The Role of the Osteocyte in Periprosthetic Osteolysis

Renee Therese Ormsby

B.Sc (Medicinal Chemistry)

Thesis by Publication and Convention submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

February 2019

Biomedical Orthopaedic Research Group

Discipline of Orthopaedics and Trauma

Adelaide Medical School

Faculty of Health and Medical Sciences

The University of Adelaide

South Australia,

Australia

THE UNIVERSITY of ADELAIDE

TABLE OF CONTENTS

Thesis Abstract _____ ***v***

Declaration _____ ***ix***

Acknowledgements _____ ***x***

Arising Publications _____ ***xi***

Published Abstracts and Scientific Communications _____ ***xii***

Previous Publications by the Author _____ ***xv***

Prizes and Awards _____ ***xvii***

Abbreviations _____ ***xviii***

Chapter 1 Literature Review _____ ***1***

Introduction _____ *6*

Wear particles _____ *7*

Metal wear particles _____ *7*

Ultra-high molecular weight polyethylene wear particles _____ *8*

Highly cross-linked PE _____ *9*

Vitamin E _____ *9*

Ceramic and PMMA particles _____ *11*

Wear particle corrosion _____ *12*

Cellular responses to wear particles _____ *12*

Monocytes/Macrophages _____ *13*

The role of Toll-like Receptor and PAMPs _____ *14*

Osteoclasts 15

Osteoblasts 18

Osteocytes 19

Osteocytic Osteolysis 20

Osteocytes and wear particles 21

Potential non-surgical treatment approaches 23

Conclusions 25

Acknowledgements 26

References 27

***Chapter 2 Evidence that osteocyte perilacunar remodelling contributes to polyethylene wear particle induced osteolysis* 52**

***Chapter 3 Examination of Changes in Osteocyte Morphology in a Murine Femoral Model of Osteolysis* 65**

Abstract 66

Introduction 67

Materials and Methods 68

Mouse femoral pump model 68

Histological analysis of mouse femurs 69

Results and Discussion 69

Conclusions 75

References 76

***Chapter 4 Osteocytes respond to particles of clinically-relevant conventional and cross-linked polyethylene and metal alloys by up-regulation of resorptive and inflammatory pathways* 79**

Chapter 5 The Effects of Vitamin E Analogues α-Tocopherol and γ-Tocotrienol on the Human Osteocyte Response to Ultra-high Molecular Weight Polyethylene Wear Particles ***93***

Abstract *97*

Background *97*

Methods *97*

Results *97*

Discussion *98*

Introduction *99*

Materials and Methods *101*

Human osteocyte-like cells *101*

Gene Expression *102*

In vitro mineralisation *102*

Results and Discussion *103*

Effects of UHMWPE and Vitamin E on the oxidative stress response *103*

Effects of UHMWPE and Vitamin E on the pro-osteoclastogenic response *104*

Effects of UHMWPE and Vitamin E on the osteocytic osteolysis response *105*

Effects of Vitamin E alone on osteocytic regulation of mineralisation *106*

Conclusion *106*

References *108*

Tables *112*

Figures *113*

Chapter 6 Evidence for gender-specific pathological bone loss mechanisms in the development of periprosthetic osteolysis ***117***

Abstract *121*

Introduction 122

Materials and Methods 124

Ethical Statement 124

Patient demographics 124

Osteocyte lacunar measurements from human bone biopsies 124

Statistical Analysis 125

Results 125

Radiographic evidence of osteolysis in patients undergoing revision THR 125

Analysis of human bone biopsies from patients undergoing Primary THR and Revision THR surgery 125

Osteocyte lacunar assessments of each primary and revision THR patient biopsy 126

Osteocyte characteristics of primary and revision THR cohorts 126

Osteocyte characteristics of Female THR and Male THR bone biopsies 127

Discussion 127

Conclusion 131

Acknowledgements 131

References 132

Tables 137

Figures 140

Chapter 7 Concluding Remarks **146**

Thesis Abstract

Total hip replacements (THR) are a common and successful procedure for relieving pain and restoring movement in patients with progressive bone disease. However, failure of the implant can occur over time, often as a result of loosening of the implant, which is commonly due to the development of osteolytic lesions. Osteolytic lesions have been shown to be associated with the production of bioactive wear particles derived from the prostheses components. Both metal from wear of the femoral stem, femoral head and acetabular cup, and ultra-high molecular weight polyethylene (UHMWPE) from the liner have been detected within the periprosthetic tissue where they are capable of stimulating biological pathways, including inducing inflammation and bone resorption. These particles have also been detected in the mineralised bone matrix. The osteocyte is the most abundant cell in the bone; however there is an incomplete understanding of how osteocytes respond to wear particles and contribute to the development of osteolysis. Therefore, in this thesis we investigated the effect of clinically relevant particles on osteocytes in both *in vitro* and *in vivo* models, and their potential role in wear particle induced osteolysis.

This study investigated the effects of clinically relevant UHMWPE and metal particles, derived from currently used prostheses, on primary human cells *in vitro*. Specifically, human osteocyte-like cells were exposed to metal and UHMWPE particles *in vitro*. Additionally, further *in vitro* experiments were performed to measure the effects of vitamin E in combination with UHWMPE particles. Gene expression analysis was employed using real time RT-PCR for all *in vitro* experiments. Analysis of biochemical

markers was also performed. Two murine models of osteolysis were analysed, including a calvarial model of osteolysis, already established by our group, as well as a mouse femoral pump model of aseptic loosening. Both models were analysed for osteoclastic resorption and osteocytic osteolysis with exposure to UHMWPE particles. Importantly, we also obtained human bone biopsies from patients undergoing either primary THR or revision THR with confirmed osteolysis, which were processed for histology and analysed for changes in osteocyte lacunar area.

Osteocyte-like cells exposed to two types of metal alloy particles, Ti6Al4V and CoCrMo showed an upregulation of genes, including MMP13, CA2 and CTSK, associated with osteocytic osteolysis. Similarly, this was observed with exposure to UHMWPE particles (conventional and XLPE), and with the addition of vitamin E. This was the first evidence of osteocytes responding to wear particles by inducing osteocytic osteolysis. Additionally, significant upregulation of inflammatory markers was observed with all particle types. In addition, evidence of a pro-osteoclastic response was observed with exposure to metal and UHMWPE particles, which was interestingly reversed with vitamin E treatment, suggesting a role for vitamin E in regulating bone resorption. Apoptosis was also observed by significantly upregulation of Caspase 3 with exposure to metal particles, an effect not observed with UHMWPE particles. Furthermore, inhibition of Caspase 3 stimulated expression of resorption markers, suggesting that inhibition of osteocyte apoptosis is important for regulating bone remodelling. Vitamin E is a known antioxidant, therefore we measured the expression of antioxidant genes, SOD1, SOD2 and Catalase. Vitamin E significantly upregulated these genes, interestingly, UHMWPE particles also induced upregulation of the antioxidant genes, which suggests UHMWPE particles may play a role in regulating oxidative stress. We investigated the effects of UHMWPE particles in two murine models of osteolysis; a calvarial model of osteolysis and a femoral

pump model with continuous UHMWPE particle delivery. We observed a loss of bone in both models with exposure to UHMWPE particles which was associated with an increase in osteoclastic resorption. However, there were distinct differences between the models with regards to osteocyte morphology. In particular, we observed osteocyte lacunae enlargement in the static calvarial model of osteolysis, in addition to the osteoclastic resorption noted by μ-CT analysis. In contrast, there was a significant reduction in osteocyte lacunar area in the femoral pump model. Conserving osteocyte lacunar structure in this model would play an important role in maintaining both the viability and the structure of the bone.

This study also included analysis of human bone biopsies obtained from revision THR surgeries with confirmed osteolysis. Histological analysis of the biopsies confirmed the presence of wear particles, as well as significant enlargement of the osteocyte lacunar areas when compared to primary THR biopsies. Further in depth analysis of the cohort revealed a significant increase in osteocyte lacunar areas in female revision THR biopsies compared to primary THR. This suggests that the female osteocyte response to particles may be preferentially directed towards osteocytic osteolysis. Whereas, male revision biopsies showed an increase in the percentage lacunar area per total bone area compared to male primary THR, which suggests a loss of bone associated with osteoclastogenesis in males. This is the first evidence of osteocytes contributing to wear particle induced osteolysis, as well as a gender specific response to wear particles. Therefore, this study suggests that female osteocytes are potentially pre-programmed to undergo osteocytic osteolysis under conditions of rapid bone loss.

In conclusion, the experiments performed and analysed in this thesis were designed to obtain mechanistic clues for the involvement of osteocytes in osteolysis with implications for the clinical translation of this work. This thesis confirms the hypothesis that osteocytes

exposed to wear particles stimulate both osteoclastic bone resorption and osteocytic osteolysis, with potential gender-specific responses. The evidence of osteocyte enlargement in human biopsies suggests that osteocytes potentially contribute to the development of osteolytic lesions. Therefore, osteocytes could play important roles as future targets for therapeutics in the prevention of osteolysis, and the consequent aseptic loosening.

Declaration

I, Renee Ormsby, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holders of those works.

I give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Signed: Date:

Acknowledgements

This thesis would not have been possible without the encouragement, support and

Help of my colleagues and supervisors at the University of Adelaide.

All this hard work could not have been accomplished without them and their support.

Needless to say, I would like to mention the support and love of my family, I

Know without them, I would not have had the confidence to pursue this degree.

Similarly, I'd like to thank my close friends and colleagues for putting up with me and taking the time to listen to me when things got tough the last year.

Odds are I wouldn't have stayed sane!

Working on this thesis for the last three years has both motivated me and inspired me to explore more ideas and think beyond the scope. It has been a challenge, nevertheless, it is not something I will ever regret. With this chapter behind me, I'm excited for new experiences and adventures pursuing my career in research.

Thanks to Dr Matt Prideaux, Dr Masakazu Kogawa, Dr Asiri Wijenayaka, Dr Yolandi Starczak, Dr Dongqing Yang, Catherine Stapledon, Shelley Hay, Dr Agatha Labrinidis, Dr Andrew Turner, Kate Barrett, Helen Tsangari, Erica Beaucage-Gauvreau, Dr Mario, Rhys Hamon and all the other people on L7 at the AHMS.

Thanks to my supervisors, Prof. Gerald Atkins, Prof. David Findlay and Prof. Bogdan Solomon.

Arising Publications

Ormsby, RT., Cantley M., Kogawa M., Solomon LB., Haynes DR., Findlay DM., Atkins GJ. *Evidence that osteocyte perilacunar remodelling contributes to polyethylene wear particle induced osteolysis*. Acta Biomaterialia. 2016 Mar; 33:242-51. doi: 10.1016/j.actbio.2016.01.016. (Published)

Ormsby R.T., Solomon, L.B., Findlay, D.M., & Atkins, G.J. (2018) *Aseptic loosening and periprosthetic osteolysis: wear now?* World Journal of Orthopedics. (In preparation for publication)

Ormsby R.T., Solomon, L.B., Yang, D., Crotti, T.N., Haynes, D.R., Findlay, D.M., & Atkins, G.J. (2019) *Osteocytes respond to clinically relevant UHMWPE and metal wear particles by up-regulation of resorptive and inflammatory pathways*. Acta Biomaterialia. (Published)

Ormsby R.T., Hosaka K., Evdokiou, A., Solomon L.B., Findlay, D.M., & Atkins, G.J. (2018) *The effects of polyethylene wear particles and vitamin E on osteocytes in vitro*. Journal of Orthopaedic Science. (In preparation for publication)

Ormsby R.T., Solomon, L.B., Findlay, D.M., & Atkins, G.J. (2018) *Evidence for gender-specific pathological bone loss mechanisms in the development of periprosthetic osteolysis*. Journal of Orthopaedic Research. (In preparation for publication)

Published Abstracts and Scientific Communications

Renee T Ormsby, Tania N Crotti, David R Haynes, L. Bogdan Solomon, David M Findlay and Gerald J Atkins. *The pro-catabolic effect of metal wear particles on osteocytes in vitro.* (2015). ASMR SA Annual Scientific Meeting, Adelaide, South Australia. (Poster)

Renee T Ormsby, Melissa Cantley, L. Bogdan Solomon, Dongqing Yang, David R Haynes, David M Findlay and Gerald J Atkins. *Evidence that osteocyte perilacunar remodeling contributes to polyethylene wear particle induced osteolysis.* (2015) ANZBMS 25th Annual Scientific Meeting, Hobart, Tasmania. (Poster)

Renee T Ormsby, Tania N Crotti, David R Haynes, L. Bogdan Solomon, David M Findlay and Gerald J Atkins. *Osteocytes And Their Role In Wear Particle Induced Periprosthetic Osteolysis* (2016). ASMR SA Annual Scientific Meeting, Adelaide, South Australia. (Oral Presentation)

Renee T Ormsby, Melissa Cantley, L. Bogdan Solomon, Dongqing Yang, David R Haynes, David M Findlay and Gerald J Atkins. *Evidence that osteocyte perilacunar remodeling contributes to polyethylene wear particle induced osteolysis*. (2016) ORS Annual Meeting (International meeting), Orlando, Florida, U.S.A. (Poster)

Renee T Ormsby, Dongqing Yang, L. Bogdan Solomon, Tania N Crotti, David R Haynes, David M Findlay and Gerald J Atkins. *Osteocytes and their role in wear particle induced periprosthetic osteolysis* (2016) ANZBMS 26th Annual Scientific Meeting, Gold Coast, Queensland, Australia. (Poster)

Renee T Ormsby, Dongqing Yang, L. Bogdan Solomon, Tania N Crotti, David R Haynes, David M Findlay and Gerald J Atkins. *Osteocytes and their role in wear particle induced periprosthetic osteolysis.* (2016). University of Adelaide, Faculty of Health and Medical Sciences 10th Florey Postgraduate Research Conference. (Poster)

Renee T Ormsby, Lucian B Solomon, Dongqing Yang, David R Haynes, David M Findlay and Gerald J Atkins. *Osteocyte responses to UHMWPE and XLPE wear particles.* (2016) ANZORS 22nd Annual Conference, Melbourne, Victoria, Australia. (PhD Award Final Oral Presentation)

Renee T Ormsby, Lucian B Solomon, Dongqing Yang, David R Haynes, David M Findlay and Gerald J Atkins. *The effects of Vitamin E and Polyethylene wear particles on osteocytes in vitro: implications for periprosthetic osteolysis.* (2017) ANZBMS 27th Annual Scientific meeting, Brisbane, Queensland, Australia. (Poster)

Renee T Ormsby, Andreas Evdokiou, Kunihiro Hosaka, Tania Crotti, David R Haynes, L. Bogdan Solomon, David M Findlay and Gerald J Atkins. *The Effects of Vitamin E and Polyethylene Wear Particles on Osteocytes In Vitro: Implications for Periprosthetic*

Osteolysis (2017) ANZORS 23rd Annual Conference, Adelaide, South Australia, Australia. (Oral Presentation)

Renee T Ormsby, Kunihiro Hosaka, Andreas Evdokiou, David R Haynes, L. Bogdan Solomon, David M Findlay and Gerald J Atkins. *The Effects of Vitamin E and Polyethylene Wear Particles on Osteocytes In Vitro* (2018) University of Adelaide, Faculty of Health and Medical Sciences 10th Florey Postgraduate Research Conference. (Poster)

Renee T Ormsby, L. Bogdan Solomon, David M Findlay and Gerald J Atkins. *Evidence For Gender-Specific Pathological Bone Loss Mechanisms In The Development Of Periprosthetic Osteolysis* (2018) Australian Orthopaedic Association SA/NT Branch Scientific Meeting. (Oral Presentation)

Previous Publications by the Author

Ito N, Wijenayaka AR, Prideaux M, Kogawa M, **Ormsby RT**, Evdokiou A, Bonewald LF, Findlay DM, Atkins GJ. *Regulation of FGF23 expression in IDG-SW3 osteocytes and human bone by pro-inflammatory stimuli.* Mol Cell Endocrinol. 2015 Jan 5;399:208-18. doi: 10.1016/j.mce.2014.10.007. Epub 2014 Oct 16. PMID: 25458698.

Prideaux M, Wijenayaka AR, Kumarasinghe DD**, Ormsby RT**, Evdokiou A, Findlay DM, Atkins GJ. *SaOS2 Osteosarcoma cells as an in vitro model for studying the transition of human osteoblasts to osteocytes.* Calcif Tissue Int. 2014 Aug;95(2):183-93. doi: 10.1007/s00223-014-9879-y. Epub 2014 Jun 12 PMID: 24916279

Ormsby RT, Findlay DM, Kogawa M, Anderson PH, Morris HA, Atkins GJ. *Analysis of vitamin D metabolism gene expression in human bone: evidence for autocrine control of bone remodelling.* J Steroid Biochem Mol Biol. 2014 Oct;144 Pt A:110-3. doi: 10.1016/j.jsbmb.2013.09.016. Epub 2013 Oct 10. Review. PMID: 24120913

Welldon KJ, Findlay DM, Evdokiou A, **Ormsby RT**, Atkins GJ. *Calcium induces pro-anabolic effects on human primary osteoblasts associated with acquisition of mature osteocyte markers.* Mol Cell Endocrinol. 2013 Aug 25;376(1-2):85-92. doi: 10.1016/j.mce.2013.06.013. Epub 2013 Jun 18. PMID: 23791847

Kogawa M, Wijenayaka AR, **Ormsby RT**, Thomas GP, Anderson PH, Bonewald LF, Findlay DM, Atkins GJ. *Sclerostin regulates release of bone mineral by osteocytes by induction of carbonic anhydrase 2*. J Bone Miner Res. 2013 Dec;28(12):2436-48. doi: 10.1002/jbmr.2003. PMID: 23737439

Atkins GJ, Rowe PS, Lim HP, Welldon KJ, **Ormsby R**, Wijenayaka AR, Zelenchuk L, Evdokiou A, Findlay DM. (2011) *Sclerostin is a locally acting regulator of late-osteoblast/preosteocyte differentiation and regulates mineralization through a MEPE-ASARM-dependent mechanism.* J Bone Miner Res. 2011 Jul;26(7):1425-36. doi: 10.1002/jbmr.345. PMID: 21312267

Kogawa M, Findlay DM, Anderson PH, **Ormsby R**, Vincent C, Morris HA, Atkins GJ. *Osteoclastic metabolism of 25(OH)-vitamin D3: a potential mechanism for optimization of bone resorption.* Endocrinology. 2010 Oct;151(10):4613-25. doi: 10.1210/en.2010-0334. Epub 2010 Aug 25. PMID: 20739402

Prizes and Awards

- 2015 University of Adelaide Faculty of Health Sciences Divisional Scholarship.
- 2016 University of Adelaide School of Medicine Research Travel Award.
- 2016 Florey Medical Research Foundation Prize for Poster presentation, presented at the University of Adelaide Faculty of Health and Medical Sciences 10th Florey Postgraduate Research Conference.
- 2017 University of Adelaide School of Medicine Research Travel Award.
- 2018 Adelaide Medical School Prize for Poster presentation, presented at the University of Adelaide Faculty of Health and Medical Sciences 120th Florey Postgraduate Research Conference.

Abbreviations

ANZBMS Society	Australian and New Zealand Bone Mineral
ANZORS	Australian and New Zealand Orthopaedic Research Society
ASMR	Australian Society for Medical Research
AOANJRR	Australian Orthopaedic Association National Joint Replacement Registry
Lac.Ar	Osteocyte Lacunar Area
BAX	BCL-2-like protein 4
BCL-2	B-cell lymphoma 2
BMD	bone mineral density
CA2	carbonic anhydrase 2
CAT	catalase
CoCrMo	Cobalt chrome molybdenum/ Vitallium
Co (II)	Cobalt (II)
COX-2	cyclooxygenase-2
CPC	cetylpyrridium chloride
Cr (VI)	Chromium (IV)
CTSK	cathepsin K
DAMPs	damage-associated molecular patterns
DLX-5	distal-less homeobox 5
DMP1	dentin matrix acidic phosphoprotein 1
DTT	Dithiothreitol

EDTA	ethylenediaminetetraacetic acid
E11	gp38 transmembrane glycoprotein
FCS	foetal calf serum
FGF23	fibroblast growth factor 23
H&E	haematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPRT1	hypoxanthine phosphoribosyltransferase 1
HXLPE/ XLPE	highly cross-linked polyethylene
IL-1	interleukin-1
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IL-8	interleukin-8
IL-11	interleukin-11
Lac.Ar	Osteocyte lacunar area
Lac.Ar/BAr (%)	Lacunar area per total bone area
LPS	lipopolysaccharide
M-CSF/CSF-1	macrophage-colony stimulating factor
MMPs	Matrix metallopeptidases
MMP9	matrix metalloproteinase-9
MMP13	matrix metalloproteinase-13
mRNA	messenger ribonucleic acid
MSX-2	muscle segment homeobox 2
NFATc1	nuclear factor of activated T-cells
NHBC	normal human bone cells

NHMRC	National Health and Medical Research Council of Australia
Ni(II)	nickel ions
NO	nitric oxide
OPG	osteoprotegerin
N.Ot/BAr (%)	Osteocyte number per bone area
OSX	osterix
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PE	polyethylene (PE)
PHEX	phosphate-regulating neutral endopeptidase
PGE2	prostaglandin E2
PMMA	polymethylmethacrylate
PPO/PO	periprosthetic osteolysis
PTH	Parathyroid hormone
RAH	Royal Adelaide Hospital
RANK	receptor activator of nuclear factor-kappa B
RANKL	receptor activator of nuclear factor-kappa B ligand
ROI	region of interest
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
RUNX2	runt-related transcription factor 2
SEM	standard error of the mean
SOST	sclerostin
SOD1	superoxide dismutase 1

SOD2	superoxide dismutase 2
TiAl6V4	Titanium aluminium vanadium
Top 20% Lac.Ar	Top 20% Osteocyte lacunar area
THR	Total Hip Replacement
TGF-β	transforming growth factor beta
TLRs	Toll like receptors
TLR-2	toll-like receptor-2
TLR-4	toll-like receptor-4
TNFα	tumour necrosis factor alpha
TRAP/ ACP5	tartrate resistant acid phosphatase
TRAP 5b	tartrate resistant acid phosphatase 5b
TWEAK	TNF-related weak inducer of apoptosis
UHMWPE	ultra-high molecular weight polyethylene
V-ATPase	vacuolar-ATPase
Wnt	wingless integration
α-MEM	Minimum Essential Medium Eagle Alpha
Modification	
µ-CT	micro-computed tomography

Chapter 1

Literature Review

Aseptic loosening and periprosthetic osteolysis: wear now?

Manuscript for submission to *World Journal of Orthopedics*, 2019

The aim of this chapter is to provide an updated review of the literature surrounding the biomaterial and biological aspects of wear particle-induced bone loss around total hip replacement (THR) prostheses. The chapter outlines the effects of the various wear particle types on cells within the bone-implant environment. This includes the effects on inflammatory cells and bone cells, including osteoclasts, osteoblasts and osteocytes, and highlights the role each cell type may play in the development of periprosthetic osteolysis.

Statement of Authorship

Title of Paper	Aseptic loosening and periprosthetic osteolysis: wear now?
Publication Status	☐ Published ☑ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Ormsby R.T., Solomon, L.B., Findlay, D.M., & Atkins, G.J. (2018) Aseptic loosening and periprosthetic osteolysis: wear now?

Principal Author

Name of Principal Author (Candidate)	Renee T Ormsby		
Contribution to the Paper	First author on the paper. Interpreted all literature obtained and drafted the manuscript.		
Overall percentage (%)	80		
Certification:	This paper reports on original research conducted during the period of my candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	25/10/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Lucian B Solomon		
Contribution to the Paper	Provided clinical insight and editing, approved final version of the manuscript.		
Signature		Date	16/08/2018

Name of Co-Author	David M Findlay		
Contribution to the Paper	Provided intellectual input and editing, approved final version of the manuscript.		
Signature		Date	16/8/2018

Name of Co-Author	Gerald J Atkins		
Contribution to the Paper	Helped plan the review, provided intellectual input, contributed to editing, approved final version of the manuscript. Senior editor on paper.		
Signature		Date	29/10/2018

Aseptic loosening and periprosthetic osteolysis: wear now?

Renee T Ormsby[1], Lucian B. Solomon[1, 2], David M Findlay[1, 2], Gerald J Atkins[1]

[1]Biomedical Orthopaedic Research Group, Centre for Orthopaedic & Trauma Research, The University of Adelaide, Adelaide, SA 5000, Australia

[2]Orthopaedic and Trauma Service, Royal Adelaide Hospital, Adelaide, SA 5000, Australia

Correspondence: Professor Gerald J Atkins

The University of Adelaide, North Terrace, Adelaide, SA 5000, Australia

Telephone: +61 8 8313 7214

E-mail: gerald.atkins@adelaide.edu.au

Running title: Wear particles and aseptic loosening

Keywords: Aseptic loosening; periprosthetic osteolysis; total hip arthroplasty; revision; polyethylene; Ti6Al4V; CoCrMo

Abstract

Total hip replacement surgeries are a common procedure designed to alleviate pain and improve movement in patients where deterioration of the joint has occurred. However, aseptic loosening caused by periprosthetic osteolysis (PPO) is a major cause of failure of these implants. The development of periprosthetic osteolytic lesions has been shown to be associated with the production of bioactive wear particles produced from the wear and fretting of the articulating surfaces of prostheses. The wear of ultra-high molecular weight polyethylene (UHMWPE) liners, the bearing surface of articulating prostheses, gives rise to bioactive particles capable of stimulating granuloma formation and bone resorption. This led to the development and widespread clinical use of highly cross-linked polyethylene (XLPE), designed to resist wear. These liners have reduced UHMWPE wear rates in hip implants, but questions remain as to their stability and the bioactivity of released particles. Other putative advances in liner technology, such as the addition of vitamin E to reduce oxidation of UHMWPE, may also influence wear particle associated disease. Additionally, metal particles, generated by fretting of the modular components of the femoral prostheses, and through electrochemical corrosion, are also capable of stimulating inflammatory and resorptive pathways, contributing to PPO. Considerable investigation into the effects of wear particles has been made at both the cellular and molecular levels, and a substantial body of literature now exists, providing evidence that multiple cell types respond to wear particulates and contribute to the process of osteolysis. This now includes cells of mesenchymal origin, such as osteocytes. Further insight into the cell and molecular mechanisms of PPO may enable non-operative treatments that will prolong the life of joint replacement prostheses. This review provides an update of our understanding of wear particle associated disease, including new knowledge regarding the participation of osteocytes in the unique category of PPO.

Introduction

Total hip replacement (THR) is a common and successful surgical procedure that improves patient quality of life by replacing the natural hip joint with an artificial prosthesis, relieving pain and restoring movement. THR is an effective treatment for a number of hip diseases that do not respond to conservative or other operative management [1, 2]. Primarily, these are osteoarthritis and rheumatoid arthritis [3], where the joint has been affected by cartilage damage and bone deformity, or fracture. Total hip prostheses consist of an acetabular cup, a femoral stem, and a femoral 'head', with or without a bearing surface of either ultra-high molecular weight polyethylene or ceramic inside the acetabular cup. Prostheses are classified as either hard-on-hard (ceramic-on-ceramic or, less commonly in modern designs, metal-on-metal), or hard-on-soft, where the metal or ceramic femoral head articulates on a polyethylene bearing surface [4]. Both the stem and cup can be implanted either uncemented and in direct contact with the host bone, or cemented with a polymethylmethacrylate (PMMA) bone cement [5]. Prostheses components have a variable lifespan, where the femoral component rather than the acetabular component is more frequently revised in older patients [6]. Data from registries tell us that good prosthesis have a survivorship of over 95% at 10 years and over 90% at 20 years [6, 7].

Failure of the implant-bone composite is a major problem associated with THR. This can be due either to failure of implant fixation leading to loosening, failure of the implant due to fracture or corrosion, or periprosthetic osteolysis (PPO) [8-12]. The Australian Orthopaedic Association National Joint Replacement Registry recorded that in 2017, 25.6% of THR implant failures were caused by loosening [7]. PPO and the resulting

implant loosening are often caused by implant wear and corrosion, leading to revision surgery, which is associated with increased complication rates and poorer outcomes, with increased morbidity and mortality [13].

Wear particles

Osteolytic lesions are associated with the production of prosthetic wear particles, formed at the articulating surfaces of implants or by fretting [14-17]. Wear particles migrate into the periprosthetic tissues [18], and have been shown to stimulate biological responses from surrounding cells [17, 19, 20]. The extent of this response is influenced by the nature of the particles, including material type, volume, size and shape. These characteristics contribute to the cellular responses induced by particles, and often result in foreign-body inflammatory reactions [21, 22]. The types of wear particles commonly found in periprosthetic tissue include ultra-high molecular weight polyethylene (UHMWPE), PMMA, ceramic and metal alloy particles [23]. In particular, both metal particles derived from the femoral head and stem and acetabular cup, and UHMWPE particles derived from the liners have been shown to be highly bioactive, affecting multiple cell types in the periprosthetic environment [4, 24, 25]. In terms of size, nano- and micro-sized particles are the most biologically active. For example, UHMWPE particles that range from 0.1-1 μm can be internalised by macrophages, stimulating an inflammatory response.

Metal wear particles

The metal alloys most frequently used in hip prostheses are stainless steel, cobalt chrome molybdenum (CoCrMo), and titanium aluminium vanadium, the latter most commonly

consisting of 6% aluminium and 4% vanadium (Ti6Al4V). Metal wear particles are produced from the femoral stem, head and acetabular components; the particles are released from the prostheses due to either poor prosthesis design, wear of the UHMWPE liner through to the metal acetabular cup [13], or fretting caused by micro-motion of modular components [26]. Metal particles that infiltrate periprosthetic tissues are characterised by blackening of the tissue surrounding the joint, causing soft tissue toxicity, termed "metallosis" [27]. These particles, typically ranging from 0.1 to 400 μm, are often released into the adjacent bone tissue, where they stimulate both the production of pro-inflammatory cytokines and osteoclastic activity, leading to increased bone resorption [28-30]. The effects of these particles are not limited to the bone, as wear particles have been reported in the lymph nodes, bone marrow, liver and spleen [31]. Accumulation of these particles in other organs can also lead to the formation of granulomas, which may require surgical excision [31]. Metal nano-sized wear particles can enter the cell by endocytosis [32], where they are capable of inducing cytotoxicity, chromosomal damage and oxidative stress [33].

Ultra-high molecular weight polyethylene wear particles

Ultra-high molecular weight polyethylene (UHMWPE) liners were introduced as a bearing surface to reduce frictional torque that occurs within metal on metal articulations [34]. However, the friction of the femoral head against the liner over time leads to the generation of UHMWPE wear particles, particularly when small abrasive particles, for example of cement, become lodged in the bearing space. A strong association exists between the rate of wear of UHMWPE liners and the progression of PPO [35]. UHMWPE wear particles, ranging in size between 0.1-5 μm, have been detected in large numbers in

the periprosthetic tissues at revision surgery [4, 25]. Macrophages are capable of phagocytosing UHMWPE particles, inducing inflammation and stimulating bone remodelling [36, 37]. Macrophages that have engulfed particles can differentiate into osteoclasts, and osteoclasts may themselves phagocytose wear particles [38-41]. Additionally, UHMWPE wear particles are able to enter the lymphatic system, and have been identified in patient lymph nodes, spleen and liver, where they can cause numerous adverse reactions [31, 42, 43].

Highly cross-linked PE

The first generation PE liners consisted of ultra-high molecular weight polyethylene (UHMWPE), formed at high temperatures to increase the strength of the polymer chains. In order to combat wear and increase stability of the UHMWPE chains, second generation liners were developed with extensive crosslinking of the UHMWPE microstructure to create highly cross-linked polyethylene (XLPE). This process alters the microstructure of the UHMWPE chains, producing a higher density of inter-chain carbon-carbon bonds, and restricts UHMWPE chain movement caused by local strain through loading and friction [44]. However, crosslinking also decreases toughness, tensile strength, yield strength and hardness of the UHMWPE. Importantly, clinical use of the XLPE liners in THR has shown an impressive decrease in wear compared to conventional UHMWPE liners [7]. Nevertheless, due to their small size and round morphology, XLPE particles appear to remain bioactive [44, 45].

Vitamin E

A further innovation in liner technology is the inclusion of the antioxidant, vitamin E. Imbuing XLPE liners with vitamin E is a process designed to reduce the formation of free radicals within the UHMWPE, as well as improving fatigue properties that would normally occur after post-irradiation melting [46], without sacrificing fatigue strength [47, 48]. Vitamin E is added by one of two methods. Firstly, vitamin E (α-Tocopherol) in liquid form is blended with UHMWPE powder prior to the irradiation process, which protects against oxidation but reduces the crosslinking efficiency [49-51]. An alternative approach is to diffuse vitamin E through the UHMWPE after radiation, preventing changes to the crosslinking efficiency [52]. The latter approach results in less vitamin E covalently bound to the UHMWPE chain, potentially allowing leaching into the surrounding tissues [53].

The clinical use of vitamin E-UHMWPE liners is still relatively limited, and very few studies have reported the effects of either vitamin E elution or vitamin E-bound UHMWPE particles on periprosthetic bone. Bichara *et al.* investigated the effects of vitamin E-UHMWPE wear particles from a clinically used liner (Biomet E1, a vitamin E imbued XLPE liner), in a murine calvarial osteolysis model. Although there was evidence of wear particle-induced osteolysis compared to the sham (no particle) control, the vitamin E-XLPE wear particles were associated with significantly reduced osteolysis compared to control particles [54]. However, this study only included young mice over a short experimental period (10 days). A five-year clinical study comparing wear rates in patients, with either the vitamin E imbued UHMWPE liner (E1) or a medium crosslinked UHMWPE (ArComXL) liner, showed no difference. However, longer follow-up is required to determine whether wear rates with both liners will remain the same [46]. Thus, further investigation is required to demonstrate if there is an advantage to using vitamin E imbued liners to reduce the development of osteolytic lesions.

Ceramic and PMMA particles

Ceramic implants are generally used for younger, more active, patients, as these implants have a high survivorship, due to a superior wear properties compared to other bearing surfaces [55, 56]. Additionally, few cases of osteolysis have been reported in association with ceramic prostheses, due to the biologic inertness of ceramic wear particles [57]. For older populations, ceramic implants have been used infrequently due to the perception that increased implant survivorship in an aged individual does not justify the increased cost of the implant. The brittle properties of ceramic is one disadvantage of this material, and microscopic imperfections can lead to cracks, which may ultimately result in implant failure [58].

Cement (PMMA) is used as a fixative in hip replacements, however loosening occurs with fatigue failure and can result in PMMA debonding from the femoral stem or bone interface [59]. PMMA particles are then derived from micro-motion between the bone and the cement, as well as fracturing of the cement mantle, with wear at the fracture surfaces [60]. PMMA wear particles have various effects on cell populations, potentially contributing to the development of osteolytic lesions [61, 62]. Bioactivity has been observed in a mouse air pouch model, where PMMA particles induced an inflammatory response, increasing membrane thickness and monocyte/macrophage number, and the expression of pro-inflammatory cytokines [63]. Additionally, various studies using *in vitro* models have shown that PMMA particles inhibit osteoblast differentiation [64] and stimulate osteoclast activity [65]. Thus, these particles appear to be highly bioactive, particularly in the 1-5 μM range [66].

Wear particle corrosion

Wear particles can also undergo corrosion when exposed to synovial or body fluids, large numbers of both metal ions and oxidative species are released by electrochemical corrosion of the metal prostheses [67-69]. These ions can have toxic and inflammatory effects, independent of particles [70]. Reactive ions produced from prostheses, including Ni(II), Cr (VI) and Co(II), are capable of inducing DNA damage and inhibiting DNA repair, as well as altering signal transduction and gene expression [33]. In order to address these effects, modification of metal implants is being investigated, including coating surfaces with either titanium nitride or titanium niobium nitride [71]. UHMWPE liners have also been shown to undergo oxidative degradation and generate reactive oxygen species (ROS) [72, 73], which in turn is associated with the stimulation of osteoclastic bone resorption [74, 75].

Cellular responses to wear particles

PPO appears to occur through stimulation of various cell types by wear particles. In addition to the osteolytic lesion, the most notable histological feature of wear particle associated disease is the presence of an invasive granuloma present within the lesion and around the implant. The granuloma is made up of a variety of cells, including macrophages, monocytes, mast cells, lymphocytes, endothelial cells and fibroblasts [4, 76]. These produce inflammatory cytokines that promote monocyte/macrophage differentiation into bone resorbing osteoclasts [41]. As depicted in **Figure 1** and discussed below, particles also have direct effects on other cell types within the bone environment, including osteoclasts, osteoblasts and osteocytes.

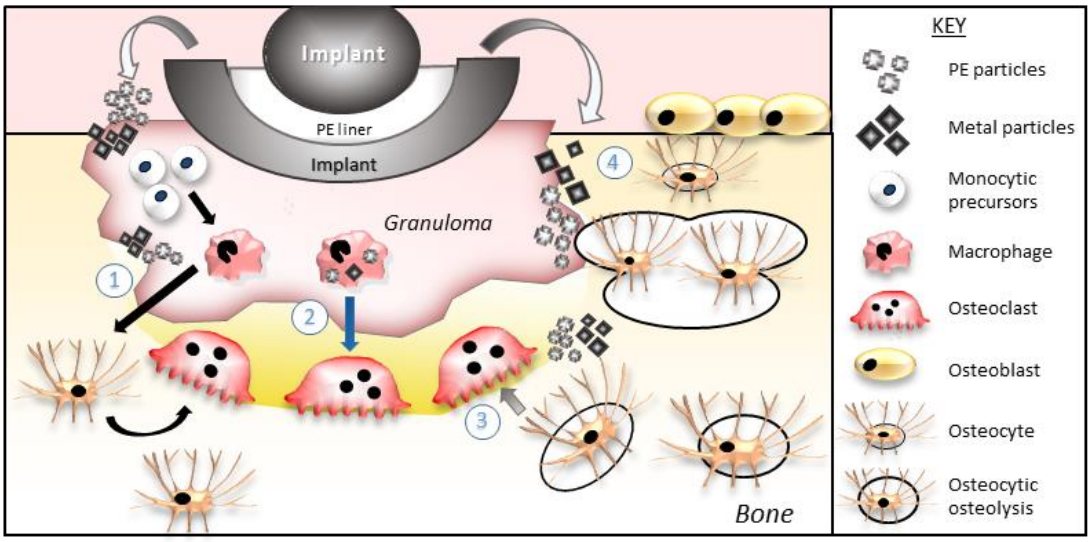


Figure 1: Schematic representation of some of the cellular pathways contributing to periprosthetic osteolysis. From left to right: White arrows show wear particles being released from the implant, including metal and polyethylene (PE). 1) Wear particles stimulating monocytic precursor cells to form activated macrophages, which produce inflammatory cytokines that stimulate osteocytes to induce osteoclastogenesis (*black arrows*). 2) Macrophages phagocytose particles stimulating their direct differentiation into osteoclasts (*blue arrow*). 3) Osteocytes are stimulated by particles, inducing osteoclastogenesis (*grey arrow*). 4) Wear particles released by implants (*white arrow*) also stimulate osteocyte peri-lacunar remodelling.

Monocytes/Macrophages

Macrophages are a key stimulatory cell type in wear particle mediated osteolysis [77]. Monocytes differentiate into resident macrophages in response to inflammation, at which point these cells selectively phagocytose dead cells and foreign materials [78]. Subsequently, the phagosome fuses with lysosomes, forming the phagolysosome, and lysosomal enzymes and free radicals attempt to digest and destroy the engulfed material

[78]. Pro-inflammatory M1 macrophages are predominantly detected in the peri-implant tissue of revision THR [79]. Macrophages infiltrate the granuloma, where they stimulate inflammatory pathways and promote bone resorption. Small wear particles are actively phagocytosed by macrophages, causing the release of an array of pro-inflammatory cytokines (TNFα, IL-1β, IL-8, IL-11 and TGF-β), and also tissue degrading enzymes, including Cathepsin K [24, 37, 80-82]. Wear particles have also been shown to induce macrophage apoptosis [83] and stimulate the release of macrophage-colony stimulating factor (M-CSF/CSF-1) and receptor activator of nuclear factor-kappa B ligand (RANKL), cytokines central to the recruitment, differentiation and bone resorbing activity of osteoclasts (see below). Additionally, both UHMWPE and metal particles induce TNFα expression in macrophages, which can synergise with RANKL to further increase osteoclast formation and activity [40, 84-87].

The role of Toll-like Receptor and PAMPs

Toll-like receptors (TLRs) play important roles as innate immune sensors, and recognise both endogenous damage-associated molecular patterns (DAMPs) and exogenous pathogen-associated molecular patterns (PAMPs) derived from bacteria and fungi [88]. Activation of TLR signalling stimulates the production of pro-inflammatory cytokines, chemokines and chemokine receptors [89]. Multiple cell types express TLRs, including monocytes/macrophages, osteoblasts, osteoclasts, and now osteocytes [90]. Importantly, TLRs have been detected in tissues surrounding both aseptic and septic loosening of THR patients [91], and are involved in the inflammatory cascade induced by wear particles [17]. TLR expression has been detected in macrophages located within periprosthetic tissues of patients with aseptic loosening [92]. Strong immunostaining of TLR-4 has been

detected in the synovial membrane of patients with aseptic loosening, this correlated with increased expression of both pro-inflammatory cytokines and chemokines [90, 93]. Animal models of wear particle induced osteolysis also show evidence of increased TLR expression [94], including a mouse calvarial model of osteolysis where both TLR-2 and TLR-4 were strongly detected with exposure to UHMWPE [95]. Deletion of the genes encoding TLR-2 and TLR-4 in mouse models of wear particle induced osteolysis resulted in reduced inflammatory responses, associated with the presence of bacterial PAMPs. The removal of Gram-negative bacterial lipopolysaccharide (LPS), a PAMP ligand recognised primarily by TLR-4, from Ti particles, reduced the expression of pro-inflammatory cytokines, including TNFα and IL-6 [92, 96]. Interestingly, oxidised alkane polymers produced from UHMWPE particles have been detected in the tissue surrounding implants, and have been shown to activate TLR-1/2 signalling. This is due to structural changes that occur with oxidation that result in recognition of these polymers as TLR ligands [97, 98]. The levels of endotoxins in patients may vary due to endotoxin clearance, as has been described in murine models of osteolysis, where accumulation and clearance of endotoxin regulate the rate of osteolysis [99]. Therefore, activation of TLR pathways likely involves multiple stimuli, and overall appears to play an important role in regulating responses to wear particles, including those mediated by attached pathogen-derived products [65, 85, 100].

Osteoclasts

Osteoclasts are haemopoietic-derived multinucleated cells, whose predominant function is bone resorption, both during development and bone homeostasis [101]. Like macrophages, osteoclasts are derived from a monocytic precursor present in bone marrow

and the peripheral blood [102] that express cell surface RANK [103]. Mature osteoclasts attach to the bone surface and form a sealed zone, into which hydrogen ions and collagenases are released. These demineralise the calcified bone matrix and degrade the collagenous organic matrix, respectively. Osteoclastic enzymes include carbonic anhydrase II and the vacuolar-ATPase (V-ATPase), which combine to acidify the resorption zone [104], and key collagenases Cathepsin K and matrix metalloproteinases, MMP-13 and MMP-9 [104]. Osteoclasts also produce tartrate-resistant acid phosphatase (TRAP/ACP5), a key histologic marker that is thought to be involved in bone matrix protein dephosphorylation [105]. Tissue biopsies from patients with periprosthetic osteolysis undergoing revision THR have been shown to express elevated levels of TRAP, Cathepsin K and MMP-9, compared to primary THR controls [106]. One study reported significantly lower levels of TRAP5b and unchanged levels of cross-laps in the serum of patients undergoing revision THR surgery for aseptic loosening, compared to primary THR recipients, and concluded that osteoclastic resorption was no longer occurring in these patients [107]. Thus, it is possible that the localised osteolysis that occurs, potentially over a long period of time, may not necessarily be detectable using systemic measures of bone remodelling.

As mentioned above, two key regulators of osteoclast formation and activity are M-CSF, and RANKL [108, 109]. M-CSF is necessary for the proliferation and maturation of monocyte precursor cells. RANKL, which is expressed by osteoblasts, osteocytes, stromal cells and T-lymphocytes, in particular T-helper 17 (Th17) cells, binds to the RANK receptor on osteoclast precursors and is essential for their differentiation into mature osteoclasts [110-114]. RANKL usually exists in a membrane-bound form but can also retain activity after cleavage from the membrane [115]. Osteoclast formation is negatively regulated by the antagonist osteoprotegerin (OPG). OPG binds to RANKL,

blocking binding to the RANK receptor, as well as preventing intracellular RANKL from translocating to the cell surface [116], therefore inhibiting osteoclast production and downregulating bone resorption. Thus, the relative levels of RANKL and OPG determine the net effect of a given stimulus on bone resorption [104, 110, 111, 117, 118].

Tissues obtained from the implant interface of patients with osteolysis show significant expression of RANKL by infiltrating macrophages in close proximity to wear particles [119, 120]. Such cells have a strong propensity to form osteoclasts, with studies showing that human and mouse macrophages containing ingested wear particles were able to differentiate into osteoclasts *in vitro* [40, 65, 100, 121-123]. The effects of wear particles on osteoclasts has been investigated *in vivo*, in particular in two rodent models, namely the air pouch model and the calvarial osteolysis model. The air-pouch model involves sub-cutaneous injection of air into the dorsal flank of a mouse, after which a particle suspension is injected into the pouch, inducing an inflammatory response resulting in osteolysis [63, 124]. The more widely used calvarial osteolysis model involves exposing the periosteum and injecting particles onto the calvarial surface to induce osteolysis [125]. Various other models have also been used, notably a femoral implant UHMWPE particle perfusion model, which was designed to mimic the motion of particles between a titanium implant and the bone surface, and also resulted in extensive osteolysis [126].

Expression of both prostaglandin E_2 (PGE_2) and the enzyme responsible for its production, cyclooxygenase-2 (COX-2), are also strongly associated with periprosthetic membranes of revision patients with PPO. Macrophages laden with wear particles in periprosthetic membranes showed significant expression of COX-2, as assessed by immunostaining [127]. Additionally, *in vitro* studies have shown that macrophages exposed to wear particles increased PGE_2 production [66, 128, 129]. COX-2/PGE_2 play

important roles in mechanical loading and in both inflammatory and osteoclastogenic pathways [130, 131].

Osteoblasts

In addition to the more 'classical' cellular pathways described above, evidence suggests that mesenchymal lineage cells are also involved in osteolysis. Mesenchymal-derived osteoblasts on the bone surface form bone by secreting a collagen-proteoglycan matrix, termed osteoid, which becomes mineralised, leading to a hydroxyapatite mineral matrix [101, 132]. Osteoblasts are differentiated under the control of key transcription factors (RUNX2, OSX, MSX-2, DLX-5), stimulated by pro-osteogenic pathways [133]. During differentiation, the osteoblast undergoes one of three fates, either differentiating into a bone lining cell [134], undergoing apoptosis [135] or becoming embedded in the osteoid. Embedded cells differentiate, firstly into immature or pre-osteocytes, and finally into mature osteocytes [136, 137]. UHMWPE particles less than 5 μm in size [138] are readily phagocytosed by osteoblasts *in vitro* [139, 140]. Particle ingestion led to a significant decrease in the production of type I collagen [141-143]. A similar response was reported in mouse osteoblastic MC3T3-E1 cells, where cells that phagocytosed UHMWPE wear particles showed a significant decrease in both cell proliferation and mineralisation [144]. There was also a significant decrease in both alkaline phosphatase activity, which is essential for bone mineral deposition, as well as osteocalcin production, a marker of osteoblast activity [140, 144, 145].

Metal wear particles and ions have also been shown to have inhibitory effects on osteoblasts, with similar suppression of type I collagen production. Particles of Ti6Al4V and CoCrMo derived from hip stem prostheses inhibited pro-collagen-α1 expression by

human primary osteoblasts *in vitro* [142]. Similarly, MC3T3-E1 cells exposed to metal particles showed decreased viability [146, 147], with apoptotic rates increasing with increased numbers of Ti particles [146, 148]. Together, the above studies suggest that exposure to wear particles would inhibit bone formation.

Wear particles have also been shown to affect MC3T3-E1 cells by upregulating expression of pro-inflammatory cytokines and chemokines, including interleukins IL-1, IL-6, IL-8, and TNFα [142, 149, 150], which are known to promote osteoclast differentiation and activity, as discussed above. Similar to effects on osteoclasts, osteoblasts were also stimulated by UHMWPE wear particles to produce PGE_2 [151, 152], which, as mentioned above, activates inflammation and osteoclastogenesis.

Osteoblasts also express key osteoclast regulatory genes in response to wear particles, including RANKL, IL-8 and M-CSF [139, 145, 153]. Human osteoblasts derived from patient bone biopsies and exposed to UHMWPE particles significantly upregulated the pro-osteoclastogenic RANKL:OPG mRNA ratio [139, 154]. Expression of both M-CSF and IL-8 were also upregulated with exposure to UHMWPE particles [139]. Collectively, these studies show that wear particles inhibit both osteoblast function and activity, and promote inflammation and bone resorption, potentially contributing to the development of PPO.

Osteocytes

The most abundant bone cell type is the osteocyte, comprising 90-95% of cells in the bone [155, 156]. The osteocyte is a mature dendritic-like cell that differentiates from bone matrix-embedded osteoblasts, forming an extensive interconnected network [157]. Through this network, osteocytes are capable of sensing mechanical and biochemical

signals, which regulate bone resorption and formation [156]. Osteocyte processes extend to the bone surface, allowing contact with bone lining cells, osteoblasts, osteoclasts, and bone marrow cells beyond the bone [158], and can also directly contact implant surfaces [159].

Osteocyte-specific genetic deletion of RANKL in mice led to osteopetrosis, suggesting that in adult bone, osteocytes rather other cell types, are the major source of RANKL for controlling osteoclastogenesis [160-162]. Osteocyte expression of sclerostin, a protein produced by mature osteocytes and a key inhibitor of the Wnt/β-catenin signaling pathway, also induces RANKL, promoting osteoclastic activity [163]. Therefore, osteocyte regulation of osteoclast differentiation and activity appears to be critical for the regulation of bone remodeling.

Osteocytic Osteolysis

The osteocyte is also capable of remodelling its perilacunar space. The term "osteocytic osteolysis" was originally coined by Belanger [164], who hypothesised that an osteocyte-specific bone removal mechanism existed, specifically, the removal of mineral and organic constituents of intercellular substance by the osteocyte through perilacunar resorption. This concept, while originally accepted, became controversial, as it was linked to the bone-flow theory, which was discredited by Parfitt [165]. However, in that influential paper, Parfitt acknowledged that perilacunar resorption by osteocytes did occur, but did so in concert with bone resorption by osteoclasts. Multiple studies emerged refuting the concept of perilacunar remodelling, specifically the inability of the osteocyte to mobilise calcium and phosphate, stimulate resorption as demonstrated on dentine or replace mineral in the bone [166-168].

Recent studies have revived the concept of osteocytic perilacunar remodelling, also known as perilacunocanalicular remodelling, where the bone mineral matrix is removed by osteocytes in a reversible manner. This process has been observed in several instances of physiologic and pathologic bone removal, including lactation and weightlessness [169-178]. A study by Tang *et al.* demonstrated the role of osteocytic osteolysis in a mouse lactation model, in which the gene encoding MMP-13 was globally deleted. Global knockdown of MMP-13 reduced osteocyte lacunar area and osteocyte interconnectivity, impaired bone remodelling and increased bone fragility in these mice [175]. This study demonstrated that osteocytic osteolysis has an important role in regulating calcium release during lactation, and homeostatic maintenance of bone matrix quality, and is dependent on MMP-13 expression. Additionally, the osteocyte product sclerostin appears to be a regulator of mineralisation of the bone matrix [179], and osteocytic osteolysis. The latter effect has been reported to involve the up-regulation of osteocytic carbonic anhydrase II, Cathepsin K, TRAP and MMP-13, based on experiments using cultured human primary osteocyte-like cultures and human trabecular bone in an *ex vivo* model [180], and in a bovine *ex vivo* bone model where the bone was mechanically loaded [181].

Osteocytes and wear particles

Few studies have reported the effects of wear particles on osteocytes. While osteocytes *in vivo* are buried within the mineral matrix, wear particles have been documented within the surrounding tissues of the implant *in vivo*. Surgical reaming of the bone surface, for example in the acetabulum, occurs prior to implant insertion, exposing the underlying trabecular bone and allowing osteocytes to contact the implant surface [159]. Through this considerable change to the bone environment, wear particles are capable of

contacting the underlying trabecular bone matrix, potentially invading the bone marrow, blood vessels and even the osteocyte lacunocanalicular network. Biopsies from deceased patients who had received a primary THR showed evidence of cobalt deposits in the mineralised bone tissue, suggesting that this is indeed possible [182].

We have previously shown that osteocytes exposed to UHMWPE wear particles *in vitro* increased the expression of RANKL and M-CSF, with downregulation of OPG [139, 183], providing evidence that osteocytes can activate bone resorption by promoting osteoclastogenesis in response to wear particles. Additionally, the expression of the gene encoding sclerostin, SOST, was also increased [139]. Sclerostin, by virtue of stimulating osteocytic RANKL expression, could therefore contribute to the pro-osteoclastogenic response in PPO [163, 181].

Osteocyte apoptosis is another key activator of bone remodelling. Apoptotic osteocytes, for example in a stress fracture model, signal neighbouring viable osteocytes to express RANKL, promoting site-specific osteoclast recruitment and activity [184]. UHMWPE wear particles do not appear to induce apoptosis in osteoblasts or osteocytes [139, 183]. However, metal wear particles have been shown to induce osteocyte death *in vitro*; MLO-Y4 cells when exposed to cobalt chrome wear particles significantly increased Caspase 3 activity, an effector caspase required for apoptosis [185]. This was similarly shown in MLO-Y4 cells with exposure to β-tri-calcium phosphate particles, where there was a reduction in cell viability [186]. Additionally, these particles induced SOST, TNFα and IL-1β expression, which play key roles in osteoclastogenesis, as discussed above. Therefore, osteocytes may stimulate osteoclastic bone resorption in response to wear particles both directly and indirectly by undergoing apoptosis.

A study by Lohmann *et al.* examined the effects of UHMWPE particle exposure on MLO-Y4 osteocyte-like cells, and reported upregulation of both NO and PGE_2 production, with a concurrent decrease in cell number [187]. As both NO and PGE_2 play important roles in regulating bone resorption [131], this could be another pathway involved in the development of osteolytic lesions. Additionally, we have shown that osteocyte-like cells derived from human trabecular bone biopsies respond to UHMWPE particles *in vitro* by inducing genes associated with osteocytic osteolysis, such as MMP-13, CTSK and carbonic anhydrase 2 [183]. Furthermore, we observed enlarged osteocyte lacunae in bone biopsies taken from patients undergoing revision THR for aseptic loosening, with radiographic evidence of periprosthetic osteolysis [183]. Wear particles were also detected in these biopsies, adjacent to the bone and within Haversian canals. As mentioned above, a study by Busse *et al*. has also shown evidence of cobalt deposits in the mineralised bone tissue of deceased patients who had undergone primary THR surgery [182]. This suggests that perilacunar enlargement could be a process induced by the osteocyte in order to clear particles from the lacunocanalicular network.

Potential non-surgical treatment approaches

There is a wealth of evidence that supports osteoclastic bone resorption as a principal cause of osteolysis, which has led to the long-held hope that non-surgical anti-resorptive approaches may be efficacious for treating PPO [188]. Therefore, multiple osteoclast-associated mediators are potential pharmacological targets. The COX-2 inhibitor, Celecoxib, was investigated in both an *in vitro* model and in mice as a potential therapeutic against the development of osteolysis. Inhibition of COX-2 in RAW 264.7 cells led to decreased osteoclast formation [189], and knockdown of COX-2 in mice

exposed to Ti particles showed reduced bone resorption, as well as a decrease in PGE_2 production [190]. While COX-2 may be a potential therapeutic target to limit the development of osteolysis, its safety in terms of cardiovascular risk has been controversial [191, 192]. As another example, treatment with the MMP-9 inhibitor, tetracycline, in a murine air pouch model of Ti particle-induced osteolysis, resulted in reduced membrane thickness caused by particle-induced inflammation. Additionally, there was a reduction in both TRAP-positive osteoclast number and RANK/RANKL expression [193]. Geng *et al*. reported using a mouse tibial implant model, that strontium ranelate (SR), a drug used for the treatment of osteoporosis, appeared to improve osseointegration and prevent osteolysis associated with the injection of Ti particles [194].

Many mediators of osteoclastic bone resorption are also produced by osteocytes during osteocytic osteolysis, and while functional studies have not yet been reported in osteocyte model systems, both osteoclast and osteocyte pathways of bone loss could be affected in *in vivo* studies. Thus, it is intriguing to speculate that osteoblast/osteocyte products may also present therapeutic options for periprosthetic osteolysis. Several of the available and pipeline anti-resorptive/pro-anabolic treatments for bone loss target osteocyte-derived products [156]. For example, Denosumab, a RANKL neutralising biologic, effectively targets an osteocyte-derived product with profound effects on osteoclast-mediated bone resorption. In addition to its widespread use in osteoporosis, Denosumab is in clinical use for the treatment of the localised, often aggressive, osteolysis associated with Giant Cell Tumour of Bone [195]. However, its use for PPO has not been reported to date. OPG-based approaches are the rodent equivalent of Denosumab; a study by Zhang and colleagues [196] tested the efficacy of viral-delivery of OPG gene expression to sites of osteolysis in a mouse tibial implant/Ti particle model and showed that increased OPG expression prevented inflammatory pseudomembrane formation and osteolysis [196].

While this study lends support to the use of Denosumab for PPO, enthusiasm for its use may be lessened by the observations of severe rebound adverse effects that can occur upon cessation of therapy for both osteoporosis [197] and Giant Cell Tumour of Bone [198]. Sclerostin is another osteocyte product currently targeted in the treatment of osteoporosis. Neutralising antibodies against sclerostin have been shown to be effective in increasing bone mass as well as inhibiting bone resorption [199]. As mentioned above, Geng *et al.* reported that strontium ranelate treatment prevented osteolysis caused by Ti particle injection, however, this was entirely dependent on the expression of sclerostin, as no effect of strontium ranelate was observed in treated mice lacking the *SOST* gene [200]. However, the effects of particles alone were not reported in this study, nor was osteolysis quantified, making these findings difficult to interpret. Therefore, it remains an open question whether a neutralizing sclerostin antibody could prevent or reverse osteolysis in the case of PPO.

Conclusions

The production of wear particles from total hip replacements, leading to PPO, remains a significant problem. Wear particles induce a multitude of effects that threaten both the structure and cellular viability of the bone, activating pathways that are key for maintaining bone homeostasis. The development of the granulomatous tissue induced by wear particles is a response by the host to recruit immune cells in an attempt to remove them. This in turn stimulates an inflammatory response to increase blood supply, as well as inducing resorption, in an attempt to remove the compromised bone. However, since the wear particles are not easily removed, further complications arise. Phagocytosis of metal and polyethylene wear particles by macrophages stimulates either bone resorption

or induces macrophage apoptosis, which then induces signaling cascades that promote osteoclast production and activity, and stimulates bone resorption.

In addition, wear particles inhibit osteogenesis; osteoblast function is compromised, preventing bone formation from occurring after the resorptive phase. This dysregulation of bone homeostasis likely contributes to the net loss of bone seen in PPO. Moreover, the actions of the osteocyte, as a regulator of both modeling and remodeling, are also compromised, or hijacked, by wear particles. Particles have been shown to induce osteocyte apoptosis, increase the expression of pro-osteoclastic resorptive markers and additionally stimulate osteocytic osteolysis, which leads to increased osteocyte lacunar area and potentially adds to the development of PPO. The addition of antioxidants, such as vitamin E, to the liner material needs to be further tested, as there is little proof of the benefits of these additives. Additionally, wear and corrosion of metal implant components is a still a common occurrence. Therefore, wear particles contribute to the development of osteolytic lesions though a multitude of pathways, and this highlights the need for more "bone friendly" prostheses and further study into the cellular mechanisms of PPO; full elucidation of these will provide an important basis for informed future therapeutic approaches.

Acknowledgements

This work was supported by funding from the National Health and Medical Research Council of Australia (NHMRC) Project Grant Scheme (Grant ID 1041456) and the Royal Adelaide Hospital Special Purposes Fund. RTO is a recipient of a University of Adelaide Post-graduate Scholarship. GJA is a NHMRC Senior Research Fellow.

References

[1] Yoon PW, Kim JI, Kim DO, Yu CH, Yoo JJ, Kim HJ, et al. Cementless total hip arthroplasty for patients with Crowe type III or IV developmental dysplasia of the hip: two-stage total hip arthroplasty following skeletal traction after soft tissue release for irreducible hips. Clinics in orthopedic surgery 2013;5:167-73.

[2] Wood AM, Brock TM, Heil K, Holmes R, Weusten A. A Review on the Management of Hip and Knee Osteoarthritis. International journal of chronic diseases 2013;2013:845015-.

[3] Konttinen YT, Xu JW, Pätiälä H, Imai S, Waris V, Li TF, et al. Cytokines in aseptic loosening of total hip replacement. Current Orthopaedics;11:40-7.

[4] Bitar D, Parvizi J. Biological response to prosthetic debris. World journal of orthopedics 2015;6:172-89.

[5] Saleh KJ, El Othmani MM, Tzeng TH, Mihalko WM, Chambers MC, Grupp TM. Acrylic bone cement in total joint arthroplasty: A review. J Orthopaed Res 2016;34:737-44.

[6] Berry DJ, Harmsen WS, Cabanela ME, Morrey BF. Twenty-five-year survivorship of two thousand consecutive primary Charnley total hip replacements: factors affecting survivorship of acetabular and femoral components. The Journal of bone and joint surgery American volume 2002;84-A:171-7.

[7] Australian Orthopaedic Association National Joint Replacement Registry Annual Report 2017. https://aoanjrrsahmricom/annual-reports-2017 2017.

[8] Malchau H, Herberts P, Ahnfelt L. Prognosis of total hip replacement in Sweden. Follow-up of 92,675 operations performed 1978-1990. Acta orthopaedica Scandinavica 1993;64:497-506.

[9] Harris WH. The problem is osteolysis. Clin Orthop 1995:46-53.

[10] Maloney WJ, Herzwurm P, Paprosky W, Rubash HE, Engh CA. Treatment of pelvic osteolysis associated with a stable acetabular component inserted without cement as part of a total hip replacement. The Journal of bone and joint surgery American volume 1997;79:1628-34.

[11] Chiang PP, Burke DW, Freiberg AA, Rubash HE. Osteolysis of the pelvis: evaluation and treatment. Clinical orthopaedics and related research 2003:164-74.

[12] Kurtz S, Mowat F, Ong K, Chan N, Lau E, Halpern M. Prevalence of primary and revision total hip and knee arthroplasty in the United States from 1990 through 2002. The Journal of bone and joint surgery American volume 2005;87:1487-97.

[13] Howie DW, Neale SD, Haynes DR, Holubowycz OT, McGee MA, Solomon LB, et al. Periprosthetic osteolysis after total hip replacement: molecular pathology and clinical management. Inflammopharmacology 2013;21:389-96.

[14] Shanbhag AS, Jacobs JJ, Glant TT, Gilbert JL, Black J, Galante JO. Composition and morphology of wear debris in failed uncemented total hip replacement. The Journal of bone and joint surgery British volume 1994;76:60-7.

[15] Shanbhag AS, Bailey HO, Hwang DS, Cha CW, Eror NG, Rubash HE. Quantitative analysis of ultrahigh molecular weight polyethylene (UHMWPE) wear debris associated with total knee replacements. Journal of biomedical materials research 2000;53:100-10.

[16] Rokkum M, Brandt M, Bye K, Hetland KR, Waage S, Reigstad A. Polyethylene wear, osteolysis and acetabular loosening with an HA-coated hip prosthesis. A follow-up of 94 consecutive arthroplasties. The Journal of bone and joint surgery British volume 1999;81:582-9.

[17] Athanasou NA. The pathobiology and pathology of aseptic implant failure. Bone Joint Res 2016;5:162-8.

[18] Chang JD, Lee SS, Hur M, Seo EM, Chung YK, Lee CJ. Revision total hip arthroplasty in hip joints with metallosis: a single-center experience with 31 cases. The Journal of arthroplasty 2005;20:568-73.

[19] Maloney WJ, Smith RL. Periprosthetic osteolysis in total hip arthroplasty: the role of particulate wear debris. Instructional course lectures 1996;45:171-82.

[20] Kadoya Y, Kobayashi A, Ohashi H. Wear and osteolysis in total joint replacements. Acta orthopaedica Scandinavica Supplementum 1998;278:1-16.

[21] Santavirta S, Konttinen YT, Bergroth V, Eskola A, Tallroth K, Lindholm TS. Aggressive granulomatous lesions associated with hip arthroplasty. Immunopathological studies. The Journal of bone and joint surgery American volume 1990;72:252-8.

[22] Santavirta S, Konttinen YT, Hoikka V, Eskola A. Immunopathological response to loose cementless acetabular components. The Journal of bone and joint surgery British volume 1991;73:38-42.

[23] Margevicius KJ, Bauer TW, McMahon JT, Brown SA, Merritt K. Isolation and characterization of debris in membranes around total joint prostheses. The Journal of bone and joint surgery American volume 1994;76:1664-75.

[24] Haynes DR, Rogers SD, Hay S, Pearcy MJ, Howie DW. The differences in toxicity and release of bone-resorbing mediators induced by titanium and cobalt-chromium-alloy wear particles. The Journal of bone and joint surgery American volume 1993;75:825-34.

[25] Liu A, Richards L, Bladen CL, Ingham E, Fisher J, Tipper JL. The biological response to nanometre-sized polymer particles. Acta biomaterialia 2015.

[26] Kretzer JP, Jakubowitz E, Krachler M, Thomsen M, Heisel C. Metal release and corrosion effects of modular neck total hip arthroplasty. Int Orthop 2009;33:1531-6.

[27] Willert HG, Buchhorn GH, Fayyazi A, Flury R, Windler M, Koster G, et al. Metal-on-metal bearings and hypersensitivity in patients with artificial hip joints. A clinical and histomorphological study. The Journal of bone and joint surgery American volume 2005;87:28-36.

[28] Grosse S, Haugland HK, Lilleng P, Ellison P, Hallan G, Hol PJ. Wear particles and ions from cemented and uncemented titanium-based hip prostheses-A histological and chemical analysis of retrieval material. Journal of biomedical materials research Part B, Applied biomaterials 2015;103:709-17.

[29] Doorn PF, Campbell PA, Amstutz HC. Metal versus polyethylene wear particles in total hip replacements. A review. Clinical orthopaedics and related research 1996:S206-16.

[30] Neale SD, Athanasou NA. Cytokine receptor profile of arthroplasty macrophages, foreign body giant cells and mature osteoclasts. Acta orthopaedica Scandinavica 1999;70:452-8.

[31] Urban RM, Jacobs JJ, Tomlinson MJ, Gavrilovic J, Black J, Peoc'h M. Dissemination of wear particles to the liver, spleen, and abdominal lymph nodes of patients with hip or knee replacement. The Journal of bone and joint surgery American volume 2000;82:457-76.

[32] Billi F, Campbell P. Nanotoxicology of metal wear particles in total joint arthroplasty: a review of current concepts. Journal of applied biomaterials & biomechanics : JABB 2010;8:1-6.

[33] Keegan GM, Learmonth ID, Case CP. Orthopaedic metals and their potential toxicity in the arthroplasty patient: A REVIEW OF CURRENT KNOWLEDGE AND FUTURE STRATEGIES. Journal of Bone & Joint Surgery, British Volume 2007;89-B:567-73.

[34] Charnley J. Arthroplasty of the hip. A new operation. Lancet 1961;1:1129-32.

[35] Howie DW, Neale SD, Stamenkov R, McGee MA, Taylor DJ, Findlay DM. Progression of acetabular periprosthetic osteolytic lesions measured with computed tomography. The Journal of bone and joint surgery American volume 2007;89:1818-25.

[36] Green TR, Fisher J, Matthews JB, Stone MH, Ingham E. Effect of size and dose on bone resorption activity of macrophages by in vitro clinically relevant ultra high molecular weight polyethylene particles. Journal of biomedical materials research 2000;53:490-7.

[37] Tamaki Y, Sasaki K, Sasaki A, Takakubo Y, Hasegawa H, Ogino T, et al. Enhanced osteolytic potential of monocytes/macrophages derived from bone marrow after particle stimulation. Journal of biomedical materials research Part B, Applied biomaterials 2008;84:191-204.

[38] Pandey R, Quinn J, Joyner C, Murray DW, Triffitt JT, Athanasou NA. Arthroplasty implant biomaterial particle associated macrophages differentiate into lacunar bone resorbing cells. Annals of the rheumatic diseases 1996;55:388-95.

[39] Sabokbar A, Fujikawa Y, Brett J, Murray DW, Athanasou NA. Increased osteoclastic differentiation by PMMA particle-associated macrophages. Inhibitory effect by interleukin 4 and leukemia inhibitory factor. Acta orthopaedica Scandinavica 1996;67:593-8.

[40] Wang W, Ferguson DJ, Quinn JM, Simpson AH, Athanasou NA. Biomaterial particle phagocytosis by bone-resorbing osteoclasts. The Journal of bone and joint surgery British volume 1997;79:849-56.

[41] Sabokbar A, Pandey R, Quinn JM, Athanasou NA. Osteoclastic differentiation by mononuclear phagocytes containing biomaterial particles. Archives of orthopaedic and trauma surgery 1998;117:136-40.

[42] Basle MF, Bertrand G, Guyetant S, Chappard D, Lesourd M. Migration of metal and polyethylene particles from articular prostheses may generate lymphadenopathy with histiocytosis. Journal of biomedical materials research 1996;30:157-63.
[43] Shea KG, Bloebaum RD, Avent JM, Birk T, Samuelson KA. Analysis of lymph nodes for polyethylene particles in patients who have had a primary joint replacement. The Journal of bone and joint surgery American volume 1996;78:497-504.
[44] Takahashi Y, Shishido T, Yamamoto K, Masaoka T, Kubo K, Tateiwa T, et al. Mechanisms of plastic deformation in highly cross-linked UHMWPE for total hip components--the molecular physics viewpoint. Journal of the mechanical behavior of biomedical materials 2015;42:43-53.
[45] Fisher J, McEwen HM, Tipper JL, Galvin AL, Ingram J, Kamali A, et al. Wear, debris, and biologic activity of cross-linked polyethylene in the knee: benefits and potential concerns. Clinical orthopaedics and related research 2004:114-9.
[46] Nebergall AK, Greene ME, Laursen MB, Nielsen PT, Malchau H, Troelsen A. Vitamin E diffused highly cross-linked polyethylene in total hip arthroplasty at five years: a randomised controlled trial using radiostereometric analysis. The bone & joint journal 2017;99-B:577-84.
[47] Brigelius-Flohe R, Traber MG. Vitamin E: function and metabolism. FASEB J 1999;13:1145-55.
[48] Costa L, Luda MP, Trossarelli L, Brach del Prever EM, Crova M, Gallinaro P. Oxidation in orthopaedic UHMWPE sterilized by gamma-radiation and ethylene oxide. Biomaterials 1998;19:659-68.
[49] Oral E, Beckos CG, Malhi AS, Muratoglu OK. THE EFFECTS OF HIGH DOSE IRRADIATION ON THE CROSS-LINKING OF VITAMIN E-BLENDED

ULTRAHIGH MOLECULAR WEIGHT POLYETHYLENE. Biomaterials 2008;29:3557-60.

[50] Oral E, Greenbaum ES, Malhi AS, Harris WH, Muratoglu OK. Characterization of irradiated blends of alpha-tocopherol and UHMWPE. Biomaterials 2005;26:6657-63.

[51] Parth M, Aust N, Lederer K. Studies on the effect of electron beam radiation on the molecular structure of ultra-high molecular weight polyethylene under the influence of alpha-tocopherol with respect to its application in medical implants. Journal of materials science Materials in medicine 2002;13:917-21.

[52] Oral E, Wannomae KK, Hawkins N, Harris WH, Muratoglu OK. Alpha-tocopherol-doped irradiated UHMWPE for high fatigue resistance and low wear. Biomaterials 2004;25:5515-22.

[53] Jarrett BT, Cofske J, Rosenberg AE, Oral E, Muratoglu O, Malchau H. In vivo biological response to vitamin E and vitamin-E-doped polyethylene. The Journal of bone and joint surgery American volume 2010;92:2672-81.

[54] Bichara DA, Malchau E, Sillesen NH, Cakmak S, Nielsen GP, Muratoglu OK. Vitamin E-diffused highly cross-linked UHMWPE particles induce less osteolysis compared to highly cross-linked virgin UHMWPE particles in vivo. J Arthroplasty 2014;29:232-7.

[55] D'Antonio JA, Capello WN, Naughton M. Ceramic bearings for total hip arthroplasty have high survivorship at 10 years. Clinical orthopaedics and related research 2012;470:373-81.

[56] Clarke IC. Role of ceramic implants. Design and clinical success with total hip prosthetic ceramic-to-ceramic bearings. Clinical orthopaedics and related research 1992:19-30.

[57] Bos I, Willmann G. Morphologic characteristics of periprosthetic tissues from hip prostheses with ceramic-ceramic couples: a comparative histologic investigation of 18 revision and 30 autopsy cases. Acta orthopaedica Scandinavica 2001;72:335-42.

[58] Bal BS, Garino J, Ries M, Rahaman MN. A review of ceramic bearing materials in total joint arthroplasty. Hip Int 2007;17:21-30.

[59] Stoffel KA, Yang DT, Arola D. The influence of surface topography on wear debris generation at the cement/bone interface under cyclic loading. Journal of materials science Materials in medicine 2008;19:1935-43.

[60] Willert HG, Bertram H, Buchhorn GH. Osteolysis in alloarthroplasty of the hip. The role of bone cement fragmentation. Clinical orthopaedics and related research 1990:108-21.

[61] Kim KJ, Chiba J, Rubash HE. In vivo and in vitro analysis of membranes from hip prostheses inserted without cement. The Journal of bone and joint surgery American volume 1994;76:172-80.

[62] Archibeck MJ, Jacobs JJ, Roebuck KA, Glant TT. The basic science of periprosthetic osteolysis. Instructional course lectures 2001;50:185-95.

[63] Wooley PH, Morren R, Andary J, Sud S, Yang SY, Mayton L, et al. Inflammatory responses to orthopaedic biomaterials in the murine air pouch. Biomaterials 2002;23:517-26.

[64] Zambonin G, Colucci S, Cantatore F, Grano M. Response of human osteoblasts to polymethylmetacrylate In vitro. Calcified tissue international 1998;62:362-5.

[65] Liu FX, Wu CL, Zhu ZA, Li MQ, Mao YQ, Liu M, et al. Calcineurin/NFAT pathway mediates wear particle-induced TNF-alpha release and osteoclastogenesis from mice bone marrow macrophages in vitro. Acta Pharmacol Sin 2013;34:1457-66.

[66] Veronesi F, Tschon M, Fini M. Gene Expression in Osteolysis: Review on the Identification of Altered Molecular Pathways in Preclinical and Clinical Studies. International journal of molecular sciences 2017;18.

[67] Sansone V, Pagani D, Melato M. The effects on bone cells of metal ions released from orthopaedic implants. A review. Clinical cases in mineral and bone metabolism : the official journal of the Italian Society of Osteoporosis, Mineral Metabolism, and Skeletal Diseases 2013;10:34-40.

[68] Jacobs JJ, Gilbert JL, Urban RM. Corrosion of metal orthopaedic implants. The Journal of bone and joint surgery American volume 1998;80:268-82.

[69] Lewis AC, Heard PJ. The effects of calcium phosphate deposition upon corrosion of CoCr alloys and the potential for implant failure. Journal of biomedical materials research Part A 2005;75:365-73.

[70] Konttinen YT, Pajarinen J, Takakubo Y, Gallo J, Nich C, Takagi M, et al. Macrophage polarization and activation in response to implant debris: influence by "particle disease" and "ion disease". J Long Term Eff Med Implants 2014;24:267-81.

[71] Chang JD. Future bearing surfaces in total hip arthroplasty. Clinics in orthopedic surgery 2014;6:110-6.

[72] Premnath V, Harris WH, Jasty M, Merrill EW. Gamma sterilization of UHMWPE articular implants: an analysis of the oxidation problem. Ultra High Molecular Weight Poly Ethylene. Biomaterials 1996;17:1741-53.

[73] Neuerburg C, Loer T, Mittlmeier L, Polan C, Farkas Z, Holdt LM, et al. Impact of vitamin E-blended UHMWPE wear particles on the osseous microenvironment in polyethylene particle-induced osteolysis. Int J Mol Med 2016;38:1652-60.

[74] Halleen JM, Raisanen S, Salo JJ, Reddy SV, Roodman GD, Hentunen TA, et al. Intracellular fragmentation of bone resorption products by reactive oxygen species

generated by osteoclastic tartrate-resistant acid phosphatase. J Biol Chem 1999;274:22907-10.

[75] Garrett IR, Boyce BF, Oreffo RO, Bonewald L, Poser J, Mundy GR. Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. J Clin Invest 1990;85:632-9.

[76] Solovieva SA, Ceponis A, Konttinen YT, Takagi M, Suda A, Eklund KK, et al. Mast cells in loosening of totally replaced hips. Clinical orthopaedics and related research 1996:158-65.

[77] Nich C, Takakubo Y, Pajarinen J, Ainola M, Salem A, Sillat T, et al. Macrophages-Key cells in the response to wear debris from joint replacements. J Biomed Mater Res A 2013;101:3033-45.

[78] Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nature reviews Immunology 2011;11:723-37.

[79] Rao AJ, Gibon E, Ma T, Yao Z, Smith RL, Goodman SB. Revision joint replacement, wear particles, and macrophage polarization. Acta biomaterialia 2012;8:2815-23.

[80] Goodman SB, Gomez Barrena E, Takagi M, Konttinen YT. Biocompatibility of total joint replacements: A review. Journal of biomedical materials research Part A 2009;90:603-18.

[81] Cobelli N, Scharf B, Crisi GM, Hardin J, Santambrogio L. Mediators of the inflammatory response to joint replacement devices. Nature reviews Rheumatology 2011;7:600-8.

[82] Rogers SD, Howie DW, Graves SE, Pearcy MJ, Haynes DR. In vitro human monocyte response to wear particles of titanium alloy containing vanadium or niobium. The Journal of bone and joint surgery British volume 1997;79:311-5.

[83] Landgraeber S, Toetsch M, Wedemeyer C, Saxler G, Tsokos M, von Knoch F, et al. Over-expression of p53/BAK in aseptic loosening after total hip replacement. Biomaterials 2006;27:3010-20.

[84] Kaufman AM, Alabre CI, Rubash HE, Shanbhag AS. Human macrophage response to UHMWPE, TiAlV, CoCr, and alumina particles: analysis of multiple cytokines using protein arrays. Journal of biomedical materials research Part A 2008;84:464-74.

[85] Holding CA, Findlay DM, Stamenkov R, Neale SD, Lucas H, Dharmapatni AS, et al. The correlation of RANK, RANKL and TNFalpha expression with bone loss volume and polyethylene wear debris around hip implants. Biomaterials 2006;27:5212-9.

[86] Haynes DR, Crotti TN, Potter AE, Loric M, Atkins GJ, Howie DW, et al. The osteoclastogenic molecules RANKL and RANK are associated with periprosthetic osteolysis. The Journal of bone and joint surgery British volume 2001;83:902-11.

[87] Sabokbar A, Kudo O, Athanasou NA. Two distinct cellular mechanisms of osteoclast formation and bone resorption in periprosthetic osteolysis. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 2003;21:73-80.

[88] Murad S. Toll-like receptor 4 in inflammation and angiogenesis: a double-edged sword. Front Immunol 2014;5:313.

[89] Takagi M, Takakubo Y, Pajarinen J, Naganuma Y, Oki H, Maruyama M, et al. Danger of frustrated sensors: Role of Toll-like receptors and NOD-like receptors in aseptic and septic inflammations around total hip replacements. J Orthop Translat 2017;10:68-85.

[90] Gu Q, Shi Q, Yang H. The role of TLR and chemokine in wear particle-induced aseptic loosening. J Biomed Biotechnol 2012;2012:596870.

[91] Takagi M, Tamaki Y, Hasegawa H, Takakubo Y, Konttinen L, Tiainen VM, et al. Toll-like receptors in the interface membrane around loosening total hip replacement implants. Journal of biomedical materials research Part A 2007;81:1017-26.
[92] Greenfield EM. Do genetic susceptibility, Toll-like receptors, and pathogen-associated molecular patterns modulate the effects of wear? Clinical orthopaedics and related research 2014;472:3709-17.
[93] Tamaki Y, Takakubo Y, Goto K, Hirayama T, Sasaki K, Konttinen YT, et al. Increased expression of toll-like receptors in aseptic loose periprosthetic tissues and septic synovial membranes around total hip implants. J Rheumatol 2009;36:598-608.
[94] Valladares RD, Nich C, Zwingenberger S, Li C, Swank KR, Gibon E, et al. Toll-like receptors-2 and 4 are overexpressed in an experimental model of particle-induced osteolysis. J Biomed Mater Res A 2014;102:3004-11.
[95] Valladares RD, Nich C, Zwingenberger S, Li C, Swank KR, Gibon E, et al. Toll-like receptors-2 and 4 are overexpressed in an experimental model of particle-induced osteolysis. Journal of biomedical materials research Part A 2014;102:3004-11.
[96] Greenfield EM, Beidelschies MA, Tatro JM, Goldberg VM, Hise AG. Bacterial pathogen-associated molecular patterns stimulate biological activity of orthopaedic wear particles by activating cognate Toll-like receptors. J Biol Chem 2010;285:32378-84.
[97] Maitra R, Clement CC, Crisi GM, Cobelli N, Santambrogio L. Immunogenecity of modified alkane polymers is mediated through TLR1/2 activation. PloS one 2008;3:e2438-e.
[98] Maitra R, Clement CC, Scharf B, Crisi GM, Chitta S, Paget D, et al. Endosomal damage and TLR2 mediated inflammasome activation by alkane particles in the generation of aseptic osteolysis. Mol Immunol 2009;47:175-84.

[99] Tatro JM, Taki N, Islam AS, Goldberg VM, Rimnac CM, Doerschuk CM, et al. The balance between endotoxin accumulation and clearance during particle-induced osteolysis in murine calvaria. J Orthopaed Res 2007;25:361-9.

[100] Haynes DR, Crotti TN, Potter AE, Loric M, Atkins GJ, Howie DW, et al. The osteoclastogenic molecules RANKL and RANK are associated with periprosthetic osteolysis. Journal of Bone and Joint Surgery-British Volume 2001;83b:902-11.

[101] Gilbert SF. Osteogenesis: The Development of Bones. 6th Edition ed2000.

[102] Miyamoto T, Suda T. Differentiation and function of osteoclasts. Keio J Med 2003;52:1-7.

[103] Atkins GJ, Kostakis P, Vincent C, Farrugia AN, Houchins JP, Findlay DM, et al. RANK Expression as a Cell Surface Marker of Human Osteoclast Precursors in Peripheral Blood, Bone Marrow, and Giant Cell Tumors of Bone. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2006;21:1339-49.

[104] Teitelbaum SL. Bone resorption by osteoclasts. Science 2000;289:1504-8.

[105] Ek-Rylander B, Flores M, Wendel M, Heinegard D, Andersson G. Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion in vitro. J Biol Chem 1994;269:14853-6.

[106] Koulouvaris P, Ly K, Ivashkiv LB, Bostrom MP, Nestor BJ, Sculco TP, et al. Expression profiling reveals alternative macrophage activation and impaired osteogenesis in periprosthetic osteolysis. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 2008;26:106-16.

[107] Maier GS, Eberhardt C, Strauch M, Kafchitsas K, Kurth AA. Is tartrate-resistant acid phosphatase 5b a potent bio-marker for late stage aseptic implant loosening? Int Orthop 2014;38:2597-600.

[108] Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, et al. Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. Proceedings of the National Academy of Sciences of the United States of America 1990;87:7260-4.

[109] Boyce BF, Xing L. Biology of RANK, RANKL, and osteoprotegerin. Arthritis research & therapy 2007;9:S1-S.

[110] Atkins GJ, Kostakis P, Pan B, Farrugia A, Gronthos S, Evdokiou A, et al. RANKL expression is related to the differentiation state of human osteoblasts. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2003;18:1088-98.

[111] Findlay DM, Atkins GJ. Relationship between serum RANKL and RANKL in bone. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 2011;22:2597-602.

[112] Pacifici R. T cells, osteoblasts, and osteocytes: interacting lineages key for the bone anabolic and catabolic activities of parathyroid hormone. Annals of the New York Academy of Sciences 2016;1364:11-24.

[113] Cardoso CR, Garlet GP, Crippa GE, Rosa AL, Junior WM, Rossi MA, et al. Evidence of the presence of T helper type 17 cells in chronic lesions of human periodontal disease. Oral Microbiol Immunol 2009;24:1-6.

[114] Komatsu N, Okamoto K, Sawa S, Nakashima T, Oh-hora M, Kodama T, et al. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. Nat Med 2014;20:62-8.

[115] Wright HL, McCarthy HS, Middleton J, Marshall MJ. RANK, RANKL and osteoprotegerin in bone biology and disease. Curr Rev Musculoskelet Med 2009;2:56-64.

[116] Honma M, Ikebuchi Y, Kariya Y, Hayashi M, Hayashi N, Aoki S, et al. RANKL subcellular trafficking and regulatory mechanisms in osteocytes. J Bone Miner Res 2013;28:1936-49.

[117] Boyce BF, Xing L. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Archives of biochemistry and biophysics 2008;473:139-46.

[118] Fazzalari NL, Kuliwaba JS, Atkins GJ, Forwood MR, Findlay DM. The ratio of messenger RNA levels of receptor activator of nuclear factor kappaB ligand to osteoprotegerin correlates with bone remodeling indices in normal human cancellous bone but not in osteoarthritis. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2001;16:1015-27.

[119] Veigl D, Niederlova J, Krystufkova O. Periprosthetic osteolysis and its association with RANKL expression. Physiol Res 2007;56:455-62.

[120] Crotti TN, Smith MD, Findlay DM, Zreiqat H, Ahern MJ, Weedon H, et al. Factors regulating osteoclast formation in human tissues adjacent to peri-implant bone loss: expression of receptor activator NFkappaB, RANK ligand and osteoprotegerin. Biomaterials 2004;25:565-73.

[121] Sabokbar A, Fujikawa Y, Neale S, Murray DW, Athanasou NA. Human arthroplasty derived macrophages differentiate into osteoclastic bone resorbing cells. Annals of the rheumatic diseases 1997;56:414-20.

[122] Sabokbar A, Fujikawa Y, Neale S, Murray DW, Athanasou NA. Human arthroplasty derived macrophages differentiate into osteoclastic bone resorbing cells. Annals of the rheumatic diseases 1997;56:414-20.

[123] Neale SD, Sabokbar A, Howie DW, Murray DW, Athanasou NA. Macrophage colony-stimulating factor and interleukin-6 release by periprosthetic cells stimulates osteoclast formation and bone resorption. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 1999;17:686-94.

[124] Edwards JC, Sedgwick AD, Willoughby DA. The formation of a structure with the features of synovial lining by subcutaneous injection of air: an in vivo tissue culture system. J Pathol 1981;134:147-56.

[125] Merkel KD, Erdmann JM, McHugh KP, Abu-Amer Y, Ross FP, Teitelbaum SL. Tumor necrosis factor-alpha mediates orthopedic implant osteolysis. The American journal of pathology 1999;154:203-10.

[126] Ma T, Huang Z, Ren PG, McCally R, Lindsey D, Smith RL, et al. An in vivo murine model of continuous intramedullary infusion of polyethylene particles. Biomaterials 2008;29:3738-42.

[127] Fujishiro T, Nishikawa T, Shibanuma N, Akisue T, Takikawa S, Yamamoto T, et al. Effect of cyclic mechanical stretch and titanium particles on prostaglandin E2 production by human macrophages in vitro. Journal of biomedical materials research Part A 2004;68:531-6.

[128] Hukkanen M, Corbett SA, Batten J, Konttinen YT, McCarthy ID, Maclouf J, et al. Aseptic loosening of total hip replacement. Macrophage expression of inducible nitric oxide synthase and cyclo-oxygenase-2, together with peroxynitrite formation, as a possible mechanism for early prosthesis failure. The Journal of bone and joint surgery British volume 1997;79:467-74.

[129] Bukata SV, Gelinas J, Wei X, Rosier RN, Puzas JE, Zhang X, et al. PGE2 and IL-6 production by fibroblasts in response to titanium wear debris particles is mediated through a Cox-2 dependent pathway. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 2004;22:6-12.

[130] Kidd LJ, Cowling NR, Wu AC, Kelly WL, Forwood MR. Selective and non-selective cyclooxygenase inhibitors delay stress fracture healing in the rat ulna. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 2013;31:235-42.

[131] Haversath M, Catelas I, Li X, Tassemeier T, Jager M. PGE(2) and BMP-2 in bone and cartilage metabolism: 2 intertwining pathways. Can J Physiol Pharmacol 2012;90:1434-45.

[132] Lian J. Cell Biology of Mineralized Tissues - Osteoblasts. Connective tissue research 1992;27:135-46.

[133] Komori T. Regulation of osteoblast differentiation by transcription factors. J Cell Biochem 2006;99:1233-9.

[134] Manolagas SC. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocrine Reviews 2000;21:115-37.

[135] Jilka RL, Weinstein RS, Bellido T, Parfitt AM, Manolagas SC. Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 1998;13:793-802.

[136] Bonewald LF. The amazing osteocyte. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2011;26:229-38.

[137] Franz-Odendaal TA, Hall BK, Witten PE. Buried alive: how osteoblasts become osteocytes. Developmental dynamics : an official publication of the American Association of Anatomists 2006;235:176-90.
[138] Ramachandran R, Goodman SB, Smith RL. The effects of titanium and polymethylmethacrylate particles on osteoblast phenotypic stability. Journal of biomedical materials research Part A 2006;77:512-7.
[139] Atkins GJ, Welldon KJ, Holding CA, Haynes DR, Howie DW, Findlay DM. The induction of a catabolic phenotype in human primary osteoblasts and osteocytes by polyethylene particles. Biomaterials 2009;30:3672-81.
[140] Lohmann CH, Schwartz Z, Koster G, Jahn U, Buchhorn GH, MacDougall MJ, et al. Phagocytosis of wear debris by osteoblasts affects differentiation and local factor production in a manner dependent on particle composition. Biomaterials 2000;21:551-61.
[141] Yao JL, CsSzabo G, Jacobs JJ, Kuettner KE, Glant TT. Suppression of osteoblast function by titanium particles. Journal of Bone and Joint Surgery-American Volume 1997;79a:107-12.
[142] Lochner K, Fritsche A, Jonitz A, Hansmann D, Mueller P, Mueller-Hilke B, et al. The potential role of human osteoblasts for periprosthetic osteolysis following exposure to wear particles. Int J Mol Med 2011;28:1055-63.
[143] Vermes C, Chandrasekaran R, Jacobs JJ, Galante JO, Roebuck KA, Glant TT. The effects of particulate wear debris, cytokines, and growth factors on the functions of MG-63 osteoblasts. The Journal of bone and joint surgery American volume 2001;83-A:201-11.

[144] Chiu R, Ma T, Smith RL, Goodman SB. Ultrahigh molecular weight polyethylene wear debris inhibits osteoprogenitor proliferation and differentiation in vitro. Journal of biomedical materials research Part A 2009;89:242-7.

[145] O'Neill SC, Queally JM, Devitt BM, Doran PP, O'Byrne JM. The role of osteoblasts in peri-prosthetic osteolysis. Bone & Joint Journal 2013;95b:1022-6.

[146] Qiu S, Zhao F, Tang X, Pei F, Dong H, Zhu L, et al. Type-2 cannabinoid receptor regulates proliferation, apoptosis, differentiation, and OPG/RANKL ratio of MC3T3-E1 cells exposed to Titanium particles. Molecular and cellular biochemistry 2015;399:131-41.

[147] Yang SY, Zhang K, Li FF, Jiang JH, Jia TH, Yang SY. Biological responses of preosteoblasts to particulate and ion forms of Co-Cr alloy. Journal of Biomedical Materials Research Part A 2015;103:3564-71.

[148] Pioletti DP, Leoni L, Genini D, Takei H, Du P, Corbeil J. Gene expression analysis of osteoblastic cells contacted by orthopedic implant particles. Journal of biomedical materials research 2002;61:408-20.

[149] O'Neill SC, Queally JM, Devitt BM, Doran PP, O'Byrne JM. The role of osteoblasts in peri-prosthetic osteolysis. The bone & joint journal 2013;95-B:1022-6.

[150] Schwarz EM, Lu AP, Goater JJ, Benz EB, Kollias G, Rosier RN, et al. Tumor necrosis factor-alpha/nuclear transcription factor-kappaB signaling in periprosthetic osteolysis. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 2000;18:472-80.

[151] Dean DD, Schwartz Z, Liu Y, Blanchard CR, Agrawal CM, Mabrey JD, et al. The effect of ultra-high molecular weight polyethylene wear debris on MG63 osteosarcoma cells in vitro. The Journal of bone and joint surgery American volume 1999;81:452-61.

[152] Dean DD, Lohmann CH, Sylvia VL, Koster G, Liu Y, Schwartz Z, et al. Effect of polymer molecular weight and addition of calcium stearate on response of MG63 osteoblast-like cells to UHMWPE particles. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 2001;19:179-86.

[153] Pioletti DP, Kottelat A. The influence of wear particles in the expression of osteoclastogenesis factors by osteoblasts. Biomaterials 2004;25:5803-8.

[154] Granchi D, Amato I, Battistelli L, Ciapetti G, Pagani S, Avnet S, et al. Molecular basis of osteoclastogenesis induced by osteoblasts exposed to wear particles. Biomaterials 2005;26:2371-9.

[155] Dallas SL, Prideaux M, Bonewald LF. The osteocyte: an endocrine cell ... and more. Endocr Rev 2013;34:658-90.

[156] Prideaux M, Findlay DM, Atkins GJ. Osteocytes: The master cells in bone remodelling. Curr Opin Pharmacol 2016;28:24-30.

[157] Noble BS, Reeve J. Osteocyte function, osteocyte death and bone fracture resistance. Molecular and cellular endocrinology 2000;159:7-13.

[158] Dallas SL, Prideaux M, Bonewald LF. The Osteocyte: An Endocrine Cell … and More. Endocrine Reviews 2013;34:658-90.

[159] Steflik DE, Corpe RS, Lake FT, Young TR, Sisk AL, Parr GR, et al. Ultrastructural analyses of the attachment (bonding) zone between bone and implanted biomaterials. Journal of biomedical materials research 1998;39:611-20.

[160] Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. Nature Medicine 2011;17:1235-41.

[161] Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nat Med 2011;17:1231-4.

[162] O'Brien CA, Nakashima T, Takayanagi H. Osteocyte control of osteoclastogenesis. Bone 2013;54:258-63.

[163] Wijenayaka AR, Kogawa M, Lim HP, Bonewald LF, Findlay DM, Atkins GJ. Sclerostin Stimulates Osteocyte Support of Osteoclast Activity by a RANKL-Dependent Pathway. PloS one 2011;6:e25900.

[164] Belanger LF. Osteocytic osteolysis. Calcif Tissue Res 1969;4:1-12.

[165] Parfitt AM. The cellular basis of bone turnover and bone loss: a rebuttal of the osteocytic resorption--bone flow theory. Clinical orthopaedics and related research 1977:236-47.

[166] Teti A, Zallone A. Do osteocytes contribute to bone mineral homeostasis? Osteocytic osteolysis revisited. Bone 2009;44:11-6.

[167] van der Plas A, Aarden EM, Feijen JH, de Boer AH, Wiltink A, Alblas MJ, et al. Characteristics and properties of osteocytes in culture. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 1994;9:1697-704.

[168] Wysolmerski JJ. Osteocytic osteolysis: time for a second look? Bonekey Rep 2012;1:229.

[169] Qing H, Bonewald LF. Osteocyte remodeling of the perilacunar and pericanalicular matrix. Int J Oral Sci 2009;1:59-65.

[170] Alcobendas M, Baud CA, Castanet J. Structural-Changes of the Periosteocytic Area in Vipera-Aspis (L) (Ophidia, Viperidae) Bone Tissue in Various Physiological Conditions. Calcified tissue international 1991;49:53-7.

[171] Rodionova NV, Oganov VS, Zolotova NV. Ultrastructural changes in osteocytes in microgravity conditions. Adv Space Res 2002;30:765-70.

[172] Kwiecinski GG, Krook L, Wimsatt WA. Annual Skeletal Changes in the Little Brown Bat, Myotis-Lucifugus-Lucifugus, with Particular Reference to Pregnancy and Lactation. Am J Anat 1987;178:410-20.

[173] Steinberg B, Singh IJ, Mitchell OG. The Effects of Cold-Stress, Hibernation, and Prolonged Inactivity on Bone Dynamics in the Golden-Hamster, Mesocricetus-Auratus. J Morphol 1981;167:43-51.

[174] Haller AC, Zimny ML. Effects of Hibernation on Interradicular Alveolar Bone. J Dent Res 1977;56:1552-7.

[175] Tang SY, Herber RP, Ho SP, Alliston T. Matrix metalloproteinase-13 is required for osteocytic perilacunar remodeling and maintains bone fracture resistance. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2012;27:1936-50.

[176] Tazawa K, Hoshi K, Kawamoto S, Tanaka M, Ejiri S, Ozawa H. Osteocytic osteolysis observed in rats to which parathyroid hormone was continuously administered. J Bone Miner Metab 2004;22:524-9.

[177] Tsourdi E, Jahn K, Rauner M, Busse B, Bonewald LF. Physiological and pathological osteocytic osteolysis. J Musculoskelet Neuronal Interact 2018;18:292-303.

[178] Wysolmerski JJ. Osteocytes remove and replace perilacunar mineral during reproductive cycles. Bone 2013;54:230-6.

[179] Atkins GJ, Rowe PS, Lim HP, Welldon KJ, Ormsby R, Wijenayaka AR, et al. Sclerostin is a locally acting regulator of late-osteoblast/preosteocyte differentiation and regulates mineralization through a MEPE-ASARM-dependent mechanism. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2011;26:1425-36.

[180] Kogawa M, Wijenayaka AR, Ormsby RT, Thomas GP, Anderson PH, Bonewald LF, et al. Sclerostin Regulates Release of Bone Mineral by Osteocytes by Induction of Carbonic Anhydrase 2. J Bone Miner Res 2013;28:2436-48.

[181] Kogawa M, Khalid KA, Wijenayaka AR, Ormsby RT, Evdokiou A, Anderson PH, et al. Recombinant sclerostin antagonizes effects of ex vivo mechanical loading in trabecular bone and increases osteocyte lacunar size. American journal of physiology Cell physiology 2018;314:C53-C61.

[182] Busse B, Hahn M, Niecke M, Jobke B, Puschel K, Delling G, et al. Allocation of nonbirefringent wear debris: darkfield illumination associated with PIXE microanalysis reveals cobalt deposition in mineralized bone matrix adjacent to CoCr implants. Journal of biomedical materials research Part A 2008;87:536-45.

[183] Ormsby RT, Cantley M, Kogawa M, Solomon LB, Haynes DR, Findlay DM, et al. Evidence that osteocyte perilacunar remodelling contributes to polyethylene wear particle induced osteolysis. Acta biomaterialia 2016;33:242-51.

[184] Cardoso L, Herman BC, Verborgt O, Laudier D, Majeska RJ, Schaffler MB. Osteocyte apoptosis controls activation of intracortical resorption in response to bone fatigue. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2009;24:597-605.

[185] Kanaji A, Caicedo MS, Virdi AS, Sumner DR, Hallab NJ, Sena K. Co-Cr-Mo alloy particles induce tumor necrosis factor alpha production in MLO-Y4 osteocytes: a role for osteocytes in particle-induced inflammation. Bone 2009;45:528-33.

[186] Zhang Y, Yan M, Yu A, Mao H, Zhang J. Inhibitory effects of beta-tricalciumphosphate wear particles on osteocytes via apoptotic response and Akt inactivation. Toxicology 2012;297:57-67.

[187] Lohmann CH, Dean DD, Bonewald LF, Schwartz Z, Boyan BD. Nitric oxide and prostaglandin E2 production in response to ultra-high molecular weight polyethylene particles depends on osteoblast maturation state. The Journal of bone and joint surgery American volume 2002;84-A:411-9.

[188] Talmo CT, Shanbhag AS, Rubash HE. Nonsurgical management of osteolysis: challenges and opportunities. Clinical orthopaedics and related research 2006;453:254-64.

[189] Han SY, Lee NK, Kim KH, Jang IW, Yim M, Kim JH, et al. Transcriptional induction of cyclooxygenase-2 in osteoclast precursors is involved in RANKL-induced osteoclastogenesis. Blood 2005;106:1240-5.

[190] Geng DC, Zhu XS, Mao HQ, Meng B, Chen L, Yang HL, et al. Protection against titanium particle-induced osteoclastogenesis by cyclooxygenase-2 selective inhibitor. Journal of biomedical materials research Part A 2011;99:516-22.

[191] Hirayama A, Tanahashi N, Daida H, Ishiguro N, Chachin M, Sugioka T, et al. Assessing the cardiovascular risk between celecoxib and nonselective nonsteroidal antiinflammatory drugs in patients with rheumatoid arthritis and osteoarthritis. Circ J 2014;78:194-205.

[192] Nissen SE, Yeomans ND, Solomon DH, Luscher TF, Libby P, Husni ME, et al. Cardiovascular Safety of Celecoxib, Naproxen, or Ibuprofen for Arthritis. N Engl J Med 2016;375:2519-29.

[193] Chen D, Zhang X, Guo Y, Shi S, Mao X, Pan X, et al. MMP-9 inhibition suppresses wear debris-induced inflammatory osteolysis through downregulation of RANK/RANKL in a murine osteolysis model. Int J Mol Med 2012;30:1417-23.

[194] Geng T, Sun S, Chen X, Wang B, Guo H, Zhang S, et al. Strontium ranelate reduces the progression of titanium particle-induced osteolysis by increasing the ratio of

osteoprotegerin to receptor activator of nuclear factor-kappaB ligand in vivo. Mol Med Rep 2018;17:3829-36.

[195] van der Heijden L, Dijkstra PDS, Blay JY, Gelderblom H. Giant cell tumour of bone in the denosumab era. Eur J Cancer 2017;77:75-83.

[196] Zhang T, Yu H, Gong W, Zhang L, Jia T, Wooley PH, et al. The effect of osteoprotegerin gene modification on wear debris-induced osteolysis in a murine model of knee prosthesis failure. Biomaterials 2009;30:6102-8.

[197] Tsourdi E, Langdahl B, Cohen-Solal M, Aubry-Rozier B, Eriksen EF, Guanabens N, et al. Discontinuation of Denosumab therapy for osteoporosis: A systematic review and position statement by ECTS. Bone 2017;105:11-7.

[198] Uday S, Gaston CL, Rogers L, Parry M, Joffe J, Pearson J, et al. Osteonecrosis of the Jaw and Rebound Hypercalcemia in Young People Treated With Denosumab for Giant Cell Tumor of Bone. J Clin Endocrinol Metab 2018;103:596-603.

[199] Cosman F, Crittenden DB, Ferrari S, Lewiecki EM, Jaller-Raad J, Zerbini C, et al. Romosozumab FRAME Study: A Post Hoc Analysis of the Role of Regional Background Fracture Risk on Nonvertebral Fracture Outcome. Journal of bone and mineral research 2018;33:1407-16.

[200] Geng T, Sun S, Yu H, Guo H, Zheng M, Zhang S, et al. Strontium ranelate inhibits wear particle-induced aseptic loosening in mice. Brazilian journal of medical and biological research 2018;51:e7414.

Chapter 2

Evidence that osteocyte perilacunar remodelling contributes to polyethylene wear particle induced osteolysis

As published in *Acta Biomaterialia*, 2016

To date there is little knowledge on the effect of wear particles on osteocytes. Preliminary work on the effect of ultra-high molecular weight polyethylene particles on osteoblasts and osteocytes has been previously published by our research group, however, the role of osteocytes in wear particle induced osteolysis is relatively unknown. Therefore, hypothesis of this thesis is that osteocytes stimulate bone remodelling in response to wear particles, contributing to the development of osteolysis, and potentially providing new targets for future clinical therapies. The work presented in Chapter 2 aimed to elucidate the effects of conventional ultra-high molecular weight polyethylene particles, Ceridust, on immature and mature osteocyte like-cells *in vitro*, and *in vivo* in a mouse calvarial model of osteolysis. Additionally, this study included analysis of *ex vivo* human bone biopsies from revision total hip replacement patients with confirmed osteolysis. Osteocyte-like cells exposed to UHMWPE *in vitro* confirmed an induction of catabolic factors that are key for bone resorption, as well as the induction of markers of perilacunar remodelling. Furthermore, perilacunar remodelling, in addition to osteoclastogenesis, was observed *in vivo*, a novel finding in this study. This study is presented in the published form of the manuscript.

Statement of Authorship

Title of Paper	Evidence that osteocyte perilacunar remodelling contributes to polyethylene wear particle induced osteolysis.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Ormsby R.T., Cantley M., Kogawa M., Solomon, L.B., Haynes, D.R., Findlay, D.M., & Atkins, G.J. (2016) Evidence that osteocyte perilacunar remodelling contributes to polyethylene wear particle induced osteolysis.

Principal Author

Name of Principal Author (Candidate)	Renee T Ormsby		
Contribution to the Paper	Performed analysis on all in vitro experiments, interpreted all data obtained and drafted the manuscript.		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	08/08/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Melissa Cantley		
Contribution to the Paper	Performed the calvarial osteolysis model, micro-CT and analysis of in vivo mouse model		
Signature		Date	09/08/2018

Name of Co-Author	Masakazu Kogawa		
Contribution to the Paper	Obtained and processed bone biopsies for analysis		
Signature		Date	10/08/2018

Name of Co-Author	L. Bogdan Solomon		
Contribution to the Paper	Provided intellectual input		
Signature		Date	16/08/2018

Name of Co-Author	David R Haynes		
Contribution to the Paper	Provided intellectual input		
Signature		Date	16/08/2018

Name of Co-Author	David M Findlay		
Contribution to the Paper	Contributed to the manuscript evaluation and the intellectual input		
Signature		Date	16/08/2018

Name of Co-Author	Gerald J Atkins		
Contribution to the Paper	Planned and supervised research work, helped with data interpretation and writing of manuscript. Senior author on paper.		
Signature		Date	29/10/2018

Acta Biomaterialia 33 (2016) 242–251

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Evidence that osteocyte perilacunar remodelling contributes to polyethylene wear particle induced osteolysis

Renee T. Ormsby [a], Melissa Cantley [b,c], Masakazu Kogawa [a], L. Bogdan Solomon [a], David R. Haynes [b], David M. Findlay [a,1], Gerald J. Atkins [a,*,1]

[a] Centre for Orthopaedic and Trauma Research, Discipline of Orthopaedics and Trauma, University of Adelaide, Adelaide, SA 5005, Australia
[b] Discipline of Anatomy and Pathology, University of Adelaide, Adelaide, SA 5005, Australia
[c] Myeloma Research Laboratory, SAHMRI, North Terrace, Adelaide, SA 5000, Australia

ARTICLE INFO

Article history:
Received 28 August 2015
Received in revised form 12 January 2016
Accepted 16 January 2016
Available online 18 January 2016

Keywords:
Polyethylene
UHMWPE
Aseptic loosening
Periprosthetic
Osteocyte
Osteocytic osteolysis

ABSTRACT

Periprosthetic osteolysis (PO) leading to aseptic loosening, is the most common cause of failure of total hip replacement (THR) in the mid- to long-term. Polyethylene (PE) particulates from the wear of prosthesis liners are bioactive and are implicated in the initiation and or progression of osteolysis. Evidence exists that cells of the osteoblast/osteocyte lineage are affected by PE particles and contribute to the catabolic response by promoting osteoclastic bone resorption. In this study, we hypothesised that osteocytes contribute directly to PO by removing bone from their perilacunar matrix. Osteocyte responses to ultra-high molecular weight PE (UHMWPE) particles were examined *in vitro* in human primary osteocyte-like cultures, *in vivo* in the mouse calvarial osteolysis model, and in the acetabulum of patients undergoing revision total hip replacement (THR) surgery for PO. Osteocytes exposed to UHMWPE particles showed upregulated expression of catabolic markers, MMP-13, carbonic anhydrase 2 (*CA2*), cathepsin K (*CTSK*) and tartrate resistant acid phosphatase (*TRAP*), with no effect on cell viability, as assessed by Caspase 3 activity. Consistent with this catabolic activity causing perilacunar bone loss, histological analysis of calvarial sections from mice exposed to UHMWPE revealed a significant ($p < 0.001$) increase in osteocyte lacunar area (Lac.Ar) compared to sham-operated animals. Furthermore, acetabular biopsies from patients with PO also showed significantly ($p < 0.001$) increased osteocyte lacunar size in trabecular bone adjacent to PE particles, compared with osteocyte lacunar size in bone from primary THR patients. Together, these findings suggest a previously unrecognised action of UHMWPE wear particles on osteocytes, which directly results in a loss of osteocyte perilacunar bone. This action may exacerbate the indirect pro-osteoclastic action of UHMWPE-affected osteocytes, previously shown to contribute to aseptic loosening of orthopaedic implants.

Statement of Significance

This study addresses the clinical problem of periprosthetic osteolysis, bone loss in response to polyethylene wear particles derived from materials used in orthopaedic implants. Periprosthetic osteolysis has been thought to be due largely to wear particles stimulating the activity of bone resorbing osteoclasts. However, in this study we demonstrate for the first time that polyethylene particles stimulate another type of bone loss, mediated by the direct activity of bone mineral embedded osteocytes, termed *osteocytic osteolysis* or *osteocyte perilacunar remodelling*. This study provides new mechanistic insight into wear-particle mediated bone loss and represents a new paradigm for the way in which bone cells, namely osteocytes, the key controlling cell type in bone, react to biomaterials.

© 2016 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Periprosthetic osteolysis (PO) combined with aseptic loosening, is the most common cause of failure of total hip replacement (THR) in the mid- to long-term [1]. Particles released from the articular

* Corresponding author at: Centre for Orthopaedic and Trauma Research, The University of Adelaide, North Terrace, Adelaide, South Australia 5005, Australia.
E-mail address: gerald.atkins@adelaide.edu.au (G.J. Atkins).
[1] Equal senior authors.

http://dx.doi.org/10.1016/j.actbio.2016.01.016
1742-7061/© 2016 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.

surfaces of prosthetic components due to repeated motion are important causative agents in PO [2,3]. Other factors, such as fluid access to the bone-implant interface and hydrostatic pressure, are also likely important [4,5]. Polyethylene (PE), in particular ultra-high molecular weight polyethylene (UHMWPE), is among the most widely used articulating surface used for hip prostheses. It is now well established that many particulates produced by prosthetic wear, including PE, metal [6], alumina [7] and polymethylmethacrylate (PMMA) [8] are bioactive, and are therefore implicated in the initiation and or progression of osteolysis. However, PE wear particles may be the most important in PO where PE linings are present [9,10]. We have reported that the extent of PE liner wear correlates strongly with osteolysis, and that THR patients with a high volumetric wear rate exhibit the greatest progression of lesion volume [11].

Sites of osteolysis are regions of high bone turnover, with active osteoclastic resorption associated with increased mineral apposition and bone formation rates. This results in an immature bone matrix with poor bone quality, containing abundant osteocytes [12]. The granulomatous tissue that develops adjacent to prostheses with PE liners contains large numbers of PE particles that are phagocytosed by macrophage-like cells [13–15]. The tissues at sites of osteolysis display a distinct catabolic profile, with abundant expression of matrix degradative enzymes, including high levels of matrix metallopeptidases (MMPs-1, 2, 3, 9 and 14 [16,17]. The interface tissues around failed prostheses have also been found to be acidic and contain high levels of the enzyme cathepsin K (CTSK) [18], associated most often with osteoclastic activity. However, it is now known that CTSK can also be expressed by osteocytes [19,20].

A body of evidence exists that cells of the osteoblast/osteocyte lineage are also affected by PE particles, both indirectly *via* mediators released by PE-activated macrophages [21], and by direct mechanisms. Exposure of osteoblast-like cells to PE induced changes in the rate of cell proliferation [22–24], decreased alkaline phosphatase activity [23], and increased the production of osteoclastogenic mediators, such as PGE_2 [23], IL-6 [25,26], GM-CSF, RANKL [27] and nitric oxide (NO) [28]. The nature of the response may be dependent on the way in which the PE particles are presented to the cells and the maturation state of the osteoblasts [28]. However, the overall effect of PE particles on osteoblasts is one of promoting a phenotype that is anti-anabolic and pro-catabolic. For example, UHMWPE particles increased the release of RANKL from human osteoblasts, while inhibiting their expression of OPG. Consistent with this, conditioned media from PE-treated osteoblasts strongly promoted osteoclastogenesis from human peripheral blood mononuclear cells [27]. We have described a 3-dimensional type I collagen gel culture system, which enables long-term culture of human osteoblastic cells in the presence of PE particles, and their differentiation into a mature osteocyte-like phenotype [29,30]. Cells exposed to PE particles in this experimental system showed an increase in mRNA expression of the late osteoblast/osteocyte markers *E11*, *DMP1* and *SOST*. At the same time, increased expression of several genes associated with osteoclast formation and activity (*RANKL*, *M-CSF* and *IL-8*), and decreased expression of *OPG* mRNA was observed [29].

Numerous studies in a wide variety of vertebrate species have demonstrated that osteocytes are capable of removing their perilacunar matrix [31], with evidence that this mechanism is involved in maintaining circulating calcium levels in hibernation [32] and in egg-laying [33]. It is now clear that osteocyte perilacunar remodelling, commonly termed osteocytic osteolysis, is a major method, by which mammals release calcium during lactation [20,34,35]. Osteocytic osteolysis also has a wider role in the maintenance of bone mineral homeostasis and quality [20,35], and is a process promoted by the osteocyte-expressed molecule, sclerostin [19]. This mechanism may also be 'hijacked' in pathologies associated with bone loss, with evidence that hyperparathyroidism is associated with enlarged osteocyte lacunae [36] that remineralise after parathyroidectomy [37]. However, the involvement of this process in other pathological events in bone is less well understood.

In this study, we report increased expression of mediators of osteocytic osteolysis by human osteocyte-like cells exposed to UHMWPE particles. We also show features consistent with PE particle induced osteocytic osteolysis in the mouse calvarial implantation model and in human bone sampled from patients undergoing revision THR for periprosthetic osteolysis. We conclude that osteocytes contribute directly to the bone loss associated with aseptic loosening.

2. Materials and methods

2.1. Osteoblast-like cells cultured with polyethylene particles

UHMWPE particles (Ceridust 3615 polyethylene powder, Clariant Company, Gersthofen, Germany) were prepared, as described previously [29]. Briefly, particles confirmed negative for endotoxin were washed and resuspended at 1 mg/ml in endotoxin-free phosphate buffered saline (PBS) containing 10% foetal calf serum (FCS). Human primary osteoblast-like cells were isolated from femoral trabecular bone taken from patients undergoing primary total hip replacement surgery and cultured *ex vivo*, as described previously [38]. Samples were obtained with informed consent and approval by the Human Ethics Committee of the Royal Adelaide Hospital. Two models of osteocyte-particle interaction were utilised; the first involved osteoblast-like cells and PE particles, which were combined and suspended together in a type I collagen gel (Cellmatrix Type 1-A, Nitta, Tokyo, Japan), with PE particles at concentrations of 100 μg/ml or 500 μg/ml. The cells were cultured in the gel for 14 and 21 days, as described previously [29]. The second model involved culturing cells under differentiating conditions for a period of 28d, which gives rise to cells that phenotypically resemble mature osteocytes and express markers of mature osteocytes, including *SOST*, *DMP1*, *PHEX* and *E11* [4]. A collagen gel containing the desired amount of PE particles was then overlaid and the cells were cultured for a further 21 days. In both systems, the collagen was mixed in a solution containing α-MEM (Gibco, Life Technologies, NY, USA), 10% FCS, (1.8 mM) potassium phosphate, dexamethasone (10 nM) and ascorbate-2-phosphate (100 μM). Medium was replenished every 3-4 days.

2.2. Preparation of RNA and gene expression analysis

Total RNA was extracted from collagen gel cultures using TRIzol (Life Technologies, NY, USA), according to manufacturer's instructions. Complementary DNA (cDNA) was synthesised using iScript™ RT kit (Bio-Rad, Hercules, CA, USA) and gene expression was analysed by real-time RT-PCR, using SYBR Green Fluor qPCR Mastermix (Qiagen, Limburg, The Netherlands), in a CFX Connect Real Time PCR System (Bio-Rad, Hercules, CA, USA). Oligonucleotide sequences for the mRNA-specific amplification of human genes *MMP13*, *CTSK*, *CA2*, *TRAP*, *RANKL*, *OPG*, *DMP1*, *SOST*, *FGF23*, *BAX* and *BCL-2* are shown in Table 1. All primers were designed in-house and were purchased from Geneworks (Thebarton, SA, Australia).

2.3. Caspase 3 activity

Human osteocyte-like cultures were assayed for Caspase 3 activity, a known marker of apoptosis. Briefly, supernatants were

Table 1
Real-time RT-PCR sequences used in this study.

Species/target gene		Primer sequence (5′–3′)	Expected product size (bp)
hMMP13	F	ggatccagtctctctatggt	95
	R	ggcatcaagggataaggaag	
hCTSK	F	ggtacagagagatccccga	138
	R	agctttcatcataatacacacctt	
hCA2	F	tctcaggacaaagcagtgctca	148
	R	gaaccaagtgaagttctgcagc	
hTRAP	F	gtgcagacttcatcctgtctcta	97
	R	gtgcagacttcatcctgtctcta	
hBAX	F	tccccccgagaggtctttt	68
	R	cggccccagttgaagttg	
hBCL-2	F	tgggatgcctttgtggaactgtacg	127
	R	tagataggcacccagggtgatgcaag	
hRANKL	F	ccacccccgatcatggt	94
	R	tcagccttttgctcatctcactat	
hOPG	F	gctcacaagaacagactttccag	106
	R	ctgttttcacagaggtcaatatctt	
hDMP1	F	agccaaatgacccttccattc	98
	R	gatcagcatcctgctcatgtt	
hSOST	F	accggagctggagaacaaca	97
	R	gctgtactcggacacgtctt	
hFGF23	F	atgctggctttgtggtgatta	99
	R	tctccgggtcgaaatagtgt	

removed and the cells were washed 2x with PBS. 50 µl of Caspase 3 lysis buffer containing 0.5 M EDTA, 1 M Tris-HCl, 10% Igepal (Sigma Aldrich, St Louis, MO, USA) was added to each well. The supernatants were centrifuged at 400 g for 10 min and returned to corresponding wells. The cells were left to lyse on ice for 20 min. The lysates were then centrifuged at 12000 g for 10 min at 4 °C. Cell lysates (20 µg) were added to a white microtitre plate with 200 µl of fluorometric caspase assay buffer (0.1 M HEPES, 1% Sucrose, 1% CHAPS, 20 mM Caspase 3 substrate (AC-DEVD-AFC, Kamiya Biomedical, Seattle, WA, USA) and 1 M DTT), covered and incubated at room temperature for 4 h. The resulting fluorescence was read at 570 nm (LS 50 Spectrofluorimeter, Perkin Elmer, San Jose, CA, USA).

2.4. Calvarial osteolysis model

The mouse calvarial osteolysis model [39] was adapted in order to test the effects of UHMWPE particles *in vivo*. All animal studies received institutional animal research ethics approval (University of Adelaide Animal Ethics Committee, approval no. M-2010-070). Nine C3H/HEJ mice were randomly separated into either control or PE treatment groups and scanned by micro-computed tomography (µ-CT) at D0. 7 days after µ-CT, the mice were anaesthetised using isofluorane anaesthetic (2% in air) and a 2 mm incision was made over the calvarial sagittal midline suture. A 1.0 × 1.0 cm area of the periosteum was exposed and lightly scratched using the tip of a plastic transfer pipette.

Animals in the control group, Group 1 ($n = 4$) were administered a 30 µl injection of PBS + 1% normal mouse serum into the incision site. Group 2 animals ($n = 5$) were administered a solution of UHMWPE particles, prepared as above and diluted in PBS+1% normal mouse serum, at a concentration of 4.6×10^7 particles in 30 µl into the incision site. The incision site was securely closed with a staple. The mice were monitored daily and at day 21 were euthanised by CO_2 inhalation and the calvariae excised and processed for histology and µ-CT.

2.5. Micro-computed tomography

As stated, calvariae were scanned at day 0 by live animal µ-CT, using a Skyscan 1076 *in vivo* µ-CT System (Skyscan, Bruker, Kontich, Belgium). Scan settings were those described previously [40–43], with 74 kV, 136 µA, pixel size of 18 µM, frame averaging = 1, rotation step = 0.5 and exposure of 590 ms. Scanning was repeated on day 21 to account for bone volume changes, with matching scan parameters. Scans were reconstructed using a filtered back-projection algorithm (NRecon software, V1.12.04, Skyscan, Bruker, Kontich, Belgium). A rectangular region of interest (ROI), centred over the skull around the midline suture where the PE particles were surgically placed, was used for analysis using CTAn (Skyscan, Bruker, Kontich, Belgium). Analysis included 525 µ-CT slice images of the skull. Global thresholding was used to separate out calvarial bone. This was used for both scan 1 (day 0) and scan 2 (day 21) and the percentage change in bone volume between the two scans was calculated. 3D models of the entire skulls were created using ANT (Skyscan, Bruker, Kontich, Belgium).

2.6. Calvaria osteocyte lacunar measurements

Following µ-CT, the calvariae were immersed in 10% neutral buffered formalin for 72 h and then decalcified in 10% EDTA for 10 weeks. Coronal 7 µm sections of each calvaria were cut, de-identified and stained with haematoxylin and eosin, and TRAP staining, the latter performed according to previously published methods [44,45]. Osteocyte lacunae from multiple sections were quantified using Image J software (U.S. National Institutes of Health, Bethesda, MD, USA). Lacunar areas were measured approximately 1 mm from the suture line, the perimeter of each individual lacunae and the total bone area were manually traced. In addition, the percentage of occupied osteocyte lacunae were quantified according to the visible number of nuclei, stained purple and located within the lacunae.

2.7. Osteocyte lacunar measurements from human bone biopsies

Trephine biopsies of bone from the acetabulum of patients undergoing either primary THR surgery for end-stage osteoarthritis, or revision THR due to periprosthetic osteolysis were obtained with informed consent and approval by the Human Ethics Committee of the Royal Adelaide Hospital. Biopsies from primary THR samples were taken from the superior, anterior and posterior acetabulum from each patient following reaming for insertion of the acetabular component. Similar biopsies from revision THR patients were taken following removal of the failed implant and granulomatous tissue, and irrigation of the exposed acetabulum in preparation for reaming. Biopsies were placed into cold sterile saline and prepared for histology within 2–18 h of the operation. Bone samples were obtained from 8 patients from each group and were fixed with 10% buffered formalin for 24 h and then slow decalcified (10% EDTA/1% paraformaldehyde). Sections (5 µm) were cut from each sample and stained with toluidine blue [19] or for TRAP, as described above. Osteocyte lacunar areas were measured as described above.

2.8. Statistical analysis

Statistical differences were assessed using one-way ANOVA, followed by Tukey's post-hoc test, or by Students T-test, as appropriate (GraphPad Prism version 6.0, GraphPad Software, La Jolla, CA, USA). Significance was taken for a value of $p < 0.05$.

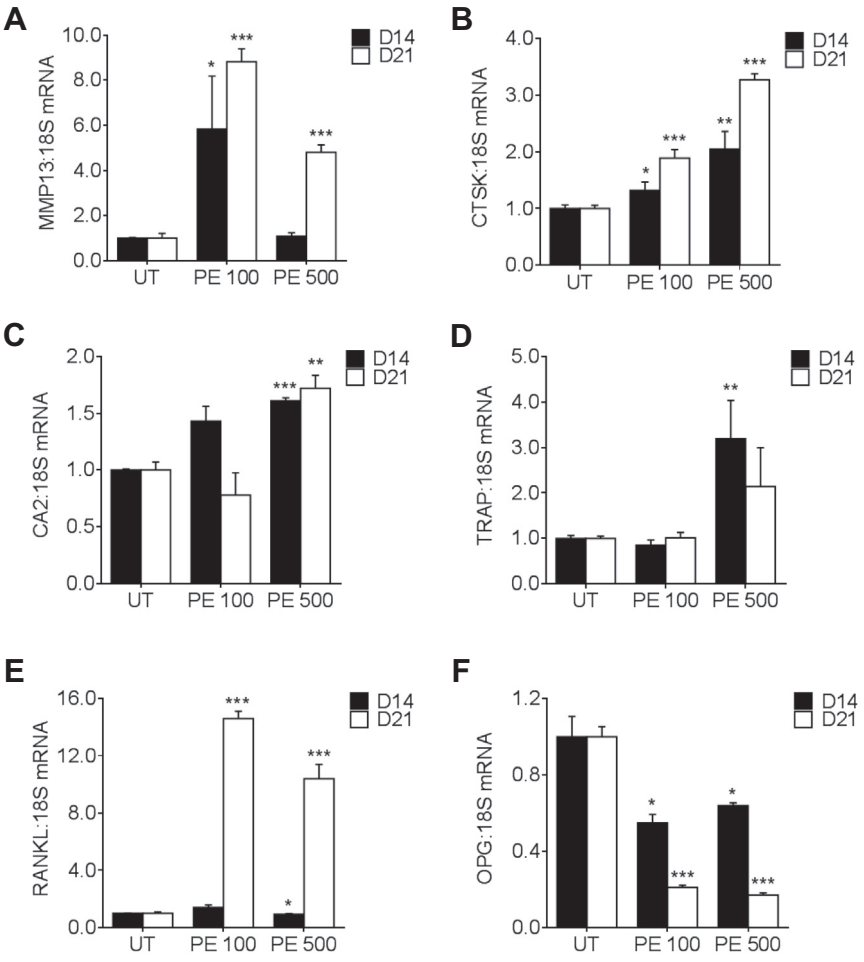


Fig. 1. The effect of UHMWPE particles on differentiating human primary osteoblasts. NHBC were co-suspended in a collagen gel either untreated (UT) or with UHMWPE particles (100 μg/ml or 500 μg/ml) and the expression of genes associated with osteocytic osteolysis *MMP13*, *CTSK*, *CA2* and *IL-6* after 14d (A–D, respectively) and 21d (E–H, respectively), was measured by real-time RT-PCR. Data shown are means ± standard error of the mean (SEM) of triplicate experiments analysed in duplicate. Significant differences are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

3.1. Effect of PE particles on osteocyte gene expression

In order to study the effect of UHMWPE particles on osteocytes *in vitro*, we utilised the differentiated NHBC model, which we have shown differentiate to an osteocyte-like stage [29,30,46,47] and to be free of contaminating haemopoietic cells [29]. NHBC were cultured with UHMWPE particles by either a co-suspension within the collagen gel or with a layer of collagen gel containing the particles on top of mature, differentiated osteocyte-like cells [29,30]. The latter model mimics more closely the *in vivo* situation, where particles are presented to bone within the granulomatous tissue that develops adjacent to sites of osteolysis. In our previous experiments, exposure of osteoblast-like cells (NHBC) to UHMWPE particles within the collagen gel resulted in a dose- and time-dependent increase in the expression of pro-osteoclastogenic mRNA species, *RANKL, M-CSF* and *IL-8*, concomitant with the acquisition of an osteocyte-like phenotype [29]. In the present experiments, we investigated whether UHMWPE particles influence expression of genes reported to be associated with osteocyte-mediated perilacunar remodelling. Messenger RNA expression of the genes *MMP13*, *CTSK*, *CA2* and *TRAP* (Fig. 1A–D) were increased in the presence of UHMWPE particles at both 14d and 21d, suggesting the induction of an osteocytic osteolysis phenotype in the differentiating cells. Consistent with our previous study [29], *RANKL* mRNA expression was increased and *OPG* mRNA suppressed in response to UHMWPE particle exposure (Fig. 1E and D).

Next, we cultured NHBC under mineralising conditions to a mature osteocyte-like stage, evidenced here by their mRNA expression of the markers *DMP1*, *SOST* and *FGF23* (Fig. 2A). These mature cultures were then exposed to UHMWPE particles in an overlying gel layer for a further 21 d. As shown in Fig. 2, these cells responded robustly to UHMWPE particles, with a dose-dependent increase in the mRNA expression of genes associated with osteocytic osteolysis, *MMP13*, *CTSK*, *CA2* and *TRAP* (Fig. 2B–E). Significant induction of *RANKL* mRNA was also seen in these cultures (Fig. 2F). Interestingly, *OPG* mRNA expression was also increased in cultures exposed to UHMWPE (Fig. 2G), suggesting that an increased bone remodelling phenotype was induced in these late stage cells.

3.2. Effect of UHMWPE particles on osteocyte viability

Polyethylene particles induced mRNA expression of both the pro-apoptotic marker, *BAX,* and the anti-apoptotic marker, *BCL-2* (Fig. 3A and B). However the ratio of *BAX:BCL-2* mRNA, which correlates with apoptosis [48], was not significantly changed with UHMWPE treatment (Fig. 3C). Supportive of this, activity of the

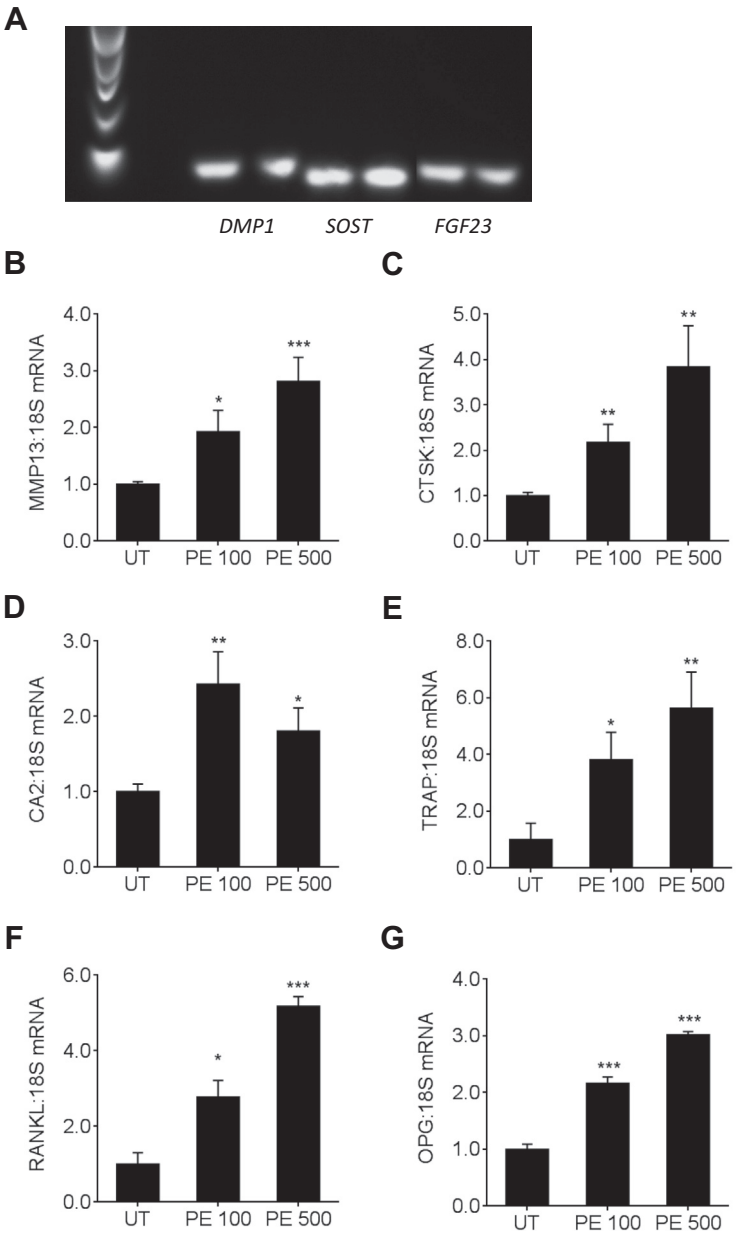


Fig. 2. The effect of UHMWPE particles on differentiated osteocyte-like cells. NHBC were differentiated for 28 d to an osteocyte-like stage, expressing the markers *DMP1*, *SOST* and *FGF23* (A). The cells were then overlaid with a collagen gel layer otherwise untreated (UT) or with gel containing UHMWPE particles (100 μg/ml and 500 μg/ml). After a further 21 d of culture, the expression of genes associated with osteocytic osteolysis, *MMP13*, *CTSK*, *CA2* and *TRAP* (B–E, respectively) or osteoclastogenesis, *RANKL* and *OPG* (F & G, respectively) was measured by real-time RT-PCR. Data shown are means ± standard error of the mean (SEM) of triplicate experiments analysed in duplicate. Significant differences are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

key apoptotic enzyme, caspase 3, showed no significant change in UHMWPE treated cells, which rather showed a decrease in caspase activity below the basal level, perhaps implying that UHMWPE particles increase osteocyte viability (Fig. 3D).

3.3. Evidence for osteoclastic resorption in response to UHMWPE particles in vivo

The μ-CT analysis of mouse calvaria exposed to UHMWPE particles revealed the presence of distinct osteoclastic resorption pits on both the outer and inner surfaces of calvariae, but no such resorption was evident on the calvarial surfaces of sham-operated animals (Fig. 4A), consistent with expectations and confirming the bioactivity of the UHMWPE preparations used. Consistent with the mice in this study growing during the course of the experiment, the calvarial percentage bone volume of a defined ROI increased in both sham control (25.58 ± 4.36 mm^3) and PE-treated (26.79 ± 4.19 mm^3) animals, however the degree of change was not significantly different between groups ($p = 0.31$). Coronal sections of calvarial showed evidence of TRAP-positive osteoclasts, as well as some TRAP-stained osteocyte lacunae, only in PE-treated animals (Fig. 4B), consistent with our findings of *TRAP* mRNA induction in PE-treated osteocyte-like cells (Fig. 1D and Fig. 2E).

3.4. Evidence of PE particles promoting an increase in osteocyte lacunar area without affecting cell viability

We next examined sections of calvariae for evidence of osteocytic osteolysis. Coronal sections were stained using haematoxylin and eosin (H&E) and examined by light microscopy (Fig. 4C).

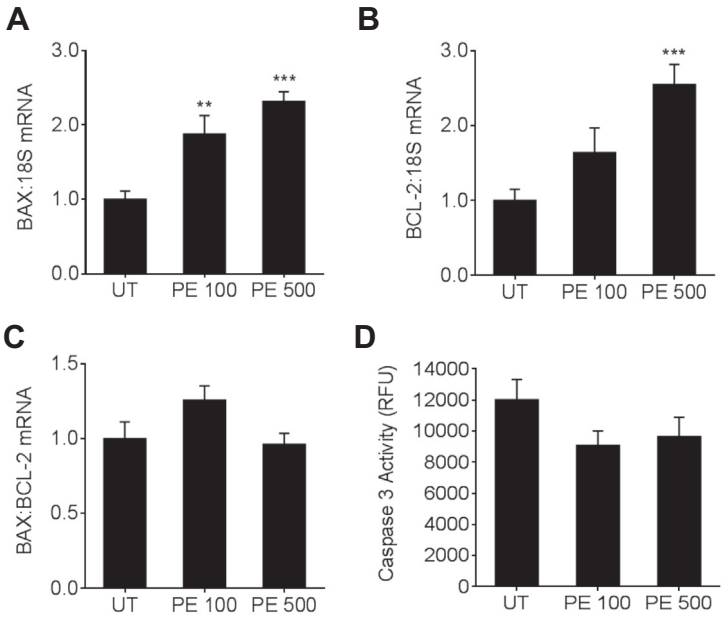


Fig. 3. The effect of UHMWPE particles on apoptotic markers. Mature differentiated osteocyte-like cells cultured with an overlaid collagen gel containing UHMWPE particles (0, 100 μg/ml and 500 μg/ml) for 21d were assessed by real-time RT-PCR for the expression of genes associated with apoptosis (A) *BAX*, (B) *BCL-2*, and (C) the associated pro-apoptotic *BAX/BCL-2* mRNA ratio. Replicate cultures were processed for the measurement of Caspase 3 activity (D). Data shown are means ± standard error of the mean (SEM) of triplicate experiments analysed in duplicate. Significant differences are denoted by $^{**}p < 0.01$, $^{***}p < 0.001$.

Measurement of osteocyte lacunar area (Lac.Ar) revealed a statistically significant increase in the UHMWPE-treated calvariae, compared with that of the sham-operated controls, indicative of osteocytic osteolysis (Fig. 4D). When the 20% largest lacunae were considered, which is assumed to represent approximate midsections through ellipsoid lacunae [20], a similar increase in the size (Top 20% Lac.Ar) of UHMWPE-exposed calvariae was observed (Fig. 4E). The percentages of occupied versus unoccupied osteocyte lacunae were calculated for both treated and control bone, and showed no significant change (Fig. 4F), suggesting that osteocyte cell death was not a feature of osteolysis in this model.

3.5. Evidence of increased osteocyte lacunar area in peri-implant bone in human patients

Bone samples were obtained from the superior, anterior and posterior acetabulum of patients undergoing either primary or revision THR surgery that was required due to peri-implant bone loss. The site behind the acetabular cup can be exposed directly to wear particles released from the joint articulations. In the samples obtained from revision samples in this study, the presence of PE particles was confirmed using polarised light microscopy (Fig. 5A). Histochemical staining revealed evidence of TRAP-stained osteocyte lacunae in revision THR samples (Fig. 5B), consistent with findings in the cell and mouse models above. Quantification of the osteocyte lacunae in these samples revealed a significant increase in the mean Lac.Ar (Fig. 5C), and the Lac.Ar expressed as a proportion of the trabecular bone area (Lac.Ar/B.Ar; Fig. 5D) in the acetabular bone of revision bone samples compared to bone from the corresponding sites in primary THR patients. These results suggest that osteocytic perilacunar bone loss occurs in human patient bone exposed to PE wear particles.

4. Discussion

While PE particles have been widely shown to stimulate osteoclast formation and activity, there have been few reports demonstrating how they affect the behaviour of cells of the osteoblastic lineage [49]. In particular, there is little information as to the effects on osteocytes, cells increasingly reported to play an important role in regulation of bone metabolism [50,51]. We previously showed that exposure of primary human osteoblast-like cells to UHMWPE particles gave rise to osteocyte-like cells with a pro-osteoclastogenic phenotype, expressing *RANKL*, *M-CSF* and *IL-8*. A similar response to PE was observed in MLO-Y4 osteocyte-like cells [28]. These reports provided the stimulus for the present investigation showing exposure of osteocytes to UHMWPE particles can contribute to peri-prosthetic osteolysis.

Here we have shown, using human osteocyte-like cells *in vitro* and the mouse calvaria model *in vivo*, a direct effect of UHMWPE particles on the osteocyte causing osteocytic osteolysis. In human osteocyte-like cells exposed to UHMWPE, the expression of the key cytosolic carbonic anhydrase isoform, *CA2*, was up-regulated. We recently reported that CA2 is responsible for the osteocytic osteolysis induced by sclerostin, due to its ability to generate protons capable of acidifying the osteocyte perilacunar matrix [19]. In addition, UHMWPE particles induced the expression of the collagenase *MMP13*, shown by Tang and colleagues to be intrinsic to osteocyte perilacunar remodelling, calcium release during lactation and the maintenance of bone quality [35]. The expression of the collagenase cathepsin K was also increased in the human osteocyte-like cells, consistent with reports of its increased expression by osteocytes during lactation [20] and in response to sclerostin [19]. The expression of *TRAP*, a marker usually associated with osteoclastic activity, was also increased by osteocytes exposed to UHMWPE particles, consistent with a role for this enzyme in osteocytic osteolysis during lactation [20,34,35], in response to sclerostin [19], and in response to continuous treatment of rats with PTH [52]. Thus, this study adds to the growing body of literature that osteocytes are able to express at least a subset of the molecular machinery of bone resorbing osteoclasts. Two model systems tested in this study, one in which immature osteoblasts were allowed to differentiate in the presence of UHMWPE particles and the other where differentiated osteocyte-like cells were exposed acutely to particles, gave similar results. It is note-

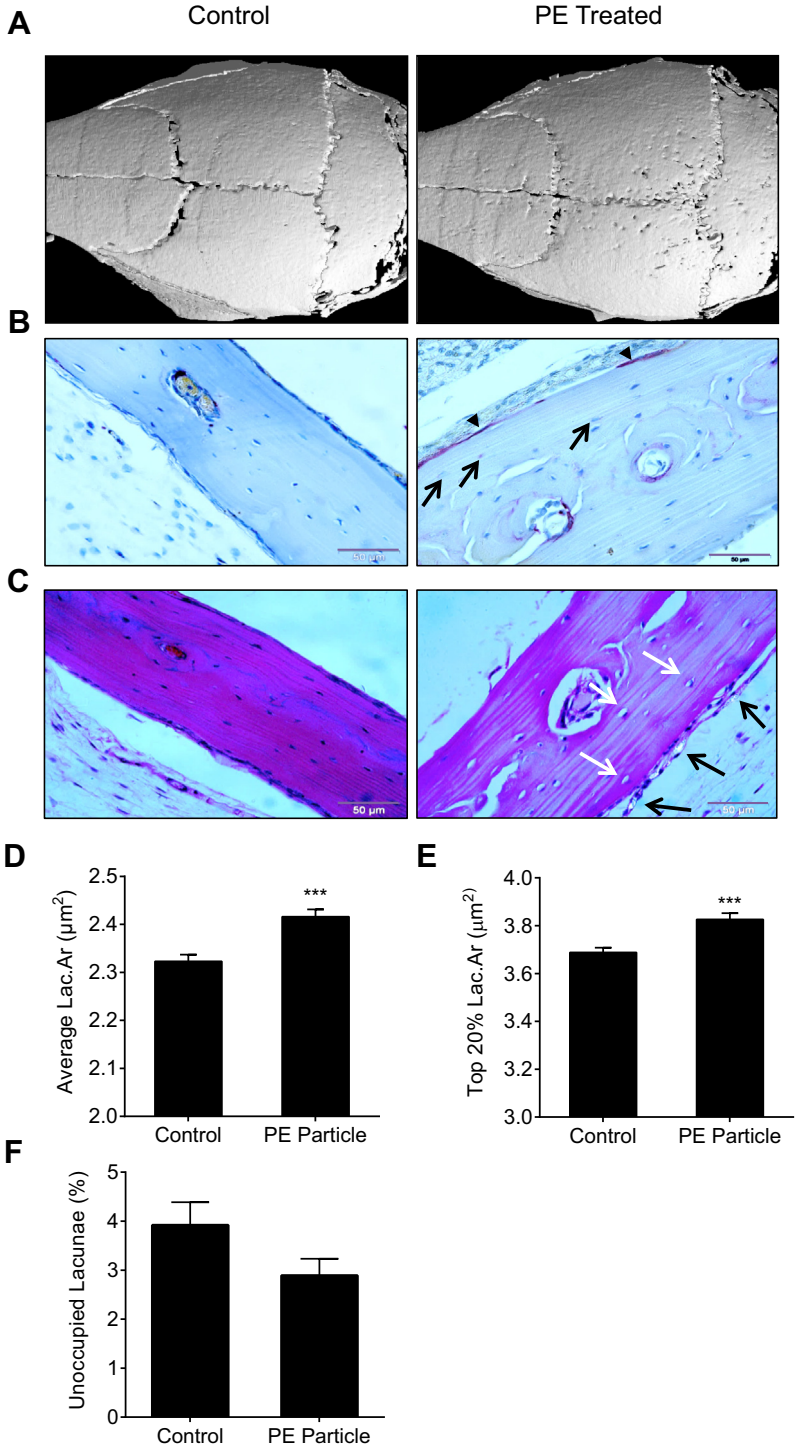


Fig. 4. Osteolysis in the murine calvarial model. (A) 3-D images (ANT, Skyscan, Bruker, Kontich, Belgium) from μ-CT scans of sham or UHMWPE particle treated mouse calvaria show evidence of resorption pits along the suture lines of the UHMWPE treated animal compared to the PBS control calvariae. Calvarial sections from control and UHMWPE treated were cut coronally in 7 μm sections and stained for (B) TRAP (arrowheads indicate TRAP-stained osteoclasts and arrows indicate TRAP-stained osteocyte lacunae) and (C) with H&E visualised under polarised light (white arrows indicate osteocyte lacunae and black arrows indicate birefringent UHMWPE particles). (D) Osteocyte lacunar area (Lac.Ar) was measured 1 μm from the suture line. (E) The top 20% Lac.Ar of control calvariae was calculated and the corresponding size range calculated in UHMWPE treated calvariae (Top 20% Lac.Ar (μm^2)). (F) The percentage of unoccupied lacunae from both control and UHMWPE treated calvaria was calculated (Unoccupied Lacunae (%)). Data shown are means ± SEM. Significant difference of UHMWPE measures to control are denoted by ***($p < 0.001$).

worthy that increases in the expression of genes associated with osteocytic osteolysis was concomitant with effects on *RANKL* and *OPG* mRNA expression, consistent with both osteocytic and osteoclastic bone loss being stimulated. These findings also suggest that wear particles induce a catabolic phenotype both as the osteoblast differentiates and also in resident mature osteocytes.

The calvarial osteolysis model is an accepted animal model of wear-particle mediated osteolysis, and is usually studied with respect to the degree of osteoclast-mediated pitting of the calvarial surface [39]. Our findings showed that in addition to UHMWPE inducing osteoclast-mediated bone loss, osteocyte lacunae were significantly enlarged with exposure to UHMWPE compared with

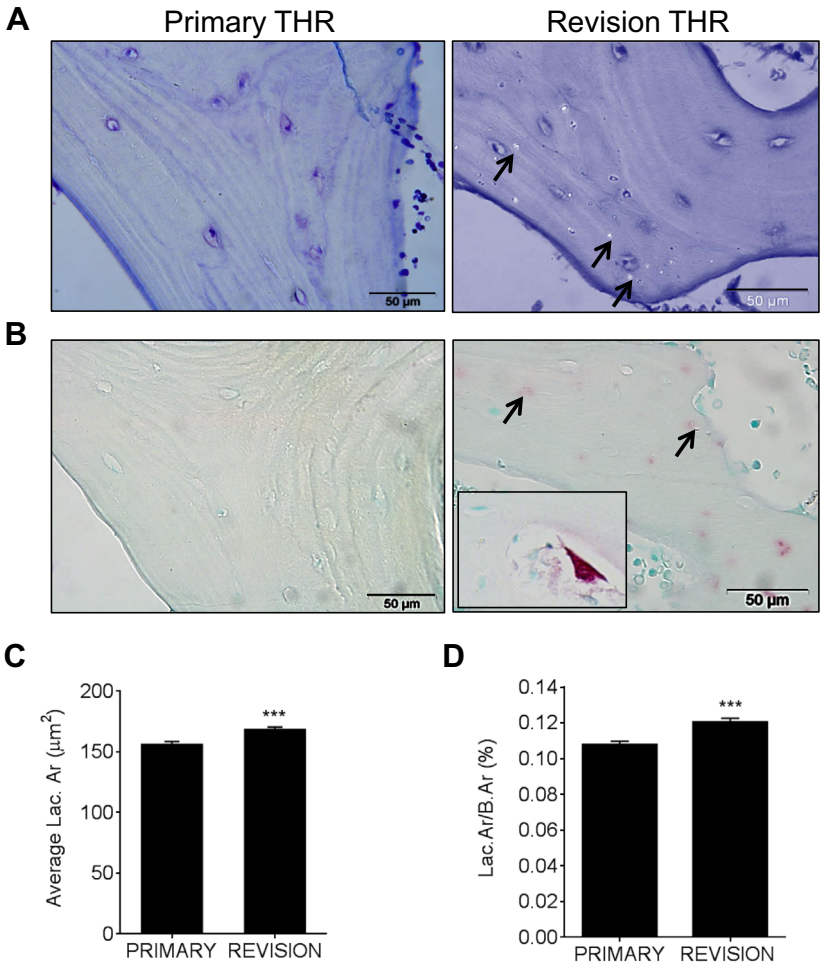


Fig. 5. Evidence for osteocyte perilacunar bone loss in patients with PO. (A) Light microscopy images of sections from an acetabular bone biopsy from patients who underwent primary THR, or revision THR, respectively, stained with toluidine blue and then visualised under polarised light, showing evidence of birefringent PE wear particles in the revision THR sample. Serial sections were also stained for TRAP activity (B); arrows show TRAP-positive osteocyte lacunae and the inset shows a rare osteoclast in these samples. Histological assessment was undertaken of bone biopsies from the acetabulum of 8 patients/group undergoing either primary THR or revision THR for aseptic loosening. Measures of (C) average osteocyte of lacunar area and (D) percentage osteocyte lacunar area per total bone area (Lac.Ar/B.Ar (%)) were calculated. Data shown are means ± SEM. Difference between groups is indicated by ***($p < 0.001$).

sham-exposed calvariae. This indicates that either osteocyte processes directly contact particles on the periosteal side, or that signals are mediated to the osteocyte indirectly through the periosteum to the adjacent osteocytes. Alternatively, secreted factors released by responding macrophages may also trigger a bone catabolic effect in osteocytes. Overall, these findings suggest that PE may have a similar effect in human hip replacement subjects undergoing revision total hip arthroplasty for aseptic loosening, where acetabular bone exposed to PE particles showed increased osteocyte lacunar area. This clinical evidence links the *in vitro* and mouse *in vivo* findings to the clinical pathology, and suggests that a component of periprosthetic bone loss may be the removal of osteocyte perilacunar bone.

The mechanism by which osteocytes respond to wear particles remains to be investigated. It is known that osteocyte processes can extend beyond the bone, particularly at the endosteal surface [53], making direct contact possible *in vivo*. Furthermore, THR involves reaming of the acetabulum prior to insertion of the acetabular component, removing the cortical bone and exposing the cancellous compartment, greatly increasing the surface area of bone that may be subsequently exposed to wear particles. Polarised light images of acetabular sections from orthopaedic implant recipients show PE particles penetrating into the trabecular bone tissue itself, suggesting that direct contact between osteocytes and wear particles is possible. Macrophages respond to wear particles by inducing recognition receptors which allow them to selectively phagocytose cell debris and foreign materials [54]. This response could be similar in osteocytes through intracellular signalling of toll-like receptors (TLRs), pattern recognition receptors shown to be expressed by osteoblasts [55,56]. Alternatively, indirect effects via cell mediators released from macrophages or osteoclasts at the sites of PO could also interact with osteocytes *in situ*. For example, we have shown that osteocytes respond to pro-inflammatory mediators, including TNFα, TWEAK [57], IL-6 and IL-1β [58].

The induction of a catabolic phenotype in osteocytes not only highlights the importance of these cells in maintaining the local bone environment but also as potential therapeutic targets in disease. The effect of particle-induced perilacunar remodelling, and perhaps perturbation of physiological osteocyte-mediated remodelling, is not yet known. Interestingly, UHMWPE particles did not appear to induce osteocyte apoptosis, as indicated by a lack of an effect on the expression of the pro-apoptotic ratio *BAX:BCL* mRNA, or on Caspase 3 activity *in vitro*, and no change in the proportion of unoccupied to occupied osteocyte lacunae *in vivo*. In fact, rather than inducing osteocyte death, UHMWPE particles appeared to maintain cell viability, with decreased caspase 3 activity relative to the basal level of expression. Thus, this study has found no evi-

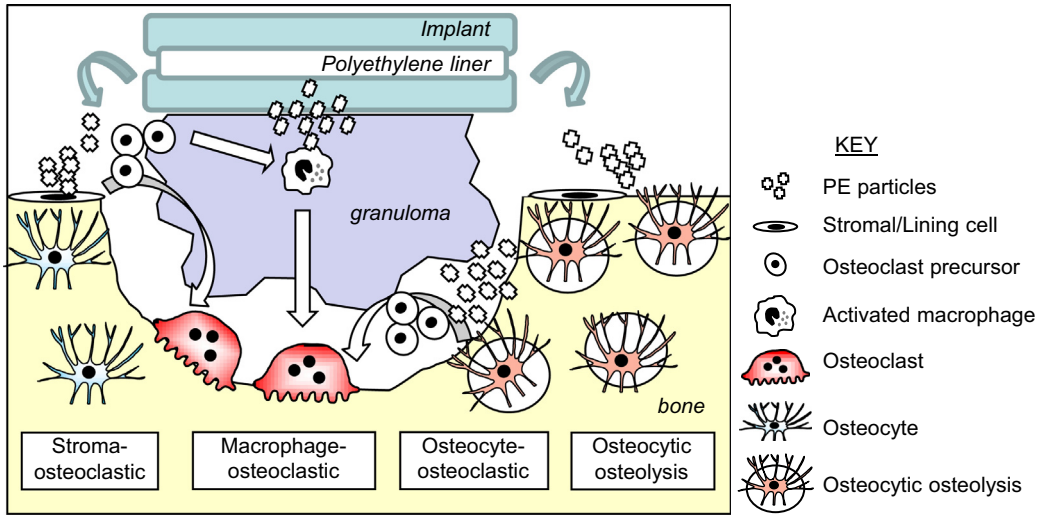


Fig. 6. Schematic representation of the cellular pathways potentially contributing to periprosthetic osteolysis: *From left to right*: PE particles released from polyethylene liners trigger stromal osteoblastic/lining cell mediated osteoclastogenesis; granulomatous tissue invades the implant/bone interface, containing inflammatory cells including macrophages, which when activated directly and indirectly by wear particles have increased potential to form bone resorbing osteoclasts; osteocytes either directly or via stromal/lining cells may trigger osteoclastogenesis through the production of factors such as RANKL; osteocytes either through direct contact with particles via their dendritic processes or indirectly from signals secreted by other cell types are triggered to undergo osteocytic osteolysis or perilacunar remodelling. Details relating to the non-osteocyte driven pathways are reviewed elsewhere [49,60].

dence that PE particles induce osteocyte apoptosis, a mechanism by which osteocytes induce osteoclast activity [59]. However, we cannot rule out that osteocyte death by either caspase 3 dependent or independent apoptosis, or by necrosis, may be a feature of the effects of wear particles on lytic lesion progression in patients with PO; further analysis of histological specimens obtained from such patients will be required to examine this. In addition, future studies should examine the effects of other particulates, such as metal alloys on the osteocyte, alone and in combination with PE.

In conclusion, the results of this study show for the first time that osteocytes respond to UHMWPE particles by resorbing their perilacunar matrix. Thus, together with our previous study, showing the induction by UHMWPE particles of pro-osteoclastic pathways by osteocytes [29], our findings show that osteocytes, in addition to the other cell types shown to be involved in this process (depicted in Fig. 6), may play key roles in the progression of peri-prosthetic osteolysis. Our findings also suggest a new paradigm for the way in which bone cells respond to biomaterials, and elucidation of the pathways involved represents an exciting new area of biomaterials-related research.

Disclosure

All of the authors have nothing to disclose.

Acknowledgements

This work was funded by a Project Grant (APP1041456) from the National Health and Medical Research Council of Australia (NHMRC) and the support of the Rebecca Cooper Foundation. RO is supported by a University of Adelaide Faculty of Health Sciences Divisional Research Scholarship. GJA is an NHMRC Senior Research Fellow.

References

[1] M.L. Wang, P.F. Sharkey, R.S. Tuan, Particle bioreactivity and wear-mediated osteolysis, J. Arthroplasty. 19 (2004) 1028–1038.
[2] Total Hip Replacement. NIH Consensus Statement, 1994;12.***
[3] D.W. Howie, D.R. Haynes, S.D. Rogers, M.A. McGee, M.J. Pearcy, The response to particulate debris, Orthop. Clin. North Am. 24 (1993) 571–581.
[4] B. Skoglund, J. Holmertz, P. Aspenberg, Systemic and local ibandronate enhance screw fixation, J. Orthop. Res. 22 (2004) 1108–1113.
[5] A. McEvoy, M. Jeyam, G. Ferrier, C.E. Evans, J.G. Andrew, Synergistic effect of particles and cyclic pressure on cytokine production in human monocyte/macrophages: proposed role in periprosthetic osteolysis, Bone 30 (2002) 171–177.
[6] D.R. Haynes, S.D. Rogers, S. Hay, M.J. Pearcy, D.W. Howie, The differences in toxicity and release of bone-resorbing mediators induced by titanium and cobalt-chromium-alloy wear particles, J. Bone Joint Surg. Am. 75 (1993) 825–834.
[7] A.M. Kaufman, C.I. Alabre, H.E. Rubash, A.S. Shanbhag, Human macrophage response to UHMWPE, TiAlV, CoCr, and alumina particles: analysis of multiple cytokines using protein arrays, J. Biomed. Mater. Res. A 84 (2008) 464–474.
[8] L.C. Jones, C. Frondoza, D.S. Hungerford, Effect of PMMA particles and movement on an implant interface in a canine model, J. Bone Joint Surg. Br. 83 (2001) 448–458.
[9] R.J. Looney, A. Boyd, S. Totterman, G.S. Seo, J. Tamez-Pena, D. Campbell, et al., Volumetric computerized tomography as a measurement of periprosthetic acetabular osteolysis and its correlation with wear, Arthritis Res. 4 (2002) 59–63.
[10] K.J. Kim, Y. Kobayashi, T. Itoh, Osteolysis model with continuous infusion of polyethylene particles, Clin. Orthop. (1998) 46–52.
[11] D.W. Howie, S.D. Neale, R. Stamenkov, M.A. McGee, D.J. Taylor, D.M. Findlay, Progression of acetabular periprosthetic osteolytic lesions measured with computed tomography, J. Bone Joint Surg. Am. 89 (2007) 1818–1825.
[12] M. Takagi, S. Santavirta, H. Ida, M. Ishii, I. Takei, S. Niissalo, et al., High-turnover periprosthetic bone remodeling and immature bone formation around loose cemented total hip joints, J. Bone Miner. Res. 16 (2001) 79–88.
[13] M.A. McGee, D.W. Howie, S.D. Neale, D.R. Haynes, M.J. Pearcy, The role of polyethylene wear in joint replacement failure, Proc. Inst. Mech. Eng. [H] 211 (1997) 65–72.
[14] T.N. Crotti, M.D. Smith, D.M. Findlay, H. Zreiqat, M.J. Ahern, H. Weedon, et al., Factors regulating osteoclast formation in human tissues adjacent to peri-implant bone loss: expression of receptor activator NFkappaB, RANK ligand and osteoprotegerin, Biomaterials 25 (2004) 565–573.
[15] A. Kobayashi, M.A. Freeman, W. Bonfield, Y. Kadoya, T. Yamac, N. Al-Saffar, et al., Number of polyethylene particles and osteolysis in total joint replacements. A quantitative study using a tissue-digestion method, J. Bone Joint Surg. Br. 79 (1997) 844–848.
[16] M. Takagi, S. Santavirta, H. Ida, M. Ishii, K. Akimoto, K. Saotome, et al., The membrane-type-matrix metalloproteinase/matrix metalloproteinase-2/tissue inhibitor of metalloproteinase-2 system in periprosthetic connective-tissue remodeling in loose total-hip prostheses, Lab. Invest. 78 (1998) 735–742.
[17] M. Takagi, S. Santavirta, H. Ida, M. Ishii, J. Mandelin, Y.T. Konttinen, Matrix metalloproteinases and tissue inhibitors of metalloproteinases in loose artificial hip joints, Clin. Orthop. Relat. Res. (1998) 35–45.
[18] Y.T. Konttinen, M. Takagi, J. Mandelin, J. Lassus, J. Salo, M. Ainola, et al., Acid attack and cathepsin K in bone resorption around total hip replacement prosthesis, J. Bone Miner. Res. 16 (2001) 1780–1786.

[19] M. Kogawa, A.R. Wijenayaka, R.T. Ormsby, G.P. Thomas, P.H. Anderson, L.F. Bonewald, et al., Sclerostin regulates release of bone mineral by osteocytes by induction of carbonic anhydrase 2, J. Bone Miner. Res. 28 (2013) 2436–2448.
[20] H. Qing, L. Ardeshirpour, P.D. Pajevic, V. Dusevich, K. Jahn, S. Kato, et al., Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation, J. Bone Miner. Res. 27 (2012) 1018–1029.
[21] J. Gallo, M. Raska, F. Mrazek, M. Petrek, Bone remodeling, particle disease and individual susceptibility to periprosthetic osteolysis, Physiol. Res. 57 (2008) 339–349.
[22] M.E. Martinez, S. Medina, M.T. del Campo, J.A. Garcia, A. Rodrigo, L. Munuera, Effect of polyethylene particles on human osteoblastic cell growth, Biomaterials 19 (1998) 183–187.
[23] D.D. Dean, Z. Schwartz, Y. Liu, C.R. Blanchard, C.M. Agrawal, J.D. Mabrey, et al., The effect of ultra-high molecular weight polyethylene wear debris on MG63 osteosarcoma cells in vitro, J. Bone Joint Surg. Am. 81 (1999) 452–461.
[24] R. Chiu, T. Ma, R.L. Smith, S.B. Goodman, Ultrahigh molecular weight polyethylene wear debris inhibits osteoprogenitor proliferation and differentiation in vitro, J. Biomed. Mater. Res. A (2008).
[25] C. Vermes, R. Chandrasekaran, J.J. Jacobs, J.O. Galante, K.A. Roebuck, T.T. Glant, The effects of particulate wear debris, cytokines, and growth factors on the functions of MG-63 osteoblasts, J. Bone Joint Surg. Am. 83-A (2001) 201–211.
[26] A.M. Rodrigo, M.E. Martinez, L. Saldana, G. Valles, P. Martinez, J.L. Gonzalez-Carrasco, et al., Effects of polyethylene and alpha-alumina particles on IL-6 expression and secretion in primary cultures of human osteoblastic cells, Biomaterials 23 (2002) 901–908.
[27] D. Granchi, G. Ciapetti, I. Amato, S. Pagani, E. Cenni, L. Savarino, et al., The influence of alumina and ultra-high molecular weight polyethylene particles on osteoblast-osteoclast cooperation, Biomaterials 25 (2004) 4037–4045.
[28] C.H. Lohmann, D.D. Dean, L.F. Bonewald, Z. Schwartz, B.D. Boyan, Nitric oxide and prostaglandin E2 production in response to ultra-high molecular weight polyethylene particles depends on osteoblast maturation state, J. Bone Joint Surg. Am. 84-A (2002) 411–419.
[29] G.J. Atkins, K.J. Welldon, C.A. Holding, D.R. Haynes, D.W. Howie, D.M. Findlay, The induction of a catabolic phenotype in human primary osteoblasts and osteocytes by polyethylene particles, Biomaterials 30 (2009) 3672–3681.
[30] G.J. Atkins, K.J. Welldon, A.R. Wijenayaka, L.F. Bonewald, D.M. Findlay, Vitamin K promotes mineralization, osteoblast-to-osteocyte transition, and an anticatabolic phenotype by {gamma}-carboxylation-dependent and -independent mechanisms, Am. J. Physiol. Cell Physiol. 297 (2009) C1358–C1367.
[31] A. Teti, A. Zallone, Do osteocytes contribute to bone mineral homeostasis? Osteocytic osteolysis revisited, Bone 44 (2009) 11–16.
[32] M.E. McGee-Lawrence, D.M. Stoll, E.R. Mantila, B.K. Fahrner, H.V. Carey, S.W. Donahue, Thirteen-lined ground squirrels (Ictidomys tridecemlineatus) show microstructural bone loss during hibernation but preserve bone macrostructural geometry and strength, J. Exp. Biol. 214 (2011) 1240–1247.
[33] A. Zambonin Zallone, A. Teti, M.V. Primavera, G. Pace, Mature osteocytes behaviour in a repletion period: the occurrence of osteoplastic activity, Basic Appl. Histochem. 27 (1983) 191–204.
[34] M.V. Clarke, P.K. Russell, D.M. Findlay, S. Sastra, P.H. Anderson, J.P. Skinner, et al., A role for the calcitonin receptor to limit bone loss during lactation in female mice by inhibiting osteocytic osteolysis, Endocrinology 156 (2015) 3203–3214.
[35] S.Y. Tang, R.P. Herber, S.P. Ho, T. Alliston, Matrix metalloproteinase-13 is required for osteocytic perilacunar remodeling and maintains bone fracture resistance, J. Bone Miner. Res. 27 (2012) 1936–1950.
[36] L. Mosekilde, F. Melsen, A tetracycline-based histomorphometric evaluation of bone resorption and bone turnover in hyperthyroidism and hyperparathyroidism, Acta Med. Scand. 204 (1978) 97–102.
[37] A. Yajima, M. Inaba, Y. Tominaga, Y. Nishizawa, K. Ikeda, A. Ito, Increased osteocyte death and mineralization inside bone after parathyroidectomy in patients with secondary hyperparathyroidism, J. Bone Miner. Res. 25 (2010) 2374–2381.
[38] G.J. Atkins, K.J. Welldon, P. Halbout, D.M. Findlay, Strontium ranelate treatment of human primary osteoblasts promotes an osteocyte-like phenotype while eliciting an osteoprotegerin response, Osteoporos. Int. (2008).
[39] X. Zhang, S.G. Morham, R. Langenbach, D.A. Young, L. Xing, B.F. Boyce, et al., Evidence for a direct role of cyclo-oxygenase 2 in implant wear debris-induced osteolysis, J. Bone Miner. Res. 16 (2001) 660–670.
[40] M.D. Cantley, P.M. Bartold, V. Marino, R.C. Reid, D.P. Fairlie, R.N. Wyszynski, et al., The use of live-animal micro-computed tomography to determine the effect of a novel phospholipase A2inhibitor on alveolar bone loss in anin vivomouse model of periodontitis, J. Periodontal Res. 44 (2009) 317–322.
[41] M.D. Cantley, P.M. Bartold, V. Marino, D.P. Fairlie, G.T. Le, A.J. Lucke, et al., Histone deacetylase inhibitors and periodontal bone loss, J. Periodontal Res. (2011).
[42] M.D. Cantley, D.R. Haynes, V. Marino, P.M. Bartold, Pre-existing periodontitis exacerbates experimental arthritis in a mouse model, J. Clin. Periodontol. 21 (2011).
[43] M.D. Cantley, P.M. Bartold, V. Marino, R.C. Reid, D.P. Fairlie, R.N. Wyszynski, et al., The use of live-animal micro-computed tomography to determine the effect of a novel phospholipase A2 inhibitor on alveolar bone loss in an in vivo mouse model of periodontitis, J. Periodontal Res. 44 (2009) 317–322.
[44] N.Z. Angel, N. Walsh, M.R. Forwood, M.C. Ostrowski, A.I. Cassady, D.A. Hume, Transgenic mice overexpressing tartrate-resistant acid phosphatase exhibit an increased rate of bone turnover, J. Bone Miner. Res. 15 (2000) 103–110.
[45] M.S. Burstone, Histochemical demonstration of acid phosphatases with naphthol AS-phosphates, J. Natl Cancer Inst. 21 (1958) 523–539.
[46] G.J. Atkins, P.S. Rowe, H.P. Lim, K.J. Welldon, R. Ormsby, A.R. Wijenayaka, et al., Sclerostin is a locally acting regulator of late-osteoblast/preosteocyte differentiation and regulates mineralization through a MEPE-ASARM-dependent mechanism, J. Bone Miner. Res. 26 (2011) 1425–1436.
[47] G.J. Atkins, K.J. Welldon, P. Halbout, D.M. Findlay, Strontium ranelate treatment of human primary osteoblasts promotes an osteocyte-like phenotype while eliciting an osteoprotegerin response, Osteoporos. Int. 20 (2009) 653–664.
[48] Z.N. Oltvai, C.L. Milliman, S.J. Korsmeyer, Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death, Cell 74 (1993) 609–619.
[49] G.J. Atkins, D. Haynes, D.W. Howie, D. Findlay, The role of polyethylene particles in peri-prosthetic osteolysis: a review, World J. Orthop. 2 (2011) 93–101.
[50] G.J. Atkins, D.M. Findlay, Osteocyte regulation of bone mineral: a little give and take, Osteoporos. Int. 23 (2012) 2067–2079.
[51] L.F. Bonewald, The amazing osteocyte, J. Bone Miner. Res. 26 (2011) 229–238.
[52] K. Tazawa, K. Hoshi, S. Kawamoto, M. Tanaka, S. Ejiri, H. Ozawa, Osteocytic osteolysis observed in rats to which parathyroid hormone was continuously administered, J. Bone Miner. Metab. 22 (2004) 524–529.
[53] H. Kamioka, T. Honjo, T. Takano-Yamamoto, A three-dimensional distribution of osteocyte processes revealed by the combination of confocal laser scanning microscopy and differential interference contrast microscopy, Bone 28 (2001) 145–149.
[54] P.J. Murray, T.A. Wynn, Protective and pathogenic functions of macrophage subsets, Nat. Rev. Immunol. 11 (2011) 723–737.
[55] R.D. Valladares, C. Nich, S. Zwingenberger, C. Li, K.R. Swank, E. Gibon, et al., Toll-like receptors-2 and 4 are overexpressed in an experimental model of particle-induced osteolysis, J. Biomed. Mater. Res. A 102 (2014) 3004–3011.
[56] Z. Bar-Shavit, Taking a toll on the bones: regulation of bone metabolism by innate immune regulators, Autoimmunity 41 (2008) 195–203.
[57] C. Vincent, D.M. Findlay, K.J. Welldon, A.R. Wijenayaka, T.S. Zheng, D.R. Haynes, et al., Pro-inflammatory cytokines TNF-related weak inducer of apoptosis (TWEAK) and TNFalpha induce the mitogen-activated protein kinase (MAPK)-dependent expression of sclerostin in human osteoblasts, J. Bone Miner. Res. 24 (2009) 1434–1449.
[58] N. Ito, A.R. Wijenayaka, M. Prideaux, M. Kogawa, R.T. Ormsby, A. Evdokiou, et al., Regulation of FGF23 expression in IDG-SW3 osteocytes and human bone by pro-inflammatory stimuli, Mol. Cell. Endocrinol. 399 (2015) 208–218.
[59] G. Kogianni, V. Mann, B.S. Noble, Apoptotic bodies convey activity capable of initiating osteoclastogenesis and localized bone destruction, J. Bone Miner. Res. 23 (2008) 915–927.
[60] D.W. Howie, S.D. Neale, D.R. Haynes, O.T. Holubowycz, M.A. McGee, L.B. Solomon, et al., Periprosthetic osteolysis after total hip replacement: molecular pathology and clinical management, Inflammopharmacology 21 (2013) 389–396.

Chapter 3

Examination of Changes in Osteocyte Morphology in a Murine Femoral Model of Osteolysis

Conventional adjunct chapter

In continuation from the previous published Chapter, where we described the effect of UHMWPE particles in a murine calvarial model of osteolysis, this adjunct chapter examines an alternative model of osteolysis, the mouse femoral pump model, in response to UHMWPE particles. The published study by Pajarinen *et al.*, from the group of Dr Stuart B. Goodman, investigated the effects of a murine model of peri-prosthetic osteolysis, where a subcutaneously implanted osmotic pump slowly and continuously delivers wear particles through a titanium rod in the distal femur over a period of 8 weeks. This model significantly results in the development of particle-induced osteolysis. As part of a collaboration between the Atkins and Goodman groups, we obtained transverse sections from previously collected mouse femoral bones analysed in this study, and investigated the effects of the continuously released UHMWPE particles on osteocyte lacunar morphology in this model.

Chapter 3: Examination of Changes in Osteocyte Morphology in a Murine Femoral Model of Osteolysis

Abstract

Periprosthetic osteolysis is a major cause of aseptic loosening of total hip replacements. Bioactive wear particles have been described as a main cause of osteolytic lesions due to the activation of biological pathways including osteoclastic and osteocytic bone resorption. Previous animal models including the calvarial osteolysis model have been frequently adapted to study osteolysis. The mouse femoral pump model of osteolysis was designed to allow slow continuous delivery of wear particles into a titanium rod implanted in the distal femur. Male mice were exposed to UHMWPE particles, released over 8 weeks, with a secondary pump exchanged to allow for continuous release. This induced osteolysis and an increase in both macrophage and osteoclast number in the peri-implant tissue. Our analysis of osteocyte parameters in this model showed a significant increase in the percentage lacunar area per total bone area but not in average lacunar area. Furthermore, the animals treated with UHMWPE particles showed a decrease in total bone area, suggesting an increase in the porosity of the bone. These findings suggest that while the increased porosity may have been contributed to by coalescing osteocyte lacunae, the loss of bone in this model mainly occurs due to an osteoclastic response to particles.

Introduction

The development of osteolysis has been identified as a key problem in total hip replacements leading to aseptic loosening. Many studies have demonstrated the involvement of multiple bone cell types in osteolysis, contributing to the pathogenesis of aseptic loosening [1]. We have described the responses of osteocytes to particles *in vitro*, where human primary osteocyte-like cultures exposed to UHMWPE particles, upregulated factors that support osteoclastogenesis [2], as well as those associated with osteocytic osteolysis [3], the latter described in the preceding Chapter. We have also showed increased osteocyte lacunar size in bone biopsies from patients undergoing revision THR with confirmed radiographic evidence of periprosthetic osteolysis. Using the murine calvarial model of osteolysis, we demonstrated the enlargement of osteocyte lacunae in response to UHMWPE particles, consistent with osteocytic osteolysis [3]. This model has been frequently used to study osteolysis, although it is acknowledged to have a number of potential limitations [4]. These include the absence of an implant, and the important differences in mechanical forces that occur in the long bones through loading and fluid pressure, which are unlikely to be fully replicated in the calvaria, although some force is imparted to the calvaria through mastication [5]. The mouse calvarial model also generates an acute response to particles, rather than the chronic effect observed in patients that occurs with long term exposure to particles [6]. Furthermore, from the point of view of studying osteocyte responses, calvarial osteocytes are derived from a neural ectoderm stem cell, whilst osteocytes of the long bones and the appendicular skeleton in general, are derived from mesenchymal stem cells. This difference in lineage results in changes to the morphology, the structural network and therefore the responses to physiological loading [7, 8], which prompted the need for a more suitable model. Therefore, the mouse femoral model of osteolysis was designed by Pajarinen *et al.*, where there is a slow

continuous delivery of wear particles into the distal femur, with the addition of a titanium rod replicating a femoral implant [9]. This model highlighted the long-term effects of wear particle exposure alongside an intramedullary implant, which induced substantial osteolysis, and a significant increase in the number of both macrophages and osteoclasts in the peri-implant tissue. Both of these factors allow this model to mimic the development of osteolysis, comparable to the clinical problem.

As in the case of particle disease in human patients, the cells within the bone environment, including osteocytes, are exposed to particles continuously in the Pajarinen model. As mentioned in Chapter 2, osteocytes play key roles in regulating both bone formation and resorption [10, 11], as well as regulating osteocytic osteolysis, also termed perilacunar remodelling [12]. Notably, this process has been to shown to play important roles during lactation and in response to PTH injection [13-17]. We have also shown that osteocytes respond to UHMWPE particles by inducing perilacunar remodelling, specifically in the calvarial model of osteolysis [3]. Therefore, to determine whether osteocytic osteolysis plays a role in alternative animal models, we analysed the effects of UHMWPE particles on osteocytes *in vivo* in the murine femoral pump model of osteolysis.

Materials and Methods

Mouse femoral pump model

A murine model of aseptic loosening was developed by employing a slow continuous delivery of wear particles into the distal femur [9]. Briefly, an osmotic pump is filled with UHMWPE particles or particle free control solution of 1% bovine serum albumin–phosphate-buffered saline (n = 3 per group). The pump and tubing were connected to a titanium rod and implanted into the distal femur where the pump slowly released the

solution over 4 weeks, after which the pump was substituted with a secondary pre-filled pump allowing particle delivery to occur for another 4 weeks [9].

Histological analysis of mouse femurs

The right femur of each mouse was dissected and then fixed (4% paraformaldehyde) for 24 hr. Each bone was then decalcified (0.5 M Ethylenediaminetetraacetic acid; EDTA) for 2 weeks and then embedded in OCT compound. For histological analysis, 8 μM bone sections were obtained from the distal metaphyseal region, above the titanium rod and the zone of osteolysis. Sections were obtained in the transverse plane and stained with toluidine blue, as described previously [3]. Bone measurements were quantified using ImageJ software (U. S. National Institutes of Health, Bethesda, MD, USA) by manually tracing each individual osteocyte lacunar area, the total bone area and as well as void areas to quantify the porosity of each section [3].

Results and Discussion

In this study, transverse bone sections were obtained from the distal femur of male mice exposed to either control PBS solution (Fig. 1A-C) or UHMWPE particles (Fig. 1D-F) for 8 weeks (n = 3 per group). Images of each section of bone show the control sections are noticeably larger in width, ranging from 2.16 to 2.66 mm, compared to the UHMWPE treated group, which ranged from 2.05 to 2.1 mm in length, suggesting a larger bone area in the control group. The sections also reveal an apparent lack of uniformity in terms of which region of the distal femur the bone was sectioned for each animal, a limitation of this study.

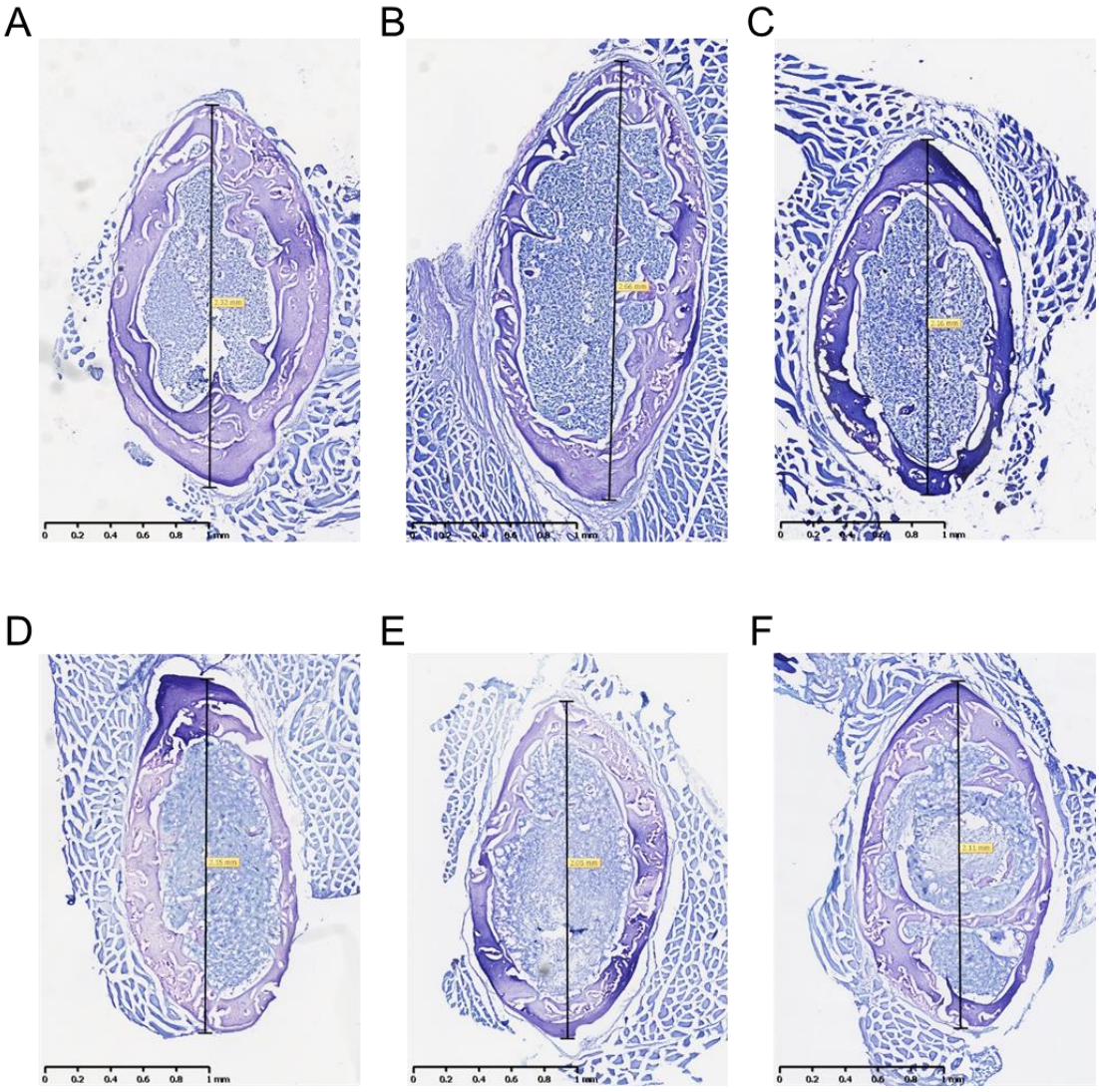


Figure 1. Mouse distal femurs were implanted with a titanium rod containing an osmotic pump that slowly released either UHMWPE particles or control PBS solution for 4 weeks. Distal femurs were then embedded in OCT compound and sectioned in the transverse plane and stained with toluidine blue. Sections above represent control (A-C) and UHMWPE (D-F) exposed bone, scale bars applied in NDP view show the width for each section. Bone sections were imaged at 20x magnification and show the extent of the porosity of the bone caused by bone loss (Fig. 2A-B).

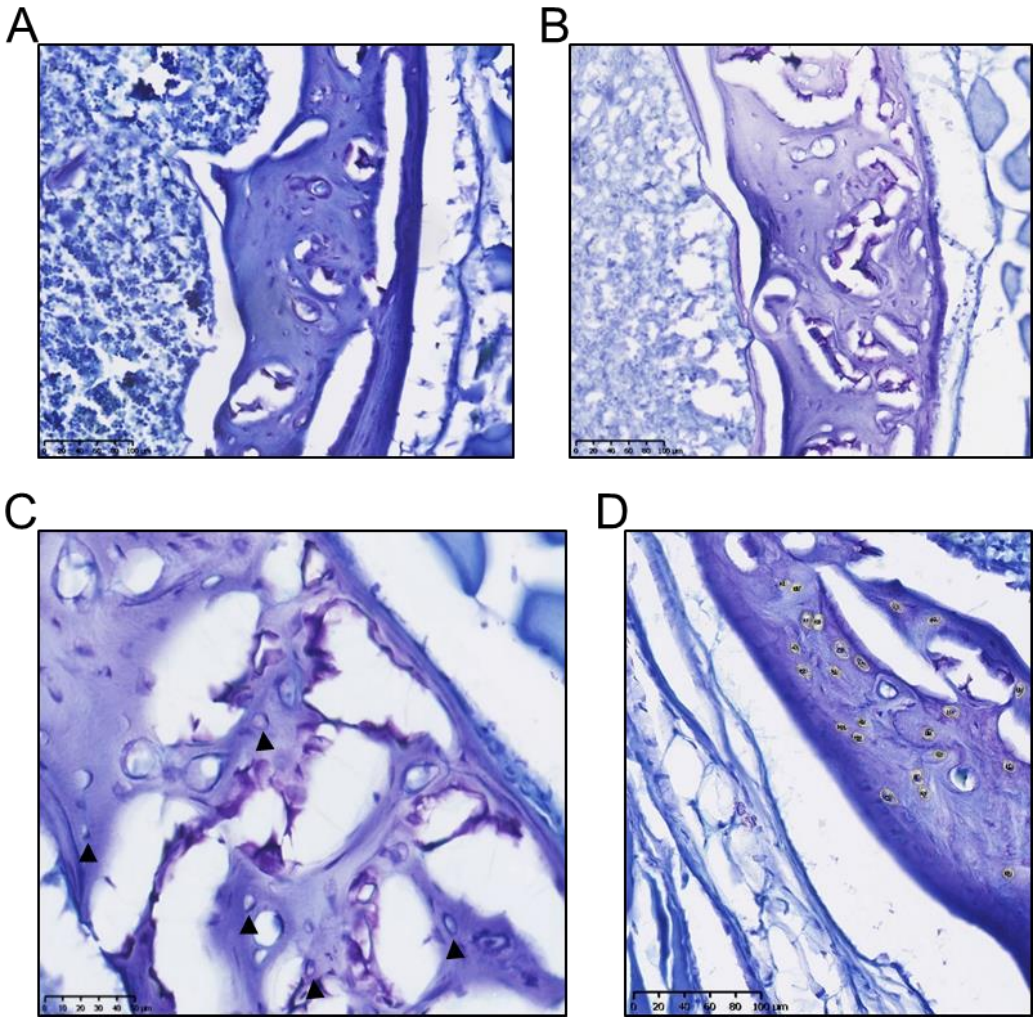


Figure 2. Transverse bone sections were imaged at 20x magnification and show the extent of the porosity of the bone, A) Control (PBS) group, B) UHMWPE particle group, C) Higher magnification (40x) shows viable osteocytes (black arrows) alongside blood vessels and other voids in the bone. D) Osteocyte lacunar areas are depicted by yellow circles around the lacunae which were quantified using ImageJ.

Sections were stained with toluidine blue and revealed a significant change in the average osteocyte lacunar area between groups (Fig. 3A). Surprisingly, the UHMWPE particle group showed a substantial decrease in average lacunar area compared to control, in apparent contrast to the findings we observed with the calvarial model [3]. In comparison,

the percentage lacunar area per total bone area was increased in the UHMWPE group (Fig. 3B). This suggests a reduction in total bone area, consistent with the change in bone width as mentioned above, which was confirmed by manually tracing around the bone area and deducting the porosity of the bone (Fig. 3C), where an area greater that 60 μm^2 was identified as a void and not an osteocyte lacunae as visualised in Figure 2D.

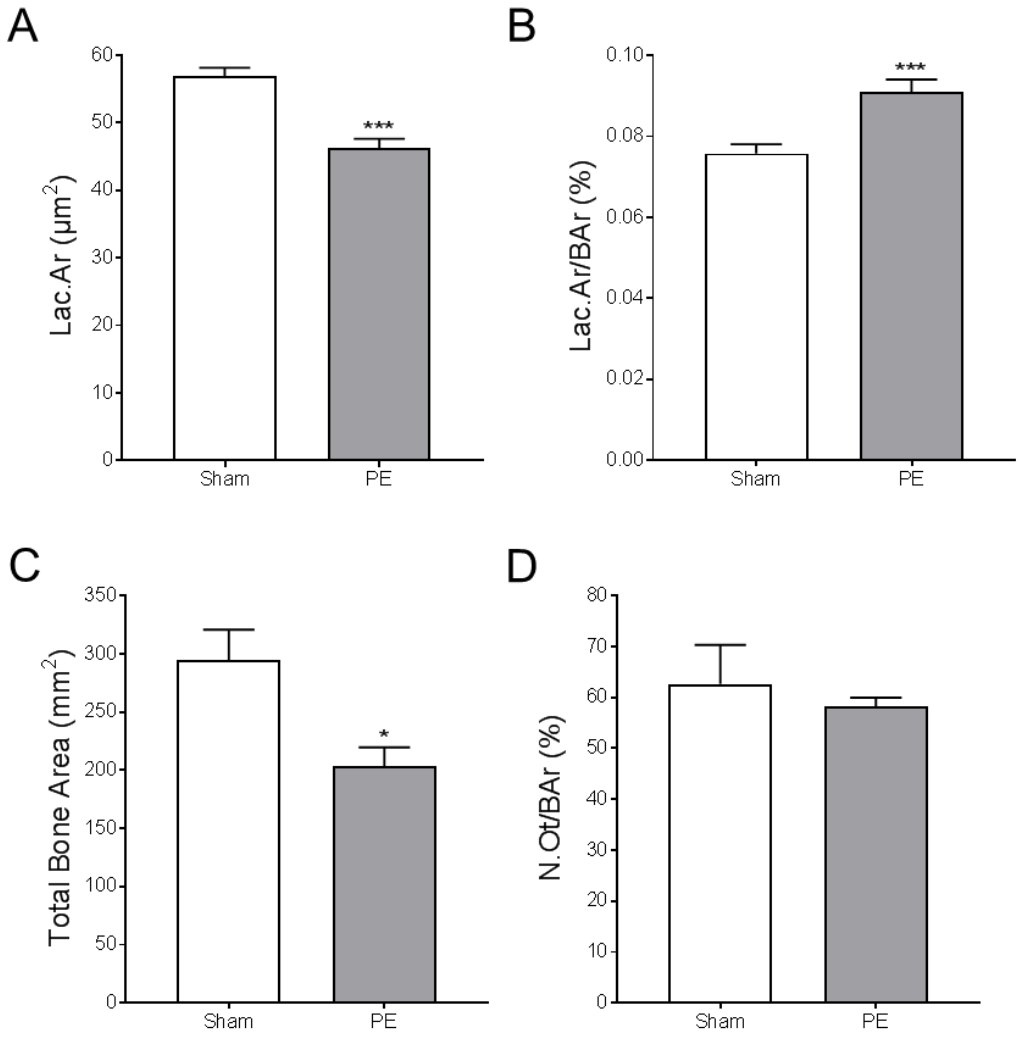


Figure 3. Quantified lacunar area for Sham and UHMWPE (PE) groups show: A) a decrease in osteocyte lacunar area in the PE group (Lac.Ar (μm^2)). B) A significant increase in % lacunar area per total bone area in the PE group compared to Sham

(Lac.Ar/BAr (%)). C) Quantification of total bone area (mm^2) showed a significant decrease in the UHMWPE group compared to sham. D) The percentage osteocyte number per bone area is not significantly different in Sham vs PE groups (N.Ot/BAr (%)). Significant data shown are means ± standard error of the mean (SEM). Significant differences are denoted by **p < 0.01, ***p < 0.001.

The published paper by Parjarinen *et al.* [9] did not report a decrease in total bone area from consecutive sections, however as depicted, the bone porosities range from large tears to smaller voids, which may not have been detectable by μ-CT. Therefore, this study replicates the loss of bone that occurs with the development of osteolysis, specifically through the active osteoclastic resorption that occurs in response to particles. It is difficult to explain our observation of significantly smaller osteocyte lacunae in particle perfused femoral bone, yet significantly larger lacunae in the calvarial model. In addition to the differences in terms of anatomical location and ontogeny of the two osteocyte populations, one potential explanation for the dissimilar findings between this and the calvarial model is the difference in time point analysed between the models. The relatively long (8 week) period of particle exposure in the Parjarinen model could have encompassed the time when osteocytic osteolysis progressed to the point of enlargement and coalescence of neighbouring osteocyte lacunae to form large pores, which is potentially contributing to the high degree of porosity of the remaining bone. We have previously observed evidence of osteocyte coalescence in human bone biopsies exposed to particles (Chapter 5). However, as the osteocyte number per total bone area is not significantly different between the groups (Fig. 3D), this suggests that the surviving osteocytes are reducing their lacunar area in response to an altered mechanical and/or biochemical environment, in order to maintain bone structural integrity and promote bone

healing. A representative image shows viable osteocytes alongside blood vessels and other voids within the section of bone (Fig. 2C). Nevertheless, these observations suggest that similar to physiological instances of osteocytic osteolysis, such as during lactation in mice [18, 19] and hibernation in certain mammals [20-22], where perilacunar bone lost to osteocytic osteolysis can be replaced, osteocytes are also capable of reducing their lacunar size under certain conditions.

Another potential explanation for the observed differences between the calvarial and femoral models is the mode of particle delivery; calvarial osteocytes were exposed to static UHMWPE particles, whereas femoral osteocytes were exposed to particles under dynamic fluid flow. The combination of fluid flow and particle stimulation may well impart different signals to the osteocyte, anabolic as opposed to catabolic. Separating out the influences of mechanical and biochemical influences on the osteocyte in terms of periprosthetic osteolysis is a subject of ongoing research in our group. It is noteworthy that this femoral model was conducted in male mice, whereas we have preferentially used female mice in our calvarial model; evidence is presented in Chapter 5, furthermore we have shown that osteocytic osteolysis in human periprosthetic osteolysis may be gender dependent. Regardless, this, to our knowledge, is the first description of osteocytes replacing bone in terms of a pathological stimulus.

A limitation of this study is the site of osteocyte lacunar analysis, which was above the titanium rod and the area of osteolysis. This may potentially explain why the osteocytes are smaller in the UHWMPE treated group, as the sections are just above the exposed area where UHMWPE particles were not detected. Therefore, further analysis from the

site of osteolysis may provide a more in depth understanding of the effects of UHMWPE particles on osteocytes in the exposed compared to the unexposed region.

Conclusions

In conclusion, the observations from this study support the loss of bone induced by chronic exposure to UHMWPE particles in the mouse femoral pump model being due to an increase in osteoclastic resorption. It is possible that the increased porosity is contributed to by coalescing osteocyte lacunae. Further investigation is required to determine if osteocytes are contributing to the development of osteolysis in this model.

References

[1] Atkins GJ, Haynes DR, Howie DW, Findlay DM. Role of polyethylene particles in peri-prosthetic osteolysis: A review. World J Orthop 2011;2:93-101.

[2] Atkins GJ, Welldon KJ, Holding CA, Haynes DR, Howie DW, Findlay DM. The induction of a catabolic phenotype in human primary osteoblasts and osteocytes by polyethylene particles. Biomaterials 2009;30:3672-81.

[3] Ormsby RT, Cantley M, Kogawa M, Solomon LB, Haynes DR, Findlay DM, et al. Evidence that osteocyte perilacunar remodelling contributes to polyethylene wear particle induced osteolysis. Acta biomaterialia 2016;33:242-51.

[4] Merkel KD, Erdmann JM, McHugh KP, Abu-Amer Y, Ross FP, Teitelbaum SL. Tumor necrosis factor-alpha mediates orthopedic implant osteolysis. The American journal of pathology 1999;154:203-10.

[5] Herring SW. Masticatory muscles and the skull: a comparative perspective. Arch Oral Biol 2007;52:296-9.

[6] Langlois J, Hamadouche M. New animal models of wear-particle osteolysis. International Orthopaedics 2011;35:245-51.

[7] Himeno-Ando A, Izumi Y, Yamaguchi A, Iimura T. Structural differences in the osteocyte network between the calvaria and long bone revealed by three-dimensional fluorescence morphometry, possibly reflecting distinct mechano-adaptations and sensitivities. Biochemical and biophysical research communications 2012;417:765-70.

[8] Vatsa A, Breuls RG, Semeins CM, Salmon PL, Smit TH, Klein-Nulend J. Osteocyte morphology in fibula and calvaria --- is there a role for mechanosensing? Bone 2008;43:452-8.

[9] Pajarinen J, Nabeshima A, Lin TH, Sato T, Gibon E, Jamsen E, et al. (*) Murine Model of Progressive Orthopedic Wear Particle-Induced Chronic Inflammation and Osteolysis. Tissue Eng Part C Methods 2017;23:1003-11.

[10] Bonewald LF. The amazing osteocyte. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2011;26:229-38.

[11] Prideaux M, Findlay DM, Atkins GJ. Osteocytes: The master cells in bone remodelling. Curr Opin Pharmacol 2016;28:24-30.

[12] Belanger LF. Osteocytic osteolysis. Calcif Tissue Res 1969;4:1-12.

[13] Tang SY, Herber RP, Ho SP, Alliston T. Matrix metalloproteinase-13 is required for osteocytic perilacunar remodeling and maintains bone fracture resistance. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2012;27:1936-50.

[14] Qing H, Ardeshirour L, Dusevich V, M. D, Wysolmerski JJ, Bonewald LF. Osteocytic perilacunar remodelling as a significant source of calcium during lactation. Journal of Bone and Mineral Research 2008;23:s401.

[15] Kogawa M, Wijenayaka AR, Ormsby RT, Thomas GP, Anderson PH, Bonewald LF, et al. Sclerostin Regulates Release of Bone Mineral by Osteocytes by Induction of Carbonic Anhydrase 2. J Bone Miner Res 2013;28:2436-48.

[16] Clarke MV, Russell PK, Findlay DM, Sastra S, Anderson PH, Skinner JP, et al. A Role for the Calcitonin Receptor to Limit Bone Loss During Lactation in Female Mice by Inhibiting Osteocytic Osteolysis. Endocrinology 2015;156:3203-14.

[17] Tazawa K, Hoshi K, Kawamoto S, Tanaka M, Ejiri S, Ozawa H. Osteocytic osteolysis observed in rats to which parathyroid hormone was continuously administered. J Bone Miner Metab 2004;22:524-9.

[18] Sharma D, Ciani C, Marin PA, Levy JD, Doty SB, Fritton SP. Alterations in the osteocyte lacunar-canalicular microenvironment due to estrogen deficiency. Bone 2012;51:488-97.

[19] Qing H, Ardeshirpour L, Pajevic PD, Dusevich V, Jahn K, Kato S, et al. Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2012;In Press.

[20] Kwiecinski GG, Krook L, Wimsatt WA. Annual skeletal changes in the little brown bat, Myotis lucifugus lucifugus, with particular reference to pregnancy and lactation. Am J Anat 1987;178:410-20.

[21] Steinberg B, Singh IJ, Mitchell OG. The effects of cold-stress. Hibernation, and prolonged inactivity on bone dynamics in the golden hamster, Mesocricetus auratus. J Morphol 1981;167:43-51.

[22] Haller AC, Zimny ML. Effects of hibernation on interradicular alveolar bone. J Dent Res 1977;56:1552-7.

Chapter 4

Osteocytes respond to particles of clinically-relevant conventional and cross-linked polyethylene and metal alloys by up-regulation of resorptive and inflammatory pathways

Accepted for publication in *Acta Biomaterialia*, 24 January 2019

As described in Chapter 2, UHMWPE particles derived from the wear of the ultra-high molecular weight polyethylene liner are a major cause of osteolysis. Osteocytes respond to UHMWPE particles by inducing inflammation, bone resorption and perilacunar remodelling. Therefore, in continuation of this study, we investigated the effects of two clinically relevant UHMWPE particles obtained from currently used liners, as well as two types of orthopaedic implant derived metal particles, Ti6Al4V and CoCrMo, on osteocytes *in vitro*. Both metal and UHMWPE particles have been previously shown to stimulate biological pathways causing inflammation, activation of bone resorption and inhibition of bone formation, however the effect of these particles on osteocytes is unknown. Therefore, the aim of Chapter 4 was to investigate the effects of both clinically relevant metal and UHMWPE particles on osteocytes *in vitro*. The findings of this study are presented in the published form of the manuscript.

Statement of Authorship

Title of Paper	Osteocytes respond to particles of clinically-relevant conventional and cross-linked polyethylene and metal alloys by up-regulation of resorptive and inflammatory pathways
Publication Status	☐ Published ☑ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Ormsby R.T., Solomon, L.B., Yang, D., Crotti, T.N., Haynes, D.R., Findlay, D.M., & Atkins, G.J. (2019) Osteocytes respond to particles of clinically-relevant conventional and cross-linked polyethylene and metal alloys by up-regulation of resorptive and inflammatory pathways.

Principal Author

Name of Principal Author (Candidate)	Renee T Ormsby		
Contribution to the Paper	First author on the paper. Generated data and performed analysis on all in vitro experiments, interpreted all data obtained and drafted the manuscript.		
Overall percentage (%)	90		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	23/10/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Lucian B Solomon		
Contribution to the Paper	Provided intellectual input		
Signature		Date	16/08/2018

Name of Co-Author	Dongqing Yang		
Contribution to the Paper	Obtained and processed bone biopsies for analysis		
Signature		Date	04/02/2019

Name of Co-Author	Tania N Crotti		
Contribution to the Paper	Provided intellectual input		
Signature		Date	17/08/2018

Name of Co-Author	David R Haynes		
Contribution to the Paper	Provided intellectual input		
Signature		Date	17/08/2018

Name of Co-Author	David M Findlay		
Contribution to the Paper	Helped with data interpretation and editing of manuscript, provided intellectual input.		
Signature		Date	16/08/2018

Name of Co-Author	Gerald J Atkins		
Contribution to the Paper	Planned and supervised research work, helped with data interpretation and writing of manuscript. Senior author on paper.		
Signature		Date	29/10/2018

Acta Biomaterialia xxx (xxxx) xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Osteocytes respond to particles of clinically-relevant conventional and cross-linked polyethylene and metal alloys by up-regulation of resorptive and inflammatory pathways

Renee T. Ormsby [a], Lucian B. Solomon [a,b], Dongqing Yang [a], Tania N. Crotti [c], David R. Haynes [c], David M. Findlay [a], Gerald J. Atkins [a,*]

[a] Biomedical Orthopaedic Research Group, Centre for Orthopaedic & Trauma Research, The University of Adelaide, Adelaide, SA 5000, Australia
[b] Orthopaedic and Trauma Service, Royal Adelaide Hospital, Adelaide, SA 5000, Australia
[c] Discipline of Anatomy and Pathology, The University of Adelaide, Adelaide, South Australia 5005, Australia

ARTICLE INFO

Article history:
Received 2 September 2018
Received in revised form 18 January 2019
Accepted 24 January 2019
Available online xxxx

Keywords:
Osteocyte
Wear particle
Polyethylene
Titanium alloy
Cobalt chrome

ABSTRACT

Periprosthetic osteolysis is a major cause of implant failure in total hip replacements. Aseptic loosening caused by osteolytic lesions is associated with the production of bioactive wear particles from the articulations of implants. Wear particles infiltrate the surrounding tissue of implants, promoting inflammation as well as bone resorption. Osteocytes have been shown to both regulate physiological osteoclastogenesis and directly remodel their perilacunar bone matrix by the process of osteocytic osteolysis. We hypothesise that osteocytes respond to wear debris of orthopaedic implant materials by adopting a pro-catabolic phenotype and thus contribute to periprosthetic osteolysis through the known pathways of bone loss. Osteocyte responses to particles derived from clinically relevant materials, ultra-high molecular weight polyethylene (UHMWPE), highly cross-linked polyethylene (XLPE) and metal alloys, Ti6Al4V and CoCrMo, were examined *in vitro* in human primary osteocyte-like cultures. Osteocyte-like cells exposed to both polyethylene and metal wear particle types showed upregulated expression of catabolic markers associated with osteocytic osteolysis, *MMP13*, carbonic anhydrase 2 (*CA2*) and cathepsin K (*CTSK*). In addition, pro-osteoclastogenesis markers *RANKL* and *M-CSF* were induced, as well as the expression of pro-inflammatory cytokines, *IL-6* and *TNFα*, albeit with different kinetics. These findings suggest a previously unrecognised action of wear particles of multiple orthopaedic materials on osteocytes, and suggest a multifaceted role for osteocytes in periprosthetic osteolysis.

Statement of Significance

This study addresses periprosthetic osteolysis, a major clinical problem leading to aseptic loosening of orthopaedic implants. It is well accepted that wear particles of polyethylene and of other implant materials stimulate the activity of bone resorbing osteoclasts. Our recent work provided evidence that commercial particles of ultra-high molecular weight polyethylene (UHMWPE) stimulated osteocytes to adopt a bone catabolic state. In this study we demonstrate for the first time that particles derived from materials in clinical use, conventional UHMWPE, highly cross-linked polyethylene (XLPE), and Ti6Al4V and CoCrMo metal alloys, all stimulate human osteocyte activities of osteocyte-regulated osteoclastogenesis, osteocytic osteolysis, proinflammatory responses, osteocyte apoptosis, albeit to varying extents. This study provides further mechanistic insight into orthopaedic wear particle mediated bone disease in terms of the osteocyte, the most abundant and key controlling cell type in bone.

© 2019 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Total hip replacement (THR) is a common and successful surgical procedure for the management of advanced osteoarthritis of the hip. In this procedure, prostheses are used to replace the

* Corresponding author at: The University of Adelaide, North Terrace, Adelaide, SA 5000, Australia.
E-mail address: gerald.atkins@adelaide.edu.au (G.J. Atkins).

https://doi.org/10.1016/j.actbio.2019.01.047
1742-7061/© 2019 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.

Please cite this article as: R. T. Ormsby, L. B. Solomon, D. Yang et al., Osteocytes respond to particles of clinically-relevant conventional and cross-linked polyethylene and metal alloys by up-regulation of resorptive and inflammatory pathways, Acta Biomaterialia, https://doi.org/10.1016/j.actbio.2019.01.047

femoral head and neck and resurface the acetabulum. The procedure aims to reduce pain and deformity, while improving patient mobility. Unfortunately, despite ongoing improvements, prosthetic implants used in THR have a limited lifespan. Periprosthetic osteolysis is a major cause of implant failure in THR, as it can lead to implant loosening [1,2] and periprosthetic fractures, two of the most common reasons for revision THR, as identified by the Australian Orthopaedic Association National Joint Replacement Registry [3]. Osteolysis is more commonly observed around the acetabular component of an implant, and is visualised on radiographs as a radiolucent area within the bone architecture [4]. At the tissue level, the osteolytic region becomes infiltrated with a granulomatous tissue, containing a wide variety of cells, including macrophages, inflammatory cells, fibroblasts and osteoclasts [5].

Bioactive wear particles are a major cause of osteolysis. Nano- and micro-scale particles produced by wear at the articulating surfaces of joint replacement prostheses infiltrate the periprosthetic tissue where they stimulate inflammatory responses that lead to bone resorption [6,7]. Wear particles are derived from the various materials comprising implant prostheses, including ultra-high molecular weight polyethylene (UHMWPE), metal, ceramic and poly-methyl methacrylate (PMMA). Particles released from metal-on-metal prostheses and metal-on-polyethylene prostheses are known to be biologically active and have been shown to cause adverse tissue reactions [8]. The small bioactive UHMWPE particles produced from the wear of the acetabular liner are capable of inducing inflammation, promoting bone resorption and inhibiting bone formation [6,7]. Importantly, it has been shown that the extent of liner wear positively correlates with the development of osteolytic lesions, and a high polyethylene liner wear rate is associated with progression of *peri*-prosthetic osteolytic lesion volume [9]. To combat the development of acetabular component polyethylene liner wear, highly cross-linked polyethylene (XLPE) liners have been employed, which have reduced wear rates and improved prosthesis revision rates [3].

The generation of metal wear particles is also implicated in the development of osteolysis. Metal particles are released by fretting at the head-stem junction in modular stem designs and when severe wear of the liner occurs, allowing direct contact of the femoral and acetabular metal components [10]. The release of metal particles is known to result in metallosis, where the metal particles infiltrate the periprosthetic tissues and stimulate a foreign body response [11]. Metal wear particles have also been shown to stimulate bone resorption through activation of the monocyte/-macrophage and other immune cells within the invading granulomatous tissue [12,13], as well as promoting the formation and activity of osteoclasts [14,15] and inhibiting osteoblast activity and viability [16–18].

Prior to implantation of the acetabular and femoral components of a hip prosthesis, the acetabulum and proximal femur are reamed to expose the underlying trabecular bone, which also potentially exposes it to subsequent wear particles. Using dark-field illumination, wear particles have been detected within the mineralised bone surrounding implants [19], adjacent to osteocytes, the major cell type embedded within the bone matrix. These cells are connected by their multiple dendritic processes, forming the lacuno-canalicular network. Osteocyte processes also enable contact with surface osteoclasts and bone lining cells [20]. Through this network, the osteocyte is capable of regulating bone formation and resorption, for example by the production of the key osteoclastogenic cytokine, receptor activator of nuclear factor kappa-B (RANKL) [21]. In addition to their pro-osteoclastic activity, osteocytes are also capable of remodelling their perilacunar space by the process of osteocytic osteolysis, also termed perilacunar remodelling [21]. During this process, the osteocyte produces key bone matrix degrading enzymes, including matrix metallopeptidases (MMPs) [22], cathepsin K/CTSK [23] and carbonic anhydrase II/CA2 [24]. Osteocytic osteolysis has been shown to play an important role in calcium release during lactation and in response to PTH injection [25]. It is now clear that osteocytes can also respond to pathological stimuli, such as Gram-negative bacterial lipopolysaccharide (LPS), Gram-positive *Staphylococcus aureus* [26] and both nano- and micro-particles of foreign bodies [27,28]. In particular, we have previously shown that micro-particles of Ceridust 3615®, an UHMWPE manufactured as a printer ink additive and commonly used to study wear particle responses, stimulated genes associated with both osteocytic osteolysis and osteoclastogenesis [29,30].

Little is known regarding the effects of orthopaedic implant-derived wear particles on osteocytes. Therefore in this study, we investigated the biological effects of two clinically relevant types of UHMWPE particles on human primary osteocyte-like cells *in vitro*; a conventional UHMWPE and a highly cross-linked polyethylene (XLPE), as well as two types of metal alloy particles, Ti6Al4V and CoCrMo, all derived from orthopaedic implants.

2. Materials and Methods

2.1. Wear particles

Polyethylene wear particles of conventional UHMWPE (Enduron®, DePuy Synthes, Warsaw, IN, USA) and XLPE (Longevity®, Zimmer Pty Ltd, Warsaw, IN, USA) were produced and characterised by BioEngineering Solutions Inc. (Oak Park, IL, USA) from the respective liners provided by our department (Orthopaedic and Trauma Service, RAH). A scanning electron microscopic based analysis was performed on 10 images of particles from each material, 5 at 1000×, and 5 at 10,000× magnification. Using Scion Image software (Scion Corporation, Frederick, MD, USA), area (A) and Ellipse Major Axis were determined for 255 individual particles. The data for each particle was 'weighted' to account for the size of each field, relative to the lowest magnification field. Roundness (R), a measure of how closely a particle resembles a circle and varies from zero to one in magnitude with a perfect circle having a value of one, was calculated by the formula $R = (4.A)/(\pi.d^2_{max})$ where d_{max} is the major diameter. The morphologies of resulting particles of UHMWPE/Enduron and XLPE/Longevity were similar, with an average R of 0.65 and 0.67, respectively, and a particle mean size of 1.22 μm (range 0.3–82.0) and 0.72 μm (range 0.2–46.7), respectively.

Metal wear particles of Titanium Aluminium Vanadium (Ti6Al4V; Tivanium®; Zimmer Pty Ltd, Warsaw, IN) and Cobalt Chrome Molybdenum (CoCrMo; Vitallium®; Howmedica), were generated from femoral head replacement prostheses by rotational milling. Particles were non-symmetrical and bell-shaped, and sized from 0.3 to 5 μm, as previously described [31,32]. All particle types were confirmed to be endotoxin-free (<0.1 EU/ml).

2.2. Human primary osteocyte-like cells

Trabecular bone biopsies were obtained from patients undergoing total hip replacement surgery for osteoarthritis of the hip with informed consent and with ethics approval by the Human Research Ethics Committees of the Royal Adelaide Hospital and the University of Adelaide. Human primary osteoblastic bone explant cells were cultured from rinsed and dissected bone, and expanded *ex vivo*, as described in detail previously [30]. Briefly, cells that grew out of bone pieces over a period of 14-21d were cultured to confluence, removed by collagenase/dispase digestion and reseeded at 50% confluence in fresh culture flasks. Once confluent, they were again removed by enzymic digestion and used for differ-

Please cite this article as: R. T. Ormsby, L. B. Solomon, D. Yang et al., Osteocytes respond to particles of clinically-relevant conventional and cross-linked polyethylene and metal alloys by up-regulation of resorptive and inflammatory pathways, Acta Biomaterialia, https://doi.org/10.1016/j.actbio.2019.01.047

entiation experiments. In order to generate human osteocytic cultures, cells derived from individual donors (passage 1) were seeded at 2×10^4 cells/cm^2 and cultured under differentiation conditions, consisting of growth in α-MEM medium containing 5% foetal calf serum (FCS), L-glutamine (2 mM), HEPES (1 mM), ascorbate 2-phosphate (100 μM) and KH_2PO_4 (1.8 mM) at 37 °C/5%CO_2 in a humidified incubator, with complete media changes every 3-4d for 28d to a mature osteocyte-like stage [24,26,29,33]. To investigate the effects of polyethylene particles, which are strongly hydrophobic in nature, we adapted our previously published method to promote cell-particle contact [29]. Thus, donor-specific osteocyte-like cultures were overlaid with a collagen gel (Cellmatrix Type 1-A, Nitta, Tokyo, Japan), containing either conventional UHMWPE or XLPE particles (50 and 100 μg/ml), or without particles. In order for to allow the cells to contact the suspended particles, these were cultured for a further 7d. To investigate the effects of metal particles, the osteocyte-like cells were similarly cultured for 28d but then overlaid directly with particles of either Ti6Al4V or CoCrMo suspended in differentiation medium (10^5, 10^6 and 10^7 particles/ml), or particle free control, for a further 48 h.

2.3. Confocal microscopy

For the purposes of imaging, cultures seeded and differentiated in 8-well chamber slides were stained with Calcein AM (Thermo Fisher; 405 nm excitation) at a final concentration of 1 μM. Alternatively, cultures were fixed in 10% buffered formalin for 10 min, then stained with CF®488A Phalloidin (Biotium; 488 nm excitation) at a final concentration of 300 nM. Cell bodies were imaged using differential interference contrast (DIC). Stained cells were examined on an Olympus FV3000 confocal microscope.

2.4. Gene expression

Total RNA was extracted using TRIZOL (Life Technologies, NY, USA) and cDNA synthesis was performed using iScript™ RT kit (Bio-Rad, Hercules, CA, USA), as per manufacturer's instructions. Real time RT-PCR was performed using SYBR Green Fluor qPCR Mastermix (Qiagen, Limburg, The Netherlands) on a CFX Connect Real Time PCR System (Bio-Rad, Hercules, CA, USA). Gene expression was normalised to *18S* mRNA, which remained constant relative to the total concentration of RNA used for the reverse transcription reaction. Oligonucleotide sequences for *18S*, *MMP13*, *CA2*, *TRAP*, *CTSK*, *RANKL* and *OPG* have been published previously [29]. Sequences for *IL6*, *TNFα, TLR4* and *COX-2* RT-PCR primers are shown in Table 1. All primers were designed to be mRNA specific by virtue of at least one primer in each pair flanking an intron-exon boundary, with specificity confirmed using non-reverse transcribed RNA controls. All primers were purchased from Geneworks (Thebarton, SA, Australia).

Table 1
Sequences of sense (S) and anti-sense (AS)-specific oligonucleotide primers and predicted PCR product sizes. Primers are human mRNA sequence-specific.

Gene	Sequence		Product size
IL6	gctatgaactccttctccacaa	Forward	120 bp
	tacatctttggaatcttctcctg	Reverse	
TNFα	agatcatcttctcgaacccc	Forward	129 bp
	tctcagctccacgccat	Reverse	
TLR4	ctgcgtggaggtggttccta	Forward	208 bp
	tgccccatcttcaattgtctg	Reverse	
COX2	ggagaaaactgctcaacaccg	Forward	118 bp
	tattcacaacgttccaaaatccc	Reverse	

2.5. Biochemical measurements

Supernatants were collected for biochemical assessment. Calcium levels were measured using the Arsenazo III method, as per the manufacturer's instructions (Thermo Fisher Scientific Inc., Middletown, VA, USA), and as described previously [24]. Briefly, 4 μl of supernatant from replicate wells was added to 196 μl of the colorimetric Calcium Arsenazo solution and incubated for 30 min at 37 °C. A standard curve was created from known calcium standards and absorbance was read on an ELISA plate reader at 630 nm.

The level of prostaglandin E_2 (PGE_2) was measured using a Prostaglandin E_2 Monoclonal ELISA Kit, as per the manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI, USA).

2.6. Caspase activity and inhibition

Caspase 3 activity was measured, as previously published [29]. Briefly, cells were lysed with 50 μl of Caspase 3 lysis buffer (Sigma-Aldrich, St Louis, MO, USA) on ice for 20 min. Supernatants were removed, centrifuged at 400g for 10 min to collect any floating cells and returned to corresponding wells to lyse. The cell lysates were then centrifuged at 12000g for 10 min at 4 °C. Protein concentrations were measured against a BSA standard (4 mg/ml BSA in PBS). Cell lysates (20 μg total protein) were incubated with 200 μl of fluorometric caspase assay buffer (0.1 M HEPES, 1% Sucrose, 1% CHAPS, 20 mM Caspase 3 substrate (AC-DEVD-AFC, Kamiya Biomedical, Seattle, WA, USA) and 1 M DTT, in the dark at room temperature for 4 h. Fluorescence was read at 570 nm (LS50 Spectrofluorimeter, Perkin Elmer, San Jose, CA, USA). In some experiments, caspase activity was blocked by the addition of the pan-caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O- methyl]-fluoromethylketone; ZVAD) (Sigma-Aldrich), at 5 μm for 48 h.

2.7. Statistical analysis

Statistical differences between *in vitro* data sets were assessed using the Mann-Whitney U nonparametric test (GraphPad v7 v0.2). Significant differences were accepted for p values < 0.05.

3. Results

3.1. Effects of UHMWPE and XLPE particles on osteocytic gene expression

To investigate the effects of clinically relevant PE particles, either conventional UHMWPE or XLPE particles were overlaid onto human mature osteocyte-like cultures in a type I collagen gel, promoting cell ingrowth and contact with these otherwise hydrophobic particles (Fig. 1). We examined the effects on inflammation by measuring the mRNA expression of two key inflammatory cytokines, interleukin (IL)-6 and TNFα, and found that both polyethylene particle types induced the expression of *IL6* (Fig. 2A), whereas only the highest concentration of HXLPE particles significantly upregulated *TNFα* (Fig. 2B). The activation of inflammatory pathways by osteocytes in response to particles could be a possible defense response against foreign bodies. We also observed a marked increase in *TLR4* mRNA expression by osteocytes in response to the particles, in particular by conventional UHMWPE (Fig. 2C).

Next, we investigated the effects of polyethylene particles on key osteoclast regulating genes. Both particle types upregulated *RANKL* mRNA expression, as well as the gene encoding osteoprotegerin/OPG, the decoy receptor (Fig. 2D and E). However, there was an overall increase in the pro-osteoclastogenic *RANKL:OPG* mRNA ratio by both particle types, particularly by conventional UHMWPE

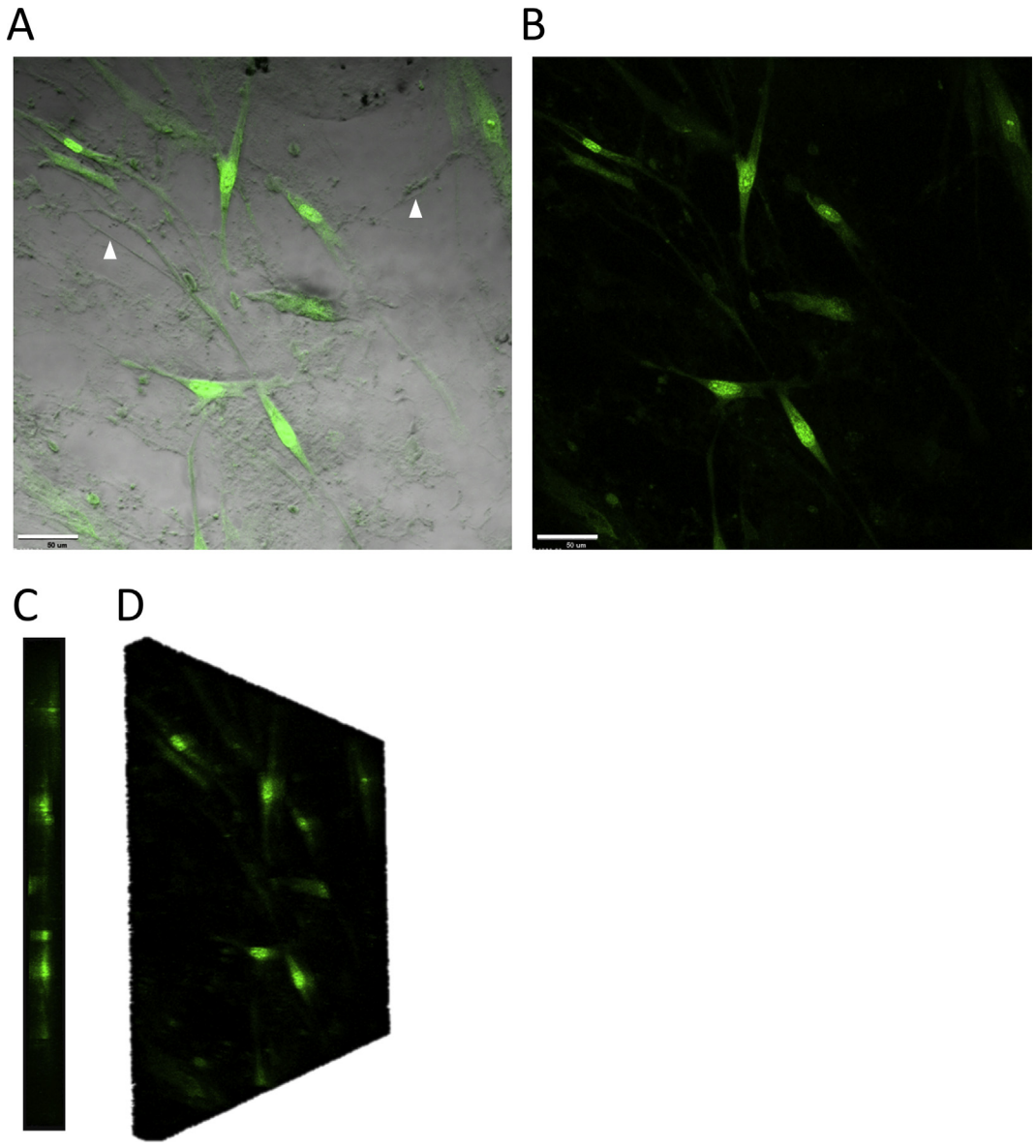


Fig. 1. Confocal microscopy analysis of osteocyte-like cells plated in type I collagen gel with Longevity particles (100 μg/ml), stained with CF®488A Phalloidin. A) Histotomography of differential interference contrast (DIC) microscopy overlayed with fluorescent confocal laser scanning (CLS) microscopy, B) CLS microscopy showing Calcein AM staining of osteocyte processes C) Side view of Z stack (Z stack = 30 μm), D) Angled view of Z stack. (30x objective). White arrowheads depict osteocyte processes; scale bars represent 50 μM.

(Fig. 2F). We also observed an increase in *MCSF* mRNA expression (Fig. 2G), consistent with our previous findings [29,30]. Osteocytic osteolysis-related genes were also observed to be upregulated with exposure to both types of polyethylene particles, including increased expression of *MMP13*, *CTSK* and *CA2* (Fig. 2H–J), consistent with our previous findings [29]. Interestingly, both concentrations of XLPE particles used markedly increased the expression of *MMP13*, the protein product of which (MMP-13) being a key enzyme involved in osteocyte-mediated matrix remodelling [22].

3.2. Effects of Ti6Al4V and CoCrMo particles on osteocytic gene expression

To study the effects of metal wear particles on osteocyte-like cells *in vitro*, the cells were overlaid for 48 h with either Ti6Al4V or CoCrMo particles (Fig. 3). We observed upregulation of expression of *IL6* and *TNFα*, by CoCrMo particles, with no change in expression induced by Ti6Al4V particles (Fig. 4A and 4B). Unlike the observed upregulation of *TLR4* by PE particles, metal particles inhibited the expression of *TLR4* (Fig. 4C).

Expression of *RANKL* mRNA was significantly increased by Ti6Al4V particles (Fig. 4D), as was *OPG* mRNA (Fig. 4E.). CoCrMo particles showed a trend towards upregulating *OPG* expression (Fig. 4E), resulting in an overall increase in the pro-osteoclastic *RANKL*:*OPG* mRNA ratio (Fig. 4F). Ti6Al4V particles also stimulated an increase in the *RANKL*:*OPG* ratio at the lowest concentration (Fig. 4F), suggesting a comparatively minor potential stimulation of osteoclastogenesis by osteocytes in response to this particle type. The expression of *MCSF* mRNA was variably regulated by both metal particle types (Fig. 4G).

Mature osteocyte-like cells exposed to both types of metal wear particles showed significant increases in the mRNA expression of the osteocytic osteolysis related genes, *CA2*, and *CTSK* (Fig. 4I and J), however, with an inconsistent effect on *MMP13* mRNA expression (Fig. 4H). Expression of *CA2* was significantly upregulated by both CoCrMo and Ti6Al4V particles, with the highest concentration of

Please cite this article as: R. T. Ormsby, L. B. Solomon, D. Yang et al., Osteocytes respond to particles of clinically-relevant conventional and cross-linked polyethylene and metal alloys by up-regulation of resorptive and inflammatory pathways, Acta Biomaterialia, https://doi.org/10.1016/j.actbio.2019.01.047

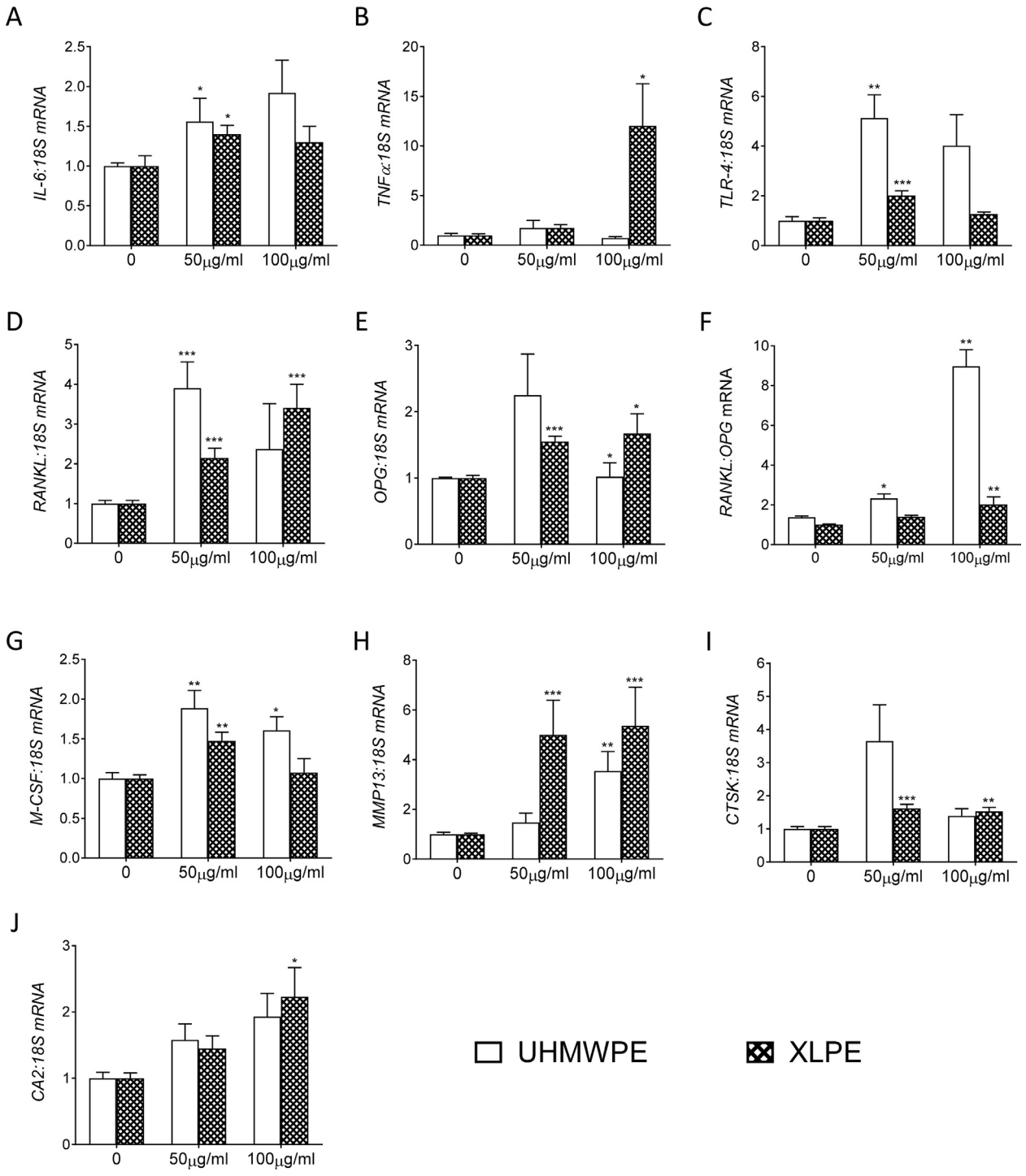


Fig. 2. The effect of UHMWPE and XLPE on differentiated osteocyte-like cells. NHBC were differentiated for 28 d to an osteocyte-like stage and then overlaid with a collagen gel layer, otherwise untreated (0) or containing a either UHMWPE or XLPE particles (50 and 100 μg/ml) for a further 7 d of culture. Relative gene expression was measured for A) *IL-6*, B) *TNFα*, C) *TLR4*, expression of genes associated with osteoclastogenesis, *RANKL*, *OPG*, *RANKL:OPG* ratio and *M-CSF* (D-G), and expression of genes associated with osteocytic osteolysis, *MMP13*, *CTSK* and *CA2* (H-J). Data shown are means ± standard error of the mean (SEM) of quadruplicate experiments analysed in duplicate. Significant differences to untreated controls are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

CoCrMo particles inducing a robust 7.6-fold increase. Interestingly, *CA2* mRNA expression was markedly higher with exposure to metal particles compared to PE.

3.3. Effects of Ti6Al4V and CoCrMo particles on calcium release

As expected, calcium deposition occurred during differentiation from an osteoblastic-like phenotype into a mature osteocyte-like phenotype over 28d in culture [34]. Calcium concentrations in the culture supernatants were higher when osteocytes were exposed to the highest concentration of Ti6Al4V particles (Fig. 5). A possible explanation for this is osteocytic mineral resorption, which is consistent with the observed increased expression of *CA2* in these cells. Calcium levels did not change in the medium of PE-treated cultures (data not shown), potentially due to the nature of the assay system, where the cell layer was overlaid with collagen gel.

Please cite this article as: R. T. Ormsby, L. B. Solomon, D. Yang et al., Osteocytes respond to particles of clinically-relevant conventional and cross-linked polyethylene and metal alloys by up-regulation of resorptive and inflammatory pathways, Acta Biomaterialia, https://doi.org/10.1016/j.actbio.2019.01.047

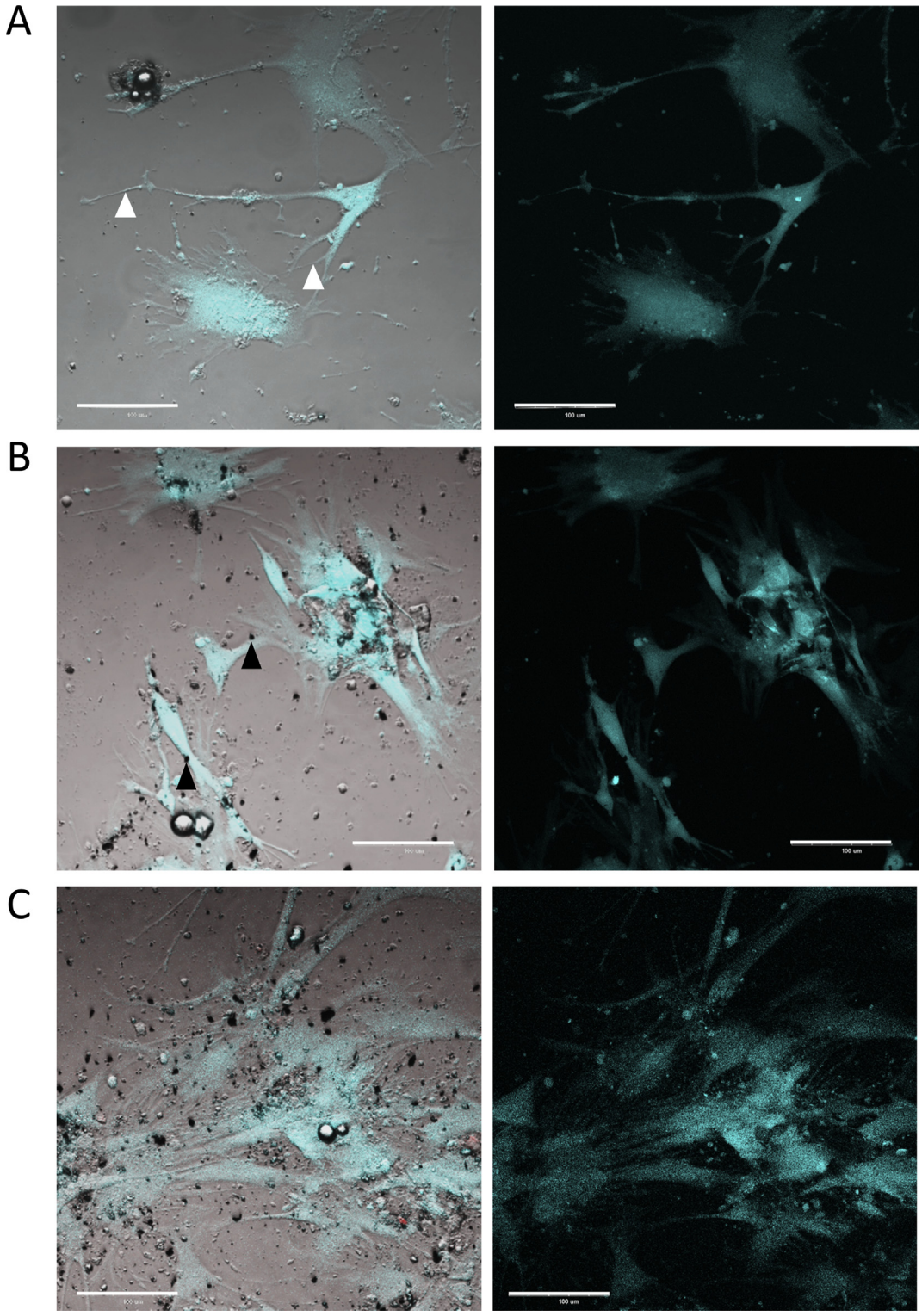


Fig. 3. Confocal microscopy analysis of osteocyte-like cells used in metal particle response experiments. A) Histotomography of DIC microscopy overlayed with fluorescent (CLS) microscopy of control osteocyte-like cells stained with Calcein AM (30x objective) (A-C). A) Osteocyte-like cells, B) Osteocyte-like cells co-cultured with Ti6Al4V particles (10^6 particles/ml), C) Osteocyte-like cells co-cultured with CoCrMo particles (10^6 particles/ml). White arrowheads depict osteocyte processes; Black arrowheads depict metal particles. Scale bars represent 100 μM.

3.4. Effects of Ti6Al4V and CoCrMo particles on prostaglandin E_2 release

To investigate other pro-inflammatory effects, we measured both the mRNA expression of cyclooxygenase 2 (*COX2*) and corresponding PGE_2 production. *COX2* expression was induced by CoCrMo but not significantly by Ti6Al4V particles (Fig. 6A). However, exposure of cells to either metal particle type significantly increased secreted PGE_2 levels in culture supernatants (Fig. 6B). No effect on *COX2* mRNA expression was observed in the PE particle treated osteocytes (data not shown).

Please cite this article as: R. T. Ormsby, L. B. Solomon, D. Yang et al., Osteocytes respond to particles of clinically-relevant conventional and cross-linked polyethylene and metal alloys by up-regulation of resorptive and inflammatory pathways, Acta Biomaterialia, https://doi.org/10.1016/j.actbio.2019.01.047

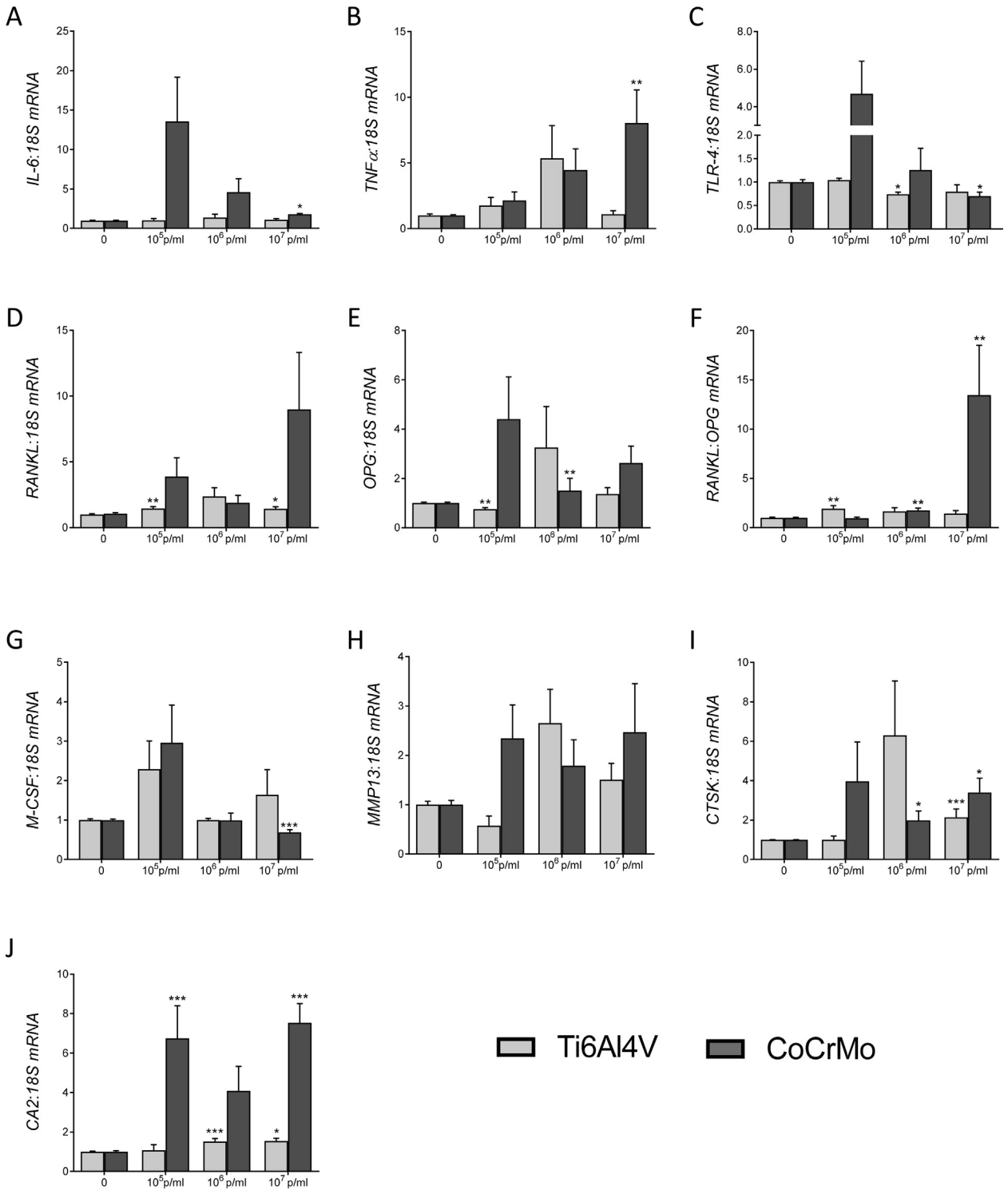


Fig. 4. The effect of Ti6Al4V and CoCrMo particles on differentiated osteocyte-like cells. Relative gene expression was measured for A) IL-6, B) TNFα, C) *TLR4*, expression of genes associated with osteoclastogenesis, *RANKL*, *OPG*, *RANKL*:*OPG* ratio and *MCSF* (D-G), and expression of genes associated with osteocytic osteolysis, *MMP13*, *CTSK* and *CA2* (H-J). Data shown are means ± SEM of quadruplicate experiments. Significant differences to untreated controls are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.5. Effects of wear particles on caspase 3 activation

To investigate the effect on cell viability with exposure to either PE or metal particles types, we measured Caspase 3 activity. Both Ti6Al4V and CoCrMo particles significantly upregulated Caspase 3 activity (Fig. 7A), indicative of a pro-apoptotic effect. In contrast, PE particles of either type had no effect on Caspase 3 activity (Fig. 7B), consistent with our previous findings that UHMWPE has no discernible effect on osteocyte-like cell viability [29].

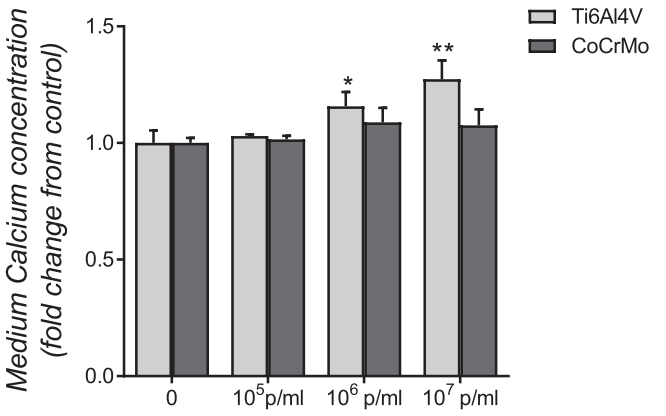


Fig. 5. The effect of osteocyte exposure to Ti6Al4V and CoCrMo particles on osteocytic mineral resorption was measured using the Calcium Arsenazo III method. Calcium concentrations in the culture supernatants were measured with exposure to either TiA6lV4 or CoCrMo particle (10^5, 10^6 and 10^7 particles/ml) or particle free control. Data shown are means ± SEM of quadruplicate experiments analysed in duplicate. Significant differences to untreated controls are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.6. Effects of Caspase 3 inhibition with metal particle exposure on osteocytes

To investigate the role of osteocyte apoptosis in this model, cells were cultured with the pan-caspase inhibitor ZVAD in the presence or absence of metal particles. ZVAD treatment significantly suppressed Caspase 3 activity in either the presence or absence of metal particles (Fig. 8A) and decreased the pro-apoptotic *BAX*:*BCL-2* mRNA ratio (Fig. 8B). CoCrMo particles upregulated both Caspase 3 activity and the *BAX*:*BCL-2* mRNA ratio, whereas Ti6Al4V particles did not. Metal particles also upregulated *RANKL* mRNA expression, which, interestingly, was observed with ZVAD alone (Fig. 8C). ZVAD also increased the pro-osteoclastogenic *RANKL*:*OPG* mRNA ratio (Fig. 8D), suggesting a link in this system between inhibition of Caspase 3 activity and a positive effect on osteoclastogenesis. Both Ti6Al4V and CoCrMo upregulated *CA2* mRNA expression, which was enhanced with ZVAD treatment (Fig. 8E). Caspase blockade also tended to exacerbate the effect of both metal types on *CTSK* expression (Fig. 8F), while effects on *MMP13* expression were mixed, with ZVAD reversing the effect of Ti6Al4V on expression but having no effect on the response to CoCrMo (Fig. 8G). Overall, these findings indicate for the first time that Caspase activity and osteocyte apoptosis may be linked to the regulation of osteocytic osteolysis.

4. Discussion

Several investigators have shown that osteoblasts, in addition to haemopoietic lineage cells, respond to UHMWPE particles [16,30,35–45]. We have previously shown that human differentiated osteocytes also respond to UHMWPE in the form of Ceridust, by adopting a pro-osteoclastogenic and osteocytic osteolytic phenotype [29]. This was consistent with enlarged osteocyte lacunae in human bone biopsies taken from patients undergoing revision THR surgery for aseptic loosening [29]. However, the responses of osteocytes to particulates of clinically utilised materials has not been previously reported. Thus, in this study we examined responses of human osteocyte-like cells to particles of Enduron™, a conventional UHMWPE liner, Longevity®, an example of a XLPE liner, as well as commonly utilised metal alloys, Ti6Al4V and CoCrMo.

Osteocytes have been shown to be a major source of the key osteoclastogenic cytokine RANKL, and to have a major role in regulating osteoclastic bone resorption in adult bone [46–48]. Consistent with our previous report [29], osteocytes responded to all particle types examined by upregulating the *RANKL*:*OPG* mRNA ratio, albeit to varying extents. In particular, the UHMWPE particles gave rise to the greatest induction of *RANKL*:*OPG* of approximately 9-fold, whereas induction by XLPE was approximately 2-fold. Additionally, both types of polyethylene particles upregulated the expression of the key pro-osteoclastogenic co-factor, *MCSF*. With regard to the metal particles, CoCrMo particles upregulated the *RANKL*:*OPG* ratio more potently than Ti6Al4V. Therefore, all particles tested stimulated a coordinated pro-osteoclastogenic effect, which we propose underlies the pro-resorptive, bone loss effect that accompanies particle generation *in vivo*.

Osteocyte apoptosis has been implicated in maintaining bone homeostasis and integrity, and is an important factor in the response to microdamage that occurs within the bone [20]. Osteocyte apoptosis can induce osteoclastic resorption as an integral part of the response to damage, with dying osteocytes inducing RANKL expression in viable, neighbouring osteocytes [49]. However, osteocyte apoptosis is not a requirement for bone resorption and these can be independent [50]. Consistent with our previous findings [29], neither UHMWPE nor XLPE altered the levels of activity of Caspase 3, a key executioner caspase. However, exposure to either Ti6Al4V or CoCrMo particles did induce significant Caspase 3 activation. Kanaji et al. have shown previously that MLO-Y4 cells, a murine osteocyte-like cell line, underwent apoptosis when exposed to CoCrMo particles [27]. It is possible therefore, that exposure to polyethylene or metal wear particles activates RANKL signalling through distinct cellular pathways. Caspase 3

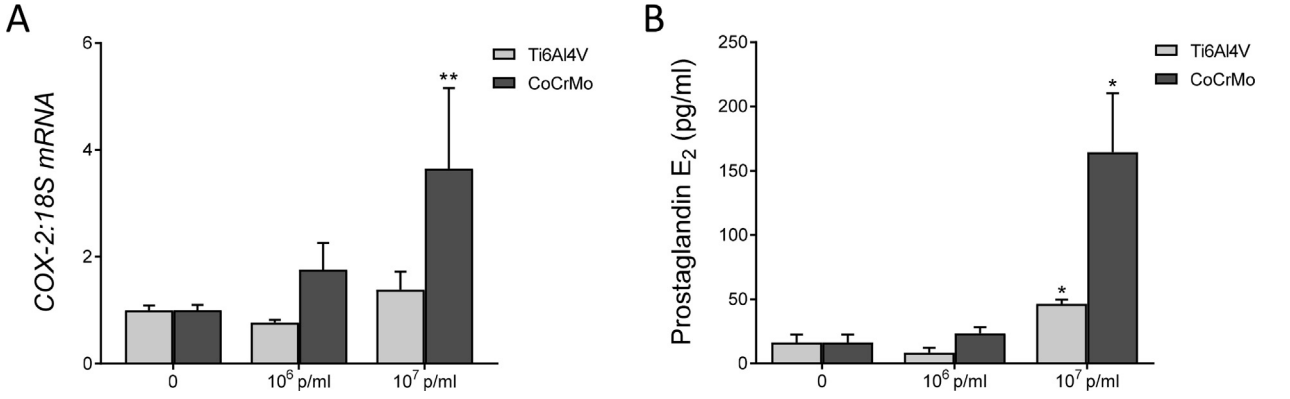


Fig. 6. The effect of osteocyte exposure to Ti6Al4V and CoCrMo particles on PGE_2 production. A) Relative *COX2* mRNA expression and B) corresponding PGE_2 concentrations in the medium. Data shown are means ± SEM of duplicate experiments analysed in duplicate. Significant differences to untreated controls are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Please cite this article as: R. T. Ormsby, L. B. Solomon, D. Yang et al., Osteocytes respond to particles of clinically-relevant conventional and cross-linked polyethylene and metal alloys by up-regulation of resorptive and inflammatory pathways, Acta Biomaterialia, https://doi.org/10.1016/j.actbio.2019.01.047

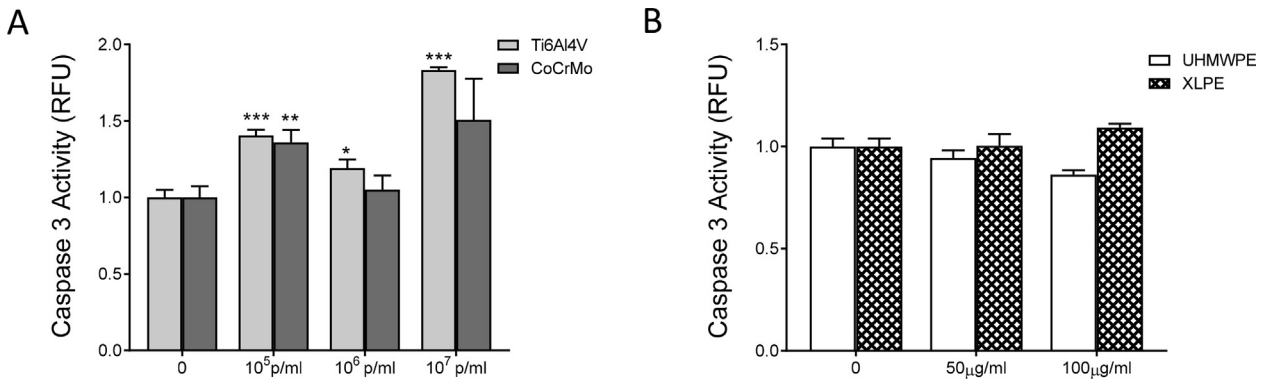


Fig. 7. The effect on osteocyte apoptosis following exposure to either: A) Ti6Al4V and CoCrMo or B) UHMWPE and XLPE particles on Caspase 3 activity (RFU). Data shown are means ± SEM of triplicate experiments analysed in duplicate. Significant differences to untreated controls are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

inhibition using the pan-caspase inhibitor ZVAD induced both a pro-osteoclastogenic effect through upregulation of *RANKL* mRNA expression and of the *RANKL*:*OPG* mRNA ratio, as well as inducing a pro-osteocytic osteolysis response, with upregulation of both *CA2* and *CTSK* expression. To our knowledge, very few studies have directly tested the effects of caspase inhibition on osteocyte behaviour. Previous studies in mice showed that administration of a pan-caspase inhibitor could prevent osteocyte apoptosis and ensuing bone loss in response to fatigue loading of the ulna [51] and ovariectomy [52]. The current findings in human cells suggest that osteocyte apoptosis and stimulation of osteoclastic bone resorption are separable. The observation that caspase blockade stimulated genes associated with osteocytic osteolysis was surprising but may be indicative of the processes of osteoclastic and osteocytic osteolysis being linked, as proposed by Baylink [53], and that one mechanism may compensate for another under certain circumstances. A potential limitation of this study is that we did not examine other possible cell fates in response to wear particles, such as necrosis [7], however the lack of any discernible effect of polyethylene particles and the clear increase in caspase 3 activation in response to metal particles suggests that apoptosis was the major mode of cell death, if any, in the models examined.

In addition to the pro-osteoclastogenic response, both metal and polyethylene wear particles stimulated an osteocytic osteolysis response, indicated by the upregulation of expression of bone matrix degrading enzymes *MMP13* and *CTSK*, and consistent with our previous findings [29]. The expression of *CA2* was also induced to a similar extent by XLPE particle types, and by both metal types, although to a much greater extent by CoCrMo. We reported that *CA2* expression by osteocytes was a key enzyme in the dissolution of bone mineral, an essential first step in the resorption of the bone matrix, and in the mechanism of osteocytic osteolysis [24]. Indeed, treatment of human bone with the carbonic anhydrase inhibitor acetazolamide prevented osteocytic osteolysis in an *ex vivo* model [24].

In addition to direct pro-resorptive effects, we found that both metal and polyethylene particles stimulated a pro-inflammatory response by osteocytes. Specifically, the production of *IL-6* and *TNFα* were significantly upregulated with osteocyte exposure to wear particles. These genes have been shown to be similarly upregulated in human patients with aseptic loosening [54,55], and inhibition of TNFα in a mouse calvarial model reduced the wear particle stimulation of osteolysis [54,56]. The osteocyte inflammatory response to polyethylene particles may be stimulated via toll-like receptor (TLR) pathways. TLRs function as sensors of foreign bodies and of pathogen associated molecular patterns (PAMPs), including LPS [57]. Similarly, TLRs have been implicated in the inflammatory cascade induced by wear particles in other cell types [58]. TLR-4, in particular, plays a key role in detecting threats from PAMPs and endogenously-derived, damage-associated molecular patterns (DAMPs). Furthermore, increased TLR-4 expression has been demonstrated in the synovial membrane of patients with aseptic loosening [59,60]. In this study, we observed an upregulation of *TLR4* mRNA expression by osteocytes with exposure to either UHMWPE or XLPE particles, consistent with osteocytes recognising these as foreign bodies, which in turn activates the inflammatory cascade. Interestingly, metal particles did not induce *TLR4* expression. However, osteocytes responded to metal wear particles but not polyethylene particles, by upregulating *COX2* expression and stimulating the production of PGE_2. Although PGE_2 plays important roles in bone metabolism, in particular in response to mechanical loading [61], differential PGE_2 receptor usage also activates inflammation and osteoclastogenesis [62], and expression of both PGE_2 and its producing enzyme COX-2 have been shown to be strongly expressed within the periprosthetic membranes of revision patients with aseptic loosening [63,64]. Notably, specific inhibition of COX-2 in a titanium particle treated osteoclast model resulted in suppression of PGE_2 production and a reduction in particle induced osteoclastogenesis [65].

The use of XLPE as a bearing surface in prostheses was designed to reduce the rate of wear compared to conventional polyethylene liners [66–69]. However, an additional finding from this study was that XLPE particles retain bioactivity and were capable of stimulating an inflammatory, pro-catabolic phenotype in the primary human osteocyte-like cells tested. In some instances, notably the stimulation of *MMP13* expression in osteocytes, XLPE particles were more potent than particles of conventional UHMWPE. Therefore, particles from XLPE liners could still play a role in the development of osteolysis in response to wear.

Our study has a number of limitations in addition to those mentioned previously. We utilised different model systems to study polyethylene and metal particles due to their respective properties, as explained in Materials and Methods. Therefore, we could not directly compare the magnitude of the effects of polyethylene versus metal, although we believe the model systems used are best suited to the study of each type of particle. In addition, while only 4 donors' cells were tested and future studies of particular responses could employ greater numbers to establish the frequency with which the observed effects occur, consistent results were obtained. A strength of the study was that the primary cells used were grown from the bone of patients undergoing THR for end-stage osteoarthritis of the hip, the actual patient group at risk of eventually developing aseptic loosening in response to wear particles, which enhances the clinical relevance to the findings. Potential variability due to individual donor comorbidities or medication regimes were likely eliminated due to the *ex vivo* growth and

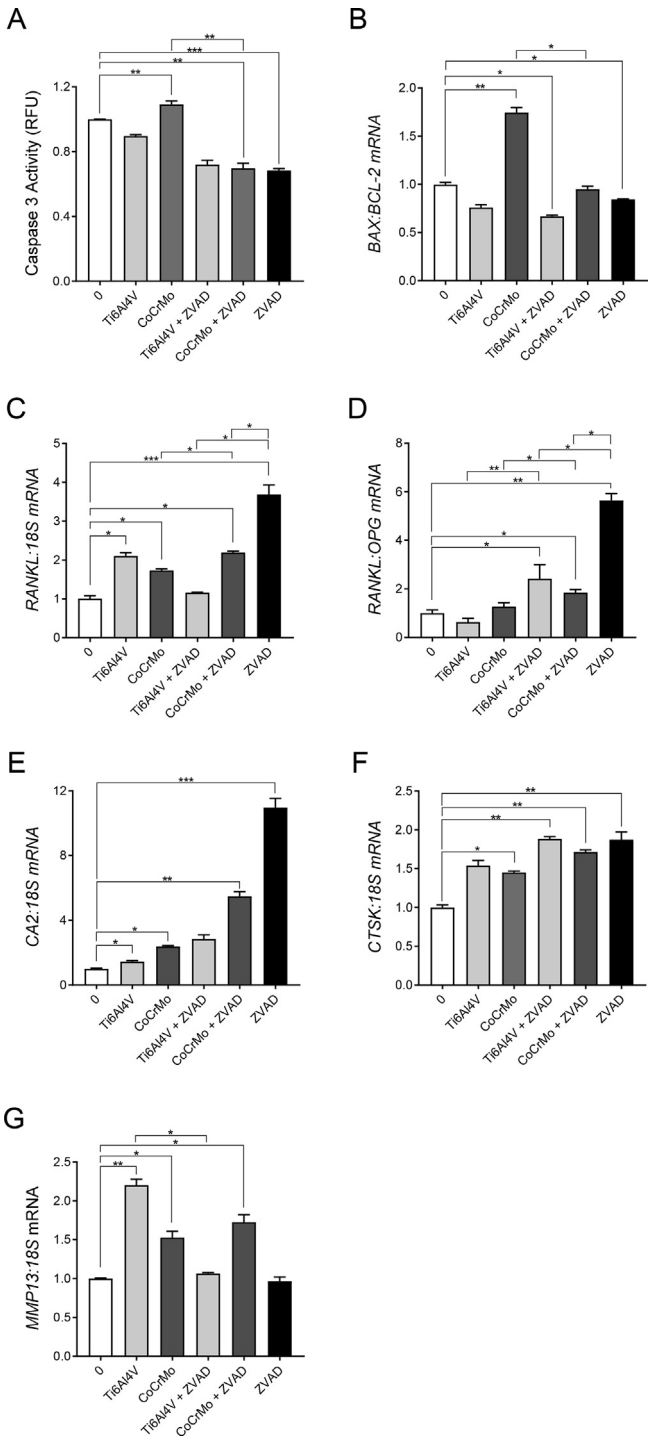


Fig. 8. The effect of Caspase 3 inhibition on the osteocyte response to metal particles. Human differentiated osteocyte-like cells were exposed either Ti6Al4V or CoCrMo particles (10^7 particles/ml) in the presence or absence of ZVAD (5 μM) for 48 h. A. Caspase 3 Activity, B. corresponding *BAX:BCL-2* mRNA ratio C. Relative *RANKL* mRNA expression, D. *RANKL:OPG* mRNA ratio, E) Relative *CA2* F) *CTSK* and G) *MMP13* mRNA levels. Data shown are means ± SEM of experimental triplicates. Significant differences between treatments are denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

expansion of the cells prior to experimentation. Furthermore, while our previous study [29] provides proof of concept of the influence of UHMWPE particles in the form of Ceridust on osteocyte behaviour *in vivo*, it would be of strong interest to investigate the current findings using a suitable *in vivo* model.

In summary, we have shown that human osteocytes are capable of sensing and responding differentially to particles of UHMWPE, XLPE, Ti6Al4V and CoCrMo. Our findings are consistent with osteocyte exposure to wear particles stimulating both osteocytic perilacunar remodelling and osteocyte-mediated osteoclastic bone resorption, with the two processes potentially being linked. Therefore, osteocytes may play a key role in contributing to the progression of periprosthetic osteolysis and this is an important consideration in the development of non-surgical approaches to the treatment of prosthesis-associated bone loss.

Acknowledgements

The authors would like to thank the nursing and surgical staff of the Orthopaedic and Trauma Service at the Royal Adelaide Hospital for their help in collecting patient bone specimens. This work was supported by funding from the National Health and Medical Research Council of Australia (NHMRC) Project Grant Scheme (Grant ID 1041456) and the Royal Adelaide Hospital Special Purposes Fund. GJA is a NHMRC Senior Research Fellow.

References

[1] Y. Abu-Amer, I. Darwech, J.C. Clohisy, Aseptic loosening of total joint replacements: mechanisms underlying osteolysis and potential therapies, Arthritis Res. Therapy 9 (Suppl 1) (2007) S6.
[2] J.J. Jacobs, K.A. Roebuck, M. Archibeck, N.J. Hallab, T.T. Glant, Osteolysis: basic science, Clin. Orthop. Relat. Res. (2001) 71–77.
[3] Australian Orthopaedic Association National Joint Replacement Registry Annual Report 2017. https://aoanjrrsahmricom/annual-reports-2017 2017.
[4] R. Dattani, Femoral osteolysis following total hip replacement, Postgrad. Med. J. 83 (2007) 312–316.
[5] S.B. Goodman, P. Huie, Y. Song, D. Schurman, W. Maloney, S. Woolson, R. Sibley, Cellular profile and cytokine production at prosthetic interfaces – Study of tissues retrieved from revised hip and knee replacements, J. Bone Joint Surgery-British 80b (1998) 531–539.
[6] G.J. Atkins, D.R. Haynes, D.W. Howie, D.M. Findlay, Role of polyethylene particles in peri-prosthetic osteolysis: a review, World J. Orthop. 2 (2011) 93–101.
[7] J. Gallo, S.B. Goodman, Y.T. Konttinen, M. Raska, Particle disease: biologic mechanisms of periprosthetic osteolysis in total hip arthroplasty, Innate Immun. 19 (2013) 213–224.
[8] S. Grosse, H.K. Haugland, P. Lilleng, P. Ellison, G. Hallan, P.J. Hol, Wear particles and ions from cemented and uncemented titanium-based hip prostheses-A histological and chemical analysis of retrieval material, J. Biomed. Mater. Res. B Appl. Biomater. 103 (2015) 709–717.
[9] D.W. Howie, S.D. Neale, R. Stamenkov, M.A. McGee, D.J. Taylor, D.M. Findlay, Progression of acetabular periprosthetic osteolytic lesions measured with computed tomography, J. Bone Joint Surg. Am. 89 (2007) 1818–1825.
[10] H.S. Kwak, J.J. Yoo, Y.K. Lee, K.H. Koo, K.S. Yoon, H.J. Kim, The result of revision total hip arthroplasty in patients with metallosis following a catastrophic failure of a polyethylene liner, Clinics Orthopedic Surg. 7 (2015) 46–53.
[11] C.H. Lohmann, G. Singh, H.G. Willert, G.H. Buchhorn, Metallic debris from metal-on-metal total hip arthroplasty regulates periprosthetic tissues, World J. Orthopedics 5 (2014) 660–666.
[12] G. Mabilleau, Y.M. Kwon, H. Pandit, D.W. Murray, A. Sabokbar, Metal-on-metal hip resurfacing arthroplasty: a review of periprosthetic biological reactions, Acta Orthop. 79 (2008) 734–747.
[13] K. Man, L.H. Jiang, R. Foster, X.B. Yang, Immunological responses to total hip arthroplasty, J. Funct. Biomater. 8 (2017).
[14] D.R. Haynes, T.N. Crotti, A.E. Potter, M. Loric, G.J. Atkins, D.W. Howie, D.M. Findlay, The osteoclastogenic molecules RANKL and RANK are associated with periprosthetic osteolysis, J. Bone Joint Surg. Br. 83 (2001) 902–911.
[15] P.E. Purdue, P. Koulouvaris, B.J. Nestor, T.P. Sculco, The central role of wear debris in periprosthetic osteolysis, Hss J. 2 (2006) 102–113.
[16] K. Lochner, A. Fritsche, A. Jonitz, D. Hansmann, P. Mueller, B. Mueller-Hilke, R. Bader, The potential role of human osteoblasts for periprosthetic osteolysis following exposure to wear particles, Int. J. Mol. Med. 28 (2011) 1055–1063.
[17] J. Yao, G. Cs-Szabo, J.J. Jacobs, K.E. Kuettner, T.T. Glant, Suppression of osteoblast function by titanium particles, J. Bone Joint Surg. Am. 79 (1997) 107–112.
[18] S.B. Goodman, T. Ma, R. Chiu, R. Ramachandran, R.L. Smith, Effects of orthopaedic wear particles on osteoprogenitor cells, Biomaterials 27 (2006) 6096–6101.
[19] B. Busse, M. Hahn, M. Niecke, B. Jobke, K. Puschel, G. Delling, A. Katzer, Allocation of nonbirefringent wear debris: darkfield illumination associated with PIXE microanalysis reveals cobalt deposition in mineralized bone matrix adjacent to CoCr implants, J. Biomed. Mater. Res. Part A 87 (2008) 536–545.
[20] L.F. Bonewald, The amazing osteocyte, J. Bone Miner. Res. 26 (2011) 229–238.

[21] M. Prideaux, D.M. Findlay, G.J. Atkins, Osteocytes: the master cells in bone remodelling, Curr. Opin. Pharmacol. 28 (2016) 24–30.
[22] S.Y. Tang, R.P. Herber, S.P. Ho, T. Alliston, Matrix metalloproteinase-13 is required for osteocytic perilacunar remodeling and maintains bone fracture resistance, J. Bone Miner. Res. 27 (2012) 1936–1950.
[23] H. Qing, L. Ardeshirpour, P.D. Pajevic, V. Dusevich, K. Jahn, S. Kato, J. Wysolmerski, L.F. Bonewald, Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation, J. Bone Miner. Res. 27 (2012) 1018–1029.
[24] M. Kogawa, A.R. Wijenayaka, R.T. Ormsby, G.P. Thomas, P.H. Anderson, L.F. Bonewald, D.M. Findlay, G.J. Atkins, Sclerostin regulates release of bone mineral by osteocytes by induction of carbonic anhydrase 2, J. Bone Miner. Res. 28 (2013) 2436–2448.
[25] E. Tsourdi, K. Jahn, M. Rauner, B. Busse, L.F. Bonewald, Physiological and pathological osteocytic osteolysis, J. Musculoskelet. Neuronal Interact. 18 (2018) 292–303.
[26] D. Yang, A.R. Wijenayaka, L.B. Solomon, S.M. Pederson, D.M. Findlay, S.P. Kidd, G.J. Atkins, Novel insights into Staphylococcus aureus deep bone infections: the involvement of osteocytes, MBio 9 (2018) e00415–e00418.
[27] A. Kanaji, M.S. Caicedo, A.S. Virdi, D.R. Sumner, N.J. Hallab, K. Sena, Co-Cr-Mo alloy particles induce tumor necrosis factor alpha production in MLO-Y4 osteocytes: a role for osteocytes in particle-induced inflammation, Bone 45 (2009) 528–533.
[28] Y. Zhang, M. Yan, A. Yu, H. Mao, J. Zhang, Inhibitory effects of beta-tricalciumphosphate wear particles on osteocytes via apoptotic response and Akt inactivation, Toxicology 297 (2012) 57–67.
[29] R.T. Ormsby, M. Cantley, M. Kogawa, L.B. Solomon, D.R. Haynes, D.M. Findlay, G.J. Atkins, Evidence that osteocyte perilacunar remodelling contributes to polyethylene wear particle induced osteolysis, Acta Biomater. 33 (2016) 242–251.
[30] G.J. Atkins, K.J. Welldon, C.A. Holding, D.R. Haynes, D.W. Howie, D.M. Findlay, The induction of a catabolic phenotype in human primary osteoblasts and osteocytes by polyethylene particles, Biomaterials 30 (2009) 3672–3681.
[31] D.R. Haynes, T.N. Crotti, M.R. Haywood, Corrosion of and changes in biological effects of cobalt chrome alloy and 316L stainless steel prosthetic particles with age, J. Biomed. Mater. Res. 49 (2000) 167–175.
[32] S.D. Rogers, M.J. Pearcy, S.J. Hay, D.R. Haynes, A. Bramley, D.W. Howie, A method for production and characterization of metal prosthesis wear particles, J. Orthop. Res. 11 (1993) 856–864.
[33] G.J. Atkins, P.S. Rowe, H.P. Lim, K.J. Welldon, R. Ormsby, A.R. Wijenayaka, L. Zelenchuk, A. Evdokiou, D.M. Findlay, Sclerostin is a locally acting regulator of late-osteoblast/preosteocyte differentiation and regulates mineralization through a MEPE-ASARM-dependent mechanism, J. Bone Miner. Res. 26 (2011) 1425–1436.
[34] G.J. Atkins, K.J. Welldon, P. Halbout, D.M. Findlay, Strontium ranelate treatment of human primary osteoblasts promotes an osteocyte-like phenotype while eliciting an osteoprotegerin response, Osteoporos. Int. 20 (2009) 653–664.
[35] C.H. Lohmann, D.D. Dean, L.F. Bonewald, Z. Schwartz, B.D. Boyan, Nitric oxide and prostaglandin E2 production in response to ultra-high molecular weight polyethylene particles depends on osteoblast maturation state, J. Bone Joint Surg. Am. (2002;84-A:) 411–419.
[36] D.D. Dean, C.H. Lohmann, V.L. Sylvia, G. Koster, Y. Liu, Z. Schwartz, B.D. Boyan, Effect of polymer molecular weight and addition of calcium stearate on response of MG63 osteoblast-like cells to UHMWPE particles, J. Orthopaedic Res.: Off. Publ. Orthopaedic Res. Soc. 19 (2001) 179–186.
[37] H. Jablonski, H. Rekasi, M. Jager, The influence of calcitonin gene-related peptide on markers of bone metabolism in MG-63 osteoblast-like cells co-cultured with THP-1 macrophage-like cells under virtually osteolytic conditions, BMC Musculoskelet Disord. 17 (2016) 199.
[38] T.H. Lin, T. Sato, K.R. Barcay, H. Waters, F. Loi, R. Zhang, J. Pajarinen, K. Egashira, Z. Yao, S.B. Goodman, NF-kappaB decoy oligodeoxynucleotide enhanced osteogenesis in mesenchymal stem cells exposed to polyethylene particle, Tissue Eng. Part A 21 (2015) 875–883.
[39] D.D. Dean, Z. Schwartz, Y. Liu, C.R. Blanchard, C.M. Agrawal, J.D. Mabrey, V.L. Sylvia, C.H. Lohmann, B.D. Boyan, The effect of ultra-high molecular weight polyethylene wear debris on MG63 osteosarcoma cells in vitro, J. Bone Joint Surg. Am. 81 (1999) 452–461.
[40] M.E. Martinez, S. Medina, M.T. del Campo, J.A. Garcia, A. Rodrigo, L. Munuera, Effect of polyethylene particles on human osteoblastic cell growth, Biomaterials 19 (1998) 183–187.
[41] R. Chiu, T. Ma, R.L. Smith, S.B. Goodman, Ultrahigh molecular weight polyethylene wear debris inhibits osteoprogenitor proliferation and differentiation in vitro, J. Biomed. Mater. Res. A 89 (2009) 242–247.
[42] C. Vermes, R. Chandrasekaran, J.J. Jacobs, J.O. Galante, K.A. Roebuck, Glant TT. The effects of particulate wear debris, cytokines, and growth factors on the functions of MG-63 osteoblasts, J. Bone Joint Surg. Am. 83-A (2001) 201–211.
[43] A.M. Rodrigo, M.E. Martinez, L. Saldana, G. Valles, P. Martinez, J.L. Gonzalez-Carrasco, J. Cordero, L. Munuera, Effects of polyethylene and alpha-alumina particles on IL-6 expression and secretion in primary cultures of human osteoblastic cells, Biomaterials 23 (2002) 901–908.
[44] D. Granchi, G. Ciapetti, I. Amato, S. Pagani, E. Cenni, L. Savarino, S. Avnet, J.L. Peris, A. Pellacani, N. Baldini, A. Giunti, The influence of alumina and ultra-high molecular weight polyethylene particles on osteoblast-osteoclast cooperation, Biomaterials 25 (2004) 4037–4045.
[45] M.E. Martinez, S. Medina, M.T. del Campo, M.J. Sanchez-Cabezudo, M. Sanchez, L. Munuera, Effect of polyethylene on osteocalcin, alkaline phosphatase and procollagen secretion by human osteoblastic cells, Calcif. Tissue Int. 62 (1998) 453–456.
[46] A.R. Wijenayaka, M. Kogawa, H.P. Lim, L.F. Bonewald, D.M. Findlay, G.J. Atkins, Sclerostin stimulates osteocyte support of osteoclast activity by a RANKL-dependent pathway, PLoS One 6 (2011) e25900.
[47] T. Nakashima, M. Hayashi, T. Fukunaga, K. Kurata, M. Oh-Hora, J.Q. Feng, L.F. Bonewald, T. Kodama, A. Wutz, E.F. Wagner, J.M. Penninger, H. Takayanagi, Evidence for osteocyte regulation of bone homeostasis through RANKL expression, Nat. Med. 17 (2011) 1231–1234.
[48] J. Xiong, M. Onal, R.L. Jilka, R.S. Weinstein, S.C. Manolagas, C.A. O'Brien, Matrix-embedded cells control osteoclast formation, Nat. Med. 17 (2011) 1235–1241.
[49] O.D. Kennedy, B.C. Herman, D.M. Laudier, R.J. Majeska, H.B. Sun, M.B. Schaffler, Activation of resorption in fatigue-loaded bone involves both apoptosis and active pro-osteoclastogenic signaling by distinct osteocyte populations, Bone 50 (2012) 1115–1122.
[50] L.I. Plotkin, A.R. Gortazar, H.M. Davis, K.W. Condon, H. Gabilondo, M. Maycas, M.R. Allen, T. Bellido, Inhibition of osteocyte apoptosis prevents the increase in osteocytic receptor activator of nuclear factor kappaB ligand (RANKL) but does not stop bone resorption or the loss of bone induced by unloading, J. Biol. Chem. 290 (2015) 18934–18942.
[51] L. Cardoso, B.C. Herman, O. Verborgt, D. Laudier, R.J. Majeska, M.B. Schaffler, Osteocyte apoptosis controls activation of intracortical resorption in response to bone fatigue, J. Bone Miner. Res. 24 (2009) 597–605.
[52] K.B. Emerton, B. Hu, A.A. Woo, A. Sinofsky, C. Hernandez, R.J. Majeska, K.J. Jepsen, M.B. Schaffler, Osteocyte apoptosis and control of bone resorption following ovariectomy in mice, Bone 46 (2010) 577–583.
[53] D. Baylink, J. Sipe, J. Wergedal, O.J. Whittemore, Vitamin D-enhanced osteocytic and osteoclastic bone resorption, Am. J. Physiol. 224 (1973) 1345–1357.
[54] E.M. Schwarz, A.P. Lu, J.J. Goater, E.B. Benz, G. Kollias, R.N. Rosier, J.E. Puzas, R.J. O'Keefe, Tumor necrosis factor-alpha/nuclear transcription factor-kappaB signaling in periprosthetic osteolysis, J. Orthopaedic Res.: Off. Publ. Orthopaedic Res. Soc. 18 (2000) 472–480.
[55] C.T. Wang, Y.T. Lin, B.L. Chiang, S.S. Lee, S.M. Hou, Over-expression of receptor activator of nuclear factor-kappaB ligand (RANKL), inflammatory cytokines, and chemokines in periprosthetic osteolysis of loosened total hip arthroplasty, Biomaterials 31 (2010) 77–82.
[56] K.D. Merkel, J.M. Erdmann, K.P. McHugh, Y. Abu-Amer, F.P. Ross, S.L. Teitelbaum, Tumor necrosis factor-alpha mediates orthopedic implant osteolysis, Am. J. Pathol. 154 (1999) 203–210.
[57] S. Murad, Toll-like receptor 4 in inflammation and angiogenesis: a double-edged sword, Front. Immunol. 5 (2014) 313.
[58] N.A. Athanasou, The pathobiology and pathology of aseptic implant failure, Bone Joint Res 5 (2016) 162–168.
[59] Y. Tamaki, Y. Takakubo, K. Goto, T. Hirayama, K. Sasaki, Y.T. Konttinen, S.B. Goodman, M. Takagi, Increased expression of toll-like receptors in aseptic loose periprosthetic tissues and septic synovial membranes around total hip implants, J. Rheumatol. 36 (2009) 598–608.
[60] Q. Gu, Q. Shi, H. Yang, The role of TLR and chemokine in wear particle-induced aseptic loosening, J. Biomed. Biotechnol. 2012 (2012) 596870.
[61] L.J. Kidd, N.R. Cowling, A.C. Wu, W.L. Kelly, M.R. Forwood, Selective and non-selective cyclooxygenase inhibitors delay stress fracture healing in the rat ulna, J. Orthopaedic Res.: Off. Publ. Orthopaedic Res. Soc. 31 (2013) 235–242.
[62] M. Haversath, I. Catelas, X. Li, T. Tassemeier, M. Jager, PGE(2) and BMP-2 in bone and cartilage metabolism: 2 intertwining pathways, Can. J. Physiol. Pharmacol. 90 (2012) 1434–1445.
[63] M. Hukkanen, S.A. Corbett, J. Batten, Y.T. Konttinen, I.D. McCarthy, J. Maclouf, S. Santavirta, S.P. Hughes, J.M. Polak, Aseptic loosening of total hip replacement. Macrophage expression of inducible nitric oxide synthase and cyclo-oxygenase-2, together with peroxynitrite formation, as a possible mechanism for early prosthesis failure, J. Bone Joint Surg. Br. 79 (1997) 467–474.
[64] S.V. Bukata, J. Gelinas, X. Wei, R.N. Rosier, J.E. Puzas, X. Zhang, E.M. Schwarz, X. Y. Song, D.E. Griswold, R.J. O'Keefe, PGE2 and IL-6 production by fibroblasts in response to titanium wear debris particles is mediated through a Cox-2 dependent pathway, J. Orthopaedic Res.: Off. Publ. Orthopaedic Res. Soc. 22 (2004) 6–12.
[65] D.C. Geng, X.S. Zhu, H.Q. Mao, B. Meng, L. Chen, H.L. Yang, Y.Z. Xu, Protection against titanium particle-induced osteoclastogenesis by cyclooxygenase-2 selective inhibitor, J. Biomed. Mater. Res. Part A 99 (2011) 516–522.
[66] Z. Mu, J. Tian, T. Wu, J. Yang, F. Pei, A systematic review of radiological outcomes of highly cross-linked polyethylene versus conventional polyethylene in total hip arthroplasty, Int. Orthop. 33 (2009) 599–604.
[67] S.M. Kurtz, H.A. Gawel, J.D. Patel, History and Systematic review of wear and osteolysis outcomes for first-generation highly crosslinked polyethylene, Clin. Orthop. Relat. Res. 469 (2011) 2262–2277.
[68] P.R. Kuzyk, M. Saccone, S. Sprague, N. Simunovic, M. Bhandari, E.H. Schemitsch, Cross-linked versus conventional polyethylene for total hip replacement: a meta-analysis of randomised controlled trials, J. Bone Joint Surgery Br. 93 (2011) 593–600.
[69] S.A. Callary, L.B. Solomon, O.T. Holubowycz, D.G. Campbell, Z. Munn, D.W. Howie, Wear of highly crosslinked polyethylene acetabular components, Acta Orthop. 86 (2015) 159–168.

Please cite this article as: R. T. Ormsby, L. B. Solomon, D. Yang et al., Osteocytes respond to particles of clinically-relevant conventional and cross-linked polyethylene and metal alloys by up-regulation of resorptive and inflammatory pathways, Acta Biomaterialia, https://doi.org/10.1016/j.actbio.2019.01.047

Chapter 5

The Effects of Vitamin E Analogues α-Tocopherol and γ-Tocotrienol on the Human Osteocyte Response to Ultra-high Molecular Weight Polyethylene Wear Particles

Manuscript submitted to the *Journal of Orthopaedic Science*, 2019

As discussed in the previous chapters, UHMWPE particles stimulate both inflammatory and resorptive responses. Recently developed acetabular liners include vitamin E, imbued within the UHMWPE chains, therefore, in this study we investigated the effects of vitamin E and UHMWPE particles on osteocytes *in vitro*. The two main analogues of vitamin E, α-Tocopherol and γ-Tocotrienol, have been described to have mixed effects on bone homeostasis. γ-Tocotrienol, in particular, has been shown inhibit bone resorption. There is only a limited amount of literature on the effects of vitamin E on osteocytes, and the *in vivo* consequences of vitamin E, either free or attached to wear particles, are unknown. Therefore, the aim of Chapter 5 was to investigate the effects of two analogues of Vitamin E, α-Tocopherol and γ-Tocotrienol, with the addition of conventional UHMWPE particles, on osteocytes *in vitro*. The findings are presented here in manuscript form.

Statement of Authorship

Title of Paper	The Effects Of Polyethylene Wear Particles And Vitamin E On Osteocytes In Vitro
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Ormsby R.T., Hosaka K., Evdokiou, A., Solomon L.B., Findlay, D.M., & Atkins, G.J. (2019) The Effects Of Polyethylene Wear Particles And Vitamin E On Osteocytes In Vitro

Principal Author

Name of Principal Author (Candidate)	Renee T Ormsby		
Contribution to the Paper	Performed analysis on all in vitro experiments, interpreted all data obtained and drafted the manuscript.		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	08/08/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Kunihiro Hosaka		
Contribution to the Paper	Generated data for the mineralisation assay and assisted with manuscript preparation		
Signature	,	Date	10/08/2018

Name of Co-Author	Andreas Evdokiou		
Contribution to the Paper	Provided intellectual input		
Signature		Date	17/08/2018

Name of Co-Author	Lucian B Solomon		
Contribution to the Paper	Provided intellectual input		
Signature		Date	16/08/2018

Name of Co-Author	David M Findlay		
Contribution to the Paper	Helped with data interpretation and editing of manuscript, provided intellectual input.		
Signature		Date	16/08/2018

Name of Co-Author	Gerald J Atkins		
Contribution to the Paper	Planned and supervised research work, helped with data interpretation and writing of manuscript. Senior author on paper.		
Signature		Date	29/10/2018

The Effects of Vitamin E Analogues α-Tocopherol and γ-Tocotrienol on the Human Osteocyte Response to Ultra-high Molecular Weight Polyethylene Wear Particles

Renee T Ormsby[1], Kunihiro Hosaka[1], Andreas Evdokiou[1], Lucian B. Solomon[1, 2], David M Findlay[1] and Gerald J Atkins[1].

[1]Biomedical Orthopaedic Research Group, Centre for Orthopaedic & Trauma Research, The University of Adelaide, Adelaide, SA 5000, Australia

[2]Orthopaedic and Trauma Service, Royal Adelaide Hospital, Adelaide, SA 5000, Australia

*Correspondence: Professor Gerald J. Atkins

The University of Adelaide, North Terrace, Adelaide, SA 5000, Australia

Telephone: +61 8 8313 7214; Facsimile: +61 8 8232 3065

E-mail: gerald.atkins@adelaide.edu.au

Running title: Vitamin E and wear particle disease

Abstract

Background

Polyethylene liners are a common bearing surface of orthopaedic prostheses. In some cases, vitamin E is added to polyethylene to prevent oxidative degradation. UHMWPE wear particles are important in the formation of periprosthetic osteolysis, a major cause of aseptic loosening. Osteocytes are an important controlling bone cell type and have been reported to respond to UHMWPE particles by inducing osteocytic osteolysis and osteoclastogenesis-related genes. Here, we examined the effects of vitamin E alone or in the context of wear particles on human osteocyte behaviour in a clinically relevant model.

Methods

Human primary osteoblasts isolated from patients undergoing hip arthroplasty for osteoarthritis were differentiated for 28d to an osteocyte-like stage. Cultures were exposed to UHMWPE wear particles with and without vitamin E analogues, α-Tocopherol and γ-Tocotrienol. Gene expression related to the oxidative response, osteocytic osteolysis and osteoclastogenesis was measured by real-time RT-PCR. Calcium release from mineralized cultures was also measured.

Results

Both vitamin E analogues upregulated expression of several antioxidant-related genes. UHMWPE particles also upregulated antioxidant gene expression, suggesting the induction of oxidative stress. UHMWPE particles upregulated the pro-osteoclastogenic *RANKL*:*OPG* mRNA ratio, however, this was reduced in the presence of vitamin E. UHMWPE particles also upregulated mRNA expression of osteocytic-osteolysis related genes. Treatment with either α-Tocopherol or γ-Tocotrienol alone and in combination

with UHMWPE particles also resulted in significant upregulation of these genes. Consistent with this, both vitamin E analogues promoted calcium release from mineralised cultures of osteocyte-like cells.

Discussion

Our findings suggest that Vitamin E may suppress osteocyte support of osteoclastogenesis in response to UHMWPE particle exposure. Vitamin E exerts an antioxidant effect on osteocytes, however, this appears to involve osteocytic osteolysis-related gene expression, which could contribute to the development of periprosthetic osteolysis. It will be important to further understand the effects of vitamin E additives to the cells of the prosthetic joint.

Word count: 300

Introduction

Implant loosening is a major problem associated with total hip replacement (THR). A main cause of aseptic loosening is the development of peri-prosthetic osteolytic lesions. Wear particles, in particular ultra-high molecular weight polyethylene (UHMWPE) wear particles, have been shown to be directly associated with the production of osteolytic lesions, where high volumetric wear rates correlate with the greatest progression of lesion volume [1]. UHMWPE wear particles are produced from the mechanical friction of the liner against the metal femoral head, exacerbated by the oxidative degradation of the polyethylene. UHMWPE wear particles have been shown to stimulate a variety of cellular pathways, including promoting bone resorption, inducing inflammation, and inhibiting bone formation [2], as well as inducing osteocyte peri-lacunocanalicular remodelling or osteocytic osteolysis [3].

The addition of vitamin E is a recent approach to improving the endurance of UHMWPE liners by preventing oxidation. The aim of imbuing UHMWPE liners with vitamin E is to reduce the formation of free radicals within the PE, as well as improving fatigue properties that would normally occur after post-irradiation melting, without sacrificing fatigue strength [4]. Vitamin E can be blended with UHMWPE powder prior to radiation, which protects against oxidation within the PE chain but reduces the crosslinking efficiency [5-7]. Alternatively, UHMWPE is diffused with vitamin E after radiation, circumventing possible changes to the crosslinking efficiency [8]. However, diffusion of vitamin E would result in relatively less vitamin E covalently bound within the PE chain compared to addition prior to radiation, potentially allowing unbound vitamin E to leach into the synovial fluid and surrounding tissues, including bone, *in vivo* [9].

Vitamin E, specifically analogues of Tocopherol and Tocotrienol, play potentially important roles in regulating bone resorption [10, 11]. Tocopherol has been shown to have varying effects on both bone formation and resorption, dependent on the dose and model system investigated [10]. For example, specific knockdown of the tocopherol transfer protein in mice, where vitamin E absorption is blocked, showed a significantly higher bone mass due to lower bone resorption and reduced osteoclast surface [12]. Tocotrienol has also been shown to reduce oxidative damage in osteoblasts, promoting osteoblast differentiation and survival [13]. Human epidemiological studies have also investigated the relationship between vitamin E and bone metabolism, with mixed results. One study, in a US population aged 50 and older, described a negative correlation between the high serum concentration of tocopherol and the femoral neck BMD [14]. Similarly, a study by Wolf *et al.* showed a negative correlation between total vitamin E intake and femoral neck BMD in an age-adjusted regression analysis of women in the USA [15]. However, a longitudinal study on Swedish subjects over the age of 50 and monitored for 19 years, has shown an association between low intake and serum concentration of α-tocopherol, and an increased rate of fracture [16].

Osteocytes are the most abundant cell in the bone, and are a key cell type in terms of controlling bone remodelling and maintaining skeletal integrity [17]. Osteocytes are capable of resorbing their perilacunar matrix by producing bone degrading enzymes, including matrix metallopeptidase 13 (*MMP13*) [18], Cathepsin K (*CTSK*) [19] and carbonic anhydrase II (*CA2*) [20]. We have previously shown that human osteocytes exposed to UHMWPE particles *in vitro* display both osteocytic osteolytic and pro-osteoclastogenic responses [3, 21]. Examination of human bone biopsies with confirmed periprosthetic osteolysis due to prosthetic wear particles also showed significantly

increased osteocyte lacunar area compared to particle-free primary THR biopsies [3, 21]. Little is known regarding the effects on osteocytes of soluble vitamin E alone, or in the presence of UHMWPE particles. Therefore in this study, we investigated the biological effects of two major analogues of vitamin E, α-Tocopherol and γ-Tocotrienol, in the presence or absence of UHMWPE particles, on human primary osteocyte-like cells *in vitro*.

Materials and Methods

Human osteocyte-like cells

Human primary osteoblasts were derived from trabecular bone biopsies, taken from four patients undergoing total hip replacements, and cultured *ex vivo*, as described previously [22]. Biopsies were obtained with informed consent and approved ethics by the Human Research Ethics Committees of the Royal Adelaide Hospital and the University of Adelaide. Human primary osteoblasts were cultured for 28 days until the cells reached a mature osteocyte-like stage, as described previously [3]. The cells were then treated for a further 7 days with either α-Tocopherol or γ-Tocotrienol (Sigma Aldrich, St Louis, MO, USA) at concentrations of 100 nM, 300 nM, 1 μM or untreated control.

Similarly, osteocyte-like cells after 28d of culture were overlaid with a collagen gel (Cellmatrix Type 1-A, Nitta, Tokyo, Japan) [3], containing UHMWPE particles (Ceridust 3615, Clariant Company) (100 μg/ml), with the addition of either vitamin E analogue, α-Tocopherol or γ-Tocotrienol (Sigma Aldrich, St Louis, MO, USA) (1 μM), or particle free and vitamin E vehicle controls, and cultured for a further 21 days. UHMWPE particles were confirmed to be endotoxin free and were sized from 0.3-10 μM. Vitamin

E (either α-Tocopherol or γ-Tocotrienol, 1 μM) was added to the culture medium every 3 days.

Gene Expression

Total RNA was extracted using TRIzol (Life Technologies, NY, USA) and complementary DNA was synthesised using iScript™ RT kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Real time RT-PCR was performed using SYBR Green Fluor qPCR Mastermix (Qiagen, Limburg, The Netherlands) on a CFX Connect (Bio-Rad, Hercules, CA, USA). Gene expression was normalised to *HPRT1*. Oligonucleotide sequences for *MMP13*, *CA2*, *CTSK*, *RANKL*, *OPG* and *M-CSF* are published [3]. Oligonucleotide sequences for *HPRT1*, *SOD1*, *SOD2* and *CAT* are listed in Table 1. All primers were designed in-house to be mRNA specific by at least one per pair flanking an intron-exon boundary, and all were purchased from Sigma Aldrich (St. Louis, MO, USA).

In vitro mineralisation

Calcium deposition was visualised using the Alizarin Red stain, as described previously [23]. Briefly, quadruplicate wells per treatment were washed with PBS and then fixed with 10% neutral buffered formalin for 1 hour at RT and then washed with PBS (3x). Each well was stained with aqueous 2% (w/v) Alizarin red solution (Sigma Aldrich) at pH 4.2, for 5 minutes at room temperature and then washed with distilled water to remove any unbound stain. Each well was imaged (Olympus SZ2-ILST, Tokyo, Japan) and then the bound stain was solubilised with 100 μl of 10% Cetylpyrridium chloride (CPC) (Sigma Aldrich) in 10 mM Sodium phosphate monobasic monohydrate (pH=7) added to

each well for 15 minutes at RT. The optical density was determined at 560 nm by spectrophotometry (Thermo Multiskan Ascent).

Results and Discussion

Effects of UHMWPE and Vitamin E on the oxidative stress response

In order to investigate the effects of vitamin E analogues and UHMWPE particles, we used a clinically relevant model where human primary osteoblasts isolated from THR implant recipients, the population at risk from developing periprosthetic osteolysis, were cultured for 28 days under pro-osteogenic conditions to generate osteocyte-like cultures [3]. Once established, the cells were overlaid with a type I collagen gel containing UHMWPE particles, with the addition of either α-Tocopherol or γ-Tocotrienol and cultured for a further 21 days.

Vitamin E has been identified as a bone supplement due to its anti-inflammatory and antioxidant activities. Therefore, we investigated the expression of key antioxidant genes, superoxide dismutases 1 and 2 (*SOD1* and *SOD2*), which are produced in response to reactive oxygen species (ROS) and act by binding to superoxide radicals, metabolising them to oxygen and hydrogen peroxide [24]. The expression of catalase (*CAT*) was also examined, which converts hydrogen peroxide into water and oxygen [24]. Osteocytes treated with either α-Tocopherol or γ-Tocotrienol significantly upregulated the expression of all three antioxidant genes (Fig. 1A-C). We observed a moderate upregulation of these genes with exposure to UHMWPE particles, suggesting an induction of oxidative stress. Importantly, both Vitamin E analogues were able to further increase the expression of these genes in the presence of UHMWPE particles, suggesting that they may confer protection from ROS in the UHMWPE -exposed cells. Previous studies have shown that

polyethylene liners and the resulting particles undergo oxidative degradation and can generate ROS [25, 26]. Therefore, upregulation of antioxidant genes could be a protective mechanism by the osteocyte in response to ROS produced by the UHMWPE particles, preventing DNA damage and potential cell death. Consistent with this protective mechanism, we have previously reported that UHMWPE particles do not induce apoptosis in human osteocyte-like cells [3].

Effects of UHMWPE and Vitamin E on the pro-osteoclastogenic response

We next investigated the expression of genes associated with osteoclastogenesis. We have previously shown that osteocytes respond to UHMWPE wear particles by upregulating the pro-osteoclastogenic *RANKL*:*OPG* mRNA ratio, as well as upregulating key osteoclastic regulatory genes, *M-CSF* and *IL-8* [3, 27]. Here, osteocytes responded to treatment with both α-Tocopherol and γ-Tocotrienol by downregulating the pro-osteoclastogenic *RANKL*:*OPG* mRNA ratio. In particular, treatment with γ-Tocotrienol suppressed *RANKL* and increased *OPG* mRNA expression in the primary human osteocytes, (Fig. 2A-B), whereas α-Tocopherol, whilst having non-significant effects on either gene alone, also resulted in reduced *RANKL*:*OPG* expression overall. The addition of α-Tocopherol had no effect on the UHMWPE response in terms of this ratio. However, γ-Tocotrienol significantly reduced the *RANKL*:*OPG* mRNA ratio in the presence of particles. UHMWPE particles also strongly upregulated *M-CSF* mRNA expression, which was effectively suppressed by either vitamin E analogue (Fig. 2D). Vitamin E analogues alone significantly downregulated *M-CSF* expression. Overall, these findings suggest that both α-Tocopherol and γ-Tocotrienol exert an anti-osteoclastogenic effect on human osteocyte-like cells, in the presence or absence of UHMWPE particles. Previous

studies have shown that mice treated with vitamin E, either α-Tocopherol or γ-Tocotrienol, have decreased bone resorption [11, 28]. Consistent with these results, a study by Bichara *et al.* [29] showed Vitamin E bound UHMWPE wear particles significantly decreased the amount of bone resorption in a mouse calvarial model of osteolysis when compared to conventional UHMWPE particles.

Effects of UHMWPE and Vitamin E on the osteocytic osteolysis response

Consistent with our previous study, UHMWPE particles induced expression of genes associated with osteocytic osteolysis, including *MMP13*, *CA2* and *CTSK* (Figure 3A-C) [3]. Similarly, UHMWPE particles increased the expression of *CA2* and *CTSK*, concurrent with the increase in antioxidant genes as described in Figure 1. Treatment with either Vitamin E analogue significantly upregulated the expression of all three genes, suggesting that Vitamin E could promote osteocytic osteolysis. Both α-Tocopherol and γ-Tocotrienol were able to further increase the expression of these genes in the presence of UHMWPE particles. As mentioned previously, UHMWPE particles are capable of producing ROS, which could be occurring in this study. ROS play a key role in osteoclastic differentiation and activity, and are involved in the resorptive process [30]. Therefore, the osteocytes in this study could be responding to ROS through one of two pathways. Firstly, the inhibition of ROS in human osteocytes by treatment with vitamin E analogues alone and in the presence of UHMWPE particles may have caused the increase in expression of genes associated with osteocytic osteolysis, *MMP13*, *CTSK* and *CA2*. Secondly, UHMWPE particles may stimulate the production of ROS by the osteocyte, inducing oxidative stress, triggering a perilacunar remodelling response. This effect would explain why there is a significant upregulation of antioxidant genes by the

osteocyte in response to UHMWPE. Therefore, removal of perilacunar bone could be one of the mechanisms used by osteocytes to relieve oxidative stress. Further investigation is required to determine if ROS produced by the osteocyte plays a role in regulating osteocytic osteolysis.

Effects of Vitamin E alone on osteocytic regulation of mineralisation

To investigate the effects of α-Tocopherol and γ-Tocotrienol on osteocytic regulation of mineralisation, osteocyte-like cultures were exposed to either analogue (0, 100 nM, 300 nM and 1 μM) in the absence of UHMWPE particles. Calcium deposition was measured using the Alizarin Red stain, as described previously [23]. Consistent with the observed regulation of osteocytic osteolysis related genes, treatment with either vitamin E analogue resulted in decreased mineralisation (Fig. 4). This is consistent with the upregulation with vitamin E treatment of genes associated with perilacunar remodelling, including *CA2*, which has been shown to be important during calcium release through osteocytic acidification of the bone matrix [20]. This suggests that vitamin E could contribute to the loss of bone that occurs during periprosthetic osteolysis specifically through promoting osteocytic removal of perilacunar bone.

Conclusion

This study demonstrates the potential effects of α-Tocopherol and γ-Tocotrienol on the regulation of bone resorption by the human osteocyte. Our data suggest that UHMWPE particles induce oxidative stress in osteocytes, which could be a potential trigger of perilacunar remodelling. Two distinct actions of the vitamin E analogues were observed: suppression of the pro-osteoclastic response, and stimulation of the osteocytic osteolysis

response. Thus, while our findings are consistent with vitamin E suppressing the osteoclastic response to UHMWPE particles, it could exacerbate an osteocytic osteolysis response, which could contribute to periprosthetic osteolysis. In conclusion, further investigation is required to determine if the lytic effects of vitamin E have potential consequences in the development of periprosthetic osteolysis and contribute to aseptic loosening.

Acknowledgements

The authors thank the nursing and surgical staff of the Orthopaedic Trauma Service at the Royal Adelaide Hospital for their help in collecting patient bone specimens. This work was supported by funding from the National Health and Medical Research Council of Australia (NHMRC) Project Grant Scheme (Grant ID 1041456) and the Royal Adelaide Hospital Special Purposes Fund. RTO was the recipient of a University of Adelaide post-graduate scholarship. GJA is a NHMRC Senior Research Fellow.

References

[1] Howie DW, Neale SD, Stamenkov R, McGee MA, Taylor DJ, Findlay DM. Progression of acetabular periprosthetic osteolytic lesions measured with computed tomography. The Journal of bone and joint surgery American volume. 2007;89:1818-25.

[2] Goodman S, Aspenberg P, Song Y, Regula D, Lidgren L. Polyethylene and titanium alloy particles reduce bone formation. Dose-dependence in bone harvest chamber experiments in rabbits. Acta orthopaedica Scandinavica. 1996;67:599-605.

[3] Ormsby RT, Cantley M, Kogawa M, Solomon LB, Haynes DR, Findlay DM, et al. Evidence that osteocyte perilacunar remodelling contributes to polyethylene wear particle induced osteolysis. Acta biomaterialia. 2016;33:242-51.

[4] Costa L, Luda MP, Trossarelli L, Brach del Prever EM, Crova M, Gallinaro P. Oxidation in orthopaedic UHMWPE sterilized by gamma-radiation and ethylene oxide. Biomaterials. 1998;19:659-68.

[5] Oral E, Beckos CG, Malhi AS, Muratoglu OK. THE EFFECTS OF HIGH DOSE IRRADIATION ON THE CROSS-LINKING OF VITAMIN E-BLENDED ULTRAHIGH MOLECULAR WEIGHT POLYETHYLENE. Biomaterials. 2008;29:3557-60.

[6] Oral E, Greenbaum ES, Malhi AS, Harris WH, Muratoglu OK. Characterization of irradiated blends of alpha-tocopherol and UHMWPE. Biomaterials. 2005;26:6657-63.

[7] Parth M, Aust N, Lederer K. Studies on the effect of electron beam radiation on the molecular structure of ultra-high molecular weight polyethylene under the influence of alpha-tocopherol with respect to its application in medical implants. Journal of materials science Materials in medicine. 2002;13:917-21.

[8] Oral E, Wannomae KK, Hawkins N, Harris WH, Muratoglu OK. Alpha-tocopherol-doped irradiated UHMWPE for high fatigue resistance and low wear. Biomaterials. 2004;25:5515-22.

[9] Jarrett BT, Cofske J, Rosenberg AE, Oral E, Muratoglu O, Malchau H. In vivo biological response to vitamin E and vitamin-E-doped polyethylene. The Journal of bone and joint surgery American volume. 2010;92:2672-81.

[10] Chin KY, Ima-Nirwana S. The effects of alpha-tocopherol on bone: a double-edged sword? Nutrients. 2014;6:1424-41.

[11] Chin KY, Ima-Nirwana S. The biological effects of tocotrienol on bone: a review on evidence from rodent models. Drug Des Devel Ther. 2015;9:2049-61.

[12] Fujita K, Iwasaki M, Ochi H, Fukuda T, Ma C, Miyamoto T, et al. Vitamin E decreases bone mass by stimulating osteoclast fusion. Nat Med. 2012;18:589-94.

[13] Fatokun AA, Stone TW, Smith RA. Responses of differentiated MC3T3-E1 osteoblast-like cells to reactive oxygen species. European journal of pharmacology. 2008;587:35-41.

[14] Zhang J, Hu X, Zhang J. Associations between serum vitamin E concentration and bone mineral density in the US elderly population. Osteoporosis Int. 2016:1-9.

[15] Wolf RL, Cauley JA, Pettinger M, Jackson R, Lacroix A, Leboff MS, et al. Lack of a relation between vitamin and mineral antioxidants and bone mineral density: results from the Women's Health Initiative. Am J Clin Nutr. 2005;82:581-8.

[16] Michaëlsson K, Wolk A, Byberg L, Ärnlöv J, Melhus H. Intake and serum concentrations of α-tocopherol in relation to fractures in elderly women and men: 2 cohort studies. The American Journal of Clinical Nutrition. 2014;99:107-14.

[17] Prideaux M, Findlay DM, Atkins GJ. Osteocytes: The master cells in bone remodelling. Curr Opin Pharmacol. 2016;28:24-30.

[18] Tang SY, Herber RP, Ho SP, Alliston T. Matrix metalloproteinase-13 is required for osteocytic perilacunar remodeling and maintains bone fracture resistance. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2012;27:1936-50.

[19] Qing H, Ardeshirpour L, Pajevic PD, Dusevich V, Jahn K, Kato S, et al. Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2012;In Press.

[20] Kogawa M, Wijenayaka AR, Ormsby RT, Thomas GP, Anderson PH, Bonewald LF, et al. Sclerostin Regulates Release of Bone Mineral by Osteocytes by Induction of Carbonic Anhydrase 2. J Bone Miner Res. 2013;28:2436-48.

[21] Ormsby RT, Solomon LB, Crotti TN, Haynes DR, Findlay DM, G.J. A. Osteocytes respond to clinically relevant UHMWPE and metal wear particles by up-regulation of resorptive and inflammatory pathways. Unpublished2018.

[22] Atkins GJ, Welldon KJ, Halbout P, Findlay DM. Strontium ranelate treatment of human primary osteoblasts promotes an osteocyte-like phenotype while eliciting an osteoprotegerin response. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2008.

[23] Kumarasinghe DD, Sullivan T, Kuliwaba JS, Fazzalari NL, Atkins GJ. Evidence for the dysregulated expression of TWIST1, TGFbeta1 and SMAD3 in differentiating osteoblasts from primary hip osteoarthritis patients. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2012;20:1357-66.

[24] Fattman CL, Schaefer LM, Oury TD. Extracellular superoxide dismutase in biology and medicine. Free Radic Biol Med. 2003;35:236-56.

[25] Premnath V, Harris WH, Jasty M, Merrill EW. Gamma sterilization of UHMWPE articular implants: an analysis of the oxidation problem. Ultra High Molecular Weight Poly Ethylene. Biomaterials. 1996;17:1741-53.

[26] Neuerburg C, Loer T, Mittlmeier L, Polan C, Farkas Z, Holdt LM, et al. Impact of vitamin E-blended UHMWPE wear particles on the osseous microenvironment in polyethylene particle-induced osteolysis. Int J Mol Med. 2016;38:1652-60.

[27] Atkins GJ, Welldon KJ, Holding CA, Haynes DR, Howie DW, Findlay DM. The induction of a catabolic phenotype in human primary osteoblasts and osteocytes by polyethylene particles. Biomaterials. 2009;30:3672-81.

[28] Abdul-Majeed S, Mohamed N, Soelaiman IN. Effects of tocotrienol and lovastatin combination on osteoblast and osteoclast activity in estrogen-deficient osteoporosis. Evid Based Complement Alternat Med. 2012;2012:960742.

[29] Bichara DA, Malchau E, Sillesen NH, Cakmak S, Nielsen GP, Muratoglu OK. Vitamin E-diffused highly cross-linked UHMWPE particles induce less osteolysis compared to highly cross-linked virgin UHMWPE particles in vivo. J Arthroplasty. 2014;29:232-7.

[30] Callaway DA, Jiang JX. Reactive oxygen species and oxidative stress in osteoclastogenesis, skeletal aging and bone diseases. Journal of bone and mineral metabolism. 2015;33:359-70.

Tables

Table 1: Sequences of sense (S) and anti-sense (AS)-specific oligonucleotide primers and predicted PCR product sizes. Primers are human sequence-specific.

Gene	Sequence (5′ – 3′)	Direction	Amplicon size
HPRT1	TGACCTTGATTTATTTTGCATACC CGAGCAAGACGTTCAGTCCT	Forward Reverse	102bp
SOD1	TGAAGAGAGGCATGTTGGAGAC TGGGCGATCCCAATTACACC	Forward Reverse	231bp
SOD2	GGCCTACGTGAACAACCTGA GCAACTCCCCTTTGGGTTCT	Forward Reverse	185bp
CAT	CCTGTGAACTGTCCCTACCG AATGCCCGCACCTGAGTAAC	Forward Reverse	218bp

Figures

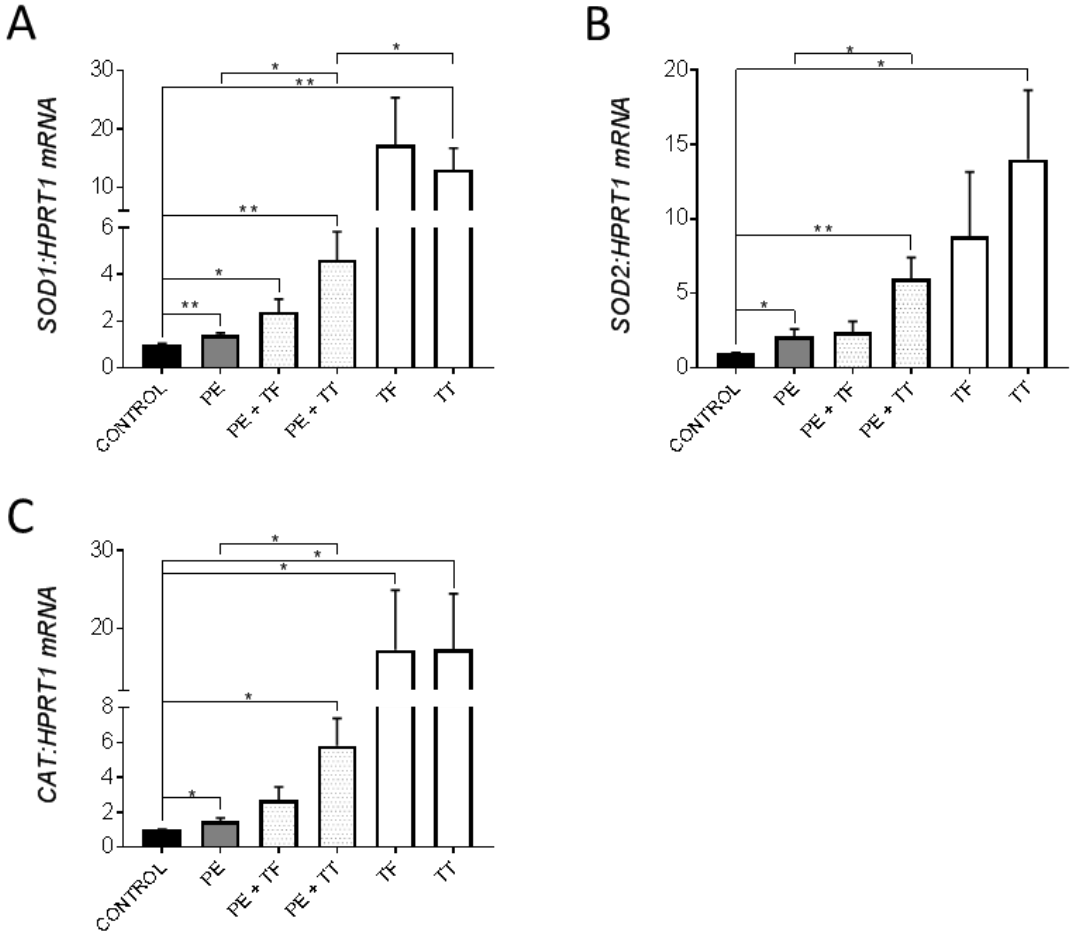


Figure 1: Human osteocyte-like cultures were overlaid with collagen gel containing UHMWPE particles and either α-Tocopherol (TF) or γ-Tocotrienol (TT), and cultured for a further 21 days. Relative gene expression was measured for key antioxidant genes *SOD1*, *SOD2* and Catalase (*CAT*) (A-C). Data shown are means ± standard error of the mean (SEM) of quadruplicate experiments analysed in duplicate. Significant differences are denoted by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

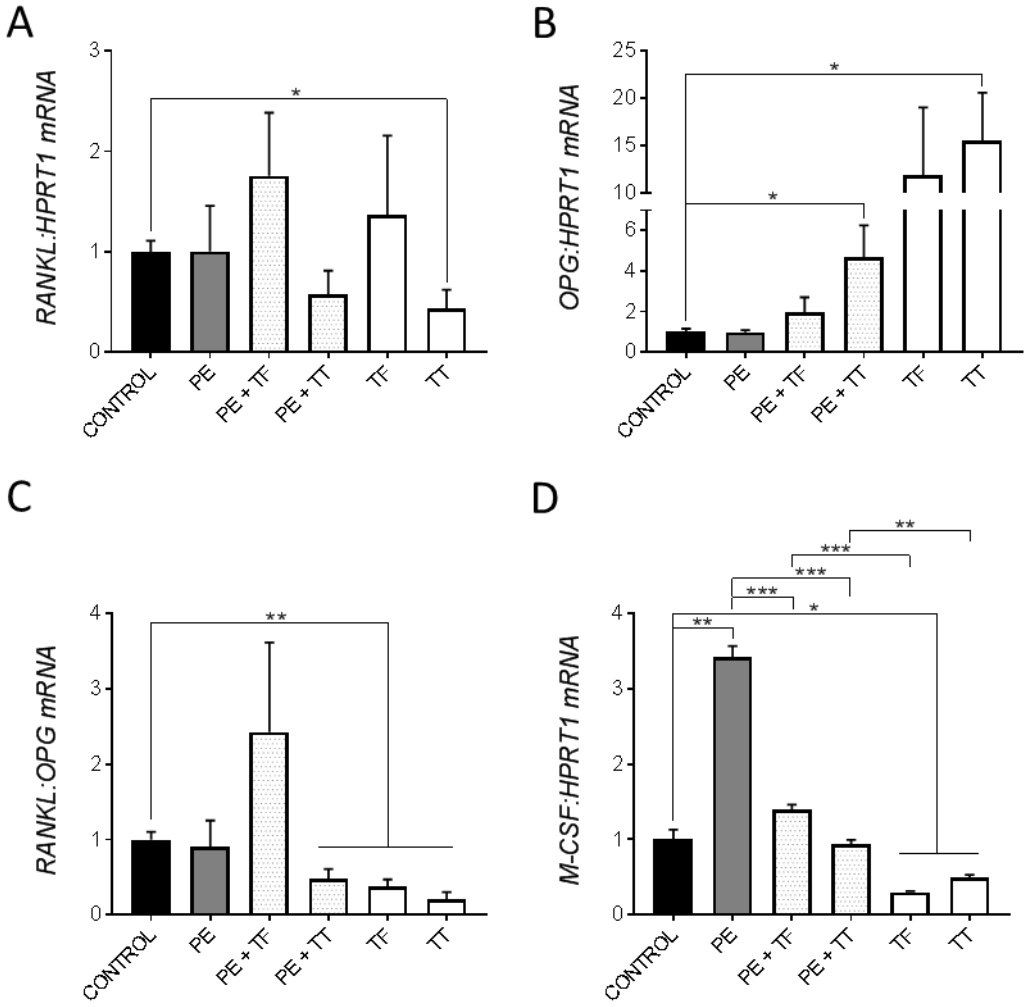


Figure 2: Relative gene expression was measured for genes associated with osteoclastogenesis: *RANKL*, *OPG*, the pro-osteoclastogenic *RANKL*:*OPG* mRNA ratio and *M-CSF* mRNA expression (A-D). Data shown are means ± standard error of the mean (SEM) of quadruplicate experiments analysed in duplicate. Significant differences are denoted by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

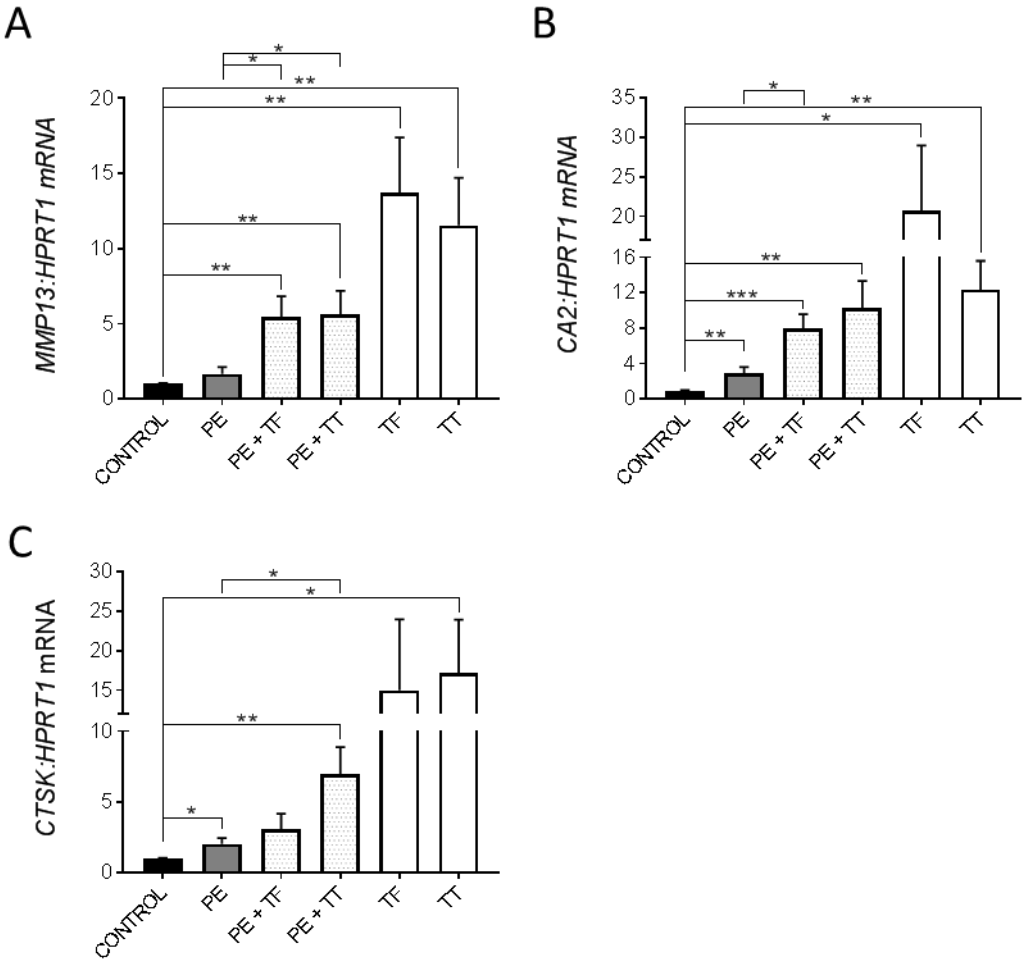


Figure 3: Relative gene expression of genes associated with osteocytic osteolysis: A. *MMP13* mRNA expression, B. *CA2* mRNA expression, C. *CTSK* mRNA expression. Data shown are means ± standard error of the mean (SEM) of quadruplicate experiments analysed in duplicate. Significant differences are denoted by *p < 0.05, **p < 0.01, ***p < 0.001.

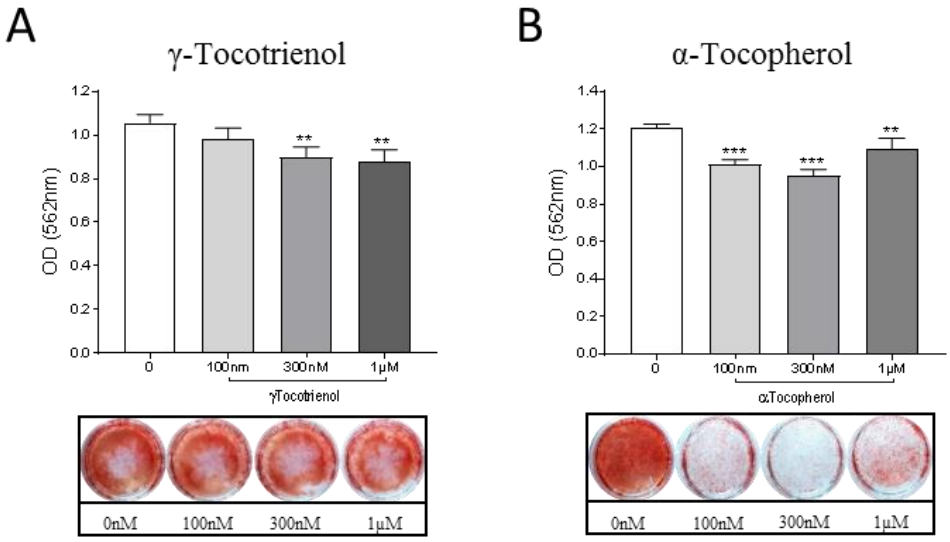


Figure 4: Human osteocyte-like cultures were treated with either α-Tocopherol (TF) or γ-Tocotrienol (100 nM, 300 nM, 1 μM) or untreated (0) for a further 6 days. Cells were then stained with Alizarin Red and quantified using Cetylpyrridium chloride to elute the bound stain. Absorbance was read at 560 nM by spectrophotometry, data shown are means ± standard deviation of quadruplicate wells. Significant differences are denoted by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Chapter 6

Evidence for gender-specific pathological bone loss mechanisms in the development of periprosthetic osteolysis

Manuscript

Work presented in Chapter 6 aimed to investigate the effects of wear particles on osteocytes *in vivo* in biopsies from patients who have undergone a revised total hip replacement due to osteolysis. As presented in Chapter 2, bone biopsies were obtained from both primary and revision THR patients, and bone parameters were analysed. Additional bone biopsies were then obtained from a larger cohort of both primary and revision THR patients, increasing the patient number from 8 to 20 per group. Revision THR biopsies showed radiographic evidence of osteolysis and biopsies from the region of osteolysis showed the presence of wear particles as detected by histological analysis. Bone parameters showed the enlargement of the osteocyte lacunar area in response to wear particles in the revision THR biopsies, as well as gender specific responses that have been previously undescribed. The findings are presented here in the form of a manuscript to be submitted to the Journal of Orthopaedic Research.

Statement of Authorship

Title of Paper	Evidence for gender-specific pathological bone loss mechanisms in the development of periprosthetic osteolysis
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Ormsby R.T., Solomon, L.B., Findlay, D.M., & Atkins, G.J. (2018) Evidence for gender-specific pathological bone loss mechanisms in the development of periprosthetic osteolysis.

Principal Author

Name of Principal Author (Candidate)	Renee T Ormsby		
Contribution to the Paper	First author on the paper. Generated data and performed analysis, interpreted all data obtained and drafted the manuscript.		
Overall percentage (%)	90		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	26/10/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Lucian B Solomon		
Contribution to the Paper	Provided intellectual input		
Signature		Date	16/08/2018

Name of Co-Author	David M Findlay		
Contribution to the Paper	Helped with data interpretation and editing of manuscript, provided intellectual input.		
Signature		Date	16/8/2018
Name of Co-Author	Gerald J Atkins		
Contribution to the Paper	Planned and supervised research work, helped with data interpretation and writing of manuscript. Senior author on paper.		
Signature		Date	29/10/2018

Evidence for gender-specific bone loss mechanisms in periprosthetic osteolysis

Renee T Ormsby[1], Lucian B Solomon[2], David M Findlay[2] and Gerald J Atkins[1*]

[1]Biomedical Orthopaedic Research Group, Centre for Orthopaedic & Trauma Research, The University of Adelaide, Adelaide, SA 5000, Australia

[2]Centre for Orthopaedic & Trauma Research, The University of Adelaide, Adelaide, SA 5000, Australia

*Correspondence: Professor Gerald J Atkins

The University of Adelaide, North Terrace, Adelaide, SA 5000, Australia

Telephone: +61 8 8313 7214

E-mail: gerald.atkins@adelaide.edu.au

Running title: Osteocytic osteolysis in aseptic loosening

Keywords: wear particles; polyethylene; UHMWPE; aseptic loosening; periprosthetic osteolysis; total hip replacement; osteocyte

Abstract

The development of osteolysis adjacent to total hip replacement (THR) prostheses is a major cause of their eventual failure. Periprosthetic osteolysis is associated with the production of bioactive particles, produced by wear of articulating prosthesis surfaces. Wear particles invade the peri-prosthetic tissue, inducing inflammation and bone resorption. Previous studies have shown that osteocytes, the most numerous cell type in the bone, can respond to wear particles of ultra-high molecular weight polyethylene (UHMWPE). Osteocytes play important roles in regulating both osteoclastic bone resorption and directly through perilacunar remodeling or osteocytic osteolysis. In this study, we performed histological analysis of bone biopsies obtained from cohorts of male and female patients undergoing either primary THR surgery, or revision THR surgery for aseptic loosening with radiographic evidence of osteolysis. Mean osteocyte lacunar area (Lac.Ar) was significantly larger in bone adjacent to THR prostheses in revision biopsies, compared to bone from similar sites in primary THR. The percentage lacunar area/bone area (% Lac.Ar/B.Ar) was also significantly larger in the revision THR biopsies. Analysis by patient gender showed significantly larger Lac.Ar in the female revision bone compared to female primary bone. In contrast, there was no significant difference in osteocyte lacunar size between the revision and primary groups in male samples. However, male revision THR biopsies displayed increased mean % Lac.Ar/B.Ar, suggesting increased osteoclastic resorptive activity at the bone surface. This parameter was unchanged between the female cohorts. These findings suggest a previously unrecognised gender-specific response in orthopaedic wear particle-induced osteolysis, and perhaps other types of pathological bone loss.

Introduction

Osteoarthritis is a common joint disorder that leads to total hip replacement (THR) surgery when non-operative treatments fail. THR alleviates pain and restores mobility to the joint, however, failure of the implant can occur due to loosening [1]. Aseptic osteolytic lesions have been identified as a main cause of loosening in implants [2, 3]. Osteolytic lesions are visualised as radiolucent areas within the bone architecture, more commonly seen in the cancellous bone above the acetabular component of an implant [4]. The lesions become devoid of bone and infiltrated with granulomatous tissue, that contain multiple cell types including macrophages, fibroblasts, osteoclasts and inflammatory cells [5].

The production of bioactive wear particles has been identified as a main cause of osteolysis [6-10]. In particular, small ultra-high molecular weight polyethylene (UHMWPE) and metal particles, which are formed by abrasive wear of bearing surfaces and fretting of modular components, have been shown to infiltrate the peri-prosthetic tissue [11]. These particles are highly bioactive and cause adverse tissue reactions [12]. Both metal and UHMWPE particles have been shown to induce biological reactions, including promoting both bone resorption and inflammatory pathways, whilst inhibiting bone formation [13-17] [13, 14, 18, 19].

Analysis of bone biopsies from patients, who have undergone revision surgery, has led to the identification of key changes in the bone environment caused by wear particles, including the upregulation of key inflammatory genes and osteoclastic markers, as well as the detection of wear particles within the mineralised bone surrounding implants [20]. We have previously identified that osteocytes also respond to wear particles, and data are emerging that these cells play active roles in periprosthetic osteolysis [21, 22]. Osteocytes are the most abundant cell type in the bone, where they are embedded in the bone matrix

and connected by a lacunocanalicular network via dendritic processes that extend between neighbouring osteocytes and into the bone marrow. Through this network, osteocytes can sense mechanical strain and chemical signals, as well as regulate both bone formation and resorption [23, 24], by sending key factors to both surface osteoclasts and bone lining cells [24]. In females, the osteocyte plays a key role to release calcium from the bone matrix during lactation, through the process of osteocytic osteolysis, also known as perilacunar remodelling. This occurs through the osteocytic production of key bone degrading enzymes, including matrix metalloproteinases (MMP's) [25], cathepsin K [26] and carbonic anhydrase II [27], which degrade both the organic and inorganic components of the bone mineral matrix, increasing the lacunar area and releasing calcium into the blood stream [28]. This process may also be regulated by calcitonin, traditionally thought to be a regulator of osteoclastic bone resorption [29].

In a previous study, we showed histological evidence of osteocytic osteolysis with increased osteocyte lacunar area in a case series of patients undergoing revision THR surgery for aseptic loosening with confirmed radiographic evidence of periprosthetic osteolysis [22]. In the current study, we examined osteocyte histology in a larger cohort of patients, undergoing revision for aseptic loosening, with confirmed radiographic evidence of periprosthetic osteolysis, and compared it to a gender and age-matched cohort of patients undergoing primary THR. We confirm here that osteocytic osteolysis is a feature of periprosthetic osteolysis, however this process appears to be restricted to females, suggesting for the first time that cellular mechanisms of pathological bone loss may be gender-specific.

Materials and Methods

Ethical Statement

Patients were recruited into this study with informed written consent and with ethics approval by the Human Research Ethics Committees of the Royal Adelaide Hospital and the University of Adelaide (RAH Approval No. 130114 and 140216a).

Patient demographics

Patients undergoing either primary THR for osteoarthritis or revision THR for aseptic loosening associated with radiographic evidence of periprosthetic osteolysis, were recruited at the Royal Adelaide Hospital. Pre-operative CT scans were obtained for 20 patients where osteolytic lesions were confirmed adjacent to the acetabular component of the prosthesis. Patient demographics are shown in Table 1, due to the advanced age of a revision THR patient, the primary THR patients were specifically included to match the age range, eliminating any significant difference between the cohort ages. Patient number also included comparable numbers of males and females. Revision THR patient implant details, shown in Table 2, show a varying selection of implant manufacturer and metal alloy type. Majority of the patient prostheses contained a conventional UHMWPE liner, however prosthetic details for four patients are unknown.

Osteocyte lacunar measurements from human bone biopsies

Intraoperative trephine biopsies were taken from the peri-acetabular bone prior to reaming for insertion of the acetabular component, in the patients undergoing primary THR, and after removal of the failed implant and the granulomatous tissue, in the patients

undergoing revision THR. The biopsies were fixed in 10% neutral buffered formalin for 48 hours and then decalcified (10% EDTA/1% paraformaldehyde) for 2 weeks. Sections (5 μm) were cut and stained with toluidine blue, as described previously [22], and imaged using NanoZoomer (Hamamatsu Photonics, Shizuoka, Japan) at 20x magnification. Osteocyte lacunar area (Lac.Ar) was quantified using Image J software (U. S. National Institutes of Health, Bethesda, MD, USA) by manual tracing around osteocyte lacunar areas and bone areas for each section, using a Bamboo Pen and Touch (Wacom, Kazo, Saitama, Japan) [22].

Statistical Analysis

Statistical differences between the osteocyte parameters, including the osteocyte number per bone area (N.Ot/B.Ar), average Lac.Ar, percentage lacunar area per total bone area (Lac.Ar/B.Ar (%)) and the top 20% Lac.Ar, were assessed using Student's t-tests (GraphPad v7 v0.2). Significant differences were accepted for p values < 0.05.

Results

Radiographic evidence of osteolysis in patients undergoing revision THR

Pre-operative CT scans were obtained for all 20 revision THR patients analysed. Figure 1 includes representative CT images showing examples of osteolytic zones in 3 individual patients, and a patient where the acetabular cup has collapsed (Fig 1D).

Analysis of human bone biopsies from patients undergoing Primary THR and Revision THR surgery

Representative images of bone morphometry of primary THR and revision THR biopsies are shown in figures 2A-C and 2D-F, respectively. Histological analysis of the bone biopsies showed metal wear particles evident within granulomatous tissue adjacent to the bone (Fig. 2D). Metal wear particles and UHMWPE particles were observed by microscopy in 15 of the 20 biopsies taken from the patients in the revision cohort, in 5 of patient biopsies both metal and UHMWPE particles were detected. Metal particles were additionally identified in Haversian canals (Fig. 2E). Evidence of osteocyte lacunae coalescence was also observed (Fig. 2F).

Osteocyte lacunar assessments of each primary and revision THR patient biopsy

Osteocyte lacunar area measurements (Lac.Ar) for each patient are shown in Figure 3. These are separate into the individual male patients, in either the primary or revision cohort (Fig. 3A-B), and the individual females within either the primary or revision cohort (Fig. 3C-D). The patient's corresponding age is shown on the x axis.

Osteocyte characteristics of primary and revision THR cohorts

We quantified the percentage of osteocytes per total bone area and found no significant difference between the primary and revision THR cohorts irrespective of gender (Fig. 4A). However, there was a significant increase in the mean Lac.Ar in the revision compared to the primary THR biopsies (Fig. 4B). Due to the 3D ellipsoid shape of an osteocyte lacunae [30], the 20% largest osteocyte lacunar area (Top 20% Lac.Ar) were considered, which correspond to the mid-cross sectional lacunae in a tissue section [31]. This measure was also significantly increased in the revision biopsies compared to the primary THR biopsies (Fig. 4C). The percentage osteocyte lacunar area per total bone

area was also significantly increased in the biopsies from revision THR (Fig. 4D). Examination of osteocyte lacunar size distribution revealed a greater number of large lacunae (60-300 μm) in biopsies from the revision THR group compared to the primary THR group (Fig. 4E).

Osteocyte characteristics of Female THR and Male THR bone biopsies

Further analysis was conducted based on patient gender. There was no difference between groups in terms of number of osteocyte per bone area (Fig. 5A). Analysis of the average lacunar area revealed significantly larger lacunae in the female revision cohort, compared to female primary THR biopsies (Fig. 5B). Similarly, the Top 20% lacunar area measure was significantly larger in the female revision THR biopsies compared to the female primary THR samples (Fig. 5C). In contrast, the percentage lacunar area per total bone area was significantly larger in the male revision THR biopsies compared to the male primary THR biopsies (Fig. 5D), an effect not seen in the female THR groups.

Discussion

There is an emerging role for osteocytes in the development of periprosthetic osteolysis [22, 32]. In this study, we analysed bone biopsies taken from sites adjacent to osteolysis in patients undergoing revision THR for aseptic loosening. Microscopic examination of bone biopsies confirmed the presence of visible particulate material in 15/20 revision THR patients examined, linking the production of wear particles and the development of osteolysis in these patients [6-10]. Histological analysis revealed significantly increased osteocyte lacunar size in the revision THR bone combining both male and female biopsies, compared to those in cases of primary THR, consistent with our previous report

[22]. Additionally, there was an overall substantial increase in the % lacunar area per total bone area in the revision THR biopsies compared to primary samples. Furthermore, we observed evidence of lacunae enlargement to the extent of coalescence, implying that osteocytic osteolysis can contribute to the macroscopic lesions characteristic of this pathology. Multiple particle types have been shown to induce the expression of osteocytic osteolytic mediators [22, 33]. Therefore, these findings further support a key role for osteocytic bone resorption in wear particle disease.

The revised patient prostheses within this cohort consisted of a variety of implant types with varying alloys, including Ti6Al4V and Vitallium (CoCrMo). Additionally, every prosthesis included a conventional UHMWPE liner. Investigation of bone biopsies obtained from this cohort showed histological evidence of metal wear particle invasion into the bone in 9/20 cases, identified primarily in the patients with a Cobalt Chrome alloy implant. Furthermore, histological analysis under polarised light also showed the presence of birefringent UHMWPE particles in 11/20 biopsies, as previously reported [22]. Interestingly, five of the 20 patients' biopsies showed evidence of both metal and UHMWPE particles. Metal particles were visible in the Haversian canals, as well as in the granulomatous surrounding the bone. The close proximity of wear particles to bone likely enables direct contact with osteocytes, causing the resulting biological responses. We have previously reported that human osteocyte-like cells were capable of physically attaching and engulfing UHMWPE particles [21]. More recently, it was demonstrated that human osteocytes internalise viable *Staphylococcus aureus* bacteria [34], which at 500-700 nm are similar in size to bioactive wear particles.

A significant finding of this study is that the osteocyte lacunae in female revision THR biopsies were significantly larger than those in the female primary THR cohort. This difference in lacunar size was not observed in the corresponding male samples,

suggesting that the female osteocyte response to particles may be preferentially directed towards osteocytic osteolysis. In comparison, the % lacunar area as a function of total bone area was significantly larger in the male revision THR biopsies compared to male primary THR, suggesting loss of bone due to increased surface bone resorption by osteoclasts in males.

The reasons for the difference in osteocyte responses between the genders are not clear. However, as the women in this cohort are all postmenopausal, this suggests that hormonal changes associated with post-menopause may play a role. A major physiological role of osteocytic osteolysis is the release of calcium from the perilacunar matrix during lactation, as previously described in mice [35]. It is possible that this process is linked to the action of the pituitary hormone prolactin, which together with the decrease in oestrogen levels, is known to be essential for the onset of lactation [36]. Additionally, it has been shown that prolactin receptors have been identified in osteoblasts, and activation of these receptors inhibits osteoblast activity and has also been shown to induce the expression of RANKL relative to its inhibitor OPG [37]. Therefore, it seems plausible that changes in prolactin levels may stimulate osteocytic responses that have not been previously described. It has also been shown that increased levels of other pituitary hormones play important roles in regulating bone mass, including follicle stimulating hormone, which has been shown to regulate osteoclastic bone resorption in mice and, importantly, correlates with increased markers of bone resorption in peri-menopausal women [38]. Thus, the hormonal changes that occur during menopause may predispose female osteocytes to induce perilacunar remodelling in general, contributing to the loss of bone [39]. In the specific case of periprosthetic osteolysis, these hormones may also interact with the effects of particles, by an unknown mechanism, to produce a catabolic phenotype in osteocytes.

There are a few limitations to this study. The patients recruited are from two separate cohorts, primary THR and revision THR for osteolysis, and the changes in osteocyte measures may have been pre-existing. Alternatively, the bone status of these two groups may have been affected by the surgical implantation of a prostheses into the joint space, specifically in the revision THR group. During the procedure, surgical reaming of the bone occurs, removing the cortical shell and exposing the underlying trabecular bone. This alone may lead to inflammatory effects that could affect osteocyte lacunar area and porosity, however there are no data published regarding this. However, we found a clear difference between males and females suggesting these possibilities are unlikely. This study is also cross-sectional; a longitudinal study would be preferable, however the unpredictable and long-term nature of this pathology and the increased use of wear-resistant materials, such as XLPE, would make this difficult. Additionally, this study is limited by the lack of "healthy" controls available for comparison. Despite this, biopsies obtained from both cohorts in this study showed no change in osteocyte lacunar occupancy number (data not shown) from viable tissue, and thus healthy, although osteoarthritic bone. Furthermore, we have not identified a basis for the gender-specific effect. Patient weight, BMI and drug status were not included this study, and underlying health issues including diabetes [40], a history of smoking [41], and obesity [42] have all been shown to affect bone health, and thus could potentially contribute to the changes observed. A larger cohort of patients with sufficient statistical power to allow multivariate analysis of all potentially contributing factors would help elucidate the influence of gender on the possible mechanisms of bone loss. Another potential confounder of this and future studies is the wide variety of hip implants and materials used, as evident in Table 2, and therefore the multiple possible types and combinations of wear particles

released. A larger cohort of patients would potentially provide further insight into which particles and combinations of particles are most clinically relevant in this context.

Conclusion

In conclusion, this study has highlighted the different modes of bone resorption in either male or female patients with confirmed aseptic loosening associated with periprosthetic osteolysis. Wear particle associated bone loss consists of both osteoclastic and osteocytic bone resorption, with osteocytes playing key roles in both pathways. However, our current findings suggest that the latter process may be specific to females. It is conceivable that female osteocytes are pre-programmed to undergo osteocytic osteolysis under conditions of rapid bone loss, or that the hormonal status of women through both pregnancy/lactation and post-menopause drives this process. However, further investigation is required to elucidate gender specificity of bone loss and the conditions under which this occurs. In summary, osteocytes may contribute to the development of osteolysis through stimulation of resorptive pathways in response to wear particles, contributing to the aseptic loosening of implants, in a gender-specific manner.

Acknowledgements

The authors would like to thank the nursing and surgical staff of the Orthopaedic and Trauma Service at the Royal Adelaide Hospital for their help in collecting patient bone specimens. This work was supported by funding from the National Health and Medical Research Council of Australia (NHMRC) Project Grant Scheme (Grant ID 1041456) and the Royal Adelaide Hospital Special Purposes Fund. GJA is a NHMRC Senior Research Fellow.

References

[1] Australian Orthopaedic Association National Joint Replacement Registry Annual Report 2017. https://aoanjrrsahmricom/annual-reports-2017. 2017.

[2] Abu-Amer Y, Darwech I, Clohisy JC. Aseptic loosening of total joint replacements: mechanisms underlying osteolysis and potential therapies. Arthritis research & therapy. 2007;9 Suppl 1:S6.

[3] Jacobs JJ, Roebuck KA, Archibeck M, Hallab NJ, Glant TT. Osteolysis: basic science. Clinical orthopaedics and related research. 2001:71-7.

[4] Dattani R. Femoral osteolysis following total hip replacement. Postgrad Med J. 2007;83:312-6.

[5] Goodman SB, Huie P, Song Y, Schurman D, Maloney W, Woolson S, et al. Cellular profile and cytokine production at prosthetic interfaces - Study of tissues retrieved from revised hip and knee replacements. Journal of Bone and Joint Surgery-British Volume. 1998;80b:531-9.

[6] Total Hip Replacement. NIH Consensus Statement. 1994;12.

[7] Bitar D, Parvizi J. Biological response to prosthetic debris. World journal of orthopedics. 2015;6:172-89.

[8] Magone K, Luckenbill D, Goswami T. Metal ions as inflammatory initiators of osteolysis. Archives of orthopaedic and trauma surgery. 2015;135:683-95.

[9] Shanbhag AS, Jacobs JJ, Glant TT, Gilbert JL, Black J, Galante JO. Composition and morphology of wear debris in failed uncemented total hip replacement. The Journal of bone and joint surgery British volume. 1994;76:60-7.

[10] Rokkum M, Brandt M, Bye K, Hetland KR, Waage S, Reigstad A. Polyethylene wear, osteolysis and acetabular loosening with an HA-coated hip prosthesis. A follow-up

of 94 consecutive arthroplasties. The Journal of bone and joint surgery British volume. 1999;81:582-9.

[11] Chang JD, Lee SS, Hur M, Seo EM, Chung YK, Lee CJ. Revision total hip arthroplasty in hip joints with metallosis: a single-center experience with 31 cases. The Journal of arthroplasty. 2005;20:568-73.

[12] Grosse S, Haugland HK, Lilleng P, Ellison P, Hallan G, Hol PJ. Wear particles and ions from cemented and uncemented titanium-based hip prostheses-A histological and chemical analysis of retrieval material. Journal of biomedical materials research Part B, Applied biomaterials. 2015;103:709-17.

[13] Haynes DR, Boyle SJ, Rogers SD, Howie DW, Vernon-Roberts B. Variation in cytokines induced by particles from different prosthetic materials. Clinical orthopaedics and related research. 1998:223-30.

[14] Revell PA, Weightman B, Freeman MA, Roberts BV. The production and biology of polyethylene wear debris. Archives of orthopaedic and trauma surgery. 1978;91:167-81.

[15] Goodman SB, Davidson JA, Song Y, Martial N, Fornasier VL. Histomorphological reaction of bone to different concentrations of phagocytosable particles of high-density polyethylene and Ti-6Al-4V alloy in vivo. Biomaterials. 1996;17:1943-7.

[16] Chiu R, Ma T, Smith RL, Goodman SB. Ultrahigh molecular weight polyethylene wear debris inhibits osteoprogenitor proliferation and differentiation in vitro. Journal of biomedical materials research Part A. 2008.

[17] Lohmann CH, Schwartz Z, Koster G, Jahn U, Buchhorn GH, MacDougall MJ, et al. Phagocytosis of wear debris by osteoblasts affects differentiation and local factor production in a manner dependent on particle composition. Biomaterials. 2000;21:551-61.

[18] Maloney WJ, Smith RL. Periprosthetic osteolysis in total hip arthroplasty: the role of particulate wear debris. Instructional course lectures. 1996;45:171-82.

[19] Kadoya Y, Kobayashi A, Ohashi H. Wear and osteolysis in total joint replacements. Acta orthopaedica Scandinavica Supplementum. 1998;278:1-16.

[20] Busse B, Hahn M, Niecke M, Jobke B, Puschel K, Delling G, et al. Allocation of nonbirefringent wear debris: darkfield illumination associated with PIXE microanalysis reveals cobalt deposition in mineralized bone matrix adjacent to CoCr implants. Journal of biomedical materials research Part A. 2008;87:536-45.

[21] Atkins GJ, Welldon KJ, Holding CA, Haynes DR, Howie DW, Findlay DM. The induction of a catabolic phenotype in human primary osteoblasts and osteocytes by polyethylene particles. Biomaterials. 2009;30:3672-81.

[22] Ormsby RT, Cantley M, Kogawa M, Solomon LB, Haynes DR, Findlay DM, et al. Evidence that osteocyte perilacunar remodelling contributes to polyethylene wear particle induced osteolysis. Acta biomaterialia. 2016;33:242-51.

[23] Prideaux M, Findlay DM, Atkins GJ. Osteocytes: The master cells in bone remodelling. Curr Opin Pharmacol. 2016;28:24-30.

[24] Bonewald LF. The amazing osteocyte. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2011;26:229-38.

[25] Tang SY, Herber RP, Ho SP, Alliston T. Matrix metalloproteinase-13 is required for osteocytic perilacunar remodeling and maintains bone fracture resistance. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2012;27:1936-50.

[26] Qing H, Ardeshirpour L, Pajevic PD, Dusevich V, Jahn K, Kato S, et al. Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation.

Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2012;In Press.

[27] Kogawa M, Wijenayaka AR, Ormsby RT, Thomas GP, Anderson PH, Bonewald LF, et al. Sclerostin Regulates Release of Bone Mineral by Osteocytes by Induction of Carbonic Anhydrase 2. J Bone Miner Res. 2013;28:2436-48.

[28] Qing H, Bonewald LF. Osteocyte Remodeling of the Perilacunar and Pericanalicular Matrix. International Journal of Oral Science. 2009;1:59-65.

[29] Clarke MV, Russell PK, Findlay DM, Sastra S, Anderson PH, Skinner JP, et al. A Role for the Calcitonin Receptor to Limit Bone Loss During Lactation in Female Mice by Inhibiting Osteocytic Osteolysis. Endocrinology. 2015;156:3203-14.

[30] Marotti G. Osteocyte Orientation in Human Lamellar Bone and Its Relevance to the Morphometry of Periosteocytic Lacunae. Metab Bone Dis Relat. 1979;1:325-33.

[31] Lane NE, Yao W, Balooch M, Nalla RK, Balooch G, Habelitz S, et al. Glucocorticoid-treated mice have localized changes in trabecular bone material properties and osteocyte lacunar size that are not observed in placebo-treated or estrogen-deficient mice. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2006;21:466-76.

[32] Atkins GJ, Haynes DR, Howie DW, Findlay DM. Role of polyethylene particles in peri-prosthetic osteolysis: A review. World J Orthop. 2011;2:93-101.

[33] Ormsby RT, Solomon LB, Crotti TN, Haynes DR, Findlay DM, G.J. A. Osteocytes respond to clinically relevant UHMWPE and metal wear particles by up-regulation of resorptive and inflammatory pathways. Unpublished2018.

[34] Yang D, Wijenayaka AR, Solomon LB, Pederson SM, Findlay DM, Kidd SP, et al. Novel Insights into Staphylococcus aureus Deep Bone Infections: the Involvement of Osteocytes. MBio. 2018;9.

[35] Qing H, Ardeshirpour L, Pajevic PD, Dusevich V, Jahn K, Kato S, et al. Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation. Journal of Bone and Mineral Research. 2012;27:1018-29.

[36] Salari P, Abdollahi M. The influence of pregnancy and lactation on maternal bone health: a systematic review. J Family Reprod Health. 2014;8:135-48.

[37] Sanz-Salvador L, Garcia-Perez MA, Tarin JJ, Cano A. Bone metabolic changes during pregnancy: a period of vulnerability to osteoporosis and fracture. Eur J Endocrinol. 2015;172:R53-65.

[38] Sowers MR, Greendale GA, Bondarenko I, Finkelstein JS, Cauley JA, Neer RM, et al. Endogenous hormones and bone turnover markers in pre- and perimenopausal women: SWAN. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2003;14:191-7.

[39] Nicks KM, Fowler TW, Gaddy D. Reproductive hormones and bone. Curr Osteoporos Rep. 2010;8:60-7.

[40] Sundararaghavan V, Mazur MM, Evans B, Liu J, Ebraheim NA. Diabetes and bone health: latest evidence and clinical implications. Ther Adv Musculoskelet Dis. 2017;9:67-74.

[41] Al-Bashaireh AM, Haddad LG, Weaver M, Kelly DL, Chengguo X, Yoon S. The Effect of Tobacco Smoking on Musculoskeletal Health: A Systematic Review. J Environ Public Health. 2018;2018:4184190.

[42] Savvidis C, Tournis S, Dede AD. Obesity and bone metabolism. Hormones (Athens). 2018;17:205-17.

Tables

Table 1: Patient demographics for Primary and Revision cohorts including age range, mean age and total number of patients. Gender specific demographics are also included.

	Age range	Mean Age	Number per group
Primary THR	70-89	75	20 (M 10, F 10)
Revision THR	69-89	80	20 (M 10, F 10)
Male Primary THR	70-84	75	10
Male Revision THR	69-87	79	10
Female Primary THR	70-89	76	10
Female Revision THR	69-91	80	10

Table 2: Revision THR patient demographics from donor biopsies including allocated number, gender, age, type of acetabular cup, cup material, liner, femoral stem name, stem material and particle type identified in histological sections (either Metal, PE, both Metal and PE, or not detected).

Patient #	**Sex**	**Age**	**Acetabular Cup name**	**Cup material**	**Liner**	**Stem name**	**Stem material**	**Particle type**
1	F	69	Trilogy	Titanium	UHMWPE	CPT	Co-Cr Alloy	Metal
2	F	74	PCA	Vitallium	UHMWPE	PCA	Vitallium	Not detected
3	F	75	Unknown	Unknown	UHMWPE	Unknown	Unknown	Not detected
4	F	76	HGP	Titanium	UHMWPE	Zimmer Anatomic II	Titanium	Both
5	F	79	Exeter	Steel	UHMWPE	Exeter	Steel	Metal
6	F	82	HG	Titanium	UHMWPE	HG	Titanium	Both
7	F	83	Exeter	All-Poly Cup	UHMWPE	Exeter polished monoblock	Stainless Steel	PE
8	F	84	None	None	None	Exeter Hemiarthroplasty	Stainless Steel	Metal
9	F	89	Muller	All-Poly Cup	UHMWPE	Muller	Co-Cr Alloy	Both
10	F	91	Unknown	Unknown	Unknown	Unknown	Unknown	Not detected
11	M	69	Howmedica All-Poly Cup	All-Poly Cup	UHMWPE	Howmedica Osteonics ODC	Co-Cr Alloy	Metal

12	M	71	Charnley	All-Poly Cup	UHMWPE	Unknown	Unknown	Not detected
13	M	73	PCA	Vitallium	UHMWPE	PCA	Vitallium	Both
14	M	76	Biomet	Titanium	UHMWPE	Unknown	Unknown	PE
15	M	77	PCA	Vitallium	UHMWPE	PCA	Vitallium	Both
16	M	81	Charnley	All-Poly Cup	UHMWPE	Charnley	Stainless Steel	PE
17	M	83	Trilogy	Unknown	UHMWPE	CPT	Co-Cr Alloy	Not detected
18	M	84	CLS	Titanium	UHMWPE	CLS	Titanium	PE
19	M	87	Meridian	Vitallium	UHMWPE	Vitaloc	Vitallium	PE
20	M	87	Unknown	Unknown	Unknown	Unknown	Unknown	PE

Figures

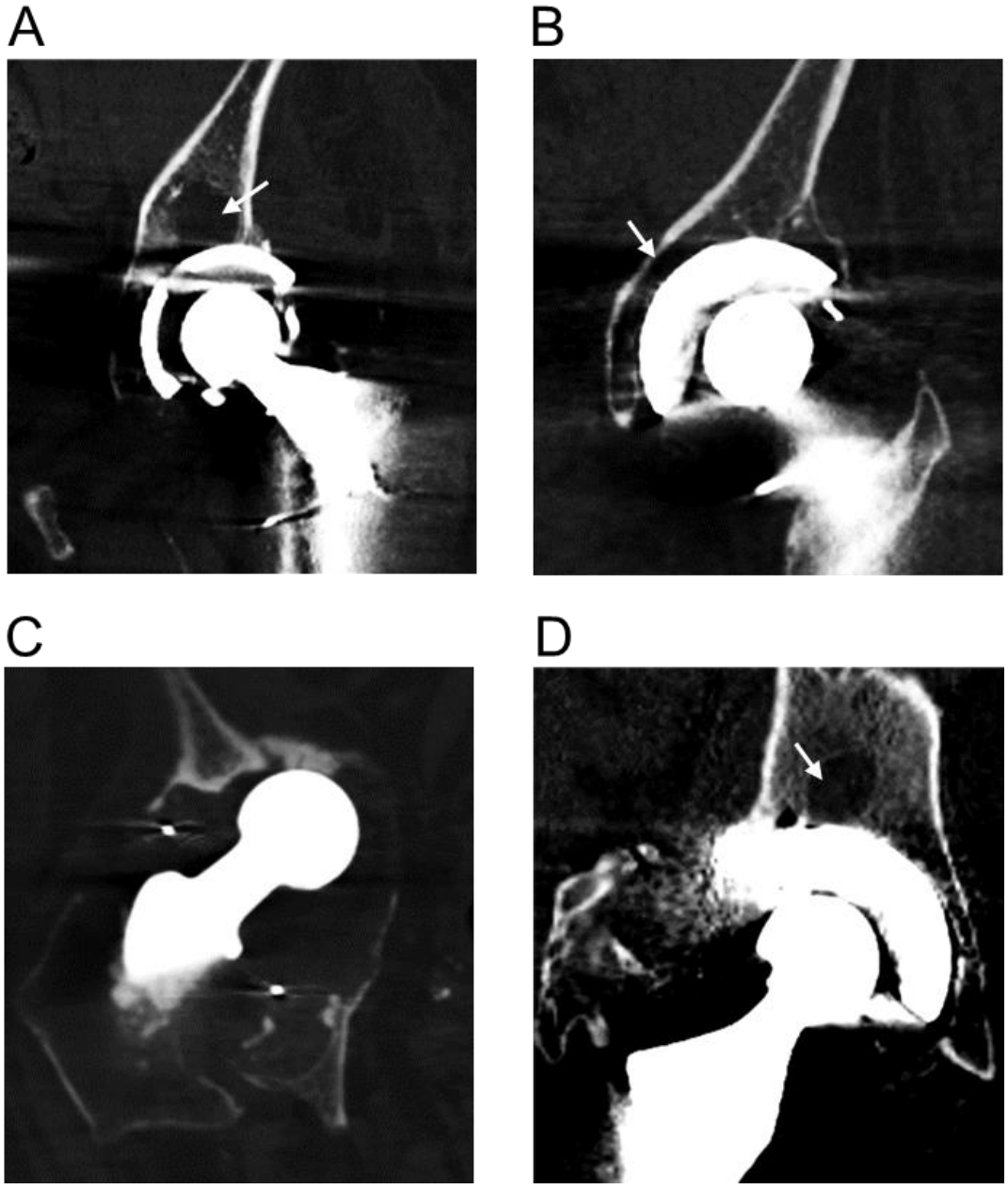


Figure 1: Representative radiographs of 4 revision patients with osteolytic lesions (white arrows). Corresponding patient and implant details are listed in Table 2 and are as follows: A) Patient 1, B) Patient 2, C) Patient 8, and D) Patient 13.

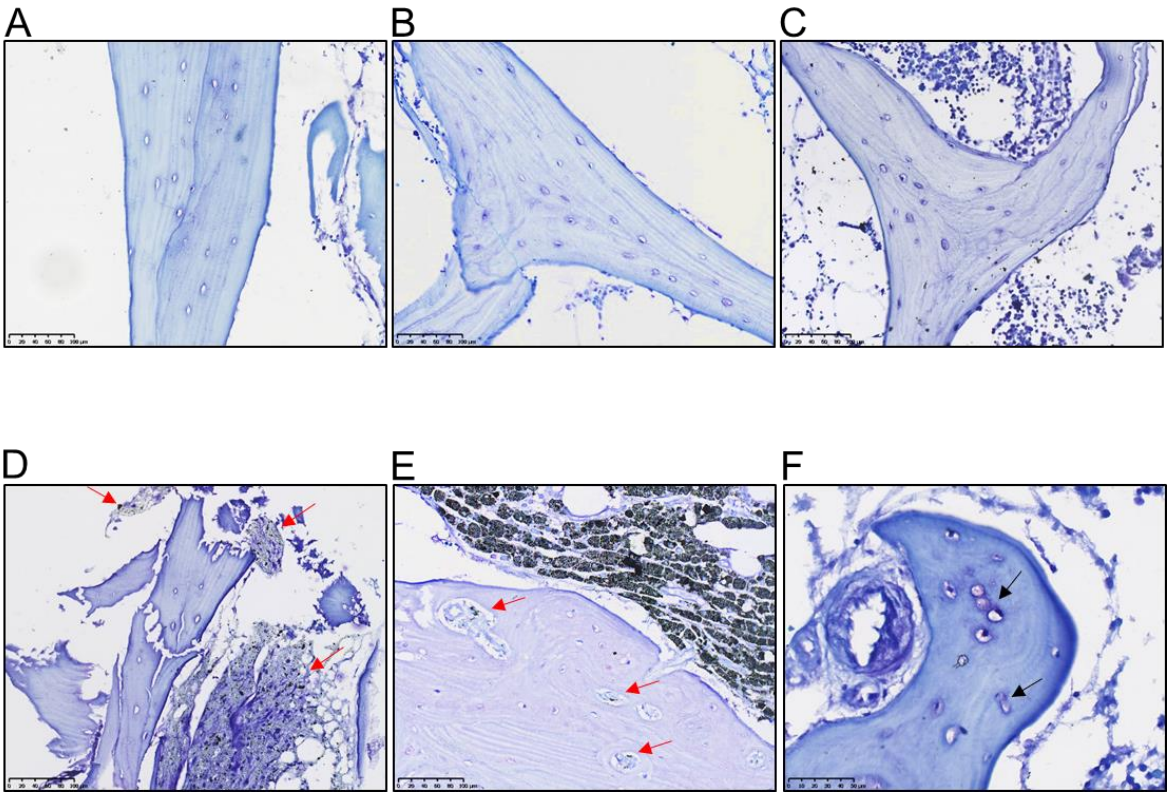


Figure 2: Bone biopsies were obtained from Primary THR (A-C) and Revision THR surgeries (D-F) and stained with toluidine blue. Images depict sections from three individual patients per group. Revision THR biopsies shown enlarged osteocyte lacunae, as well as the presence of both metal particles and granuloma in 2D and E (red arrows). Evidence of osteocyte coalescence is discernible in 2F, denoted by black arrows.

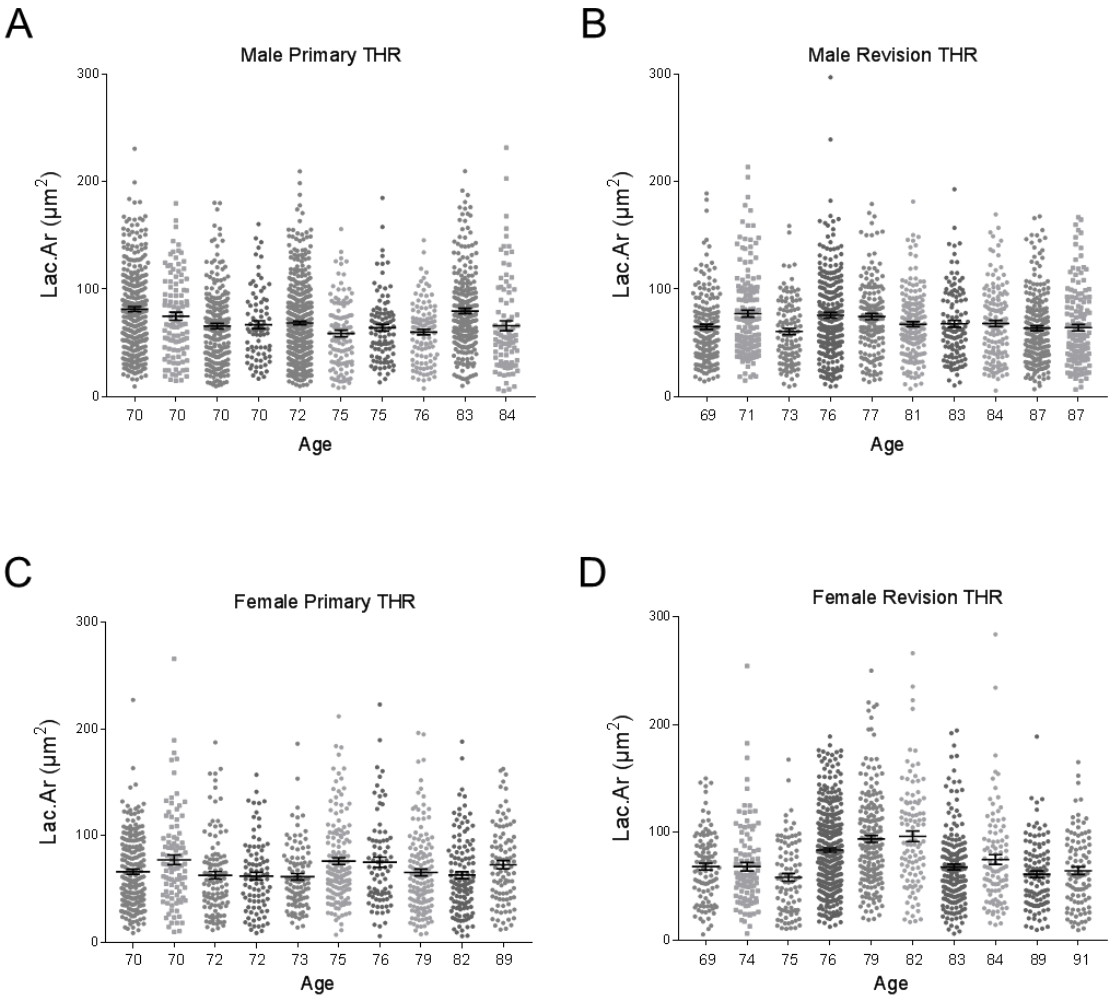


Figure 3: Column scatter plots of quantified osteocyte lacunar area for each patient biopsy with corresponding patient age. A) Male Primary THR patient biopsies. B) Male Revision THR patient biopsies. C) Female Primary THR patient biopsies and D) Female Revision THR biopsies.

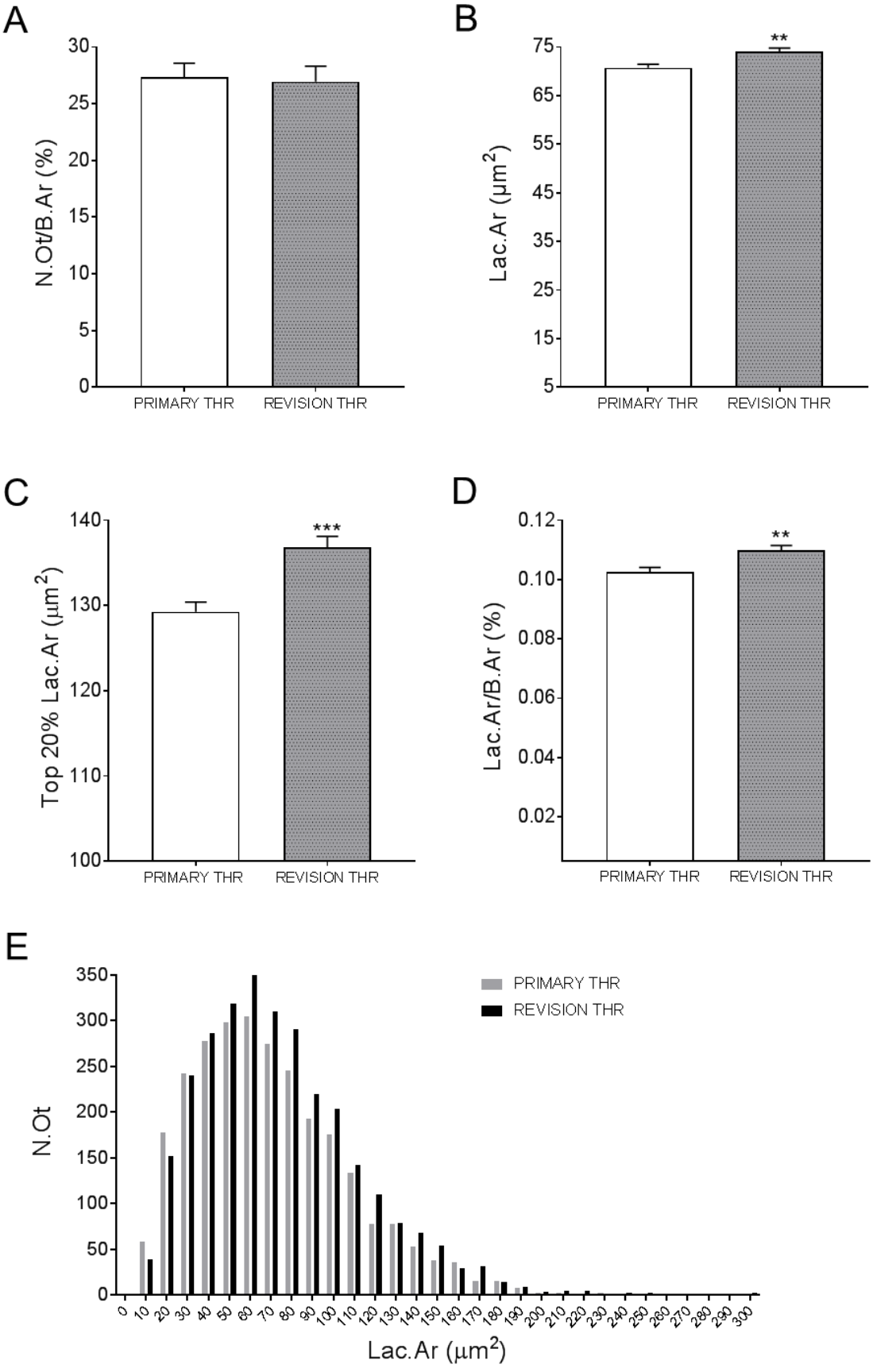

A
N.Ot/B.Ar (%)
30
25
20
15
10
5
0
PRIMARY THR
REVISION THR
B
Lac.Ar (µm2)
75
65
55
45
35
25
15
5
**
PRIMARY THR
REVISION THR
C
Top 20% Lac.Ar (µm2)
140
130
120
110
100
***
PRIMARY THR
REVISION THR
D
Lac.Ar/B.Ar (%)
0.12
0.10
0.08
0.06
0.04
0.02
**
PRIMARY THR
REVISION THR
E
N.Ot
350
300
250
200
150
100
50
0
PRIMARY THR
REVISION THR
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300
Lac.Ar (µm2)

Figure 4: Osteocyte characteristics for human Primary THR compared to Revision THR biopsies. A) Osteocyte number per bone area (N.Ot/B.Ar (%)) of Primary compared to Revision THR biopsies. B) Average osteocyte lacunar area (Lac.Ar (μm^2)) of Primary compared to Revision THR biopsies. C) The top 20% lacunar areas of Primary THR biopsies compared to Revision THR (Top 20% Lac.Ar (μm^2)). D) The percentage lacunar area per total bone area (Lac.Ar/B.Ar (%)) of Primary THR biopsies compared to Revision THR biopsies. E) Histogram of the osteocyte lacunar size distribution of Primary THR biopsies compared to Revision THR, ranging from 60 to 300µm. Significant differences are denoted by $^{**}p < 0.01$, $^{***}p < 0.001$.

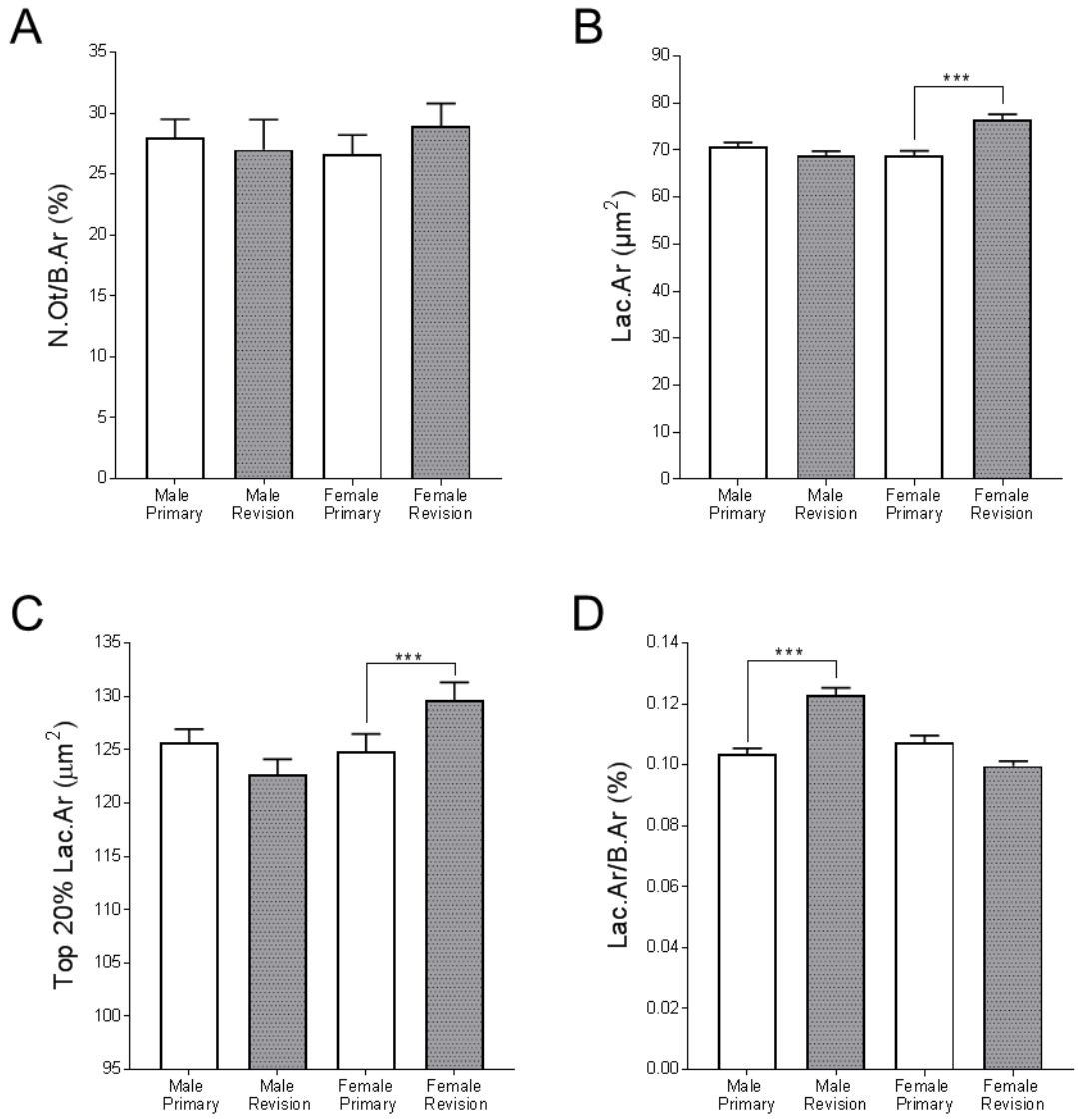


Figure 5: Gender-specific analysis of osteocyte lacunar properties. A) Osteocyte number per bone area (N.Ot./B.Ar (%)) for Male Primary THR, Male Revision THR, Female Primary THR and Female Revision THR biopsies. B) Average lacunar area (Lac.Ar (μm^2)). C) The top 20% lacunar area (Top 20% Lac.Ar (μm^2)) for each cohort. D) The percentage lacunar area per total bone area in all cohorts (Lac.Ar/B.Ar (%)). Data shown are means ± standard error of the mean (SEM). Significant differences are denoted by $^{**}p < 0.01$, $^{***}p < 0.001$.

Chapter 7

Concluding Remarks

The development of osteolytic lesions adjacent to joint replacement prostheses is an ongoing problem that can lead to the aseptic loosening of total hip replacements. Wear particles have been identified as a main cause of this form of osteolysis, through the stimulation of biological responses that lead to the ultimate destruction of bone, as described in Chapter 1. We and others have shown evidence of particles adjacent to and within the mineralised bone surrounding implants. Therefore osteocytes, cells embedded in the bone matrix, are exposed to wear particles. This thesis investigated the hypothesis that osteocytes are exposed to and respond to particles *in vivo*.

Preliminary work in this thesis examined the effect of wear particles on osteocytes in *in vitro* experiments, as shown in Chapters 2 and 4. These chapters describe the osteocyte responses to both clinically relevant metal alloy particles and ultra-high molecular weight polyethylene particles (UHMWPE), in particular, through cell contact. Using confocal microscopy shown in Chapter 4, both types of particles are visualised alongside cell bodies in either 2D culture or 3D collagen gel. Through this contact, both UHMWPE and metal particles stimulated a variety of cellular responses, as described in Chapters 2 and 4. This included the upregulation of key osteoclastogenic genes, RANKL and M-CSF. Osteocytes have been described as a key regulator of osteoclastogenesis, at least in adult mice, and play a major role in regulating osteoclastic bone resorption in adult bone. Therefore, wear particles stimulate resorption by acting directly on osteoclasts, as described in the literature review, Chapter 1, but importantly, we have shown that

osteocytes also regulate the pro-osteoclastic response to wear particles, contributing to osteolysis. Work described in Chapter 4 additionally shows the upregulation of pro-inflammatory pathways in particle-stimulated osteocytes. In particular, we have shown osteocytes can interact with UHMWPE particles through activation of the toll-like receptor pathway, inducing a foreign body response to particles and contributing to the inflammation observed with particle exposure. This furthers our understanding of the osteocyte and its capability of detecting foreign bodies, which has been unreported previously.

Chapter 4 also describes the effect of metal particles on osteocyte viability *in vitro*, where metal, but not UHMWPE particles, induced apoptosis. This finding suggests that the production of metal particles in particular, is highly detrimental to bone health, as osteocyte apoptosis can induce osteoclastic resorption, potentially contributing to the bone loss observed in the presence of prosthesis wear. Additionally, this finding is of interest as metal particles have been shown to cause apoptosis/necrosis in periprosthetic tissue of failed implants. Thus, the *in vitro* model used appears to closely mimic the clinical problem, validating this study. A limitation of this chapter is the lack of investigation into other markers of apoptosis or other cell fates such as necrosis. However, the robust increase in caspase 3 activation stimulated by metal particles suggests that apoptosis was the major mode of cell death in this study.

Chapters 2 and 4 also describe the upregulation of bone-degrading enzymes in response to particles. These enzymes, including MMP-13, CA2 and CTSK, are known to be involved in resorbing the bone mineral matrix. Perilacunar remodelling has been described as a mechanism orchestrated by the osteocyte to release bone mineral calcium,

for example during lactation, however as discussed in Chapters 2 and 6, we have found clear evidence that osteocytes also respond to wear particles by inducing perilacunar remodelling, or osteocytic osteolysis. As mentioned in Chapter 1, the concept of osteocytic osteolysis, despite initial acceptance, became controversial. The ability to resorb the bone was apportioned only to the osteoclast, and *in vitro* studies where osteocytes were cultured on dentine yielded no substantial resorption. More recent studies have analysed changes in osteocyte lacunar area *in vivo*, in particular during lactation and weightlessness, in various mammalian models. These studies have provided substantial evidence that the osteocyte is capable of not only releasing calcium from the bone mineral matrix through perilacunar remodelling during lactation and weightlessness, but is also capable of replacing bone, for example during weaning. Therefore, in this study the ability of the osteocyte to remove and replace the bone mineral matrix is acknowledged as a key mode of action, acting in concert with osteoclasts to remodel, and in the case of osteolysis, resorb the bone.

In the murine model of osteolysis described in Chapter 2, we have shown that osteocytes respond to UHMWPE particles by significantly increasing their lacunar area through osteocytic osteolysis. This model of wear particle induced osteolysis was predominantly associated with the induction of osteoclastic resorption, nonetheless, in this study we have shown both pathways are activated in response to UHMWPE particles. The adjunct chapter, Chapter 3, where we investigated the response of UHMWPE particles in a mouse femoral pump model, appeared to demonstrate the reverse action of osteocytic osteolysis, where osteocytes, distal from the zone of osteolysis, reduced their perilacunar size. Therefore, this suggests that osteocytes, in this situation are capable of laying down mineral to maintain bone structure and viability. Our evidence in this Chapter suggests

for the first time that osteocytes exposed to UHMWPE particles can drive osteocytic osteolysis to the point of lacunae coalescing, however the substantial loss of bone in this model also promotes osteocytic bone formation in order to retain structural integrity, with no change in osteocyte number per total bone area. This finding is potentially due to the osteocyte response to specific mechanical stresses, altered by the adjacent zones of osteocytic osteolysis, and highlights an interesting concept that is worthy of further investigation.

An important aspect of this thesis, as discussed in Chapters 2 and 6, was the analysis of human biopsies from revision total hip arthroplasty patients with confirmed osteolysis. This study showed evidence of osteocyte lacunar enlargement, which is only achievable by active perilacunar remodelling. Importantly, wear particles were observed in a significant proportion of the revision bone biopsies analysed. This is the first evidence of osteocytes responding to particles by inducing perilacunar remodelling in clinical cases, a concept only described previously in rodent studies. This key finding suggests that osteocytes in human patients are stimulating osteocytic osteolysis, contributing to the development of osteolytic lesions. In addition to this, this thesis also describes the highly novel concept, that the osteocyte response to wear particle derived osteolysis could be gender-specific. As previously mentioned, studies have shown the role of osteocytes in removing and replacing calcium during lactation and weaning, which suggests that osteocytes in females may be programmed to remove bone mineral in response to certain stresses. This action is likely to be linked to hormonal changes that occur during pregnancy and lactation, and perhaps also with menopause. Hormonal changes in postmenopausal women include decreases in oestrogen and changes in prolactin levels, and the upregulation of pituitary hormones, which have been linked to increased bone

resorption in premenopausal women. Therefore, the changes in hormone levels may predispose osteocytes in females to undergo osteocytic osteolysis and contribute to the loss of bone. Further investigation is warranted to elucidate the mechanisms of gender-specific bone loss, and the conditions under which this could occur. With respect to this, there are limitations to this study, including the small patient number and unmatched samples. A larger cohort is needed to expand upon the gender-specific response as described in Chapter 6. Also, the ability to match primary and revision samples in a prospective longitudinal study would be desirable. However, as evidence of osteolysis occurs usually over a long period of time, many factors make this difficult to achieve, including maintaining contact with patients, the relatively low frequency of occurrence of osteolysis and the increase in the use of prosthetic materials resistant to wear (XLPE). As mentioned in Chapter 6, the health and metabolic status of the patients in these cohorts is another limitation. Health issues such as diabetes, a history of smoking and obesity have all been shown to have negative effects on bone health and therefore could contribute to the changes in the bone observed in this study. Therefore, expansion of the cohort and further in-depth, multivariate analysis of patient factors would lead to stronger findings that would support the gender-specific results described in this study.

Furthermore, a closer investigation is needed to describe the effects of the variety of patient implants, shown in Table 2 of Chapter 6, and therefore the multiple possible types of wear particles. This would require a larger cohort of patients to effectively analyse all particle types from clinically relevant prostheses, including the variety of cobalt chrome alloys, stainless steel and ceramic particles not investigated in this study. This would also potentially provide information regarding the osteoclastogenic response and apoptotic response to wear particles. Additionally, a closer observation of the histiocytic response

to each particle type would potentially yield more information regarding the inflammatory response to specific particles.

In Chapter 5, we also reported the effects of vitamin E on osteocytes, to elucidate the effects of vitamin E imbued in UHMWPE liners. One key finding was the reduction of osteoclastic markers with vitamin E treatment, however, vitamin E alone and in combination with particles appeared to strongly induce markers of osteocytic osteolysis. This study also suggested that osteocytic osteolysis may be associated with oxidative stress and the production of reactive oxygen species (ROS) by the osteocyte. Nevertheless, this new concept requires further investigation to determine if vitamin E is in fact inducing osteocytic osteolysis by inhibiting ROS, and to clarify the specific roles ROS may play within the osteocyte.

Future work regarding the role of the osteocyte in peri-prosthetic osteolysis should include analysis of osteocytes within regions of osteolysis in a larger cohort of human subjects. This would include further investigation of biopsies from pre and post-menopausal women to determine if the changes in hormone levels are contributing to the development of osteolysis, with blood biochemistry to measure hormone levels as well as dietary calcium and vitamin D status. Additionally, further *in vitro* experiments could include a larger cohort of patient derived osteoblast-like cells to determine whether either age or gender affects the expression of key osteocyte genes, including the bone degrading enzymes discussed in this thesis.

Other work could include the use of inhibitors of osteocytic osteolysis in both *in vitro* and *in vivo* experiments, including a murine model of osteolysis, to determine if prevention

of perilacunar remodelling could safely combat osteolysis. Another study could include the use of the mouse model of ovariectomy with the addition of wear particles to see if hormonal changes *in vivo* stimulates changes in osteocyte lacunar area. Similarly, another potential study could be the investigation into the effect of wear particles in an ageing mouse model with OVX, which may mimic the clinical problem with respect to changes in hormones in humans. Additionally, investigation into mouse orchiectomy surgeries would provide information regarding the loss of reproductive hormones and any potential effects in males with respect to osteocyte responses. Therefore, further investigation is required to determine if the osteocyte could be targeted as a potential non-surgical approach to combating wear induced osteolysis in future therapies.

Due to the complex issues associated with periprosthetic osteolysis, including mechanical strain, material and biological responses, it is worthy to note that the results in this thesis have limitations with respect to these factors. However, the evidence provided by both the *in vitro* and *in vivo* studies show supporting evidence that the osteocyte response to wear particles stimulates perilacunar remodelling, which would have consequences for periprosthetic osteolysis.

In conclusion, the work presented in this thesis substantiates the concept that osteocytes respond to clinically relevant wear particles by activating resorptive pathways. This has been shown both in animal models, clinically-relevant human *in vitro* models and, importantly, in human bone biopsies. Furthermore, we have shown novel evidence that in female joint replacement patients, osteocytes may contribute to the development of peri-prosthetic lesions through osteocytic osteolysis, potentially associated with hormonal changes that occur with age in women. Together, the results from this study

show that osteocytes play an important role in contributing to osteolysis and thus may provide specific targets for future therapies to prevent the wear particle induced bone loss that leads to aseptic loosening.