings suggest for further investigation into a potential link between neurovascular abnormalities and the pathophysiological mechanism of tHO.

There is reported evidence of HO formation in stroke patients, and interestingly, elevated serum concentrations of bone turnover markers, including osteoprotegerin, sclerostin, OPN and dickkopf-related protein 1, were displayed in patients during the first few days following an acute stroke event [479]. Injury-induced hypoxia is proposed to activate angiogenesis and osteogenic precursor cells and initiate HO formation [480, 481]. Furthermore, it is believed that hypoxic induction of VEGF further promotes brain oedema. This notion has been demonstrated in a murine stroke model whereby an exacerbation of neuroinflammation and ischaemic cerebral injury causing substantial secondary damage resulted from induction of experimental fracture shortly before stroke [482]. These results support the proposed 'efferent' signalling pathway between brain and bone that, when dysregulated, may be responsible for perpetuating the pathogenesis of tHO [368].

Mild traumatic brain injury also includes concussion and is the most common TBI affecting military personnel exposed to explosive blast [483]. Mckee et al. [483] describes histopathological parallels between concussion and blast-related neurotrauma, suggesting similarities in their underlying biomechanical mechanisms. Many military personnel develop tHO from the combination of trauma and neurogenic injuries following blast injury, and cases of tHO have been described in association

with very mild TBI, such as concussion injury [9, 484-486]. Bajwa [35] discusses the long-term consequences of TBI on the skeletal system and suggests that secondary to injury, the disruption of the hormonal signals and neural circuits that originate in the hypothalamus may induce adverse skeletal effects. Experimental evidence in adolescent mice reported a negative effect of glucocorticoids on endochondral ossification in the growth plate, constraining longitudinal and appositional bone growth [487]. Leptin can also indirectly modulate bone formation through effectors downstream of the hypothalamus such as estrogen, cortisol, IGF-1 and parathyroid hormone, and activation of local adrenergic signalling at the osteoblast level via β2 adrenergic receptors [398]. Several studies have outlined the central effects of leptin on bone mass via central osteo-modulatory signalling and discuss the transmission of the regulatory signals beginning with circulatory leptin and ending with altered bone cell activity [488, 489].

Not only ageing but also obesity, a high-fat diet, and type-2 diabetes (T2DM) can affect the function of MSCs. Medial artery calcification is common in individuals with T2DM, neo-angiogenesis arising from the inflamed adventitial-medial junction can upregulate mural BMP-Wnt signalling, leading to the trans-differentiation of myofibroblasts towards a fibrogenic and osteogenic phenotype [261]. It has been reported that diabetes may promote the onset and development of neurodegenerative diseases by impairing the integrity of the neurovascular unit. Thus, diabetes could be a novel mechanism of the neurodegeneration [490]. Hyperglycaemia and hyperinsulinemia influence the microenvironment of the MSC niche resulting in MSC dysfunction and reducing osteogenic differentiation. However, intensified adipogenesis increases the concentration of leptin, which regulates bone formation and can support osteogenesis. Supporting this hypothesis, ventromedial hypothalamic neurons have been identified as playing a pivotal role in bone formation, with chemical lesioning of these neurons resulting in a high bone mass phenotype in mice [491]. This was thought to occur via the ablation of leptin receptors, which are densely populated in this region, thus inhibiting the osteogenic effect of leptin [491].

Proteinopathies, persistent oxidative stress, and chronic neuroinflammation may be the pivotal intermediary pathological processes that give rise to progressive neuropathology in CNS disorders. Therefore, whether this confluence of multiple secondary injury processes that lead to progressive, long-term neurodegeneration is also acutely responsible for triggering pathological ectopic bone formation must be considered. The widespread effects of burn injury (inflammatory insult on the nerves), SCI, TBI and COVID-19 virus on neuronal homeostasis and regulatory functions suggest that

tHO may be a maladaptive response to the early pathophysiological changes in CNS and or PNS microstructures following injury.

Appendix 2: Supplementary Material (Chapter 4)

Appendix 2A: Flow Diagram of Patient Identification, Screening Protocol and Selection Process

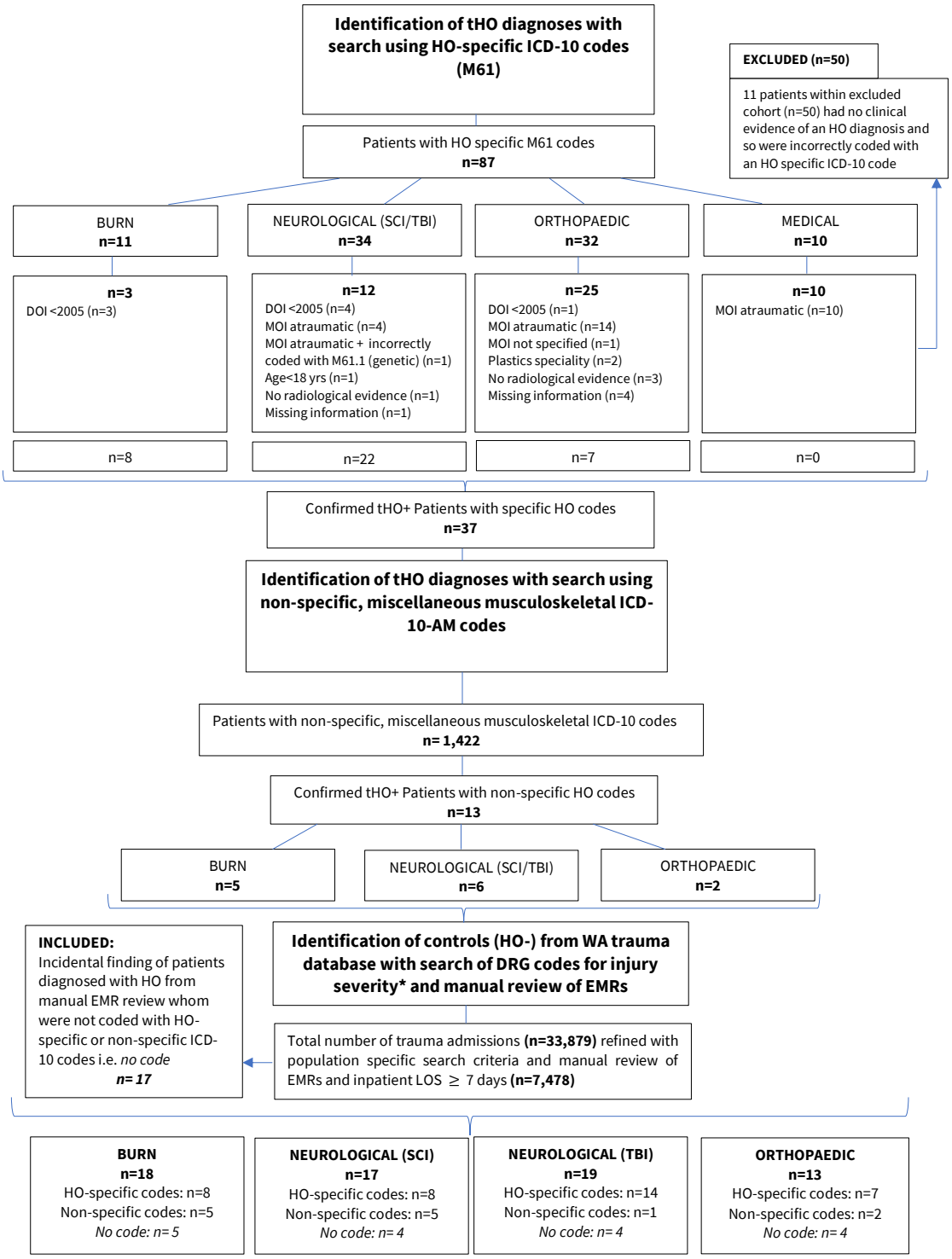
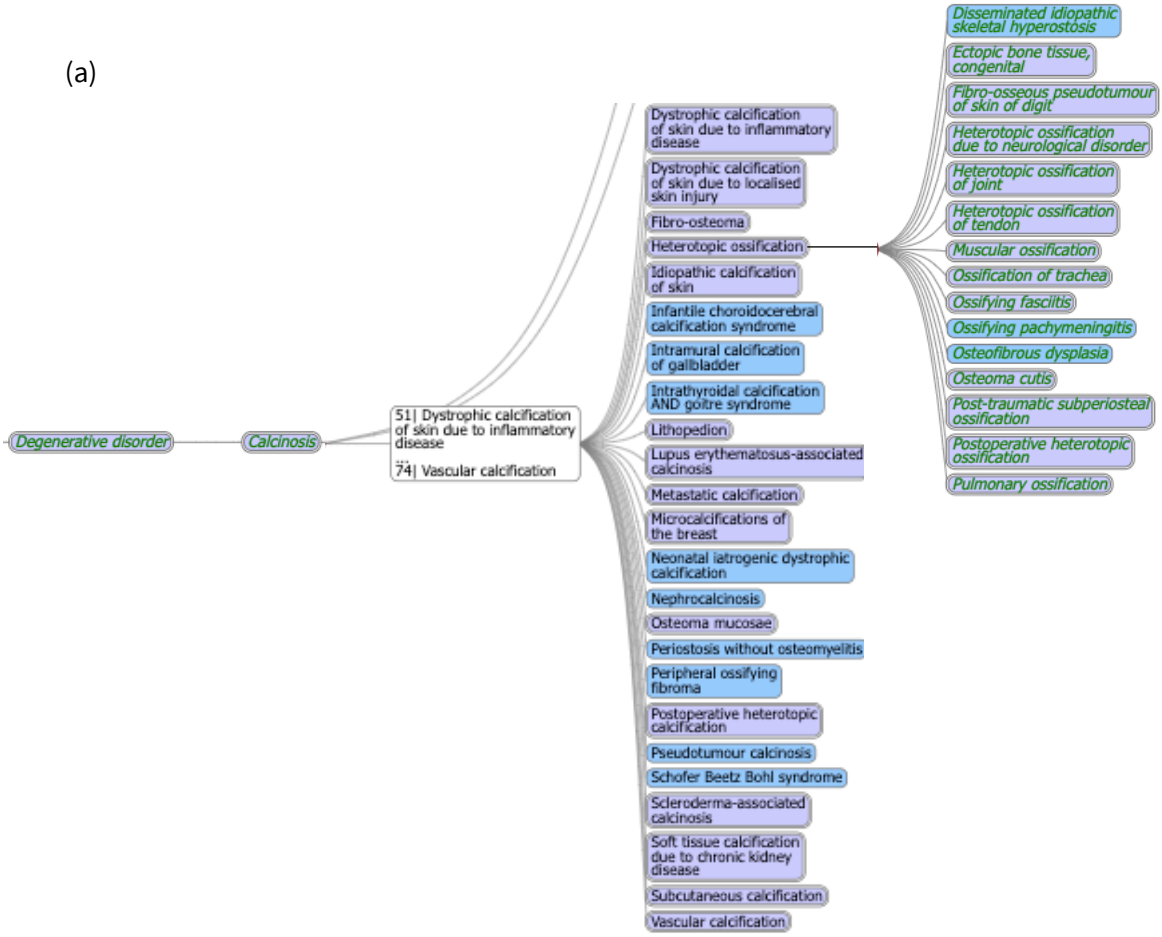


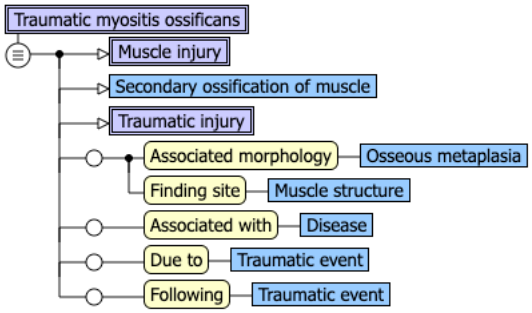
Figure A2.1. Flow diagram of patient identification, screening protocol and selection process

Appendix 2B: Current Systematised Nomenclature of Medicine Clinical Terms Australian Release (SNOMED-CT-AU) for Heterotopic Ossification

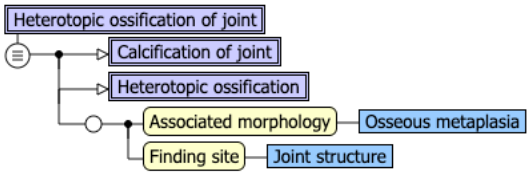
(a)



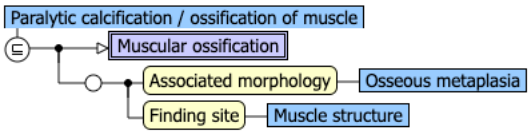
(b)



(c)



(d)



(e)

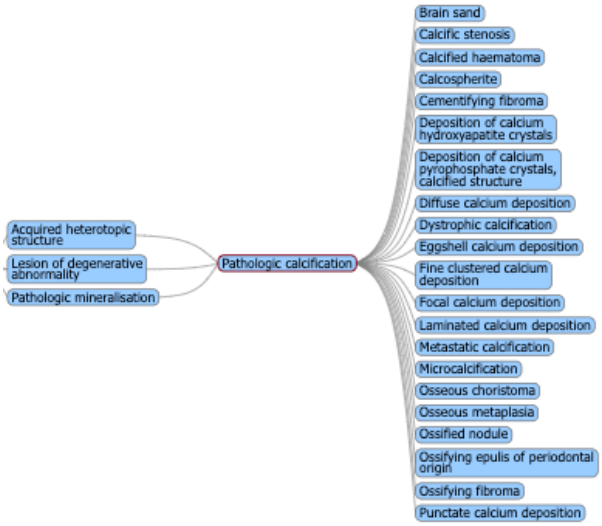


Figure A2.2. Current Systematised Nomenclature of Medicine (SNOMED) clinical terms (Australian release) for heterotopic ossification. (a) Heterotopic ossification (b) Traumatic myositis ossificans. (c) Heterotopic ossification of joint (d) paralytic calcification/ossification of muscle. (e) Pathologic calcification (clinical finding). For which an acceptable synonym includes heterotopic calcification however, no traumatic mechanism of injury is specified in the code descriptions.

Appendix 2C. Diagnosis Episode of Care and ICD-10-AM Code Distribution for Inpatient and Outpatient Diagnoses

Table A2.1. Diagnosis episode of care and ICD-10-AM code distribution for inpatient and outpatient tHO diagnoses by primary injury cohort.

	BURN	**SCI**	**TBI**	**ORTHO**	**TRAUMA (TOTAL)**	***p***
Diagnosis episode of care						
n	18	17	19	13	67	
Inpatient	17 (25.3%)	13 (19.4%)	17 25.3%)	1 (1.4%)	48 (71.6%)	<0.001
Outpatient	1 (1.4%)	4 (5.9%)	2 (2.9%)	12 (17.9%)	19 (28.4%)	
ICD-10-AM code specificity for inpatient and outpatient tHO diagnoses						
n	18	17	19	13	67	
HO-specific (M61)	8 (11.9%)	8 (11.9%)	14 (20.8%)	7 (10.4%)	37 (55.2%)	0.457
Non-specific	5 (7.4%)	5 (7.4%)	1 (1.4%)	2 (2.9%)	13 (19.4%)	
No code	5 (7.4%)	4 (5.9%)	(5.9%)	4 (5.9%)	17 (25.4%)	

n: no. of subjects. tHO: traumatic heterotopic ossification, SCI: spinal cord injury, TBI: traumatic brain injury, Ortho: orthopaedics, *p:* p-value

Appendix 2D: Description and Distribution of Coding Method Used for Identification of True Inpatient and Outpatient tHO Diagnoses by Primary Injury Cohort

Table A2.2. Description and distribution of coding method used for identification of true inpatient (n=48) and outpatient (n=19) tHO+ diagnoses by primary cohort.

Diagnosis episode of care				**Primary injury cohort**				**Total**
				BURN	**SCI**	**TBI**	**ORTH**	
Inpatient n=48	**ICD code specificity**	**M61 ICD-10-AM code**	***n***	**8**	**8**	**13**	**0**	**29**
			% within ICD code specificity	27.6%	27.6%	44.8%	0.0%	100.0%
			% within injury category	47.1%	61.5%	76.5%	0.0%	60.4%
			% of total	16.7%	16.7%	27.1%	0.0%	60.4%
		Non-specific ICD-10-AM code	***n***	**5**	**3**	**1**	**0**	**9**
			% within ICD code specificity	55.6%	33.3%	11.1%	0.0%	100.0%
			% within injury category	29.4%	23.1%	5.9%	0.0%	18.8%
			% of total	10.4%	6.3%	2.1%	0.0%	18.8%
		No code	***n***	**4**	**2**	**3**	**1**	**10**
			% within ICD code specificity	40.0%	20.0%	30.0%	10.0%	100.0%
			% within injury category	23.5%	15.4%	17.6%	100.0%	20.8%
			% of total	8.3%	4.2%	6.3%	2.1%	20.8%
	Total		***n***	**17**	**13**	**17**	**1**	**48**
			% within ICD code specificity	35.4%	27.1%	35.4%	2.1%	100.0%
			% within injury category	100.0%	100.0%	100.0%	100.0%	100.0%
			% of total	35.4%	27.1%	35.4%	2.1%	100.0%
Outpatient			***n***	**0**	**0**	**1**	**7**	**8**

n=19	**ICD code specificity**	**M61 ICD-10-AM code**	% within ICD code specificity	0.0%	0.0%	12.5%	87.5%	100.0%
			% within injury category	0.0%	0.0%	50.0%	58.3%	42.1%
			% of total	0.0%	0.0%	5.3%	36.8%	42.1%
		Non-specific ICD-10-AM code	***n***	**0**	**2**	**0**	**2**	**4**
			% within ICD code specificity	0.0%	50.0%	0.0%	50.0%	100.0%
			% within injury category	0.0%	50.0%	0.0%	16.7%	21.1%
			% of total	0.0%	10.5%	0.0%	10.5%	21.1%
		No code	***n***	**1**	**2**	**1**	**3**	**7**
			% within ICD code specificity	14.3%	28.6%	14.3%	42.9%	100.0%
			% within injury category	100.0%	50.0%	50.0%	25.0%	36.8%
			% of total	5.3%	10.5%	5.3%	15.8%	36.8%
	Total		***n***	**1**	**4**	**2**	**12**	**19**
			% within ICD code specificity	5.3%	21.1%	10.5%	63.2%	100.0%
			% within injury category	100.0%	100.0%	100.0%	100.0%	100.0%
			% of total	5.3%	21.1%	10.5%	63.2%	100.0%

n = no. of patients. tHO: traumatic heterotopic ossification, SCI: spinal cord injury, TBI: traumatic brain injury, ortho: orthopaedic

Appendix 2E: Description and Distribution of Coding Method Used for Identification of Total tHO+ Diagnoses By Primary Injury Cohort

Table A2.3. Description and distribution of ICD-10-AM coding method used for identification of total inpatient and outpatient diagnoses of true tHO+ (n=67) by primary injury cohort.

			Primary injury cohort				Total
			BURN	**SCI**	**TBI**	**ORTH**	
ICD code specificity	**M61 ICD-10-AM code**	***n***	**8**	**8**	**14**	**7**	**37**
		% within ICD code specificity	21.6%	21.6%	37.8%	18.9%	100.0%
		% within injury category	44.4%	47.1%	73.7%	53.8%	55.2%
		% of total	11.9%	11.9%	20.9%	10.4%	55.2%
	Non-specific ICD-10-AM code	***n***	**5**	**5**	**1**	**2**	**13**
		% within ICD code specificity	38.5%	38.5%	7.7%	15.4%	100.0%
		% within injury category	27.8%	29.4%	5.3%	15.4%	19.4%
		% of total	7.5%	7.5%	1.5%	3.0%	19.4%
	No code	***n***	**5**	**4**	**4**	**4**	**17**
		% within ICD code specificity	29.4%	23.5%	23.5%	23.5%	100.0%
		% within injury category	27.8%	23.5%	21.1%	30.8%	25.4%
		% of total	7.5%	6.0%	6.0%	6.0%	25.4%
Total		***n***	**18**	**17**	**19**	**13**	**67**
		% within ICD code specificity	26.9%	25.4%	28.4%	19.4%	100.0%
		% within injury category	100.0%	100.0%	100.0%	100.0%	100.0%
		% of total	26.9%	25.4%	28.4%	19.4%	100.0%

n = no. of patients. tHO: traumatic heterotopic ossification, SCI: spinal cord injury, TBI: traumatic brain injury, ortho: orthopaedic

Appendix 2F: Description and Distribution of Specific tHO ICD-10-AM (M61) Codes for Confirmed tHO+ Cases Including Inpatient and Outpatient Diagnoses

Table A2.4. Description and distribution of HO-specific ICD-10-AM (M61) codes for confirmed tHO+ cases including inpatient and outpatient diagnoses.

		Primary injury cohort (*n*=37)												Total N (Code count)	
		BURN tHO+ *n*=8			SCI tHO+ *n*=8			TBI tHO+ *n*=14			ORTH tHO+ *n*=7				
		N	% within cohort	% total	N	% within cohort	% total	N	% within cohort	**% total**	N	% within cohort	% total	N	**%**
M61 Calcification and ossification of muscle	**M61.0** Myositis ossificans traumatica	1	8.3%	**11.1%**	1	10.0%	**11.1%**	5	31.3%	**55.5%**	2	25.0%	**22.2%**	**9**	**19.6%**
	M61.2 Paralytic calcification and ossification of muscle	0*	0.0%	**0.0%**	5**	50.0%	**100%**	0*	0.0%	**0.0%**	0*	0.0%	**0.0%**	**5**	**10.9%**
	M61.3 Calcification and ossification of muscles associated with burns	9**	75.0%	**100%**	0*	0.0%	**0.0%**	0*	0.0%	**0.0%**	0*	0.0%	**0.0%**	**9**	**19.6%**
	M61.4 Other calcification of muscle	0	0.0%	**0.0%**	0	0.0%	**0.0%**	1	6.3%	**100%**	0	0.0%	**0.0%**	**1**	**2.2%**
	M61.5 Other ossification of muscle	2**	16.7%	**10.5%**	3	30.0%	**15.7%**	9*	56.3%	**47.3%**	5*	62.5%	**26.3%**	**19**	**41.3%**
	M61.9 Unspecified calcification and ossification of muscle	0	0.0%	**0.0%**	1	10.0%	**33.3%**	1	6.3%	**33.3%**	1	12.5%	**33.3%**	**3**	**6.5%**
Total		**12 (26%)**			**10 (21.7%)**			**16 (33.3%)**			**8 (17.4%)**			**46 (100%)**	

n=total number of patients per primary injury cohort. N = frequency of used code in each primary injury category (code count). For patients coded with >1 code, the total number of codes per patient was included in the final frequency calculation**.** **denotes a subset of HO cohort categories whose column proportions differ significantly from *each other at the .05 level. tHO: traumatic heterotopic ossification, SCI: spinal cord injury, TBI: traumatic brain injury, ortho: orthopaedic

Appendix 2G: Description and Distribution of Non-Specific, Miscellaneous Musculoskeletal ICD-10-AM Codes for Confirmed tHO Cases

Table A2.5. Description and distribution of tHO non-specific, miscellaneous musculoskeletal ICD-10-AM codes for true tHO cases (n=13), including inpatient and outpatient diagnoses.

		Primary injury cohort (n=13)								Total (code count)	
		BURN tHO+ *n*=5		SCI tHO+ *n*=5		TBI tHO+ *n*=1		ORTH tHO+ *n*=2			
		N	%	N	%	N	%	N	%	N	%
Non-specific, misc., ICD-10-AM codes	M89.8	1	12.5%	1	12.5%	0	0.0%	1	50.0%	3	12.0%
	M79.89	0	0.0%	2	25.0%	0	0.0%	0	0.0%	2	8.0%
	M89.32	1	12.5%	0	0.0%	0	0.0%	0	0.0%	1	4.0%
	M85.83	1	12.5%	0	0.0%	0	0.0%	0	0.0%	1	4.0%
	S79.9	0	0.0%	0	0.0%	1	14.3%	0	0.0%	1	4.0%
	M25.02	0	0.0%	1	12.5%	0	0.0%	0	0.0%	1	4.0%
	M86.96	1	12.5%	0	0.0%	0	0.0%	0	0.0%	1	4.0%
	M25.46	0	0.0%	1	12.5%	0	0.0%	0	0.0%	1	4.0%
	M89.81	1	12.5%	0	0.0%	0	0.0%	0	0.0%	1	4.0%
	M25.75	0	0.0%	0	0.0%	1	14.3%	0	0.0%	1	4.0%
	M24.65	0	0.0%	0	0.0%	1	14.3%	0	0.0%	1	4.0%
	M67.85	0	0.0%	0	0.0%	1	14.3%	0	0.0%	1	4.0%
	M85.86	1	12.5%	0	0.0%	0	0.0%	0	0.0%	1	4.0%
	M67.93	0	0.0%	0	0.0%	0	0.0%	1	50.0%	1	4.0%
	M86.97	1	12.5%	0	0.0%	0	0.0%	0	0.0%	1	4.0%
	M21.95	0	0.0%	0	0.0%	1	14.3%	0	0.0%	1	4.0%
	M75.5	0	0.0%	1	12.5%	0	0.0%	0	0.0%	1	4.0%
	M89.85	1	12.5%	0	0.0%	0	0.0%	0	0.0%	1	4.0%
	M79.85	0	0.0%	1	12.5%	0	0.0%	0	0.0%	1	4.0%
	T93.1	0	0.0%	0	0.0%	1	14.3%	0	0.0%	1	4.0%
	M21.80	0	0.0%	0	0.0%	1	14.3%	0	0.0%	1	4.0%
	M85.80	0	0.0%	1	12.5%	0	0.0%	0	0.0%	1	4.0%
TOTAL		**8**	**100.0%**	**8**	**100.0%**	**7**	**100.0%**	**2**	**100.0%**	**25**	**100.0%**

n=total number of patients per primary injury cohort. N = frequency of used code in each primary injury category (code count). For patients coded with >1 code, the total number of codes per patient was included in the final frequency calculation. Misc: miscellaneous, tHO: traumatic heterotopic ossification, SCI: spinal cord injury, TBI: traumatic brain injury, ortho: orthopaedic

Appendix 2H: Frequency of Descriptive Terms Used by Health Professionals in Clinical Documentation Relating to tHO Diagnoses

Table A2.6. Frequency of descriptive terms related to tHO used by health professionals in clinical documentation for 67 cases of tHO across the network of hospitals. A total of 69 individual descriptive terms for tHO were identified, highlighting the wide variation in descriptors for tHO.

Descriptive terms for tHO	Count of Descriptive terms for tHO
Heterotopic ossification	39
Myositis ossificans	24
Heterotopic bone formation	13
Heterotopic calcification	11
Heterotrophic ossification	10
Heterotopic bone	8
Soft tissue calcification	8
Ossification	5
Hypertrophic ossification	5
Soft tissue ossification	5
Heterotropic ossification	4
Calcification	4
Dystrophic calcification	4
Focus of ossification	4
Calcifications	3
Heterotopic new bone formation	2
Calcific foci	2
Heterotrophic soft tissue calcification	2
Heterotrophic bone	2
myositis ossification	1
HO bone formation	1
Post traumatic bony ossicles	1
Extensive exuberant callus	1
Increased bone formation	1
Extensive irregular ossification	1
Ossified fragment	1
Extra osseous calcification	1
Extensive dystrophic ossification	1
Foci of calcification	1

Calcific density	1
Foci of ossification	1
Muscular dystrophic ossification	1
Focus of calcification	1
Calcific focus	1
Periarticular heterotopic calcification	1
Amorphous calcification	1
Small calcified lump	1
Bone island	1
Heterotropic ossifications	1
Heterotopic bony formation	1
Hypertrophic bone	1
Bony heterotopia	1
Hypertropic ossification	1

Appendix 3: Supplementary Material (Chapter 5, Part 1)

Appendix 3A: Margins Plot (95% CI) of the Effect of tHO on Total Hospital Length of Stay

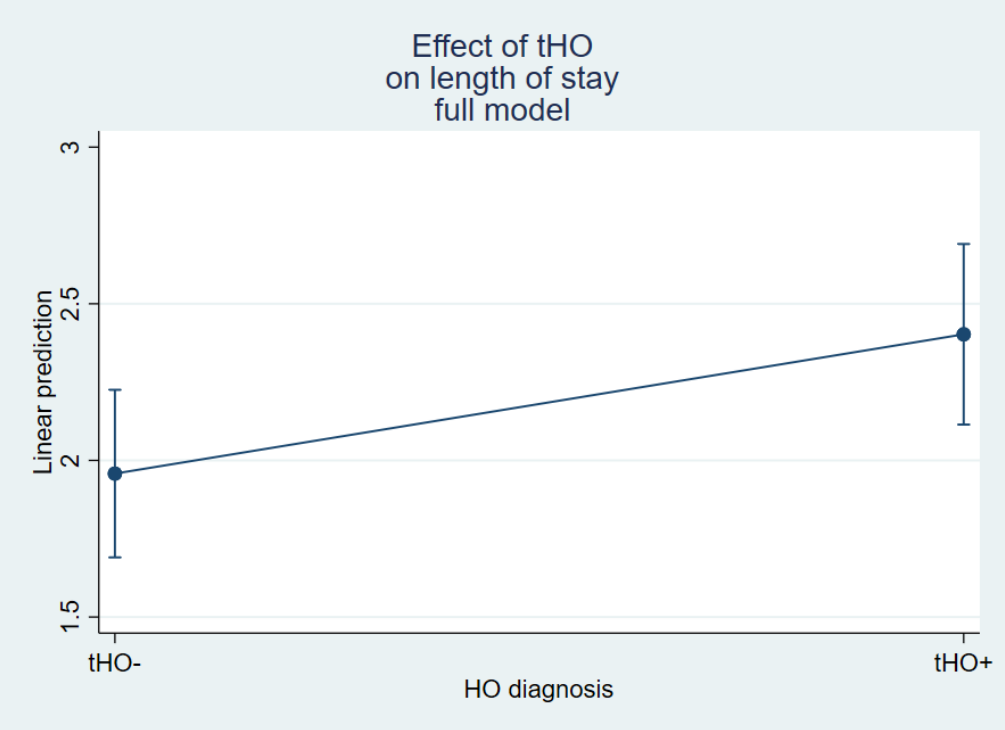


Figure A3.1. Margins plot (95% CI) of the effect of tHO on total hospital length of stay. The differences in the main effects were observed by evaluating the Estimated Marginal Means.

Appendix 3B. Baseline Demographic and Injury Severity Characteristics of the tHO+ and tHO- Groups According to Primary Injury Cohort

Table A3.1. Comparison of tHO+ and tHO- groups according to matching criteria; age, gender, and population-specific injury severity factors.

	Burn		
	tHO+ n= 17	tHO- n= 51	Total
Injury age (years)	38 (31-48)	34 (26-43)	37 (27-44)
Gender			
Male	15 (37.5%)	44 (37.0)	59 (86.7%)
Female	2 (28.6%)	7 (31.8%)	9 (13.2%)
TBSA (%)	48.2±23.3	39.4±18.1	41.6±19.7
AIS-Burn			
1	1 (5.9%)	2 (3.9%)	3 (4.4%)
2	2 (11.8%)	6 (11.8%)	8 (11.8%)
3	2 (11.8%)	7 (13.7%)	9 (13.2%)
4	0 (0%)	7 (13.7%)	7 (10.3%)
5	11 (64.7%)	30 (68.8%)	41 (60.3%)
6	0 (0%)	0 (0%)	0 (0%)
	Neurological (SCI)		
	tHO+ n= 13	tHO- n= 39	Total
Injury age (years)	35 (25-40)	45 (30-58)	41 (28-56)
Gender			
Male	11 (27.5%)	33 (27.7%)	44 (84.6%)
Female	2 (28.6%)	6 (27.3%)	8 (15.4%)
ISNCSCI			
Cervical	8 (25.8%)	23 (74.2%)	31 (59.6%)
Thoracic	5 (23.8%)	16 (76.2%)	21 (40.4%)
Lumbar	0 (0%)	0 (0%)	0 (0%)
AIS-Spine			
1	0 (0%)	0 (0%)	0 (0%)
2	0 (0%)	0 (0%)	0 (0%)
3	0 (0%)	0 (0%)	0 (0%)
4	6 (46.2%)	18 (46.2%)	24 (46.2%)

5	6 (46.2%)	18 (46.2%)	24 (46.2%)
6	1 (7.6%)	3 (7.6%)	4 (7.6%)
	Neurological (TBI)		
	tHO+ n= 17	tHO- n= 51	Total
Injury age (years)	25 (22-47)	30 (24-47)	30 (23-47)
Gender			
Male	14 (35%)	42 (35.3%)	56 (82.3%)
Female	3 (42.9%)	9 (40.9%)	12 (17.6%)
TBI severity			
Mild	0 (0%)	0 (0%)	0 (0%)
Moderate	0 (0%)	0 (0%)	0 (0%)
Severe	9 (52.9%)	22 (43.1%)	31 (45.6%)
Extremely severe	8 (47.1%)	29 (56.9%)	37 (54.4%)
AIS-Head			
1	0 (0%)	0 (0%)	0 (0%)
2	1 (5.9%)	6 (11.8%)	7 (10.3%)
3	7 (41.2%)	32 (62.7%)	39 (57.4%)
4	8 (47%)	13 (25.5%)	21 (30.8%)
5	1 (5.9%)	0 (0%)	1 (1.5%)
6	0 (0%)	0 (0%)	0 (0%)

tHO: traumatic heterotopic ossification, SCI: spinal cord injury, TBI: traumatic brain injury, TBSA: total burn surface area, ISNCSCI: The International Standards for Neurological Classification of Spinal Cord Injury, ASIA: American Spinal Injury Association. AIS: Abbreviated Injury Score.

Appendix 3C: Univariate Analysis of tHO Diagnostic Characteristics

Table A3.2. Univariate analysis of tHO diagnostic characteristics for inpatient and outpatient tHO diagnoses by primary injury category.

	Trauma (TOTAL)	Burn	SCI	TBI	*p*
Time to radiological evidence of tHO	53 (38-81)	56 (88-42)	65 (117-48)*	38 (58-33)*	0.019
Diagnosis inpatient episode of care					
n	30	-	13	17	0.003
Acute care	10 (33.3%)	-	0 (0.0%)	10 (33.3%)	
Rehabilitation	20 (66.6%)	-	13 (43.3%)	7 (23.3%)	

Categorical variables are presented as n (%). Continuous variables are presented as median (IQR). Due to model of care differences for burn and neurological trauma patients, LOS outcomes for acute and rehabilitation episodes of care are comparable only in the neurological cohorts. *n*: no. of subjects. *Signifies statistically significant subgroup comparisons at $p<0.05$. tHO: traumatic heterotopic ossification, SCI: spinal cord injury, TBI: traumatic brain injury, *p*: p-value

Univariate results

Within the neurological cohorts, tHO+ patients had a significantly longer stay in the acute care setting than tHO- patients (median tHO+, 32 [IQR 24-54] *vs.* tHO-, 23 [IQR 17-31] days; p<0.001), with the highest median LOS of 35 (IQR 30-59) identified in the TBI tHO+ group. Similarly, LOS on a rehabilitation ward was significantly longer for tHO+ patients following neurological injury (median 114 *vs.* 64 days; $p=0.006$). Rehabilitation LOS was the highest in the tHO+ cohort after SCI (median 126 days) however, a significant difference in rehabilitation LOS was only identified between the two tHO groups following TBI (median tHO+, 110 [IQR 34-212] days *vs.* tHO-, 37 [IQR 18-64] days; $p=0.003$).

Appendix 3D: Initial Model for Multivariate Negative Binomial Regression Analysis

Table A3.3. Initial model for multivariate negative binomial regression analysis.

VARIABLES ASSOCIATED WITH TOTAL LOS	IRR	95% CI	*p*
tHO diagnosis	1.54	1.33-1.79	0.000
ICU admission	1.37	1.08-1.74	0.009
LOS ICU	1.00	0.99-1.01	0.684
Mechanical ventilation (hours)	1.00	0.99-1.00	0.201
Long bone fracture	1.19	0.97-1.44	0.090
Injury to hip region and thigh	1.38	1.12-1.68	0.002
CNS or PNS injury	1.01	0.86-1.19	0.859
Other ossification disorder	1.30	1.12-1.50	0.000
UTI	0.99	0.82-1.17	0.931
Pressure injury	1.36	1.14-1.61	0.000
DVT	1.21	1.01-1.43	0.037
Sepsis	0.91	0.76-1.08	0.280

tHO: traumatic heterotopic ossification, ICU: intensive care unit, LOS: length of stay, CNS: central nervous system, PNS: peripheral nervous system, UTI: urinary tract infection, DVT: deep vein thrombosis, IRR: incident rate ratios, 95% CI: 95% confidence intervals, *p*: p-value.

Univariate results

Covariates significantly associated ($p<0.05$) with an increasing total LOS to be included in the initial model for backwards stepwise multivariate logistic regression analyses were identified by univariate analysis and are shown in **Table A3.3.** ICU admission, ICU LOS, mechanical ventilation hours; concomitant injuries at admission: long bone fracture, injury to the hip region or thigh area, CNS or PNS injury; comorbidities: other ossification disorder; complications during hospital stay: pressure injury, deep vein thrombosis (DVT), urinary tract infection (UTI), sepsis.

Median ICU LOS was significantly higher in the tHO+ group; 14 (IQR 7-24) days *vs.* 7 (IQR 3-11) days in the non-tHO group. Burns patients who developed tHO had a significantly longer ICU LOS than

burn injury controls (median tHO+, 21 [IQR 17-34] *vs.* tHO-, 5 [IQR 0-11] days; $p<0.001$) however, ICU stay was similar between groups after SCI and TBI.

In the final multivariate regression model, the factor most strongly associated with increasing LOS was tHO diagnosis (IRR 1.56; 95% CI 1.35-1.79, $p<0.000$). Following tHO, occurrence of individual concomitant injuries and critical care variables were independently influential on LOS: injury to the hip region and thigh (IRR 1.48; 95% CI 1.24 – 1.76, $p<0.000$), ICU admission (IRR 1.38; 95% CI 1.09-1.74, $p<0.007$), pressure injury (IRR 1.34; 95% CI 1.15-1.57, $p<0.000$), other ossification disorder (IRR 1.33; 95% CI 1.16-1.53, $p<0.000$), DVT (IRR 1.20; 95% CI 1.09-1.01-1.42, $p<0.035$) and mechanical ventilation hours (IRR 1.00; 95% CI 1.0002 – 1.0005, $p<0.000$).

The resulting IRRs indicate that patients who develop tHO during hospitalisation are predicted to stay in hospital 56% longer (IRR 1.56, 95% CI, 1.35-1.79, $p<0.000$) than trauma patients who do not develop tHO. The CI of 1.33-1.79 indicates the range of values that the true IRR is likely to fall between. This interval does not include 1 (which is the null value for the IRR), and we can conclude that the effect of tHO diagnosis on the length of hospital stay is statistically significant at the 95% level of confidence.

Appendix 4: Supplementary Material (Chapter 5, Part 2)

Appendix 4A: Comparison of Clinical Variables for tHO+ Patients According to Primary Injury Cohort

Table A4.1. Comparison of clinical variables for tHO+ patients (n=47) according to primary injury cohort.

	Total tHO+ (n=47)		**Burn tHO+ (n=17)**		**SCI tHO+ (n=13)**		**TBI tHO+ (n=17)**		***p***
	mean /n	**Sd /%**	**Mean /n**	**Sd /%**	**Mean /n**	**Sd /%**	**Mean /n**	**Sd /%**	**-**
Days to radiological evidence	66.4	44.3	64	28	**89***	54	**52***	46	**0.019**
tHO+ diagnosis modality									**0.000**
USS	2	4.2	1	5.9	1	7.7	0	0.0	
XR	**37**	**78.7**	15	88.2	8	61.5	14	82.4	
CT	5	10.6	0	0.0	2	15.4	3	17.6	
MRI	0	0.0	0	0.0	0	0.0	0	0.0	
3-phase bone scan	3	6.3	1	5.9	2	15.4	0	0.0	
tHO Site									**0.000**
Unilateral shoulder	0	0.0	0	0.0	0	0.0	0	0.0	
Bilateral shoulders	1	2.1	1	5.9	0	0.0	0	0.0	
Unilateral elbow	7	14.9	3	17.6	0	0.0	4	23.5	
Bilateral elbows	6	12.8	**6***	**35.3**	0	0.0	0	0.0	
Unilateral forearms	1	2.1	0	0.0	1	7.7	0	0.0	
Bilateral forearms	0	0.0	0	0.0	0	0.0	0	0.0	
Unilateral hip	9	19.1	0	0.0	2	15.4	**7***	**41.2**	
Bilateral hips	5	10.6	0	0.0	3	23.1	2	11.8	
Unilateral knee	3	6.4	0	0.0	3	23.1	0	0.0	
Bilateral knees	2	4.3	1	5.9	1	7.7	0	0.0	
Unilateral foot	1	2.1	1	5.9	0	0.0	0	0.0	
Bilateral feet	0	0.0	0	0.0	0	0.0	0	0.0	
Unilateral multiple (>2) sites	2	4.3	0	0.0	1	7.7	1	5.9	
Bilateral multiple (>2) sites	10	21.3	5	29.4	2	15.4	3	17.6	
Fracture site vs tHO site									0.246
Yes**,** correlates with tHO site	15	51.7	3	37.5	2	33.3	10	66.7	

*Indicates statistically significant difference between primary injury cohorts. n: number of subjects, Sd: standard deviation, *p*: p-value. tHO: traumatic heterotopic ossification, SCI: spinal cord injury, TBI: traumatic brain injury, USS: ultrasound, XR: x-ray, CT: computerised tomography, MRI: magnetic resonance imaging.

Univariate results

Trauma patients were most commonly diagnosed with tHO in more than 2 bilateral anatomical sites (n=10, 21.3%) than in multiple joints unilaterally (n=2, 4.3%) **(Figure 6)**. Symmetrical joint involvement most frequently appeared in the elbows after burn injury (35.3%), with the TBI cohort

demonstrating a greater frequency of unilateral elbow cases than the burns cohort (23.5% *vs.* 17.6%). Conversely, no cases of elbow involvement and only a single case involving the forearm were identified in the SCI cohort. TBI patients predominantly developed tHO unilaterally in the hip (41.2%) and similarly, tHO primarily affected the hip region in the SCI cohort with zero cases of hip involvement after burn injury.

Appendix 4B: Complete List of Demographic and Clinical Variables Recorded for tHO+ And tHO- Subjects

Table A4.2. Complete list of all demographic and clinical variables recorded for tHO+ (n=47) and tHO- (n=141) subjects.

	TOTAL (n=188)		tHO+ (n=47)		tHO- (n=141)		*p*
Variable name	**Mean /n**	**Sd /%**	**mean /n**	**Sd /%**	**mean /n**	**Sd /%**	**-**
Injury age	37.5	14.6	36	14.1	38	14.8	0.4
TBSA	41.7	19.8	48.2	23.4	39.5	18.2	0.1
ICU LOS	11.4	14.2	19.3	21.3	9	10.1	**0.001**
ICU admission	155	84.2	39	90.7	116	82.3	0.2
Total LOS	104.2	102.7	167	149.2	83.3	70.7	**0.000**
Mechanical ventilation (hours)	203.9	356.9	375.3	534.2	154.8	270.1	**0.005**
Gender							0.9
Female	29	15.4	7	14.9	22	15.6	
Male	159	84.6	40	85.1	119	84.4	
Smoke	94	50	18	38.3	76	53.9	0.07
Primary Injury							1.0
Burn	68	36.2	17	36.2	51	36.2	
SCI	52	27.7	13	27.7	39	27.7	
TBI	68	36.2	17	36.2	51	36.2	
Mechanism of injury							**0.019**
Burn (flame)	54	28.7	9	19.1	45	31.9	
Burn (explosion)	29	15.4	4	8.5	25	17.7	
Burn (contact/scald)	1	0.5	1	2.1	0	0.0	
Burn (electrical)	5	2.7	3	6.4	2	1.4	
Burn (chemical)	3	1.6	0	0.0	3	2.1	
Assault	10	5.3	1	2.1	9	6.4	
Low fall (same level or <1m)	3	1.6	1	2.1	2	1.4	
High fall (≥2m)	17	9.0	4	8.5	13	9.2	
Land transport crash: MVA	37	19.7	11	23.4	26	18.4	
Land transport crash: Pedestrian	3	1.6	2	4.3	1	0.7	
Land transport crash: MBA	13	6.9	5	10.6	8	5.7	
Land transport crash: Quad bike	3	1.6	2	4.3	1	0.7	
Land transport crash: Bicycle	2	1.1	1	2.1	1	0.7	
Sporting accidents	1	0.5	1	2.1	0	0.0	

Water related accidents	1	0.5	1	2.1	0	0.0	
Attempted hanging	1	0.5	1	2.1	0	0.0	
Horse related injury	4	2.1	0	0.0	4	2.8	
Other or unspecified injury	1	0.5	0	0.0	1	0.7	
CONCOMITANT INJURIES							
Long bone fracture	80	42.6	29	61.7	51	36.2	**0.003**
Fracture management							**0.010**
Conservative mx	33	17.6	8	17	25	17.7	
Surgical mx	36	19.1	16	34	20	14.2	
Injury to hip region and thigh	55	29.3	24	51.1	31	22	**0.000**
Type of injury to hip region and thigh							**0.000**
Fracture	31	16.5	7	14.9	24	17	
Dislocation +/- fracture	6	3.2	6	12.8	0	0	
Contusion	4	2.1	1	2.1	3	2.1	
Amputation	3	1.6	2	4.3	1	0.7	
Soft tissue injury incl. haematoma, laceration	8	4.3	6	12.8	2	1.4	
Other or unspecified	2	1.1	1	2.1	1	0.7	
Injury to CNS or PNS	40	21.3	18	38.3	22	15.6	**0.001**
CO-MORBIDITIES AND COMPLICATIONS DURING ADMISSION							
Other ossification disorders	86	45.7	26	55.3	60	42.6	0.1
UTI	88	48.9	28	62.2	60	44.4	**0.041**
Pressure injury	84	45.9	25	54.3	59	43.1	0.2
DVT	21	11.2	5	10.6	16	11.3	0.9
Sepsis	60	31.9	24	51.1	36	25.5	**0.001**
Streptococcus infection	46	24.9	14	31.8	32	22.7	0.2
Staphylococcus aureus infection	106	57.3	25	56.8	81	57.4	0.9
Other ***Staphylococcus*** infection	44	24.4	17	38.6	27	19.9	**0.012**
Other infection	158	84.9	42	93.3	116	82.3	0.07
Gram-positive bacteria							
Bacillus species	2	1.1	1	2.3	1	0.7	0.4
Bacillus cereus	19	10.6	6	13.6	13	9.6	0.4
Clostridium innocuum	1	0.6	0	0	1	0.7	0.6
Corynebacterium striatum	1	0.6	1	2.3	0	0	0.08
Enterococcus avium	1	0.6	1	2.3	0	0	0.08
Enterococcus faecalis	24	13.3	9	20.5	15	11	0.1
Enterococcus faecium	5	2.8	3	6.8	2	1.5	0.06
Enterococcus species	11	6.1	5	11.4	6	4.4	0.09
Enterococcus faecalis	4	2.2	0	0	4	2.9	0.2
Paenibacillus	3	1.7	0	0	3	2.2	0.3
Pediococcus species	1	0.6	1	2.3	0	0	0.07

	Peptostreptococcus anaerobius	1	0.6	0	0	1	0.7	0.6
	Propionibacterium acnes	6	3.3	2	4.5	4	2.9	0.6
Gram-negative bacteria								
	Aeromonas hydrophilia	1	0.6	0	0	1	0.7	0.6
	A coliform organism	6	3.3	2	4.5	4	2.9	0.6
	Acinetobacter calcoaceticus - baumanii complex	11	6.1	6	13.6	5	3.7	**0.017**
	Acinetobacter baumannii	8	4.4	5	11.4	3	2.2	**0.010**
	Acinetobacter species	5	2.8	2	4.5	3	2.2	0.4
	Bacteroides fragilis	1	0.6	0	0	1	0.7	0.6
	Bacteroides thetaiotaomicron	1	0.6	1	2.3	0	0	0.08
	Citrobacter freundii complex	1	0.6	1	2.3	0	0	0.08
	Citrobacter koseri	4	2.2	0	0	4	2.9	0.2
	Clostridium difficile	10	5.6	4	9.1	6	4.4	0.2
	Cronobacter sakazakii	2	1.1	1	2.3	1	0.7	0.4
	Escherichia coli	55	30.6	15	34.1	40	29.4	0.5
	Enterobacter cloacae complex	26	14.4	13	29.5	13	9.6	**0.001**
	Enterobacter aerogenes	13	7.2	3	6.8	10	7.4	0.9
	Enterobacter other	2	1.1	1	2.3	1	0.7	0.4
	Haemophilus influenzae	25	13.9	8	18.2	17	12.5	0.3
	Klebsiella pneumoniae	43	23.9	10	22.7	33	24.3	0.8
	Klebsiella ornithinolytica	2	1.1	0	0	2	1.5	0.6
	Klebsiella oxytoca	8	4.4	4	9.0	4	2.9	0.09
	Klebsiella variicola	5	2.8	0	0	5	3.7	0.2
	Morganella morganii	2	1.1	1	2.3	1	0.7	0.4
	Moxarella catarrhalis	3	1.7	2	4.5	1	0.7	0.09
	Proteus mirabilis	5	2.8	2	4.5	3	2.2	0.4
	Pseudomonas species	5	2.8	2	4.5	3	2.2	0.4
	Pseudomonas aeruginosa	70	38.9	23	52.3	47	34.6	**0.036**
	Pseudomonas stutzeri	2	1.1	1	2.3	1	0.7	0.4
	Pseudomonas putida	3	1.7	1	2.3	2	1.5	0.7
	Serratia marcescens	9	5	3	6.8	6	4.4	0.5
	Shewanella algae	1	0.6	0	0	1	0.7	0.6
	Stenotrophomonas maltophilia	7	3.9	2	4.5	5	3.6	0.6
Fungi								
	Aspergillus terreus	1	0.6	1	2.3	0	0	0.08
	Aspergillus niger	2	1.1	1	2.3	1	0.7	0.4
	Aspergillus fumigatus	3	1.7	1	2.3	2	1.5	0.7

Candida	31	17.2	12	27.3	19	14	**0.042**
Fusarium species	1	0.6	1	2.3	0	0	0.08
Penicillium species	1	0.6	0	0	1	0.7	0.6
Parasite							
Blastocystis hominis	2	1.1	0	0	2	1.5	0.4

n: number of subjects, Sd: standard deviation, *p*: p-value. TBSA: total burn surface area, ICU: intensive care unit, LOS: length of stay, SCI: spinal cord injury, TBI: traumatic brain injury, CNS: central nervous system, PNS: peripheral nervous system, UTI: urinary tract infection, DVT: deep vein thrombosis.

Univariate results

Patients with tHO had a significantly longer ICU LOS (mean tHO+, 19.3±21.3 vs. tHO-, 10.1±11.4, p<0.001) and required prolonged mechanical ventilation (mean hours 375.3±534.2 vs. 154.8±270.1, p=0.005). The average total LOS in the tHO+ group was 167±149.2 and 83.3±70.7 in the non-tHO group (p<0.001).

Concomitant injuries significantly associated with tHO were injuries to the CNS or PNS (38.3% *vs.* 15.6%, p=0.001), to the hip region and thigh (51.1% *vs.* 22%, *p*<0.001) and the type of injury to the hip region and thigh (*p*<0.001) with hip dislocation and/or soft tissue injury (including haematoma or laceration) being reported more often in the tHO+ group. Lower limb fracture was more frequently in the tHO- group (17%) than the tHO+ group (14.9%). The presence of a long bone fracture was most frequently observed in the tHO+ group (61.7% *vs.* 36.2, *p*=0.003), with a significantly greater proportion of tHO+ patients managed with surgical fixation than conservatively (34% *vs.* 14.2%, *p*=0.010). In the tHO+ cohort, the fracture site only correlated with the site of tHO formation in 51.7% of cases (*p*=0.247).

Complications during hospital admission that were significantly associated with tHO were urinary tract infection (p=0.041) and sepsis (p=0.001). Evaluation of local and systemic infective agents identified no significant association between tHO and *Streptococcus* (*p*=0.222) and *Staphylococcus aureus* (*S. aureus*) (p=0.941) infections. However, the presence of other staphylococcus infections (excluding *S. aureus*), were detected as significantly different between groups (tHO+, 38.6% *vs.* tHO, 19.9%, *p*=0.012). Infectious pathogens identified to be significantly associated with tHO+ were *Acinetobacter baumannii* (*p*=0.010), *Enterobacter cloacae complex* (*p*=0.001), *Pseudomonas aeruginosa* (*p*=0.036) and Candida (p=0.042)**.**

Appendix 4C: Variables from Univariate Analysis Included in Initial Model for Logistic Regression Analyses with Backward Stepwise Elimination

Table A4.3. Variables from univariate analysis included in initial model for logistic regression analyses with backward stepwise elimination.

	OR	*p*	95% CI
LOS total	1.01	0.000	1.00–1.01
LOS ICU	1.05	0.001	1.02–1.09
Mechanical ventilation (hours)	1.00	0.005	1.000-1.002
UTI	2.05	0.041	1.03-4.11
Sepsis	3.04	0.001	1.53-6.04
Injury to hip region and thigh	3.70	0.000	1.84-7.45
Long bone fracture	2.84	0.003	1.43-5.62
CNS or PNS injury	3.36	0.001	1.60-7.06

ICU: intensive care unit; LOS: length of stay; UTI: urinary tract infection; CNS: central nervous system; PNS: peripheral nervous system, OR: odds ratio, *p*: p-value, 95% CI: 95% confidence interval.

Appendix 4D: Initial Model for Logistic Regression Analysis Examining Significant Predictor variables of tHO

Table A4.4. Initial model for logistic regression analysis examining significant predictors of tHO.

	OR	***p***	**95% CI**
LOS ICU	1.01	0.867	0.90-1.12
LOS total	**1.01**	**0.014**	**1.00-1.02**
Mechanical ventilation (hours)	1.00	0.978	0.99-1.00
UTI	1.74	0.458	0.40-7.53
Sepsis	2.20	0.285	0.52-9.37
Long bone fracture	1.92	0.510	0.27-13.40
Injury to hip region and thigh	4.42	0.133	0.64-30.8
CNS or PNS injury	2.18	0.214	0.64-7.50

ICU: intensive care unit; LOS: length of stay; UTI: urinary tract infection; CNS: central nervous system; PNS: peripheral nervous system, OR: odds ratio, *p*: p-value, 95% CI: 95% confidence interval.

Appendix 5: Supplementary Material (Chapter 6)

Appendix 5A: Longitudinal Dynamics of Plasma Alkaline Phosphatase and Liver Markers for tHO+ Patients Following Burn Injury

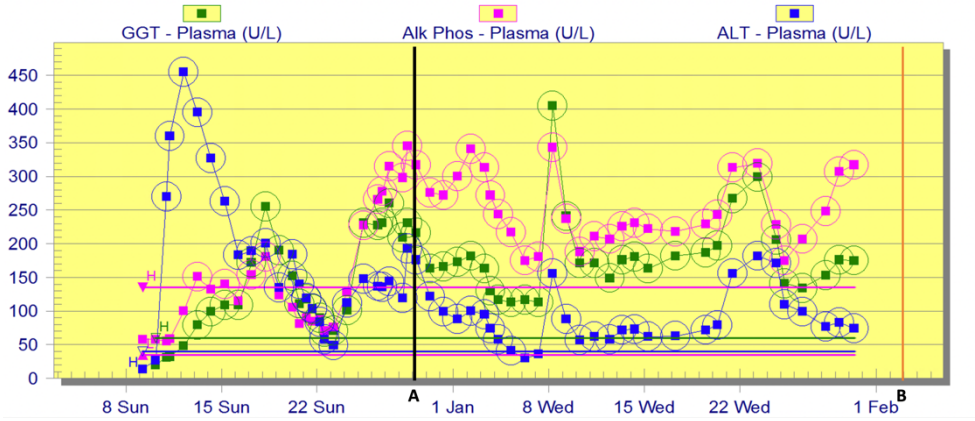


Figure A5.1A. (A) Onset of tHO specific symptoms (localised pain in bilateral elbow joints) reported 23 days post burn injury and showing correlation with first 'peak' of ALP activity. ALP remains persistently higher than ALT indicating that assessment of the ALP:ALT ratio may be useful trigger to warrant further investigation. **(B)** First radiographic evidence of tHO in bilateral elbows on plain x-ray at day 57.

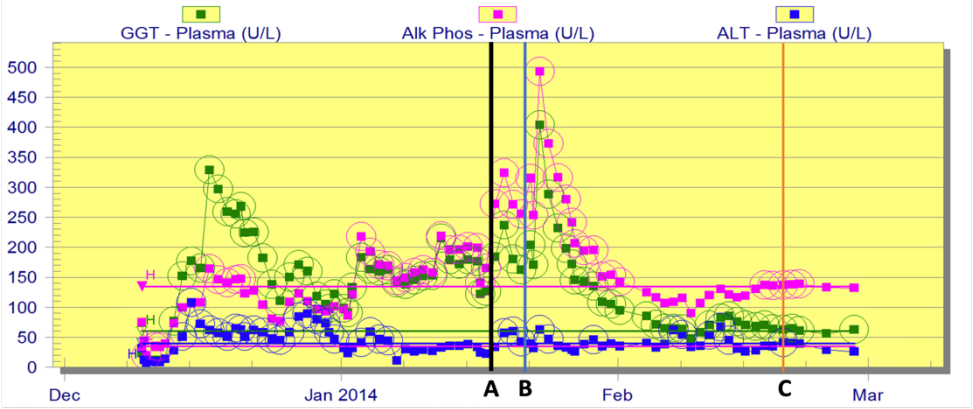


Figure A5.1B. (A) Onset of tHO specific symptoms (reduced ROM, stiffness, and pain in L elbow, followed by reduced ROM in right elbow) reported 40 days post burn injury showing correlate with first ALP 'peak'. Persistent elevation of ALP is noted, which is markedly higher than ALT, more suggestive that ALP rise is from bone origin **(B)** No radiographic evidence of tHO on plain x-ray at 42 days post injury **(C)** First radiographic evidence of tHO in bilateral elbows on plain x-ray at day 73.

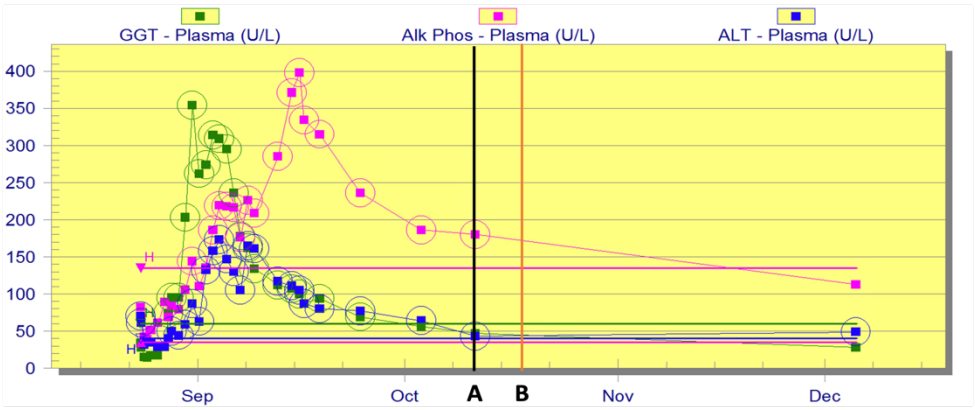


Figure A5.1C. (A) Onset of tHO specific symptoms (patient noticed 'hard lump' in right groin region) reported 50 days post burn injury **(B)** First radiographic evidence of tHO in right groin region only 6 days later. In this case, it is likely that the palpable mass represents already established tHO formation and thus, it is likely that onset of the disease process occurred well before this time point, potentially correlating with preceding spike in ALP activity, which is markedly more elevated than ALT, likely indicating active bone turnover followed by the evidence decrease in activity reflective of mature bone formation.

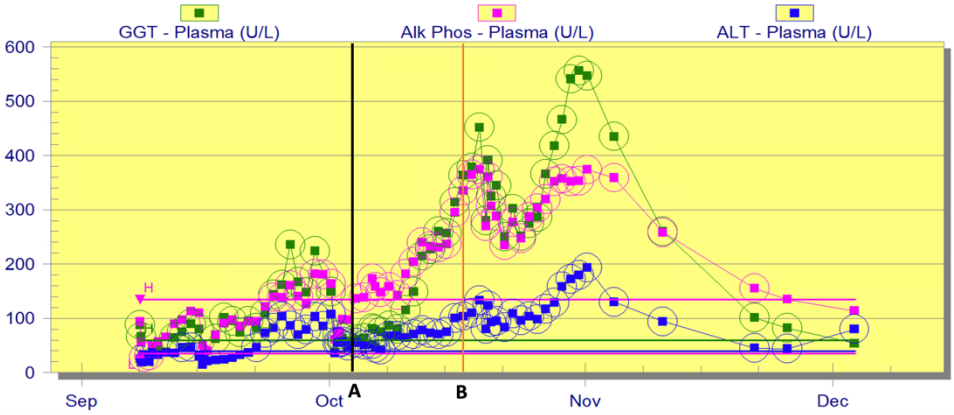


Figure A5.1D. (A) Onset of tHO specific symptoms (localised pain and reduced ROM in bilateral elbows) reported 26 days post burn injury, correlating with rising levels of ALP **(B)** First radiographic evidence of tHO in bilateral elbows on plain x-ray at 39 days. Assessment of ALP:ALT ratio shows the ALP rise is significantly more elevated with minimal change in ALT, more suggestive of bone origin.

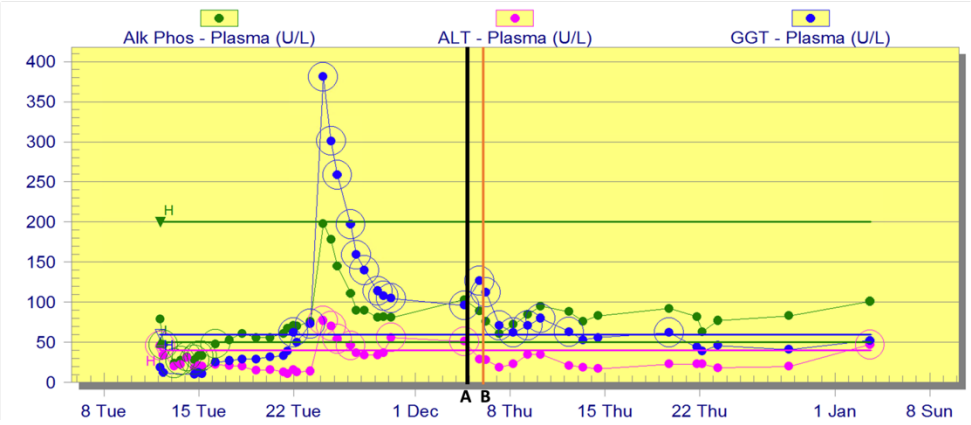


Figure A5.1E. (A) Onset of tHO specific symptoms; stiffness at 23 days with onset of localised pain at 38 days post injury. **(B)** tHO of the right elbow was evident on plain x-ray the following day (day 24), suggesting maturation of disease and again, the preceding spike in ALP with minimal change in ALT levels may indicate active bone formation.

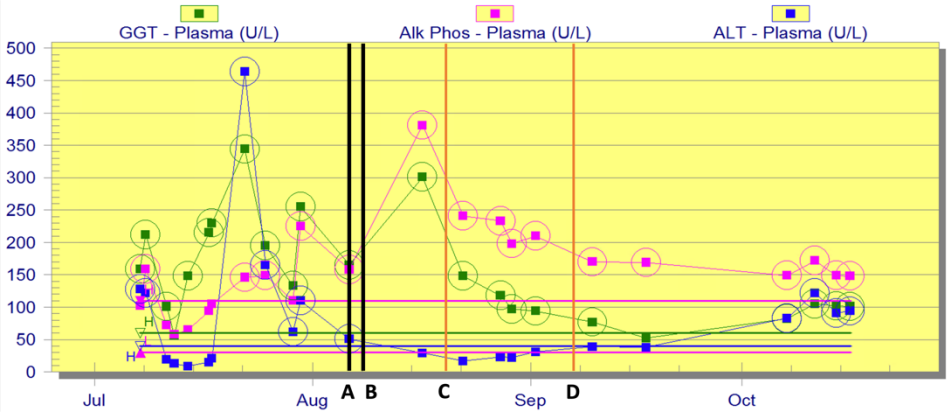


Figure A5.1F. (A) tHO symptom onset; localised pain followed by reduced ROM in L elbow at day 33 and **(B)** onset of right femoral stump pain reported 35 days post injury. **(C)** Formal diagnosis of left elbow tHO and in (**D)** bilateral femoral stumps (amputation sites) by plain x-ray on day 47 and 65, respectively. There appears to be similar temporal association of ALP and ALT as shown in **Figure S1D**, with first report of HO-specific symptoms post first rise in ALP and followed by a more significant peak in ALP activity, which remains significantly more elevated over time than ALT, suggesting a likely bone origin.

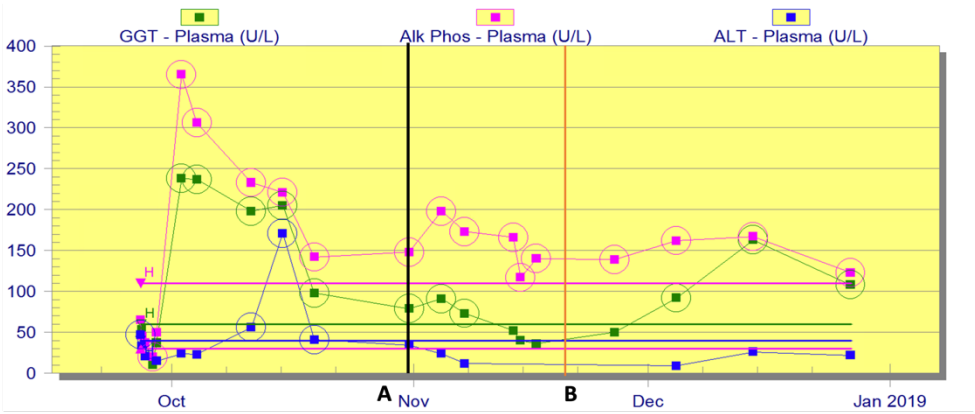


Figure A5.1G. (A) First report of tHO specific symptoms in bilateral elbows at 35 days post burn injury **(B)** 3-phase bone scan shows evidence of tHO in bilateral elbows by day 55. Plasma ALP remains persistently elevated over and levels are consistently higher than plasma ALT and GGT, more indicative of ALP levels that are of bone origin.

Appendix 5B: 136 Significantly Differentially Expressed Genes Identified from RNA-Seq Analysis with Annotated Key Functions

Table A5.1. RNAseq – 136 DEGs and their functions with annotated function.

GI accession number	Log2FC	padj	S1	Gene symbol	Gene name	Category function	Function
ENSG00000158022	-6.417	0.01		TRIM63	tripartite motif containing 63	Bone / osteogenesis Muscle	- Encodes a member of the RING zinc finger protein family found in striated muscle and iris. - This protein plays an important role in the atrophy of skeletal and cardiac muscle and is required for the degradation of myosin heavy chain proteins, myosin light chain, myosin binding protein, and for muscle-type creatine kinase - Glucocorticoid-induced gene tripartite motif-containing 63 (TRIM63) promotes differentiation of osteoblastic cells: cells over expressing exogenous TRIM63 showed increased expression of an osteoblastic differentiation marker gene, alkaline phosphatase, with reduced proliferation. These results suggest that TRIM63 is a candidate for genes mediating the glucocorticoid-induced promotion of osteoblastic differentiation [492] - Diseases associated: hypertrophic cardiomyopathy - the clinical profile of these patients (moderate to severe hypertrophy, high incidence of ventricular arrhythmias, extensive fibrosis, and frequent LV systolic dysfunction).
ENSG00000185053	-6.128	0.00		SGCZ	Sarcoglycan zeta	Bone / osteogenesis Muscle	- Protein coding gene. The sarcoglycans are part of the dystrophin-associated glycoprotein complex (DGC), an oligomeric complex spanning the plasma membrane of skeletal and cardiac muscle fibres [493] - May play a role in the maintenance of striated muscle membrane stability by bridging the inner F-actin cytoskeleton and the extra-cellular matrix, conferring structural stability to the sarcolemma and protecting muscle fibres from mechanical stress during muscle contraction [494] - Mutations in any of the sacroglycan genes cause destabilisation of the complex, resulting in different forms of limb-girdle muscular dystrophy [493] - Associated diseases: Hallucinogen Abuse and Generalized Epilepsy with Febrile Seizures
ENSG00000002745	-5.424	0.00		WNT16	Wnt family member 16	Bone / osteogenesis Muscle	- A certain level of Wnt16 is required for bone homeostasis and a positive regulator of bone mass [495] - A key regulator in periosteum bone formation – WNT16 signalling increases osteoblast differentiation and bone formation while WNT16 inhibition leads to decreased bone formation [495] [496] - Osteoblast specific overexpression of human Wnt16 increases cortical and trabecular bone mass and structure in mice [497]

							- Periosteal bone formation rate and mineral apposition was reduced in Wnt16 knockout vs wild-type mice [498] - Highly expressed in skin. Non-canonical signalling by Wnt16 is involved in the specification of hematopoietic stem cells, the senescence of MRC5 fibroblasts and the proliferation of human keratinocytes [499]
ENSG00000148488	-5.074	0.01		ST8SIA6	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6		- Protein coding gene. Encodes a member of the glycosyltransferase 29 protein family. Members of this protein family synthesize sialylglycoconjugates - Sialylation may contribute to multidrug resistance in cancer cells via PI3k/Akt pathway [495] - Diseases associated: cancer (serum ST8SIA6-AS1 diagnostic biomarker in carcinoma), myeloid leukaemia, neuroblastoma [495]
ENSG00000179776	-4.884	0.01		CDH5	cadherin 5		- Calcium-dependent call adhesion protein encoded by CDH5 gene [500] - Type 2 or VE-cadherin (vascular endothelial), CD144, is expressed specifically in endothelial cells, and mediates calcium dependent homophilic binding at adherens junctions between cells [500] - The adhesive function and expression levels of VE-cadherin at endothelial contacts play a central role in the control of vascular permeability and leukocyte recruitment into tissue [501] - Gene ontology: calcium ion binding, beta-catenin binding, BMP receptor binding. - Diseases associated: breast and prostate cancer, epithelia sarcoma, peroapical granuloma. - Pathways: ERK signalling, MSCs and lineage-specific markers. GWAS: PHF-Tau measurement
ENSG00000198203	-4.441	0.03	x	SULT1C2	sulfotransferase family 1C member 2		- A protein coding gene. Sulfotransferase enzymes catalyse the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds. These cytosolic enzymes are different in their tissue distributions and substrate specificities. - Physiological functions of gene are largely unclear - Diseases associated: colorectal tumour (via interaction with Vitamin D receptor). - Pathways: Vitamin D receptor pathway
ENSG00000169174	-4.407	0.00	x	PCSK9	proprotein convertase subtilisin/kexin type 9	Nervous system	- An enzyme encoded by the PCSK9 gene. - Plays a role in cholesterol and fatty acid metabolism, regulates neuronal apoptosis via modulation of LRP8/APOER2 levels and related anti-apoptotic signalling pathways. - Diseases associated: Clinical marker for increased incidence and recurrent CAD events, autosomal dominant familial hypercholesterolemia - Pathways: Evolocumab mechanism
ENSG00000253677	-4.382	0.04		UBE2HP	Ubiquitin conjugating enzyme E2 H pseudogene 1		- Pseudogene

ENSG00000198797	-4.376	0.01	x	BRINP2	BMP/retinoic acid inducible neural specific 2	Nervous system	- BRINP2 is a neural-specific protein, protein coding gene. - Expressed in brain, adrenal, testis. Role in inhibiting neuronal cell proliferation by negative regulation of the cell cycle transition. - Retinoids (and BMPs) are essential for chondrocyte maturation during endochondral bone formation. Retinoic acid rapidly and dramatically stimulated accumulation of BMP-2 and BMP-6 messenger RNA [502] - Diseases associated: spinocerebellar ataxia. - GO BP: cellular response to retinoid acid, positive regulation of neutron differentiation. GO CC: active in dendrite, neuronal cell body. Important paralog of gene: BRINP3. [503]
ENSG00000137675	-4.249	0.00	x	MMP27	matrix metallopeptidase 27	Skin/fibrosis ECM	- MMP27 is a protein coding gene. Involved in the breakdown of extracellular matrix in normal physiological processes, such as tissue remodelling. In the adult, MMP-27 mRNA is mostly abundant in anti-IgG/IgM stimulated B lymphocytes, bone, and kidney [504] - Associated diseases: osteoarthritis (unregulated in cartilages), metastasis (expressed by CD163/CD206 macrophages in endometriotic lesions). - Pathways: MMP, integrin pathway - GO: calcium ion binding, MMP activity. Important paralog of this gene is MMP10.
ENSG00000121769	-4.246	0.00	x	FABP3	fatty acid binding protein 3	Nervous system	- FABP3 is an intracellular protein, and its function is to arrest growth of mammary epithelial cells (modulation of cell growth and proliferation). - Expression: mainly heart, also skeletal muscle, skin, brain (variety of neurons), adrenal glands etc. Diseases associated: cancer (candidate tumour suppressor gene for breast Ca), pulmonary embolism, acute myocardial infarction - Role in pathogenesis/potential biomarker of synucleopathies: FABP3 binds to aSyn aggregates - abundant in damaged dopaminergic neurons. Elevated CSF levels associated with future dementia in PD patients [505] - Pathways: MSCs and lineage-specific markers, lipoprotein metabolism - GO: transporter activity, cytoskeletal protein binding.
ENSG00000166473	-4.201	0.00	x	PKD1L2	polycystin 1 like 2 (gene/pseudogene)		- PKD1L2 is a protein coding gene which may function as a component of cation channel pores - Also, a polymorphic pseudogene in humans - Expressed in fat, heart. Diseases associated: polycystic kidney disease - Pathways: MAPK-Erk pathway. GO: calcium ion binding
ENSG00000203446	-4.071	0.00		SUGCT-AS1	SUGCT antisense RNA 1		- RNA gene, affiliated with the lncRNA class
ENSG00000188393	-4.006	0.05		CLEC2A	C-type lectin domain family 2 member A	Nervous system	- CLEC2A is a protein coding gene and plays a role in modulating the extent of T-cell expansion, enhances the expansion of TCR-stimulated T-cells, facilitates dedicated immune recognition of keratinocytes. - Expression: skin - high expression in CD8(+), B-lymphocytes and naive CD4(+) T-cells. - Restricted mostly to proliferating lymphocytes. - Diseases associated: Skin Squamous Cell Carcinoma. GO: natural killer cell mediated cytotoxicity. GWAS: multiple sclerosis, neurofibrillary tangles measurement.
ENSG00000103316	-3.953	0.02	x	CRYM	crystallin mu		- Also known as NADP-regulated thyroid-hormone-binding protein encoded by CRYM gene. Specifically catalyses the reduction of imine bonds in brain substrates that may include cystathionine ketimine (CysK) and lanthionine ketimine (LK).

							- Binds thyroid hormone which is a strong reversible inhibitor. Presumably involved in the regulation of the free intracellular concentration of triiodothyronine and access to its nuclear receptors [506] - Expression: heat, brain. Diseases associated: deafness. Pathways: lysine degradation II (pipecolate pathway) and Peptide chain elongation. - GO: protein homodimerization activity and NADP binding.
ENSG00000186417	-3.864	0.01	x	GLDN	gliomedin	Nervous system	- GLDN is a protein coding gene that contains olfactomedin-like and collagen-like domains. - Promotes formation and maintenance of the nodes of Ranvier in the peripheral nervous system and mediates interaction between Schwann cell microvilli and axons via its interactions with NRCAM and NFASC [507] - Diseases associated: Lethal Congenital Contracture Syndrome 11 and Polyhydramnios. - GO: protein binding involved in heterotypic cell-cell adhesion. GWAS: bone mineral density
ENSG00000231123	-3.853	0.05	x	SPATA20P1	Spermatogenesis Associated 20 Pseudogene 1		- Pseudogene
ENSG00000206384	-3.795	0.01	x	COL6A6	collagen type VI alpha 6 chain	Skin/fibrosis	- COL6A6 encodes a large protein that contains multiple von Willebrand factor domains and forms a component of the basal lamina of epithelial cells. May regulate epithelial cell-fibronectin interactions. - Expression: lung, fat. - Diseases associated: Nail Disorder, Nonsyndromic Congenital, 8 and Ullrich Congenital Muscular Dystrophy 1. - Pathways: collagen formation and RET signaling. GWAS: PHF-tau measurement, thoracic aortic calcification measurement, deleterious mutation may contribute to development of thoracic OPLL [508]
ENSG00000102575	-3.725	0.00	x	ACP5	acid phosphatase 5, tartrate resistant	Bone/ osteogenesis Skin/fibrosis	- TRAcP 5b is an osteoblast enzyme and a marker of bone resorption. - ACP5 encodes tartrate-resistant acid phosphatase (TRAP) - TRAcP 5b is an osteoblast enzyme and a marker of bone resorption - Involved in osteopontin/bone sialoprotein dephosphorylation - TRAP deficiency increases levels of phosphorylated osteopontin, which causes dysregulated endochondral ossification - ACP5 deficiency protected mice from BLM-induced lung injury and fibrosis and reduced the differentiation and proliferation of fibroblasts [509] - Increased expression in certain pathological states Gaucher and Hodgkin diseases, the hairy cell, the B-cell, and the T-cell leukemias. - Diseases associated: Spondyloenchondrodysplasia (islands of chondroid tissue within bone) with Immune Dysregulation and Hairy Cell Leukemia. - Pathways: metabolism of water-soluble vitamins and cofactors and Osteoclast Signaling. - GO: hydrolase activity, ferric iron binding, ossification**.**
ENSG00000140297	-3.724	0.04		GCNT3	glucosaminyl (N-acetyl)		- Protein coding gene predicted to act upstream of or within intestinal absorption; kidney morphogenesis; and tissue morphogenesis. - Diseases associated: colorectal Cancer.

					transferase 3, mucin type		- Pathways are O-linked glycosylation of mucins and Metabolism of proteins. GO: acetylglucosaminyltransferase activity and N-acetyllactosaminide beta-1,6-N-acetylglucosaminyltransferase activity
ENSG00000132622	-3.677	0.02		HSPA12B	heat shock protein family A (Hsp70) member 12B		- Protein coding gene that functions in regulation of HSF1-mediated heat shock response and is required for protection of vascular endothelial cells, repair of myocardium, and inhibition of inflammatory response. - Protective role in vascular endothelial barrier dysfunction by preserving endothelial permeability during sepsis, facilitates lung tumour growth, expressed in tumour cells. HSPA12B gene is downstream of and potentially regulated by uc.454.
ENSG00000196666	-3.671	0.01	x	FAM180B	family with sequence similarity 180 member B		- Protein coding gene. - Expression: fat, skin. - Diseases associated: include Borderline Leprosy and Mosaic Variegated Aneuploidy Syndrome. - GO: enables protein binding, located in extracellular region.
ENSG00000152092	-3.641	0.03	x	ASTN1	astrotactin 1	Nervous system	- Neuronal adhesion molecule that is required for normal migration of young postmitotic neuroblasts along glial fibres, especially in the cerebellum.
ENSG00000115592	-3.579	0.02	x	PRKAG3	protein kinase AMP-activated non-catalytic subunit gamma 3	Muscle	- The protein encoded by this gene is a regulatory subunit of the AMP-activated protein kinase (AMPK). - Dominantly expressed in skeletal muscle and may play a key role in the regulation of energy metabolism in skeletal muscle [510] - Diseases associated: Skeletal Muscle Glycogen Content and Metabolism Quantitative Trait Locus and Wolff-Parkinson-White Syndrome. Pathways: mTOR signalling, TP53 regulates metabolic genes, AMPL signalling, angiopoetin-like protein 8 regulatory pathway, meformin, SERBP signalling. - GO: protein kinase binding and adenyl nucleotide binding
ENSG00000127954	-3.557	0.02	x	STEAP4	STEAP4 metalloreductase	Bone / osteogenesis	- A metalloreductase and expression in human tissue is highest in bone marrow - Protein coding gene plays a role in systemic metabolic homeostasis, integrating inflammatory and metabolic responses (by similarity). - Upregulated during osteoclast differentiation. - Knocking down STEAP4 expression in macrophages inhibits osteoclast formation and decreases cellular ferrous iron concentration, which in turn, reduces mitochondria ROS production and RANKL-induced CREB activation [511] - Associated with obesity and insulin-resistance, involved in inflammatory arthritis, through the regulation of inflammatory cytokines. Inhibits anchorage-independent cell proliferation. - Diseases associated: Hepatocellular Carcinoma and Arthritis. Pathways are Copper homeostasis. - GO: phosphogluconate dehydrogenase (decarboxylating) activity and ferric-chelate reductase (NADPH) activity.

ENSG00000126861	-3.548	0.00	x	OMG	oligodendrocyte myelin glycoprotein	Nervous system	- Protein Coding gene. Diseases associated: Neurofibromatosis, Type I and Primary Progressive Multiple Sclerosis. - Pathways: p75(NTR)-mediated signaling, RET signalling, spinal cord injury - GO: identical protein binding
ENSG00000161381	-3.535	0.01	x	PLXDC1	plexin domain containing 1		- Protein Coding gene. Plays a critical role in endothelial cell capillary morphogenesis. - Diseases associated: Mulchandani-Bhoj-Conlin Syndrome and Osteogenic Sarcoma.
ENSG00000188001	-3.280	0.00	x	TPRG1	tumor protein p63 regulated 1	Nervous system	- Protein Coding gene - Is a presynaptic protein that is differentially expressed across brain areas and synapse types - Expression: oesophagus, skin. Diseases associated: Mixed Liposarcoma
ENSG00000184350	-3.241	0.01	x	MRGPRE	MAS related GPR family member E		- Protein Coding gene. Orphan receptor - May regulate nociceptor function and/or development, including the sensation or modulation of pain [512] - Diseases associated: Mixed Sleep Apnoea and Isolated Growth Hormone Deficiency, Type Ib. GO: G protein-coupled receptor activity
ENSG00000271216	-3.196	0.02		LINC01050	Long intergenic non-protein coding RNA 1050		- RNA gene
ENSG00000138449	-3.188	0.01	x	SLC40A1	solute carrier family 40 member 1		- The protein encoded by this gene is a cell membrane protein that may be involved in iron export from duodenal epithelial cells. - Defects in this gene are a cause of hemochromatosis type 4 (HFE4).
ENSG00000106066	-3.170	0.02		CPVL	carboxypeptidase vitellogenic like		- Protein coding gene. May be involved in the digestion of phagocytosed particles in the lysosome, participation in an inflammatory protease cascade, and trimming of peptides for antigen presentation - Exact function of this protein has not been determined Diseases associated: Noonan Syndrome 6 and Behcet Syndrome - GO: serine-type carboxypeptidase activity
ENSG00000143387	-3.132	0.02	x	CTSK	cathepsin K	Bone / osteogenesis	- The protein encoded by this gene is a lysosomal cysteine proteinase involved in bone remodelling and resorption. - This protein, which is a member of the peptidase C1 protein family, is predominantly expressed in osteoclasts. - The encoded protein is also expressed in a significant fraction of human breast cancers, where it could contribute to tumour invasiveness. - Diseases associated: Pycnodysostosis and Osteochondrodysplasia. - Associated pathways: Innate Immune System and Activated TLR4 signalling. - GO: cysteine-type endopeptidase activity and collagen binding.
ENSG00000214402	-3.084	0.02		LCNL1	lipocalin like 1		- The encoded protein is the primary lipid binding protein in tears and is overproduced in response to multiple stimuli including infection and stress.

							- Diseases associated: Dry Eye Syndrome and Lateral Displacement of Eye. Among its related pathways are Transport of vitamins, nucleosides, and related molecules and Transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds - Gene Ontology (GO) annotations related to this gene include cysteine-type endopeptidase inhibitor activity
ENSG00000064886	-3.079	0.04	x	CHI3L2	chitinase 3 like 2	Bone / osteogenesis	- The encoded protein is secreted and is involved in cartilage biogenesis - Gene Ontology (GO) annotations related to this gene include carbohydrate binding and chitin binding
ENSG00000266995	-3.052	0.05		TERF1	Telomeric Repeat Binding Factor (NIMA-Interacting) 1		- Pseudogene
ENSG00000137869	-3.042	0.01	x	CYP19A1	cytochrome P450 family 19 subfamily A member 1		- This protein localizes to the endoplasmic reticulum and catalyses the last steps of estrogen biosynthesis. - Mutations in this gene can result in either increased or decreased aromatase activity; the associated phenotypes suggest that estrogen functions both as a sex steroid hormone and in growth or differentiation. - Diseases associated with CYP19A1 include Aromatase Deficiency and Aromatase Excess Syndrome. - Associated pathways are Integrated Breast Cancer Pathway and Follicle Stimulating Hormone (FSH) signaling pathway - GO annotations related to this gene include iron ion binding and electron transfer activity
ENSG00000228203	-3.023	0.00		RNF144A-AS1	RNF144A antisense RNA 1		- Non-protein coding gene - Upregulation of 15 Antisense Long Non-Coding RNAs in Osteosarcoma
ENSG00000003989	-2.904	0.03		SLC7A2	solute carrier family 7 member 2		- Protein coding gene that functions as permease involved in the transport of the cationic amino acids (arginine, lysine, and ornithine). Diseases associated: Lysinuric Protein Intolerance. - Associated pathways are Amino acid transport across the plasma membrane and Transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds. - GO annotations related to this gene include amino acid transmembrane transporter activity and basic amino acid transmembrane transporter activity
ENSG00000250722	-2.849	0.01		SELENOP / SEPP1	selenoprotein P	Nervous system	- This gene encodes a selenoprotein that is predominantly expressed in the liver and secreted into the plasma - It has been implicated as an extracellular antioxidant, and in the transport of selenium to extra-hepatic tissues via apolipoprotein E receptor-2 (apoER2). Mice lacking this gene exhibit neurological dysfunction, suggesting its importance in normal brain function [513] - Diseases associated with SELENOP include Keshan Disease and Colorectal Adenoma - Associated pathways are Integrin-mediated Cell Adhesion and Response to elevated platelet cytosolic Ca2+.
ENSG00000187950	-2.838	0.00		OVCH1	ovochymase 1		- Protein Coding gene

							- Unknown function - GO annotations related to this gene include serine-type endopeptidase activity
ENSG00000287315	-2.809	0.00		NA	NA		- RNA gene affiliated with the lncRNA class
ENSG00000253227	-2.755	0.02		NA	NA		- RNA gene affiliated with the lncRNA class
ENSG00000155962	-2.747	0.00	x	CLIC2	chloride intracellular channel 2	Muscle	- A member of the p64 family; the protein is detected in fetal liver and adult skeletal muscle tissue - This gene maps to the candidate region on chromosome X for incontinentia pigmenti
ENSG00000018236	-2.742	0.01	x	CNTN1	contactin 1	Nervous system	- Protein coding gene. - Mediates cell surface interactions during nervous system development and may play a role in the formation of axon connections in the developing nervous system. Involved in the formation of paranodal axo-glial junctions in myelinated peripheral nerves and in the signaling between axons and myelinating glial cells via its association with CNTNAP1 [514] - Participates in oligodendrocytes generation by acting as a ligand of NOTCH1. Its association with NOTCH1 promotes NOTCH1 activation through the released notch intracellular domain (NICD) and subsequent translocation to the nucleus. Interaction with TNR induces a repulsion of neurons and an inhibition of neurite outgrowth (By similarity). - Diseases associated with CNTN1 include Myopathy, Congenital, Compton-North and Demyelinating Polyneuropathy. Among its related pathways are NOTCH2 Activation and Transmission of Signal to the Nucleus and Developmental Biology. - GO annotations related to this gene include carbohydrate binding. Potential biomarker/therapeutic target in gastric cancer
ENSG00000115648	-2.714	0.02	x	MLPH	melanophilin		- Protein Coding gene. Rab effector protein involved in melanosome transport. Serves as link between melanosome bound RAB27A and the motor protein MYO5A. - Diseases associated with MLPH include Griscelli Syndrome, Type 3, and Griscelli Syndrome. - Associated pathways are Deregulation of Rab and Rab Effector Genes in Bladder Cancer - GO annotations related to this gene include actin binding and myosin binding
ENSG00000187955	-2.711	0.01		COL14A1	collagen type XIV alpha 1 chain	Skin / fibrosis	- Protein coding gene. Plays an adhesive role by integrating collagen bundles. - Diseases associated with COL14A1 include Palmoplantar Keratoderma, Punctate Type Ia, and Punctate Palmoplantar Keratoderma - Associated pathways are ERK Signalling and Phospholipase-C Pathway - GO annotations related to this gene include extracellular matrix structural constituent. An important paralog of this gene is COL12A1.
ENSG00000089041	-2.696	0.04	x	P2RX7	purinergic receptor P2X 7		- Protein coding gene - Receptor for ATP. P2X receptors are members of the ligand-gated ion channel family that open in response to extracellular ATP. Responsible for ATP-dependent lysis of macrophages through the formation of membrane pores permeable to large molecules. May function in both fast synaptic transmission and the ATP-mediated lysis of antigen-presenting cells - Diseases associated with P2RX7 include Tularemia and Extrapulmonary Tuberculosis - Associated pathways are CREB Pathway and Innate Immune System - GO annotations related to this gene include protein homodimerization activity

ENSG00000055732	-2.656	0.02		MCOLN3	mucolipin 3		- Protein coding gene - Nonselective ligand-gated cation channel probably playing a role in the regulation of membrane trafficking events - Acts as Ca(2+)-permeable cation channel. Mediates release of Ca(2+) from endosomes to the cytoplasm, contributes to endosomal acidification and is involved in the regulation of membrane trafficking and fusion in the endosomal pathway - Diseases associated with MCOLN3 include Mucolipidosis and Mucolipidosis Iv - Among its related pathways are TRP channels and Ion channel transport.
ENSG00000108551	-2.651	0.02	x	RASD1	ras related dexamethasone induced 1	Nervous system	- Protein coding gene. The encoded protein is an activator of G-protein signaling and acts as a direct nucleotide exchange factor for Gi-Go proteins - This protein interacts with the neuronal nitric oxide adaptor protein CAPON, and a nuclear adaptor protein FE65, which interacts with the Alzheimer's disease amyloid precursor protein - This gene may play a role in dexamethasone-induced alterations in cell morphology, growth, and cell-extracellular matrix interactions - Epigenetic inactivation of this gene is closely correlated with resistance to dexamethasone in multiple myeloma cells [515] - Diseases associated with RASD1 include Malt Worker's Lung and Smith-Magenis Syndrome - Among its related pathways are MAP Kinase Signalling and Neuroscience - GO annotations related to this gene include GTP binding and GTPase activity
ENSG00000172901	-2.642	0.05	x	LVRN	laeverin		- Protein coding gene. Over expression of LVRN impedes the invasion of trophoblasts by inhibiting epithelial-mesenchymal transition - Diseases associated with LVRN include Astigmatism - Gene Ontology (GO) annotations related to this gene include metallopeptidase activity - An important paralog of this gene is ANPEP.
ENSG00000251323	-2.576	0.03		LINC02728	long intergenic non-protein coding RNA 2728		- RNA gene
ENSG00000102445	-2.562	0.04	x	RUBCNL	rubicon like autophagy enhancer		- Protein coding gene. This gene encodes a cysteine-rich protein that contains a putative zinc-RING and/or ribbon domain - The encoded protein is related to Run domain Beclin-1-interacting and cysteine-rich domain-containing protein, which plays a role in endocytic trafficking and autophagy - In cervical cancer cell lines, this gene is expressed at low levels and may function as a tumour suppressor - Diseases associated with RUBCNL include Cervical Cancer and Cervix Uteri Carcinoma in Situ - An important paralog of this gene is RUBCN
ENSG00000103811	-2.548	0.00		CTSH	cathepsin H		- Protein Coding gene. Important for the overall degradation of proteins in lysosomes - Increased expression of this gene has been correlated with malignant progression of prostate tumours - Diseases associated with CTSH include Narcolepsy 1 and Retinitis Pigmentosa 46 - Among its related pathways are Innate Immune System and Metabolism of proteins - Gene Ontology (GO) annotations related to this gene include peptidase activity

ENSG00000262898	-2.540	0.02		NA	NA		- Novel transcript, LncRNA gene
ENSG00000164344	-2.481	0.04	x	KLKB1	kallikrein B1	Skin / fibrosis ECM	- Protein Coding gene. This gene encodes a glycoprotein that participates in the surface-dependent activation of blood coagulation, fibrinolysis, kinin generation and inflammation - Diseases associated with KLKB1 include Prekallikrein Deficiency and Malignant Essential Hypertension - Associated pathways are Formation of Fibrin Clot (Clotting Cascade) and Degradation of the extracellular matrix - Gene Ontology (GO) annotations related to this gene include serine-type endopeptidase activity and heme binding.
ENSG00000136040	-2.471	0.05		PLXNC1	plexin C1	Nervous system	- Protein Coding gene - Role in regulating axon guidance, cell motility and migration, and the immune response - The encoded protein and its ligand regulate melanocyte adhesion, and viral semaphorins may modulate the immune response by binding to this receptor - Receptor for SEMA7A, for smallpox semaphorin A39R, vaccinia virus semaphorin A39R and for herpesvirus Sema protein. Binding of semaphorins triggers cellular responses leading to the rearrangement of the cytoskeleton and to secretion of IL-6 and IL-8 [516] - Diseases associated with PLXNC1 include Polycystic Kidney Disease 4 With or Without Polycystic Liver Disease and Smallpox. - Associated pathways are Semaphorin interactions and Developmental Biology - GO annotations related to this gene include signalling receptor binding.
ENSG00000099953	-2.468	0.03	x	MMP11	matrix metallopeptidase 11	Skin / fibrosis ECM	- Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix such as tissue remodelling during various physiological processes, as well as in disease processes, such as arthritis and metastasis [517] - Diseases associated include Dermatofibrosarcoma Protuberans and Cutaneous Fibrous Histiocytoma - Among its related pathways are Colorectal Cancer Metastasis and Degradation of the extracellular matrix - GO annotations related to this gene include calcium ion binding and metallopeptidase activity.
ENSG00000159674	-2.451	0.04	x	SPON2	spondin 2	Nervous system	- Protein Coding gene - Cell adhesion protein that promotes adhesion and outgrowth of hippocampal embryonic neurons - Binds directly to bacteria and their components and functions as an opsonin for macrophage phagocytosis of bacteria - Diseases associated with SPON2 include Pharyngitis - Among its related pathways are ERK Signaling and Diseases of glycosylation - GO annotations include antigen binding and lipopolysaccharide binding
ENSG00000164776	-2.408	0.02	x	PHKG1	phosphorylase kinase catalytic subunit gamma 1	Nervous system	- Protein coding gene. Catalytic subunit of the phosphorylase b kinase (PHK), which mediates the neural and hormonal regulation of glycogen breakdown (glycogenolysis) by phosphorylating and thereby activating glycogen phosphorylase [518] - Diseases associated with PHKG1 include Glycogen Storage Disease, Type Ixd and Glycogen Storage Disease Ixb. - Associated pathways are Activation of cAMP-Dependent PKA and Glycogen Metabolism

							- GO annotations related to this gene include transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity
ENSG00000105894	-2.388	0.03		PTN	pleiotrophin	Nervous system Skin / fibrosis	- Protein coding gene. The protein encoded by this gene is a secreted heparin-binding growth factor - The protein has significant roles in cell growth and survival, cell migration, angiogenesis, and tumorigenesis in several tissues namely neuron and bone [519] - Binds ALK and promotes cell survival and cell proliferation through MAPK pathway activation - Diseases associated with PTN include Peyronie's Disease and Nasal Cavity Olfactory Neuroblastoma - Among its related pathways are Development Slit-Robo signaling and p70S6K Signaling - GO annotations related to this gene include growth factor activity and protein phosphatase inhibitor activity.
ENSG00000187098	-2.366	0.00	x	MITF	melanocyte inducing transcription factor	Skin / fibrosis	- Protein Coding gene. he encoded protein regulates melanocyte development and is responsible for pigment cell-specific transcription of the melanogenesis enzyme genes - Diseases associated with MITF include Tietz Albinism-Deafness Syndrome and Melanoma, Cutaneous Malignant 8 - Associated pathways are SUMOylation and Kit receptor signaling pathway - GO annotations related to this gene include DNA-binding transcription factor activity and RNA polymerase II proximal promoter sequence-specific DNA binding
ENSG00000232656	-2.286	0.00		IDI2-AS1	IDI2 antisense RNA 1		- RNA Gene, and is affiliated with the lncRNA class. Diseases associated with IDI2-AS1 include Dilated Cardiomyopathy.
ENSG00000197635	-2.265	0.02	x	DPP4 / CD26	dipeptidyl peptidase 4	Skin/fibrosis	- Dimeric transmembrane exopeptidase found mostly on surface of endothelial, epithelial, and immune cells - Suggested that DPP4 decreases osteoblastogenesis - Dpp4 affects osteoblastic differentiation in MSC - treatment with ana- gliptin (DPP4i) increased the expression of RUNX2, BMP-2, ALPL, OCN, BGLAP and OPN – that increased differentiation into osteoblasts and matrix mineralisation. These effects were mediated by activation of the Wnt/B-catenin pathway [520] [521] - DPP4i treatment has a positive effect on bone mass density and bone microstructure in animal models of obesity [521]
ENSG00000166278	-2.251	0.00		C2	complement C2		- Protein Coding gene - Deficiency of C2 has been reported to associated with certain autoimmune diseases and SNPs in this gene have been associated with altered susceptibility to age-related macular degeneration - Diseases associated with C2 include Macular Degeneration, Age-Related, 14 and Complement Component 2 Deficiency - Associated pathways are Immune response Lectin induced complement pathway and Creation of C4 and C2 activators - GO annotations related to this gene include serine-type endopeptidase activity
ENSG00000284959	-2.231	0.05		NA	NA		- Novel Transcript, Antisense To TSHR

ENSG00000189058	-2.225	0.01	x	APOD	apolipoprotein D		- Protein Coding gene. Occurs in the macromolecular complex with lecithin-cholesterol acyltransferase - It is involved in the transport and binding of bilin. - Diseases associated with APOD include Breast Cyst and Androgen Insensitivity Syndrome. - Associated pathways are Transport of vitamins, nucleosides, and related molecules and Transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds - GO annotations related to this gene include transporter activity and cholesterol binding.
ENSG00000154175	-2.207	0.02		ABI3BP	ABI family member 3 binding protein		- Protein Coding gene. Diseases associated with ABI3BP include Agnathia-Otocephaly Complex and Mood Disorder. - GO annotations related to this gene include heparin binding and glycosaminoglycan binding.
ENSG00000286153	-2.169	0.01		NA	NA		- Novel Transcript, Antisense to RUNX1 - RNA gene affiliated with the IncRNA class
ENSG00000019991	-2.167	0.01		HGF	hepatocyte growth factor	Skin/fibrosis	- HGF is involved in the modulation of the inflammatory response i.e. HGF induced anti-inflammatory mechanisms. Anti-inflammatory effects of HGF in adipose tissue including the reduction in the levels of chemoattractants (MCP-1 and CXCL2), inflammatory cytokines (TNF-α and iNOS) [522] [523] - Up-regulation of HGF following mechanical stretch or injury may be related to discovery that implicates this growth factor in inflammatory resolution, which may potentially be linked with inhibition of the inflammatory master regulator NF-κB/p65 [136] - Coudriet et al. [524] demonstrated that HGF could decrease the acute phase of the inflammatory response by attenuating the production of IL-6 - HGF is an important component of the fibroblast secretome, and has been shown to stimulate cancer cell invasiveness, and promote the epithelial-mesenchymal transition (EMT) and migration [525] - HGF induces Wnt signaling in colon cancer cells [526]
ENSG00000151692	-2.166	0.04		RNF144A	ring finger protein 144A	Nervous system	- Protein Coding gene. This gene encodes a member of a family of RING finger domain containing E3 ubiquitin ligases that also includes parkin and parc - The expression of this gene is induced by DNA damage Mediates the ubiquitination and degradation of the DNA damage kinase PRKDC - Diseases associated with RNF144A include Feingold Syndrome 1 and Parkinson Disease, Late-Onset - Associated pathways are Protein ubiquitination and Metabolism of proteins - GO annotations related to this gene include ligase activity.
ENSG00000214814	-2.120	0.02		FER1L6	fer-1 like family member 6	Nervous system	- Protein Coding gene. Diseases associated with FER1L6 include Cerebellar Ataxia Type 43 and Miyoshi Muscular Dystrophy - Gene Ontology (GO) annotations related to this gene include ATPase activity, coupled to transmembrane movement of substances.
ENSG00000182612	-2.115	0.05		TSPAN10	tetraspanin 10		- Protein Coding gene. Regulates maturation of the transmembrane metalloprotease ADAM10

							- Diseases associated with TSPAN10 include Exudative Vitreoretinopathy 5 and Accommodative Esotropia - GO annotations related to this gene include enzyme binding
ENSG00000157873	-2.114	0.00	x	TNFRSF14	TNF receptor superfamily member 14		- The encoded protein functions in signal transduction pathways that activate inflammatory and inhibitory T-cell immune response
ENSG00000180113	-2.077	0.04		TDRD6	tudor domain containing 6		- This gene encodes a tudor domain-containing protein and component of the chromatoid body, a type of ribonucleoprotein granule present in male germ cells
ENSG00000163590	-2.066	0.02		PPM1L	protein phosphatase, Mg2+/Mn2+ dependent 1L		- The protein encoded by this gene is a magnesium or manganese-requiring phosphatase that is involved in several signaling pathways - The encoded protein downregulates apoptosis signal-regulating kinase 1, a protein that initiates a signaling cascade that leads to apoptosis when cells are subjected to cytotoxic stresses. May be involved in adiposity since it is upregulated in adipose tissues [527] - Diseases associated include rheumatoid arthritis
ENSG00000269688	-2.046	0.01		NA	NA		- Novel Transcript, Sense Intronic To HNRNPL - RNA gene affiliated with the IncRNA class
ENSG00000197971	-2.045	0.03		MBP	myelin basic protein	Nervous system	- The protein encoded by the classic MBP gene is a major constituent of the myelin sheath of oligodendrocytes and Schwann cells in the nervous system - MBP-related transcripts are also present in the bone marrow and the immune system - Diseases associated with MBP include Demyelinating Disease and Secondary Progressive Multiple Sclerosis.
ENSG00000167191	-2.040	0.01		GPRC5B	G protein-coupled receptor class C group 5 member B		- The encoded protein may modulate insulin secretion and increased protein expression is associated with type 2 diabetes.
ENSG00000112796	-2.022	0.02	x	ENPP5	ectonucleotide pyrophosphatase /phosphodiesterase family member 5	Bone / osteogenesis	- This gene encodes a type-I transmembrane glycoprotein. - Abnormal NPP expression (mainly E-NPP1) is involved in pathological mineralization of bone and cartilage, crystal depositions in joints, invasion and metastasis of cancer cells, and type 2 diabetes in rat suggest the encoded protein may play a role in neuronal cell communications.
ENSG00000130653	-2.014	0.00		PNPLA7	patatin like phospholipase domain containing 7		- Human patatin-like phospholipases, such as PNPLA7, have been implicated in regulation of adipocyte differentiation and have been induced by metabolic stimuli

ENSG00000100979	-1.939	0.00	x	PLTP	phospholipid transfer protein		- The protein encoded by this gene is one of at least two lipid transfer proteins found in human plasma - The encoded protein transfers phospholipids from triglyceride-rich lipoproteins to high density lipoprotein
ENSG00000180353	-1.919	0.00		HCLS1	hematopoietic cell-specific Lyn substrate 1		- Substrate of the antigen receptor-coupled tyrosine kinase. Plays a role in antigen receptor signalling for both clonal expansion and deletion in lymphoid cells
ENSG00000225855	-1.913	0.03	x	RUSC1-AS1	RUSC1 antisense RNA 1	Nervous system	- Antisense RNA for RUSC1 - Putative signalling adapter which may play a role in neuronal differentiation - May be involved in regulation of NGF-dependent neurite outgrowth.
ENSG00000134324	-1.903	0.01	x	LPIN1	lipin 1		- Acts as a magnesium-dependent phosphatidate phosphatase enzyme which catalyzes the conversion of phosphatidic acid to diacylglycerol during triglyceride, phosphatidylcholine and phosphatidylethanolamine biosynthesis and therefore controls the metabolism of fatty acids at different levels
ENSG00000132196	-1.875	0.05	x	HSD17B7	hydroxysteroid 17-beta dehydrogenase 7	Bone / osteogenesis	- Diseases associated with HSD17B7 include Ck Syndrome and Congenital Hemidysplasia with Ichthyosiform Erythroderma and Limb Defects (affects bone) - Among its related pathways are super pathway of steroid hormone biosynthesis and cholesterol biosynthesis I.
ENSG00000230918	-1.874	0.03		DPP4-DT	DPP4 divergent transcript	Skin / Fibrosis	- DPP4-DT (DPP4 Divergent Transcript) is an RNA Gene and is affiliated with the lncRNA class.
ENSG00000185339	-1.871	0.03	x	TCN2	transcobalamin 2		- This gene encodes a member of the vitamin B12-binding protein family - Alternatively referred to as R binders, is expressed in various tissues and secretions - This plasma protein binds cobalamin and mediates the transport of cobalamin into cells.
ENSG00000283930	-1.851	0.01		PLD5P1	Phospholipase D Family Member 5 Pseudogene 1		- Pseudogene
ENSG00000092068	-1.817	0.03	x	SLC7A8	solute carrier family 7 member 8		- Sodium-independent, high-affinity transport of small and large neutral amino acids - Plays an essential role in the reabsorption of neutral amino acids from the epithelial cells to the bloodstream in the kidney.
ENSG00000177842	-1.813	0.03		ZNF620	zinc finger protein 620		- Among its related pathways are Herpes simplex virus 1 infection
ENSG00000112137	-1.806	0.03		PHACTR1	phosphatase and actin regulator 1	Muscle ECM	- The protein encoded by this gene is a member of the phosphatase and actin regulator family of proteins - This family member can bind actin and regulate the reorganization of the actin cytoskeleton

ENSG00000159640	-1.774	0.02		ACE	angiotensin I converting enzyme		- This gene encodes an enzyme involved in blood pressure regulation and electrolyte balance.
ENSG00000011465	-1.734	0.05		DCN	decorin	Bone / osteogenesis Skin / fibrosis ECM	- Component of ECM of connective tissues: skin, tendon, bone, and cartilage - In bone: regulation of collagen fibril diameter and fibril orientation, and possibly the prevention of premature osteoid calcification - A role in matrix mineralization by modulating collagen assembly is suggested by *in vitro* studies [528] - Bone matrix decorin has been reported to bind TGFβ and enhance its inhibitory effect on the proliferation of osteoblastic cells and on monocytes [529] • in other systems TGFβ induction of biglycan synthesis was inhibited by decorin - As TGFβ stimulates matrix protein production by chondrocytes and osteoblasts, a matrix store of DCN may be involved in an early response to stimulate cells for tissue repair.
ENSG00000177363	-1.713	0.05		LRRN4CL	LRRN4 C-terminal like		- Protein coding gene and an integral component of membrane
ENSG00000156869	-1.709	0.00	x	FRRS1	ferric chelate reductase 1	Nervous system	- Reduces ferric to ferrous iron before its transport from the endosome to the cytoplasm - Diseases associated with FRRS1 include Microcephaly 13, Primary, Autosomal Recessive and Primary Autosomal Recessive Microcephaly
ENSG00000184557	-1.708	0.05		SOCS3	suppressor of cytokine signalling 3		- This gene encodes a member of the STAT-induced STAT inhibitor (SSI), also known as suppressor of cytokine signalling (SOCS), family - SSI family members are cytokine-inducible negative regulators of cytokine signalling.
ENSG00000262902	-1.704	0.03		MT-CO1P40	MT-CO1 pseudogene 40		- Pseudogene
ENSG00000189002	-1.693	0.03		PROS2P	protein S (beta) pseudogene		- Pseudogene
ENSG00000233237	-1.587	0.02		LINC00472	long intergenic non-protein coding RNA 472		- Diseases associated with LINC00472 include Lung Cancer Susceptibility 3 and Ovarian Cancer.
ENSG00000157601	-1.585	0.04		MX1	MX dynamin like GTPase 1		- Diseases associated with MX1 include Influenza and Viral Encephalitis - Associated pathways are Interferon gamma signalling and Viral mRNA Translation.
ENSG00000121064	-1.577	0.00	x	SCPEP1	serine carboxypeptidase 1	Nervous system	- Diseases associated with SCPEP1 include Galactosialidosis (affects bone) and Epilepsy, Familial Temporal Lobe, 4. - GO annotations related to this gene include serine-type carboxypeptidase activity.

ENSG00000186952	-1.572	0.04	x	TMEM232	transmembrane protein 232		- Diseases associated with TMEM232 include Failure of Tooth Eruption, Primary (bone)
ENSG00000229739	-1.569	0.03		LOC102724919	uncharacterized LOC102724919		- Function poorly defined
ENSG00000267632	-1.531	0.02		NA	NA		- Novel transcript - RNA gene affiliated with the IncRNA class
ENSG00000225313	-1.529	0.00		NA	NA		- Novel transcript - RNA gene affiliated with the IncRNA class
ENSG00000184500	-1.512	0.00	x	PROS1	protein S		- This gene encodes a vitamin K-dependent plasma protein that functions as a cofactor for the anticoagulant protease, activated protein C (APC) to inhibit blood coagulation - GO annotations related to this gene include calcium ion binding and endopeptidase inhibitor activity
ENSG00000151414	1.533	0.03	x	NEK7	NIMA related kinase 7		- Diseases associated with NEK7 include Erysipeloid - Associated pathways are DNA Damage and Transport of the SLBP independent Mature mRNA - GO annotations related to this gene include transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity.
ENSG00000182873	1.534	0.03	x	PRKCZ-AS1	PRKCZ antisense RNA 1		- Diseases associated with PRKCZ-AS1 include Lung Cancer Susceptibility 3.
ENSG00000135269	1.766	0.03	x	TES	testin LIM domain protein	ECM	- This protein is a negative regulator of cell growth and may act as a tumor suppressor. - This scaffold protein may also play a role in cell adhesion, cell spreading and in the reorganization of the actin cytoskeleton.
ENSG00000241684	1.794	0.00		ADAMTS9-AS2	ADAMTS9 antisense RNA 2		- Diseases associated with ADAMTS9-AS2 include High Grade Glioma and Renal Cell Carcinoma, Nonpapillary.
ENSG00000157570	1.796	0.03		TSPAN18	tetraspanin 18		- Predicted to be integral component of plasma membrane - Novel regulator of thrombo-inflammation.
ENSG00000182253	1.825	0.03		SYNM	synemin	Muscle ECM Nervous system	- Diseases associated with SYNM include Alexander Disease and Myopathy, Myofibrillar, 1. - Among its related pathways are Cytoskeleton remodelling Neurofilaments.
ENSG00000178752	1.891	0.03		ERFE	erythroferrone		- Protein Coding gene - Diseases associated with ERFE include Congenital Dyserythropoietic Anemia (blood disease, also associated with skeletal abnormalities) and Beta-Thalassemia.
ENSG00000197915	1.971	0.01	x	HRNR	hornerin		- HRNR (Hornerin) is a Protein Coding gene. Diseases associated with HRNR include Epidermolysis Bullosa Simplex with Nail Dystrophy and Ichthyosis Vulgaris - Among its related pathways are Innate Immune System. - Gene Ontology (GO) annotations related to this gene include calcium ion binding.

ENSG00000154319	2.153	0.02		FAM167A	family with sequence similarity 167 member A		- Diseases associated with FAM167A include Maturity-Onset Diabetes of The Young, Type 11 and Maturity-Onset Diabetes of The Young.
ENSG00000182261	2.490	0.03	x	NLRP10	NLR family pyrin domain containing 10		- This protein likely plays a regulatory role in the innate immune system - Diseases associated with NLRP10 include Type 1 Diabetes Mellitus 24 and Irritant Dermatitis.
ENSG00000111057	2.546	0.01	x	KRT18	keratin 18	Skin / fibrosis	- KRT18 encodes the type I intermediate filament chain keratin 18. Keratin 18, together with its filament partner keratin 8, are perhaps the most commonly found members of the intermediate filament gene family - Associated conditions include cleft palate.
ENSG00000261335	2.595	0.04		LOC105274304	uncharacterized LOC105274304		- Function poorly defined.
ENSG00000101096	2.641	0.04	x	NFATC2	nuclear factor of activated T cells 2	Transcription factor Bone / osteogenesis	- Transcription factor that plays a role in osteoclastogenesis - Nfat proteins suppress the differentiation and function of cells of the osteoblastic lineage - Nfatc2 activation inhibited alkaline phosphatase activity and mineralized nodule formation in bone marrow stromal cell cultures [530] - Nfatc2 activation in osteoblasts inhibits bone formation and causes cancellous bone osteopenia [530] - NFAT signalling may co-ordinately alter Wnt4, Frizzled9, and DKK2 expression, providing a potential mechanism by which NFAT signalling regulates osteoblast proliferation [338]
ENSG00000130751	2.973	0.05	x	NPAS1	neuronal PAS domain protein 1	Nervous system	- Studies of a related mouse gene suggest that it functions in neurons - The exact function of this gene is unclear, but it may play protective or modulatory roles during late embryogenesis and postnatal development.
ENSG00000147180	3.028	0.01		ZNF711	zinc finger protein 711		- This gene encodes a zinc finger protein of unknown function. It bears similarity to a zinc finger protein which acts as a transcriptional activator - This gene lies in a region of the X chromosome which has been associated with cognitive disability.
ENSG00000172156	3.472	0.05		CCL11	C-C motif chemokine ligand 11		- In response to the presence of allergens, this protein directly promotes the accumulation of eosinophils, a prominent feature of allergic inflammatory reactions
ENSG00000143355	3.473	0.02		LHX9	LIM homeobox 9	Transcription factor Nervous system	- This gene encodes a member of the LIM homeobox gene family of developmentally expressed transcription factors - Involved in developmental processes like neuronal and gonadal development - Associated with heel bone mineral density and neuroticism

ENSG00000182985	3.486	0.03	x	CADM1	cell adhesion molecule 1	Bone / osteogenesis	- A cell adhesion molecule - Negatively regulates osteoclast differentiation and function - CADM1-deficient mice exhibited significantly reduced bone mass compared with wild-type mice, which was due to the increased osteoclast differentiation, survival, and bone-resorbing activity in Cadm1-deficient osteoclasts [531] - Associated with retroperitoneal fibrosis, a disorder in which inflammation and extensive fibrosis occurs in the back of the abdominal cavity.
ENSG00000261573	3.656	0.02		NA	NA		- Novel transcript - RNA gene affiliated with the lncRNA class
ENSG00000175315	3.901	0.01	x	CST6	cystatin E/M		- The type 2 cystatin proteins are a class of cysteine proteinase inhibitors found in a variety of human fluids and secretions, where they appear to provide protective functions.
ENSG00000250056	3.948	0.02	x	LINC01018	long intergenic non-protein coding RNA 1018		- Diseases associated with LINC01018 include Hepatocellular Carcinoma.
ENSG00000183715	4.162	0.00	x	OPCML	opioid binding protein/cell adhesion molecule like		- Among its related pathways are Metabolism of proteins and post-translational modification-synthesis of GPI-anchored proteins.
ENSG00000166897	4.305	0.03	x	ELFN2	extracellular leucine rich repeat and fibronectin type III domain containing 2		- Protein phosphatase-1 (PP1) is one of the main eukaryotic serine/threonine phosphatases. - Located in extracellular space - The protein encoded by this gene binds to the catalytic subunit of PP1, strongly inhibiting phosphatase activity - Related pathways: Beta-Adrenergic signalling and apoptotic pathways in synovial fibroblasts - Knock down of ELFN2 inhibits the growth and metastasis of gastric cancer cells
ENSG00000141052	4.318	0.01	x	MYOCD	myocardin	Transcription factor Muscle	- Master regulator, transcriptional co-activator of serum response factor (SRF) and modulates expression of cardiac and smooth muscle-specific SRF-target genes, and thus may play a crucial role in cardiogenesis and differentiation of the smooth muscle cell lineage.
ENSG00000115468	5.530	0.02	x	EFHD1	EF-hand domain family member D1	Bone / osteogenesis ECM	- A member of the EF-hand super family of calcium binding proteins (calcium ion channel), which are involved in a variety of cellular processes including mitosis, synaptic transmission, and cytoskeletal rearrangement. - Associated with Acromelic Frontonasal Dysostosis (rare bone disease)
ENSG00000124107	5.614	0.04		SLPI	secretory leukocyte	Bone / osteogenesis	- This gene encodes a secreted inhibitor which protects epithelial tissues from serine proteases. - Associated with Renal Osteodystrophy (bone disease)

					peptidase inhibitor		
ENSG00000204792	6.873	0.00	x	LINC01291	long intergenic non-protein coding RNA 1291		- Promotes the aggressive properties of melanoma
ENSG00000237515	7.298	0.00	x	SHISA9	shisa family member 9	Bone/ Osteogenesis Nervous system	- A type-I transmembrane protein that is localized post-synaptically. - Expression in brain > skeletal muscle > bone marrow - Associated with bone density (osteoporosis), bone inflammation. Regulator of short-term neuronal synaptic plasticity in the dentate gyrus. Associated with AMPA receptors (ionotropic glutamate receptors) in synaptic spines and promotes AMPA receptor desensitization at excitatory synapses (by similarity). - Associated diseases: Paget's disease (bone formation disease), T2DM - Modulators of both FGF and Wnt signaling (a Wnt inhibitory regulator) [532]
ENSG00000158022	-6.417	0.01		TRIM63	tripartite motif containing 63	Bone / osteogenesis	- Encodes a member of the RING zinc finger protein family found in striated muscle and iris. - Glucocorticoid-induced gene tripartite motif-containing 63 (TRIM63) promotes differentiation of osteoblastic cells: cells over expressing exogenous TRIM63 showed increased expression of an osteoblastic differentiation marker gene, alkaline phosphatase, with reduced proliferation. These results suggest that TRIM63 is a candidate for genes mediating the glucocorticoid-induced promotion of osteoblastic differentiation [492] - Associated diseases: hypertrophic cardiomyopathy - the clinical profile of these patients (moderate to severe hypertrophy, high incidence of ventricular arrhythmias, extensive fibrosis, and frequent LV systolic dysfunction).

GO: gene ontology, S1: site 1, 'x' indicates gene also significantly differentially expressed in tHO patients at proximal site 1 compared to control fibroblasts.

Appendix 5C: Gene Primers for Quantitative RT-PCR

Table A5.2. Gene primers for quantitative RT-PCR.

Primer	Assay ID	Product size / Amplicon length
TATA-box binding protein (TBP)	TaqMan™ Gene Expression Assay Hs00427620_m1 (Thermo Fisher Scientific, USA)	Size S (250 rxns) / 91
18S	TaqMan™ Gene Expression Assay Hs99999901_s1 (Thermo Fisher Scientific, USA)	Size S (250 rxns) / 187
Nuclear factor of activated T-cells 2 (NFATC2)	TaqMan™ Gene Expression Assay Hs00905451_m1 (Thermo Fisher Scientific, USA)	Size XS (75 rxns) / 102
Wnt family member 4 (Wnt4)	TaqMan™ Gene Expression Assay Hs01573505_m1 (Thermo Fisher Scientific, USA)	Size S (250 rxns) / 66
dipeptidyl peptidase 4 (DPP4)	TaqMan™ Gene Expression Assay Hs00897386_m1 (Thermo Fisher Scientific, USA)	Size S (250 rxns) / 69
cell adhesion molecule 1 (CADM1)	TaqMan™ Gene Expression Assay Hs00942509_m1 (Thermo Fisher Scientific, USA)	Size XS (75 rxns) / 77
Six transmembrane epithelial antigen of the prostate 4 (STEAP4)	TaqMan™ Gene Expression Assay Hs01026584_m1 (Thermo Fisher Scientific, USA)	Size XS (75 rxns) / 75
Matrix extracellular phosphoglycoprotein (MEPE)	TaqMan™ Gene Expression Assay Hs00220237_m1 (Thermo Fisher Scientific, USA)	Size XS (75 rxns) / 81

ID: identification, rxns: reactions

Appendix 5D: Summary of Human Biospecimens Collected within Australia during Research Programme

Table A5.3A. Summary of tHO+ biospecimens from burns patients collected within Western Australia over the course of this research programme.

tHO+ Subject ID	Blood	Urine	Bone	Tissue biopsy	Fibroblast culture		Sample sent for RNAseq		
					Site 1	Site 2	Sample name	Vol (µl)	Conc (ng/µl)
P1	✓	✓	x	✓	✓	x	HO+ P1 S1	20	108
P2	✓	✓	x	✓	✓	✓	HO+ P2 S1 HO+ P2 S2	20 20	199 802
P3	✓	✓	✓	✓	x	✓	HO+ P3 S2	20	232
P4	✓	✓	x	✓	✓	✓	HO+ P4 S1 HO+ P4 S2	20 20	356 214
P5	x	✓	x	✓	✓	✓	HO+ P5 S1	20	114
P6	✓	✓	x	✓	✓	✓	HO+ P6 S1 HO+ P6 S2	20 20	466 181
P7	✓	✓	x	✓	✓	✓	HO+ P7 S1 HO+ P7 S2	20 20	294 123
P8	✓	✓	x	✓	✓	✓	HO+ P8 S1 HO+ P8 S2	20 20	80.4 118
P9	✓	✓	x	✓	✓	✓	HO+ P9 S1 HO+ P9 S2	20 20	92.4 484
TOTAL	**8**	**9**	**1**	**9**	**8**	**8**	**15**	**-**	**-**

tHO: traumatic heterotopic ossification, ID: identification, RNAseq: RNA sequencing, RNA vol: volume, conc: concentration

Table A5.3B. Summary of human tHO+ biospecimens collected from burns patients within New South over the course of this programme of research

tHO+ Subject ID	**Blood**	**Urine**	**Bone**	**Tissue biopsy**
P10	Withdrew	-	-	-
P11	✓	✓	x	✓
P12	✓	✓	✓	✓
P13	✓	✓	x	✓
P14	✓	✓	x	x
P15	✓	✓	x	x
P16	✓	✓	x	x
P17	✓	✓	x	✓
TOTAL	**7**	**7**	**1**	**3**
P3: tissue biopsy taken near the tHO site				
P2 and 7: tissue biopsy taken from a convenient site				

tHO: traumatic heterotopic ossification, ID: identification

Table A5.3C. Summary of human tHO+ biospecimens collected from burns patients within Queensland over the course of this programme of research

tHO+ Subject ID	**Blood**	**Urine**	**Bone**	**Tissue biopsy**
P18	✓	✓	**x**	✓
P19*				
P20*				
TOTAL	**1**	**1**	**0**	**1**
*P19 and P20: recruited – awaiting sample collection				

tHO: traumatic heterotopic ossification, ID: identification

Table A5.3D. Summary of human tHO- biospecimens collected from burns patients within Western Australia over course of this programme of research

tHO- Subject ID	Blood	Urine	Bone	Tissue biopsy	Fibroblast culture	Sample sent for RNAseq		
						Sample name	Vol (µl)	Conc (ng/µl)
C1	✓	✓	x	✓	✓	HO- C1	20	280
C2	✓	✓	x	✓	x	-	-	-
C3	✓	✓	x	✓	✓	HO- C3	20	140
C4	✓	✓	x	✓	✓	HO- C4	20	350
C5	x	✓	x	✓	✓	HO- C5	20	300
TOTAL	**4**	**5**	**0**	**5**	**4**	**4**	**-**	**-**

tHO: traumatic heterotopic ossification, ID: identification, RNAseq: ribonucleic acid (RNA) sequencing, vol: volume, conc: concentration

Appendix 5E: Summary of Completed Experiments and Included Cell Lines

Table A5.4. Summary of experiments completed and included cell lines

CELL LINE (tHO+)	ALP assay	ARS assay	RNA extraction		Flow cytometry (gene)	qRT-PCR (gene)
			Day	Conc (ng/µl)		
tHO+ P1 S1	Exp_3	Exp_3	-	-	Exp_2 (CD26+)	
tHO+ P2 S1	Exp_2.2 Exp_7.1	Exp_2.2 Exp_7.1	0	78.7	Exp_3 (CD26+)	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	234.3		
			21	136.8		
tHO+ P2 S2	Exp_2.2	Exp_2.2	0	70.7	Exp_3 (CD26+)	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	246.1		
			21	187.4		
tHO+ P3 S2	Exp_4 Exp_6	Exp_4 Exp_6	0	71.1		Exp_1 (CD26, NFATC2)
tHO+ P4 S1	- Exp_4 Exp_6	Exp_1 Exp_4 Exp_6	0	242.0	Exp_1 (CD26+)	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	537.3		
			21	404.3		
tHO+ P4 S2	- Exp_4 Exp_6	Exp_1 Exp_4 Exp_6	0	196.9	Exp_1 (CD26+)	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	396.8		
			21	310.9		
tHO+ P5 S1	-	-	0	78.2	-	Exp_1 (CD26, NFATC2)
tHO+ P5 S2	-	-	0	43.5	-	Exp_1 (CD26, NFATC2)
tHO+ P6 S1	-	-	-	-	-	
tHO+ P6 S2	-	-	-	-	-	
tHO+ P7 S1	Exp_3 Exp_6	Exp_3 Exp_6	0	265.1	-	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	401.9		
			21	296.3		
tHO+ P7 S2	Exp_3 Exp_6	Exp_3 Exp_6	0	248.7	-	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	365.6		
			21	230.7		
tHO+ P8 S1	Exp_5	Exp_5	0	36.7	-	
tHO+ P8 S2	Exp_5	Exp_5	0	77.2	-	
tHO+ P9 S1	Exp_5 Exp_7.2	Exp_5 Exp_7.2	0	85.6	-	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	-		

			21	180.1		
tHO+ P9 S2	Exp_5 Exp_7.2	Exp_5 Exp_7.2	0	112.9	-	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	479.3		
			21	215.5		
tHO+ Osteoblasts	Exp_3 Exp_4 Exp_5 Exp_6 Exp_7.2	Exp_3 Exp_4 Exp_5 Exp_6 Exp_7.2	0	142.4	-	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	195.7		
			21	*3.1		
CELL LINE (tHO- and Normal fibroblasts)	**ALP assay**	**ARS assay**	**RNA extraction**		**Flow cytometry (gene)**	**qRT-PCR (gene)**
			Day	**Conc (ng/µl)**		
HO- C1	Exp_3 Exp_7.1	Exp_3 Exp_7.1	0	81.2	Exp_2 (CD26+)	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	497.6		
			21	277.3		
HO- C3	Exp_4 Exp_6	Exp_4 Exp_6	0	59.9	-	Exp_1 (CD26, NFATC2) Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	413.8		
			21	318.3		
HO- C4	- Exp_4	Exp_1 Exp_4	-	-	Exp_1 (CD26+)	
HO- C5	Exp_2.2 Exp_5	Exp_2.2 Exp_5	-	-	Exp_3 (CD26+)	
Normal fibroblasts	Exp_2.2	Exp_2.2	0	99.3	Exp_2 (CD26+) Exp_3 (CD26+)	
Normal fibroblasts 1	Exp_6	Exp_6	0	246.5	-	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	403.3		
			21	360.1		
Normal fibroblasts MiHa	Exp_3 Exp_4 Exp_7.1	Exp_3 Exp_4 Exp_7.1	0	111.8	-	Exp_1 (CD26, NFATC2) Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	295.5		
			21	277.9		
Normal fibroblasts HaSt	Exp_4 Exp_6	Exp_4 Exp_6	0	143.5	-	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	287.8		
			21	214.9		
Normal fibroblasts NaBe	Exp_5 Exp_7.2	Exp_5 Exp_7.2	0	80.4	-	Exp_2 (CD26, STEAP4, CADM1, WNT4, NFATC2)
			14	304.0		
			21	340.7		
Normal fibroblasts MoRa	Exp_5	Exp_5	-	-	-	-

Appendix 5F: Standard Operating Procedure for Collection, Storage, and Processing of Human Fibroblasts from 3mm Skin Punch Biopsies for RNA Extraction

Collect 3mm skin punch biopsy:

- All participants will have biopsies on enrolment using local anaesthesia
- In the study group, samples will be collected on one occasion for each participant, as paired punch biopsies from 2 sites: tissue site over/in close proximity to formation of ectopic bone and from tissue site away from site of HO formation
- A single biopsy will be collected for each participant in the control group.

Biopsies are collected using disposable 3mm punch biopsy

- Prepare "biopsy kit"
    - 1x punch biopsy
    - 1x 1.5mL plastic nunc tube
    - 5 mL syringe
    - 25 gauge needle
    - Lignocaine with Adrenaline
    - Centrimide / Chlorhexidine
    - Dressing Pack
    - Algistie & Fixomull
    - Procedure consent form
    - Complete procedure consent form and sign by patient
    - Clean area to be biopsied with Chlorhex wash
    - Using 5mL syringe, inject biopsy site with local anaesthetic
    - After approx. 5 mins, use punch biopsy to take sample
    - Dress wound with Algisite & Fixomull
    - Place biopsy into 1.5mL plastic tube
- Label with study label, ID code, collection date, and time and no addressograph
- Send the research urine sample to the lab (Burn Injury Research Unit, at the University of Western Australia, Crawley Campus/Harry Perkins Institute of Medical Research, North Campus) for processing, storage, and analysis

Process 3mm skin punch biopsy:

Culture of human fibroblasts from heterotopic ossification 3mm punch biopsies for RNA extraction

Andrew Stevenson, 2020

Fibroblasts from the skin biopsies were cultured using a standard explant method [293] detailed below.

1. Immediately place biopsy in a tube full of explant cell media - DMEM/F-12, GlutaMAX™ supplement (Life Technologies, Cat. No. 10565042) with 10% Foetal Bovine Serum, 5% Penicillin-Streptomycin (Life Technologies, 10,000 U/mL, Cat. No. 15140122), 2.5ug/ml Amphotericin B (Life Technologies, 250 ug/ml, Cat. No. 15290018), 1% Kanamycin Sulfate (Life Technologies, 15160-054). Transport tissue to the lab where and place in a petri dish
2. Slice into three equal sized potions
3. Place pieces of the biopsy dermis side down in a T-25 (25 cm^2) (Greiner Bio-One, Germany) culture flask without any media
4. Leave to incubate at 37°C for 30 minutes to allow them to stick to the base of the flask
5. After 30 minutes, tilt flask to an upright position and check the tissue pieces have adhered to the flask. If not adhered, incubate for a further 30 minutes at 37°C
6. Once the biopsies have adhered, rotate flask to an upright position and add 5ml explant media
7. Slowly rotate to a horizontal position, taking care to make sure the biopsies remained adhered to the base of the flask.
8. Incubate in normal cell culture conditions (37°C, 5% CO_2) until fibroblasts begin to migrate out of tissue and divide. Change media every 48hrs
9. Once cells reach confluence in the T-25 cell culture flask, trypsinise and place into a T-75 (75 cm^2) (1:3 split) (Greiner Bio-One, Germany), and mark as passage 1 (p1). Biopsy tissue can be discarded

Appendix 5G: Standard Operating Procedure for Urine Collection, Processing, and Storage

Urine Collection, Processing and Storage Version 1
Method based on Mark Fear recommendations 2020

Andrew Stevenson, 2020

Collect Urine

- Collect at time of enrolment
- Provide patient with yellow top urine specimen jar
- A mid-stream urine sample required
- Using a disposable pipette, transfer 1ml to each of three 1.5mL plastic screw-top tubes
- Label tubes with study label, ID code, collection date, and time and no addressograph
- Send the research urine sample to the lab (Burn Injury Research Unit, at the University of Western Australia, Crawley Campus/Harry Perkins Institute of Medical Research, North Campus) for processing, storage, and analysis

Process Urine

- Store at 4°C and ***process within 24 hours***
- Record collection and processing date and time
- Transfer to LoBind centrifuge tubes and label
- Centrifuge at 855g for 3 minutes at room temp
- Without disturbing cell and cell debris pellet, transfer supernatant evenly across at least 2 tubes at 50µl-1000µl per tube
- Label with unique code

For Biomarker use the following:

  - 53_23_U_20190408 (Perth_Patient 23_Urine_yyyymmdd)

For Biobank use the following:

  - 2_02012020_U (Patient 2_ddmmyyyy_Urine)

- Record total urine fluid volume and number of tubes
- Store samples at -80°

Reagents:

Item	Supplier	Code	Price (2019)
2ml LoBind Eppendorf tubes	Sigma	Z666513-100EA	$35.90/100
Alternatively use 1.5ml LoBind tubes	Sigma	Z666505-100EA	$35.90/100
Lab Book	Dynamic Stationary	128 page lined note	$14.50

Appendix 5H: Standard Operating Procedure for Blood Collection, Processing, and Storage

Blood collection, processing, and storage

Mark Fear, 2019

1. Purpose

This SOP describes the methodology for the collection and processing of blood samples, from participants who have consented to the study, **Exploring the mechanisms of Traumatic Heterotopic Ossification**

Longitudinal metabolite profiling

Blood (plasma) samples will be processed and stored at -80°C within 2 hours from collection in accordance with best practice recommendations for studies of the metabolome. Stored samples will permit extended studies of the biochemical mechanisms underpinning the patient phenotypes (for example, additional measures of inflammation including longitudinal measures of cytokine levels, or broader protein/metabolite analyses). All patient samples that are in excess of the requirement for this analysis will be used for method development purposes where required, or stored for a maximum of ten years, after which they will be destroyed by incineration.

2. Aim

To collect and process blood specimens to obtain Peripheral Blood Mononuclear Cells (PBMC) and plasma. These samples will then be frozen for later analysis.

3. Reagents and Consumables

- Vacutainers containing lithium heparin (e.g., Becton Dickinson green 10mL Cat #367874)
- Eppendorf Protein Lo-bind tubes Cat#30108094
- Lymphoprep or Ficoll-paque Plus – brought to room temperature
- RPMI containing 2% Heat-inactivated fetal calf serum – brought to room temperature
- 15mL and 50mL polypropylene tubes
- SepMate tubes – 15mL (Stemcell Technologies Cat#85415) and 50mL (Cat#85450)
- Sterile, filtered pipette tips
- Pipettes – P200 and P1000
- Electronic pipette aid and serological pipettes (2mL – 25mL)
- Vacuum suction and glass pipettes
- DMSO
- Cryovials
- CoolCell/MrFrosty
- Site-specific participant code book

- De-identified blood sample logbook

Standard Personal Protective Equipment (PPE) must be worn at all times when collecting and processing biological specimens, including gloves and safety glasses during collection and gloves, safety glasses and a laboratory gown when processing the samples in the laboratory. Any staff member or student who is involved in the processing of biological samples must be immunised against Hepatitis B.

4. Procedure – Plasma and PBMC
    1. Collect blood into vacutainers containing lithium heparin (the BD vacutainers we use specify "17 international units of heparin/mL of blood"). Aim to collect between 8-10mL of blood. Label tube with participant unique identifier code and collection date and time. **Store at room temperature and process within 24 hours.**
    2. Record the collection and processing date and time in your site-specific participant code book. Centrifuge blood at 1700 x g for 10min. Aliquot the plasma supernatant into 3-4 x 2mL Eppendorf Lo-bind tubes and label these with the unique patient code from your site-specific patient code book.
    Record the collection date and total plasma volume in the blood sample logbook. Store plasma aliquots in a -80 freezer.
    3. Dilute the remaining blood cells up to 4x in room temperature RPMI. It will probably be necessary to make a 2x dilution first, transfer to a 10 or 15mL tube, then dilute 2x again.
    4. Prepare 2 x SepMate 15mL tubes by pipetting the correct volume of room temperature Ficoll-paque Plus (or Lymphoprep) through the centre of the insert. See below for volumes:

Table 1. Sample and Density Gradient Medium Volumes

SEPMATE™ TUBE	INITIAL SAMPLE (mL)	DENSITY GRADIENT MEDIUM (mL)
15	0.5 - 4.0	4.5
15	> 4 - 5	3.5
50	4 - 17	15

    5. Split the diluted blood equally between the two tubes, carefully pipetting down the sides of the tubes. Some mixing may occur at the edge of the insert; this is fine.
    6. Centrifuge the sample at 1000 x g for 20min at room temperature with the **brake on**. If there are red cells above the insert repeat this step again.
    7. Pour off the top layer (containing PBMCs) in a quick, smooth motion, being sure not to hold the tube upended for more than a few seconds. Collect into 2 x 15mL tubes.
    8. Make each tube up to 15mL with RPMI + 2% FCS. Centrifuge at 300 x g for 8min, brake on.
    9. Discard the supernatants. Resuspend the pellet from one tube in 1mL of RPMI + 2% FCS and transfer to the other tube. Resuspend the cells in 15mL of RPMI + 2% FCS and centrifuge at 120 x g for 10min, **brake off.** This will deplete the platelets from the samples.
    10. Discard the supernatant and resuspend the cells in 1mL RPMI + 2% FCS. Take an aliquot for counting.

11. The method used to count the cells is not important as long as it is accurate and allows for live/dead discrimination. We will count by taking a 1:10 dilution of cells, further diluting them 1:2 in 0.4% trypan blue, then counting manually using a haemocytometer.
12. Cells will be stored at approximately 2 x 10^6 cells/vial at a concentration of 1 x 10^6 cells/mL, label enough cryovials with the participant identifier code and place them on ice.
13. Dilute the cells with RPMI + 2% FCS to a concentration of approximately 2 x 10^6 cells/mL.
14. Place the cells on ice and add an equal volume of pre-chilled RPMI + 15% DMSO; take at least 1min to add the first mL of 15% DMSO drop-by-drop, and slowly add the rest.
15. Transfer 2mL aliquots to the labelled cryovials.
16. Move cryovials into a CoolCell/MrFrosty (at room temp) and store in a -80 freezer, at least overnight.
17. Transfer cryovials to liquid nitrogen storage. Write details of the number of cryovials in the blood sample logbook.

Appendix 5I: Standard Operating Procedure for Alkaline Phosphatase Activity Assay

Alkaline Phosphatase Activity Assay Procedure

Lucy Barrett, 2021

1. 1 x BCIP/NBT tablets dissolved in 10ml ddH20 (used in 2 hours)
2. Make up wash buffer = 1.5ml of 1% tween20 added to 23.75ml PBS
3. Aspirate media
4. Wash cells carefully w 500ul/well PBS + remove PBS
5. Add 300ul/well 4% PFA + leave 15mins
6. Wash cells with 500ul/well wash buffer + remove
7. Add 300ul/well BCIP/NBT solution
8. Incubate for 15mins @ room temp in foil in drawer
9. Wash with 500ul/well wash buffer + remove
10. Add 500ul/well PBS
11. Leave in PBS, cover plates with foil and store in fridge

Appendix 5J: Standard Operating Procedure for Osteoblast Mineralisation Assay

WESTERN AUSTRALIA UNIVERSITY
Bone Biology & Disease Laboratory

School of Biomedical Sciences

SOP NO Version 1	**TITLE: Osteoblasts Mineralisation Assay**
MADE BY: Audrey Chan	**Director Approval:** Nathan Pavlos **Head of Lab. Approval:** Nathan Pavlos
VALID FROM: 23/10/2015	**REVISION DATE: 01/04/2018**

1. Purpose:

The purpose of this SOP is to provide details about the Osteoblast Mineralisation Assay Method. The operator must carry out this procedure to ensure that Good Laboratory Practices are followed throughout.

2. Scope:

This SOP applies to the Osteoblasts Mineralisation Assay in the Centre for Orthopaedic Research. School of Surgery.

3. Responsibilities

- Lab managers and/or line managers in each section are responsible for ensuring that users have been trained and are competent to carry out their work safely and in accordance with this SOP.

- All Staff are responsible for the carrying out the process in a safe working practice.

- All Staff are responsible for using the most recent working practice for this procedure.

- All staff must refer to the appropriate risk assessments.

4. Procedure:

4.1- Solutions

Osteogenic media: complete αMEM, supplemented with 50μg/mL ascorbate, 5mM β-glycerophosphate

Control: Osteogenic media

PTH: Osteogenic media + PTH (final concentration 50nM)

BMP2: Osteogenic media + BMP2 (final concentration 30ng/mL)

Making 5mg/mL L-Ascorbate（AA can be used only once after thawing out）

C6H8O6; FW 176.1 (3 bottles in chemical room) (Sigma-Aldrich, L4544, 25g)

Add 0.15g "L-Ascorbate" to 30mL autoclaved 1X PBS

Filter sterilise through 0.2μm filter

Aliquot into 0.6mL tubes in T/C hood and store at -20°C (long term storage) or 4°C (immediate use)

Add fresh to media prior to use at a final concentration of 50μg/mL (100μL / 10mL media)

Making 0.4M β-glycerophosphate

C3H7O6PNa2 ; FW 216.0 (Sigma-Aldrich, G6251, 100g)

Add 1.7g β-glycerophosphate in 20mL autoclaved 1X PBS

Filter sterilise through 0.2μM filter

Aliquot into 0.6mL tubes in T/C hood and store at -20°C (long term storage) or 4°C (immediate use)

Add fresh to media prior to use at a final concentration of 2mM (50μL / 10mL media

Making 10-3 dexamethasone (Do not use with mice Osteoblast)

Dilute in ethanol and store in -80°C

Dilute to 10-3 for storage, and 10-4 as a working dilution

Add fresh to media prior to use at a final concentration of 10-8M (1μL / 10mL media)

Making 200µM PTH

$C_{181}H_{29}N_{55}O_{51}S_2$ MW 4117.72

Solution A: 0.1M Acetic Acid 57.2µL glacial acetic acid

+ 10mL sterile MilliQ H2O

200M PTH stock

1.125mL 1X PBS (autoclaved)

+ 100µL 0.1M acetic acid

+1mg PTH (pre-weighed from commercial company)

Need to dilute to 50µM stock using Solution A

Aliquot into 20defL and store at -20°C (long term storage)

Add fresh to media prior to use (final concentration 50nM) i.e. 1µL (50µM PTH) / mL media

Able to be boosted to 100nM PTH for short time stimulations

Making 10µg/mL BMP2

(R&D Systems, 355-BM 50µg)

Solution B: 4mM HCl + 0.1% BSA (sterile) 40µL 0.1M HCl

+ 960µL sterile MilliQ H2O

+ 0.01g (0.1%) BSA

= 1mL

OR

100µL 0.4M HCl

+ 9.9mL sterile MilliQ H2O

+ 0.1g (0.1%) BSA

= 10mL

10µg/mL BMP2 in 4mM HCl + 0.1% BSA

10µg BMP2

+ 1mL 4mM HCl + 0.1% BSA (sterile)

Aliquot in 20μL and store at -20°C (long term storage) or 4°C (immediate use)

Add fresh to media prior to use (final concentration 30ng/mL) i.e. 3μL (10μg/mL BMP2) / mL media

4.2- Methods

4.2.1- Osteoblast Culture:

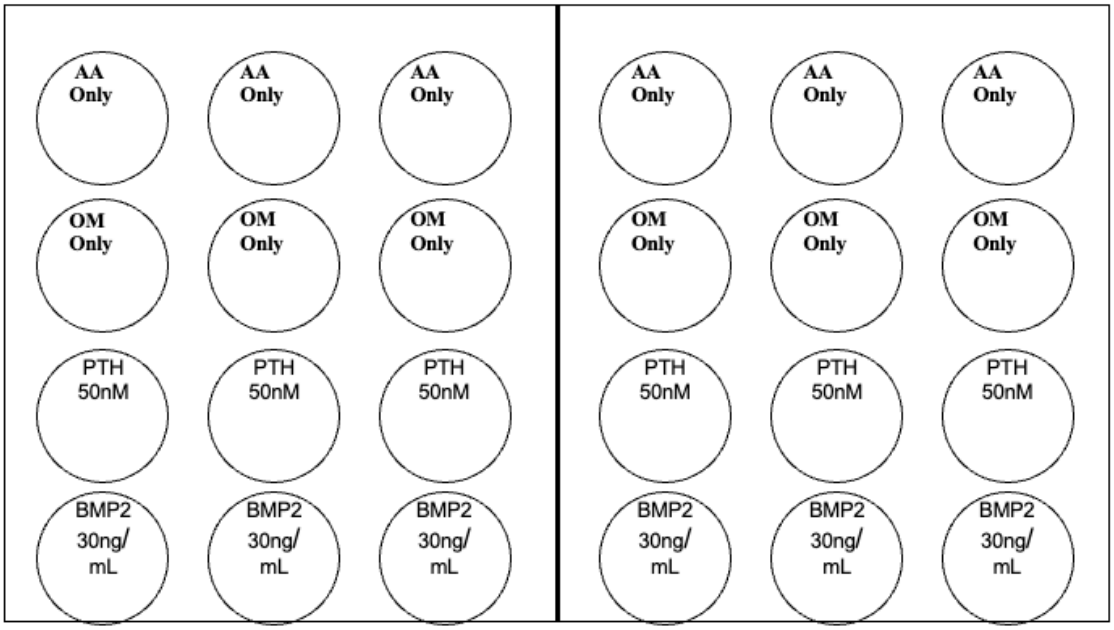
Mineralisation Assay and Quantification with Cetylpyridinium Chloride

1. Grow osteoblasts in complete αMEM supplemented with 50μg/mL AA
2. Seed osteoblasts in 24 well format at 2 - 8 x 104 cells/well.
3. Culture for 3 days before any stimulation. Each well should have between 250μL – 500μL media (optimal = 350μL). We use 500μL / well

For example:

AA = ascorbic acid

OM = osteogenic media



1. Per plate, prepare **osteogenic media**:
   10mL (or 15 mL) complete MEM (10% FBS, 1% P/S, 1% Glu)
   + 100 µL (or 150 µL) ascorbate (5mg/mL, final concentration 50µg/mL)
   + 75µL (or 112.5 µL) ⍺-glycerophosphate (0.4M, final concentration 3mM) For Human and Rat, add:
   + 1µL dexamethasone (10^{-4}M, final concentration 10^{-8})
2. Divide osteogenic media into 3.5 mL aliquots:
   a. 3.5 mL osteogenic media
      + 3.5 µL PTH (50µM, final concentration 50nM)
   b. 3.5 mL osteogenic media
      + 10.5 µL BMP2 (10µg/mL, final concentration 30ng/mL)
      Vortex well!
3. Remove and add appropriate media one condition at a time to avoid confusion
4. Stimulate wells every 2-3 days for 28 days.

Fixation using 4% PFA or 10% Formalin:

1. Gentle pipette medium from wells
2. Rinse gently twice in pre-warmed 1X PBS (autoclaved)
3. Remove last rinse gently but completely.
4. Work in fume hood. Remember, a Biosafety hood is not a fume hood.
5. Fix in 300 - 500 µL 4% PFA or 10% Formalin (pre-warmed) for 15 minutes at RT.
6. Gently remove Fixative solution.
7. Wash well twice by filling with MilliQ water or with 1X PBS if you want to store the plate at this point.

Solutions required:

Alizarin Red Solution (ARS) 40mM MW 360.28

Dissolve 0.7g of alizarin red in 50mL deionised H2O (MilliQ)

Adjust pH to 4.1-4.3 using ammonium hydroxide (10%w/v), or HCl as a second preference

Sodium Phosphate (pH 7.0)

10mM Sodium Phosphate Monobasic:

Dissolve 1.2g sodium phosphate monobasic in 1L of deionised H2O (MilliQ)

10mM Sodium Phosphate Dibasic:

Dissolve 1.42g sodium phosphate dibasic in 1L of deionised H2O (MilliQ)

Take 500mL of 10mM Sodium Phosphate Dibasic and pH to 7.0 using 10mM Sodium Phosphate Monobasic (takes about 400mL)

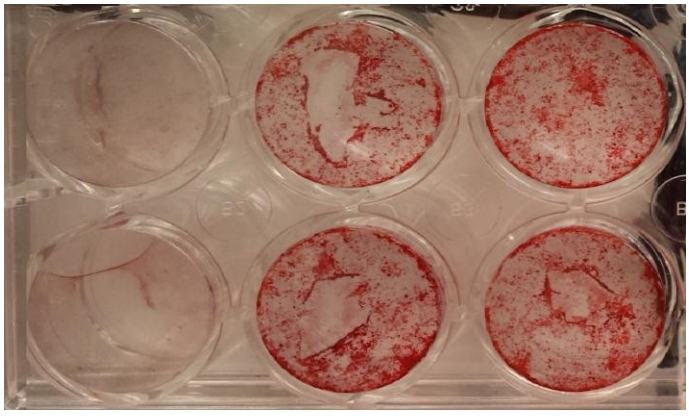
10% w/v Cetylpyridinium Chloride in 10mM Sodium Phosphate (pH 7.0)

Dissolve 50g cetylpyridinium chloride (poisons cupboard) in 100mL 10mM Sodium Phosphate (pH 7.0) (more if necessary, but no more than 450mL). Make to a final volume of 500mL once dissolved completely. This takes a while to go into solution so do not panic!

Mineralisation staining Procedure:

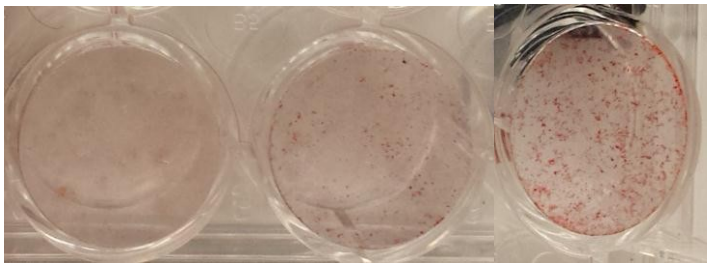
1. Plates can be originally stored in 1X PBS at 4°C after fixation
2. Remove PBS, wash well but carefully twice in MilliQ H2O
3. Add 500µL ARS/well, incubate for 20 minutes at RT (rocking platform gently)
4. Gentle pipette out the dye solution.
5. Rinse x3 in MilliQ H2O (add 1mL MilliQ H2O to each well, swirl gently and remove) if osteoblasts are too confluent and are peeling off. If they're sturdy enough, able to wash x4 for 5 minutes each on rocking platform.
6. Leave plates to air dry in a safe place
7. Scan plates for own records

OB from 8 days mouse (p2) 2.104 cells / well - 24 plate



AA OM PTH 50nM

WT



AA OM PTH 50Nm

KO

De-Staining: Quantification

1. Add 1mL 10% w/v cetylpyridinium chloride (in 10mM sodium phosphate, pH 7.0) to each 24 well.
2. Incubate at RT, rocking/shaking for 30 minutes
3. In the meantime, make standards:

Concentration (**mM**)	10mM ARS (µ**L**) (Dilute stock 40mM ¼)	10% w/v cetylpyridinium chloride in 10mM sodium phosphate, pH 7.0 (**mL**)
0	0	2
0.05	10	1.99
0.1	20	1.98
0.2	40	1.96
0.3	60	1.94
0.4	80	1.92
0.5	100	1.90
0.75	150	1.85
1	200	1.80
1.5	300	1.70
2	400	1.60
3	600	1.40

4. Scan plate for own records
5. Harvest de-stained solution into 2mL tubes
6. Add 100µL of each standard and sample (in duplicate) in 96 well plate
7. Read at 595nm
8. Able to store plates at -20°C for long term storage or on the bench for short term storage.

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I warrant that I have obliged, where necessary, permission from the copyright owners to use any third-party copyright material reproduced in this thesis. Significant effort has been made to ensure that the work of individuals throughout this programme of research has been rightfully acknowledged. I would be happy to discuss with parties who believe that their work has been omitted or incorrectly acknowledged.

Appendix 7: Ethics Approvals

Ethics approval was granted by the South Metropolitan Health Human Research Ethics Committee (RGS3452) and from The University of Notre Dame, Fremantle (2020-013F). Site Governance approval was granted by South Metropolitan Health Service (Fiona Stanley Hospital), East Metropolitan Health Service (Royal Perth Hospital) and North Metropolitan Health Service (Sir Charles Gairdner Osborne Park Health Care Group), Concord Repatriation General Hospital, New South Wales, Australia, Royal Brisbane and Women's hospital, Queensland, Australia.

THE UNIVERSITY OF NOTRE DAME AUSTRALIA

19 Mouat St (PO Box 1225) Fremantle WA 6959
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28 April 2021

A/Prof Dale Edgar & Nichola Foster
School of Physiotherapy
The University of Notre Dame Australia
Fremantle Campus

Dear Dale and Nichola,

Reference Number: 2020-013F

Project title: "Traumatic Heterotopic Ossification: Exploring Prevalence, Risk Factors, Prevention and Treatment - Phase 3."

Thank you for submitting the above project for review. It is noted that you have ethics approval and amendment approval for this project from South Metropolitan Health Service HREC, reference number RGS3452. Your application has been assessed as qualifying for a Cross-Institutional approval and is therefore exempt from HREC review. I am pleased to advise that ethical clearance has been granted for this proposed study.

Other researchers identified as working on this project are:

Name	School / Centre	Role
W/Prof Fiona Wood	Fiona Stanley Hospital	Co-Supervisor
Dr Mark Fear	Fiona Wood Foundation	Co-Supervisor
A/Prof Nathan Pavlos	University of Western Australia	Co-Supervisor
Dr Edward Raby	Fiona Stanley Hospital	Co-Supervisor
Dr Aaron Tay	Sir Charles Gairdner Hospital	Co-Supervisor
Elizabeth Capell	The Alfred Hospital	Co-Investigator
Dr Frank Li	Concord Repatriation Hospital	Co-Investigator
Dr Jason Brown	Royal Brisbane & Women's Hospital	Co-Investigator
A/Prof Leila Cuttle	Queensland University of Technology	Co-Investigator

All research projects are approved subject to standard conditions of approval.

Please read the attached document for details of these conditions.

Should you have any queries about this project, please contact me at #2964 or Natalie.Giles@nd.edu.au.

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17 December 2019

A/Prof Dale Edgar & Nichola Foster
School of Physiotherapy
The University of Notre Dame Australia
Fremantle Campus

Dear Dale and Nichola,

Reference Number: 019186F

Project title: "Traumatic Heterotopic Ossification: Exploring Prevalence, Risk Factors, Prevention and Treatment."

Thank you for submitting the above project for review. It is noted that you have ethics approval for this project from South Metropolitan Health Service HREC, reference number RGS3452.

Your application has been assessed and I am pleased to advise that ethical clearance has been granted for Phases 1 and 2 of the study. Phase 3 requires review by the full HREC as per UNDA Research Ethics Policy.

Other researchers identified as working on this project are:

Name	School	Role
Winthrop Prof Fiona Wood	Fiona Stanley Hospital	Co-Supervisor
Dr Mark Fear	Fiona Wood Foundation	Co-Supervisor
A/Prof Nathan Pavlos	University of Western Australia	Co-Supervisor
Dr Edward Raby	Fiona Stanley Hospital	Co-Supervisor
Dr Aaron Tay	Sir Charles Gairdner Hospital	Co-Supervisor

All research projects are approved subject to standard conditions of approval.

Please read the attached document for details of these conditions.

Should you have any queries about this project, please contact me at #2964 or Natalie.Giles@nd.edu.au.

Yours sincerely,

Dr Natalie Giles
Research Ethics Officer
Research Office

cc: Dr Merv Travers, SRC Chair, School of Physiotherapy

South Metropolitan Health Service Human Research Ethics Committee
Level 2, Education Building, Fiona Stanley Hospital
14 Barry Marshall Parade
MURDOCH WA 6150

22 November 2019

Miss Nichola Foster
Fiona Stanley Hospital
11 Robin Warren Drive
MURDOCH WA 6150

Dear Miss Foster

PRN: RGS0000003452
Project Title: Traumatic Heterotopic Ossification: Exploring Prevalence, Risk Factors, Prevention and Treatment.

Thank you for submitting the above research project for ethical review. This project was considered by the South Metropolitan Health Service Human Research Ethics Committee at its meeting held on 12 November 2019. To find the original letter and any possible attachments, click here when logged into RGS.

I am pleased to advise you that the above research project meets the requirements of the *National Statement on Ethical Conduct in Human Research (2007)* and ethical approval for this research project has been granted by South Metropolitan Health Service Human Research Ethics Committee.

The nominated participating sites in this project are:

- Fiona Stanley Hospital (WA)
- Osborne Park Hospital (WA)
- Royal Perth Hospital (WA)
- Sir Charles Gairdner Hospital (WA)
- Alfred Health (VIC)
- The Royal Brisbane and Women's Hospital (QLD)
- Concord Repatriation General Hospital (NSW)

[Note: If additional sites are recruited prior to the commencement of, or during the research project, the Coordinating Principal Investigator is required to notify the Human Research Ethics Committee (HREC). Notification of withdrawn sites should also be provided to the HREC in a timely fashion.]

The approved documents include:

Document	Version	Version Date
Human Research Ethics Application (HREA): Application ID: NF00042	1	28/10/2019
Western Australia Specific Module (WASM)	1	29/10/2019

DATA TRANSFER AGREEMENT

This Data Transfer Agreement ("Agreement") is entered into between

Sydney Local Health District {hereafter referred to as "the Disclosing Party"}
Level 11, KGV Building
Missenden Road
Camperdown, NSW, 2050
ABN: 17 520 269 052

And

The University of Western Australia {hereafter known as "the Recipient"}
35 Stirling Highway
Perth, WA 6009
ABN: 37 882 817 280

1. RECITALS

1.1. The Disclosing Party and Recipient are both study sites in the clinical trial research study outlined in clause 2.1.
1.2. The purpose of this Agreement is to provide the Recipient with access to Data for use in the following titled research project:

2. STUDY DETAILS

2.1. **Study Title "Study": Traumatic Heterotopic Ossification: Exploring Prevalence, Risk Factors, Prevention and Treatment**

2.2. **Description of Data transferred to Recipient "Data":** Phase 1: Patient level clinical data relevant for patient groups relating to the prevalence of traumatic heterotopic ossification (HO) will be collected. Phase 2: demographic, injury, intervention and clinical investigation data will be collected for patients with confirmed HO following neurological, orthopaedic or burn injury/trauma and for patients who were diagnosed with a neurological, orthopaedic or burn/injury trauma but did not develop heterotopic ossification will constitute the control group

2.3. **Permitted Use of Data "Purpose":** In line with the protocol for the study approved by South Metropolitan Health Service Human Research Ethics Committee ref numbers:

RGS0000003452

2020/STE00650 - Traumatic Heterotopic Ossification: Exploring Prevalence, Risk Factors, Prevention and Treatment

MATERIAL TRANSFER AGREEMENT
FOR MATERIALS PROVIDED BY QUT

THIS AGREEMENT is made

BETWEEN **QUEENSLAND UNIVERSITY OF TECHNOLOGY**, ABN: 83 791 724 622, of 2 George Street, Brisbane, QLD 4000 (**"QUT"**)

AND **THE UNIVERSITY OF WESTERN AUSTRALIA**, ABN: 37 882 817 280, of 35 Stirling Highway, Nedlands, Perth, WA 6009 (**"Recipient"**)

BACKGROUND

A. QUT is in possession of the QUT Material.

B. The Recipient has asked QUT to provide the QUT Material to the Recipient for the Purpose, and QUT has agreed to do so on the terms of this Agreement.

C. QUT may also disclose Confidential Information to the Recipient for the Purpose.

D. The QUT Material and the Confidential Information are of unique value, and may be the basis of applications for patents.

E. QUT will be prejudiced by any unauthorised use of the QUT Material and/or any unauthorised use or disclosure of the Confidential Information, including being precluded from being granted patents or suffering financial loss.

OPERATIVE PROVISIONS

1. DEFINITIONS

1.1 In this Agreement, the following words have the following meanings:

Agreement means this document entitled '*Material Transfer Agreement for Materials provided by QUT*' including any schedules.

Confidential Information means all information disclosed by QUT for the Purpose which at the time of disclosure has not been published including ,any information about the QUT Material and any inventions; discoveries; facts; data; ideas; manner, method or process of manufacture; method or principle of construction; chemical composition or formulation; techniques; products; prototypes; processes; names; know how; routines; specifications; drawings; trade secrets; technology methods; computer programs; circuit board layouts; and other knowledge, disclosed in connection with this Agreement.

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Schedule 2 – Template Project Schedule

BRISBANE DIAMANTINA HEALTH PARTNERS PROJECT SCHEDULE

This Project Schedule to the Umbrella Research Agreement dated 1 December 2020 incorporates the relevant Terms of the Umbrella Research Agreement and upon execution, constitutes a separate agreement between the Collaborators [and Third Party Collaborators] named below.

Mouse over the ⓘ symbol to view instructions for completing each section.	
Project Title	**Exploring the mechanisms of traumatic heterotopic ossification**
Project Description ⓘ	Project aims to explore the mechanism of Heterotopic Ossification (OH) following traumatic injury, a rare complication of Burns injury. There is limited information related to the cellular processes, diagnosis, prophylaxis and treatment of HO following burn injury. The study aim to explore the prevalence, risk factors, prevention and treatment of HO in a multicentre setting; Specific aims for RBWH: Do patients who develop HO following burns, neurological or orthopaedic injury/trauma share a common genetic lineage predisposing of ectopic bone formation that differs to patients who do not develop HO following trauma? The findings will hep better understand the mechanism of HO following burn injury and contribute to diagnostics and treatments.
Partners ⓘ	☐ Children's Health Queensland Hospital and Health Service ☐ The Commonwealth Scientific and Industrial Research Organisation ☐ Mater Misericordiae Limited ☒ Metro North Hospital and Health Service ☐ Metro South Hospital and Health Service ☐ QIMR Berghofer Medical Research Institute ☐ The State of Queensland acting through Queensland Health ☒ Queensland University of Technology ☐ The University of Queensland ☐ Translational Research Institute ☐ West Moreton Hospital and Health Service

Appendix 8: Published Article (Chapter 3)

Burns Open 7 (2023) 126–138

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journal homepage: www.sciencedirect.com/journal/burns-open

IBM Watson AI-enhanced search tool identifies novel candidate genes and provides insight into potential pathomechanisms of traumatic heterotopic ossification

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ABSTRACT

Background: Traumatic heterotopic ossification (tHO) is the pathological formation of ectopic bone in soft tissues that can occur following injury to the skin, nervous system, or direct musculoskeletal trauma. Relatively high rates of tHO are expected after damage to neural structures. In clinical practice, diagnosis, prevention, and treatment of tHO are highly variable, partly due to a limited understanding of the pathophysiology. Identifying critical molecular contributors to the development of tHO remains challenging, limiting the development of effective diagnostics and treatment.

IBM Watson for Drug Discovery (WDD) uses machine learning and natural language processing to interrogate a literature repository encompassing private and public data sources. This study used WDD to identify plausible new genes and pathways that may be involved in tHO.

Methods: A three-stage process centred around the disease agnostic WDD repository was applied during this study. Firstly, WDD was used to pool and target search the scientific literature involving heterotopic ossification arising from burns, orthopaedic trauma, and neurological injury populations. This training of the WDD natural language processing algorithms using known entities was used to discover novel intercepts in the network of semantic relationships evident in the published literature to 2019. Indications of plausible relationships were sought by triangulating biological concepts such as genes and diseases. In this step, using the WDD predictive analytics engine, the study identified and ranked 233 candidate genes that may be associated with pathological ectopic ossification, utilising a set of 100 genes with previously defined associations with tHO. Finally, a search of the WDD-linked literature related to the top 25 genes identified from the rank product analysis was conducted to validate WDD's predictions of potential novel candidate genes.

Results: Of the top 25 ranked genes, six genes (MMRN1, MSC/MyoR, ITGAM/CD11b, PDGF-D, GREM-1 and NELL-1) were identified to have evidence of likely association with tHO. These candidate genes had previously defined roles in inflammation, aberrant tissue repair and regeneration, extracellular matrix remodelling and mineralisation, endochondral or intramembranous bone formation and injury-associated bone reactions, as well as functions in WNT and BMP signalling that are known to be important in osteogenic differentiation.

Conclusions: Using a machine-learning approach, this study identified a novel set of plausible candidate gene targets associated with tHO. Machine-learning methods may effectively support target discovery and understanding of pathophysiology in complex disease states.

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1. Introduction

Traumatic heterotopic ossification (tHO) is a pathological process characterised by the production of mature, lamellar bone at non-skeletal sites, such as within muscle and connective tissue [1]. Post-traumatic HO [2], traumatic HO [3] or acquired HO [4] are used interchangeably in today's literature. Well known to burn clinicians, tHO is a debilitating sequela of local and systemic inflammatory insult [5]. Other accounts of tHO have been reported following high-velocity blast injury [6,7]; traumatic brain injury and spinal cord injury [8-10]; and after fracture, dislocation and soft tissue trauma [11,12]. The rate of disability is variable, with some tHO lesions considered clinically irrelevant. In contrast, others may incur significant patient morbidity, and patient quality of life can be compromised by movement dysfunction and severe pain [13,14].

Over recent years, a body of research has emerged to elucidate the pathophysiological processes underlying tHO. The contemporary understanding of tHO pathogenesis describes a process induced by central or peripheral nervous system (CNS, PNS) injury [15-19]. It is thought that tHO arises due to aberrant tissue repair in the presence of persistent neuroinflammatory dysregulation, leading to ectopic bone formation at the peripheral injury site via endochondral or intramembranous ossification [15-19]. Multipotent cellular contributors of tHO have been identified from a host of tissues; specifically, the multipotency of local mesenchymal stem cell (MSC) populations - identified as chondro-osseous progenitors that appear to arise from different local soft tissues and, or peripheral nerves [6,20-22]. However, despite recent efforts to elucidate the pathological processes underlying tHO, the precise mechanisms by which injury initiates tHO formation are poorly understood [23]. No genetic mutation has been found to be causally linked to the development of tHO in trauma cases [3].

New technologies such as cognitive computing offer a means by which the assimilation of novel answers to challenging research questions can be discerned [24]. With the increasing volume and diversity of biomedical literature sources, manual assimilation of the body of knowledge is becoming increasingly challenging for human practitioners [25]. This creates a gap in the ability to design future studies based on the sum of knowledge generated to date and may increasingly hamper future research, particularly in complex and rare conditions such as tHO. Cognitive computing provides a new method to rapidly retrieve pertinent information from a wide corpus of the biomedical literature, making it easier to uncover and extract new insights from existing data [26,27]. Integrating diverse literature datasets, including clinical trials, patents, and other sources, into the searchable repository can further enhance the utility of cognitive computing approaches to assimilate research reports and assist in the development of novel hypotheses or identification of potential therapeutic targets [24,26].

The capability of the IBM Watson for Drug Discovery (WDD) platform to facilitate the generation of new hypotheses about the relevance, function, or linkage between genes, drugs, and diseases of interest has been validated through recent scientific discoveries [28-30]. By combining natural language processing (NLP) with expert-curated data sources found in the literature, the cognitive-based computing platform, embodied by WDD may offer an effective solution to accelerate basic science discovery in tHO [24].

In this study, we utilised WDD to interrogate the literature and identify candidate genes and pathways that may play a role in tHO. To the best of our knowledge, this is the first time this strategy has been applied to develop new hypotheses to investigate the pathological processes underlying the genesis and propagation of traumatic HO.

2. Methods

2.1. General methodology and approach

IBM Watson for Drug Discovery (WDD) is a discovery platform that uses cognitive computing and NLP to read large bodies of text and then apply predictive analytics to text to help researchers identify and rank promising gene and protein candidates for further evaluation. WDD's functionality has been described in detail previously [28,30,31]. The WDD platform enables two main types of capabilities. First, large volumes of information from unstructured natural language text can be read in a manner with a nuanced understanding of the syntax and meaning of complex biological relationships between genes, diseases, and drugs. WDD can produce interactive visualisations of these relationships (biological relationship network extraction). Semantic relations are discovered using rule-based and machine-learning-based approaches to understand mentions of two distinct entities according to their context, which co-occur in the same sentence of a document [24]. WDD is also disease agnostic in that it will identify common gene and drug relationships across conditions, thereby enabling researchers to further understand a genes role through publications in completely different therapeutic areas. By incorporating data from both unstructured and external structured sources, including data presented in figures and tables, WDD is able to provide a broader domain understanding [24].

The second primary function is that WDD enables the evaluation of a potential list of candidate genes, diseases, or drugs through a sophisticated predictive ranking model (predictive analytics). This function leverages features of text to suggest, for example, similarities between genes whose connection may never have been explicitly identified in the literature to have a role in, or link to, a given disease. Or it may have the same potential role that a group of genes already known to have that linkage based on individual features. The WDD Predictive Analytics engine requires two key inputs: a list of known entities (genes, drugs, diseases, or chemicals) and a list of potential candidate entities to map a concept space based on linguistic similarity [24]. Instead of using known biology, the predictive analytics algorithms look for patterns in how entities of interest are described in reports [24].

The general application of WDD to this specific study was

1. To use WDD's biological relationship network extraction analysis function to search the literature and identify common themes and biological relationships of relevance to tHO with different aetiology.
2. To use the predictive analytics function of WDD to identify new candidate genes that may be implicated in tHO.

2.2. Biological relationship network extraction

The directed relationships-based biological network analysis function of WDD was utilised first to extract the known semantic relationships between biological concepts from the scientific literature. Here, "Heterotopic ossification" was used as the initial search entity (condition) to identify known biological interaction types and establish explicit, directional relations between entity types, i.e., heterotopic ossification to genes and conditions/diseases. The strength of the relationship between two entities is depicted by value, representing the total number of connections to HO identified by WDD in the literature (Table A.2).

Flexible, simple network visualisations of the genes and conditions based on the strength of association to heterotopic ossification were produced, with the ability to select the relationship of interest between HO (target type; condition) and a gene (source type). For example, a gene (source) with a predisposition or a regulation relationship with HO (target). The interactive relationship network allowed the user to select individual links in the network and drill down into the linked literature to understand how and confirm the plausibility of the relationship that connected the two entities was discovered. To validate WDDs predictions of genes that may be associated with tHO, a thorough search and review of the relevant linked literature supporting each association were conducted [31]. In addition to gene relationships, utilising the disease-agnostic capabilities of WDD, literature synthesis and review of

Table 1
Combined Rank top 25 gene list.

Gene	Name	Rank	Score (GD)	Evidence of association with tHO prior to 2019
MMRN1/ EMILIN-4	Multimerin 1/Elastin microfibril interfacer 4	1	0.081	No
IL-1α	Interleukin 1 Alpha	2	0.064	Yes
IL-15	Interleukin 15	3	0.049	Yes
MSC/ MyoR	Musculin/Myogenic Repressor	4	0.049	No
ITGAM/ CD11b	Integrin Subunit Alpha M/ Cluster of Differentiation molecule 11B	5	0.048	No
PDGF-D	Platelet Derived Growth Factor D	6	0.046	No
SOST	Sclerostin	7	0.045	Yes
GREM1	Gremlin 1	8	0.044	No
CD14	Cluster of differentiation 14	9	0.043	Yes
CD8A	CD8a molecule	10	0.042	Yes
SPARC/ON	Secreted protein acidic and rich in cysteine/ osteonectin	11	0.042	Yes
MMP2	Matrix metalloproteinase-2	12	0.041	Yes
CDKN1A/ p21	Cyclin-dependent kinase inhibitor 1A	13	0.041	Yes
EPO	Erythropoietin	14	0.041	No
NOS2/ iNOS	Nitric Oxide Synthase 2/ Inducible nitric oxide synthase	15	0.04	Yes
IL1RN	Interleukin 1 Receptor Antagonist	16	0.04	Yes
SMAD7	SMAD family member 7	17	0.04	Yes
DKK1	Dickkopf WNT signalling pathway inhibitor 1	18	0.039	Yes
ANG1	Angiopoietin 1	19	0.039	Yes
VWF	Von Willebrand factor	20	0.039	Yes
TIMP1	Tissue inhibitor of metalloproteinases 1	21	0.038	Yes
KITLG/SCF	KIT ligand/Stem cell factor	22	0.038	Yes
IL2RA/ CD25	Interleukin 2 receptor subunit alpha	23	0.038	Yes
NFATC1	Nuclear Factor of Activated T cells 1	24	0.037	Yes
NELL1	NEL-like molecule-1	25	0.037	No

IBM Watson predictive analytics process and literature summary of top 25 ranked genes.

Table 2
Results of the custom validation test.

Validation set	Rank
GDF2	3
PTHLH	6
ACVR1	7
BMP2R	10
TGFB3	11

Fishers Exact Test P-value: 0.000151834.
Wilcoxon Rank Sum P-value: 0.000116413.
Validation rating is **HIGH.**

associated conditions of interest were also carried out (Table A.3).

2.3. Predictive analytics process

Having established that IBM Watson methodology is valid and capable of identifying genes and proteins likely to be involved in tHO, the predictive analytics application was then used to predict new interactions between genes and heterotopic ossification.

2.4. Candidate and known gene sets for predictive analytics

Two sets of entities were uploaded for predictive analytics analysis, one with known similarities or properties (the Known set) and the other with unknown similarities or properties (the Candidate set). A set of 100 known genes was created based on the genes' previously defined associative roles in pathological ectopic bone formation. This known gene set (Table A.5) was generated through WDD's biological relationship network analysis function as previously described and was used to interrogate the candidate gene list. A candidate gene set containing 233 genes with a potential role in ectopic bone formation, obtained from a previous study, was included (Table A.4) [32].

All entered entities were then queried over known medical literature available to the end of 2019, including 29 million MEDLINE abstracts, all the Open-Source PMC full-text journal articles and over a million licensed medical journal articles, patents, and public government reports to find the most relevant articles containing mentions of these entities in the literature. The WDD platform then calculated a similarity matrix containing a similarity index for each pair of entities, presented as a distance network (Fig. A.2) or similarity tree, providing a visual representation of the similarity between the candidate set and known genes entered into the search. Using a graph diffusion algorithm, Watson computed a predictive similarity score based on the similarity matrix, measuring each entity's similarity to all the known entities [32]. The predictive analytics output delineated a ranked list of 233 candidate entities based on their computed similarity score. The top 50 ranked genes from this list are shown in Table A.6. A final list of the top 25 ranked genes (Table 1) most likely to be involved in HO was subsequently produced from the rank product analysis. An in-depth review of the relevant literature was conducted to identify any previous association with tHO before 2020.

2.5. Statistical validation

To validate the predictive power of the model generation by WDD, a custom validation method was performed. The top five ranked entities (BMPR2, ACVR1, PTHLH, GDF2, TGFB3) in our known set comprised the validation set, which was subsequently placed in the candidate set. This set was used to verify how similar WDD ranked these known entities compared to others in the candidate set. Watson then supplied a rating to the overall candidate validation based on the yielded p-values (1-(low)-0.05(medium)-0.01-(High)-0). This rating is based on two statistical significance tests: the Fisher's Exact Test and the Wilcoxon Rank Sum, as shown in Table 2.

2.6. An augmented literature search using WDD

An in-depth review of the available literature up to the end of 2019 was carried out to assess (i) the ranked list of 100 genes and associated conditions with HO identified using the biological network analysis function, and (ii) the top 25 ranked genes produced from the rank product analysis. Firstly, to validate potentially novel entities (genes) identified by WDD, a search was conducted which included the (gene name) including gene symbol and other known aliases, with the following search terms, used in different combinations; "ossification", "calcification", "ectopic ossification" and "ectopic bone" and "osteogenesis". To identify texts that focused on the pathophysiological mechanisms, additional searches were conducted using the following terms: (gene name) AND "pathophysiology", "physiology", "pathogenesis", and "molecular mechanisms". To capture the depth and breadth of data, searches were not restricted by publication dates.

Articles were eligible for inclusion if they were published in English and full text, peer-reviewed primary research reports investigating each gene function(s) and, or pathophysiological role(s) in various disease

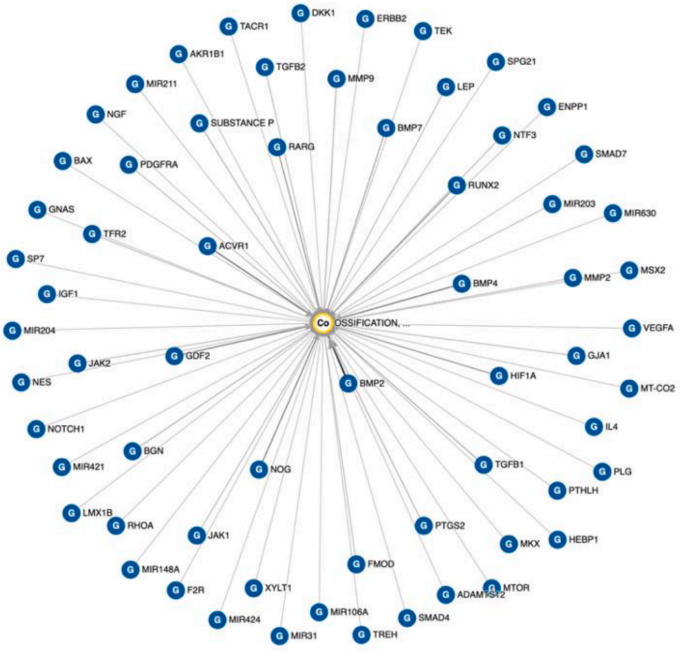


Fig. 1. Biological entity network visualisation of potential gene relationships to heterotopic ossification. The searched entity (heterotopic ossification, condition [Co]) is represented by a white circle and connected entities (genes [G]) by blue circles. Distance from the searched entity (heterotopic ossification) represents the number of documents (value) supporting the connection: nearer circles are connected by relationships in more documents than farther circles [24]. The depicted values for each associated gene are shown in (Table A.2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

states. In the absence of higher-level evidence, case series/reports allow the identification of rare clinical conditions and can provide a thorough review of important topics [33]. Thus, animal studies, case studies and case series have been included. As the pathophysiology of HO in burns children was likely to differ from that in adults, paediatric studies were excluded. Conference proceedings and editorials were also excluded. The references of selected studies were pursued for articles that may have been missed via the electronic search, and full-text articles were retrieved where possible. Findings from each study were then analysed and developed into key domains to enable the recognition of recurring relationships across data.

3. Results

3.1. Biological entity network visualisations

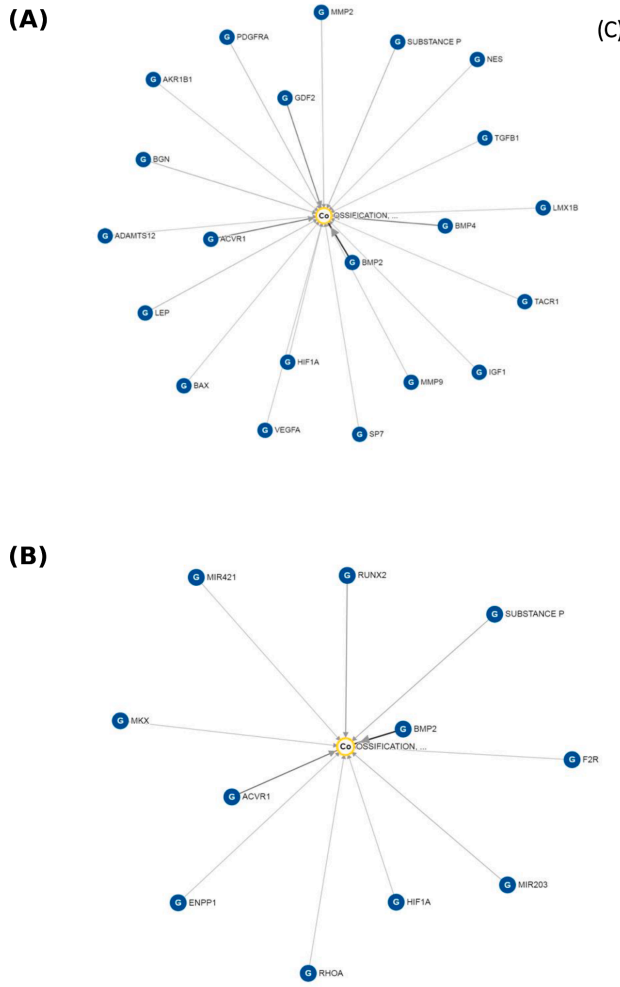
WDD automatically extracted relationship networks and illustrated the extracted genes with specific relationships between them. This was achieved by representing the sentence-level gene-condition connections identified earlier as a matrix. One hundred genes associated with heterotopic ossification were mentioned in at least one document published before the end of 2019. They comprised the relationship network illustrated in Fig. 1 [30]. Table A.2 shows Watson's ranking of 100 genes based on the strength of association to heterotopic ossification and the depicted value for each associated gene. Bone morphogenic protein 2 (BMP2) and Bone morphogenic protein 4 (BMP4) were identified as the top candidates by WDD, ranking 1 and 2 respectively and thus, representing the closest connected gene entities to HO as the search entity. Comparatively, Neural cell adhesion molecule 1 (NCAM1) and Collagen type II alpha 1 (COL2A1), with ranks 66 and 65, respectively, are presented in farther circles, representing a less significant association with HO.

The semantic relationships between HO and genes identified by WDD and extracted as interaction networks of biological entities are illustrated in Fig. A.1. Evaluation of each connection in detail reveals sentence-level extractions, which in aggregate create a list of directional relations between two known entities. This relationship can be direct or indirect and is linked through a domain-relevant verb occurring in the same sentence, termed a trigger word. Table A.1 provides examples of biologically relevant trigger words curated and used by WDD for semantic relationship extraction between genes (source) and heterotopic ossification (target). For instance, WDD extracted a negative regulation relationship between Runt-related transcription factor 2 (RUNX2) and HO that contained a direct relation via the trigger word 'inhibit'. The curated evidence supporting the semantic relationships identified by Watson guided an in-depth literature review to validate WDD's predictions of genes associated with HO.

The network of conditions that Watson has produced in real-time by using its annotators to extract relationships between HO and other associated conditions is shown in Table A.3. Conditions ranked highly by WDD for an association to HO included age-related or degenerative diseases such as arthritis and osteoporosis, as well as fibrosis and multiple bone related cancers. Notably, conditions with an increased prevalence of HO previously reported in the literature, including burns [34], spinal cord and brain injury [8] and fractures [35], were all ranked in the top 30 conditions by WDD.

3.2. Predictive analytics

The predictive analytics application was used to expand research targets in HO and prioritise genes most likely to be of interest, where the corpus of data analysed by Watson was restricted to literature published up to the end of 2019. WDD ranked 233 candidate genes (Table A.4) that may be associated with pathological ectopic ossification using a known



(C)

Gene with Predisposition Relationship	Confidence (%)
TGFB1	81
BMP2	79
HIF1A	79
BMP4	77
SP	77
GDF2	76
LEP	76
ACVR1	75
BGN	72
PDGFRA	72
IGF1	69
BAX	66
NES	65
TACR1	63
AKR1B1	63
LMX1B	60
ADAMTS12	59
MMP9	77
SP7	61
VEGFA	69
MMP2	10

Fig. A.1. Genes extracted by Watson that are considered to have a (A) predisposition relationship and (B) regulation relationship to HO. Relationships are depicted by Watson so that a user can see their relationship (E.g. positive or negative regulation, association, predisposition, modification) to heterotopic ossification without having to read all associated documents supporting the connection. (C) confidence (%) represents the level of certainty WDD is of the relationship.

set of 100 genes (Table A.5) identified by WDD's biological relationship network analysis function as having previously defined associations with HO.

Watson ranked each candidate gene based on the semantic similarity of the gene to the 100 known genes, producing a final ranked list of candidate genes most likely to be involved in HO (Table A.6). The predictive similarity score generated by WDD for each gene, as shown in Table A.6, is a measure of an entity's similarity to all the known entities and ranked highest to lowest. A similarity score of 0.081 was generated for Multimerin-1 (MMRN1) as the highest-ranked entity, indicating it is the most similar of the set. The ranked list of candidate entities was further analysed through visualisations. For example, the distance network in Fig. A.2 considers the values between all entities in the similarity matrix and represents the distance between any two entities.

The top 25 results of this ranking are shown in Table 1. For nineteen of the top 25 genes predicted by Watson to be associated with tHO, a thorough literature search demonstrated these genes had been previously investigated before 2019 and identified as likely candidates in tHO. Six of the top 25 genes were determined to have no apparent link to tHO before 2019 including MMRN1, Musculin/Myogenic repressor (MSC/MyoR), Integrin Alpha M/Cluster of Differentiation 11b (ITGAM/CD11b), Platelet-Derived Growth Factor D (PDGFD), and Gremlin-1 (GREM1) and NEL-like molecule-1 (NELL1). Further interrogation of the literature highlighted that these candidate genes had previously defined roles in inflammation, aberrant tissue repair and regeneration, extracellular matrix remodelling and mineralisation, endochondral or intramembranous bone formation and injury-associated bone reactions. Additionally, the genes had functional roles in pathways of osteogenic

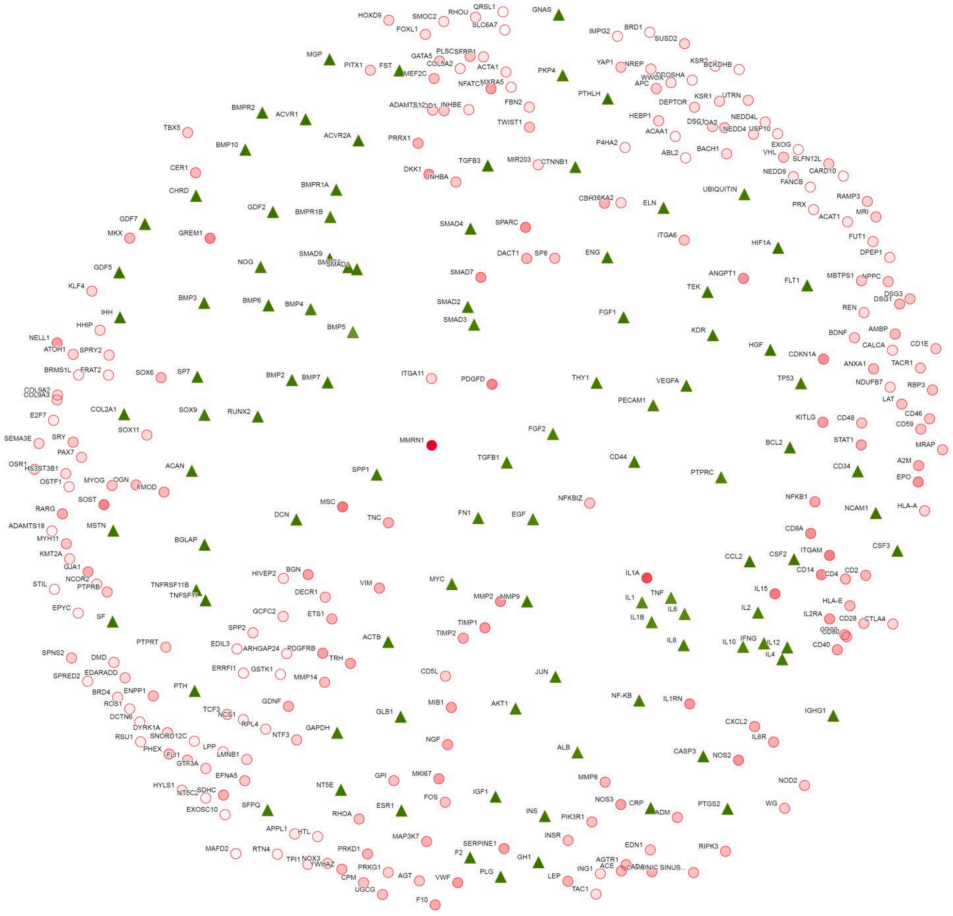


Fig. A.2. The Distance network visualization is optimal when you want to see the closest nodes to a node of interest. Entities that were entered in the first entity group field (the Known set) appear as green triangles, and entities that were entered in the second entity group field (the Candidate set) appear as red circles. In the Watson program when hovering on a Candidate entity, the five most similar Known entities are indicated. When hovering on a Known entity, the five most similar Candidate entities are indicated [24]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

differentiation, including BMP and WNT signalling and or defined roles in pathways relevant to HO and highlighted in the known gene list. Thus, these genes represented attractive candidates and were assessed further with an in-depth literature review.

3.3. Validation of predictive analytics data

The results of the custom validation test showed how highly Watson ranked the validation entities compared with the remaining candidate set, demonstrating that Watson's predictive model for heterotopic ossification was highly accurate. Table 2 shows WDD's ranking of five genes comprising the validation set when removed from the known set and placed into the candidate set. All five genes ranked within the top 15 places out of 233 candidate genes. Based on two statistical significance tests, the Fisher's Exact Test and the Wilcoxon Rank Sum, the yielded p-values were between 0 and 0.01, and the rating Watson supplied to the overall candidate validation was high.

4. Discussion

This unbiased, evidence-based predictive approach of IBM Watson identified six candidate genes as potential new research targets in tHO, MMRN1, MSC/MyoR, ITGAM/CD11b, PDGFD, GREM1 and NELL-1. Scientific validation of these entities may contribute to understanding pathological changes identified in tHO.

Although systematic reviews are considered the gold standard in knowledge synthesis, they are, by definition, limited in scope and clinical or research applicability. This is particularly so where a paucity of high-quality studies are not available. There remains a substantial repository of knowledge that is difficult to assimilate in a systematic manner. Typically, the execution of the methodology is a time-demanding process and is often focused on a narrow clinical question. However, in a clinical condition where rigorous searches and synthesis of refined evidence is yet to elucidate the unique pathophysiologic process, alternative approaches to interrogate large data sets may be

Table A.1
An excerpt generated by WDD of the supporting literature linked to each associated source (gene) and it's relationship to heterotopic ossification.

Document title	Source	Source type	Relationship type	Trigger word (verb)	Target (Text)	Snippet	Field title	Document type	Document publication date	Publication Name	Digital object identifier
Substance P signaling mediates BMP-dependent heterotopic ossification.	SUBSTANCE P	GENE	Regulation	mediates	heterotopic ossification	Substance P signaling mediates BMP-dependent heterotopic ossification.	Title	Medline	Oct 1, 2011	Journal of cellular biochemistry	https://doi.org/10.1002/jcb.23259
Synergistic inhibition of endochondral bone formation by silencing Hif1α and Runx2 in trauma-induced heterotopic ossification.	RUNX2	GENE	Regulation Negative	inhibit	HO	The results showed that lacking of Runx2 and Hif1α could inhibit HO formation.	Abstract	Medline	Aug 1, 2011	Molecular therapy: the journal of the American Society of Gene Therapy	https://doi.org/10.1038/mt.2011.101
Recombinant human bone morphogenetic protein 2-induced heterotopic ossification of the retroperitoneum, psoas muscle, pelvis and abdominal wall following lumbar spinal fusion.	BMP2	GENE	Regulation Positive	induced	heterotopic ossification	Recombinant human bone morphogenetic protein 2-induced heterotopic ossification of the retroperitoneum, psoas muscle, pelvis and abdominal wall following lumbar spinal fusion.	Title	Medline	May 1, 2010	Skeletal radiology	https://doi.org/10.1007/s00256-010-0890-8
Thoracic myelopathy caused by ossification of ligamentum flavum of which fluorosis as an etiology factor	TGFB1	GENE	Association	played	ectopic ossification	TGF-β1 could have played a role in chondroid metaplasia and ectopic ossification in OLF.	Document Body	Pmcoa	Nov 2, 2006	Journal of Orthopaedic Surgery and Research	https://doi.org/10.1186/1749-799X-1-10
Anaplerotic Accumulation of Tricarboxylic Acid Cycle Intermediates as Well as Changes in Other Key Metabolites During Heterotopic Ossification	MMP9	GENE	Association	linking	heterotopic ossification	Activation of MMP9 is a complex mechanism that requires the presence of plasmin, the active form of plasminogen, thus, linking heterotopic ossification with platelet activation and recruitment to the site of new bone formation.	Document Body	Pmcoa	Dec 31, 2015	Journal of Cellular Biochemistry	https://doi.org/10.1002/jcb.25454
Analysis of the mechanism by which nerve growth factor promotes callus formation in mice with tibial fracture	NGF	GENE	Regulation negative	reduced	heterotopic ossification	Local injection of exogenous NGF facilitated the complete healing of the fracture and reduced the formation of heterotopic ossification.	Document Body	Pmcoa	Feb 7, 2017	Experimental and Therapeutic Medicine	https://doi.org/10.3892/etm.2017.4108

Table A.2
A set of 100 genes with associated value extracted by Watson to have a relationship with heterotopic ossification, as shown in the visual representation (Fig. 1). The value represents the strength of the relationship i.e. the number of documents supporting the connection.

Rank	Entity name	Value	Rank	Entity name	Value
1	BMP2	595	**51**	AKT1	92
2	BMP4	321	**52**	IL10	92
3	ACVR1	305	**53**	CASP3	90
4	VEGFA	289	**54**	NT5E	90
5	BGLAP	278	**55**	SF	89
6	TNF	278	**56**	CSF2	88
7	RUNX2	263	**57**	GLB1	88
8	BMP7	255	**58**	TEK	88
9	FGF2	228	**59**	PLG	87
10	PTH	218	**60**	BMPR1A	86
11	IL6	213	**61**	IL4	84
12	FN1	204	**62**	BMP3	83
13	GAPDH	193	**63**	BMP5	83
14	TGFB1	190	**64**	PECAM1	83
15	ALB	185	**65**	IGHG1	80
16	INS	183	**66**	SMAD2	78
17	EGF	179	**67**	F2	77
18	PKP4	179	**68**	TGFB3	77
19	SMAD1	174	**69**	BMPR2	76
20	PTGS2	168	**70**	CCL2	76
21	SPP1	166	**71**	MSTN	75
22	IGF1	164	**72**	JUN	73
23	IL1	162	**73**	TNFRSF11B	72
24	BMP6	156	**74**	TP53	72
25	SMAD5	153	**75**	ACVR2A	71
26	SMAD9	140	**76**	CSF3	71
27	CRP	134	**77**	FGF1	70
28	NOG	132	**78**	HGF	70
29	SP7	132	**79**	MYC	70
30	PTPRC	127	**80**	IHH	69
31	CD34	125	**81**	GNAS	67
32	IL1B	125	**82**	IL12	67
33	ACAN	124	**83**	BCL2	66
34	GH1	124	**84**	BMP10	66
35	ACTB	122	**85**	CHRD	66
36	GDF2	119	**86**	FST	66
37	ELN	114	**87**	NCAM1	66
38	SOX9	109	**88**	COL2A1	65
39	NF-KB	108	**89**	SMAD3	65
40	IFNG	107	**90**	SMAD4	65
41	TNFSF11	107	**91**	HIF1A	64
42	THY1	105	**92**	DCN	63
43	IL8	104	**93**	GDF7	63
44	ESR1	103	**94**	FLT1	62
45	GDF5	103	**95**	PTHLH	62
46	IL2	103	**96**	UBIQUITIN	62
47	CTNNB1	100	**97**	BMPR1B	61
48	ENG	96	**98**	KDR	61
49	CD44	94	**99**	MGP	61
50	SFPQ	94	**100**	MMP9	61

Table A.3
A network of conditions that Watson has produced in real time. Watson uses it's annotators to extract relationships between heterotopic ossification and other associated conditions demonstrating its power as a disease agnostic.

Rank	Condition	Value
1	OSSIFICATION, HETEROTOPIC	6156
2	PAIN	1973
3	WOUNDS AND INJURIES	1728
4	ARTHRITIDES, DEGENERATIVE	1117
5	CANCER	1023
6	DISLOCATIONS	936
7	FIBRODYSPLASIA OSSIFICANS PROGRESSIVA	817
8	ARTHRITIS	616
9	AGE RELATED OSTEOPOROSIS	585
10	ASEPTIC NECROSIS OF BONE	492
11	SPINAL CORD INJURIES	484
12	HEMORRHAGE	437
13	RHEUMATOID ARTHRITIS	397
14	BENIGN CHONDROMA OF BONE	382
15	LYTIC BONE LESION	346
16	NECROSIS	346
17	DIABETES MELLITUS	341
18	ANKYLOSES	336
19	FRACTURES, BONE	329
20	BONE RESORPTION	326
21	BONE CANCER	321
22	DEEP THROMBOPHLEBITIS	313
23	FIBROSIS	310
24	BRAIN INJURY	304
25	METASTASES	295
26	POSTOPERATIVE COMPLICATIONS	293
27	BURNS	285
28	HEMATOMA	285
29	INFLAMMATORY RESPONSE	281
30	ABNORMALITY, CONGENITAL	273
31	ANASARCA	257
32	BED SORE	257
33	HYPERTROPHY	254
34	HIP DISLOCATION	252
35	ANKYLOSING SPONDYLARTHRITIDES	250
36	CEREBROVASCULAR ACCIDENT	250
37	CICATRIX	219
38	OBESE	217
39	ANOXIA	212
40	CHRONIC KIDNEY DISEASE	208
41	BONE DISEASE, METABOLIC	207
42	ARTERIOSCLEROSES	205
43	BONE DISEASE	199
44	BREAST CANCER	197
45	CRANIOCEREBRAL TRAUMA	196
46	BONE PAGET DISEASE	195
47	WOUND INFECTION	192
48	BONE MARROW DISEASE	190
49	HYPERTENSIVE VASCULAR DISEASE	186
50	DYSPLASIA	185

beneficial to guide the identification of relevant genes, proteins and, or drugs of interest.

This study identified six new genes of interest in a list of 25 top-ranked genes, including 19 genes with evidence in the literature of a role in tHO. Of the six genes, further literature investigations demonstrated roles for these proteins in physiological and pathological processes that are known to be involved in tHO. Recent literature that has been published since 2019 supports several of the lower-ranked genes in our results [36]. Among the Top 6 genes, MMRN1 (ranked 1), also known as EMILIN-4, is a glycoprotein stored in platelets, endothelial cells (ECs) and megakaryocytes that is deposited into the extracellular matrix (ECM) [37]. MMRN1 is differentially expressed in several cancer cell lines [37,38], inflammatory diseases [39,40], and bacterial and viral infections [41,42]. It may be necessary to maximise platelet adhesion at vascular injury sites by binding to collagen [43]. Recently, upregulation of MMRN1 was observed in serum exosomes from burn patients, a population at risk of tHO development [44].

Similarly, *MMRN1* upregulation in the vasculature in response to increased Vascular endothelial growth factor (VEGF)-A and TGF-β signalling in the tumour microenvironment has been reported, suggesting a possible role in angiogenesis and VEGF signalling, which are involved in tumorigenesis and implied in the pathogenesis of tHO [45-49]. Currently, there are few studies relating MMRN1 to bone remodelling. Liron et al. [50] reported that the Mmrn1 gene is expressed in bone tissue, pre-osteoclasts and non-differentiated osteoblasts. The authors observed a 55% lower bone volume in mutant mice attributable to the deletion of *Mmrn1*, suggesting a physiological role of *Mmrn1* in bone metabolism.

Further research is required to elucidate the contribution of other distinct ECM influencers, such as MMRN1 and other members of the multimerin family, in the control of tissue microenvironments and hematopoietic stem cell niche function that can favour osteogenesis leading to pathological ossification. Considering that circulating Mmrn1 levels have been detected in the blood plasma, serum, urine, and saliva of cancer patients, Mmrn1 may serve as a potential biomarker for tHO.

Table A.4
Candidate gene set used for interrogation using WDD's predictive analytics function. The list contains 233 genes with a potential role in ectopic bone formation, extracellular matrix production and fibrosis.

RTN4	PRRX1	A2M	MXRA5	ERRFI1
UGCG	PRX	TIMP1	MIB1	WWOX
BACH1	HLA-A	ACE	EDARADD	SP8
NGF	HYLS1	CD40	SUSD2	FBN2
NCS1	MRAP	CD86	SMOC2	IL6R
BDNF	NDUFB7	DROSHA	SOX6	INSR
GJA1	OGN	PRKG1	UTRN	SEMA3E
GDNF	RPL4	NPPC	CPM	E2F7
MMP2	SPARC	ADM	ANXA1	PIK3R1
BGN	FANCB	IL1A	MYH11	MEF2C
FMOD	EPO	NT5C2	PTPRB	RIPK3
KMT2A	BRD1	RHOA	DSG3	RPS6KA2
CD59	COL9A2	ROS1	MKI67	NOX3
TRH	ACTA1	STIL	EPYC	SFRP1
DPEP1	HLA-E	TCF3	PLSCR4	PRKD1
LEP	WG	TNC	DSC1	CARD10
MIR203	DMD	VWF	INHBA	RSU1
DKK1	IL1RN	AGTR1	YWHAZ	NEDD4L
SOX11	MYOD1	BCKDHB	DSG1	OSR1
SMAD7	NOS2	CD46	LPP	NCOR2
YAP1	MMRN1	CD80	IMPG2	MBTPS1
TAC1	MYOG	CDKN1A	ADAMTS18	DEPTOR
TACR1	APPL1	COL9A3	GCFC2	KSR2
CD2	DECR1	CTLA4	RBP3	NEDD4
RARG	F10	CXCL2	HS3ST3B1	SPNS2
HEBP1	GSTK1	EDN1	PTPRT	USP10
ANGPT1	SERPINE1	EXOSC10	OSTF1	NCOA2
NTF3	NOD2	GPI	NEDD9	RAMP3
ENPP1	SNORD12C	MMP14	KITLG	ING1
PAX7	VIM	MMP8	DCTN6	NFKBIZ
CALCA	ACAA1	PDGFRB	KLF4	IL15
CD48	MAFD2	REN	SPRY2	HIVEP2
FUT1	SDHC	TPI1	FRAT2	LMNB1
CDH1	CD1E	ACAT1	DACT1	MSC
APC	EXOG	AMBP	FOXL1	NFATC1
VHL	ITGAM	CD28	MAP3K7	NFKB1
CD5L	SLC6A7	CD8A	FLI1	GTF3A
ABL2	SOST	ITGA6	QRSL1	AGT
PHEX	CER1	TIMP2	RHOU	FOS
HHIP	GREM1	COL5A2	EFNA5	NELL1
BRD4	HTL	ITGA11	SPRED2	MKX
MRI	CD14	NREP	GATA5	ADAMTS12
LAT	IL2RA	PDGFD	ARHGAP24	DYRK1A
SRY	NOS3	EDIL3	SLFN12L	KSR1
STAT1	TWIST1	P4HA2	ATOH1	HOXD9
TBX5	ADA	SPP2	PITX1	
INHBE	CD4	ETS1	BRMS1L	

Table A.5
Known set used for Predictive analytics. A set of 100 genes with previously defined associations with heterotopic ossification was used to interrogate the candidate gene list.

Known set	
BMP2	IL10
BMP4	CASP3
ACVR1	NT5E
VEGFA	SF
BGLAP	CSF2
TNF	GLB1
RUNX2	TEK
BMP7	PLG
FGF2	BMPR1A
PTH	IL4
IL6	BMP3
FN1	BMP5
GAPDH	PECAM1
TGFB1	IGHG1
ALB	SMAD2
INS	F2
EGF	TGFB3
PKP4	BMPR2
SMAD1	CCL2
PTGS2	MSTN
SPP1	JUN
IGF1	TNFRSF11B
IL1	TP53
BMP6	ACVR2A
SMAD5	CSF3
SMAD9	FGF1
CRP	HGF
NOG	MYC
SP7	IHH
PTPRC	GNAS
CD34	IL12
IL1B	BCL2
ACAN	BMP10
GH1	CHRD
ACTB	FST
GDF2	NCAM1
ELN	COL2A1
SOX9	SMAD3
NF-KB	SMAD4
IFNG	HIF1A
TNFSF11	DCN
THY1	GDF7
IL8	FLT1
ESR1	PTHLH
GDF5	UBIQUITIN
IL2	BMPR1B
CTNNB1	KDR
ENG	MGP
CD44	MMP9
SFPQ	IL10
AKT1	

However, further study is required [51].

Myogenic repressor (MyoR), also known as Musculin, is another candidate gene predicted by WDD to have a potential association with tHO. MyoR was initially identified as a transcriptional repressor of muscle differentiation, and its expression is restricted to precursors of the skeletal muscle lineage [52]. MyoR is expressed at high levels in proliferating myoblasts and is downregulated early during myogenesis at the onset of differentiation. It has been speculated whether MyoR may be critical for selectively delaying the expression of certain muscle-specific genes during primary myogenesis or inhibiting myogenesis by inducing cell death or stimulating cell proliferation [53 53]. Gagan et al. [54] showed that miR-378, a microRNA that is upregulated during differentiation by Myogenic differentiation 1 (MyoD), promotes the transition from proliferating myoblasts to differentiating myotubes through targeting MyoR and repressing myogenic differentiation via inhibition of MyoD transcriptional activity. Along this line, it is worth noting the finding of Hupkes et al. [55]. This study demonstrated an effect of miR-378 on promoting BMP-2-induced osteogenic differentiation of C2C12 myoblast-like cells [55]. In addition to the critical role(s) of MyoR in skeletal myogenesis, tissue repair, differentiation, and regeneration [52,56], accumulated evidence has also expanded MyoR function to the regulation of the inflammatory reaction and immune function in the context of trauma and disease [57-60]. Yu et al. [60] suggests that MyoR may act as a novel regulatory factor for maintaining the balance between excessive inflammatory reaction and tissue repair in the intestinal epithelium, whereby MyoR deficiency enhances inflammation via excessive secretion of Interleukin-22, leading to aggravated colonic epithelial injury [60]. A more comprehensive understanding of the mechanistic actions of MyoR and how it affects the differentiation of myogenic and non-myogenic cells during adult tissue regeneration and its interactive feedback in the context of acute traumatic injury is warranted.

Ranked five by Watson was CD11b/ITGAM, a receptor originally described on neutrophils and macrophages that is responsible for supporting adhesion and molecular cross-talk of these cells with ECM proteins [61]. CD11b has been implicated in the development of several inflammatory diseases [62,63] and has an oncogenic role where it is

Table A.6
IBM Watson predictive analytics process of top 50 ranked genes. A predictive similarity score is generated by Watson that measures an entities similarity to all known entities. A graph diffusion (GD) score (similarity score) is assigned by Watson to each gene/protein based on semantic similarity of the candidate entity to the known set [28]. The higher the number, the more similar an entity is to the set of known entities.

Rank	Gene	Graph diffusion (GD) score
1	MMRN1	0.081
2	IL1A	0.064
3	IL15	0.049
4	MSC	0.049
5	ITGAM	0.048
6	PDGFD	0.046
7	SOST	0.045
8	GREM1	0.044
9	CD14	0.043
10	CD8A	0.042
11	SPARC	0.042
12	MMP2	0.041
13	CDKN1A	0.041
14	EPO	0.041
15	NOS2	0.04
16	IL1RN	0.04
17	SMAD7	0.04
18	DKK1	0.039
19	ANGPT1	0.039
20	VWF	0.039
21	TIMP1	0.038
22	KITLG	0.038
23	IL2RA	0.038
24	NFATC1	0.037
25	NELL1	0.037
26	MKI67	0.037
27	PDGFRB	0.037
28	BGN	0.036
29	SERPINE1	0.036
30	NOS3	0.036
31	NFKB1	0.035
32	MIB1	0.034
33	F10	0.033
34	A2M	0.033
35	RARG	0.033
36	CXCL2	0.033
37	CD40	0.033
38	IL6R	0.033
39	STAT1	0.032
40	TRH	0.032
41	ADA	0.032
42	NGF	0.032
43	TNC	0.031
44	VIM	0.031
45	SDHC	0.031
46	TIMP2	0.031
47	GJA1	0.031
48	CDH1	0.031
49	LEP	0.031
50	PRRX1	0.03

expressed on myeloid lineage osteoclast precursors [64]. However, a direct association between CD11b and pathological ossification has yet to be established. The findings of Park-Min et al. [65] identified CD11b as a negative regulator of the earliest states of osteoclast differentiation. These authors demonstrated that *CD11b*-deficient mice exhibited decreased bone mass associated with increased osteoclast numbers and reduced bone formation. Ehirchiou et al. [61] found that CD11b is expressed in human chondrocytes and the ECM of articular cartilage from patients with osteoarthritis (OA). The authors established a novel role of CD11b signalling in preventing chondrocyte hypertrophy and chondrocyte mineralisation *in vitro* [61]. More so, primary murine CD11b KO chondrocytes exhibited greater alkaline phosphatase (*Alp*) gene expression levels and production of pro-mineralising cytokine Interleukin-6 and monocyte chemoattractant protein 1 (MCP-1), sustaining the loop between inflammation and calcification [61]. Thus, it is possible that down-regulation of these calcification factors, via cross-talk with CD11b-dependent signalling pathways such as c-Jun N-terminal Kinase (JNK) and Nuclear factor-kappa B (NF-kB), maybe a potential mechanism by which CD11b exerts its role as a negative regulator of mineralisation in chondrocytes [61].

Notably, the presence of soluble CD11b has been confirmed in human plasma [62] and in the synovial fluid of OA patients [61], postulating that the primary source could be synovial cells and infiltrating inflammatory cells. These results suggest that CD11b may participate in mineral deposition, and it may be worth exploring the interaction of CD11b integrin signalling in the regulatory mechanisms that govern pathological mineralisation. Modulating CD11b integrin signalling may be an effective strategy to protect traumatised joints from developing inflammation-associated peri-articular ossifications.

Platelet-derived Growth Factors (PDGF) are potent mitogens and chemoattractants for cells of mesenchymal origins [66] and play an indirect role in facilitating bone formation via induction of MSC migration to the site of bone regeneration and by supporting the expansion of osteoprogenitor cells and promoting angiogenesis [66]. PDFG-D is mainly expressed in endothelial cells, smooth muscle cells and macrophages and plays a role in wound healing [67], fibrotic processes [68] and in disease processes including various cancers [69,70] and atherosclerosis [71]. However, little is known about the role of PDGF-D in regulating physiological and pathological bone formation [72]. Wang and colleagues [73] showed that the downregulation of PDGF-D led to the inactivation of Notch-1 and NF-kB DNA binding activity, which resulted in the downregulation of target genes such as VEGF and the activity of Matrix Metalloproteinase 9 (MMP-9). Huang et al. [69] revealed that Pdgf-d regulates osteoclastic differentiation and promotes intraosseous tumour growth associated with increased osteoblastic bone responses in mice. These authors highlighted a novel function of PDGF-D in early bone remodelling. It was demonstrated that PDGF-D-specific signal transduction upregulated the expression of Nuclear Factor of Activated T cells 1 (NFATc1), a master transcription factor for osteoclast differentiation [66]. Thus, PDGF-D may be considered an osteoinductive factor that can modulate osteogenic capacity in a proinflammatory microenvironment and contribute to aberrant tissue healing and injury-associated bone reactions.

Gremlin 1 (GREM-1) is a secreted glycoprotein and a BMP antagonist that preferentially binds to BMP-2/4/7 ligands in the ECM, opposing BMP effects on osteoblastic differentiation and function *in vitro* and *in vivo* [74,75]. Thus, it has been proposed that GREM1 may play a key role in regulating endochondral bone formation [76]. Gazzerro et al. [74] demonstrated that deletion of *Grem1* in the bone microenvironment led to sensitisation of BMP and Wnt/β-catenin signalling and activity, enhanced ALP expression and increased bone formation *in vivo*. Moreover, the downregulation of Gremlin in osteoblastic cells increased the BMP-2 stimulatory effect on the Suppressor of Mothers Against Decapentaplegic (SMAD) signalling, ALP activity and enhanced expression of osteogenic markers (osteocalcin and Runx2). As Grem1 can block BMP activity, it likely influences SMAD 1/5/8 signalling and, in turn, the expression of hypertrophic markers. It has been proposed that bone-derived Grem-1 may work in conjunction with BMP-4 to initiate a catabolic and tissue remodelling program in hypertrophic chondrocytes and osteoblasts that favours the pathological remodelling of the osteochondral junction in OA subchondral bone [76]. Aberrant expression of Grem1 has also been associated with rheumatoid arthritis [77], fibrosis of the lung [78,79] and kidney [80], in various cancers [81,82] and other skeletal and connective tissue disorders [83,84]. However, until recently, an association between Grem1 and tHO was not reported. During our WDD investigation, a paper by Yu et al. [85] showed that Grem1 expression is decreased in the early stages of tHO in an Achilles tendon tenotomy rat model. Further investigation into the signalling mechanisms engaged by GREM1 under pathological conditions associated with ectopic ossification is warranted.

NELL-1 (NEL-like molecule-1; NEL [a protein strongly expressed in

neural tissue encoding epidermal growth factor-like domain]), ranked highly by WDD as a potential candidate gene in tHO, is reported to control skeletal ossification [86]. NELL-1 is recognised as an osteo-specific growth factor with anti-adipogenic activities, capable of promoting osteochondrogenic cell differentiation and mineralisation *in vitro* [87-90]. The molecular mechanisms underlying NELL1-induced osteogenic differentiation are not fully understood; however, findings suggest that the osteoinductive activity of NELL-1 is particular to the osteochondral lineage [87,89,91]. NELL-1 mediates critical downstream effects of master osteogenic regulator RUNX2 and plays a role in intramembranous and endochondral ossification [87,92-94]. Aghaloo et al. [89] demonstrated that Nell-1 promoted osteogenesis in MC3T3-E1 osteoblasts and induced *in vivo* bone regeneration equivalent to BMP2. The ability of NELL-1 to direct BMP2-treated cells toward osteogenesis and repress adipogenesis requires intact Wnt signalling, and it has been proposed that Nell1- may also regulate Runx2 via canonical Wnt signalling [94]. In a mouse intramuscular transplantation model, bone marrow stromal cells transduced with *the Nell-1* gene formed mature bone via an endochondral ossification mechanism *in vivo*. Nell-1 was found to act synergistically with BMP to increase the responsiveness of myoblastic C2C12 cells to BMP-2 stimulation and promote bone regeneration. However, Nell-1 could not promote osteoblastic differentiation leading to ectopic bone formation independently of BMP-2 [95]. These findings demonstrate that Nell-1 activates Runx2 via a mechanism independent of the BMP/SMAD pathways and functions selectively on cells in the osteochondrogenic lineage or multipotent cells to undergo osteochondral differentiation and bone formation [87]. In an *in vivo* ectopic bone formation assay, overexpression of Nell-1 significantly enhanced mineralisation and maturity of BMP-9-induced bone formation of MSCs, whilst effectively suppressing BMP-9-induced adipogenesis [90]. Targeting the synergistic bone formatting activity of NELL-1 and osteogenic BMPs (such as BMP-2 and BMP-9) may be a viable therapeutic target in treating undesirable heterotopic bone formation.

In summary, this study has applied a cognitive computing approach to identify genes to be factors influencing trauma-induced heterotopic bone formation. The six most promising genes identified in this study may aid in accelerating focused research on specific candidates for laboratory assessment and validation. To aid this approach, WDD should be supplemented with known manually curated pathway information, e.g., Kyoto Encyclopedia of Genes and Genomes (KEGG), to augment what we extract from the text. Further assessment and laboratory validation of potentially novel targets identified will be necessary to conclusively assess the utility of the WDD [32]. Understanding the critical cellular events and pathways responsible for aberrant cell fate in tHO might inform other disease processes such as muscle fibrosis, vascular calcification and other pathological processes involving the aberrant bone formation and allow improved targeted therapies that are amendable for therapeutic interventions of tHO.

5. Limitations

While the use of WDD in tHO proved successful in identifying a novel set of plausible candidate genes that may participate in the pathogenesis of tHO, our approach has some limitations. Firstly, as Watson uses only sentence-level information to identify associations to a known set of proteins, analysis based on semantic similarity may pose challenges when evaluating particularly complex biological relationships, relying on a deeper understanding of the surrounding context and nuance of individually written sentences identifying the association. To mitigate this limitation, this study employed a manual review of the curated evidence supporting the semantic relationships identified by Watson to validate its predictions further. It is a potential limitation that at the time of this study, we did not have the technology, or scope to complete full paper translations, and papers and abstracts written in languages other than English had to be excluded. In addition, the use of specific known genes is required to identify candidate genes of interest using the predictive analytics method. In contrast to gene ontologies or standard pathway analyses, given that the current input is likely based on literature evidence, this approach is only valuable for investigating genetic data with a priori hypothesis. It is even more pertinent to consider this when investigating conditions such as tHO with minimal high-level evidence in the current literature of implicated biological processes and genetic influences [28]. These limitations will likely be primarily overcome with advancements in AI platforms, algorithms, and the computing power available.

6. Conclusion

This study illustrated the utility of machine learning approaches to support the exploration, identification and prioritisation of promising gene relationships and targets in rare diseases like tHO. The genes identified in this study (MMRN1, MSC/MyoR, ITGAM/CD11b, PDGF-D, GREM-1 and NELL-1) are potential new gene candidates for future studies investigating the pathobiology of tHO. Combining whole genome approaches and more extensive molecular-driven studies with advanced analytics and machine learning approaches can significantly accelerate research, thus streamlining pathways from basic science to clinical translation.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Consent/ethical approval

Not required.

Appendix A

See Figs. A.1 and A.2, Tables A.1-A.6.

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Appendix 9: Published Article (Chapter 4)

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Evaluation of the accuracy of diagnostic coding and clinical documentation for traumatic heterotopic ossification diagnoses in Western Australian hospitals

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ABSTRACT

Background: Traumatic heterotopic ossification (tHO) refers to the pathological formation of ectopic bone in soft tissues that can occur following burn, neurological orthopaedic trauma. As completeness and accuracy of medical diagnostic coding can vary based on coding practices and depend on the institutional culture of clinical documentation, it is important to assess diagnostic coding in that local context. To the authors' knowledge, there is no prior study evaluating the accuracy of medical diagnostic coding or specificity of clinical documentation for tHO diagnoses across Western Australia (WA) trauma centres or across the full range of inciting injury and surgical events.

Objective: To evaluate and compare the clinical documentation and the diagnostic accuracy of ICD-10-AM coding for tHO in trauma populations across 4 WA hospitals.

Methods: A retrospective data search of the WA trauma database was conducted to identify patients with tHO admitted to WA hospitals following burn, neurological or orthopaedic trauma. Patient demographic and tHO diagnostic characteristics were assessed for all inpatient and outpatient tHO diagnoses. The frequency and distribution of M61 (HO-specific) and broader, musculoskeletal (non-specific) ICD-10-AM codes were evaluated for tHO cases in each trauma population.

Results: HO-specific M61 ICD-10-AM codes failed to identify more than a third of true tHO cases, with a high prevalence of non-specific HO codes (19.4 %) and cases identified via manual chart review (25.4 %). The sensitivity of M61 codes for correctly diagnosing tHO after burn injury was 50 %. ROC analysis showed that M61 ICD-10-AM codes as a predictor of a true positive tHO diagnosis were a less than favourable method (AUC=0.731, 95 % CI=0.561–0.902, $p = 0.012$). Marked variability in clinical documentation for tHO was identified across the hospital network.

Conclusion: Coding inaccuracies may, in part, be influenced by insufficiencies in clinical documentation for tHO diagnoses, which may have implications for future research and patient care. Clinicians should consistently

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employ standardised clinical terminology from the point of care to increase the likelihood of accurate medical diagnostic coding for tHO diagnoses.

Introduction

Traumatic heterotopic ossification (tHO) refers to the development of extra-skeletal bone in muscle and soft tissues following tissue insult secondary to trauma [1]. Cases of tHO are broadly classified into three aetiological categories: neurogenic HO resulting from traumatic brain injury (TBI) and spinal cord injury (SCI); orthopaedic HO developing after fracture, dislocation and soft tissue trauma; and HO following burns and high-velocity blast injury [2,3]. Traumatic HO typically manifests as a progressive condition marked by rapid movement loss at affected joints and severe pain [4]. Each aetiology of tHO creates a unique clinical picture of prevalence, risk profile, and area of formation, all of which must be considered by treating clinicians [3].

The International Classification of Diseases (ICD) codes, currently in its tenth revision with Australian modification (ICD-10-AM), is a standard diagnostic coding system used by healthcare facilities in Australia to classify diseases and other health problems and are essential for monitoring disease prevalence [5–8]. Coding accuracy directly impacts the quality of decisions based on codes such as quality, costs, and effectiveness of care. It thus has widespread use for supporting essential funding, clinical, and research decisions [5,6]. However, ICD-10-AM diagnostic classifications typically do not provide the detail needed to capture patient data at the point of care for meaningful use in clinical and operational improvement and retrospective research [9]. The Systematised Nomenclature of Medicine - Clinical Terms, Australian release (SNOMED CT-AU), is an extensive dictionary of clinical terminology designed to record clinical information that has been adopted by health systems, especially those with electronic medical records (EMR), to facilitate consistent clinical documentation at the point of care, and the aggregation and exchange of clinical data between facilities that has a commonly understood meaning [9].

Assessing the accuracy of diagnostic coding is pivotal to ensuring the validity and reliability of administrative diagnostic data [8]. As completeness and accuracy of coding can vary based on coding practices and depends on the institutional culture of clinical documentation, it is essential to assess diagnostic coding in that local context [5]. There are no prior studies evaluating the accuracy of medical diagnostic coding for the diagnosis of tHO across Western Australia (WA) trauma centres or traumatic injury populations. Thus, the primary objective of this study was to evaluate and compare the clinical documentation and accuracy of ICD-10-AM coding for the diagnosis of tHO across four major WA hospitals.

Methods

Study participants

A retrospective data audit sought to identify patients with tHO admitted to four WA hospitals in April 2020. Adult patients were included if admitted at age 18 or over and discharged between 1st May 2005 and 1st May 2019 following neurological (TBI and SCI), burn or orthopaedic trauma. Subjects were excluded if death occurred or comfort care/palliation was instigated during their hospital stay.

A flow chart of patient identification, screening protocol and selection is shown in **Figure S1**. Potential tHO cases were identified in a cascading, three-tier ICD-10-AM coded WA trauma database search. Both primary diagnosis codes and secondary conditions were captured and given equal weight. Tier one included patients with HO-specific code M61 (calcification and ossification of muscle) and its subclassifications [10]. Tier two included patients with additional miscellaneous 'non-specific' musculoskeletal codes, including previously defined codes, to capture all patients with a possible diagnosis of tHO [10]. The third tier included all trauma admissions with an inpatient hospital length of stay (LOS) ≥ 7 days to identify any confirmed cases of tHO not identified by medical coders and without an HO-specific or non-specific ICD-10-AM code, i.e. 'no code'. Time to tHO diagnosis has been reported to occur as early as four weeks from injury onset and in patients with greater injury severity and a prolonged hospital LOS [4]. Therefore, the filter criteria of LOS ≥ 7 days ensured an injury cohort at higher risk of developing tHO was captured.

A screening protocol was conducted on a case-by-case basis to confirm or exclude a tHO diagnosis and traumatic injury mechanism. This included a manual review by a single investigator (NF) of patient EMRs, medical and allied health clinical documentation, discharge summaries and imaging reports. The presence of radiological and clinical evidence of a tHO diagnosis and a preceding traumatic injury mechanism classified a true case of tHO (tHO+).

Statistical analysis

The frequency and distribution of HO-specific M61 and non-specific ICD-10-AM codes and tHO diagnostic characteristics were assessed for each trauma population across the overall hospital network (Table 1). Nominal parameters were evaluated using Pearson's Chi-square test and are shown as frequencies and percentages. In the burns cohort, the predictive performance of the HO-specific (M61) diagnostic codes in discriminating true tHO cases from those without tHO was evaluated using receiver operating characteristic (ROC) curve analysis. The

Table 1
Diagnostic characteristics for inpatient and outpatient tHO diagnoses by primary injury category.

Diagnosis characteristics of tHO+ cohort	BURN	SCI	TBI	ORTHO	TRAUMA (TOTAL)	*p*
Diagnosis episode of care						
n	18	17	19	13	67	
Inpatient	17 (25.3 %)	13 (19.4 %)	17 (25.3 %)	1 (1.4 %)	48 (71.6 %)	<0.001
Outpatient	1 (1.4 %)	4 (5.9 %)	2 (2.9 %)	12 (17.9 %)	19 (28.4 %)	
Diagnosis inpatient episode of care						
n	8	13	17	1	39	
Acute care	5 (12.8 %)	0 (0.0 %)	10 (25.6 %)	0 (0.0 %)	15 (38.4 %)	0.003
Rehabilitation	3 (7.6 %)	13 (33.3 %)	7 (17.9 %)	1 (2.5 %)	24 (61.5 %)	
ICD-10-AM code specificity for inpatient and outpatient tHO diagnoses						
n	18	17	19	13	67	
HO-specific (M61)	8 (11.9 %)	8 (11.9 %)	14 (20.8 %)	7 (10.4 %)	37 (55.2 %)	0.457
Non-specific	5 (7.4 %)	5 (7.4 %)	1 (1.4 %)	2 (2.9 %)	13 (19.4 %)	
No code	5 (7.4 %)	4 (5.9 %)	4 (5.9 %)	4 (5.9 %)	17 (25.4 %)	

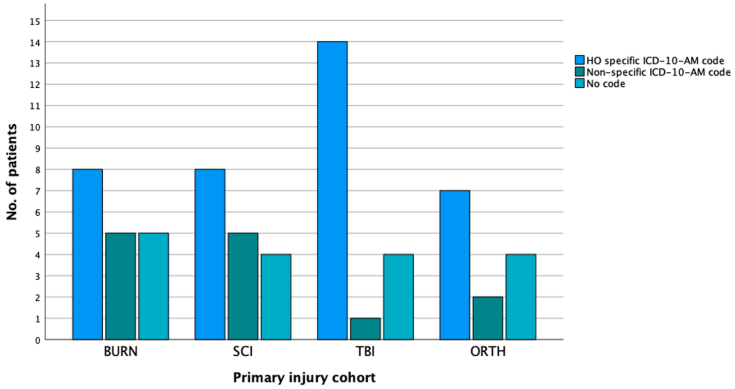

Fig. 1. The frequency and distribution of ICD-10-AM codes for inpatient and outpatient tHO diagnoses (n = 67) according to primary injury cohort.

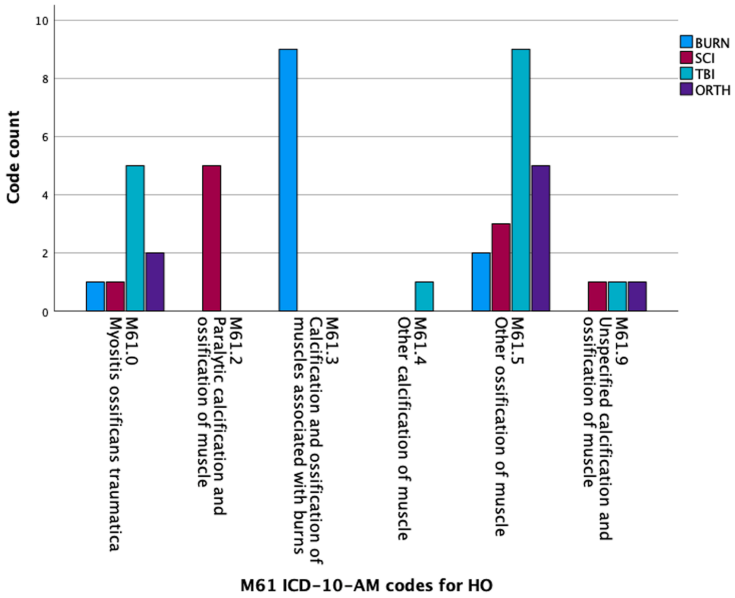

Fig. 2. Bar graph showing frequency (code count) of HO-specific ICD-10-AM codes (N = 46) for true inpatient and outpatient tHO diagnoses (n = 37 patients), stratified by primary injury cohort. For patients coded with > 1 code, the total number of codes per patient was included in the final frequency calculation.

discriminative ability of M61 codes was evaluated using the area under the curve (AUC) for the conversion outcome, where an AUC $\geq$ 0.8 would indicate that a test has favourable sensitivity and specificity characteristics [11]. Statistical significance was defined at the conventional 5 % level. All computations were performed using IBM SPSS Statistics for Macintosh, version 29.0 (IBM Corp, Inc.).

Ethical considerations

Ethics approval was granted by the South Metropolitan Health Human Research Ethics Committee (RGS3452) and from The University of Notre Dame, Fremantle (2020–013F).

Results

Patient cohort

Eighty-seven patients were identified in tier one to have M61 ICD-10-AM codes for tHO. After chart review, only 37 patients were confirmed to have clinical and radiographic evidence of tHO with a primary traumatic injury mechanism. The remaining 50 patients were excluded; 11 had no clinical or radiographical evidence of tHO and thus were deemed incorrectly coded. Tier two captured a cohort of 1422 patients with non-specific, miscellaneous musculoskeletal ICD-10-AM codes, of which 13 were identified as true tHO+ cases. Therefore, of the 1509

Table 2
Description and distribution of coding method used to identify true inpatient tHO+ diagnoses ($n = 48$ patients) by primary cohort. n: no. of patients, tHO: traumatic heterotopic ossification, SCI: spinal cord injury, TBI: traumatic brain injury, ortho: orthopaedics.

ICD-10-AM code specificity for inpatient tHO diagnoses		BURN tHO+	SCI tHO+	TBI tHO+	ORTHO tHO+	TRAUMA tHO+ (TOTAL)
HO-specific (M61)	*n*	**8**	**8**	**13**	**0**	**29**
	Primary injury%	47.1 %	61.5 %	76.5 %	0 %	–
	Total %	27.6 %	27.6 %	44.8 %	0 %	**60.4 %**
Non-specific	*n*	**5**	**3**	**1**	**0**	**9**
	Primary injury %	29.4 %	23.1 %	5.9 %	0 %	–
	Total %	55.6 %	33.3 %	11.1 %	0 %	**18.75 %**
No code	*n*	**4**	**2**	**3**	**1**	**10**
	Primary injury %	23.5 %	15.4 %	17.6 %	100 %	–
	Total %	40 %	20 %	30 %	10 %	**20.8 %**
TOTAL		**17 (35.4 %)**	**13 (27.1 %)**	**17 (35.4 %)**	**1 (2.1 %)**	**48**

patients identified from the HO-specific and non-specific ICD-10-AM code search, the true prevalence of tHO was determined to be 3.3 % (50 of 1509). Tier three captured a further 7478 patients, including 17 true tHO cases with neither HO-specific nor non-specific ICD-10-AM codes amongst trauma admissions with a LOS $\geq$ 7 days.

Analysis of ICD-10-AM coding for traumatic heterotopic ossification

A final cohort of 67 patients was confirmed to have clinical and radiographic evidence of tHO+ with a preceding traumatic injury mechanism. Thirty-seven patients (55.2 %) were coded with an HO-specific code, and 13 with a non-specific code (19.4 %). Patients with radiographical and clinical evidence of tHO, missing an HO-specific and non-specific code ($n = 17$), constituted a quarter of total true tHO cases (Fig. 1 and Table 1). The frequency and distribution of HO-specific and non-specific ICD-10-AM codes for all inpatient and outpatient tHO diagnoses, stratified according to primary injury cohort, are shown in Tables **S1 to S4.**

The frequency (code count) of HO-specific (M61) ICD-10-AM codes used for inpatient and outpatient diagnoses ($n = 37$) of tHO is shown in Fig. 2 and **Table S3.** Only a single SCI patient was identified to be incorrectly coded with the M61.1 code for fibrodysplasia ossificans progressive (FOP) and, in addition, had an atraumatic MOI and was subsequently excluded from the analysis. Therefore, in the final trauma cohort, there were zero tHO cases identified with the M61.1 code, suggesting that the ICD-10-AM coding for post-traumatic HO was used and distinguished correctly from FOP, a genetic form of pathological ectopic bone formation.

For patients with an inpatient diagnosis of tHO, only 60.4 % of patients were correctly identified with an M61 code (Table 2). Over a third of true tHO cases were identified from non-specific codes only ($n = 9$) or incidentally through manual chart review, i.e. no code ($n = 10$). A quarter of the burns cohort was not coded with the M61 code attributing tHO to the burn injury i.e. M61.3 (Calcification and ossification of muscles associated with burns). Similarly, half of the SCI cohort were coded with 'other' or 'unspecified' M61 codes instead of M61.2 (Paralytic calcification and ossification of muscle).

In a sub-cohort analysis, the reported sensitivity of M61 codes for correctly diagnosing tHO after burn injury was 50 %. Thus, for half the true tHO cases, the M61 code was not used when it should have been, returning a high rate of false negatives. Only a single false positive case was identified, equating to a 96.3 % specificity rate. The positive and negative predictive values of an M61 code were 88.9 % and 76.5 %, respectively. ROC analysis showed that M61 ICD-10-AM codes as a predictor of a confirmed positive tHO diagnosis was 0.731 (95 % CI = 0.561-0.902, $p = 0.012$), indicating only fair performance [11].

Evaluation of clinical documentation for tHO

A manual review of EMRs identified considerable variation in clinical documentation for 67 tHO cases, revealing 69 different descriptive terms used for tHO across the hospital network (Fig. 3 **and Table S5)**. A tHO diagnosis was not documented on the medical discharge summary for almost a third of patients with inpatient tHO diagnoses ($n = 48$). Thirty-eight patients had an inpatient tHO diagnosis stated on the medical discharge summary; however, 43.8 % had no evidence of tHO recorded as a 'complication' or 'co-morbidity' under secondary diagnoses. For patients under acute and rehabilitation specialities, 29.2 % of cases were not coded under the correct episode of care the initial tHO diagnosis was given.

Discussion

This study brings attention to the marked variability and inaccuracies in ICD-10-AM coding and clinical documentation for the diagnosis of tHO after burn, neurological and orthopaedic trauma. Across four major WA hospitals, 1509 patients were screened from the ICD-10-AM code search, and tHO prevalence was found to be 3.3 %; a relatively conservative finding compared to other previously published figures [12–14]. Notably, the manual chart review revealed 17 true tHO cases that were not coded with appropriate HO-specific or non-specific codes, meaning that almost half the tHO cases in the final cohort were incorrectly coded. These data support that the low overall tHO prevalence captured by ICD-10-AM codes search may, in part, be attributable to the integrity of clinical coding and documentation for the complication of tHO [10,15].

As the term heterotopic ossification refers only to the environment in which the bone process occurs, the term myositis ossificans (MO) is commonly used in the literature to classify aberrant bone formation within skeletal muscle [16]. However, tHO is not entirely specific to muscle and can develop within other soft tissue types such as joint structures, periosteum, ligaments, and tendons [17]. However, the HO-specific M61 ICD-10-AM codes are not attributable to calcification or ossification of other commonly involved soft tissue and peri-articular structures. Therefore, the inclusion of an HO-specific ICD-10-AM code that is distinct from MO of skeletal muscles, e.g. 'Calcification and ossification of the joint region, [site]' may be necessary for distinguishing MO from other sub-forms of tHO with no intramuscular involvement.

Unlike the M61.2 and M61.3 codes for tHO associated with burns and SCI/TBI, there is currently no M61 code that specifies an association with orthopaedic injury or procedures. Including an M61 code that encapsulates tHO patients treated under the orthopaedic speciality would be beneficial for a more accurate classification of tHO that is distinguishable between trauma populations. Additionally, there would be further use in the inclusion of an ICD-10-AM code for post-operative HO, which may help distinguish between tHO and HO associated with elective surgical procedures without a traumatic mechanism of injury, e.g. joint replacement – an orthopaedic population observed to have high rates of HO [18–20].

It is known that post-operative tHO following fracture is frequently asymptomatic and incidentally detected during routine post-operative

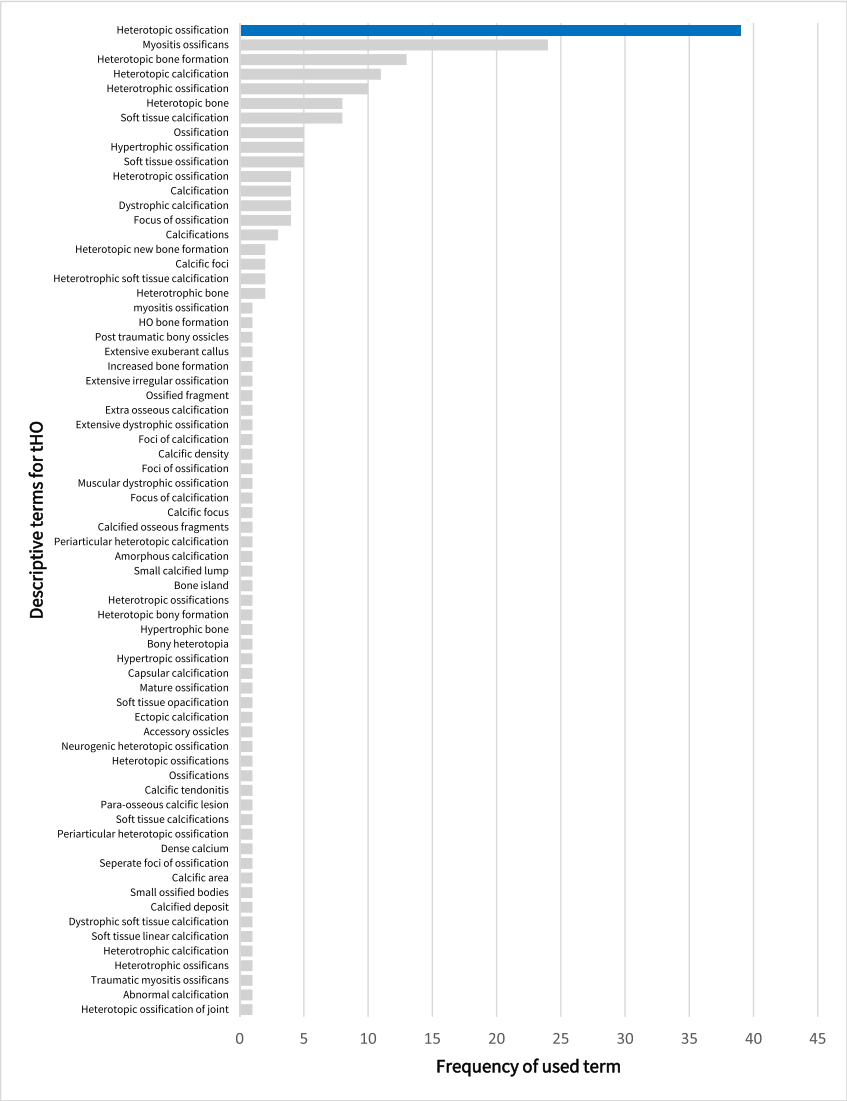

Fig. 3. Descriptive terms for tHO in decreasing order of frequency. A total of 69 individual descriptive terms relating to tHO were used by health professionals in clinical documentation for 67 true tHO cases across the network of hospitals.

radiographs [21]. A reported incidence of asymptomatic tHO was 18.78 % in a cohort of patients with elbow fractures requiring surgical fixation [22]. In the present study, the higher frequency of outpatient tHO diagnoses after orthopaedic injury could be an explanatory factor contributing to the relatively conservative estimates of orthopaedic tHO. More so, subclinical cases of asymptomatic tHO may not be captured as systematically in the outpatient setting than if they were to be routinely surveilled as an inpatient. These findings highlight the need to implement surveillance guidelines in both inpatient and outpatient settings to achieve cost-effective screening and early diagnosis of symptomatic and asymptomatic tHO cases.

This investigation noted considerable variation in documentation for tHO by clinicians across the hospital network. Clinical coders rely on the accuracy of discharge summaries completed by treating medical officers as one of the primary sources of clinical documentation for verifying clinical concepts and justifying code assignment for patients' episode(s) of care [5]. As such, using accurate and consistent documentation for tHO between clinicians and across institutions may improve the

specificity of coding for injury-specific classifications of infrequent events like traumatic HO.

Another commonly used code set in health is the SNOMED Clinical Terms [23]. SNOMED terminology and ICD-10-AM code classification enable standardisation and semantic interoperability between healthcare systems [9]. Standardised clinical languages and concepts are pivotal to this occurring [24]. However, as evidenced in the present findings, there is a deficiency of a structured and standardised vocabulary of terms and concepts used by health professionals in patient EMRs, for the naming and identification of tHO. This may have ramifications for the accurate sharing of clinical information relating to tHO diagnoses between healthcare systems, without a loss of detail or change to meaning [9]. Additionally, the analysis of clinical data relies on consistent data entry through standardised identifiers [24]. To improve the standardised data recording for tHO at the point of care, such as for patient discharge summaries, clinicians should implement SNOMED CT-AU as a reference set for consistency in using clinical terms relating to tHO.

These data lead us to suggest that clinical documentation by health professionals should inform and distinguish between the following categories of clinical information relating to tHO and referenced against SNOMED clinical terms **(Figure S2)**:

1. Primary aetiology: Traumatic vs. atraumatic vs. genetic HO (FOP)
2. Preceding injury category: neurogenic (paralytic) tHO associated with traumatic spinal cord and brain injury vs. tHO associated with burns vs. tHO related to orthopaedic trauma
3. Primary anatomical site(s): joint region vs. intramuscular site (MO)
4. Anatomical structures involved: skeletal muscle (MO) vs. other soft tissues i.e. ligament/tendon/extra-capsular/intra and extra-articular
5. Spectrum of pathological calcium deposition: calcification vs. ossification

For tHO with articular involvement, a measure of tHO severity based on existing site-specific classification schemes that appear to correlate with joint function can be used in conjunction with consistent and distinguishable clinical documentation relating to tHO diagnoses [25–27].

Although the multicentre design of this study may improve the generalisability of our findings, this study is of retrospective design, and conclusions must be confirmed in a larger, prospective investigation. Due to the large number of orthopaedic admissions without HO-specific or non-specific ICD-10-AM codes in this study ($n = 5151$), performing individual chart reviews to confirm or rule out a tHO+ diagnosis definitively was not feasible. However, the incidental identification of true tHO+ cases from manual chart review of burn and neurological admissions suggests the reported rate of orthopaedic tHO may be underestimated and, thus, a potential limitation of this study. Furthermore, as tHO determination was not uniform by a classification system and the methods of detecting tHO varied, the different tHO rates between tertiary sites could be due to interobserver error. For this reason, consensus must be made on the definition of tHO, and a standard method or classification system must be consistently implemented to reduce the heterogeneity across studies and institutions.

Overall, the present findings allude to the poor specificity of medical coding for tHO diagnoses across the WA hospital network, which may have implications for future retrospective research reliant on accurate injury diagnostic coding. In the burn cohort, the reported sensitivity of M61 codes for correctly diagnosing tHO was only 50%, indicating that using M61 diagnostic codes is less than an acceptable method to classify tHO cases after burn injury accurately. The implications of coding inaccuracies may be of even greater significance to rare disease populations such as tHO, where the true event rate and impact of risks and complications of diagnoses may be inappreciable due to poor coding practices, resulting in unreliable data for outcomes-based research [10]. Consequentially, clinician and hospital reimbursement may be impacted, which has potentially detrimental effects on the quality of future care for trauma patients.

Conclusion

This study highlights the inaccuracies in medical diagnostic coding and inconsistencies in clinical documentation for the diagnosis of tHO within the WA hospital network, which may have implications for future research and patient care. Clinicians should consistently employ standardised clinical terminology from the point of care to increase the likelihood of accurate medical diagnostic coding for tHO diagnoses. The poor specificity of M61 codes in identifying true tHO cases should be considered in future retrospective studies utilising an ICD-10-AM code search, which should incorporate a broadened search criteria to include non-specific musculoskeletal codes for identifying tHO patients.

Data availability

The data supporting this study cannot be publicly shared for ethical or privacy reasons and may be shared upon reasonable request to the corresponding author if appropriate.

CRediT authorship contribution statement

Nichola Foster: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. **Edward Raby:** Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing, Project administration, Resources. **Fiona M. Wood:** Project administration, Resources, Supervision, Validation, Writing – review & editing, Investigation, Writing – original draft. **Mark Fear:** Supervision, Validation, Writing – review & editing. **Nathan Pavlos:** Supervision, Writing – review & editing. **Dale W. Edgar:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.injury.2024.111329.

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Appendix 10: Published Article (Chapter 5, Part 1)

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Trauma patient heterotopic ossification diagnosis is associated with increased hospital length of stay

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ABSTRACT

Background: Traumatic heterotopic ossification (tHO) refers to the development of extra-skeletal bone in muscle and soft tissues following tissue insult secondary to surgery or trauma. This presents a persistent clinical concern associated with significant patient morbidity and expense to diagnose and treat. Traumatic HO is a substantial barrier to rehabilitation for trauma-injured patients. As such, the development of tHO after burn and other trauma is hypothesised to prolong inpatient length of stay (LOS) and thus increase health care costs.
Objective: To investigate the association between an inpatient tHO diagnosis and hospital LOS in trauma patients.
Methods: A retrospective audit of trauma patients over a 14-year period was completed using data from four WA hospitals. Burn and neurological trauma patients diagnosed with tHO as an inpatient (tHO+) and control subjects (tHO-), matched (1:3) by age, gender, and injury severity factors, were identified using medical diagnostic codes. Data relating to patient and injury-related determinants of LOS from tHO+ and tHO- subjects were analysed to model the association of tHO on total hospital length of stay.
Results: 188 identified patients were hospitalised due to traumatic injury; 47 patients with tHO following burn injury ($n = 17$), spinal cord injury ($n = 13$) and traumatic brain injury ($n = 17$), and 141 control patients. Those who developed tHO during hospitalisation had a significantly higher median LOS than matched trauma patients who did not develop tHO (142 days *vs.* 61 days). Multivariate regression analyses identified the following independent predictive factors of a prolonged hospital LOS: tHO diagnosis, mechanical ventilation hours, injury to the hip region and thigh area, other ossification disorder, pressure injury, admission to intensive care unit and deep vein thrombosis. Trauma patients diagnosed with tHO during their hospital admission stayed 1.6 times longer than trauma patients matched for injury severity without a tHO diagnosis (IRR 1.56, 95% CI 1.35–1.79, $p<0.001$).
Conclusion: Traumatic heterotopic ossification is an independent explanatory factor for increased hospital LOS in patients following burns, spinal cord, and traumatic brain injury. Early diagnosis may assist in reducing the impact of tHO on acute hospital stay after trauma.

Introduction

Traumatic heterotopic ossification (tHO) refers to the pathological formation of mature, lamellar bone at non-skeletal sites, such as within muscle and connective tissue [1–3]. Cases of tHO have been reported following high-velocity blast injury [3,4]; traumatic brain injury (TBI) and spinal cord injury (SCI) [5–7]; and after fracture, dislocation and soft tissue trauma [8]. Post-traumatic HO [9], traumatic HO [10] or

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acquired HO [11] are used interchangeably in today's literature.

Traumatic HO can be a substantial barrier to rehabilitation for trauma-injured patients. Typically, tHO manifests as a progressive condition marked by rapid movement loss at affected joints and severe pain [12]. Secondary complications such as nerve entrapment and muscle weakness are associated with ectopic bone formation. Long-standing joint dysfunction is often compounded by secondary scar and soft tissue contracture, joint arthrosis, muscle atrophy and articular cartilage degeneration [12]. The myriad of symptoms further impacts the rehabilitation trajectory for these already functionally compromised individuals after trauma, accounting for impaired patient quality of life and significant caregiver burden [12,13]. Due to the functional impairments associated with tHO, increasing evidence indicates a relationship between tHO and a prolonged hospital length of stay (LOS).

The length of hospital stay is an essential metric for assessing the quality of trauma care, and it is a marker of costs and resource utilisation in trauma centres [14]. Decreased LOS has been associated with a reduced risk of hospital-acquired infections and improved treatment outcomes and patient quality of life [15]. To ensure trauma units can operate efficiently, it is vital to determine causes for and suggest potential solutions to the long-term occupation of beds [16]. Injury severity, ICU admission, surgical interventions, injury complications and comorbidities have previously been identified as predictors of prolonged hospital stay in trauma patients [14,17,18]. However, the independent influence of tHO on LOS after burn and neurological trauma has not been evaluated. Reliable predictions of LOS can enable hospitals to plan and allocate resources efficiently, tailor individual care for patients, and set reasonable patient expectations. Thus, the primary aim of this study was to assess the relationship between tHO and hospital LOS. It was hypothesised that tHO would be associated with an increased index hospital LOS compared to patients of the same injury severity with no tHO diagnosis.

Methods

Study participants

A retrospective data audit was conducted, including patients admitted to three WA tertiary hospitals: Sir Charles Gairdner Hospital (SCGH), Fiona Stanley Hospital (FSH) and Royal Perth Hospital (RPH), and a trauma rehabilitation facility, Osborne Park Hospital (OPH). The inclusion criteria were adult patients following neurological (TBI and SCI) or burn injury and or tHO diagnosed during inpatient hospital stay; patients aged 18 years or over at the time of admission; discharge was recorded between 1st May 2005 and 1st May 2019. Subjects were excluded if death occurred or comfort care/palliation was instigated during their hospital stay.

Identification of tHO+ cohort

To identify HO diagnoses in various tissues, a limited data set request was conducted using the WA trauma database for patients with HO-specific ICD-10-AM (*International Statistical Classification of Diseases and Related Health Problems, Tenth Revision, Australian Modification*) codes (M61 for HO). Additional miscellaneous musculoskeletal 'non-specific' HO codes were also included in the broadened search to capture all patients with a possible diagnosis of tHO. Both primary and secondary diagnostic codes were eligible for inclusion. For all patients with specific (n = 87) and non-specific HO diagnosis codes (n = 1422), individual medical records and imaging were screened by a single reviewer (NF) to confirm an inpatient tHO diagnosis and traumatic injury mechanism. The anatomical site(s) of tHO were recorded, and the distribution of affected site(s) is shown in Table S4. Including patients with an inpatient rather than an outpatient diagnosis of tHO was assumed to be the most indicative of influence on total LOS. A total of 67 patients were confirmed to have clinical and radiographic evidence of tHO with a preceding traumatic injury mechanism, of which 48 patients were diagnosed with tHO as inpatients. A final cohort of 47 tHO+ patients met the inclusion criteria and comprised the study group.

Identification of tHO- cohort

A search of the WA trauma database was conducted using injury-specific search criteria diagnosis-related group codes for injury severity and via manual chart review to identify control subjects: patients with evidence of a traumatic injury mechanism and no diagnosis of tHO, matched (1:3) by age, gender, and injury severity factors. A total of 33,879 trauma admissions were captured in the initial search and then filtered to identify trauma patients with an inpatient hospital LOS $\geq$7 days. Time to tHO diagnosis has been reported to occur as early as two months from injury onset and in patients with greater injury severity and a prolonged hospital LOS [19,20]. Therefore, the filter criteria of a LOS $\geq$7 days ensured an injury cohort at higher risk of developing tHO was captured. A cohort of 7478 trauma patients with a LOS $\geq$7 days was subsequently screened to identify subjects according to the matching criteria, comprising the control group (tHO-, n = 141). All patient demographic and clinical variables that comprised the matching criteria per injury cohort were crosschecked via medical record and imaging review.

Classification of injury severity

Comparison of tHO+ and tHO- groups according to matching criteria is shown in Table 1. In addition to injury-specific severity indices, injury severity was also compared between groups using the abbreviated injury scale (AIS). The AIS is an anatomically-based injury severity scoring system that classifies each injury by body region on a six-point ordinal scale [21,22]. The AIS was calculated for index admission for the respective trauma-related injury according to the primary injury category: AIS-burn, AIS-spine and AIS-head (Table 1). Rather than relying on ICD-10 discharge codes, the medical records and images were analysed to ensure accuracy, and AIS scores were assigned using AIS 2008 scoring paradigms [22,23].

The severity of burn injury was indicated by Total Body Surface Area (TBSA) at the time of injury, and patients were matched according to their TBSA (%). Burn-injured patients were also scored according to the AIS related to TBSA and depth of burn injury, denoted as AIS-burn (Table 1 and Table S1) [24].

For SCI patients, the level and extent of a neurological deficit are measured by the Neurological Level of Injury (NLI), The International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) and the American Spinal Injury Association (ASIA) impairment scale, scored at the time of hospital admission [25]. These data were supplemented with AIS coding data; the score for the spine body region was retrieved and denoted as AIS-spine. However, as with burn injury, the AIS has limitations as a predictor of mortality after SCI [24,26]. Consistent with AIS codes, SCI patients were classified as complete or incomplete injuries at the cervical (subclassified: C3 or above, C4 or below), thoracic, or lumbar spine, with or without associated fractures or dislocations at the same level as the traumatic SCI [27] (Table 1 and Table S2).

Traumatic brain injury refers to brain injury acquired through a traumatic event, and the severity of the injury can be categorised as mild, moderate, severe and extremely severe [28]. The severity indices used in this study were the Glasgow coma scale (GCS) and duration of post-traumatic amnesia (PTA), determined using a validated screening tool, [29]. Head AIS, in combination with GCS, has been used to rate the severity of head injury for selecting and stratifying patients for clinical studies of TBI [30–32]. Thus, the severity of TBI was further defined by an AIS-head score (AIS 1 to 2, 3 to 4 and 5–6 indicate mild, moderate and severe TBI, respectively [33] (Table 1 and Table S3).

Table 2
Univariate analysis of length of stay variables for tHO+ and tHO- subjects. Categorical variables are presented as n (%). Continuous variables are presented as median (interquartile range). Due to model of care differences for burn and neurological trauma patients, LOS outcomes for acute and rehabilitation episodes of care are comparable only in the neurological cohorts. LOS: length of stay, tHO: traumatic heterotopic ossification, SCI: spinal cord injury, TBI: traumatic brain injury, ICU: intensive care unit, p: p-value.

	Burn			Neurological (SCI)			Neurological (TBI)			TRAUMA (TOTAL)		
	tHO+	tHO-	*p*	tHO+	tHO-	*p*	tHO+	tHO-	*p*	tHO+	tHO-	*p*
ICU												
n	17	51		12	39		14	51		43	141	
LOS	21 (17–34)	5 (0–11)	<0.001	9 (2–22)	7 (3–11)	0.482	11 (7–13)	9 (6–12)	0.423	14 (7–24)	7 (3–11)	<0.001
Acute care												
n	–	–	–	13	38		17	51		30	140	
LOS	–	–	–	24 (14–52)	19 (16–26)	0.265	35 (30–59)	26 (19–34)	<0.001	32 (24–54)	23 (17–31)	<0.001
Rehabilitation												
n	–	–	–	13	39		17	51		30	141	
LOS	–	–	–	126 (86–162)	122 (76–156)	0.684	110 (34–212)	37 (18–64)	0.003	114 (46–206)	64 (29–118)	0.025
Total LOS												
n	17	51		13	39		17	51		47	141	
LOS	123 (67–154)	30 (19–52)	<0.001	178 (144–235)	142 (101–180)	0.108	113 (69–196)	61 (44–97)	0.001	142 (74–178)	61 (31.5–113)	<0.001

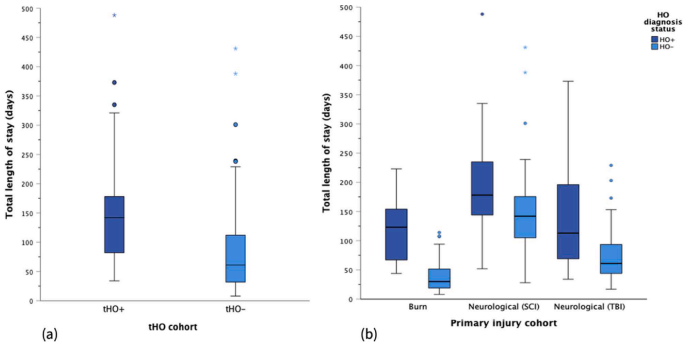

Fig. 1. (a) Box plots of the total length of hospital stay (LOS) stratified by HO diagnosis status and (b) by primary injury cohort.

injury (median 114 *vs.* 64 days; $p = 0.006$). Rehabilitation LOS was the highest in the tHO+ cohort after SCI (median 126 days); however, a significant difference in rehabilitation LOS was only identified between the two tHO groups following TBI (median tHO+, 110 [IQR 34–212] days *vs.* tHO-, 37 [IQR 18–64] days; $p = 0.003$).

Covariates significantly associated ($p<0.05$) with an increasing total LOS to be included in the initial model for backwards stepwise multivariate logistic regression analyses were identified by univariate analysis and are shown in Table S6; ICU admission, ICU LOS, mechanical ventilation hours; concomitant injuries at admission: long bone fracture, injury to the hip region or thigh area, central nervous system (CNS) or peripheral nervous system(PNS) injury; comorbidities: other ossification disorder; complications during hospital stay: pressure injury, deep vein thrombosis (DVT), urinary tract infection (UTI) and sepsis. After backward stepwise elimination, the relationships between total LOS and ICU LOS, long bone fracture, UTI and sepsis were insignificant. Following the elimination process, the final model presents the influential factors for longer LOS, demonstrating that the diagnosis of tHO is an important factor for LOS when other relevant factors are accounted for (Table 3).

The resulting incidence rate ratios indicate that patients who develop tHO during hospitalisation are predicted to stay in hospital 56% longer (IRR 1.56, 95% CI, 1.35–1.79, $p<0.000$) than trauma patients who do not develop tHO. The Estimated Marginal Means corresponded to an average increase in adjusted total LOS for a tHO+ case of 2.4 days compared to 1.9 days for matched trauma controls (Figure S1).

Table 3
The final model for multivariate negative binomial regression examining predictors of increased hospital length of stay. tHO: Traumatic heterotopic ossification, ICU: intensive care unit; DVT: deep vein thrombosis, IRR: incidence rate ratio, CI: confidence interval, p: p-value.

VARIABLES ASSOCIATED WITH TOTAL LOS	IRR	95% CI	*p*
tHO diagnosis	1.56	1.35 – 1.79	<0.001
Mechanical ventilation (hours)	1.00	1.0002 – 1.0005	<0.001
Injury to hip region and thigh	1.48	1.24 – 1.76	<0.001
Other ossification disorder	1.33	1.16 – 1.53	<0.001
ICU admission	1.38	1.09 – 1.74	0.007
Pressure injury	1.34	1.15 – 1.57	<0.001
DVT	1.20	1.01 – 1.42	0.035

When ordered by IRR, the factor most strongly associated with increasing LOS was HO diagnosis (IRR 1.56; 95% CI 1.35–1.79, $p<0.000$). Following tHO, occurrence of individual concomitant injuries and critical care variables were independently influential on LOS: injury to the hip region and thigh (IRR 1.48; 95% CI 1.24 – 1.76, $p<0.000$), ICU admission (IRR 1.38; 95% CI 1.09–1.74, $p<0.007$), pressure injury (IRR 1.34; 95% CI 1.15–1.57, $p<0.000$), other ossification disorder (IRR 1.33; 95% CI 1.16–1.53, $p<0.000$), DVT (IRR 1.20; 95% CI 1.09–1.01–1.42, $p<0.035$) and mechanical ventilation hours (IRR 1.00; 95% CI 1.0002 – 1.0005, $p<0.000$).

Discussion

This is the first study to show that tHO is independently associated with greater in-hospital LOS for patients following burn, spinal cord, and traumatic brain injury. Patients diagnosed with tHO during hospitalisation stayed 56% longer than matched trauma patients who did not develop tHO. Thus, the study also demonstrated the value of pooling comparative epidemiological data on LOS outcomes from individual tHO populations across four trauma centres. After adjustment for age, gender, injury severity, critical care factors and the presence of multiple co-morbidities, the seven determinants of hospital LOS were diagnosis of tHO, injury to the hip region and thigh, ICU admission, pressure injury, other ossification disorders, and mechanical ventilation hours. This study presents a novel quantification of the impact of acute tHO on the health care system and is a first step towards identifying the health resource costs associated with the complication of tHO.

Trauma patient LOS is highly variable and is closely governed by factors beyond the diagnoses, injury severity and procedures, including pre-existing co-morbidities, post-operative and injury-related complications, rehabilitative needs and resources or options for discharge disposition [14,34,35]. No prior study has robustly assessed the independent contribution of a tHO diagnosis to the LOS trajectory in burn and neurological trauma patients. Previous data have only allowed a superficial examination of such factors that may be associated with a prolonged hospital stay in tHO cohorts and have shown only a weak association or no attempt was made to subject these factors to a multivariate analysis owing to the inclusion of a relatively small sample size. As such, this study differs from others assessing the association between tHO and length of hospital stay.

Previous work has emphasised the contribution of critical card factors such as ICU admission and the requirement for mechanical ventilation to a prolonged LOS in trauma patients [36,14]. Our results demonstrate similar effects of an ICU admission (IRR 1.37, $p = 0.007$) and mechanical ventilation hours (IRR 1.00, $p<0.001$) on increasing LOS. However, in contrast to previous work, tHO was determined to be a significant predictor of an increased total LOS, independent of the effects of an increasing ICU LOS and mechanical ventilation time [19,37,38]. Thus, a prolonged ICU admission is not necessarily a causal factor in increased total LOS in the presence of a tHO diagnosis.

There are several possible explanations for these findings. Traumatic HO increases the risk of health morbidities, such as acute and chronic pain, pressure ulcers and impaired wound healing [12]. More so, HO-specific symptomology shares similarities with other acute inflammatory conditions such as DVT, cellulitis, and osteomyelitis, posing challenges for early, definitive tHO diagnosis [39]. Without accurate and reliable early diagnosis, there is little change of early intervention, and late detection fails to limit the unabated progression of tHO. As such, tHO further impedes patients' physical and psychosocial functioning through their rehabilitation [12]. Loss of movement is associated with poor functional outcomes, and a lack of normal movement negatively impacts areas such as personal care, participation in social roles, and returning to independent daily living [12]. Reduced functional independence increases the reliance on supportive others when transitioning home from the rehabilitation facility, complicating discharge planning and likely delaying time to discharge [12]. These factors support the argument that tHO prolongs hospital LOS and increases medical care utilisation and associated costs.

Other ossification disorders captures co-morbid conditions, including pre-existing or new inflammatory conditions of muscle and bone, *e.g.*, myositis, osteomyelitis, and previous fractures. Vascular calcification, renal and bladder calculi, and other pathological calcification and mineralisation diseases such as osteophyte formation associated with osteoarthritis, enthesopathies and ankylosing spondylitis [40]. The evaluation of mechanisms related to such disease states that resulted in increased length of hospital stay was not primarily investigated in the present research. However, these might confound the relationship between tHO and prolonged hospital LOS. Screening for co-morbidities (other ossification disorders) and concomitant injuries (injury to the hip region and thigh) on admission and monitoring of complications (pressure injury and DVT) may help improve LOS outcomes for patients hospitalised due to traumatic injury.

Factors influencing outcomes have variable influences at different times during hospitalisation, emphasising that different phases of care may have different optimal therapies [41]. Univariate analysis showed that TBI patients with tHO had a significantly longer rehabilitation LOS than controls and other primary injury cohorts. However, time to diagnosis was the earliest in this group (38 days [IQR 33–58], $p = 0.020$), which is similar to previous reports [38,42,43]. Differences between injury cohorts may be attributable to differentiations in spatiotemporal patterns of disease onset and progression between primary injuries. Early disease onset after TBI may lead to a more established and clinically significant disease progression during rehabilitation, affecting functional mobility and prolonging LOS in the rehabilitation unit. Or, it may be attributable to the consistency and implementation of systematic screening protocols and therapeutic strategies for tHO in respective acute and rehabilitation facilities. However, no established clinical guidelines are available for clinicians in WA hospitals to guide early, routine surveillance of tHO in trauma patients.

It is worth noting that patients who encounter severe burns experience long periods of reduced consciousness and narcotic and sedating medications and, thus, are likely to have difficulties processing and communicating information, such as their experience of tHO symptoms [44]. Similarly, TBI patients experience protracted periods of PTA, often compounded with cognitive and language deficits. These communication barriers are of particular relevance for detecting clinical suspicion of tHO in burn and neurological trauma patients, as the onset of tHO and experience of symptoms appears to occur prior to evidence on conventional radiographs [12]. This suggests a possible benefit from clinical trials involving the conduction of an early surveillance program comprising early risk stratification by clinicians and routine screening of high-risk patients utilising diagnostics sensitive for detecting early or potential tHO lesions such as bedside ultrasound or three-phase bone scintigraphy [39,45].

Overall, the findings of this study emphasise the importance of addressing tHO diagnoses at specific points in hospitalisation. Identifying critical care junctures can guide interventions for improved quality of care and patient outcomes in at-risk trauma populations. There is a need for standardised patient management protocols for early surveillance and interventions targeting tHO prevention, and it is important to consider tHO patients as a distinct population when allocating resources or planning quality improvement interventions. Early diagnosis through increased clinician awareness, patient education, surveillance, and improved diagnostic algorithms can facilitate prompt treatment and slow disease progression. This approach may effectively improve patients' functional outcomes and reduce the requirement for an extensive rehabilitation stay and the risk of readmission.

Limitations

This study has several limitations. The determination of tHO was not uniform within the classification system and the methods of detecting tHO varied. As such, in the investigation of images, the detection sensitivity and determination of tHO may differ amongst the various institutes. Although purposeful multi-centre and multi-diagnosis study design may improve the generalisability of our findings to other burn and trauma centres, this retrospective-cohort study has limited causative conclusions, and results need to be confirmed in future prospective clinical trials. Finally, despite controlling for many known confounding variables through matching criteria and choice of statistical tests for analysis, it is possible that other variables with an effect on hospital LOS were not included in the results.

Future research

It is understood that patients with a longer LOS have significantly higher odds of readmission, and increased readmission rates may reflect different processes of care [46]. The present study did not explicitly investigate re-admission rates associated with tHO due to the high frequency of patients receiving tHO-related care in the private sector; thus, tHO-related re-admission rates could not be accurately captured within the included network of hospitals. Due to the chronicity of primary tHO symptoms and associated secondary morbidities, tHO patients are at higher risk of re-hospitalisation [12]. Further, the magnitude of the financial cost associated with tHO has not been thoroughly investigated in trauma populations. Some insight may be gained from an international survey examining the burden of illness of fibrodysplasia ossificans progressive (FOP). The mean cost of care (~$164,000 U.S. dollars per year) highlights the increasing financial burden of severe FOP-related mobility restrictions [13]. To fully elucidate the burden of illness associated with tHO and ensure comprehensive measurement of LOS outcomes, tHO-related re-admission rates and the associated financial costs should be considered in future investigations.

Conclusion

This study showed that a diagnosis of traumatic heterotopic ossification during hospitalisation independently prolonged hospital LOS for burn and neurotrauma patients. This investigation presents a novel first step towards understanding the economic impact of acute tHO. It offers valuable insight into healthcare service utilisation and resource costs associated with LOS outcomes in tHO patients.

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CRediT authorship contribution statement

Nichola Foster: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Lisa Martin:** Formal analysis, Methodology, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **Edward Raby:** Supervision, Validation, Writing – review & editing, Writing – original draft. **Fiona M. Wood:** . **Mark Fear:** Project administration, Supervision, Writing – review & editing. **Nathan Pavlos:** Supervision, Validation, Writing – review & editing. **Dale W. Edgar:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

None.

Data availability

The data supporting this study cannot be publicly shared for ethical or privacy reasons and may be shared upon reasonable request to the corresponding author if appropriate.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.injury.2024.111328.

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