THE GENETICS OF OSTEOLYSIS AND HETEROTOPIC OSSIFICATION AFTER TOTAL HIP ARTHROPLASTY

BY

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THESIS

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STATEMENT OF ORIGINALITY

I hereby certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not previously been submitted for any degree or other purposes.

I declare that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

STATEMENT OF ATTRIBUTION

Subject recruitment and DNA extraction had been carried out for the candidate gene study described in chapter 4 prior to the commencement of my PhD candidature, my contribution to this study involved: 1. Processing of the stored DNA samples for genotyping; 2. Use of haploview software and HapMap to establish tagging SNP lists; 3. Generation of FASTA files; 4. Association analyses and meta-analyses.

My contribution to the HO GWAS described in chapter 5 included: 1. Subject recruitment; 2. DNA extraction and processing for genotyping; 3. Radiographic evaluation for case/control characterisation and HO grading in the cases; 4 Post-genotyping quality control measures and association analyses which involved learning to write UNIX command lines and shell scripts; 5. Filtering of analyses output files and identification and collation of signals.

Recruitment for the whole genome replication cohort described in chapter 6 commenced prior to starting my PhD candidature, however I was involved in the recruitment process. This involved visiting the Norwegian Arthroplasty Registry on several occasions. I was personally involved in the mailing out of invites and saliva samples/documentation to those agreeing to participate. I also helped co-ordinate the recruitment at various time points throughout the recruitment process. I was involved in the extraction of DNA from saliva samples however three-quarters of the samples were extracted and quantitated by a colleague who I trained in these processes and supervised throughout. All data analysis was carried out myself.

My contribution to the osteolysis GWAS described in chapter 7 included: 1. Subject recruitment in both discovery and replication cohorts; 2. DNA extraction and processing for genotyping; 3. Radiographic evaluation for case/control characterisation and polyethylene wear measurements using EBRA software; 4. Post-genotyping quality control measures and whole genome association analyses using UNIX command lines and shell scripts; 5. Filtering of analyses output files and identification and collation of signals; 6. Replication SNP selection; 7. Replication association analyses and meta-analyses.

DEDICATION

To my wife Chloe and to my children Alexander, William and Anna who I now promise to spend more time with.

To my mother, Jacqueline MacInnes, who passed away this year and would have been so pleased and proud that I have completed this thesis

“I’m hoping that these next 20 years will show what we did 20 years ago in sequencing the first human genome, was the beginning of the health revolution that will have more positive impact on people’s lives than any other health event in history”

J. Craig Venter, Human Genome Project, 2015.

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PUBLICATIONS AND PRESENTATIONS

Publications

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Presentations

International

MacInnes, SJ; Fenstad, AM; Panoutsopoulou, K; Southam, L; Hallan, G; Dale, H; Furnes, O; Zeggini, E; Wilkinson, JM. *A Genome-Wide Association Study of Osteolysis Following Total Hip Arthroplasty.* Late Breaking Poster presentation at the Orthopaedic Research Society meeting, Las Vegas, 2015.

MacInnes, SJ; Panoutsopoulou, K; Southam, L; Zeggini, E; Wilkinson, JM. *A Whole Genome Association Study of Heterotopic Ossification Following Total Hip Arthroplasty.* Late Breaking Poster presentation at the Orthopaedic Research Society meeting, Las Vegas, 2015.

MacInnes SJ. *Current concepts in the genetic association of implant failure*. This was an invited podium presentation at the EFORT meeting, London, June 2014.

MacInnes, SJ; Fenstad, AM; Michael, A; Buckle, C; Furnes, O; Hallan, G; Havelin, L; Fevang, BT; Dale, H; Høl, PJ; Wilkinson, JM. *Using a National Joint Register Dataset and Postal Methodology to Develop a Large DNA Archive for Musculoskeletal Disease.* Poster presentation at the Orthopaedic Research Society Meeting, New Orleans, March 2014.

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Anne M. Fenstad, Ove Furnes, Geir Hallan, Leif I. Havelin, Bjørg-Tilde Fevang, Håvard Dale, Paul Johan Hø[2], Scott J. Macinnes, J. Mark Wilkinson. *Using the Norwegian Arthroplasty Register to Generate a DNA Biobank.* Podium presentation to the International Congress of Arthroplasty Registries, June 2013.

MacInnes SJ, Del Vescovo E, Gordon A, Wilkinson JM. *Genetic Variation in Inflammasome and Bone Turnover Pathways and Risk of Heterotopic Ossification following Total Hip Arthroplasty.* Poster presented at the Orthopaedic Research Society meeting, San Francisco, Feb 2012.

Del Vescovo E, MacInnes SJ, Ollier WER, Kay P, Gordon A, Greenfield EM, Wilkinson JM. *Genetic Variation in Inflammasome and Bone Turnover Pathways and Risk of Osteolysis following Total Hip Arthroplasty*. Poster presented at the Orthopaedic Research Society meeting, San Francisco, Feb 2012.

National

MacInnes, SJ; Fenstad, AM; Panoutsopoulou, K; Southam, L; Hallan, G; Dale, H; Furnes, O; Zeggini, E; Wilkinson, JM. *A Genome-Wide Association Study of Osteolysis Following Total Hip Arthroplasty.* Podium presentation at BORS session at British Hip Society Annual meeting, London, 2015.

MacInnes, SJ; Panoutsopoulou, K; Southam, L; Zeggini, E; Wilkinson, JM. *A Whole Genome Association Study of Heterotopic Ossification Following Total Hip Arthroplasty.* Poster presentation at British Hip Society Meeting, London, 2015.

MacInnes SJ, Del Vescovo E, Gordon A, Wilkinson JM. *Genetic Variation in Inflammasome and Bone Turnover Pathways and Risk of Heterotopic Ossification following Total Hip Arthroplasty.* Presented at the British Hip Society Meeting, Manchester, March 2012.

Prizes

Journal of Orthopaedic Research Editor's pick: MacInnes SJ, Del Vescovo E, Kiss-Toth E, Ollier WE, Kay PR, Gordon A, Greenfield EM, Wilkinson MJ*Genetic Variation in Inflammatory and Bone Turnover Pathway and Risk of Osteolytic Responses to Prosthetic Materials, J Orthop Res* 2015 Feb;33(2):193-8

Poster Presentations Highlight: *A Whole Genome Study of Heterotopic Ossification Following Total Hip Arthroplasty,* British Hip Society Annual Meeting, London, March 2015

Getty Plate for 1st prize oral presentation: *A Whole Genome Study of Heterotopic Ossification Following Total Hip Arthroplasty,* South Yorkshire Orthopaedic Registrars day, July 2015

3rd Prize Winner 3rd Year Oral Presentations: *A Genome Wide Association Study of Osteolysis Following Total Hip Arthroplasty*, University of Sheffield 13 July, 2015

ABSTRACT

Aseptic loosening, the clinical end point of osteolysis, is the most common complication resulting in revision surgery of total hip arthroplasty (THA). It is the result of focal peri-prosthetic inflammatory bone loss at the prosthesis-host interface, and is driven by particulate wear debris generated primarily at the articular bearing surface. There is variation between individuals in their inflammatory response to wear debris suggesting a genetic component. Candidate gene studies have shown that susceptibility to osteolysis associates with polymorphic variation in genes encoding several inflammatory cytokines and bone regulatory molecules. Better understanding of the genetic component is required to address the pathogenesis of aseptic loosening. Advances in high throughput genotyping and mapping of genomic variation has made it possible to examine common genetic variation or quantitative traits as possible risk factors of disease through genome wide association studies (GWAS).

This thesis which forms my PhD candidature, describes several studies undertaken to understand the biological processes contributing to osteolysis. We have followed the advances in genotyping and bioinformatics progressing from a candidate gene study to undertaking whole genome analysis.

We describe the largest candidate gene study to date looking at tagging SNPs in genes thought to play important roles in bone turnover and inflammatory pathways. We have undertaken the first GWAS for osteolysis susceptibility and time to prosthesis failure following THA in over 3,700 patients. During the recruitment process of the replication cohort from the Norwegian arthroplasty register we have proved the feasibility of using such registries for recruitment to answer research questions and establish linked biobanks for the study of musculoskeletal disease.

Heterotopic ossification is also a common complication following THA and similarly its pathogenesis is poorly understood. We also describe a GWAS looking for variants associated with the development of HO and its severity following THA.

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ABBREVIATIONS

A	Adenine
AP	Antero-posterior
BETA:	regression coefficient
BMD	Bone Mineral Density
BMP	Bone Morphogenetic Protein
BMU	Basic Multicellular Unit
Bp:	base pairs
C	Cytosine
CATK	Cathepsin K
CEU:	Caucasian European in Utah
CHR:	Chromosome
CI:	Confidence interval
CNV	Copy Number Variant
CRP	C Reactive Protein
CTX-1	C-telopeptides of type-I collagen
DAMP:	Danger associated molecular patterns
dbSNP:	SNP database
DKK-1	Dickkopf 1
DNA:	Deoxyribonucleic acid
dsDNA:	double stranded deoxyribonucleic acid

EA	Effect Allele
EAF	Effect allele frequency
EBRA	Ein Build Roentgen Analyse
EDTA:	Ethylene Diamine Tetraacetic Acid
FOP	Fibrodysplasia Ossificans Progressiva
G	Guanine
GWAS	Genome Wide Association Study
HO	Heterotopic Ossification
HWE:	Hardy Weinberg equilibrium
IFN:	Interferon
IL:	Interleukin
kB:	kilo bases
LD:	Linkage disequilibrium
LOD	Logarithm of the Odds
LPS:	Lipopolysaccharide
LRP5/6	low density lipoprotein receptor related protein 5/6
MAF:	Minor allele frequency
M-CSF	Macrophage Colony Stimulating Hormone
MMP	Matrix Metalloproteinase
mRNA:	messenger RNA
MSC	Mesenchymal Stem Cell
MW:	Molecular weight
MyD88:	Myeloid differentiation primary response gene (88)

NALP3: NACHT, LRR and PYD domains-containing protein 3

NEA Non Effect Allele

NFkB: Nuclear factor kappa Beta

NLR NOD-like Receptor

NOD: nucleotide-binding oligomerization domain

OA: Osteoarthritis

OPG: Osteoprotegerin

OPPG Osteoporosis Pseudoglioma syndome

OR: Odds ratio

PAMP: Pathogen associated molecular patterns

PCR: Polymerase chain reaction

PINP N-terminal propeptide of type –I collagen

POH Progressive osseous Heteroplasia

PRR: Pattern recognition receptor

PTH Parathyroid Hormone

QC: Quality control

QQ: Quantitative-quantitative

QTL: Quantitative trait locus

r^2: squared correlation coefficient of determination for recombination

RANK: Receptor activator of nuclear factor k B

RANKL: Receptor activator of nuclear factor kB ligand

RAP: Regional association plot

ROS Reactive Oxygen Species

RNA:	Ribonucleic acid
rs	Reference SNP
RT-PCR:	Real time-Polymerase chain reaction
SD:	Standard deviation
SE:	Standard error
sFRP	Secreted Frizzled Related Protein
SNP:	Single nucleotide polymorphism
SOST	Sclerostin
T	Thymine
TE:	Tris EDTA
THA:	Total hip arthroplasty
TIRAP:	TIR domain-containing adapter molecule
TLR:	Toll like receptor
TNF:	Tumour necrosis factor
TRAM:	TRIF-related adaptor molecule
TRAP	Tartrate Resistant Acid Phosphatase
TRIF:	TIR-domain-containing adapter-inducing interferon-β
UHMWPE	Ultra-High Molecular Weight Polyethylene
UTR:	Untranslated region
VEGF	vascular endothelial growth factor
WnT:	wingless signalling pathway

CHAPTER 1

INTRODUCTION

1.1 Overview

Aseptic loosening following total hip arthroplasty (THA) describes mechanical failure of the prosthesis-host interface that arises as a result of periprosthetic inflammatory bone loss. This inflammatory bone loss, termed osteolysis, is caused by the host response to wear debris generated from the prosthetic surfaces. There is variation in individual's response to wear debris and a number of environmental factors have been implicated. Our group have previously shown that genetic variation contributes to the development of osteolysis and this work aims to explore this further. In order to understand the content of this thesis this chapter outlines the impact of aseptic loosening following THA. The pathophysiological manifestation of osteolysis will be described including the biological and molecular pathways responsible for normal and pathological bone turnover. The genetic basis for osteolysis will also be discussed, outlining the justification for the methods in this study. The secondary aim of this thesis is to investigate the genetic contribution to heterotopic ossification formation following THA. The impact, pathogenesis and genetic basis will also be discussed.

1.2 Bone

1.2.1 Function

Bone is a highly active tissue which has both mechanical and metabolic functions. It forms the skeleton which provides support, protects viscera and provides the framework required for body movement and locomotion. As the principal reservoir of calcium in the body, bone plays a vital role in calcium homeostasis. Tight regulation of calcium metabolism relies on hormonal interaction with bone, kidneys and the small intestine. Undifferentiated pluripotent stem cells in bone marrow are the precursors of all blood cell types with the exception of lymphocytes.

1.2.2 Cells and Matrix

The osteoblast

Osteoblasts are highly active mononuclear cells which are derived from the mesenchymal cell lineage (Heino and Hentunen, 2008). Differentiation of mesenchymal stem cells to preosteoblasts and subsequently mature osteoblasts relies on a number of transcription and growth factors (Ducy et al., 2000). Mature osteoblasts acquire the ability to secrete non-mineralized extracellular matrix composed of type I collagen and known as osteoid, which they subsequently mineralize. Osteoblasts respond to a number of hormones (fig 1.12). The life span of the osteoblast has been estimated at three months, after which it has one of three fates. It can either become embedded in its own matrix as an osteocyte, undergo apoptosis or become an inactive lining cell (Franz-Odendaal et al., 2006).

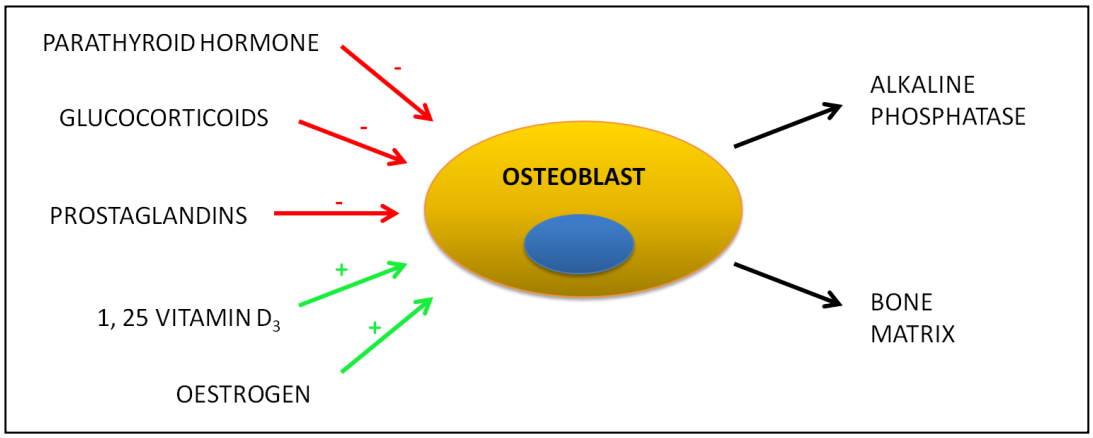


Figure 1.1 Hormonal control of osteoblast activity

The osteocyte

The osteocyte is the most abundant cell in bone accounting for 95% of all bone cells (Franz-Odendaal et al., 2006). The differentiation of the osteocyte from the osteoblast takes 3 days and involves a decrease in cell size and increase in size of cell processes (pseudopodia) (Knothe Tate et al., 2004). This transforms the large round bodied osteoblast into a more stellate shaped cell. The nascent osteocyte has thick elongating pseudopodia which radiate and are believed to be involved in the extrusion of the deposited matrix (Knothe Tate et al., 2004). Mature osteocytes have much thinner and longer processes which connect to other osteocytes and osteoblasts via gap junctions. These connections are important for intercellular communication.

The osteocyte is thought to be the key regulator of adult bone remodelling, through RANKL and sclerostin signalling (Xiong, 2011) (Nakashima, 2011). The mechanosensory role of osteocytes is necessary for the maintenance of the bony matrix and will be discussed in a later section.

The osteoclast

Osteoclasts are large multinucleated cells that resorb bone. They are derived from haemopoeitic stem cells and follow the same cell lineage as macrophages (Suda et al., 1992). There are a number of processes adopted by osteoclasts which lead to the efficient resorption of bone mineral and matrix. The area immediately surrounding the finger like projections of the ruffled border are known as Howship's lacunae. The plasma membrane of the osteoclast binds tightly to the bone matrix creating a sealing zone. The underside of the plasma membrane forms the ruffled border which creates a large surface area for optimal bone resorption. Secretion of hydrochloric acid through the ruffled border then dissolves the hydroxyapatite crystals. The subsequent secretion of the proteolytic enzymes tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CATK) lead to the degradation of the collagen matrices. Degradation products are removed from the resorption lacunae through a vesicular transport system within the osteoclast (Vaananen et al., 2000).

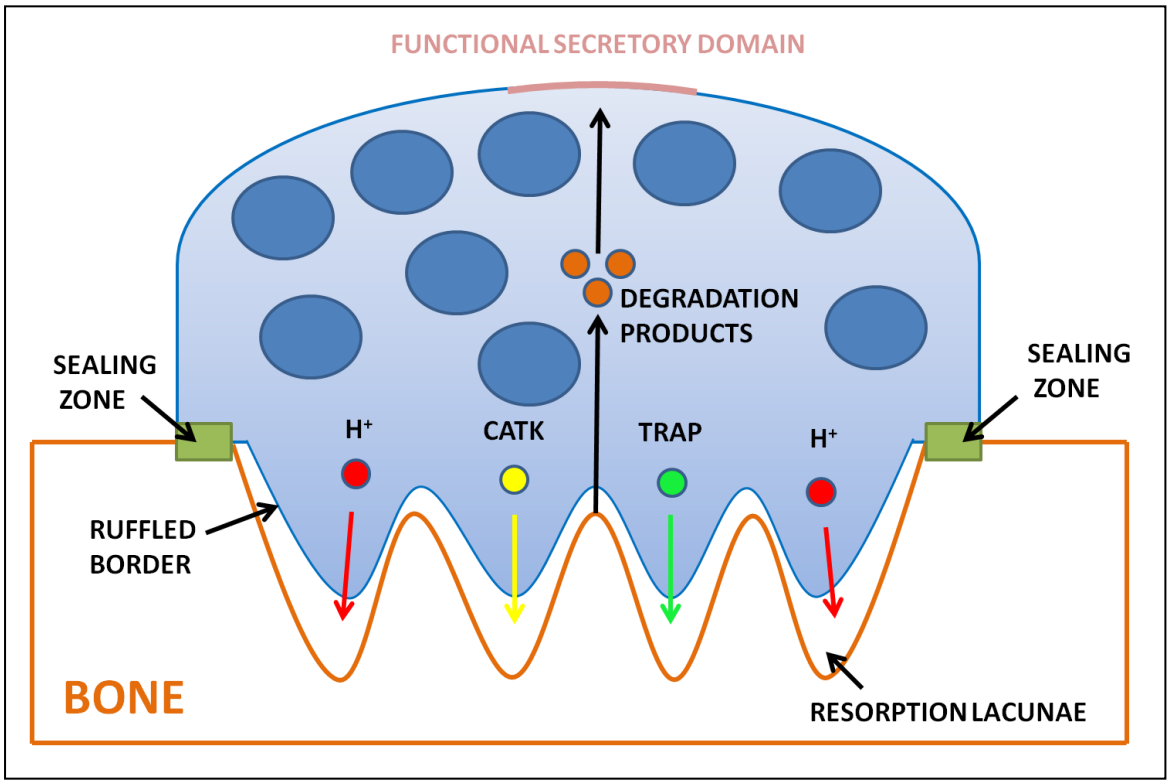


Figure 1.2 Osteoclast function (Adapted from Vaananen et al, 2000)

Bone extracellular matrix

The extracellular matrix comprises organic and non- organic components. The majority of the matrix is formed by bundles of type I collagen fibrils. These fibrils run in parallel creating lamellae. Between the fibrils are pores containing the inorganic components. The inorganic constituent is mostly calcium hydroxyapatite with lesser amounts of carbonate, magnesium and phosphate (Clarke, 2008). A smaller proportion of the non- collagenous proteins including osteocalcin are also present and are involved in calcium binding, mineral stabilisation and bone regulation (Ducy et al., 1996).

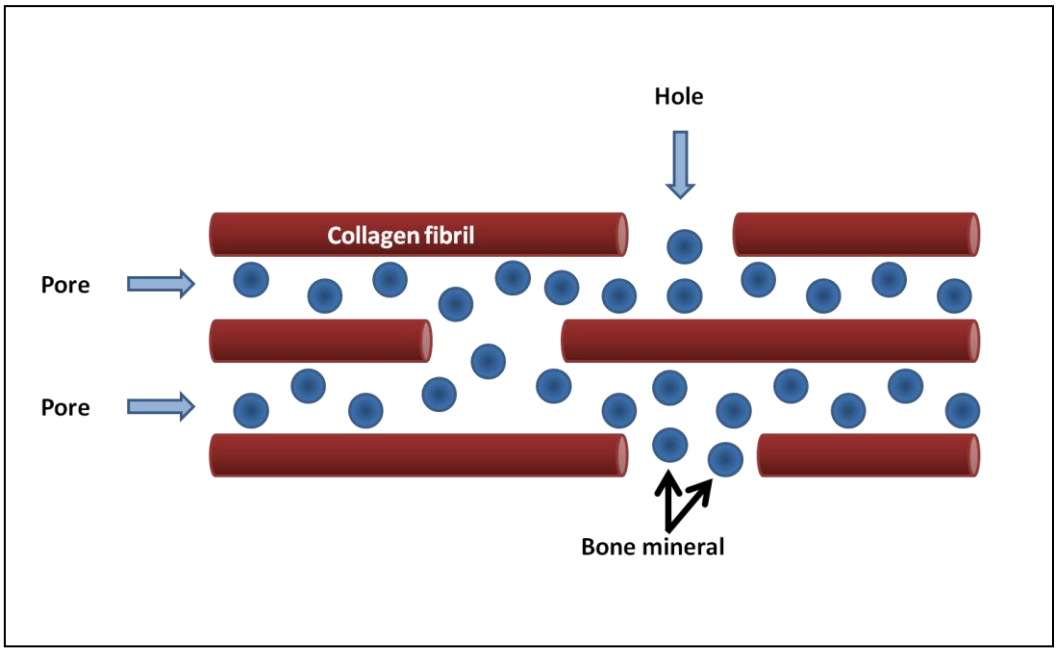


Figure 1.3 Structure of extracellular matrix in lamellar bone (Adapted from Miller, Review of Orthopaedics, 4th edition, 2004)

1.3 Bone modelling and remodelling

Bone is a remarkable, specialised connective tissue. By undergoing constant resorption and formation it is able to adapt to a host of physiological and environmental

circumstances. Bone loss occurs during periods of prolonged immobility (Bauman et al., 1999) and also during long term microgravity (Vico et al., 2000). In contrast, excessive loading of bone results in net bone formation (Souminen, 1993). This functional adaption was first recognised by Wolff in 1892 (Wolff, 1892). Almost a century later, Frost described two distinct mechanisms for bone adaption to mechanical loading (Frost, 1990a) (Frost, 1990b):

> *Modelling: involves osteoclast activation resulting bone resorption or osteoblast activation leading to bone formation, crucially, not at the same location. An obvious example of this is skeletal growth and development during childhood. In adulthood, this may occur in various disease states and in altered mechanical loading.*
>
> *Remodelling: involves coupled bone resorption and formation occurring at the same location in a defined temporal sequence of resorption followed by formation.*

Due to a large surface to volume ratio, 20% of cancellous bone is undergoing remodelling at any one time (Hill, 1998) compared to only 2 – 5% of cortical bone each year (Hadjidakis, 2006). Bone remodelling is carried out by a team of cells known collectively as a “Basic Multicellular Unit” (BMU). The leading region of the BMU contains around ten osteoclasts which create a resorption tunnel in the dominant loading direction (Petrtyl, 1996). As the BMU moves across the surface of bone the thousands of osteoblasts residing in the tail deposit osteoid filling the tunnel (Parfitt, 1994).

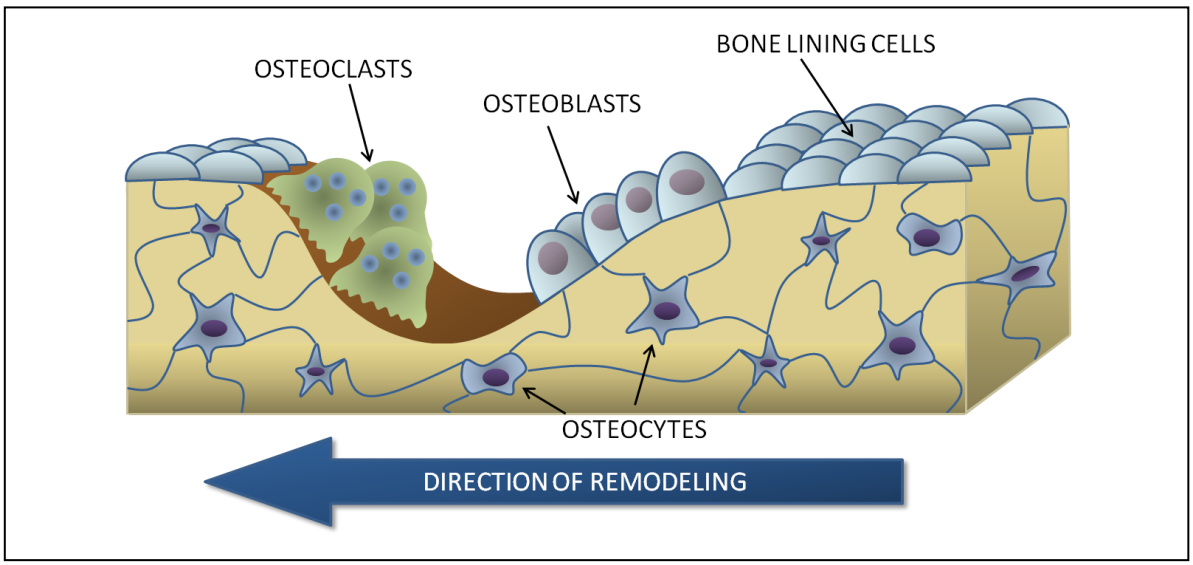


Figure 1.4 The basic molecular unit for bone. Adapted from (Seeman and Delmas, 2006)

The resorption phase lasts around 3 weeks, whereas the osteoblastic refilling takes around 3 – 4 months (Martin TJ, 2008). The remodelling process always follows the same cyclic sequence of quiescence, activation, resorption, reversal and again returning to quiescence (Hill, 1998).

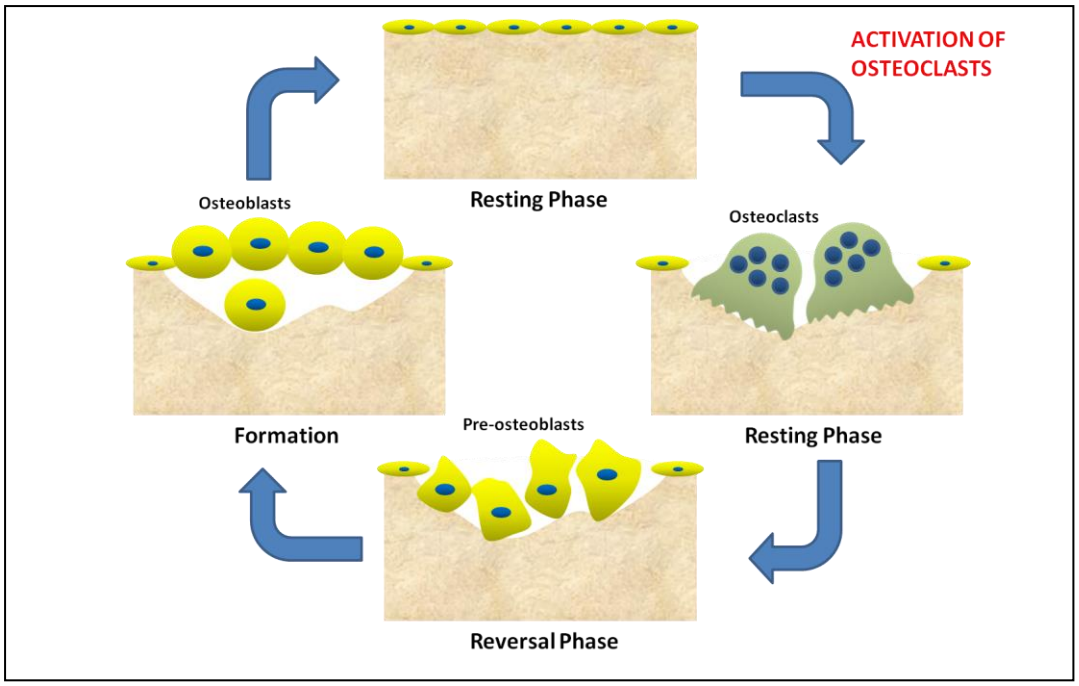


Figure 1.5 Bone remodelling cycle. Resorptive phase: activated osteoclasts resorb bone matrix. Reversal phase: osteoblast precursors migrate to resorption lacuna. Formation phase: Osteoblasts lay down new matrix. Resting phase: osteoblasts mature into lining cells and osteocytes. Adapted from (Hill, 1998).

1.3.1 Molecular control of bone remodelling

The cellular coupling involved in bone remodelling was first proposed by Frost in 1964 (Frost, 1964). Our understanding of the cellular and molecular aspects of bone remodelling has improved greatly over the last ten years with the discovery of RANKL and WnT signalling.

Osteoclasts are derived from haematopoietic stem cells. Their differentiation into mature polykaryons relies on macrophage colony stimulating factor (M-CSF) and activation of receptor activator of nuclear factor κ B (RANK) by its ligand (RANKL), a member of the tumour-necrosis factor superfamily. (Figure 1.6).

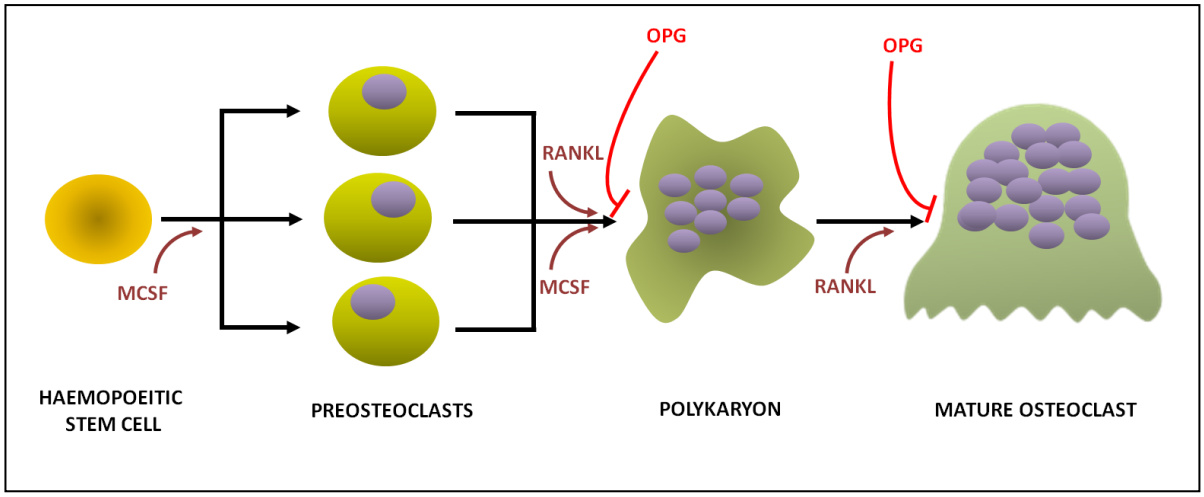


Figure 1.6 Molecular control of osteoclast differentiation (adapted from Boyle W Nature 2003)

The discovery of the importance of these mediators in osteoclastogenesis was due to a number of breakthroughs and provided a molecular mechanism for coupling. Rodan and Martin first proposed that osteoblasts may play an important role in osteoclast regulations due to their reaction to the bone resorbing hormones PTH, vitamin D3 and prostaglandins (Rodan GA, 1981). A study by Takahashi noted that a culture of both haemopoeitic and stromal cells lead to the production of osteoclasts, whereas these cells cultured separately did not (Takahashi et al., 1988). The discovery of osteoprotegerin (OPG), a TNF receptor-related protein, was vital in our understanding (Simonet et al., 1997). It was found to block osteoclast formation in vitro (Yasuda et al., 1998). The relation of OPG to the TNFR family identified RANKL as the key cytokine regulating osteoclastogenesis. The bone resorbing hormones induce RANKL expression in (Boyle WJ, 2003) osteoblasts which regulates adjacent osteoclasts in the BMU (Udagawa et al., 2000). Recent evidence now suggests that osteocytes embedded in the bone matrix express a much higher amount of RANKL than osteoblasts implicating them as the leading source of RANKL contributing to bone remodelling (Nakashima, 2011) (Xiong, 2011).

The differentiation of osteoblasts relies on expression of Runx2, osterix (Osx) and the wingless (WnT) signalling pathway (Robling et al., 2006) (Figure1.18). The WnTs are a family of glycoproteins now known to play a pivotal role in the regulation of bone formation, remodelling and development (Goldring and Goldring, 2007). WnT proteins exert their effects through two signalling pathways known as the canonical and non-canonical pathways. Canonical WnT signalling promotes osteoblast proliferation, mineralisation and inhibits apoptosis. It also increases the OPG/RANKL ratio, thereby inhibiting osteoclastogenesis (Martin 2008 Ost Int). In canonical signalling the binding of WnT ligands, such as WnT3a, to the LRP5/6:Frizzled coreceptor complex promotes the accumulation of cytoplasmic β catenin and its translocation to the nucleus enhancing transcription factors (Kobayashi et al., 2008).

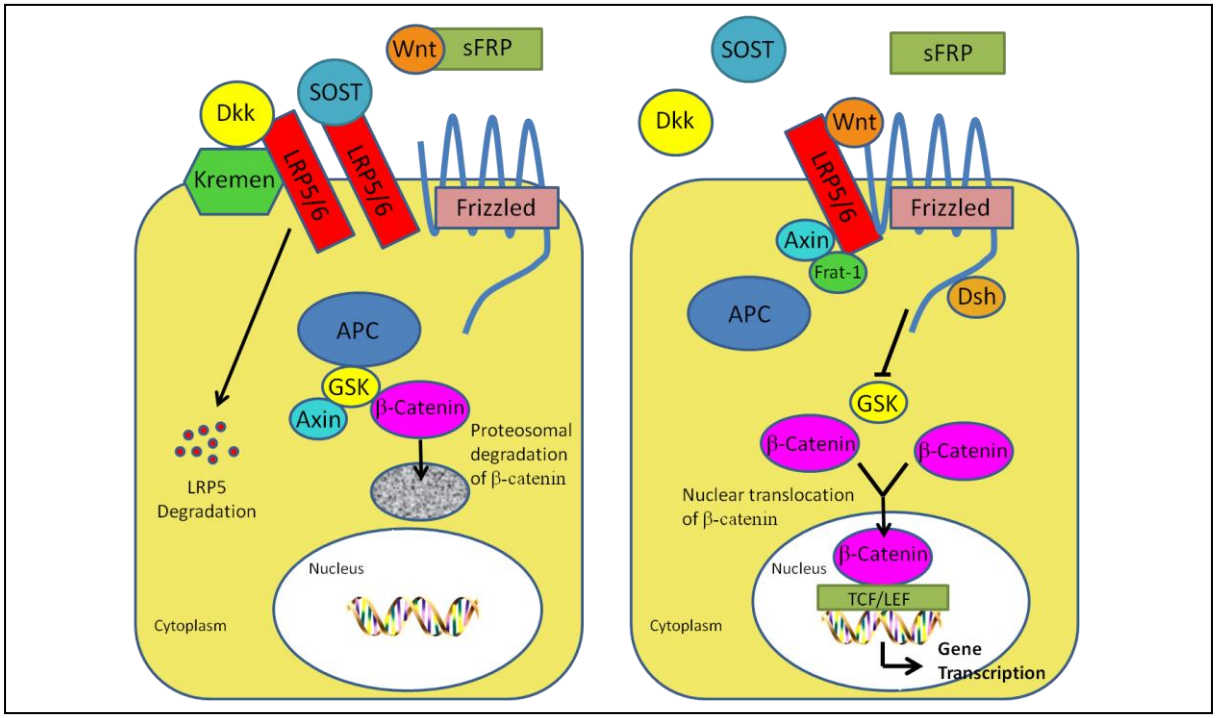


Figure 1.7 The WnT signalling pathway (Adapted from Martin Osteoporosis Int 2008)

The understanding of the importance of WnT signalling in bone was greatly improved with the discovery of a mutation in the human low density lipoprotein receptor related protein 5 (LRP5) a WnT coreceptor. The autosomal recessive disorder OPPG, where patients develop early osteoporosis, was found to be caused by loss of function in the LRP5 gene (Boyden et al., 2002). A genome wide association study has also linked LRP5 polymorphisms BMD and fracture risk (Richards et al., 2008). In addition, a

mouse model blocking DKK-1 (a known inhibitor of WnT) was found to reduce osteoclast number and impair bone formation (Diarra et al., 2007).

Non-canonical WnT signalling regulates bone homeostasis independently of β-catenin. The non-canonical WnT ligand WnT5a binds to the Ror2:frizzled co-receptor complex promoting osteoblast differentiation through Runx2 via JnkNK and PPARg signalling (Tu et al., 2007) (Takada et al., 2007). Maeda et al showed that osteoblast precursors also express WnT5a which increases RANK expression in osteoclasts, increasing their sensitivity to RANKL and thereby promoting osteoclastogenesis and bone resorption (Maeda et al 2012). WnT5a has also been shown to activate the planar cell polarity pathway through RhoGTPase signalling (Kobayashi et al., 2008).

GTPases of the Rho subfamily act as molecular switches which play a pivotal role in the regulation of actin cytoskeleton, membrane transport and transcription factor activity (Etienne-Manneville et al, 2002). Of the 20 family members, the best studied are RhoA, Rac1 and Cdc42. Rho GTPases are now known to be key downstream regulators of both canonical and noncanonical WnT signalling (Schlessinger et al, 2009) (Zhu et al, 2013). Activation of the small GTPase RhoA is required for WnT3a induced canonical osteoblastogenesis (Rossol-Allison et al, 2009). RhoA has also been shown to be important in the motility of osteoclasts and their ability to resorb bone (Chellaiah et al, 2000). RhoGTPases have been shown to control a number of cellular responses in non-canonical WnT signalling acting as regulators downstream of Frizzled. (Schlessinger et al, 2009).

1.3.2 Biomechanical control of bone remodelling

As mentioned previously bone has the remarkable ability to adapt to loading. The osteocytes are the principle cells involved in mechanotransduction control of modelling. They exclusively express the SOST gene which encodes the protein sclerostin. This protein inhibits the WnT signalling pathway by binding LRP5 resulting in the inhibition of bone formation. Linkage studies of families with the dysplastic bone disorders sclerosteosis and van Buchem disease, both characterised by an increase

amount of bone, and caused by loss of function mutations of SOST, lead to its discovery (Balemans et al., 2001). Conversely, mechanical unloading of bone leads to sclerostin expression and consequently reduced bone mineral density (Lin et al., 2009).

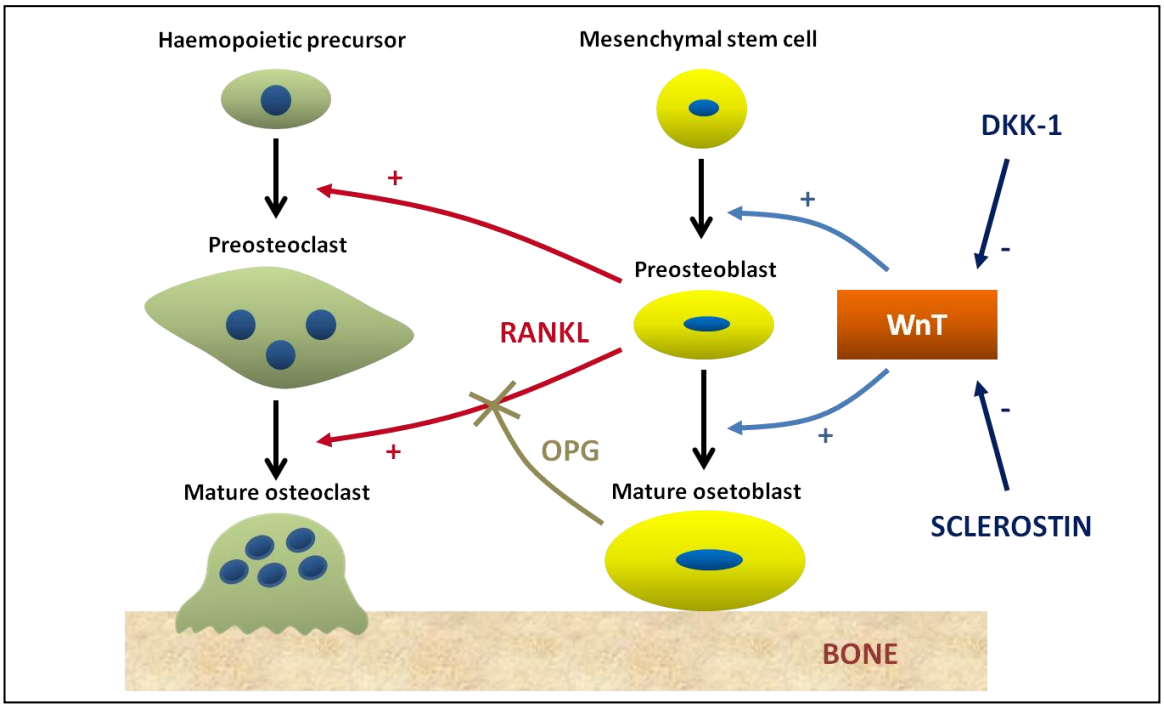


Figure 1.8 Cellular and molecular coupling in bone remodelling (Adapted from Goldring, 2007).

1.3.3 Bone loss after THA

Bone loss following THA may be broadly categorised as occurring early or late.

1. Early: The insertion of a prosthesis alters the local strain environment in the surrounding bone due to the difference in elastic modulus between the prosthesis and bone, and due to difference in the site of load transfer. An example of this is

resorption of bone at the proximal femur that is well recognised and has been attributed to this process (Charnley and Cupic, 1973). As the elastic modulus of metal is much greater than that of bone, load is transferred through the prosthesis distally. Subsequently disuse osteoporosis occurs at the proximal end of the prosthesis (Oh and Harris, 1978). A study by McCarthy found that 3 years after THA there was an average loss of 40% in bone mineral content inside the lesser trochanter and 28% average loss distally in the medial cortex of the femur (McCarthy et al., 1991). A point of equilibrium does appear to be reached with resorption predominantly occurring in the first 2 years following arthroplasty (Bugbee et al., 1997).

2. Late: During use, wear particles are generated from the prosthesis bearing surfaces. This triggers a foreign body chronic granulomatous inflammatory response resulting in osteoclast activation leading to periprosthetic bone resorption (Goldring SR, 1983). This process is discussed in greater detail in the next section.

Bone loss after THA also occurs as a result of normal ageing, at a rate of approximately 1-2% per year. This cause of periprosthetic bone loss will not be considered further in this thesis, but may contribute to prosthetic failure through periprosthetic fracture.

1.4 Osteolysis

1.4.1 Impact of osteolysis

Throughout this thesis the term osteolysis will be used to describe the bone loss that occurs as a result of the inflammatory process initiated by wear particulate debris; and the term aseptic loosening will be used to describe the mechanical failure of the prosthesis-bone construct that results from the osteolytic process.

THA is one of the most successful orthopaedic procedures and has relieved pain and improved hip function in millions of patients worldwide. Ninety percent of patients have either a good or excellent long-term outcome (Harris and Sledge, 1990). It is an extremely cost effective procedure (Faulkner, 1998). The number of primary THA procedures is increasing every year with 98,211 carried out in England and Wales in the year ending 31st December 2015 (www.njrcentre.org.uk, 2016).

Despite the success of modern prosthetic designs and bearing surfaces, around 10% of THA prostheses still fail within 10 years (Kurtz SM, 2007). Improvements in surgical technique and prosthesis design have decreased the incidence of deep sepsis, dislocation and fracture, however aseptic loosening, the clinical end point of osteolysis, remains the most frequent complication and in the UK accounts for 60% of all revision surgery (Table 1.1) (www.njrcentre.org.uk, 2016). Prosthesis loosening results in pain and disability, requiring revision surgery. Revision THA is associated with a 3 to 8-fold greater in-hospital mortality, poorer functional outcome, longer hospital stay, and higher cost than primary surgery (Kurtz SM, 2007, Mahomed NN, 2003, Doro C, 2006, Zhan C, 2007).

Advances in materials, design and surgical technique has improved the wear performance of prostheses, which will decrease the future incidence of osteolysis.

However, an ageing population combined with younger more active patients now undergoing joint arthroplasty suggests that osteolysis and resulting prosthesis loosening will continue to be the major complication of THA.

National Joint Registry Hip Annual Report Data 2016		
	Number	**%**
Revision procedures	88,822	
Indication for revision		
Aseptic Loosening	40,992	46%
Osteolysis	12,988	14%
Pain	18,407	20.7%
Infection	11,189	12.5%
Dislocation/ subluxation	12,117	13.6%

Table 1.1 Summary of hip surgery data from 13th Annual Report National Joint Registry for England and Wales (www.njrcentre.org.uk, 2016)

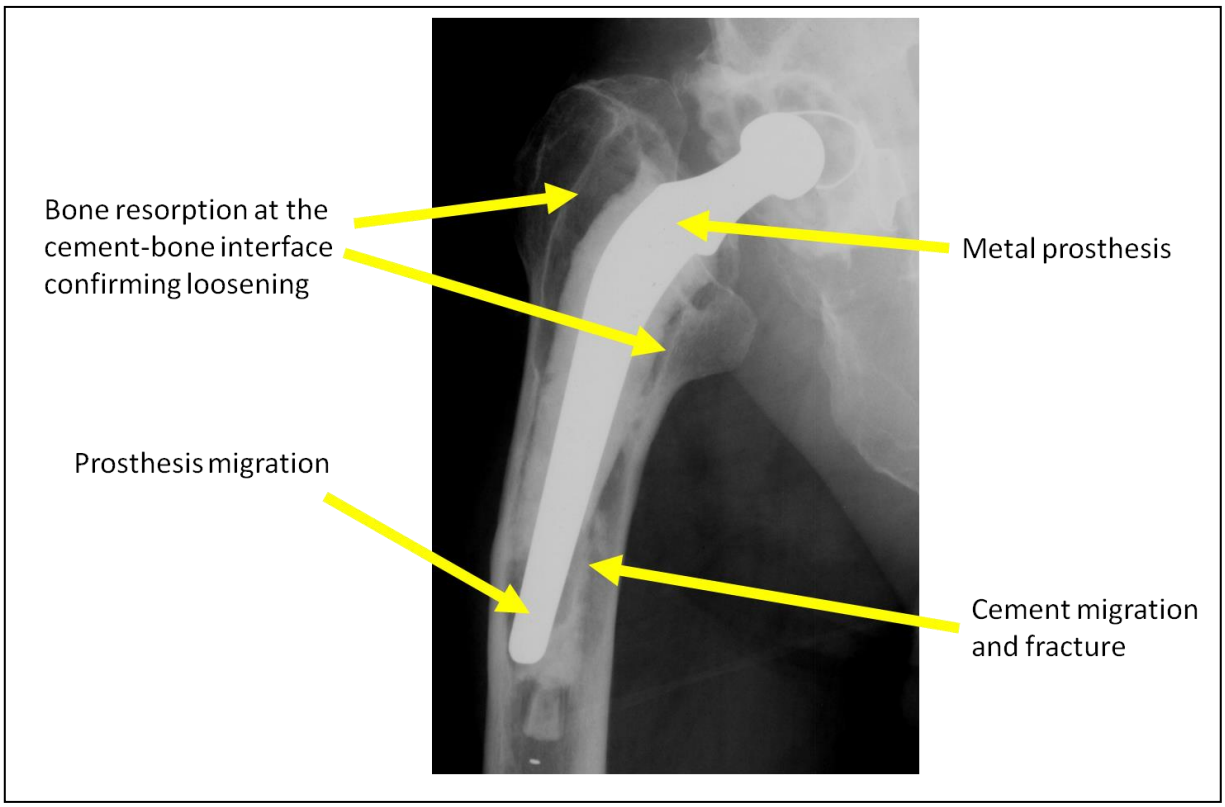


Figure 1.9 Radiograph of hip replacement showing aseptic loosening

1.4.2 Pathophysiology of Osteolysis

The term aseptic loosening describes mechanical failure of the prosthesis-host interface, and arises primarily as the end result of focal periprosthetic inflammatory bone loss occurring at this interface. This pro-inflammatory microenvironment is driven by particulate wear debris, which is generated primarily at the articular bearing surface and at other non-articular prosthesis or cement surfaces (Goldring SR, 1983). Willert first proposed the involvement of prosthetic debris in the development of osteolysis. He identified a resultant foreign body reaction and granuloma formation which included macrophages and multinucleated giant cells (Willert HG, 1977). This foreign body reaction has subsequently been reproduced in animal models (Goodman SB, 1990). Once particulate wear debris has been dispersed into the joint fluid it may initiate a

foreign body reaction at contact surfaces with the host tissues. Schmalzried coined the term “effective joint space” to describe all areas where open communication with the joint pseudo-capsule may allow circulation of the joint fluid and particulate debris (Schmalzried TP, 1992). The effective joint space is thus dynamic and may advance along a tissue plane as osteolysis progresses. Variations in pressure in the joint space during activity may contribute to this circulation (Schmalzried TP, 1992).

1.4.3 The Biology of Osteolysis

The Fibrous membrane

The process of aseptic loosening is characteristically accompanied with the development of a fibrous membrane at the bone-cement interface. Histological analysis of this membrane has shown a synovial-like fibrovascular tissue containing cells including macrophages, fibroblasts and foreign body giant cells (Goldring SR, 1983, Harris WH, 1976).

Molecular signalling pathways involved in osteolysis

The predominant cell types driving osteolysis, the macrophage and fibroblast, signal through various pro-inflammatory cytokines (including the interleukins, TNF alpha, and vascular endothelial growth factor VEGF) following either phagocytosis of the particles or through surface contact (Tuan RS, 2008).

The biological process through which wear particles induce this inflammatory response is still not fully understood. It has become clear that the innate immune system is involved in the initiation of the biological response. The innate immune system is the body’s first defence against foreign pathogens. Its ability to recognize and eliminate pathogens relies on pattern recognition receptors (PRR). PRRs are

expressed by several cells in the monocyte cell lineage and include toll-like receptors (TLR) and the NOD-like receptors (NLR). These subfamilies evoke an inflammatory response either directly through the activation of transcription factors upregulating pro-inflammatory mediators or through the formation of inflammasomes.

The inflammasome

Caspases are cystein proteins which are important regulators of cell apoptosis and inflammation by cleaving interleukin precursors into their active forms. A number of the NLRs have been shown to activate caspase-1 within multiprotein complexes known as inflammasomes (Martinon F, 2009). One of the best characterised inflammasomes is the NLRP3 inflammasome. The complex consists of NLRP3, the adaptor molecule ASC and the cystein protease caspase-1 (Cassel et al., 2009). Activation of the NLRP3 inflammasome can occur as a result of (1) Danger associated molecular patterns (DAMPs) following cellular injury, (2) Pathogen associated molecular patterns (PAMPS) released from the cell walls of bacteria, (3) Particulate phagocytosis, (4) Reactive oxygen species (ROS) overproduction during cellular stress and infection.

NLRP3 activation then allows caspase-1 to cleaveIL-1β to its mature and active form, IL-1β. Although NLRP3 inflammasome activation is required for the maturation of IL-1β it is not solely sufficient. Pro-IL-1b must first be primed either through circulating toll like receptor activation by PAMPs and DAMPs and circulating IL-1β.

The inflammasome in osteolysis

There is now evidence that the NLRP3 inflammasome is involved in the inflammatory response to wear particles through IL-1β production. The presence of PAMPs has been confirmed in the periprosthetic tissue of patients undergoing revision surgery for aseptic loosening (Nalepka JL, 2006). Using RNA gene sequencing, the presence of

bacteria in the periprosthetic biofilm surrounding loose prostheses has also now been confirmed (Dempsey KE, 2007). It has been shown both in vitro and in animal models that PAMPs adherent to particulate debris activate PRRs on macrophages, increasing the biological activity of wear particles (Greenfield EM, 2008).

Caicedo et al demonstrated in vitro that metal implant debris stimulated an inflammatory response in macrophages through inflammasome signalling (Caicedo et al., 2009). Maitra et al found that in vitro UHMWPE wear particles are phagocytosed causing intracellular activation of NLRP3 through cathepsin B release. In addition, alkane polymers generated by UHMWPE activate TLRs in cytokine release (Maitra R, 2009). St Pierre *et al* showed in a mouse model that titanium particles induce an inflammatory response through the activation of the NLRP3 inflammasome (St Pierre CA, 2010).

Osteoclast activation

The indirect pathway

The released pro-inflammatory cytokines, by macrophages, modulate the activation of other cell types in the periprosthetic environment, including osteoblasts. Osteoblasts closely interact with osteoclasts in coupled bone remodelling, regulating bone resorption through the activation of osteoclasts (Rodan GA, 1981). Activated osteoblasts stimulate the monocyte / macrophage cell lineage through activation of receptor activator of nuclear factor κ B (RANK) by its ligand (RANKL) and macrophage colony stimulating factor (M-CSF). Together these induce expression of genes required for the development and maturation of polykaryon osteoclasts and activation of their function of bone resorption (Boyle WJ, 2003). This upregulation of periprosthetic bone resorption results in failure of the integrity of the prosthesis-host construct and loosening of the prosthesis. Activated macrophages also produce matrix metalloproteinases (MMPs) that directly degrade demineralised collagen matrix.

The direct pathway

Fibroblasts are the most frequent cell type found in the loosening membrane, and also play a role in the pathogenesis of osteolysis. They produce the fibrous collagenous matrix which surrounds the prosthesis and in addition, secrete RANKL and IL-6, as a direct response to wear particles, which are both osteoclastogenic and stimulate the formation of multinucleated giant cells (Wei X, 2005, Sakai H, 2002). In addition to upregulation of the osteoclastic response, particulate debris suppresses differentiation of mesenchymal stem cells (MSC) into mature functioning osteoblasts and reduces synthetic activity of mature osteoblasts further shifting turnover balance in favour of net bone loss (Wang ML, 2003).

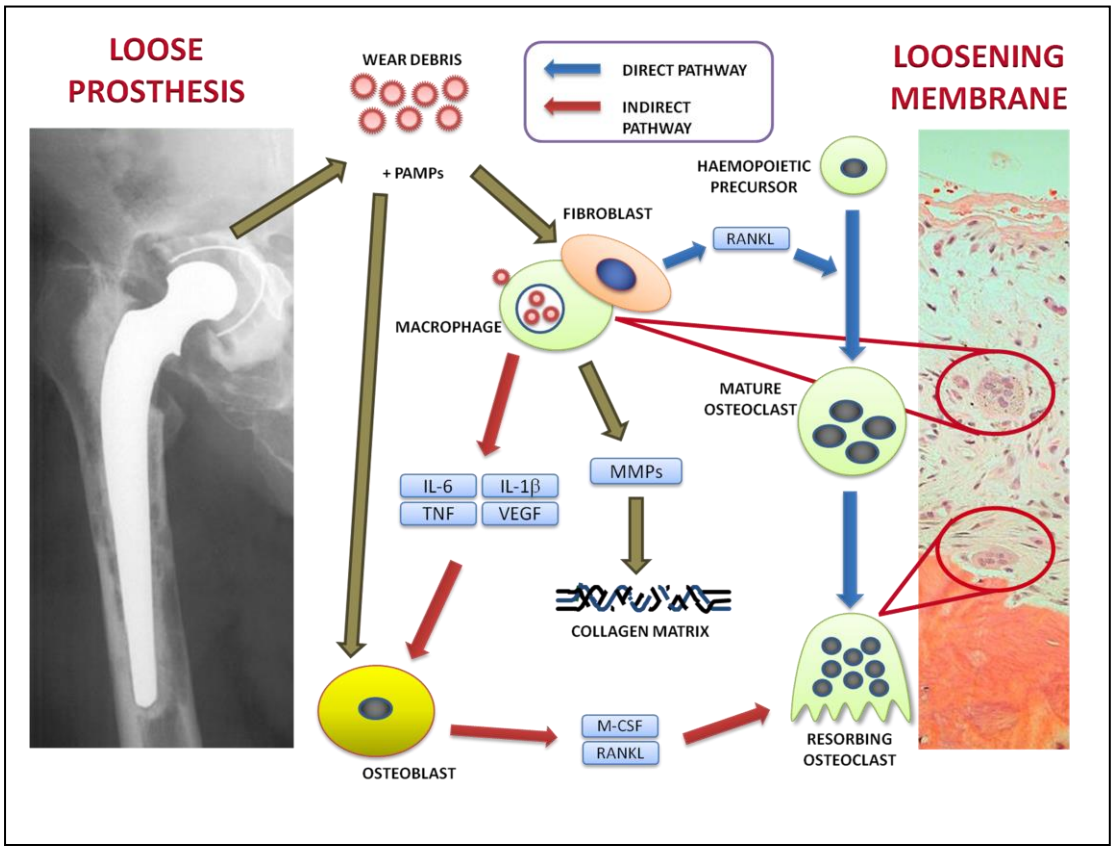


Figure 1.10 Summary of biological response to wear debris. Recruitment and activation of osteoclasts may occur directly through the production of RANKL by fibroblasts, or indirectly through the production of pro-inflammatory cytokines that stimulate the production of RANKL by the osteoblast. TNF may stimulate osteoclast differentiation and activation though both routes.

Other cells types may also be involved in the inflammatory response to wear particulate debris. These include lymphocytes and mast cells. The presence of lymphocytes suggests involvement of the adaptive immune system. It is suggested that particulate debris may undergo opsonisation which allows them to be targeted by B and T lymphocytes. Degranulated mast cells have been found in the periprosthetic tissue surrounding loose prostheses confirming their activation in the process of osteolysis (Solovieva SA, 1996)

Variability in subject responses to wear debris

Patients vary in their osteolytic response to particulate wear debris. Some show little bone resorption in the presence of marked prosthesis wear whereas others undergo marked osteolysis following a small amount of prosthesis wear (Figure 1.11) (Wilkinson JM, 2005). Macrophage responsiveness to in-vitro polyethylene and ceramic particulate debris stimulation has been shown to vary between individuals (Matthews JB, 2000) (Hatton, 2003). An in vitro study by Gordon et al found that monocytes (PBMCs) from patients with a susceptibility to osteolysis exhibited quantitatively greater inducible cytokine responses to particulate compared to patients without this susceptibility (Gordon, 2008) . It has therefore been suggested that patient variability in osteolytic response to particulate debris has a genetic basis. In support of this there is increasing evidence, through candidate gene studies, that genetic variants in cytokines and effector proteins involved in the pathogenesis of osteolysis are involved.

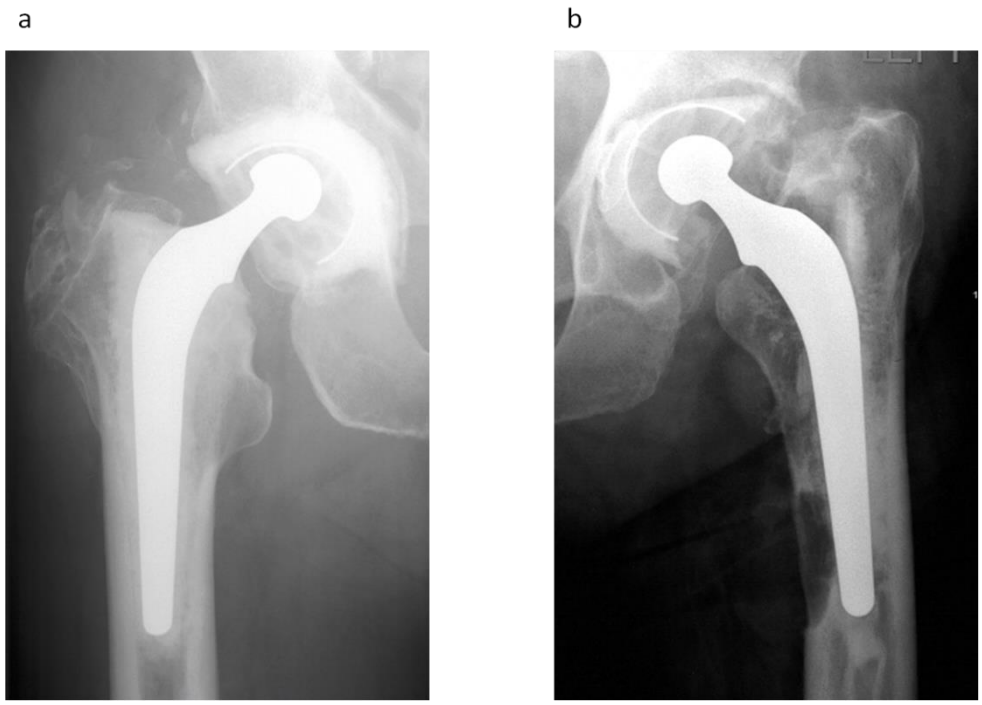


Figure 1.11 Patients exhibit variable osteolytic responses to wear debris. a) radiograph showing marked polyethylene wear, but no osteolytic response, b) radiograph showing mild wear but pronounced femoral and acetabular osteolysis with prosthesis loosening.

1.5 Heterotopic ossification

1.5.1 The clinical impact of heterotopic ossification

Heterotopic ossification (HO) is the pathological formation of lamellar bone in extra-skeletal tissues that do not normally ossify (figure 1.12). HO can be differentiated histologically from calcification, by the presence of trabeculae (Balboni et al., 2006). It was first described by the German physician Reidel in 1883 and in 1918, Dejerne and Ceiller observed its formation in First World War soldiers who had sustained spinal cord injury (Dejerne and Ceiller, 1918). It is a common complication following THA with a variable reported incidence of between 5 and 90% (Charnley, 1972, Newman et al., 2015, DeLee et al., 1976, Rosendahl et al., 1973). HO has been shown to complicate

up to 25% of fractures, and occurs in up to 65% of blast injured amputees (Potter et al., 2007, Forsberg et al., 2009, Mitchell et al., 2010). It's incidence following spinal cord injury has been reported as 30% (Stover et al., 1991).

There is an increased incidence of HO in males (DeLee et al., 1976, Pavlou et al., 2012), those with a previous history of HO (Ritter and Vaughan, 1977), ankylosing spondylitis (Bisla et al., 1976), diffuse idiopathic skeletal hyperostosis (Blasingame et al., 1981) and hypertrophic osteoarthritis (Sawyer et al., 1991). Rheumatoid arthritis has been shown to be protective against HO but it is unclear whether this is due to anti-inflammatory medication prescribed for this group of patients (Zhu et al., 2015). It has been suggested that surgical technique plays a role in the incidence of HO formation. This may be, in part, due to the amount of intraoperative tissue trauma evidenced by the increased rate of HO in revision surgery and longer operative procedures (Hierton et al., 1983). There remains controversy whether surgical approach influences the formation of HO. Bischoff et al and Ashton et al found lower rates of HO using a posterior approach versus an anterolateral approach (Bischoff et al., 1994, Ashton et al., 2000). It has been suggested that less abductor retraction and subsequent ischaemia with a posterior approach may be responsible (Corrigan et al., 2015). Morrey et al found no difference between anterolateral, trans-trochanteric or posterior approaches (Morrey et al., 1984). Similarly, Corrigan et al showed no difference in the HO incidence following hip hemiarthroplasty using anterior, anterolateral and posterior approaches (Corrigan et al., 2015). Martin et al found no reduction in HO rate using a minimally invasive anterolateral approach compared to a direct lateral approach (Martin et al., 2011). There is also debate whether use of cemented or cementless influences HO rate. Pavlou et al found that fully cemented implant were more likely to form HO, and proposed that release of bone debris during reaming may be a contributing factor (Pavlou et al., 2012). In contrast, a prospective randomised controlled trial found no difference between cemented or cementless THA (Nayak et al., 1997)

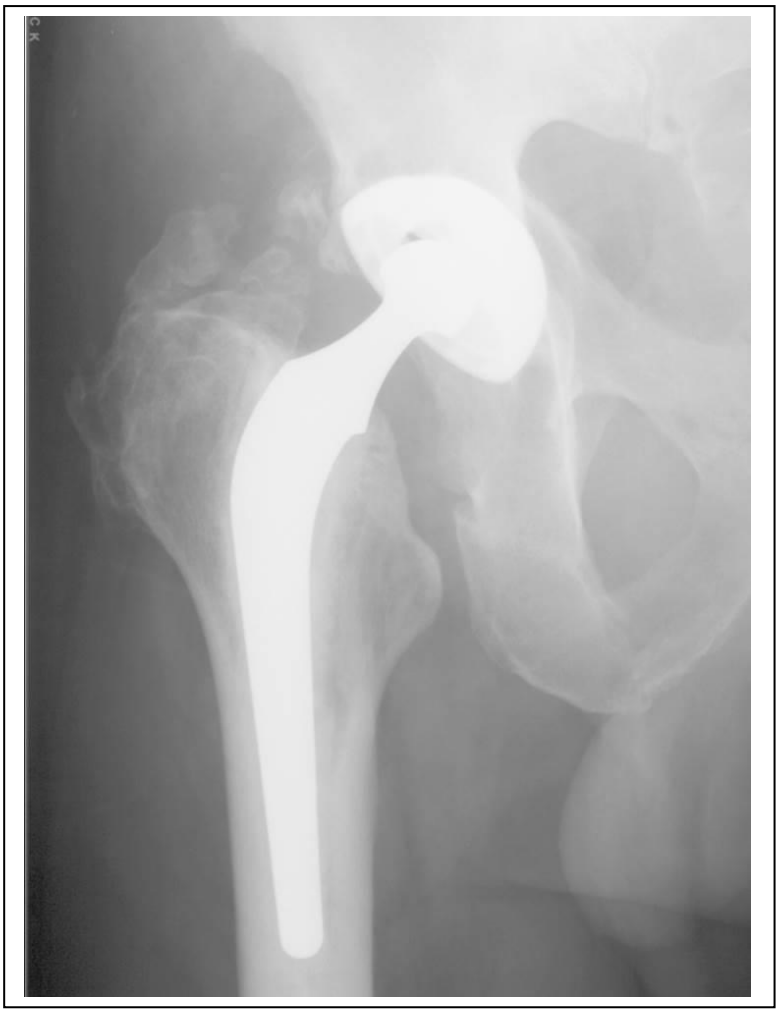


Figure 1.12 HO following cemented Charnley THA

The majority of cases of HO following THA are asymptomatic and Charnley deemed its occurrence as a matter only of ”academic interest”(Charnley, 1972). There are a number of reports, however, associating poorer post-operative functional outcome with increasing severity of HO (Nollen and Slooff, 1973, Pohl et al., 2005). The incidence of clinically significant HO has been reported to be between 3 and 7% of THA cases (Board et al., 2007). The most common symptom of HO is pain (Garland, 1991). In addition to pain, restriction of joint motion may occur, as well as neural or vascular compression (Garland, 1991, Hierton et al., 1983, Brooke et al., 1991). Other symptoms include localised warmth, swelling and erythema clinically mimicking infection (Orzel and Rudd, 1985). Pyrexia may also occur (Macfarlane et al., 2008).

Early detection of HO is difficult. It's formation usually occurs within the first 2 postoperative weeks with radiographic evidence appearing after 6 weeks. Symptoms may not manifest until 12 weeks (Orzel and Rudd, 1985, Mavrogenis et al., 2011). A number of studies have looked at biomarkers for early detection of HO. Wilkinson et al found higher levels of N-terminal propeptide of type –I collagen (PINP), osteocalcin and C-telopeptides of type-I collagen (CTX-I) in patients who developed HO following THA compared with those who did not (Wilkinson et al., 2003). Sell and Schleh reported significantly higher post-operative CRP levels in patients who developed HO (Sell and Schleh, 1999). Three phase technetium 99m isotope bone scanning is the most sensitive method for early detection (Freed et al., 1982). Its expense and the associated radiation exposure preclude it as a useful screening tool.

Once HO has formed, a process that is usually complete by 12 months following surgery, treatment options are limited and usually consist of surgical resection. This is further complicated by recurrence rates which have been reported to be as high as 100% **(**Mavrogenis et al., 2011)

1.5.2 Aetiopathogenesis of heterotopic ossification

The exact aetiopathogenesis of HO is still not fully understood. It can be broadly classified into traumatic, neurogenic or genetic in cause, with that following THA included in the traumatic group (Ekelund et al., 1991). It has been proposed that 3 prerequisites are required for the formation of HO: 1. Triggering event leading to release of osteogenic induction agents; 2. Osteogenic precursor cells; 3. An environment permissive of bone formation (Chalmers et al., 1975). Although it remains unclear whether both the acquired and genetic forms share an exact pathogenesis, there do seem to be a number of common pathways (Winkler et al., 2015). Interplay between inflammatory, osteogenic, and neurogenic pathways has been suggested (Wang et al., 2015).

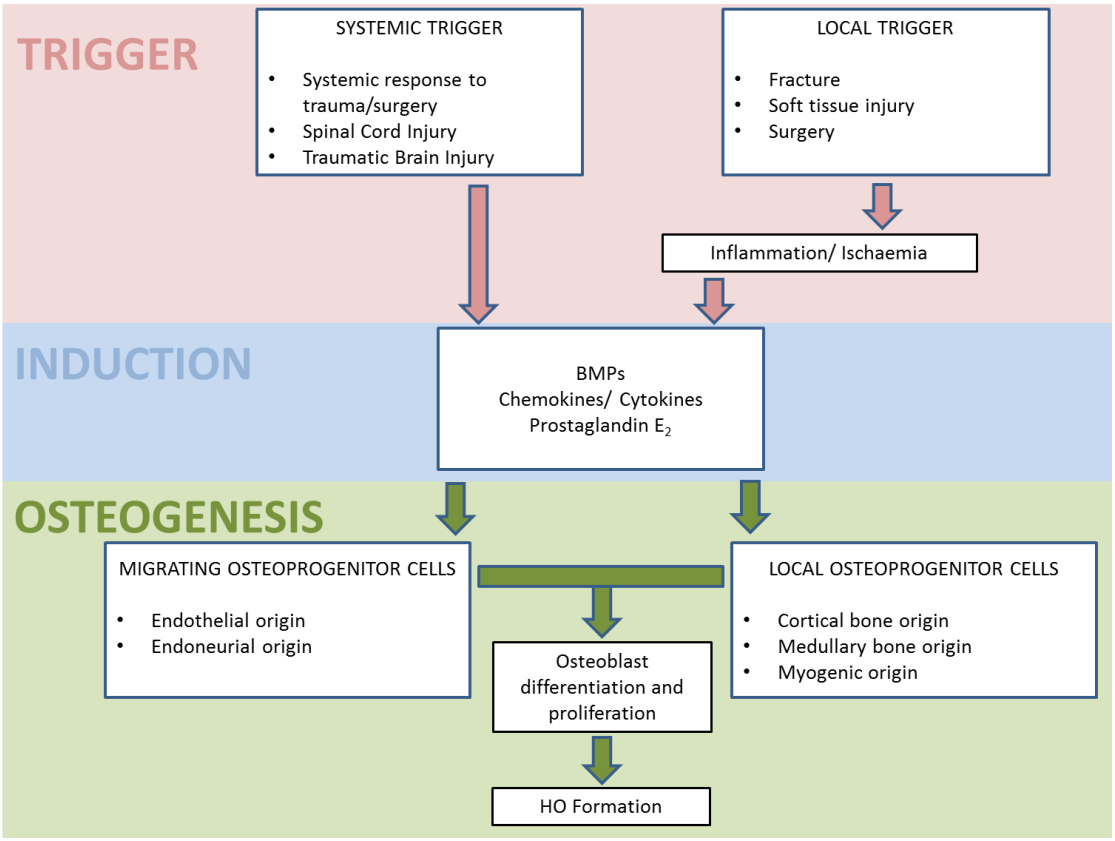


Figure 1.13 HO pathogenesis (adapted (Edwards and Clasper, 2015))

An established trigger for HO involves an initial inflammatory response (Sawyer et al., 1991). This inflammatory and hypoxic microenvironment has been shown to result in localised myocyte cell death (Shore and Kaplan, 2010). This is then followed by angiogenesis and the differentiation of osteogenic precursor cells (Kaplan et al., 1993). The origin of these precursor cells is uncertain. Lineage tracing studies have suggested that local myocyte osteoprogenitor cells contribute only minimally to the formation of HO (Lounev et al., 2009). Cortical bone derived stem cells and medullary mesenchymal stem cells are able to differentiate into osteoblasts and their release during fracture or during THA surgery may contribute to HO (Puzas et al., 1989, Winkler et al., 2015). It has been shown that osteoprogenitor cells with an endothelial or endoneurial origin may migrate to the region of ectopic bone formation (Lounev et al., 2009, Lazard et al., 2015).

Urist discovered in 1965 that demineralised bone matrix induced heterotopic bone formation following trauma in a rabbit model (Urist, 1965). He postulated that this osteogenesis was controlled by bone morphogenetic proteins (BMPs) (Urist and Strates, 1971). The role of BMPs signalling in the formation of HO is well established and their use as primary inducers forms the basis for many animal models used in the study of HO (Winkler et al., 2015). BMP signalling has been shown to control osteoprogenitor migration and differentiation (Shore and Kaplan, 2010). There is a contribution of a neuro-endocrine pathway in the formation of HO. Neural inflammation induced by BMP2 results in the release of osteogenic precursors from peripheral sensory neurones (Salisbury et al., 2011). The endoneurial derived progenitors migrate through newly formed endoneurial vessels to the site of bone formation (Lazard et al., 2015). A number of cytokines/ chemokines capable of promoting osteogenesis have been propose and include platelet derived growth factor, fibroblast growth factor, transforming growth factor-β, insulin-like growth factor, epidermal growth factor, IL-6 and IL-10 (Edwards and Clasper, 2015). The use of indomethacin in HO prophylaxis has led to the proposal that prostaglandin-E_2 may be an important systemic factor (Ahrengart et al., 1988).

The permissive environment required for ectopic bone formation is also not clear. Studies have shown that an environment with low oxygen tension induces chondrocyte differentiation leading to endochondral ossification (Schipani et al., 2001, Mobasheri et al., 2005). Olmstead-Davies et al showed that BMP induced generation of brown adipocytes in the region of soft tissue injury leads to a low oxygen tension environment permissive of ectopic bone formation (Olmsted-Davis et al., 2007).

1.5.3 Classification of HO following THA

The two recognised patterns of HO following THA are found in the peri-femoral neck region and within the abductors. It most commonly occurs in the abductors (Board et al., 2007). Brooker et al described a classification system for the ossification following THA on AP radiographs (figure 1.14) (Brooker et al., 1973):

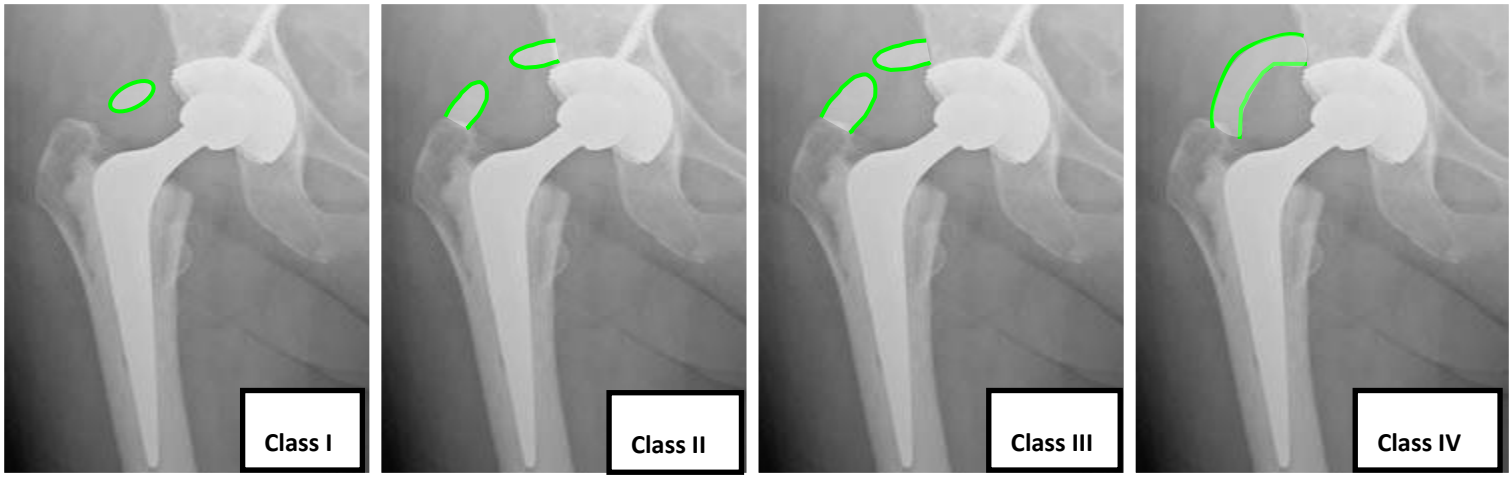


Figure 1.14 The Brooker grading of HO around the hip:

Class 1: a small island of bone

Class 2: bone spurs from pelvis and proximal femur leaving at least 1cm between opposing surfaces

Class 3: bone spurs from pelvis and proximal femur leaving gap less than 1cm

Class 4: apparent ankylosis of the hip

1.6 The Genetic Basis of Disease

The process of bone formation and resorption is governed by the interaction of a number of bone regulatory and inflammatory proteins. The quantity and quality of each of these proteins is determined by the underlying genes which encodes them. Variation within the genetic code can lead to an alteration in the structure, function or expression of regulatory proteins and this section will therefore outline the basics of genetics and its variation.

1.6.1 The structure of DNA

The structure of DNA (deoxyribonucleic acid) was first recognised by Watson and Crick in 1953 (Watson JD and Crick FH, Nature 1953, 171: 737-738). DNA consists of two helical chains of nucleotide bases covalently bound to a sugar-phosphate. Two types of nucleotide bases exist: those derived from purine and those derived from pyrimidine. The purine bases are adenine (A) and guanine (G). The pyrimidine bases are cytosine (C) and thymine (T). Purines always pair with pyrimidines, bound by hydrogen bonds in the following pairs: A-T or C-G (Passarge, Colour atlas of genetic, 3rd edition, Thieme, 2006).

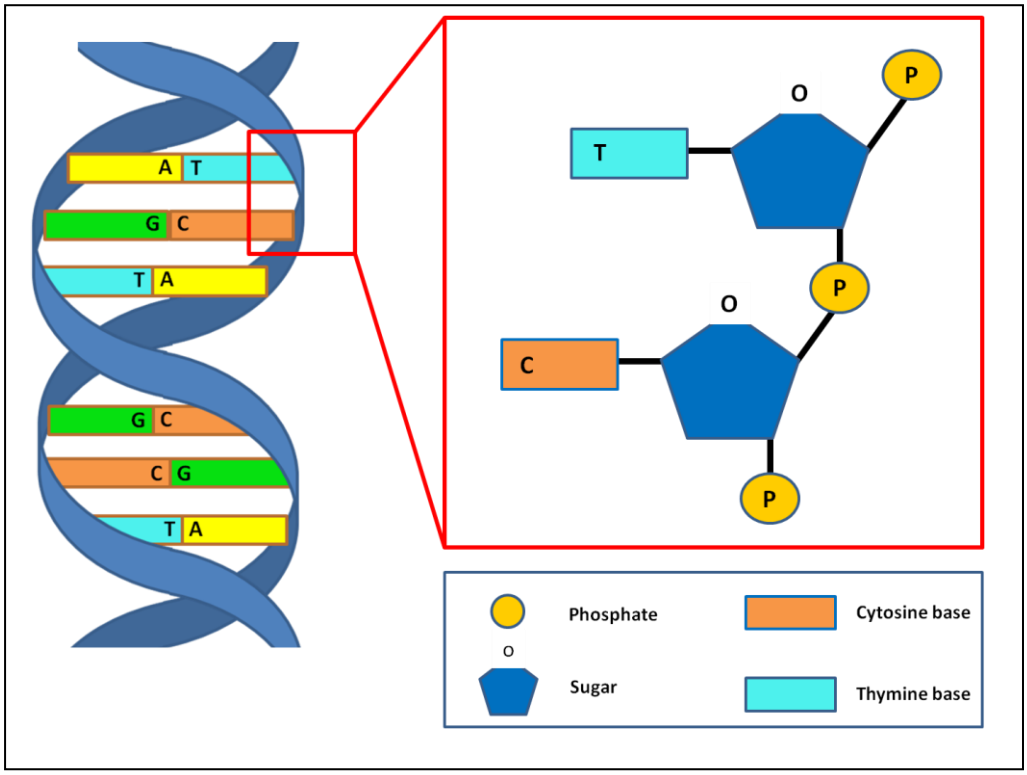


Figure 1.15 Structure of DNA

The sequence of base pairs confers the genetic information. Each triplet of bases (known as a codon) code for a specific amino acid. A total of 64 triplets are possible. As there are only 20 amino acids much of the DNA strand is non-coding. Coding regions are known as exons and non-coding regions are known as introns. Although introns were once thought to be redundant, it is now understood that they may contain promoter regions involved in gene expression (Williams Optometry 2001). The transcribing RNA is spliced, removing the introns, and forms mature mRNA ready for translation (Passarge, Colour atlas of genetics).

Replication of DNA occurs with the breakage of the hydrogen bonds joining each base pair. Each strand acts as a template to generate a new strand. A complimentary strand of RNA transcribes the sequence. The transcribing RNA is then spliced, removing the introns, and forms mature mRNA ready for translation (Passarge, Colour atlas of genetics).

1.6.2 Genetic variation

The Human genome project successfully sequenced the human genome by 2004 (IHGSC, 2004). It consists of 3.3 billion base pairs with only 3% representing coding regions (Burton et al., 2005). Within a given ethnic population, the sequence of DNA between individuals is 99.9% identical (Feuk et al., 2006). The relatively small proportion of variation within the code confers the phenotypic heterogeneity within a population. These variants occur at approximately every 1000 nucleotide base pairs of the code; thus the human genome differs by about 20 to 30 million bases pairs between individuals (Jorde and Wooding, 2004). Genetic variation is not only responsible for evolutionary advantage; it is also a potential cause of disease. The individual specific risk of common diseases is thought to be influenced by the sum of many genetic variations, each potentially causing small changes in biological function and consequently subtle changes in phenotype (Misch EA, 2008).

There are two main sources of genetic variation: mutation and recombination. Mutations can occur as a result of environmental mutagens including a number of chemicals and ionising radiation or, less commonly, may occur spontaneously. Recombination is the exchange of genetic material between two chromosomes. When a chromosomal pair is lined up during meiosis there may be crossing over resulting in the exchange. This is known as homologous recombination and is an important evolutionary process. It allows the restructuring of genetic material increasing variability and helps to eliminate unfavourable mutations (Passarge, 2007). Double strand breaks in DNA can be repaired by homologous recombination where a homologous chromosome is used as an accurate template. DNA end joining (non-homologous recombination) is a process that ligates the DNA ends without homology. It is therefore more susceptible to result in mutation.

Types of variation

Human genetic sub microscopic variants can be divided into 2 nucleotide composition classes (Frazer et al., 2009):

1. Single nucleotide variants

A Single nucleotide polymorphism (SNP) is a single base change in a nucleotide sequence. Two copies of each chromosome are inherited (one maternal and one paternal). This creates a possible 2 alleles at any given locus. An individual can be either heterozygous or homozygous for the allele. The minor allele frequency (MAF) denotes the frequency of the less common allele in a population. It has been estimated that the human genome contains 11 million SNPs. Around 7 million of these occur with a MAF of over 5% and the remainder of at least 1% with which they are defined (Frazer et al., 2009).

2. Structural variants

Structural variants are all variants that larger than 1 kb. It is believed they account for 20% of all genetic variation and underlie over 70% of the variant bases (Frazer et al., 2009). They include insertion or deletion variants, block substitutions, inversions and copy number variants (CNV). CNVs occur when segments of identical sequences are repeated in some chromosomes but not others. Recent evidence suggests that they may account for 13% of the human genome (Stankiewicz and Lupski, 2010).

Implications of genetic variation

Synonymous variation

Not all variations confer a change in phenotype. If the variation does not alter the polypeptide product of a gene a synonymous (or silent) variation occurs. Thus, as long as a change in the DNA sequence creates a codon which codes for the same amino acid, a structural but not functional change will occur.

Missense variation

A missense variation occurs when a sequence variation results in a change of the encoded polypeptide. This change in amino acid may alter the function of a protein leading to a reduction or even loss of its biological activity.

Promoter variation

Each cell differs in its expression of certain genes and polypeptide product. This control of transcription is regulated by the promoter region which is responsible for the activation of RNA polymerase. The promoter region is typically within 200bp upstream of its corresponding gene (Meuller, 2003), but may extend further or be on a non-contiguous DNA section. Any variation in this region can alter the binding of transcription factors which in turn can alter gene expression.

Nonsense variation

If a substitution leads to the introduction of a stop codon there may be a premature termination of translation of a peptide. The resulting protein is unlikely to retain normal function.

Frame-shift variation

These variations occur as a result of insertion or deletion. This alters the codon reading frame and greatly changes the translated protein which is unlikely to retain normal functional.

Genetic variation has the potential to alter phenotype through the gain or loss of function. Alterations in transcription and translation of DNA result in changes in protein expression and structure respectively. Variation within coding regions has an obvious effect on gene structure but variation in non-coding regions also has the potential to alter phenotype through changes in gene regulation.

Epigenetics

In addition to allelic variation, gene expression can be influenced without a change in genotype. The study of heritable changes in phenotype without an underlying change in nucleotide sequence is termed epigenetics. Epigenetic modification occurs as a result of environmental influence. There are three methods in which epigenetic modification can occur: DNA methylation, histone modification and RNA associated silencing (Egger et al, 2004).

DNA methylation is a process whereby methyl groups bind to the C5 position of cytosine bases. This normally occurs where there is an adjacent guanine base; known as a CpG (5'-C-phosphate-G-3') site. 5-Methylcytosine in a promoter CpG site binds methylated CpG binding proteins which leads to inhibition of transcription initiation by preventing the binding of transcription factors (Jones et al, 2001).

DNA is packaged around intracellular histone octamers forming a complex known as chromatin. Histones can be epigenetically modified by acetylation and methylation, consequently altering the formation and function of chromatin. Histone methylation has been shown to regulate transcription and influence stem cell differentiation (Varini

et al, 2013). In contrast to histone methylation, which has been associated with both promotion and suppression of transcription, histone acetylation has been shown to activate transcription. Acetylation leads to altered chromatin folding which allows promoters to be more accessible to RNA polymerases thereby promoting gene expression (Eberharter et al, 2001).

Non-coding RNAs, such as siRNAs (small inhibitory RNAs) and miRNAS (microRNAs), are now thought to play an important role in the regulation of epigenetic mechanisms of gene expression. These small non-coding RNAs act by promoting mRNA degradation and inhibiting translation (Phillips, 2008). More recently, non-coding RNAs have been shown to regulate cytosine methylation and histone modifications (Piletic et al, 2016).

1.6.3 Genetic epidemiology

Genetic epidemiology is the "science which deals with the aetiology, distribution, and control of disease in groups of relatives and with inherited causes of disease in populations" (Morton, 1982). The study of genetic variation was undertaken even before the discovery of DNA through the use of Mendel's laws of inheritance (Stern C, 1966) (Fisher, 1918). Advances in technology and understanding of molecular biology led to the evolution of the epidemiological evaluation of the genetic basis of disease. All genetic epidemiological studies ask the same questions: Is there a genetic component to the disorder and what are the genes responsible for this disorder?

The framework for genetic epidemiology

The first step in the approach to identifying whether a disease has a genetic component is through the study of families. This is usually in the form of observational studies on sibling/twin concordance or parent-offspring concordance. Segregation analysis is then used to determine the mode of inheritance within the family. Linkage analysis then allows us to localise a chromosomal area containing shared genetic

markers which are linked to the causative variant. Association analyses are then used to investigate specific alleles within this region under the linkage signal

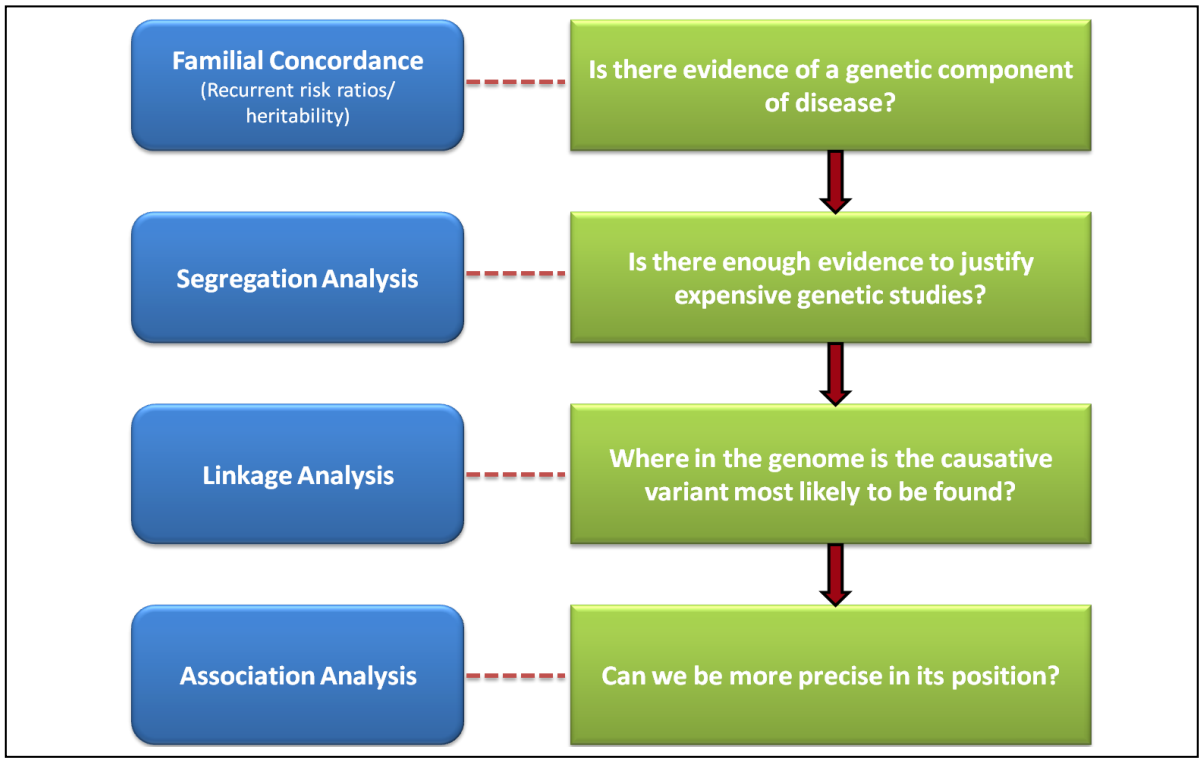


Figure 1.16 Traditional framework for identification of causative genetic variants (adapted from Burton PR, Lancet 2005).

Families have traditionally been used to study traits as it is difficult to obtain large representative cohorts from the general population with the desired expressed phenotype of study. Family studies were useful and very successful in the determination of various Mendelian inherited disorders (Beaudet et al., 1989), (Gusella et al., 1983). The large effect size of single variations in disease causing alleles made these relatively straight forward to identify through the principle of genetic linkage. Common complex diseases, however, have polygenic inheritance with each causative allele contributing a small effect to the expressed phenotype. Linkage analysis in families has been used to study complex diseases, however, has had limited success,

due to inadequate power (Risch and Merikangas, 1996). This has led to the use of large population association analyses.

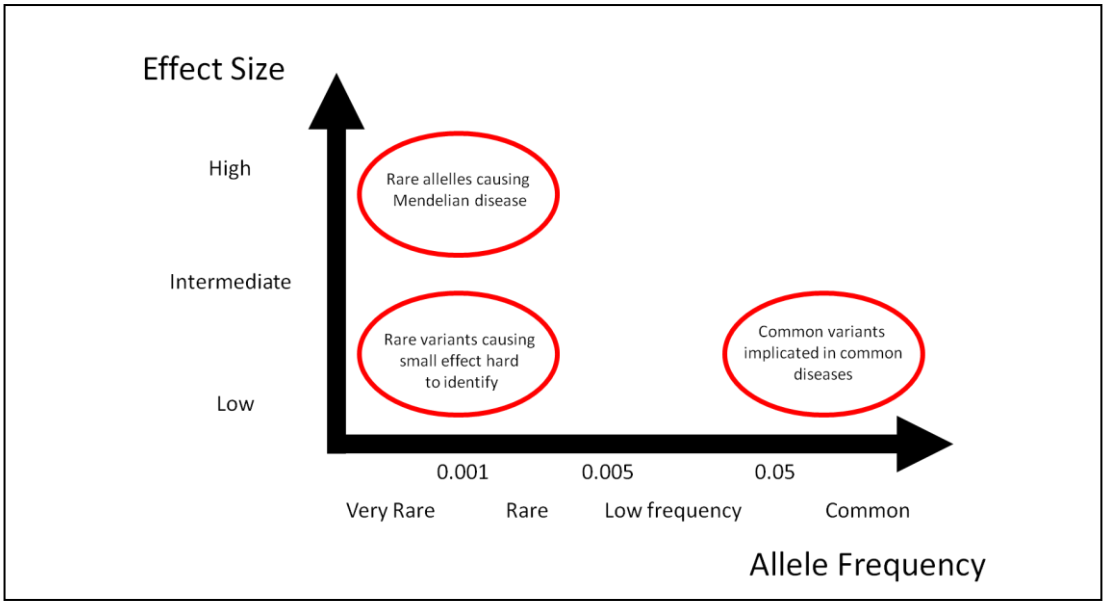


Figure 1.17 Genetic architecture of variants. Adapted from (Manolio et al., 2009)

1.6.4 Linkage analysis studies

Linkage analysis follows the passage of genetic markers within haplotypes through the generations (Williams et al., 2011). If a marker consistently accompanies the phenotype of interest, this suggests a gene that is in close to proximity to that marker may confer causation (Burton et al., 2005). In practice, haplotypes cannot be traced further than a few generations and affected siblings are therefore commonly used to compare haplotypes. An important factor in linkage analysis is the rate of recombination segregating two loci. This is known as the recombination fraction (Θ) and describes how far apart genetic loci are by quantifying the probability of

recombination between them (Williams et al., 2011). Loci that are close to each other on the same chromosome are more likely to be segregated together than loci on separate chromosomes (Teare, 2005). If Θ is ≥50% then the loci are just as likely to recombine as not as they are far apart or on different chromosomes (Williams et al., 2011) Θ is used to calculate the logarithm of the odds (LOD) score which is the likelihood of linkage between two loci. The higher the LOD, the greater the evidence that two loci co-segregate and are linked (Teare, 2005). Using this method, linkage analysis can narrow down a genomic location containing the gene of interest (Williams et al., 2011), (Teare, 2005).

1.6.5 Association analysis studies

Genetic association studies look for association between genetic variants and a phenotype in a study population of unrelated individuals. They compare the frequency of alleles between affected individuals and a control group of unaffected individuals. They are therefore usually case-control studies. Association analysis has greater power than linkage studies to detect variants with small effects and are therefore popularly used to study common complex diseases (Cordell, 2005). Identified variants can either be causative loci (directly associated) or markers in linkage disequilibrium (LD) with the causative loci (indirectly associated). Two loci are deemed to be in LD if, they are present together on a haplotype more frequently than would be expected when tested across many individuals (Jorde, 2000). As it is likely that a number of the causative variants will be in non-coding regions it is difficult to select candidates for association analysis. Therefore, indirect association analyses are most commonly used despite being weaker than direct analyses. (Palmer, 2005). In order to reduce the risk of false negatives in indirect association studies we need adequate mapping of variants and their LD markers. This genetic mapping is being carried out by the International HapMap Project. The International HapMap (haplotype mapping) project established in 2002 provides an online open source database which catalogues SNP frequencies, genotypes and haplotypes data for 4 genetically diverse populations (Yoruba in Ibadan, Nigeria (YRI), Han Chinese in Beijing, China (CHB), Japanese individuals from Tokyo, Japan (JPT) and European ancestry living in Utah from the

Centre d'Etude du Polymorphisme Human database (CEU)) (International HapMap Consortium, 2005). The website contains a genome browser which allows the user to find SNPs in any region of interest and can be used to determine tag SNPs for use in association studies. The advancement of SNP mapping and LD patterns together with technological advances allows investigators to now select a set of genetic markers that efficiently assays most common genetic variations (Skol et al., 2006). The 1000 genome project was an international collaboration which was established in 2008 with the aim of creating a comprehensive description of common human genetic variation. The final phase of the project, published in 2015, characterised over 84 million SNPs in 2,504 individuals from 26 populations around the world and provides one benchmark for studies of human genetic variation (Auton et al., 2015).

1.6.6 Genome wide association studies (GWAS)

The reduced cost of genotyping, advancements in genomic, increasing SNP databases and LD pattern mapping has allowed us to carry out association analyses across the whole genome. GWAS can be undertaken across large populations using a hypothesis-free approach. It therefore allows us to identify susceptibility loci without first having to undertake a linkage scan. The scanning of loci across the whole genome aims to identify clusters of SNPs in association with a particular phenotype. Clustering would imply that markers are in LD with other and therefore close to the susceptibility locus (Williams et al., 2011). Many early studies are often said to be underpowered and robust study design including population stratification, adequate genotyping and analysis is paramount (Hattersley, 2005). In order to reduce the risk of false positive results and increase the power of these studies, replication and meta-analyses should be undertaken.

1.6.7 The genetic association with osteolysis

A number of candidate gene studies have shown that genetic variation in genes encoding various inflammatory cytokines is associated with osteolysis. Wilkinson *et al*

showed an association between variability within the DNA encoding the tumour necrosis factor (TNF) promoter region (dbSNP rs361525) and risk of osteolysis following THA (Wilkinson, 2003). Subjects with osteolysis were approximately 1.7 times as likely to carry the variant DNA code as those subjects with no osteolysis. This association has been replicated in an independent population by Ambruzova *et al (Ambruzova Z, 2006)*. Gordon *et al* have reported genetic variation within the genes encoding Interleukin-1 receptor antagonist (IL-1RN) and IL-6 is also associated with osteolysis (Gordon A et al., 2008). Similar associations have also been identified in other populations (Kolundzic, 2006, Gallo, 2009, Malik, 2007).

Variation within genes that regulate bone turnover also associate with osteolysis. Gordon *et al* showed that carriage of the dbSNP rs288326 variant in the *FRZB* gene encoding secreted frizzled-related protein-3 (Frp3), a regulatory glycoprotein within the osteogenic Wnt signalling pathway that modulates mesenchymal stem cell differentiation of osteoblasts (Martin TJ, 2008), associated with susceptibility to osteolysis following THA (Gordon A et al., 2007). Its carriage also associated with the development of heterotopic ossification following THA. Malik *et al* have also shown associations between aseptic loosening and other candidate loci within the genes encoding matrix metalloproteinase 1 and the vitamin D receptor (Malik, 2007), mannose-binding lectin (Malik MH et al., 2007), and the RANK/OPG pathway (Malik MH et al., 2006).

Recent studies using beadchip assays have shown that many genes are differentially expressed in wear debris-induced cells and tissues (Garrigues GE, 2005, Shanbhag AS, 2007, Koulouvaris P, 2008), and have highlighted our limited understanding of the spectrum of biological mediators involved in the pathogenesis of osteolysis. The identification of further risk loci is required to further understanding of the pathogenesis of aseptic loosening. This would potentially allow for the development of screening tools, and provide investigational targets for prophylaxis or treatment with the aim of reducing the need for revision surgery, and its associated morbidity.

The reducing cost of genotyping, advances in genomic technology, and increasing SNP mapping now allows us to carry out cost-efficient association analyses across the whole genome in large populations, and is the next step that will allow a more complete understanding of the contribution of genetic factors to osteolysis.

1.6.8 The genetic association with HO

The increased incidence of HO in bone forming disorders and the heterogeneity in HO manifestation in patients with similar injury patterns suggests a genetic predisposition. To date, the majority of genetic research has looked at the rare heritable causes of HO and few screening studies have been carried out in other populations.

The rare genetic forms of HO, fibrous dysplasia ossificans progressiva (FOP) and progressive osseous heteroplasia (POH), are characterised by progressive extra-skeletal bone formation following minor trauma (Kaplan and Shore, 2000). FOP is caused by an autosomal dominant mutation in the *ACVR1* gene. *ACVR1* encodes the bone morphogenetic protein (BMP) type I receptor activating-like kinase2 (ALK2) (Shore et al., 2006). This gain of function mutation has been shown in the knock-in mouse model to promote the spontaneous and injury induced formation of HO (Kaplan et al., 2012). Bone formation typically starts in the neck and thoracic spine but progresses over time eventually bridging the joints of the axial and appendicular skeleton (Shore and Kaplan, 2010). This leads to complete immobilisation of the body and has led to the colloquial term “stone man syndrome.” Like FOP, POH leads to extensive ectopic ossification but is differentiated by a number of clinical differences. The heterotopic ossification originates in the dermis and progresses into the deeper soft tissues (Kaplan et al., 1994). POH is caused by an inactivating mutation in *GNAS*, which encodes the stimulatory G-protein alpha subunit that activates adenylyl cyclases and is involved in bone formation (Shore et al., 2002).

Larson et al found that patients with HLA-B27 were more likely to develop HO following traumatic spinal cord injury (Larson et al., 1981). The absence of HLA-B27, however, does not preclude the development of HO. Candidate gene studies have associated variants in genes encoding the $\beta_2$adrenergic receptor, toll-like receptor (TLR) 4, complement factor H, and secreted frizzled related protein 3 (sFRP3) with susceptibility to HO (Gordon A et al., 2007, Mitchell et al., 2010). As with osteolysis, no genome wide analyses for HO have been carried out to date. This hypothesis free approach has the potential to identify variants in genes which have never before been implicated in its pathogenesis. This will further our understanding of the biological pathways involved which currently elude us.

1.7 Study Hypotheses

1. We hypothesise that genetic variation contributes to osteolysis susceptibility and time to prosthesis failure following THA.
2. We hypothesise that genetic variation contributes to heterotopic ossification susceptibility and severity following THA.
3. We hypothesise that national joint registries can be utilised for large scale recruitment and postal collection of saliva samples provides adequate DNA yield to establish a DNA biobank for high throughput genotyping.

1.8 Study Aims

1.8.1 Primary Aims

1. To conduct a candidate gene study to determine whether variants in genes involved in bone turnover and inflammatory pathways contribute to osteolysis susceptibility and time to prosthesis failure following THA.
2. To conduct a GWAS to identify susceptibility loci for osteolysis and time to prosthesis failure following THA.

1.8.2 Secondary Aims

1. To conduct a replication study of osteolysis GWAS results and meta-analysis of both stages.
2. To conduct a single stage GWAS to identify susceptibility loci for heterotopic ossification (HO) after THA.
3. To conduct a single stage GWAS for severity of HO after THA.

CHAPTER 2

METHODS

2.1 Overview

This chapter outlines the various methodological approaches, their principles and application as used in the thesis. Specific methods, as they apply to individual studies are outlined briefly within each results chapter, as the applications differ for each experiment.

2.2 Phenotype Definition

2.2.1 Osteolysis case definition

Available radiographs were examined to identify presence of aseptic loosening. The criteria of Harris and McGann were used to define loosening of the femoral prosthesis (Harris and McGann, 1986):

Definition of loosening	Radiographic morphology
Definitely loose	1. Migration of cement 2. Fracture of cement 3. Lucent line at the cement stem interface
Probably loose	Continuous (100%) line at the cement bone interface without prosthesis migration
Possibly Loose	Incomplete lucent line (50 - 90%) at the cement bone interface

The criteria of Harris and Penenberg were used to define loosening of the acetabular cup prosthesis (Harris and Penenberg, 1987):

Definition of loosening	Radiographic morphology
Definitely loose	1. Migration of the acetabular cup 2. Fracture of cement
Impending loosening	Continuous (100%) line at the cement bone interface that is nowhere less than 2mm wide

Femoral gradings: definitely loose and probably loose and acetabular gradings: definitely loose and impending loosening were included in this study as cases. Possibly loose grades were excluded from the study.

2.2.2 Prosthesis survival time

Prosthesis survival time was measured in the cases as the time between the date of primary surgery to the date of radiographic diagnosis of osteolysis (or date of revision surgery if the date of diagnosis was not known). In the controls, it was determined as the time between the date of primary surgery and the date of the most recent radiograph.

2.2.3 HO Identification and grading

Presence of HO was assessed on AP plain radiographs of not less than 1-year post THA. Cases comprised subjects with radiographic evidence of post-operative HO and were graded (0-4) using the Brooker classification as described earlier in this thesis.

2.3 Ein Bild Roentgen Analyse (EBRA) measurement of wear

2.3.1 Principles of EBRA

Polyethylene wear rate is an important predictor of osteolysis. It is therefore important to have reliable methods for measuring polyethylene wear radiographically. Good correlation between radiographic measurements of wear and measurements made on retrieved cups has been shown (Livermore et al., 1990). The EBRA method, developed at the University of Innsbruck, Austria, was originally used to assess acetabular cup and femoral stem migration (Wilkinson et al., 2002). The software was later modified allowing wear measurements to be made (Ilchmann et al., 1995). A grid of transverse and longitudinal tangents is drawn between prominent pelvic structures determining the position of the pelvis (Figure 2.1).

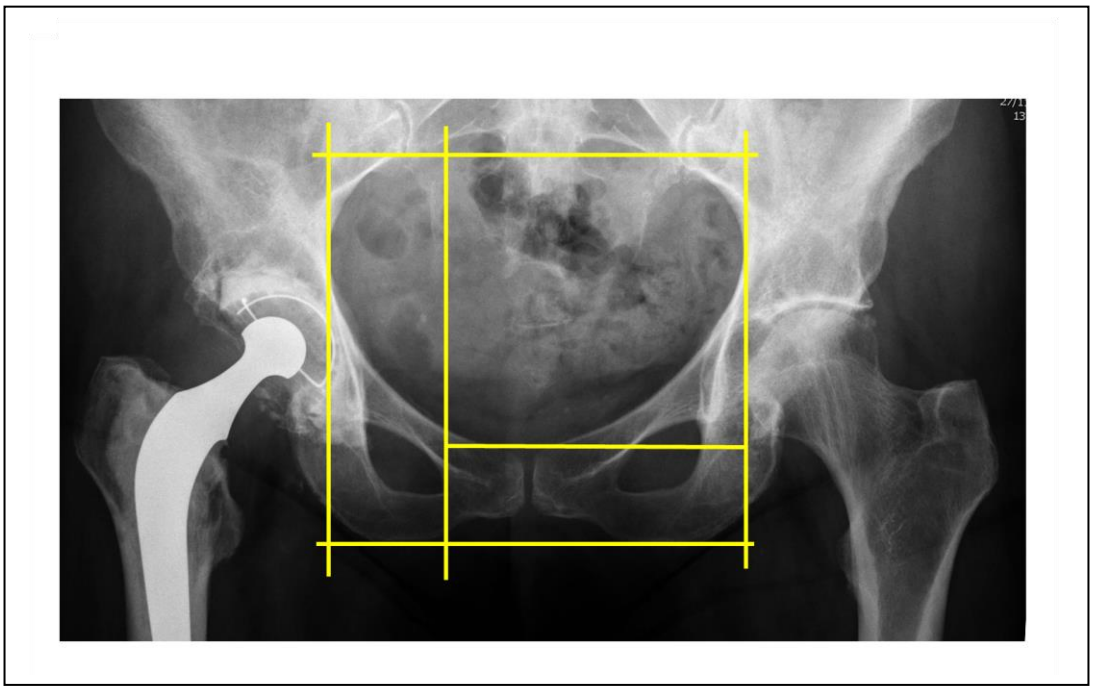


Figure 2.1 Reference lines of EBRA.

Following this, the software allows the size and position of the femoral head and the acetabular cup contrast wire to be measured. The software then measures the distance between the centre of the femoral head and the centre of the cup on the x and y axes, thereby calculating a vector of linear wear of the polyethylene cup by the femoral head.

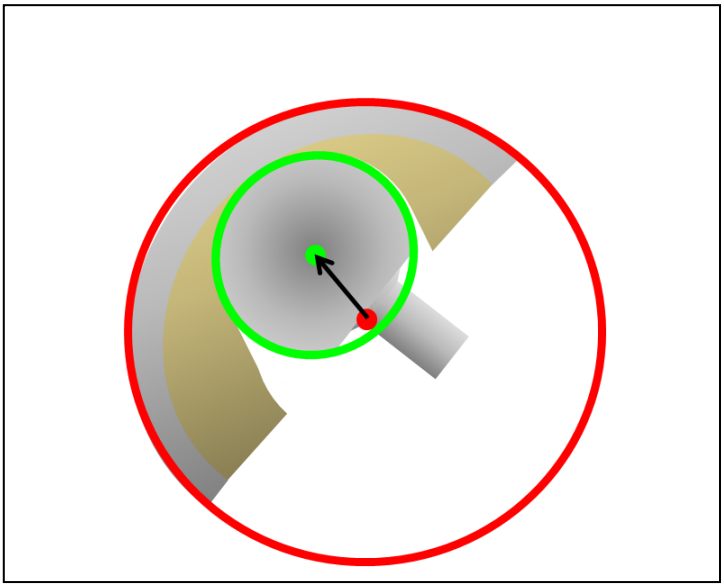


Figure 2.2 Schematic representation of the wear vector as measured by EBRA. It is calculated by measuring the distance on the x and y axis of the centre of the femoral head to the centre of the acetabular cup.

EBRA has good accuracy when compared to other plain radiographic measurement methods (Ilchmann et al., 1995).

2.3.2 Discovery cohort wear measurements

EBRA software was used to calculate wear measurements for patients with available and appropriate radiographs. For the osteolysis group, measurements were obtained from radiographs obtained immediately prior to revision surgery. The control group measurements were taken from the most recently available radiographs. Wear vectors were used as a covariate in the osteolysis susceptibility and time to failure

quantitative trait analyses. Subjects without radiographs were allocated interpolated wear rates calculated by the mean of wear rates in their group (cases or controls). As EBRA had been used to measure linear wear for subjects previously recruited in the Sheffield genetic hip study, this method was continued for our newly recruited subjects. Although we accept of lack of volumetric wear measurement using EBRA, we deemed linear wear an acceptable measure as the largest femoral head diameter was 29mm.

2.4 DNA Sampling

2.4.1 Sources of DNA

Genomic DNA can be extracted from a number of human sources where nucleated cells are present. Blood is the most commonly used source of DNA for genetic testing and it yields large amounts of high quality DNA. Its collection does, however, require study subjects to attend for an invasive procedure, and collection needs to be carried out by trained phlebotomists Large scale genetic epidemiological studies require large numbers of study subjects and therefore require more convenient methods of DNA sample collection. Saliva has been shown in various studies to be a viable alternative source of genomic DNA to whole blood for genetic epidemiological studies and whole genome scanning (Ng, 2005), (Rogers et al., 2007), (Nishista, 2009) (Rylander-Rudqvist et al., 2006). Both blood and saliva were used in this study. All DNA samples from extant subjects were extracted from whole blood and have been stored at -80^{o}C. Prospectively recruited patients have been recruited through postal invites and therefore all DNA samples have been extracted from saliva samples received through this route.

2.4.2 DNA collection

All saliva samples were collected using the Oragene OG-500DNA self-saliva collection kit (DNA Genotek, Inc., Ottawa, Ontario, Canada). This is an all in one system for the collection, stabilisation, transportation and purification of saliva. Each kit contains (1) Collection tube: The lid of which contains 2mls of DNA stabiliser and purifier (to prevent bacterial growth and degradation of DNA), (2) Sealing cap, (3) Instructions for use**.**

2.4.3 Sample collection

The instructions for saliva donation, as recommended by DNA Genotek, are:

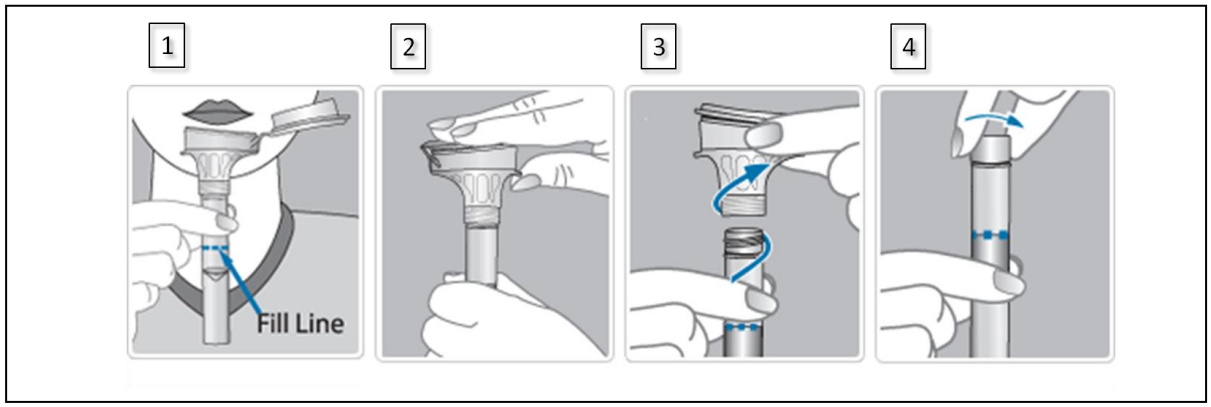


Figure 2.3 Collection instructions

1. *Spit into the collection tube until the level of saliva has reached the fill line after the bubbles have settled.*
2. *Close the lid pushing hard until a click is heard, thus breaking the seal releasing the DNA stabiliser and purifier.*
3. *Unscrew the funnelled lid from the collection bottle as it does not have a satisfactory seal for storage and transportation.*
4. *Tightly screw small cap provided onto the collection tube for transportation and storage.*

It is important not to eat, drink, smoke or chew gum for 30 minutes before providing a saliva sample.

2.4.4 DNA extraction

Equipment and reagents required:

1. *Water bath at 50^{o}C*
2. *Centrifuge that accommodates 15 mL tubes and capable of 4500 rpm.*
3. *Micro-centrifuge that accommodates 2 mL Eppendorf tubes and capable of 12,000 rpm*
4. *15 mL conical Falcon tubes*
5. *2 mL micro-centrifuge tubes*
6. *100% ethanol at room temperature*
7. *70% ethanol at room temperature*
8. *TE DNA storage buffer (10 mM TrisHCl, 1 mM EDTA)*
9. *prepIT^{TM}·L2P DNA purifier reagent*
10. *3 mL sterile pastette pipettes*

DNA extraction procedure (CIGMR SOP and Oragene DNA laboratory protocol)

1. *Mix saliva sample in collection tube by gently shaking to ensure sample is properly mixed with Oragene DNA solution.*
2. *Incubate sample at 50^{o}C in a water bath for at least 1 hour to maximize DNA yield and ensure nucleases are permanently inactivated.*
3. *Transfer the entire sample (4 mL) into the 15 mL Falcon tube noting the sample volume and colour.*
4. *Add 160 µL of prepIT DNA purifier reagent and mix by vortexing for a few seconds. The sample should become turbid as impurities are precipitated.*

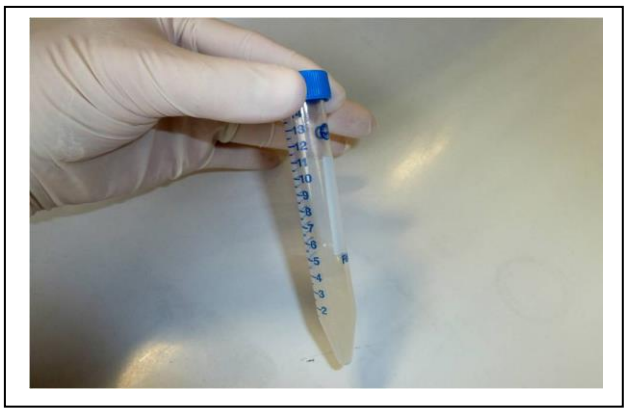


Figure 2.4 Turbid impurities

5. *Incubate in -20 ^{o}C freezer for 5 minutes, remove, shake and place back in freezer for another 5 minutes to assist impurity removal.*
6. *Centrifuge at room temperature at 4500 rpm for 15 minutes. Following this step a pellet of impurities will be seen at the bottom of the tube. The supernatant should be clear and will contain DNA in solution*

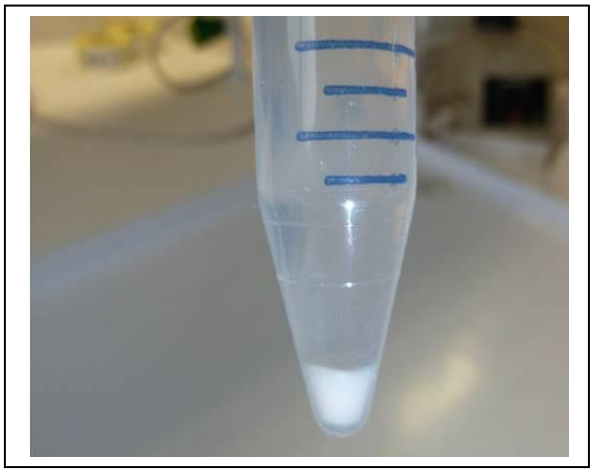


Figure 2.5 Impurity pellet

7. *Remove the supernatant with a 3 mL pastette pipette carefully avoiding disturbance of the pellet. If any disturbance of the pellet occurs the sample must be re-centrifuged.*

8. *Add 4 mL of 100% ethanol at room temperature and gently mix by inverting tube 10 times. The precipitated DNA should now become visible in the tube as a string or clot of white fibres.*

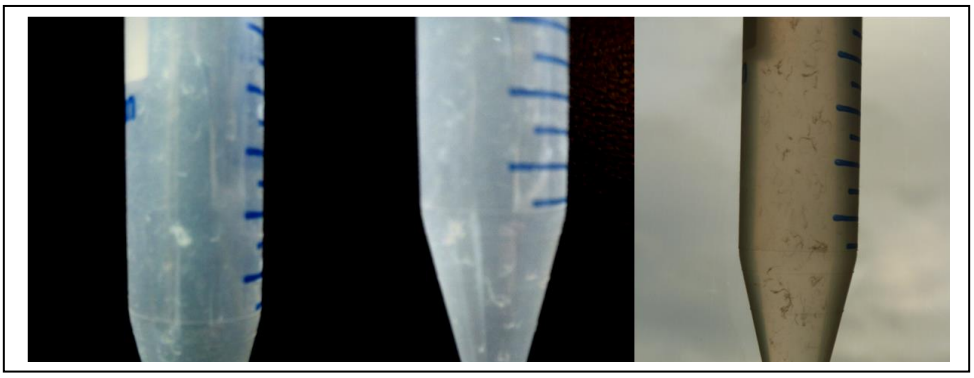


Figure 2.6 Visible DNA

9. *The sample should be left to stand at room temperature allowing full precipitation of DNA.*
10. *Centrifuge the sample at room temperature at 4500 rpm for 15 minutes. Following this step a pellet of DNA should be seen at the bottom of the tube.*
11. *Pour out the 100% ethanol (the DNA pellet should be stable at the bottom of the tube).*
12. *Add wash of 1 mL of 70% ethanol to ensure any residual inhibitors are removed.*
13. *Carefully removed the DNA pellet with the 70% ethanol and place into a 2 mL micro-centrifuge tube.*
14. *Centrifuge at 12000 rpm for 15 minutes*
15. *Pour out ethanol (again DNA pellet should be stable at bottom of tube) and leave tube to stand at room temperature for 20 minutes allowing remaining ethanol to evaporate and pellet to fully dehydrate.*
16. *Rehydrate pellet by adding 1 mL of TE buffer and vortex sample for 30 seconds. The sample should be placed on orbital plate shaker overnight to ensure complete rehydration of DNA.*

2.4.5 Sample storage

Appropriate storage of DNA samples is important to ensure optimum yield and quality. The storage factors that can potentially affect DNA are storage tube material, storage temperature, and freeze thaw cycles (Lee, 2010).

TE buffer is used in the rehydration and storage of extracted DNA. Cell lysis during DNA extraction can alter the pH of the solution. Tris buffers the solution keeping the pH to near neutral and protecting the pH sensitive DNA. EDTA helps inactivate nucleases by chelating the cations required for their normal function.

Isolated DNA may be stored at 4^{o}C for several weeks, -20^{o}C for several months and -80^{o}C for years (Holland et al., 2003). An EU workshop for Biobanks has recommended that DNA samples are frozen to prevent bacterial growth and reduce risk of sample loss through evaporation (EU_Workshop, 2003). The UK Biobank recommends storing samples at -20^{o}C to inhibit nucleases (UK Biobank 2004). A study by Smith et al comparing optimal storage conditions found that the highest quantity of DNA remained in the samples stored at -80^{o}C (Smith and Morin, 2005). Freeze-thawing causes double strand breakages of DNA (Grecz et al., 1980). DNA Genotek claim that Oragene DNA samples can be freeze-thawed 3 times without evidence of DNA degradation (www.dnagenotek.com).

The majority of our samples were stored initially at room temperature until DNA was extracted following manufacturer's recommendations. A small number of samples were stored at -20^{o}C prior to extraction. All samples were stored at -20^{o}C following extraction. Long term storage following processing was at -80^{o}C.

2.5 DNA quality control measures

2.5.1 Summary of control process

DNA samples included in the candidate gene study had previously undergone quality control measures using picogreen quantification and gel electrophoresis analysis. The

quality control process before whole genome scanning included visual inspection, volume measurement, Sequenom MassARRAY iPLEX assay quality control (QC), picogreen quantification and gel analysis.

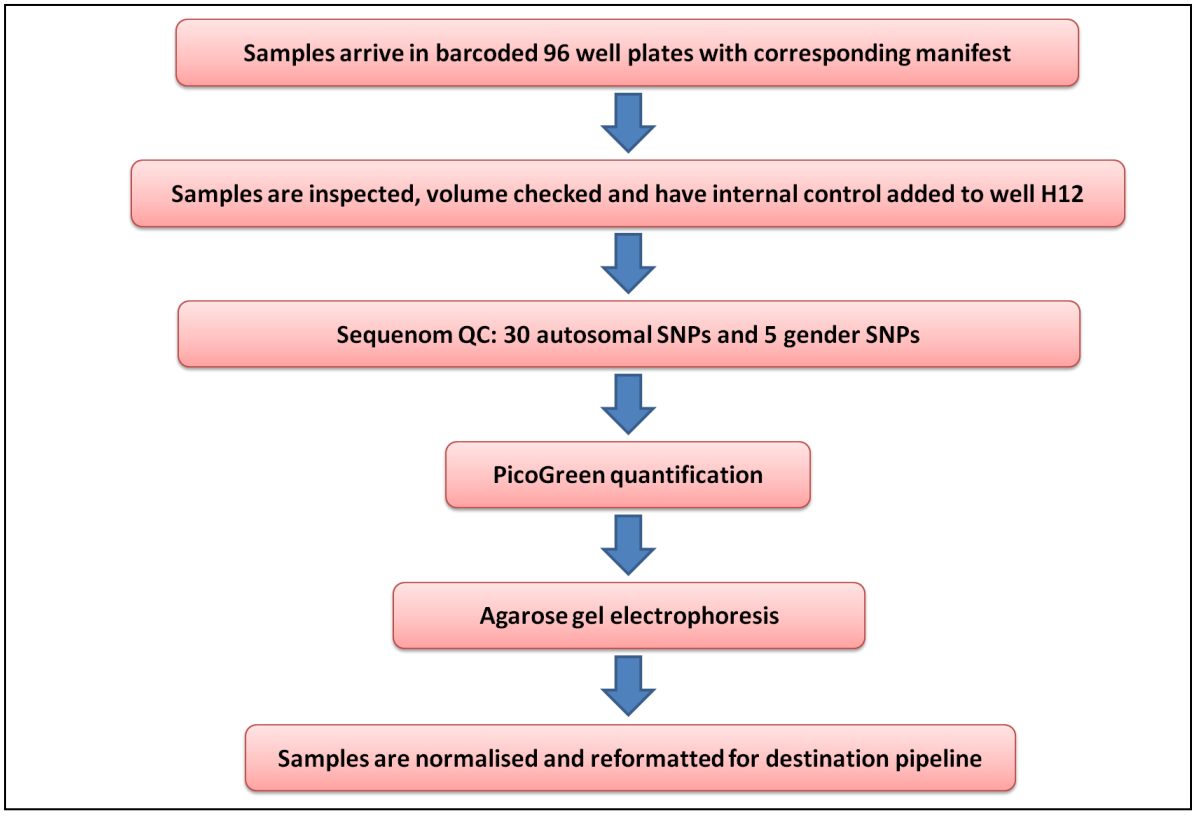


Figure 2.7 Quality control process used in these studies

2.5.2 Visual inspection

Each sample plate is inspected to ensure no damage had occurred on transportation and that no samples were of uncharacteristic colour.

2.5.3 Sequenom MassARRAY iPLEX assay quality control

The Sequenom MassARRAY iPLEX assay QC plex contains 30 autosomal SNPs and 5 gender SNPs. It allows the identification of plate rotations and ensures that the gender information in the accompanying manifest is reflected in the physical plate.

2.5.4 Visualisation by electrophoresis on an agarose gel

Principles of DNA gel electrophoresis

Electrophoresis is a method used to separate DNA strands based on their rate of movement while under the influence of an electric field (Sharp et al., 1973). Agarose is a natural polysaccharide purified from seaweed. The agarose gel used is porous and permits passage of the DNA strands. The negatively charged phosphate ions forming the DNA backbone are attracted to the positive electrode at one end of the gel tray initiating movement of the DNA strands. The rate of movement of a DNA fragment is directly proportional its size (Southern, 1979).

Equipment required:

1. *Agarose powder*
2. *Microwave*
3. *500µg/mL ethidium bromide*
4. *Gel casting tray and comb well divider*
5. *1X TE buffer*
6. *Power supply*
7. *Distilled water*
8. *Xylene cyanol loading dye*

Agarose gel electrophoresis method*:*

Ethidium bromide is mutagenic and should be handled with extreme caution.

1. *Make a 1% agarose gel by mixing 1g of agarose powder in 100ml of 1X TE buffer. Place in the microwave for 2 minutes until all powder crystals have dissolved. Add 2µL of ethidium bromide and swirl. Pour in to a gel casting tray with well divider in place and leave for at least an hour to cool.*
2. *Once cooled, transfer the solidified gel into a distilled water basin and pull out well divider to reveal wells.*
3. *Put 4µL of the DNA ladder standard into well number 1.*
4. *Mix 2µL xylene cyanol loading dye with 8µLof of each DNA sample to be tested. The xylene loading dye gives colour to the samples and also renders them denser that the buffer causing them to sink in the wells. Like DNA, it is negatively charged, and moves towards the positive electrode. It can therefore be used is used to assess how fast the gel is running.*
5. *Pipette each 10 µL DNA sample into each well.*
6. *Slide the top on the water basin and run gel for 30 minutes.*
7. *Place gel into UV reader*

Electrophoresis for DNA extraction technique validation

Fourteen test samples of volunteer DNA were extracted from 2ml saliva samples. Test samples were run on an electrophoresis agarose gel in conjunction with a 1Kb DNA standard ladder in order to validate the extraction method, (figure 2.8). All samples showed high molecular weight DNA (greater than 10Kbp), with no evidence of degradation.

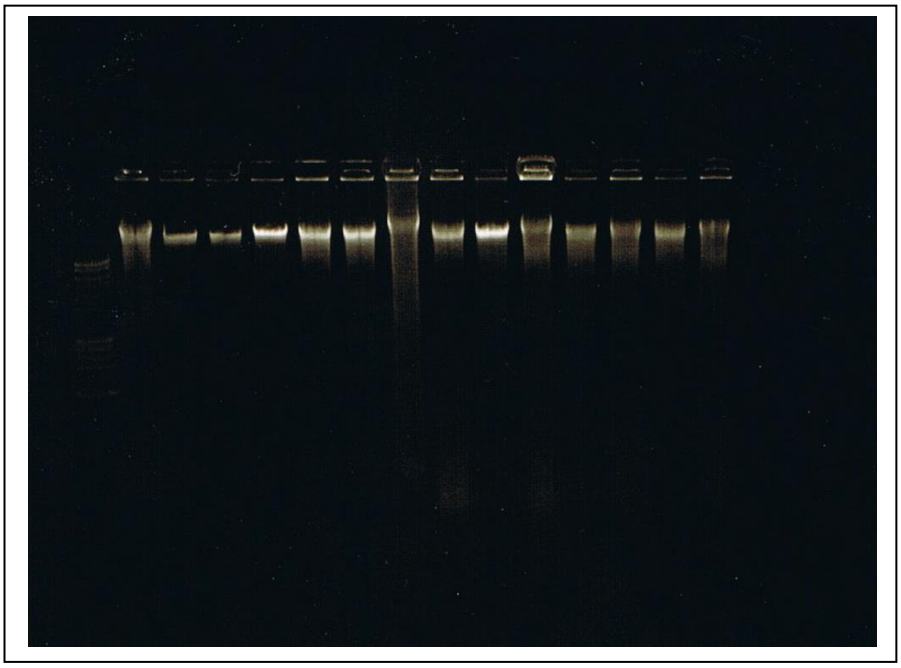


Figure 2.8 Agarose electrophoretic gel of 14 test samples of showing extracted high molecular weight DNA.

Electrophoresis as part of Sanger quality control measures:

300ng of each sample was separated via gel electrophoresis on a 2% gel. DNA fragments were identified using ethidium bromide. Degraded samples, identified as missing or very faint bands, were excluded from the study.

2.5.5 DNA Quantitation

Determining DNA concentration extracted from human tissue samples is an important quality control step for the success of genotyping assays. Commonly practiced methods include spectrophotometry (nanodrop) and fluorometry (picogreen/ Qubit fluorometry).

Principles of PicoGreen quantitation

The main disadvantage of spectrophotometry is the inability to distinguish between double stranded DNA (dsDNA), single stranded DNA (ssDNA) and RNA. In addition, the presence of proteins and phenols in the sample can alter the UV readings (Blotta et al., 2005). Spectrophotometry tends, therefore, to over-estimate the concentration of double stranded DNA in a sample.

The introduction of cyanine dyes improved the specificity of nucleic acid quantitation. PicoGreen is a sensitive fluorochrome that selectively binds dsDNA. When bound to dsDNA, fluorescence enhancement is very high and due to its specificity, presence of ssDNA and RNA has little effect on the quantitation result (Ahn S. Nucleic acids research 1996). The concentration of dsDNA in a test sample can be measured by plotting fluorescence on a linear range determined by known standard DNA concentration.

Picogreen quantitation as part of Sanger quality control measures

Picogreen quantitation was carried out by the genotyping team at the Wellcome Trust Sanger Institute (WTSI), Cambridge. Double stranded DNA (dsDNA) concentration was measured using the Invitrogen Quant-iT PicoGreen dsDNA assay. Samples with a concentration of less than 35 µL were excluded from the GWAS study.

Principles of Qubit Fluorometry

In addition to picogreen quantitation carried out at the WTSI, we undertook prior quantitation of suspended DNA samples using Qubit™ dsDNA BR Assay Kits and the Qubit™ 2.0 Fluorometer (Invitrogen Ltd, Paisley, UK). Qubit fluorometry uses the

same principles as picogreen. The dyes used selectively bind dsDNA. This binding alters the fluorescence of the dye increasing its fluorescence by several orders of magnitude. The manufacturers have shown that the QubitdsDNA assays have comparable accuracy to Invitrogen Quant-iT PicoGreen dsDNA assay.

Qubit™ 2.0 Flourometric Quantitation method:

1. *Pre-label the 0.5ml PCR Tubes (Axygen PCR-05-C tubes, Part No. 10011-830, VWR) with the Patient ID of the samples you in intend to quantitate. Label 2 additional tubes for the DNA standards 1 and 2. Note: The Sample tubes should always be kept in the same linear order as your spreadsheet; this will help with re-entering the values into the spreadsheet later on.*

2. *Prepare the Working Solution by mixing the Qubit dsDNA BR Reagent with the Qubit dsDNA BR Buffer in a 1:200 ratio. Invert 10 times to mix. The exact amounts needed to make up the working solution can be calculated using the following formulae.*

   - *200 x* n = t
   - t - n = b

   Where *n* = the number of samples to be quantitated + 2.
   Also, *n* = the amount of Qubit dsDNA BR Reagent needed (µl)
   b = *the amount of Qubit dsDNA BR Buffer needed (µl)*
   t = *the total volume of the working solution*

3. *Load 190µl of the Qubit working solution into each of the two standards tubes and load 10µl of the corresponding standard into each tube.*

4. *Load 198µl of the Qubit working solution into each of the tubes intended for the samples. Try to avoid creating bubbles. If processing a large number of samples in one run (>100), it is recommended that you load the working solution in batches of 50 at a time, this reduces the risk of contamination and evaporation from the assay tubes.* ***Note:*** *When using the Fluorometer you can load anything between 1µl and 20µl of sample into each tube as long as the final assay tube volume is*

made up to 200µl, therefore the amount of Qubit Working solution needed in each tube depends on the amount of sample you want to use. However, when quantitating following DNA extraction using Oragene -500 DNA Self Collection Kits and ethanol precipitation, the optimal amount of sample needed is 2µl, as this way you are less likely to find that the concentration of DNA in your assay tube is too high or too low for the fluorimeter to read.

5. *Load 2µl of each sample into its corresponding tube, vortexing the stock tube briefly prior to each load. Try to avoid creating bubbles. Pipetting accuracy is very important.*

6. *Once all of the assay tubes have been made up to 200 µl with the working solution and the DNA sample, shake the samples to mix and remove any bubbles.*

7. *Allow the Assay tubes to incubate at room temperature for 2 minutes.*
   ***Note:** After this incubation period, the fluorescence signal is stable for 3 hours only at room temperature.*

8. *Turn on the Qubit 2.0 Fluorometer and select DNA, then dsDNA Broad Range*

9. *The fluorimeter will prompt you to read new standards, follow the instructions for reading these standards. New standards should be set every time you perform a batch of quantitations. Always check the standards with the 'Check Stds.' Tab, one should be low concentration with a value of approximately 100, and the other should be high concentration at around 10,000.*

10. *Quantitate the first sample as prompted by the fluorimeter, this involves placing the sample into the well, closing the lid, then pressing 'read'. You should always remove any bubbles prior to reading by gently flicking the assay tube. Try not to hold the tubes in your hand for too long as raising the temperature will affect the reading.*

11. *After the first sample has been read, press 'Calculate Stock Conc.'. Select the appropriate Sample volume from the wheel (in our case 2 µl) and this will calculate the concentration of the sample in your stock tube. Choose the units 'ng/µl'. This will help with entering the data into your spreadsheet.*

12. *Proceed to read all of the samples. It is very important that the order is kept the same and sample readings aren't missed or duplicated as the order that the samples are read are the only way of matching them back to their corresponding patient ID numbers.*

13. *Once all read, double check that you have the correct number of readings, then save the data to a memory card.*

14. *The total DNA yield for each sample was calculated with the following formula: v (c x d) where v = stock volume, c = concentration of DNA in assay tube, d = dilution factor.*

2.6 DNA Genotyping

2.6.1 Candidate gene approach

SNP Tagging

Tagging SNP selection was performed using Hapmap Gene Browser (release #24, phase 1 and 2 – full dataset, www.hapmap.ncbi.nlm.nih.gov) and Haploview software (v4.2, www.broadinstitute.org/haploview/haploview) using a pairwise tagging approach (r^2=0.8). Common variants (MAF≥0.05) within the gene of interest, and extending 5Kb upstream and 2Kb downstream to include variants within the adjacent regulatory flanking sequences, were tagged using this approach.

FASTA file configuration

Once tagging SNPs were identified using the HapMap *Gene Browser (release #24, phase 1 and 2 – full dataset, www.hapmap.ncbi.nlm.nih.gov)* was used to generate a FASTA file for genotyping. A FASTA file consists of the variant of interest and its

flanking sequence which is used on the probe design to allow hybridisation and identification of the variant in question.

Principles of KASPar assay genotyping

Genotyping for the candidate gene study described in chapter 4 was outsourced to KBioscience Ltd (Hoddeston, Herts, UK) using competitive allele specific PCR (KASP). KASPar is run initially as a standard polymerase chain reaction (PCR). The primer mix consists of 2 allele specific oligonucleotide forward primers and a common reverse primer. During the first round of PCR the allele-specific primer binds to the region directly upstream of the SNP of interest while the 3' end of the primer binds to its complementary base on the SNP. Simultaneously the reverse primer binds the complementary strand. The KASP Taq DNA polymerase extends each strand thereby copying the region of DNA containing the SNP of interest. There are two oligonucleotides in the assay mix which are fluorescently labelled at their 5' ends. These sequences are identical to the tail sequence of the allele specific primers and are labelled with either FAM or VIC. During the annealing stage the fluorescently labelled oligonucleotides binds to its complementary tail sequence and is further extended by the KASP Taq. Further PCR amplification produces more fluorescently labelled templates. The fluorescent signal generated allows differentiation of heterozygosity or homozygosity for an allele.

2.6.2 Principles of high throughput genotyping

A DNA microarray is a solid slide which is “arrayed” with multiple microscopic spots, known as features. Each spot contains picomoles of oligonucleotides of specific DNA sequences known as probes (Manoj Kumar, 2009). These probes are attached to the solid surface by a covalent bond. Each probe is used to hybridize a complimentary DNA strand allowing a specific locus (allele) to be examined through fluorescence based detection (Schena et al., 1995).

A genotyping array must have the ability to interrogate loci associated with the phenotype of interest. This may be either directly, by including genetic variants responsible or indirectly, by including SNPs in sufficient linkage disequilibrium with the causative variants (Carlson et al., 2003). Data from the international hap map project allows the indirect approach to be implemented and forms the basis for SNP selection of genotyping arrays.

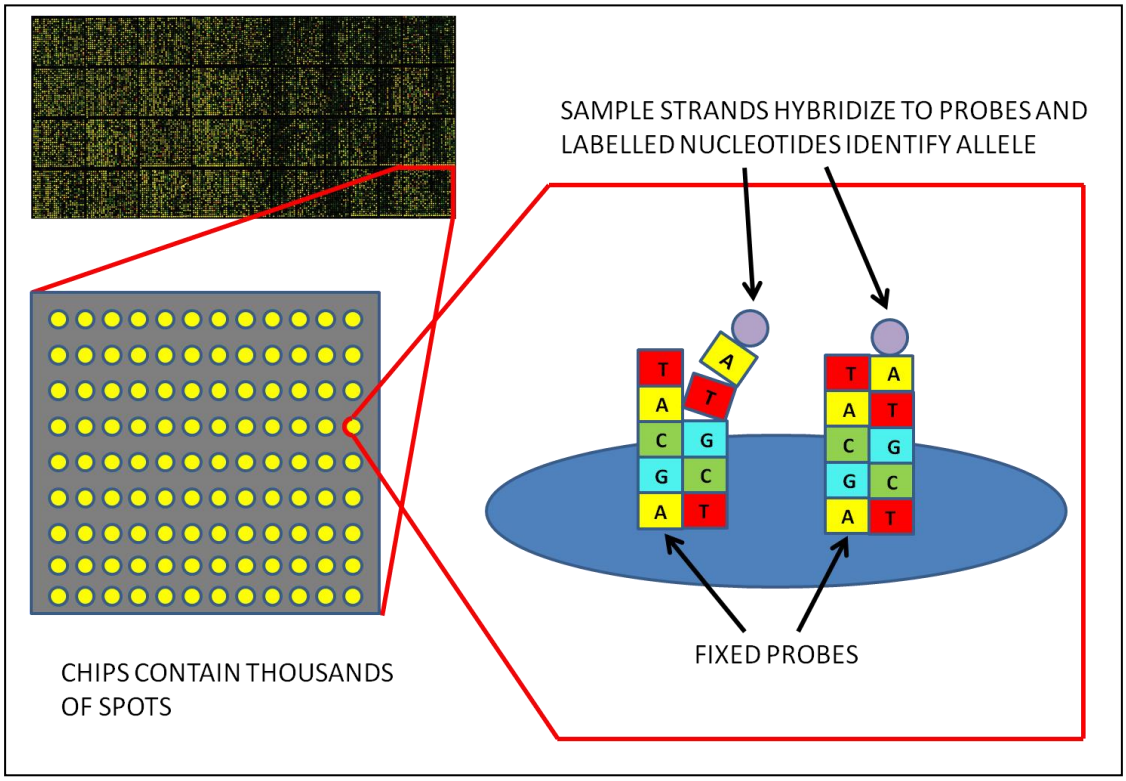


Figure 2.9 Principle of microarrays

2.6.3 Microarray chip selection

There are a number of commercially available microarray chips, each providing better coverage in some genomic regions than others (Saccone). Ideally one would choose the chip that best matches the study population. The most popular criterion for this is

global coverage, defined as the fraction of common SNPs tagged by the SNPs on the chip (Li et al., 2008).

2.6.4 Discovery cohort GWAS genotyping *Illumina Human610-Quad BeadChip:*

Genotyping for the discovery cohorts described in chapters 5 and 7 was carried out using the Illumina Human610-Quad Beadchip. It uses tag SNPs to the coverage of 624,000 randomly selected SNPs. It provides 89% coverage of the Caucasian European (CEU) HapMap loci (MAF>5%, LD r^2>0.8) using tags based on the Caucasian HapMap population (Illumina DNA analysis, www.illumina.com).

2.6.5 Replication cohort GWAS genotyping *Sequenom MassARRAY iPLEX assay MassARRAY iPLEX platform:*

The replication cohort genotyping was performed using the Sequenom MassARRAY iPLEX assay Sequenom MassARRAY iPLEX assay MassARRAY iPLEX assay and the MassARRAY® System (Agena Bioscience Inc, Hamburg, Germany). Sequenom MassARRAY iPLEX assay's MassARRAY software designs extension primers for SNPs of interest. It is able to perform high throughput genotyping using a multiplexed PCR approach using a single extension primer and is capable of >100,000 genotypes per day (Gabriel et al., 2009).

2.7 Genome data analysis

2.7.1 PLINK

PLINK (version 1.07) is an open-source whole genome association analysis toolset which can be download from the psychiatric and neurodevelopmental genetic unit website affiliated with the Harvard Medical School (www.pngu.mgh.harvard.edu/~purcell/plink). It was developed by Shaun Purcell at the Centre for Human Genetic Research (CHGR), Massachusetts General Hospital and the Broad Institute of Harvard (Purcell et al., 2007). It was designed to deal with the many challenges of whole genome study analysis. It has the ability to manage with large GWAS datasets. It can deal with confounding caused by population stratification and non-random genotype failure. It has the ability to perform a number of association analyses allowing for covariates. It can also utilise common SNP panels to examine chromosomal regions in order to identify unexpected relatedness as a cause for multiple rare variants. It can be run on an MS-DOS platform as a command line program. It focuses purely on the analysis of genotype/ phenotype data in the form of PED and MAP files.

PED Files

The PED file is a white space delimited file and contains phenotypic and genotypic data. The first 6 columns are mandatory and contain: Family ID, Individual ID, Paternal ID, Maternal ID, Sex, Phenotype.

No blanks can be left in the file. If any data is unknown (such as sex or paternal/ maternal ID) they are recorded as 0. Phenotypic data must be entered as either a binary or continuous (quantitative trait) value. PLINK automatically recognises which.

The following columns contain the allele data at each locus for each individual.

MAP Files

The MAP file is also a white space delimited file. It contains the genetic information. Each line describes a single marker and contains 4 columns: Chromosome, Reference SNP (rs) number, Genetic distance (morgans), Base pair position (bp units).

Each marker must represent those in each genotypic column on the PED file. The minimum data required on a MAP file for an analysis to be carried out is the rs number. Data for each rs number is available on the HapMap website (www.hapmap.ncbi.nlm.nih.gov).

PLINK quality control measures

Prior to association analyses sample and SNP quality controls measures were undertaken.

Missingness

The missingness command identifies missing data from the MAP and PED files. Individuals or SNPs with missing data are excluded from analyses.

Minor allele frequency (MAF)

A list of minor allele frequencies can be generated for each SNP. A MAF threshold can be selected during association analyses. Variants with MAF <0.05 were not included in analyses.

Hardy-Weinberg Equilibrium

The Hardy-Weinberg principle states that for a population, the allele and genotype frequencies remain constant unless less there is non-random mating, mutations,

evolutionary selection, limited population size, migration (Hardy, 1908). Deviation from this equilibrium may potentiate bias in allele frequencies in a population and checking Hardy-Weinberg equilibrium is now common practice in association studies (Wigginton, 2005).

The Hardy Weinberg equation can be used to estimate the frequency of alleles in a population.

For a population in genetic equilibrium: $p^2 + 2pq + q^2 = 1$

Where p = frequency of the dominant allele and q = frequency of the recessive allele.

A list of genotype counts and Hardy-Weinberg test statistics can be generated for each SNP using PLINK. A p value threshold is calculated as per Bonferroni (0.05/ number of markers). Any marker with a p value below 0.0001 was excluded from analyses.

Association analyses

Standard case/control association analysis can be carried out using χ^2 test. In order to adjust for covariates a linear regression model is used. The minor allele frequency threshold of >0.05 and confidence interval of 95% was adjusted and implemented in each analysis. For continuous phenotypic data a linear regression model analysis for quantitative trait loci was carried out. Again, covariates were adjusted for.

2.7.2 GWAS Power calculation

The power calculation for our GWAS was made using Quanto v1.2 (Gauderman and Morrison, 2006). Our discovery analysis had ≥80% power to detect a variant with risk allele frequency 0.35 with an allelic OR of 1.65. The replication analysis had ≥80% power to detect a variant with risk allele frequency of 0.45 and allelic OR of 1.45. We attempted to reduce the impact of modest power by looking only at common variants (MAF ≥0.05) but consequently we are unable to predict the importance rarer variants may have on osteolysis.

2.7.3 SNPTEST whole genome association analyses

SNPTEST

SNPTEST is an open-source program for single SNP association in genome wide analyses. The software is part of the Oxford University genome-wide analysis software suite (OGWASS) and can be downloaded from https://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest. To use SNPTEST all commands were created using the UNIX common operating system. A knowledge of the appropriate UNIX command lines was required to run this software and create the shell scripts required for analyses. Due to the large volume of data and statistical tests run in GWAS all analyses were run using the Sanger Institutes platform load sharing facility (LSF) known as "The Farm." This workload management platform uses multiple LSF clusters to efficiently run association analyses and other data-intensive processes.

Sample and SNP Quality Control

We undertook post quality control checks at sample and SNP level. Samples were excluded if they failed gender check, had a call rate <95%, or had excess homozygosity or heterozygosity out with 3 SD of the mean (using autosomal SNPs) Genome-wide pair-wise identity by descent (IBD) for each sample was used to identify duplicates and related samples. We removed any sample with a $\pi\hat{} > 0.2$. Ancestry was validated by multidimensional scaling (MDS) and principle component analysis (PCA) using Goldsurfer (Pettersson et al., 2008) after autosomal data from the discovery cohort was merged with HapMap 3 data (The_International_HapMap_Consortium, 2007). Samples identified by genotype to be from individuals of non-UK European ancestry were excluded from association analyses. SNP QC was carried out on autosomal SNPs. SNPs were excluded if they had a minor allele frequency (MAF) ≥5% and a call rate <95%, or a MAF <5% and call

rate <99%. Monomorphic SNPs were excluded. SNPs with an exact Hardy Weinberg Equilibrium (HWE) p<0.0001 were excluded.

1000 Genome Imputation

Genotype imputation is the process of predicting genotypes which haven't been directly assayed. This is an in silico process which uses data from a reference panel to infer missing genotype data into a study dataset. This increases the number of SNPs that can be tested for association thereby increasing power (Spencer et al., 2009). It also has the potential of improving fine mapping of an associated region (Marchini and Howie, 2010). We imputed the GWAS dataset using the European reference panel from the 1000 Genomes Project (Dec 2010 phase I interim release) (1000 Genomes consortium et al, 2010), and using IMPUTE2 (Marchini et al., 2007).

SNP Association Analysis Plan

1. *Create a folder for association analyses which includes the following files: i) Sample file (needs to be a space delimited text file), ii) Gen file (separate file for each chromosome, iii) snptest program.*
2. *Create shell script to run snptest association analysis.*
3. *The analysis will generate 3 output files per chromosome (text, log, and error). Check that all error files are empty and check in log files that correct number of samples and covariates has been included in analysis.*
4. *Create a new folder named "snptest output" and copy all text output files into folder*
5. *Use the merge perl script to merge all files into a single output file.*
6. *Use the filter perl script to filter merge output file (change MAF and info scores to desired threshold on script before running).*
7. *Using R scripts, create Manhattan and QQ plots for the filtered output file*

8. *In order to identify low p value snps, run the perl filter script again but this time include a p value threshold of 9 x 10^{-5}.*
9. *Open new file in excel spreadsheet and sort in p value order (smallest to largest).*
10. *Sort by chromosome order and add sorting level for chromosomal position (smallest to largest).*
11. *Add new column and name "signal."*
12. *Looking at the chromosomal positions group snps into signals within 300Kb of each other.*
13. *Now resort by signal and add sorting level for p value. The lowest p value snp in each signal is the index snp.*
14. *Using Locuszoom software, create regional association plots for each signal.*

Whole genome association analyses

Osteolysis susceptibility case-control analysis and a quantitative trait locus (QTL) association analysis for time to prosthesis failure was undertaken on >10 million variants under the additive model using method score implemented in SNPTESTv2. Phenotype data was normalised where required and we performed a linear regression to adjust for age, gender, fixation method, osteolysis-free survival time. Residuals were then transformed to z-scores. Association analyses were undertaken with and without and polyethylene wear as a covariate.

An HO susceptibility case-control analysis and an evaluation of disease severity using a binomial analysis of Brooker grades 1 and 2 vs grades 3 and 4 in the cases was undertaken under the additive model using the score test in SNPTEST v2.3.0 (University of Oxford, Oxford, UK). Association analyses were adjusted for age and gender as these have previously been described as known risk factors for HO (DeLee et al., 1976, Hierton et al., 1983). SNPTEST output files were further filtered to exclude SNPs with a frequentist info score <0.4 and a minor allele frequency (MAF) <0.05.

2.7.4 Osteolysis replication analyses

Index SNPs from signals with $p<9x10^{-5}$ were prioritised for replication. Genotype-calling intensity plots were examined and SNPs with poorly clustering plots were not taken forward. Only associating SNPs with an imputation information score >0.4 and a minor allele frequency (MAF) >0.05 were progressed for the replication association analyses.

Samples failing gender check or with call rate <80% were excluded. SNPs were removed if their call rate was <80% or with HWE $p <10^{-4}$. Association analyses were undertaken using logistic regression in PLINK v1.07. Z-standardised residuals were generated on phenotype data requiring normalisation and regressed on age, gender, implant type, fixation method and osteolysis-free survival time.

2.7.5 Meta-analysis

Genome Wide Association Meta-Analysis (GWAMA)

GWAMA is an open source software developed by the Wellcome Trust Centre for Human Genetics, University of Oxford, which was designed to perform meta-analysis for GWAS (Magi and Morris, 2010). The software along with source files and sample data can be downloaded at http://www.well.ox.ac.uk/GWAMA. The following information is required for each SNP to be meta-analysed: (i) the marker identifier; (ii) the allelic effect estimate and corresponding standard error (or an allelic odds ratio and 95% confidence interval in the case of a dichotomous trait); and (iii) the allele for which the effect has been estimated and the complimentary non-reference allele. GWAMA aligns all studies to the same reference allele at each SNP and meta-analysis is performed for each SNP by combining allelic effects weighted by the inverse of their variance.

Summary statistics from both the osteolysis discovery and replication analyses were combined and meta-analysed using the fixed-effects model implemented in GWAMA v2.1 (Magi and Morris, 2010).

CHAPTER 3

STUDY POPULATIONS

3.1 Overview

This chapter outlines the subject recruitment strategies, inclusion criteria, and population demographics for the UK populations studied in this thesis. The subject recruitment and population demographics for the Norwegian replication population are described separately in chapter 6.

3.2 Discovery Population

3.2.1 Inclusion criteria

All subjects recruited in the GWAS discovery cohort comprised unrelated men and women recruited in the United Kingdom and of North European ancestry who had previously undergone primary cemented or hybrid (cemented femur) THA for idiopathic osteoarthritis. All subjects have received a prosthesis using a metal-on-conventional polyethylene bearing couple.

3.2.2 Exclusion criteria

Any subject not of North European ancestry was excluded to prevent genetic admixture. Patients with alternative bearing couples such as metal on metal, ceramic on polyethylene, or ceramic on ceramic were also excluded. This ensured that the particulate exposure hazard was similar in all cases. Fully cementless implants were also excluded. This was because subjects already recruited through the Sheffield genetic hip study had all undergone fully cemented or hybrid THA and we wanted to reduce the number of variables in our complete cohort. Any subject who had undergone THA for a diagnosis other than primary osteoarthritis, such as inflammatory arthropathy or neck of femur fracture, was excluded. This exclusion included subjects with an indication of osteoarthritis secondary to another pathology such as avascular necrosis, developmental hip dysplasia or Perthes' disease.

3.2.3 Case definition

Osteolysis

The cases comprised subjects with radiographic evidence of aseptic loosening or osteolysis affecting either the acetabular or femoral prosthetic component, as defined in the previous chapter, whether the prosthesis had been revised or not. Revisions for indications such as dislocation, or infection were excluded; as were revisions of fully cementless implants and revisions for liner wear in the absence of aseptic loosening or osteolysis.

Heterotopic ossification

Cases comprised subjects with radiographic evidence of post-operative HO following THA and were graded (0-4) using the Brooker classification (see Figure 1.14).

3.2.4 Control definition

Osteolysis

The controls comprised subjects who had primary THA for idiopathic osteoarthritis ≥7 years previously, were asymptomatic from the replaced hip, and free from documented complications relating to osteolysis or aseptic loosening following primary surgery and with radiographic evidence of being osteolysis-free.

Heterotopic ossification

Controls comprised subjects who had no evidence of HO on plain AP radiographs of the pelvis taken not less than 1 year following primary THA.

3.2.5 Subject recruitment

All UK subjects were recruited as part of 2 research protocols approved by a research ethics committee:

1. Samples recruited under the ethics approval of the Sheffield Musculoskeletal Biobank (Oxfordshire Research Ethics Committee, REC reference number 10/H0606/20, 28th May 2010; approval letter in appendix A). This approval was renewed on 24th April 2015, REC reference number 15/SC/0132; approval letter in appendix B)
2. Samples recruited under the ethics approval of the arcOGEN study (Oxfordshire Research Ethics Committee, REC reference number 07/H0606/150, 3rd January 2008; approval letter in appendix C).

Each subject provided written consent for DNA samples to be collected for research purposes.

Sheffield Musculoskeletal Biobank Samples

Subjects under Biobank ethics approval comprise 2 extant cohorts, and one prospective collection:

1. Sheffield Genetic Hip study cohort
2. Wrightington Genetic hip study cohort

3. Newly recruited patients from Sheffield Teaching Hospital NHS Foundation Trust, Sheffield

Sheffield Genetic Hip Study Cohort

A total of 684 patients were recruited between April 2000 and April 2006 as part of a series of case-control candidate gene studies looking at the association between osteolysis and genetic variants. All subjects were unrelated Caucasian men and women of North European origin having previously undergone primary cemented THA with a metal-on-conventional polyethylene bearing couple for idiopathic osteoarthritis.

631 patients from this cohort with available DNA were included in the case-control candidate gene study discussed in chapter 4.

A total of 602 subjects with available DNA from this cohort were included in the GWAS study. Three hundred and seventy-five subjects from this cohort have already undergone GWAS genotyping as part of the arcOGEN study (discussed below). A further 226 DNA samples extracted from whole blood from subjects in this cohort, and stored in the Sheffield Musculoskeletal Biobank, were forwarded for GWAS genotyping. Two hundred and ten (93%) passed quality control. Following pre-genotyping QC, it was noted that one of these patients had already undergone genotyping as part of arcOGEN. The remaining 209 were genotyped. Of those who failed QC, 2 had too low a concentration, 9 were degraded samples unsuitable for genotyping and 5 had a gender fail.

Wrightington Genetic Hip Study Cohort

312 patients were recruited from Wrightington Hospital between August 2002 and August 2006 for a series of case-control candidate gene studies looking at the

association between osteolysis and genetic variants. All subjects were of European Caucasian origin and had undergone total hip arthroplasty with a Charnley monoblock femoral stem and either a Charnley or Ogee flanged conventional polyethylene acetabular cup. Only patients with a primary diagnosis of osteoarthritis were included.

One hundred and thirty patients from this cohort were suitable for inclusion in this study having met the inclusion criteria and having available DNA samples. All included subjects had extracted DNA samples from whole blood stored at the Sheffield Musculoskeletal Biobank.

One hundred and twenty-five (96%) passed quality control and underwent genotyping. Five failed on gender quality control. Of those who passed QC, 49 were male and 40 had either undergone revision surgery for aseptic loosening or had radiographic evidence of osteolysis.

Newly recruited Subjects

Patient arthroplasty databases from Sheffield Teaching Hospitals NHS Foundation Trust were examined using the above inclusion and exclusion criteria. Subjects were then cross referenced with the Patient Focused Information (PFI) database to check they were still alive and had available contact details. Four hundred and eighty-three patients were identified and invited to participate in the study. Three hundred and thirteen patients replied to the invitation (response rate of 65%). One hundred and sixty-three (34%) did not respond after a reminder letter. Of those who replied, 265 (55%) agreed to donate a saliva sample to the Biobank. All responders who agreed to participate in the study were sent a consent form, a questionnaire (appendix D) and an Oragene OG-500 saliva collection kit with a return mailer kit. Two hundred and twenty subjects returned the saliva kit with a donated sample (completer rate of 45%).

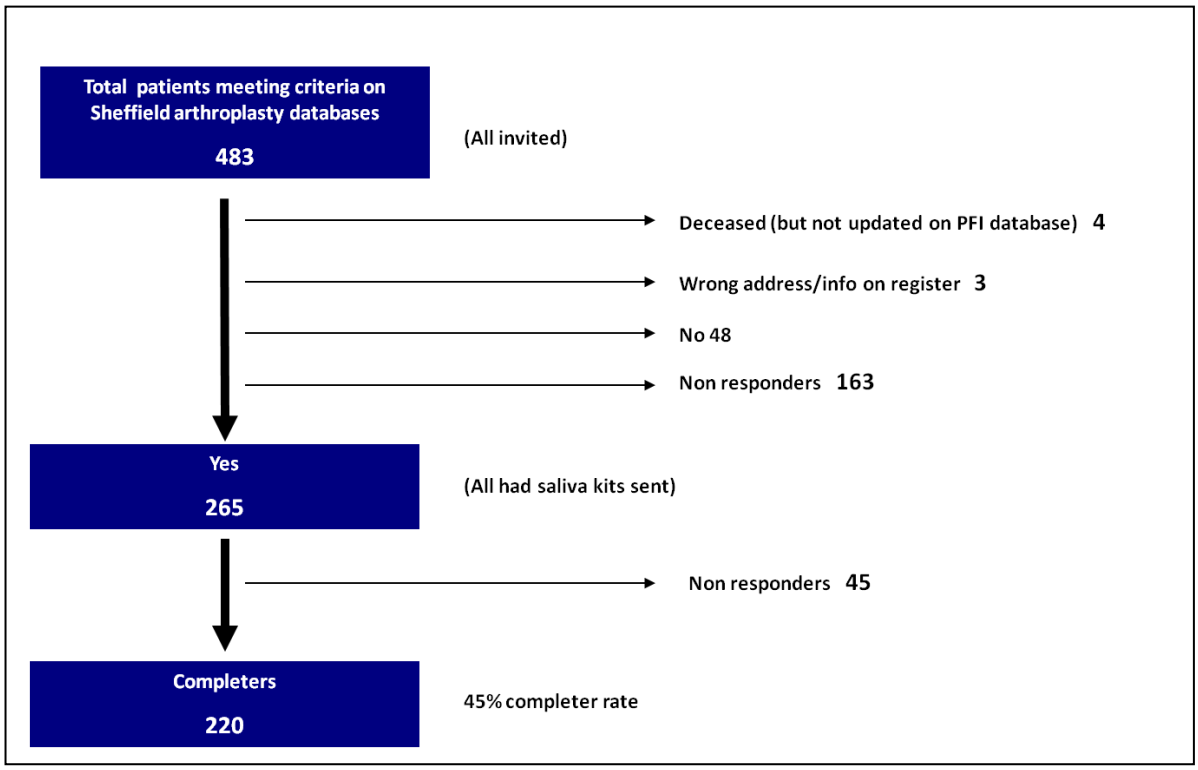


Figure 3.1 Response rates of Sheffield recruitment

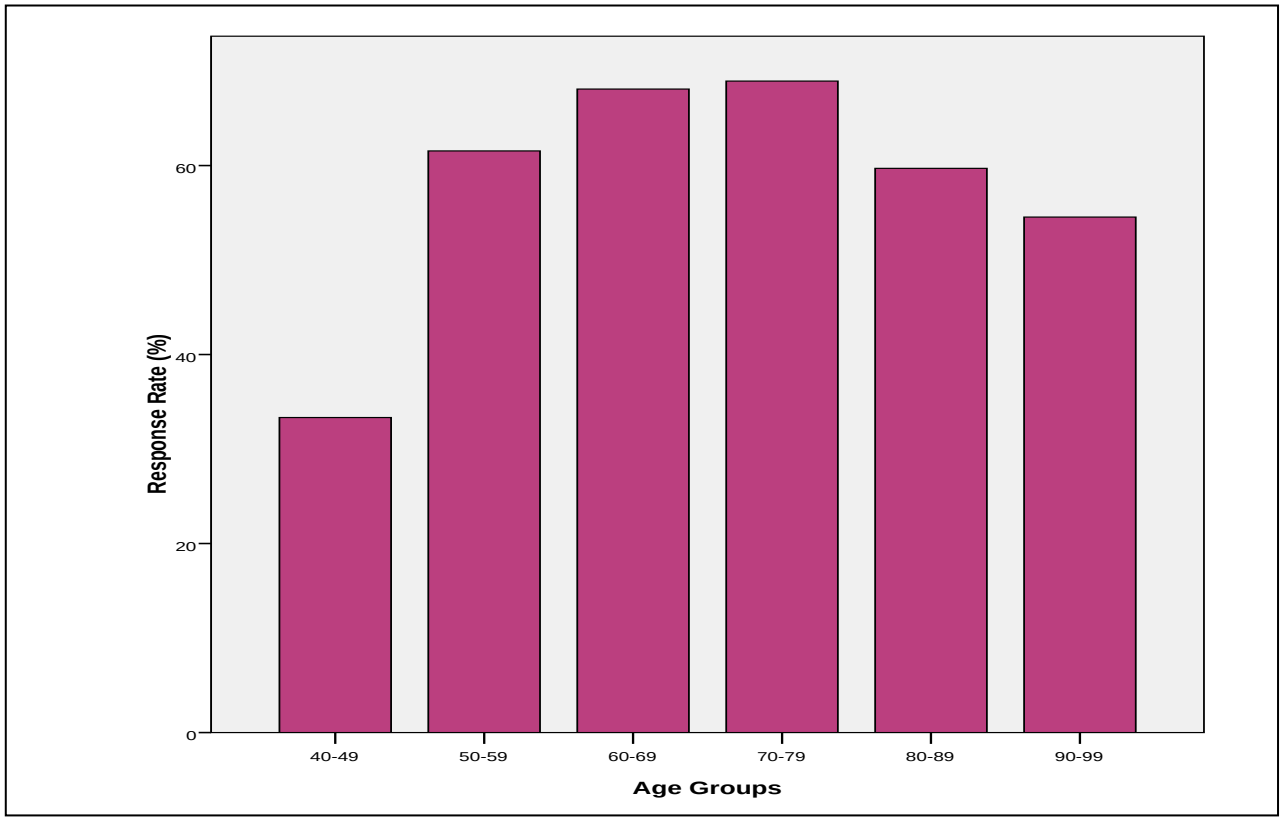


Figure 3.2 Bar chart outlining response rates (%) for each age group

No statistically significant difference was found between age and response rate (ANOVA with a post hoc Sheffe comparison, p >0.05). No statistically significant difference was found between gender and response rate (ANOVA with a post hoc Sheffe comparison, p >0.05). One of the saliva samples from the completers leaked in transit and was not suitable for further processing. The remaining 219 saliva samples from completers were processed and DNA extracted. The mean volume of samples was 1.76ml (SD 0.43, range 0.5 – 3ml). Overall mean yield of DNA from extracted saliva samples was 159.1µg (range 0.03 to 1220.8µg, SD 231.6) using picogreen. In 8 samples the DNA content was below the detection limit. No correlation was found between volume of saliva donated and yield of DNA (Spearman ranks correlation $r_s = 0.03$, P>0.05). No correlation was found between age and DNA concentration (Spearman ranks correlation $r_s = -0.09$, P>0.05).

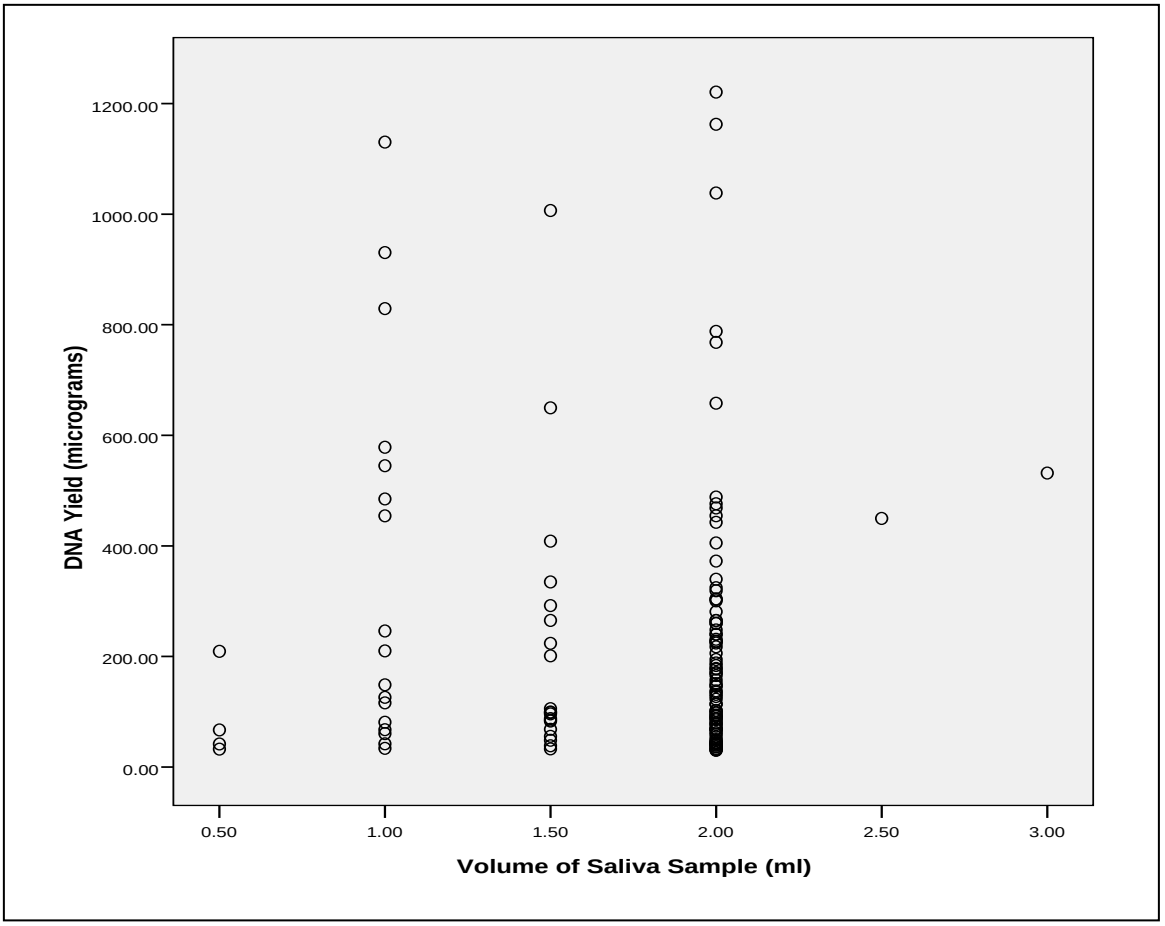


Figure 3.3 Scatter plot comparing saliva volume with DNA yield

Electrophoretic analysis of DNA samples showed degradation in two (0.9%) samples. Seventy (32%) samples were found on picogreen assay to have a concentration below the threshold of 35ng/µL required for GWAS genotyping. One sample failed on Sequenom MassARRAY iPLEX assay gender QC.

Ten subjects were subsequently found to have incorrect data entered onto the arthroplasty database and did not meet the inclusion criteria giving a current total of 131 subjects (60 male) suitable for genotyping. Sixty-three of these patients had either undergone revision surgery for aseptic loosening or had radiographic evidence of osteolysis.

3.2.6 arcOGEN study population

The arcOGEN study was a 2 stage genome wide association study of 7,410 subjects with hip and/or knee OA recruited from 9 UK centres (Sheffield, Nottingham, Oxford, London, Edinburgh, Newcastle, Southampton, Worcester and Wansbeck). Inclusion criteria were radiographic OA with Kellgren Lawrence grade ≥2 or OA sufficiently symptomatic to require total joint arthroplasty.

Sheffield

All extant Sheffield genetic hip study subjects were donated to the arcOGEN project. 375 of these samples passed QC and underwent GWAS genotyping. Of these, 172 were male and 151 had either undergone revision surgery for aseptic loosening or had radiographic evidence of osteolysis. All subjects were suitable for inclusion in this study.

Other centres

121 (46 male) subjects from London, Edinburgh, Newcastle and Oxford meeting our inclusion criteria were identified from the arcOGEN database. All subjects had already undergone genotyping as part of the arcOGEN study. Twenty-nine had radiographic evidence of osteolysis.

3.2.7 Total recruitment for discovery cohort

1071 subjects from the above cohorts met the inclusion criteria for this study and had available DNA samples which were sent to the WTSI.

961 (433 male) subjects were genotyped having passed Sanger pre-genotyping QC. Of these, 357 were osteolysis cases. Subjects in the osteolysis group were younger and a greater proportion were male versus subjects in the control group (Table 3.1).

Characteristic	*Control Group (n= 604)*	*Osteolysis group (n= 357)*	*p value*
Age at primary THA (years)[a]	66±9	61±9	< 0.001
Sex (male/female)[b]	242/362	191/166	<0.001

Table 3.1 Characteristics of discovery study subjects

[a] *Student's t-test*

[b] χ^2 test

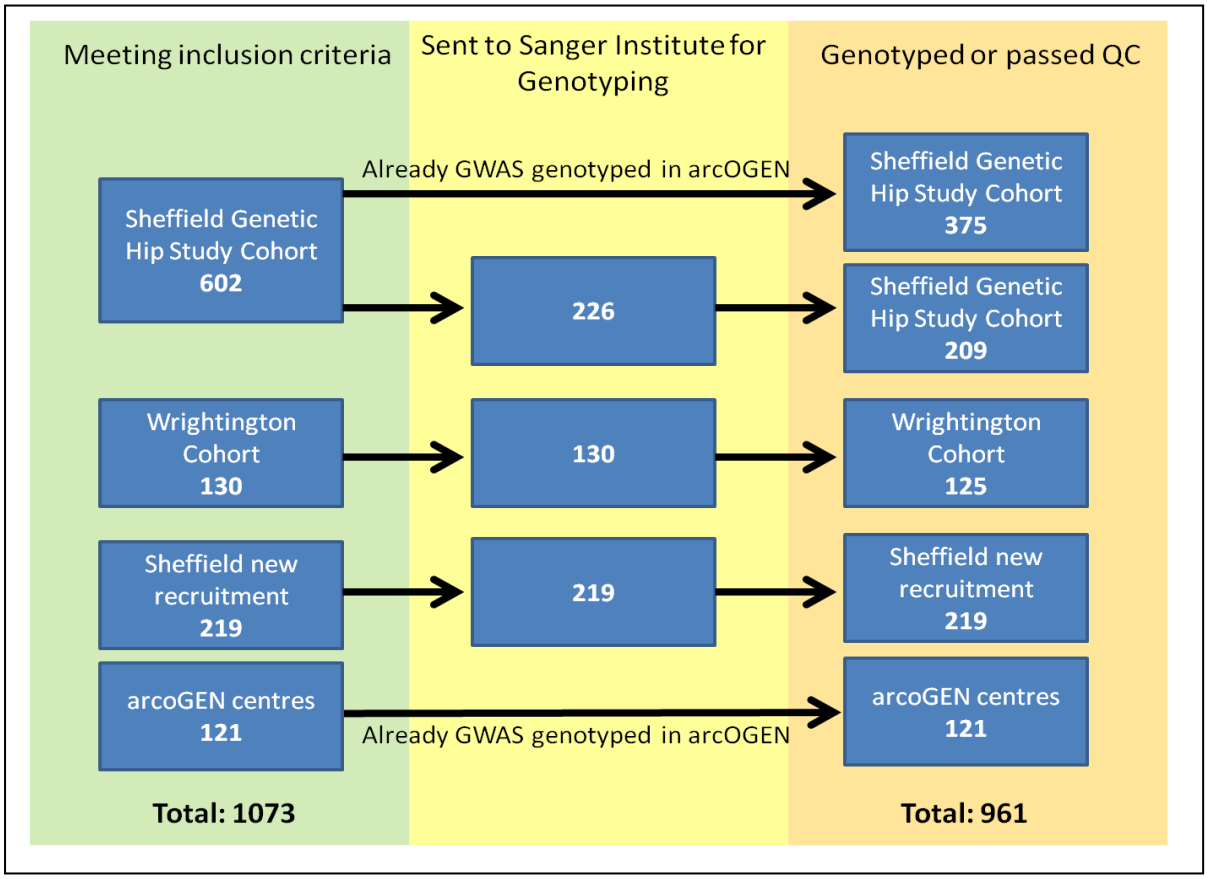


Figure 3.4 Discovery cohort recruitment summary flowchart

3.2.8 Post genotyping sample QC exclusions

Further sample QC was carried out as described earlier in section 2.8.3. Of the 961 samples passing pre-genotyping QC a further 67 were excluded: 5 for gender fail, 1 for % heterozygosity, 6 for ethnicity,12 for relatedness. 14 were further found to be duplicates, and 29 were found to have incorrect data entered onto the phenotype database. The total number of subjects passing all QC steps and with both complete phenotype and genotype data suitable for whole genome association analyses was 894 (402 were male and 317 were osteolysis cases).

3.2.9 Discussion

The discovery cohort comprised a combination of prospective recruitment with the collation of extant cohorts. There was subject overlap between the Sheffield cohorts and careful database cross referencing and multiple quality control steps were carried out to prevent duplication of subjects.

The study population reflected known epidemiological risk factors for osteolysis with a higher number of male subjects and subjects of younger age seen in the osteolysis group (Eskelinen et al., 2006) (Furnes et al., 2001) (Roder et al., 2003) (Bordini et al., 2007).

A previous study using postal collection of saliva samples found the lowest response rates in the most elderly patients (Rylander-Rudqvist et al., 2006). Our results did not reflect this. The trend suggested that the youngest group had the lowest response rates however this did not reach statistical significance in our population size. The one saliva sample that leaked during postage occurred because the saliva collection instructions had not been followed properly. All other samples survived transit having been collected and packaged as per manufacturer's instructions.

We found that the DNA concentration varied greatly between saliva samples, ranging from 0.3ng/µL to 1220.8ng/µL. The mean yield of 150µg from our samples fell within the expected range outlined by the manufacturers. It is higher than that achieved by other epidemiological studies (Rylander-Rudqvist et al., 2006, Ng, 2005) and similar to the median yield achieved by (Rogers et al., 2007). 32% of samples yielded a concentration of less than the 35ng/µL required for GWAS genotyping. This is a similar percentage found by Rylander–Rudqvist (Rylander-Rudqvist et al., 2006). Nishista et al found that a larger saliva volume correlated with a larger DNA yield (Ng et al, 2005). We did not find a statistically significant correlation between saliva volume and DNA yield.

We found that postal recruitment of subjects identified using hospital datasets achieves reasonable response rates. Postal collection of saliva samples was an effective, non-invasive and convenient method of obtaining DNA samples. Although DNA extracted from saliva can be used for whole genome analysis, the large variation between saliva samples in our study suggests that some samples may require concentrating before GWAS genotyping. This should be considered when planning recruitment in any epidemiological study using saliva as a source of DNA.

CHAPTER 4

GENETIC VARIATION IN INFLAMMATORY AND BONE TURNOVER PATHWAYS AND RISK OF OSTEOLYTIC RESPONSE TO PROSTHETIC MATERIALS

4.1 Overview

This chapter describes a 2-stage case-control association study using a candidate gene approach. Previous candidate SNP and candidate gene studies looking at variation within genes encoding inflammatory cytokines and protein involved in the regulation of bone turnover and their association with osteolysis formed the basis for this study. Here, we examined two cohorts, comprising 758 (347 male) Caucasian subjects who had undergone THA with a metal on polyethylene bearing couple; 315 of whom had developed osteolysis. Key genes within inflammatory, bone resorption, and bone formation pathways were screened for common variants by pairwise-SNP tagging. In the discovery cohort four SNPs within RANK, and one each within *KREMEN2*, *OPG*, *SFRP1*, and *TIRAP* ($p < 0.05$) were associated with osteolysis susceptibility. Two SNPs within *LRP6*, and one each within *LRP5*, *NOD2, SOST, SQSTM1, TIRAP*, and *TRAM* associated with time to implant failure ($p < 0.05$). Five SNPs showed the same effect direction in the replication analyses but none reached statistical significance. Meta-analysis of the two cohorts identified four SNPs within *RANK*, and one each within *KREMEN2*, OPG, *SFRP1*, and *TIRAP* associated with osteolysis susceptibility ($p < 0.05$). Our results suggest that variation within inflammatory signalling and bone turnover pathways may play a role in susceptibility to osteolysis

4.2 Introduction

Several investigators have shown that particulate debris from prosthetic materials initiate inflammatory signalling through pattern recognition receptors (PRR). Furthermore, these PRRs are expressed in osteolytic membrane taken from patients with failing prostheses (Takagi et al., 2007, Tamaki et al., 2009). Bone turnover is closely regulated by the interplay between the Wnt and RANK signalling pathways (Boyle WJ, 2003, Goldring and Goldring, 2007). Candidate gene studies also show that variants within several pro-inflammatory cytokines associate with osteolysis susceptibility (Wilkinson, 2003, Ambruzova Z, 2006, Gordon A et al., 2008, Kolundzic, 2006, Gallo, 2009, Malik, 2007). Similarly, variations within several genes involved in the regulation of bone turnover associate with osteolysis susceptibility (Malik, 2007, Gordon A et al., 2007, Malik MH et al., 2007, Malik MH et al., 2006). These studies have focused on specific candidate variants within genes that are known to regulate inflammatory responses or bone turnover, and thus have not explored other potential variants within or adjacent to candidate genes.

Here we used a 2-stage case-control association study design to identify susceptibility loci for osteolysis and quantitative trait loci (QTL) for time to prosthesis failure within pivotal genes that modulate inflammatory signalling and bone turnover. We applied a SNP-tagging approach to systematically and economically screen for common variants both within and in the flanking regulatory regions of the genes studied.

4.3 Methods

Subject recruitment for this study is described in chapter 3. Subjects recruited for the Sheffield genetic hip study and the Wrightington genetic hip study were included in this candidate gene study.

4.3.1 Discovery Cohort

The discovery cohort included the Sheffield recruitment population recruited from Northern England between April 2000 and April 2006. The definitions of cases and controls, inclusion and exclusion criteria are described in Chapter 2 Briefly, cases comprised patients undergoing revision surgery for osteolysis or aseptic loosening, and controls were patients who had undergone THA greater than 7 years previously and had no current radiographic evidence of osteolysis or prosthesis loosening. Polyethylene wear in both patient groups was quantitated as previously described in Chapter 2 (Wilkinson et al., 2005, Wilkinson et al., 2002) and used as an analysis covariate.

4.3.2 Replication Cohort

The ‘replication' population comprised the Wrightington cohort, recruited from the North West of England between 2002 and 2004. The definitions of cases and controls, inclusion and exclusion criteria are described in Chapter 2 and elsewhere (Malik, 2007). Briefly, cases comprised patients undergoing revision surgery for osteolysis or aseptic loosening within six years of implantation, and controls were patients who had undergone THA at least 10 years previously and were currently asymptomatic, and had no radiographic evidence of osteolysis or aseptic loosening.

4.3.3 Genotyping

DNA was extracted from peripheral whole blood using methods previously described. Genotyping was carried out by competitive allele specific PCR (KASP), (LGC Genomics, Hoddesdon, UK), as described in Chapter 2. Tagging SNP selection was performed using Hapmap Gene Browser (release #24, phase 1 and 2–full dataset, www.hapmap.ncbi.nlm.nih.gov) and Haploview software (v4.2, www.broadinstitute.org/haploview/haploview) using a pairwise tagging approach ($r^2 = 0.8$). Common variants (MAF ≥ 0.05) within the gene of interest, and extending 5 Kb upstream and 2 Kb downstream to include variants within the adjacent regulatory flanking sequences, were tagged using this approach. The following genes were selected for genotyping: *MD2, MSK1, MSK2, MyD88, NOD1, NOD2, P2Y1, P2Y6, P2 × 7, SQSTM1, TLR1, TLR2, TLR4, TLR5, TLR6, TLR9, TRAM, TIRAP, TRIF* (inflammatory signalling); *DKK1, KREMEN2, LRP5, LRP6, SFRP1, SOST, Wnt3A* (bone formation); *TNFRSF11A* (encoding RANK), *TNFRSF11B* (encoding OPG), and *TNFSF11* (encoding RANK) (bone resorption). A total of 318 SNPs were directly genotyped using this approach (full tagging SNP details given in Supplementary Table 4.1).

Candidate gene	Gene ID	Tag SNP
RANK	TNFRSF11A	rs10469252, rs12165104, rs12158117, rs12956925, rs12970081, rs17069901, rs17069902, rs17069904, rs17720953, rs4303637, rs4426449, rs4429388, rs4485469, rs4524033, rs4524035, rs4941125, rs65672721, rs6567276, rs7226991, rs7236060, rs7237982, rs7239667, rs8083511, rs8089829, rs8094884, rs8099222, rs9646629, rs9951012, rs9960450, rs17069845, rs8086340
OPG	TNFRSF11B	rs3134067, rs11573847, rs11573905,, rs7463176, rs7464496, rs3102735, rs3134056, rs6469783, rs1994276, rs7820642, rs3134058, rs2875845, rs11573869, rs1485286, rs3134063, rs11573901, rs3134053, rs1032129, rs11573871, rs53102724, rs1095591,
RANKL	TNFS11	rs4942143, rs346574, rs1054016, rs2148072, rs875625, rs4338693,, rs9562414, rs931273
Sequestosome 1	SQSTM1	rs10277,rs7711505,rs513235,rs502729, rs4797,rs2241349,rs155788,rs3734007,rs515110,rs513165
MD2	LY96	rs11783456, rs4738414, rs7839393, rs10808798, rs10504554, rs17226566, rs1905045, rs1991262
CD14	CD14	rs2569193, rs2569190, rs4914
NOD1	NOD1	rs4272257, rs2709803, rs2907749, rs2256023, rs6949758, rs11536450, rs17770244, rs1558068, rs4720004, rs3823773, rs7789045
NOD2	NOD2	rs3135499, rs17313265, rs13339578, rs5743291, rs8056611, rs574328
TLR1	TLR1	rs5743565, rs5743611, rs574359
TLR2	TLR2	rs1816702, rs5743708, rs11938228, rs1898830, rs5743704, rs7656411, rs3804100, rs3804099
TLR4	TLR4	rs2149356, rs5030728, rs7044464, rs10759932, rs12377632, rs1927906, rs1554973, rs11536857, rs11536897, rs11536889, rs11536869
TLR5	TLR5	rs2241097, rs851139, rs2241096, rs2353476
TLR6	TLR6	rs3775073, rs6531668, rs5743810, rs5743794
TLR9	TLR9	rs187084, rs352143, rs352140
TRAM	TICAM2	rs256997, rs9326969, rs10079000, rs2288384, rs17473484, rs419939, rs11957931, rs256946,
TRIF	TICAM1	rs8120, rs1046673, rs4807650, rs4807651, rs7255265
TIRAP	TIRAP	rs7932766, rs8177382, rs8177375, rs4937114, rs1786704, rs591163, rs8177352, rs6853, rs7744
P2Y1R	P2RY1	rs701265, rs17451266, rs12497578
PRY2R	P2RY2	rs1790081, rs1783596, rs4944831, rs12364461, rs949141, rs508859, rs17244555, rs557451
P2y6r	P2y6	rs12276627, rs3741152, rs11235714, rs12798517, rs3741153, rs7925649, rs1806516, rs2027765, rs12803970, rs1790063

P2X7R	P2RX7	rs2567989, rs2686369, rs17434809, rs654856, rs12821688, rs500930, rs208292, rs2230911, rs17434640, rs12314721, rs17434647, rs1653609, rs208294, rs208302, rs11065464, rs6489794, rs1718134, rs503720, rs2857585, rs7137837, rs208296, rs12829218, rs1186055, rs2230912, rs7958311, rs504677, rs1718125, rs3751143, rs35933842, rs28360457, rs1653624
DKK1	DKK1	rs1528877, rs1896367, rs2241529, rs2288335
KREMEN2	KREMEN 2	rs4238844, rs2285829, rs731721, rs7184777, rs4786361
LRP5	LRP5	rs314750, rs312779, rs3781590, rs1784235, rs3781586, rs312024, rs545382, rs606989, rs312781, rs676318, rs314779, rs3781596, rs3781600, rs312023, rs11228202, rs638051, rs4988327, rs11826287, rs312009, rs314756, rs12417014, rs901824, rs2242340, rs312014, rs3781579, rs4930573, rs4988331, rs624947
LRP6	LRP6	rs7957531, rs2417086, rs718403, rs2075241, rs1012672, rs11054738, rs7980903, rs7966410, rs10772542, rs7304561, rs12310020, rs2284396, rs17302049, rs11054704, rs12833575, rs10845493, rs12309338, rs10743980
Myd88	Myd88	rs4988457, rs6767684, rs7744, rs6796045
Sfrp1	Sfrp1	rs11786592, rs9693456, rs7833518, rs4736964, rs10106678, rs7832767, rs968428, rs4736959, rs6651363, rs3242, rs921142, rs9694405, rs17574424
Sclerostin	SOST	rs1234612, rs865429, rs851062, rs851056
MSK1	RPS6KA5	rs4904742, rs1018548, rs1286098, rs10150820, rs1286112, rs3783834, rs8013649, rs11848326, rs11159989, rs1286264, rs2401952, rs1286092, rs11620665, rs12590018, rs17261092, rs7151354, rs1957386, rs1286060, rs6575165, rs9944098, rs1286148, rs7151724, rs1286127, rs10150585, rs17722981, rs1152431, rs10134356
MSK2	RPS6KA4	rs10897487, rs612448, rs3782101

Table 4.1. Tagging SNPs used to screen each gene, including 5Kb upstream and 2Kb downstream. Tags were generated using Hapmap Genome Browser (release #24, phase 1 and 2 – full dataset, www.hapmap.ncbi.nlm.nih.gov) and Haploview software (v4.2, www.broadinstitute.org/haploview/haploview)

4.3.4 Association Analysis

Quality control (QC) and association analyses were carried out using PLINK v1.07 (www.pngu.mgh.harvard.edu/~purcell/plink). Two genotype-phenotype association analyses were performed: A case-control association analysis for susceptibility loci for osteolysis, and a quantitative trait analysis (QTL) for time to prosthesis failure (cases only and defined as the time from insertion to osteolysis diagnosis) using linear regression. Signals reaching a statistical significance threshold of $p < 0.05$ in the

discovery cohort after adjustment for age, sex, time since surgery, and annual prosthesis wear rate were taken forward for analysis in the replication cohort. Finally, case-control and time-to-failure meta-analyses of signals genotyped in both cohorts were performed using GWAMA v1.4 (WTSI).

4.4 Results

Seven hundred and fifty-eight subjects were included in the analyses, of whom 315 formed the osteolysis group. The discovery cohort comprised 631 patients (275 osteolysis cases). The replication cohort comprised 127 patients (40 osteolysis cases). 130 subjects, as described in section 3.2.5, were initially included but 3 were later excluded due to gender fail at quality control. Subjects in the osteolysis group were younger, a greater proportion were male and they had higher annual polyethylene wear rate versus the control subjects (Table 4.1, $p < 0.05$). These findings are consistent with known risk factors for osteolysis and were included as covariates in the association analysis.

Subject characteristics	Control Group	Osteolysis Group	P value
Discovery cohort	*n=356*	*n=275*	
Age at THA (years ±SD)[a]	65±8	59±9	<0.001
Sex (male/female)[b]	147/209	148/127	0.002
Osteolysis free survival (years±SD)[c]	12±4	10±5	<0.001
Polyethylene wear (mm)	0.77(0.49 to 1.23)	1.18 (0.75 to 1.91)	<0.001
Replication cohort	*n=87*	*n=40*	
Age at THA (years±SD)[a]	72±8	69±7	0.03
Sex (male/female)[b]	29/58	23/17	<0.001
Osteolysis free survival (years±SD)[c]	18±6	4±1	<0.001

Table 4.2. Characteristics of study subjects. Analysis is cases versus controls within each cohort by [a]Student's t-test, [b]Chi-squared test, or [c]Mann-Whitney U test, as appropriate.

4.4.1 Osteolysis Susceptibility

In the discovery case-control association analysis 4 SNPs within *TNFRSF11A*, including rs4524033, rs9960450, rs7226991, and rs4485469, and one each within *KREMEN2* (rs4786361), *SFRP1* (rs921142), *TIRAP* (rs8177375), and *TNFRSF11B* (rs11573847), met the significance threshold of $p < 0.05$ for carriage forward into the replication analysis (Table 4.2). At replication, four of these SNPs showed an association in the same direction as for the discovery cohort (rs4524033, rs7226991, and rs4485469, all in *TNFRSF11A*; and rs921142 in *SFRP1*), but none reached statistical significance. Meta-analysis of the two cohorts identified eight SNPs which

were associated with susceptibility to osteolysis (Fig. 4.1, $p < 0.05$). Four of these signals lay within *TNFRSF11A*, and 1 each within *TNFRSF11B, SFRP1, KREMEN2*, and *TIRAP*.

Gene	Chromosome	SNP	Minor allele	OR	OR 95%L	OR 95%U	P value
TNFRSF11A	18	rs4524033	A	1.693	1.241	2.309	0.0009
SFRP1	8	rs921142	G	1.323	1.036	1.691	0.025
TNFRSF11A	18	rs9960450	C	0.500	0.273	0.918	0.025
TNFRSF11B	8	rs11573847	G	1.668	1.061	2.621	0.027
KREMEN2	16	rs4786361	A	0.745	0.574	0.969	0.028
TNFRSF11A	18	rs7226991	A	0.733	0.552	0.972	0.031
TIRAP	11	rs8177375	G	0.646	0.426	0.980	0.040
TNFRSF11A	18	rs4485469	G	0.765	0.588	0.995	0.046

Table 4.3. Discovery cohort osteolysis susceptibility loci meeting the replication threshold (P<0.05). Analysis is cases (n=275) versus controls (n=356) using PLINK (version 1.07).

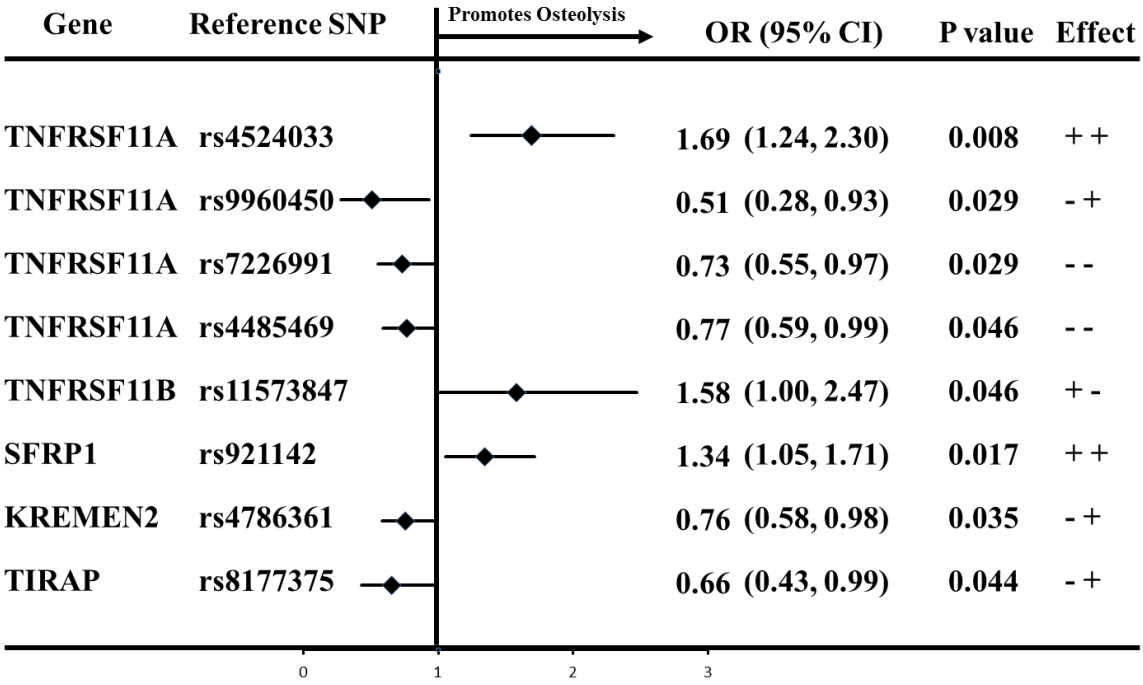


Figure 4.1. Meta-analysis discovery and replication cohorts of susceptibility loci for development of osteolysis. Analysis is cases (n=315) verses controls (443) across discovery and replication cohorts and made using GWAMA software (version 1.4). The effect column indicates the effect direction of the given allele in the stage 1 and stage 2 cohorts.

4.4.2 Time to Failure

Two SNPs within *LRP6* (rs10743980 and rs2417086), and one each within *LRP5* (rs606989) *NOD2* (rs5743289), *SOST* (rs851056), *SQSTM1* (rs155788), *TIRAP* (rs1786704), and *TRAM* (rs10079000) associated with time to implant failure in cases at $p < 0.05$, and were carried forward (Table 4.3). At replication one SNP in *TIRAP* (rs1786704) showed the same direction of association with the discovery cohort, but none reached statistical significance, and no SNPs were associated with time to failure following meta-analysis of the two cohorts (Fig. 4.2, $p > 0.05$).

Gene	Chromosome	SNP	Minor allele	BETA	BETA 95%L	BETA 95%U	P
LRP6	12	rs10743980	T	-0.913	-1.687	-0.139	0.022
SOST	17	rs851056	G	-0.865	-1.620	-0.109	0.026
TRAM	5	rs10079000	A	-0.912	-1.726	-0.099	0.029
SQSTM1	5	rs155788	C	-0.907	-1.716	-0.098	0.029
NOD2	16	rs5743289	T	1.034	0.104	1.965	0.030
LRP6	12	rs2417086	G	-0.836	-1.601	-0.071	0.033
TIRAP	11	rs1786704	C	-0.952	-1.851	-0.052	0.039
LRP5	11	rs606989	T	1.601	0.073	3.129	0.041

Table 4.4. Discovery cohort quantitative trait loci associated with time to prosthesis failure in cases (n=275) meeting replication threshold (P<0.05). Analysis made by linear regression using PLINK (version 1.07).

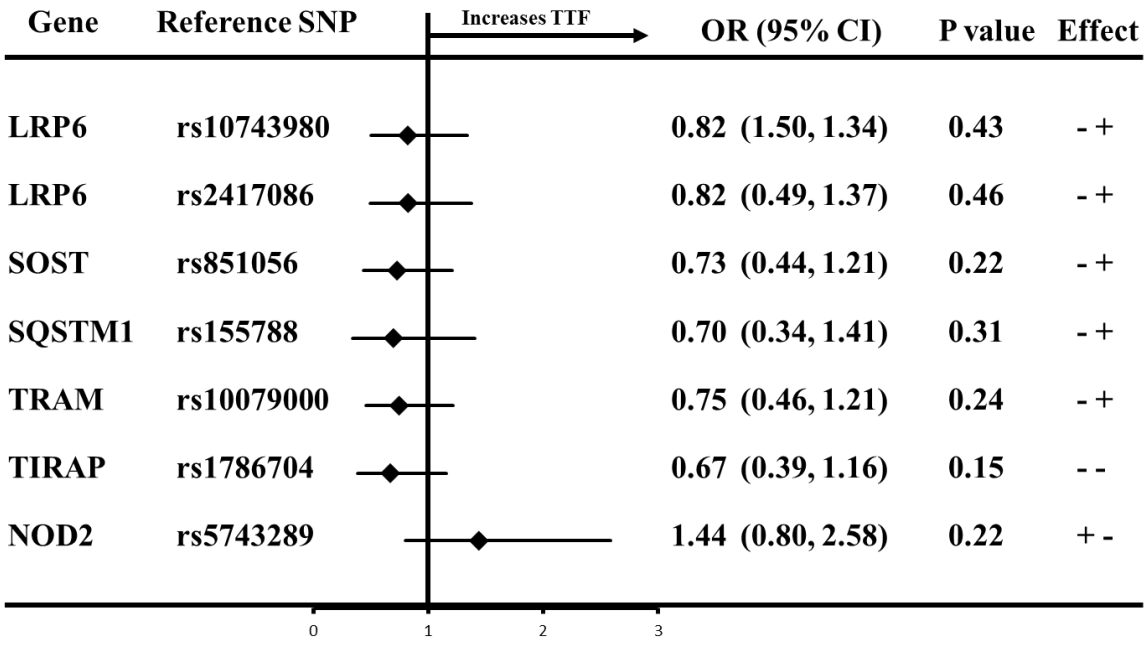


Figure 4.2. Meta-analysis discovery and replication cohorts of quantitative trait loci associated with time to prosthesis failure. Analysis is within cases only (n=315) across discovery and replication cohorts and made using GWAMA software (version 1.4). The effect column indicates the effect direction of the given allele in the stage 1 and stage 2 cohorts.

4.5 Discussion

We conducted a 2-stage case-controlled association study to identify common genetic variants that associate with susceptibility to osteolysis, and with time to diagnosis in cases. At meta-analysis of the two stages we identified eight SNPs that were weakly associated with osteolysis susceptibility. Seven of these lay within bone resorption (six signals) or bone formation (two signals) pathways, and one within genes that regulate inflammatory signalling (*TIRAP*).

Variants within *TNFRSF11A* (encoding RANK) and *TNFRSF11B* (encoding OPG) showed the strongest association with susceptibility to osteolysis, and are consistent

with the function of these genes in regulating osteoclast differentiation and activation. The functional role of these variants is uncertain, as all lie in non-coding regions of the gene. However, they may act as markers for coding SNPs or regulate transcription and translation through effects on mRNA stability, splicing, or binding of miRNAs.

Variation within bone formation pathways was associated with osteolysis, and previously showed variation within *SFRP3* was associated with both osteolysis and heterotopic ossification susceptibility (Gordon A et al., 2007). *SFRP1* is also a Wnt antagonist. *SFRP1*-/- mice have high trabecular bone mass, (Bodine et al., 2004) and inhibition of *SFRP1* activity associates with increased bone formation (Gaur et al., 2009). The rs921142 variant within *SFRP1* lies in the 5'UTR region and may thus affect stability of the RNA transcript Kremen2 is a transmembrane protein that blocks the LRP6 receptor inhibiting Wnt signalling, and Kremen2 deficient mice have increased bone formation (Schulze et al., 2010). Our finding of an association between osteolysis and variation within Kremen-2 is previously unreported.

We found fewer than anticipated associations within the inflammatory signalling pathways, given their reported importance to osteolysis. The only consistent association found was between the *TIRAP* rs8177375 variant and susceptibility to implant failure. This variant also showed a trend toward association with time to prosthesis failure in the osteolysis cases (β—1.12, $p = 0.08$), but failed to meet the threshold for genotyping in the replication cohort. TIRAP is an adaptor protein that modulates many inflammatory pathways, including inflammatory signalling through TLR-4, and also transduces signals from TLR2 (Greenfield et al., 2010). This *TIRAP* variant is located in the 3' UTR and associates with risk of sepsis-induced lung injury (Song et al., 2010).

We used knowledge of the haplotype architecture of candidate genes to conduct a screen for common variants across the whole of each gene, including its regulatory

flanking sequences. We used a 2-stage design to reduce the likelihood of false positive associations, carrying forward to replication only those signals reaching a significance threshold of $p < 0.05$ in the discovery cohort. This approach also has several limitations. Although the presented sample size combines the two largest cohort studies of the genetics of osteolysis reported to date, (Gordon A et al., 2008, Malik, 2007) it remains small in genetic association study terms. We aimed to address the issue of multiple testing and false positive associations through a 2-stage analysis process, followed by meta-analysis of the datasets for signals undergoing replication analysis. Our study only had power to detect common variants with relatively large effects sizes and with modest *p*-values. For example this design had 80% power (at $p < 0.0005$, joint analysis, multiplicative model, CaTS), (Skol et al., 2006) to detect a variant with an OR of 1.5 with a MAF of 0.35, but only 7% power to detect a variant with similar effect size and a MAF of 0.05. Although our design aimed to reduce the number of candidate SNPs genotyped without loss of sensitivity for identifying disease loci by using Hapmap data on their haplotype structure, candidate pathways and genes were selected based on a-priori knowledge of their importance to the pathogenesis of osteolysis.

CHAPTER 5

A WHOLE GENOME ASSOCIATION STUDY OF SUSCEPTIBILITY TO HETEROTOPIC OSSIFICATION FOLLOWING TOTAL HIP ARTHROPLASTY

5.1 Overview

This chapter describes a discovery-stage genome wide association study of heterotopic ossification susceptibility and severity following THA. A replication cohort for this phenotype is currently being collected. The aetiopathogenesis of HO is still not fully understood and the majority of genetic research has looked at the rare heritable causes of HO. Only a few screening studies have been carried out in other populations. In this study we aimed to identify genetic risk loci by conducting a genome-wide association study comprising 891 Caucasian European patients (410 HO cases). All had undergone THA for primary osteoarthritis and were recruited from the United Kingdom. Genotyping was undertaken using the Illumina 610 beadchip followed by imputation using the European reference panel from the 1000 Genome Project. An HO susceptibility case-control analysis and an evaluation of disease severity in those with HO was undertaken using SNPTEST v2.3.0. 8 signals were associated with HO susceptibility and 11 with HO severity at $p<9.9x10^{-6}$.in this discovery set. The most significant signal that was associated with HO susceptibility lay in flanking region of *ARHGAP18* (index SNP rs59084763, $p=2.48x10^{-8}$) and reached genome wide significance. Two other signals approaching genome wide significance lay in the flanking region of *BMP-2* (index SNP rs11699612, $p=9.3x10^{-8}$) and within *LGI1* (index SNP rs10882328, $p=3.8x10^{-7}$). The most significant signal that was associated with HO severity lay within *KIF26B* that encodes the kinesin like protein KIF26B (rs35338958, $p=1.65x10^{-6}$). Signal replication in an independent cohort will be required to confirm these associations.

5.2 Introduction

Heterotopic ossification (HO) is a common complication following total hip arthroplasty (THA) with a reported incidence of up to 61% (Newman et al., 2015). In addition to restriction of joint motion HO can lead to pain, as well as neural or vascular compression (Garland, 1991, Hierton et al., 1983, Brooke et al., 1991). The exact pathogenesis of HO remains unknown and investigators in recent years have attempted to identify the osteoprogenitor cell origin and signalling pathways involved both in the acquired and hereditary forms.

The increased risk of HO in patients with ankylosing spondylitis (Bisla et al., 1976), diffuse idiopathic skeletal hyperostosis (Blasingame et al., 1981) and hypertrophic osteoarthritis (Sawyer et al., 1991) together with the heterogeneity in HO manifestation in patients with similar injury patterns suggests a significant genetic predisposition. To date, the majority of genetic research has looked at the rare heritable causes of HO (FOP and POH) and few screening studies have been carried out in other populations. Candidate gene studies have associated variants in genes encoding the $\beta_2$adrenergic receptor, toll-like receptor (TLR) 4, complement factor H, and secreted frizzled related protein 3 (sFRP3) with susceptibility to HO (Gordon A et al., 2007, Mitchell et al., 2010). Here we aimed to identify genetic risk loci associated with the formation of HO following THA by conducting a genome-wide association study.

5.3 Subjects and Methods

5.3.1 Subject Recruitment and Phenotype Characterisation

The cohort examined in this study consisted of the recruited GWAS discovery population. Controls comprised subjects who had no evidence of HO on plain AP radiographs of the pelvis taken not less than 1 year following primary THA. Cases

comprised subjects with radiographic evidence of post-operative HO and were graded (0-4) using the Brooker classification. As described in section 1.5.3 (Brooker et al., 1973).

5.3.2 Genotyping and Association Analysis

Genomic DNA was extracted from either whole blood or saliva and genotyped using the Illumina 610k beadchip (Illumina, San Diego, CA). We imputed the GWAS data using the European reference panel from the 1000 Genome Project. We undertook post genotyping quality control checks as described in section 2.8.3.

An HO susceptibility case-control analysis and an evaluation of disease severity. We were unable to normalise the HO grade data and therefore undertook a binomial analysis comparing grades 1 and 2 versus grades 3 and 4 in the cases under the additive model using the score test in SNPTEST v2.3.0 (University of Oxford, Oxford, UK). Association analyses were adjusted for age and gender as these have previously been described as known risk factors for HO (DeLee et al., 1976, Hierton et al., 1983). SNPTEST output files were further filtered to exclude SNPs with a frequentist info score <0.4 and a minor allele frequency (MAF) <0.05.

5.4 Results

5.4.1 Patient characteristics

Eight hundred and ninety-one subjects were analysed (401 males). Of these, 410 subjects formed the HO group. The mean age of subjects was 63.5 (SD 8.9) and no difference in mean age was found between the groups (63.65$\pm$8.9 vs 63.42$\pm$8.9, p=0.7). There was a higher proportion of males in the HO group versus the control

group (p<0.0001). The Brooker grading of HO in the cases was as follows: grade 1 n=207, grade 2 n=133, grade 3 n=69, grade 4 n= 1.

5.4.2 HO Susceptibility Analysis

We identified 8 independent signals with index SNPs at $p<9.9x10^{-6}$ that were associated with HO susceptibility (table 5.1). The most significant signal lies 1Kb downstream of *ARHGAP18* and reached genome wide significance (rs59084763, effect allele T, effect allele frequency (EAF) 0.194, odds ratio OR 1.87, 95% CI 1.48 to 2.38, $p=2.48x10^{-8}$) (figure 5.1). Two signals approached genome-wide significance: variant rs11699612 (effect allele T, EAF 0.25, OR 1.74, 95% CI 1.4 to 2.16, $p=9.3x10^{-8}$) lies 300Kb upstream of *BMP2* (figure 5.2). Variant rs10882328, (effect allele A, EAF 0.287, OR 0.57, 95% CI 0.47 to 0.71, $p=3.8x10^{-7}$) lies within an intronic region of *LGI1* (figure 5.3).

Index SNP	Chr	EA	NEA	EAF	OR	OR 95L	OR 95U	P value	Gene
rs59084763	6	T	C	0.194	1.872	1.475	2.375	2.48E-08	Downstream of *ARHGAP18*
rs11699612	20	T	C	0.246	1.735	1.395	2.157	9.39E-08	Upstream of *BMP2*
rs10882328	10	A	G	0.287	0.577	0.468	0.713	3.87E-07	*LGI1*
rs1059129	7	G	A	0.152	1.904	1.463	2.477	2.60E-06	*PMS2P1*
rs35946190	9	G	A	0.182	0.573	0.446	0.736	3.93E-06	*ASTN2*
rs3768863	2	G	A	0.550	0.661	0.548	0.798	6.82E-06	*BIN1*
rs6463103	7	G	T	0.773	0.622	0.498	0.778	9.15E-06	Upstream of *LOC105375249*
6-137128553	6	A	C	0.061	2.239	1.495	3.354	9.32E-06	Upstream of *PEX7*

Table 5.1. Results of the HO susceptibility analysis. This table includes all independent SNPs with $p<9.9x10^{-6}$. Chr = chromosome, EA = affect allele, NEA = non affect allele; OR = odds ratio, OR_95L/95U = lower and upper margins of 95% CI.

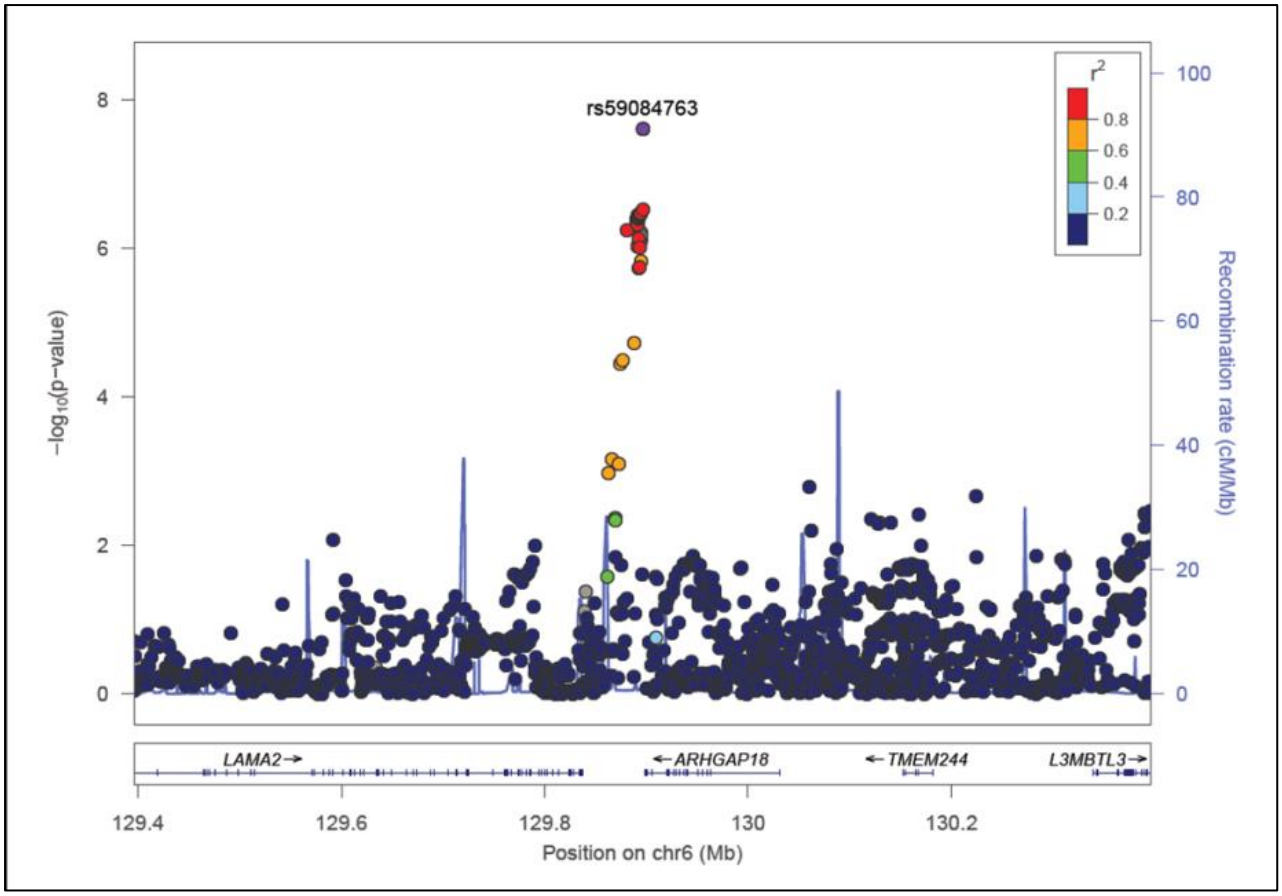


Figure 5.1. Regional association plot for rs59084763 (that lies downstream of *ARHGAP18*) from the HO susceptibility analysis. The index SNP is denoted by the purple marker. The colour reflects the pairwise r^2 with index SNP. The region extends 500kb upstream and downstream of the index SNP.

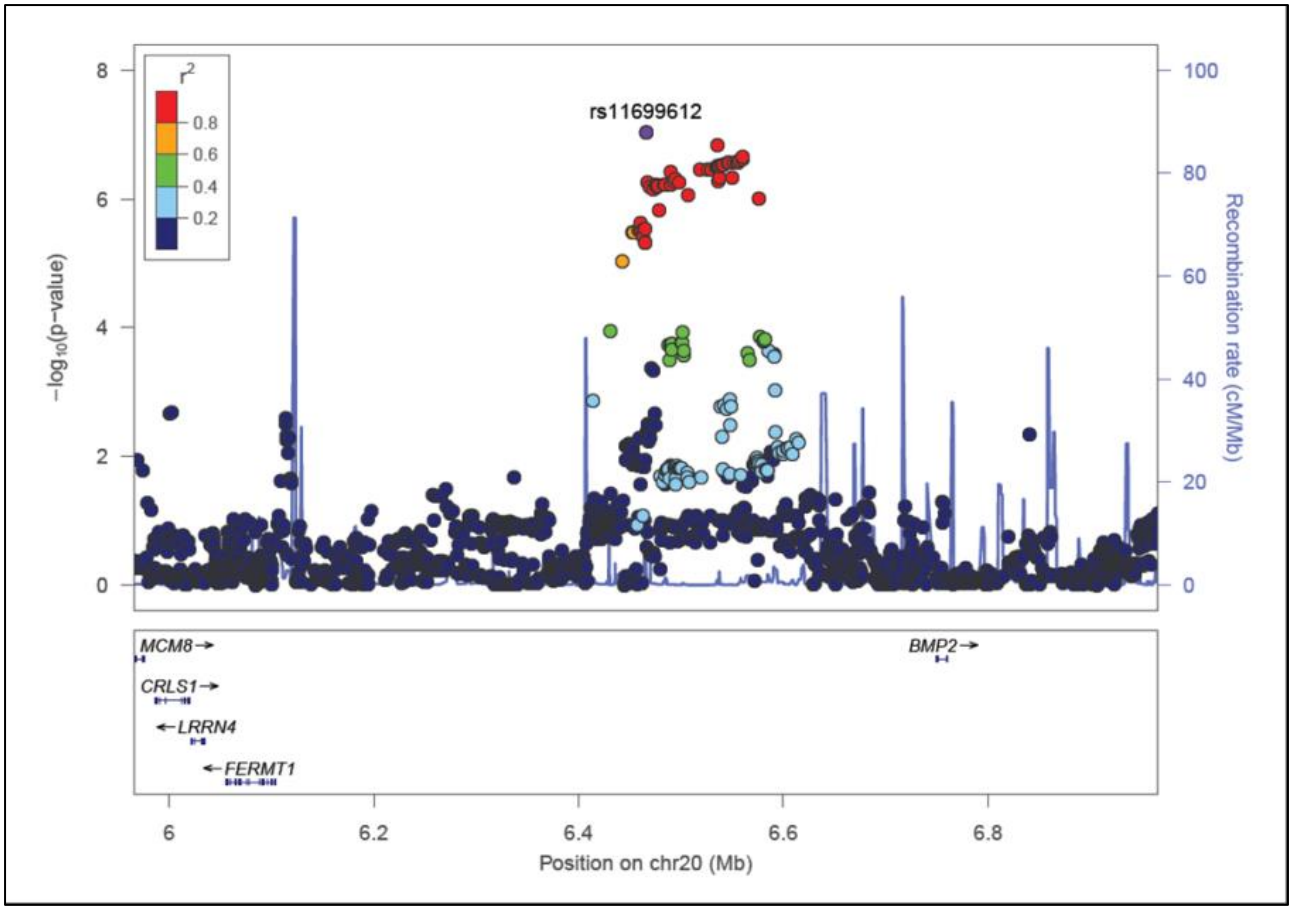


Figure 5.2. Regional association plot for rs11699612 (that lies upstream of *BMP2*) from the HO susceptibility analysis. The index SNP is denoted by the purple marker. The colour reflects the pairwise r^2 with index SNP. The region extends 500kb upstream and downstream of the index SNP.

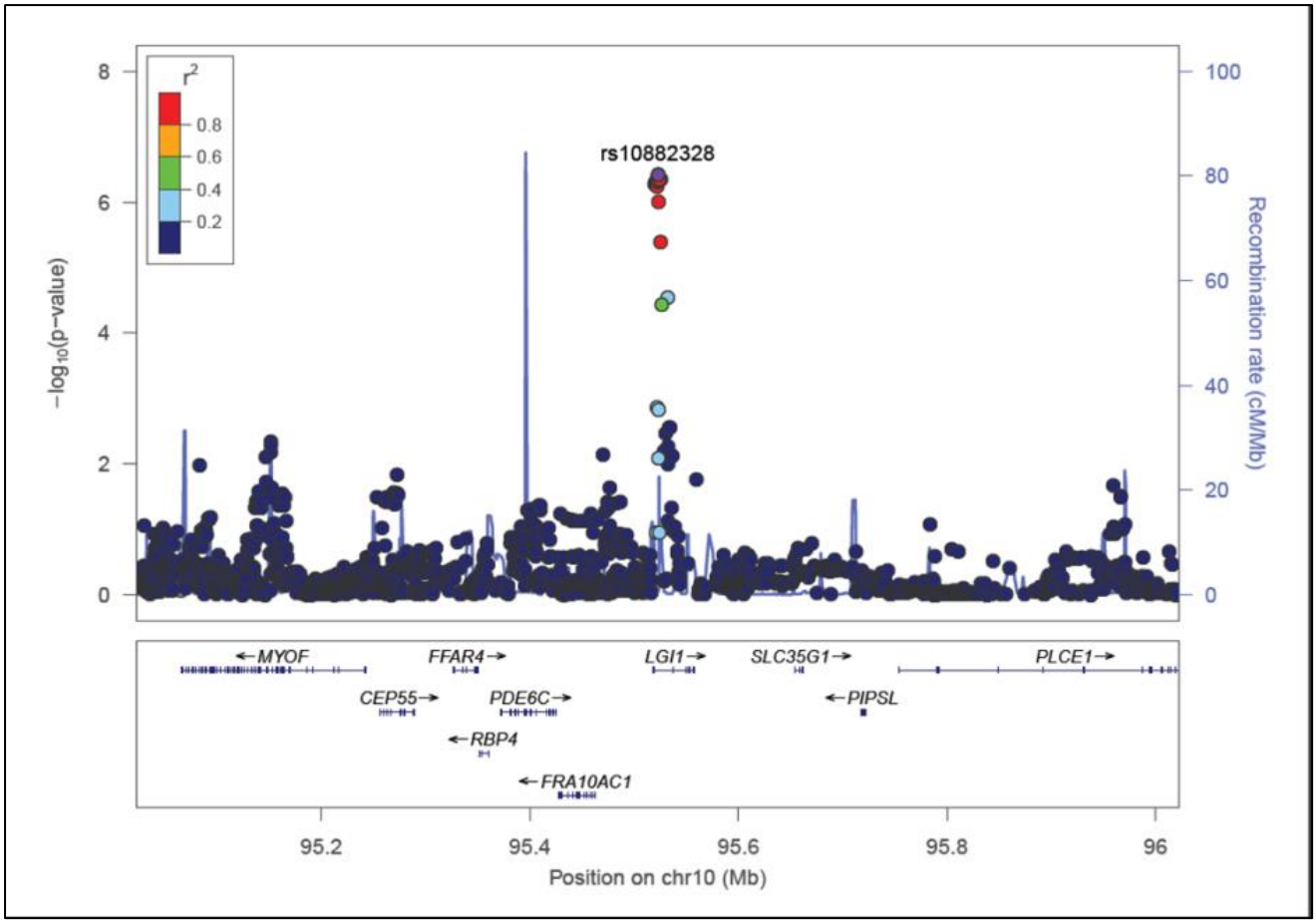


Figure 5.3. Regional association plot for rs10882328 (that lies within *LGI1*) from the HO susceptibility analysis. The index SNP is denoted by the purple marker. The colour reflects the pairwise r^2 with index SNP. The region extends 500kb upstream and downstream of the index SNP.

5.4.3 HO Severity Analysis

Eleven signals were associated with HO severity with index SNPs at $p<9.9x10^{-6}$ (table 5.2). No signals in the severity association analysis reached genome wide significance. The most significant signal lay within an intronic region of *KIF26B*. The index SNP within this signal was rs35338958 (OR 3.0, EAF 0.099, 95% CI 1.85 to 5.01) at $p=1.65x10^{-6}$ (figure 5.4).

Index SNP	Chr	EA	NEA	EAF	OR	OR 95L	OR 95U	P value	Gene
rs35338958	1	T	C	0.099	3.044	1.850	5.008	1.65E-06	*KIF26B*
rs13109480	4	C	T	0.071	2.896	1.634	5.135	1.88E-06	Upstream of *HSP90AB2P*
rs11953126	5	G	A	0.474	0.426	0.289	0.629	2.42E-06	*LOC105374705*
rs2300936	9	G	A	0.087	3.355	1.994	5.642	3.86E-06	*C5*
rs491425	18	G	A	0.663	2.632	1.667	4.158	5.02E-06	*FHOD3*
rs1367425	2	T	C	0.218	0.437	0.301	0.636	5.07E-06	no nearby genes
rs11880610	19	C	G	0.098	2.788	1.684	4.617	5.98E-06	*ZNF331*
rs4612038	5	A	G	0.300	2.351	1.621	3.411	6.00E-06	*EDIL3*
rs34242676	9	C	T	0.288	2.220	1.525	3.232	6.15E-06	Downstream of *GRIN3A*
rs13235561	7	A	C	0.333	2.148	1.485	3.110	6.19E-06	*LOC105375122*
rs10058296	5	T	C	0.087	2.373	1.489	3.783	6.59E-06	Upstream of *HTR1A*

Table 5.2. Results of the HO severity analysis. This table includes all independent SNPs with $p<9.9x10^{-6}$. Chr = chromosome, EA = affect allele, NEA = non affect allele; OR = odds ratio, OR_95L/95U = lower and upper margins of 95% CI.

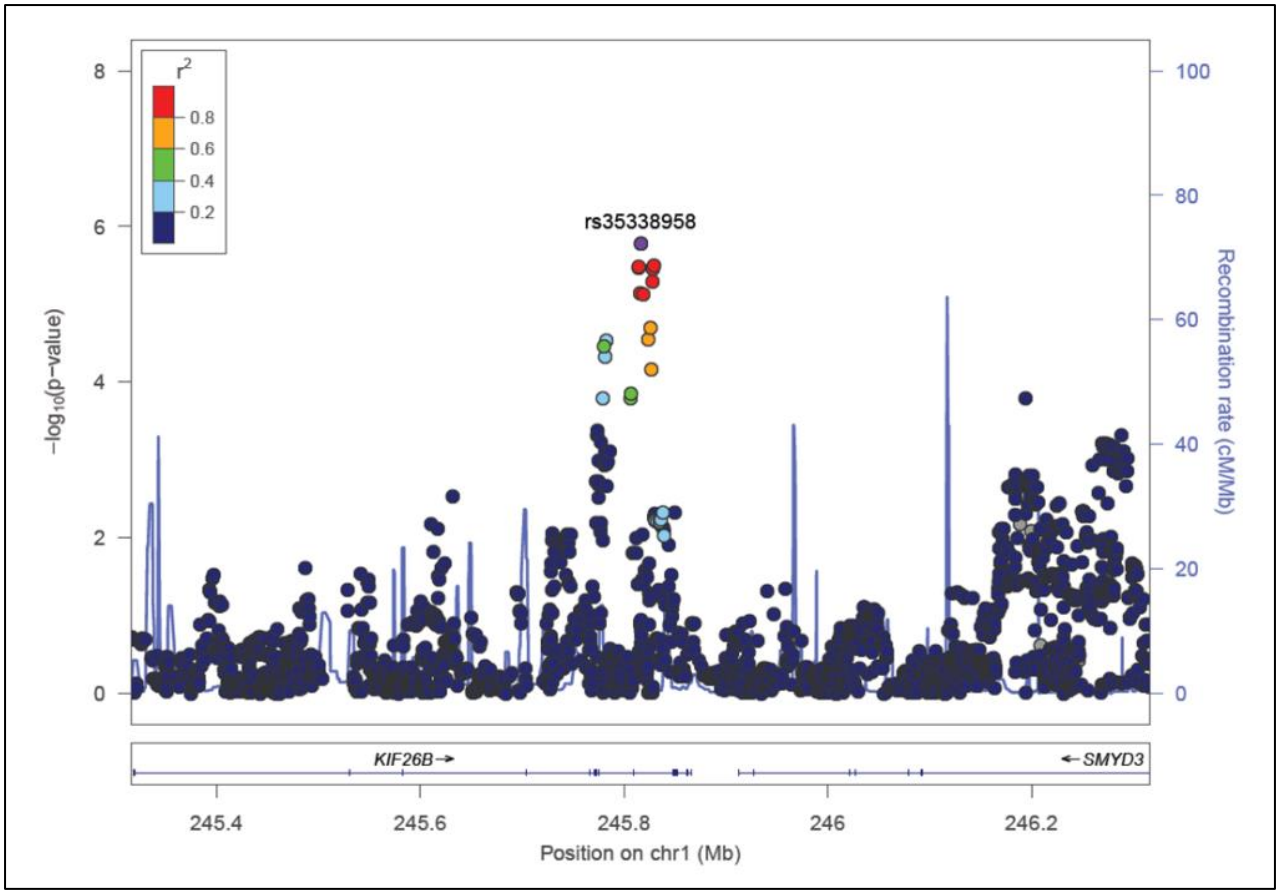


Figure 5.4. Regional association plot for rs35338958 (that lies within *KIF26B*) from the HO severity analysis. The index SNP is denoted by the purple marker. The colour reflects the pairwise r^2 with index SNP. The region extends 500kb upstream and downstream of the index SNP.

5.5 Discussion

We have undertaken the first GWAS examining HO susceptibility and severity following THA. We have identified a number of possible association signals within or in the flanking regions of *ARHGAP18, BMP2, LGI1* and *KIF26B* that warrant further investigation by replication in suitable cohorts.

The variant rs59084763 associated with susceptibility to HO and lies 1Kb downstream of *ARHGAP18*. Although this signal is not within *ARHGAP18*, downstream flanking regions may contain downstream promoter elements (DPE) which regulate the initiation of transcription. Variation within such a region has the capacity to alter the level of RNA transcription and subsequent gene activity. The *ARHGAP* gene family, encodes the RhoGAP proteins, which activate GTPase, modulating cell signalling. The Rho family of GTPases play an important role in cell differentiation, proliferation and migration (Symons, 1996). *ARHGAP18* has been suggested as one of the crucial factors for the regulation of RhoA for the control of cell spreading, and migration (Maeda et al., 2011). Our identification of SNP associations in the flanking region of *ARHGAP18* may prove promising in light of recent evidence suggesting the progenitor cells for HO likely migrate to the site of bone formation (Lazard et al., 2015). RhoA has been shown to influence mesenchymal stem cells into an osteogenic lineage (Arnsdorf et al., 2009, Chen et al., 2011). *ARHGAP18* may therefore be important in both the migration and osteogenic lineage of HO progenitor cells and is a plausible associated candidate for replication and follow on functional studies.

The intergenic variant rs11699612 associated with HO susceptibility lies 300Kb upstream of *BMP2*. Upstream regions contain promoter and enhancer regions that regulate transcriptional activity of the gene. Variants within these regions have the potential to influence RNA transcriptional regulation by altering the binding of RNA polymerase and other transcription factors. The bone morphogenetic proteins belong to the transforming growth factor beta (TGFβ) superfamily, a group of growth factors involved in embryogenesis and tissue healing (Termaat et al., 2005). The role of BMPs in heterotopic ossification is well established. Their discovery by Urist in the 1960s

was through the induction of bone in the skeletal muscle of rabbits (Urist and Strates, 1971). Overexpression of BMPs have been linked to HO development (Kaplan et al., 2004). Evans et al found greater transcript levels and expression of BMP2 in war wounds which developed HO (Evans et al., 2014). BMP2 is now commonly used as an inducer in many rodent models of HO (Engstrand et al., 2008). The osteoinductive properties of BMP-2 has led to its use as an adjunct in spinal fusion (Burkus et al., 2016). Like ARHGAP18, BMP2 has been shown to influence the activation of RhoA in early osteogenesis. Wang et al identified two polymorphisms, Ser87Ser (rs1049007) and Ser37Ala (rs2273073), in *BMP2* that associated with susceptibility to ossification of the posterior longitudinal ligament (Wang et al., 2008). As well as direct osteoinduction in HO, BMP-2 signalling has been linked to both neural and inflammatory pathways. MacDonald et al showed that the administration of BMP-2 can induce inflammation (MacDonald et al., 2010). BMP2 and BMP3 (osteogenin) have also been shown to be chemoattractants for monocytes (Convente et al., 2015). Interestingly, Kaplan et al showed that reduction of the immune response using transgenic mice did not prevent the formation of HO (Kaplan et al., 2007). This suggests that in addition to the immune system, other mechanisms such as neuronal pathways are involved. Salisbury et al showed that BMP-2 causes peripheral nerves to express osterix (Salisbury et al., 2011). Osterix is a zinc finger containing transcription factor which plays an important role in osteoblast differentiation (Nakashima et al., 2002). BMP-2 also induces substance P release from neurones which leads to recruitment and degranulation of mast cells leading to HO and further links the neuronal and inflammatory pathways (Salisbury et al., 2011). Lazard et al demonstrated that BMP-2 causes neural derived progenitor cells (likely neural crest stem cells) to undergo osteogenic differentiation and migrate to the site of HO (Lazard et al., 2015). The evidence for the involvement of BMP-2 in the formation of HO suggests it is a plays a key role in the interplay of various contributing pathways.

The intronic variant rs10882328 associating with HO susceptibility was found within *LGI1*. *LGI1* was originally discovered in gliomas but has also been linked to an autosomal dominant form of epilepsy (Chernova et al., 1998, Poza et al., 1999). This gene encodes the Leucine-rich, glioma inactivated 1 (LGI1) protein. The LGI1 protein is thought to regulate the strength of synaptic transmission by bridging between the

disintegrin and metalloprotease 22 and 23 (ADAM22 and ADAM23) transmembrane proteins (Fukata et al., 2010). Yu et al found that absence of LGI1 causes an increase in synaptic transmission (Yu et al., 2010). The association of *LGI1* with HO is novel and its function in neuronal signalling is still not fully understood. It may provide a link for the neuronal involvement in the development of HO.

The signal that was most strongly associated with HO severity lay within *KIF26B*. The Kinesin Superfamily Proteins (KIFs) are molecular motors responsible for intracellular transport of proteins and signal transduction (Hirokawa et al., 2009). *KIF26B* which grouped with *KIF26A* form the Kinesin-11 family and are important in limb development. The association of *KIF26B* with HO following THA is novel, however, it has previously been associated with ectopic calcification in a rodent knee injury model (Rai et al., 2015). This makes *KIF26B* of interest in the further study of HO.

Interestingly the two most significant signals associating with HO susceptibility are linked through common biological processes. Both ARHGAP18 and BMP2 are known regulators of RhoA. The Rho GTPases are regulators of osteogenesis through their involvement in both canonical and non-canonical WnT signalling (Schlessinger et al, 2009). RhoA has been shown to regulate osteogenesis through its effect on both osteoblasts and osteoclasts (Yoshida et al 2009) (Chellaiah et al, 2000). Ohnaka et al, studying the effects of pitavastatin on bone, discovered that BMP2 expression was regulated by RhoA (Ohnaka et al). The stimulation of RhoA in MSCs by BMP2 is necessary for osteogenesis through its effect on cell spread and shape (Wang et al 2012). ARHGAP18 is localised to the leading edge of migrating cells and controls cell shape through its regulation of RhoA (Maeda et al 2011).

The association of BMP2 and ARHGAP18 with RhoA signalling suggests a possible common pathway in the development of ectopic bone. The effects of altered regulation of RhoA through transcriptional variation of *BMP2* and *ARHGAP18* are potentially threefold: 1. Migration of progenitor cells to area of bone formation, 2. Actuation of MSCs down an osteogenic lineage through altered regulation of WnT signalling, 3. Enhanced osteogenesis through stimulation of further BMP2 signalling. As BMP2 and RhoA signalling have been implicated in a number of biological

processes their exact contribution to the pathogenesis of HO remains unclear. Follow on functional studies of ARHGAP18, BMP2 and the RhoGTPases are required to explore this further.

This study has limitations. The sample size was small, and this may have led to fewer genome wide significant associations ($p<9x10^{-8}$), especially for the rarer loci. We attempted to minimise this effect by including only common variants with MAF>0.05. A number of the allele effect sizes were quite large and may represent type 1 statistical errors, highlighting the importance of a further replication study. Further, we used the Brooker classification for HO. This is the accepted standard method, but provides only a semi-quantitative measure. An alternative approach using cross-sectional imaging would provide volumetric quantitation, but is not used in routine clinical practice.

In conclusion, we have identified several possible signal associations with HO susceptibility and severity. However, this data represents only a discovery cohort, and replication in independent cohorts is essential before these candidate signals can be robustly confirmed.

CHAPTER 6

USING A NATIONAL JOINT REGISTER DATASET AND POSTAL METHODOLOGY TO DEVELOP A LARGE DNA ARCHIVE FOR MUSCULOSKELETAL DISEASE

6.1 Overview

This chapter outlines the osteolysis replication population recruitment. Local recruitment of sufficient numbers of patients for large scale epidemiological studies is difficult and time consuming, especially when studying low incidence phenotypes. National joint registries provide large repositories of clinical data and the linking of such datasets with patient biological material offers new opportunities to study musculoskeletal disease. We examined the response rates for postal saliva collection to build a DNA biobank for patients who had undergone primary and revision total hip arthroplasty (THA). The quality and quantity of DNA collected was also examined. The DNA and clinical datasets were then linked to examine patient demographic factors that associated with DNA yield. This study highlights the possible extended role of arthroplasty registries for large scale recruitment of subjects for the study of musculoskeletal disease. It also demonstrates the feasibility of establishing DNA biobanks in conjunction with national registries and the successful linking of datasets.

6.2 Introduction

National arthroplasty registers allow the auditing of orthopaedic prosthetic implants and their outcomes and were introduced for care quality purposes. They provide an excellent repository of clinical information about the patient experience of joint replacement. The linking of such clinical datasets with patient biological samples can extend the role of these registries to answer research questions. The value of linking of national arthroplasty datasets with other clinical dataset (Hospital episode statistics, HES) has already been realised (Sibanda et al., 2008). Cancer registry data is also now being utilised to answer biological questions about disease (Govindaraju et al., 2008, John et al., 2004). Many of these registries are now establishing their own biobanks of DNA and tissue samples (Goodman, 2009). To date there have been no biobanks established for national arthroplasty registries.

Traditionally, whole blood has been used for obtaining DNA samples, however, this method has logistical limitations, especially when applied to the recruitment of large cohorts. Study subjects need to attend a healthcare facility for venepuncture and, due to its invasive nature, is disliked by patients. Additionally, blood samples require refrigerated storage and timely processing. Saliva is a viable alternate source of DNA for large genetic epidemiological studies (Ng, 2005, Quinque et al., 2006, Rylander-Rudqvist et al., 2006, Rogers et al., 2007, Nishista, 2009, Bahlo et al., 2010). Saliva sample return rates have been shown to be higher than those of blood but these rates are highly variable between studies (Hansen et al., 2007, Bhutta et al., 2013). The postal collection of saliva is a cost effective, non-invasive, and convenient method of obtaining human DNA.

In this study, we aimed to build a repository of DNA from patients after primary and revision total hip arthroplasty (THA recruited from a national arthroplasty registry) using postal saliva collection. We evaluated response rates, and assessed whether the quality and quantity of DNA collected was adequate for GWAS.

6.3 Methods

6.3.1 Recruitment environment

All replication study subjects were recruited from the Norwegian Arthroplasty Register (Haukeland University hospital, Bergen, Norway). This project had the collaborative support of the staff and facilities of the register, led by Professor Ove Furnes. They provided access to the necessary number of subjects needed for the stage 2 recruitment. The register has collected information on primary and revision THA procedures performed in Norway since 1987 now has over 200,796 recorded total hip prosthesis operations (Norwegian Arthroplasty Annual Report, June 2016, http://nrlweb.ihelse.net/Rapporter). The Norwegian Arthroplasty Register employs data entry and management clerks, and statistical epidemiologists that have a large experience of conducting follow up postal questionnaire and clinical studies.

6.3.2 Inclusion and exclusion criteria

As in the discovery recruitment outlined in chapter 2, subjects recruited in the replication cohort comprised unrelated men and women of North European ancestry who had previously undergone primary cemented or hybrid (cemented femur) THA for idiopathic osteoarthritis. Any subject with a fully cementless implant and those who had undergone THA for a primary diagnosis other than osteoarthritis, such as inflammatory arthropathy or osteoarthritis secondary to another pathology, was excluded.

6.3.4 Case and control definition and matching

Cases comprised subjects who have undergone revision surgery for aseptic loosening or osteolysis affecting either the acetabular or femoral prosthetic component.

Revisions for indications such as dislocation, or infection were excluded; as were revisions of fully cementless implants and revisions for liner wear in the absence of aseptic loosening or osteolysis. Although subjects who underwent revision for liner wear in the absence of osteolysis could have been considered an interesting group of controls, numbers were not sufficient to warrant inclusion. Controls comprised subjects identified to have had primary THA for idiopathic osteoarthritis ≥10 years previously, and no recorded revision surgery episodes.

A number of environmental factors affect the development of osteolysis. In the UK cohort case-control association study we adjusted for these risk factors using logistic regression in order to determine that component of risk for osteolysis which is associated with the candidate loci. The Norwegian cohort recruitment strategy was planned to minimise differences in known risk factors between the cases and controls a-priori. The revision subjects were recruited first and the control group subjects individually matched to be of the same age (± 2 years), sex, implant brand, bearing couple material and size, implant fixation method, and year of primary surgery (± 2 years). A total of 9 identified potential controls were matched per revision in order to achieve 3 recruited controls per revision, allowing for estimated response rates. The large pool of subjects available for recruitment in the control group (38,000) made this approach feasible, and the most robust method for minimising environmental risk factor variance between the groups. Two limitations of this approach are that we did not have access to wear measurements in the replication cohort, nor radiographic evidence of freedom from osteolysis in the control group. In mitigation of the first confounder, we adjusted for polyethylene wear as a risk factor in the discovery cohort when selecting the signals to progress for replication. To help mitigate against the second confounder, we used a screening questionnaire (appendix E) to confirm that all control subjects were asymptomatic from the replaced hip at the time of recruitment. This will leave a potential group of subjects with silent osteolysis that are misclassified in the control group.

6.3.6 Subject recruitment

All recruited subjects were provided with an information sheet and signed a study specific consent form. Stage 2 recruitment and saliva collection was ethically approved in Norway (West Norway Research Ethics Committee, REK reference 2008/100 18-OYSV, 08.09.08; approval letter in appendix F). This collection was also reviewed and approved for transfer to the UK for DNA extraction and genotyping by the Norwegian Biobank authorities (08/8916, 20.10.08). Recruitment was carried out in a number of stages.

6.3.7 Revision Recruitment

Revisions were recruited first in order to adequately match the controls. Recruitment started in April 2009 and subjects registered before this time were identified for inclusion, and comprised the first mail outs of invitations. Following this, at year end it was possible to identify and include revisions registered in 2009. Revisions carried out in 2010 formed the third round of invitation mail outs.

6.3.8 Control Recruitment

Control recruitment was undertaken in two batches. The first group of controls were matched against recruited revision subjects up to and including 2009. Following, responses from revision subjects having surgery in the year 2010, further controls were matched and invites mailed out in a second recruitment batch in December 2011.

6.3.9 Subject Recruitment and saliva collection

Patients enrolled in the Norwegian arthroplasty register who were identified as still living in Norway and meeting the inclusion criteria were invited to participate in the biobank. Patients were sent an invitation letter, questionnaire and consent form by post. All subjects who returned a signed consent form agreeing to participate in the study received, by post, an Oragene OG-500 DNA Self Collection Kit (DNA Genotek, Inc, Ottawa, Canada) with detailed instructions on its use. A prepaid return envelope was supplied to return samples. Non-responders who had been sent a saliva collection kit received a further reminder letter by mail (figure 6.1).

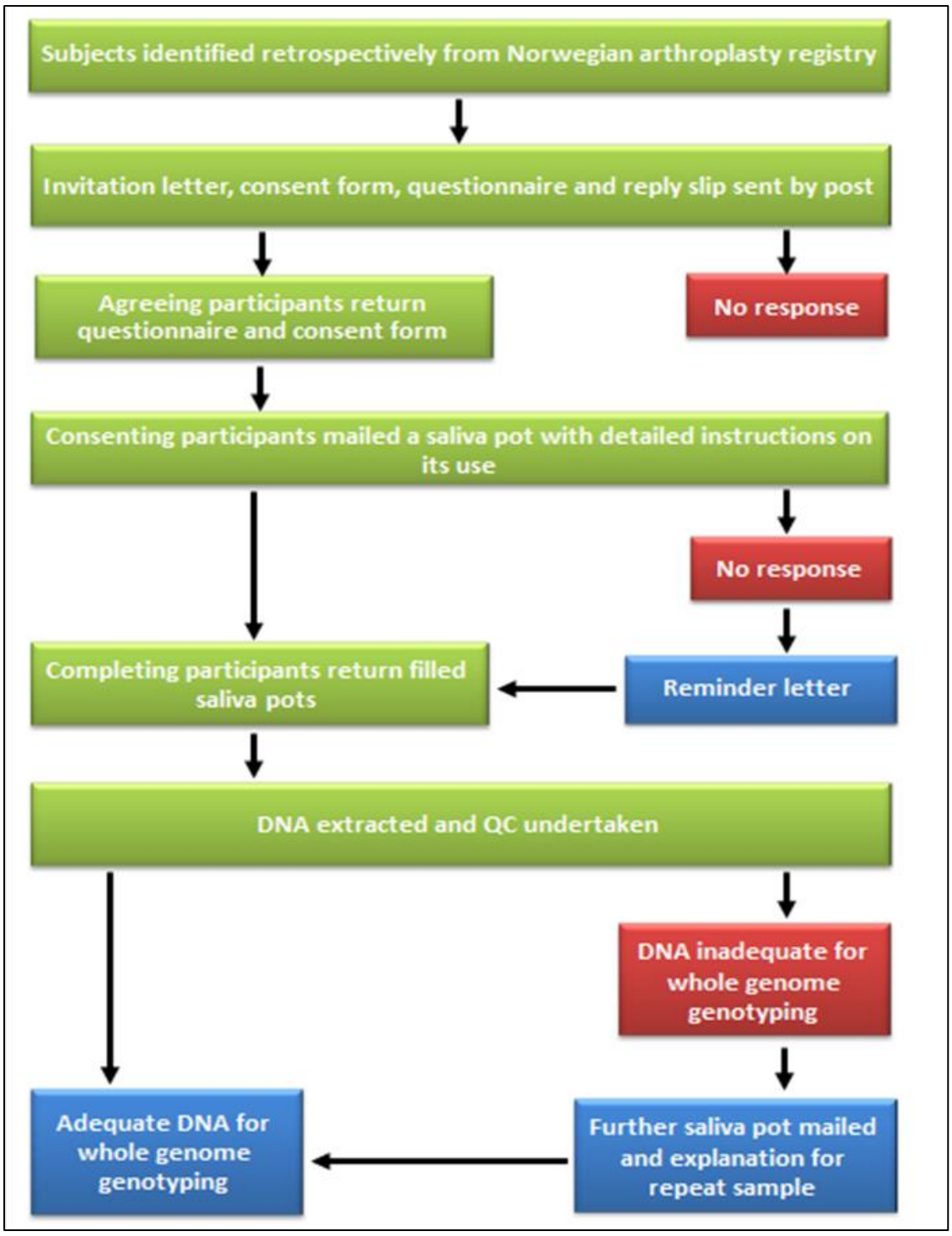


Figure 6.1. Schematic diagram outlining the recruitmant strategy

6.3.10 Laboratory methods

Saliva samples were stored at room temperature before processing. A small subset was stored at -20ºC prior to DNA extraction. Prior to DNA extraction all samples were visually inspected to ensure no damaged had occurred on transportation. The volume of the saliva was determined by subtracting the 2mls of DNA stabiliser. The colour of the sample was noted as either clear, light brown or dark brown representing contaminants.

DNA was extracted manually by precipitation in ethanol using the manufacturer's protocol for manual purification of DNA from 4.0mL, PD-PR-015 Issue 2.0. These methods are described in detail in chapter 2.2.4.

DNA samples were quantitated using the Qubit dsDNA BR assay kit (Product Code: Q32853, Invitrogen) the concentration of DNA was measured using the Qubit 2.0 fluorimeter (Product Code: Q32866, Invitrogen.) as described in chapter 2.5.5. DNA quality was evaluated in a sample subset, by gel electrophoresis as described in section 2.5.4 to measure DNA fragment length, using the TapeStation 2200 (Agilent Technologies, CA, US). DNA samples were stored at -20ºC immediately following extraction and then transferred to -80ºC for long-term storage. Subjects who had provided samples with a DNA concentration below that required for genotyping were contacted and sent another saliva kit to provide a repeat sample.

6.3.11 Data Linkage

Clinical data was collected from the register database and linked to the subsequent DNA collection by a unique identifier. This data linkage facilitated the examination of patient demographic factors that associated with DNA yield.

6.4 Results

6.4.1 Subject recruitment and response rates

There were 121,613 primary THAs and 10,255 revision THA cases identified on the Norwegian Arthroplasty Registry at the time of study. 8,396 subjects (6,367 primary THAs, 2,029 revision THAs, mean age 66.5, 36.5% males) met the inclusion criteria and were invited to participate. 3,710 agreed, a response rate of 44%. 3,068 (2,276 primary THAs, 792 revision THAs, saliva samples were subsequently received from agreeing participants (completer rate 36.5%; 83% of responders) (figure 6.2). Characteristics of completer subjects can be seen in table 6.1. The completer rate was found to be higher in those subjects who had undergone revision surgery than those who had undergone primary surgery (39% vs 35.7% respectively, p=0.001). The completer rate was higher in men than women (41.5% vs 33.7% respectively, p=0.0001).

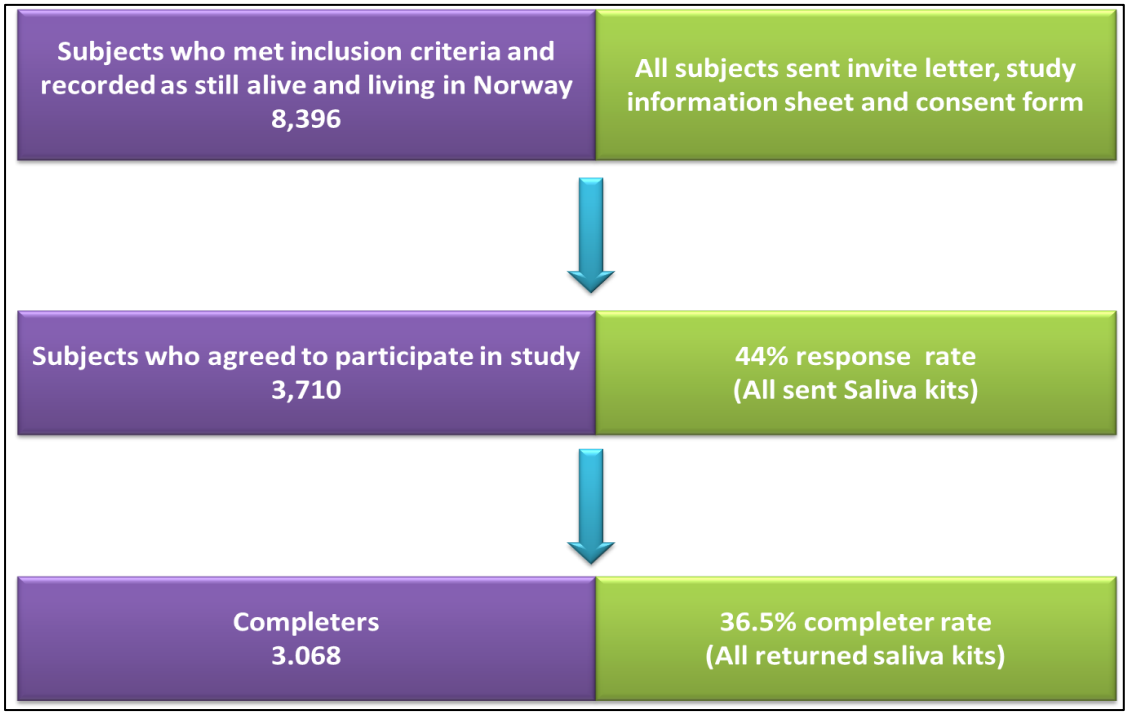


Figure 6.2. Overview of subjects recruited

Characteristic	Primary Arthroplasty Completers	Revision Arthroplasty Completers	Total
Mean Age (years)	65.7 (SD 7.1)	64.3 (SD 7.05)	65.3 (SD 7.1)
Gender (male/female)	937/1339	334/458	1271/1797
Response Rate	35.7%	39%	36.5%

Table 6.1. Completing subject's demographic characteristics

6.4.2 Saliva sample characteristics

3,104 saliva samples were received. This larger number compared to number of completer subjects is due to 36 repeat samples being received from subjects who had initially provided low yield DNA samples. Of these, 31 samples had leaked during shipping, 10 samples were contaminated during processing and 7 were lost during processing (figure 6.3). The remaining 3,056 samples were suitable for processing.

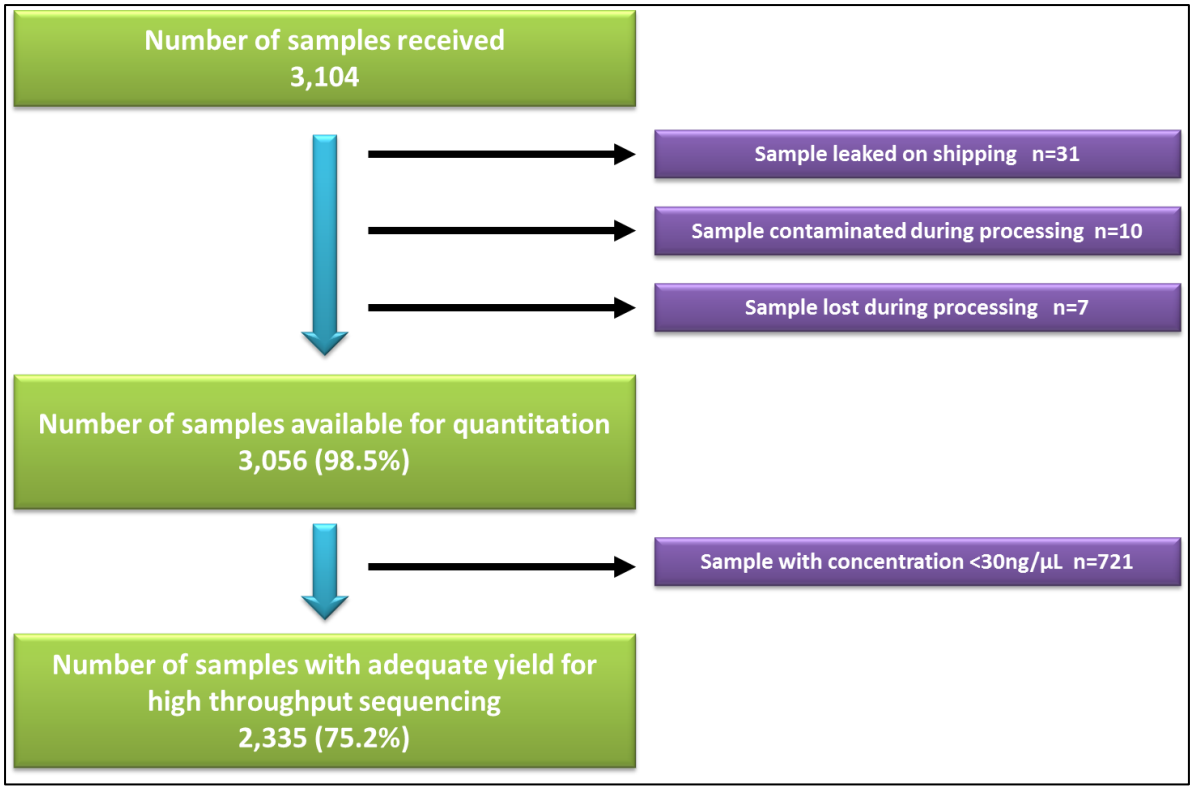


Figure 6.3. Overview of sample processing

Received saliva sample volumes ranged from 0.5ml – 4ml. The mean saliva volume was 2.0mL (SD 0.68). The volume of saliva sample provided was inversely proportional to the age of the patient (linear regression, r = -0.09, p = 0.01). The colour of sample was not recorded in 47 samples. Of the remaining, 67.4% samples were clear, 28.2% light brown in colour and 4.4% were dark brown and assumed to be heavily contaminated.

6.4.3 DNA Quantitation

The median DNA yield was 52.1µg (IQR 22.65µg – 103.5µg) (figure 6.4). The median stock concentration was 69.05ng/µl (IQR 30.2ng/µl – 136.75ng/µl). 2335 (75.2%) samples had extracted DNA concentrations >30ng/µl recommended for high-throughput genotyping. 80% of extracted DNA had a fragment length of >60 Kilobases (Kb) (figure 6.5). Of the duplicate samples received, the median DNA concentration

was 36.2ng/µl with 65% of samples achieving the above required concentration for high throughput genotyping. This was an increase in median concentration in this group of subjects by 22.85ng/µl (13.35 - 36.2ng/µl). There was no difference in DNA yield between men and women (p=0.9). Samples stored at room temperature prior to DNA extraction had a greater yield of DNA than those stored at -20^{o}C (84.22 µg vs 69.6 µg, respectively, p=0.001). The total yield of DNA had a weak positive correlation with the volume of the saliva sample provided (Pearson correlation bivariate two-tailed, r = 0.17, p= 0.01) (figure 6.6). The darker (presumed contaminated) saliva samples were found to provide higher yields of DNA (ANOVA, F = 101.39, p = 0.0001).

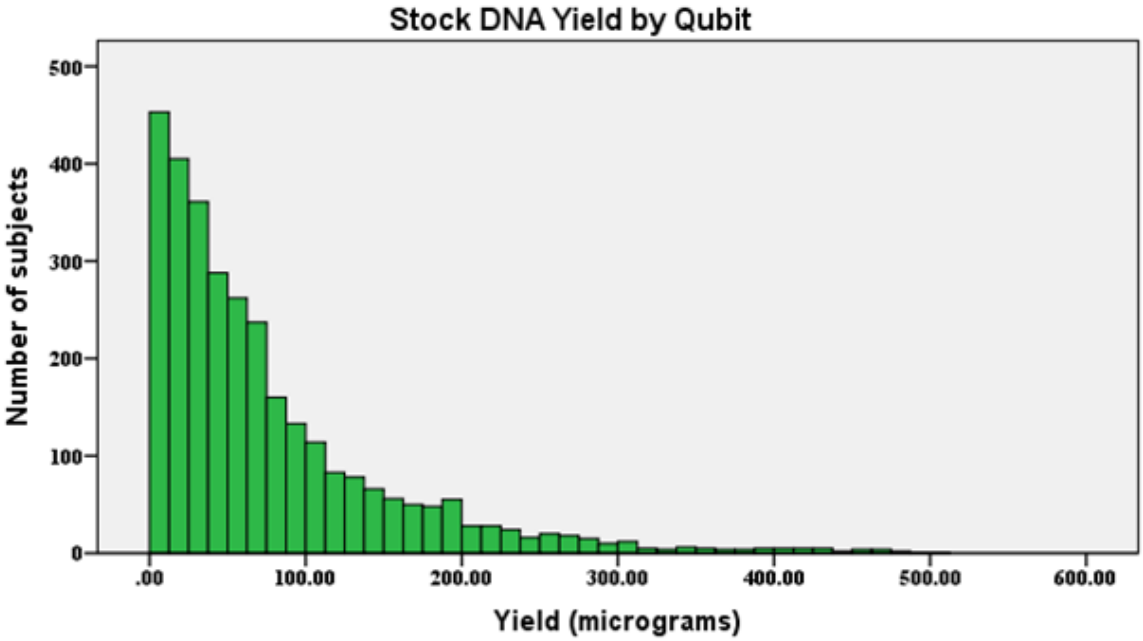
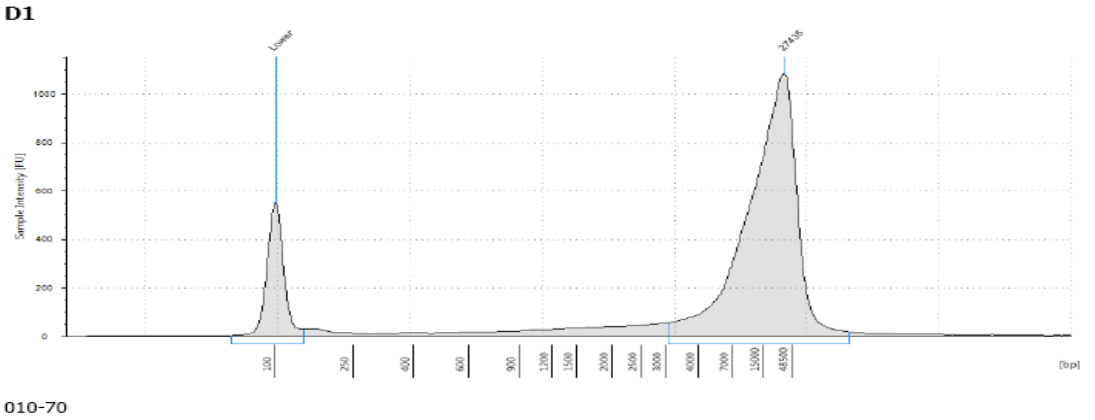


Figure 6.4. Histogram outlining the Stock DNA Yield by Qubit



Peak Table

Size [bp]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	% Integrated Area	Peak Comment	Observations	From [bp]	To [bp]
100	8.50	8.50	-		Lower Marker	59	138
27435	48.8	-	99.59			3081	>60000
-	-	-	-		Sample Well	-	-

Figure 6.5. Gel Electrophoresis on a D1 agarose gel demonstrating DNA quality. The y axis represents fluorescence intensity of the DNA fragments and the x axis shows the length of the DNA fragments. The peak on the left represents a DNA size standard (100bp). The peak on the right represents the DNA samples tested. The lack of spread in the peak on the right indicates numerous large DNA fragments.

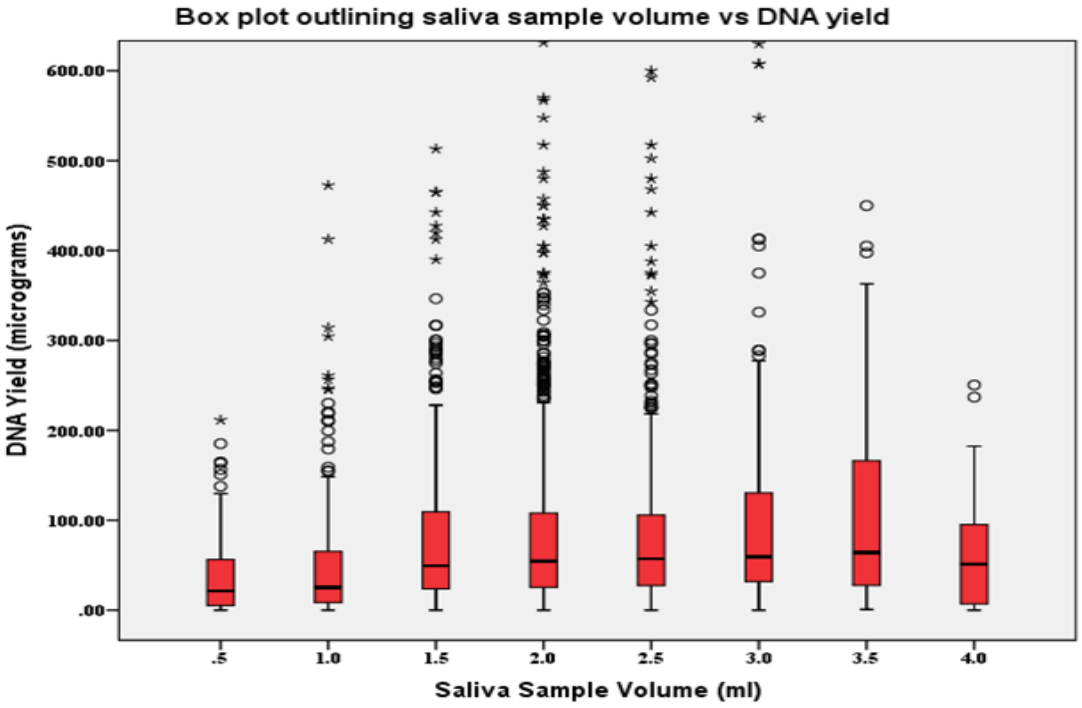


Figure 6 6. Box plot outlining saliva sample volume vs DNA yield

6.5 Discussion

In this study, we have demonstrated that postal recruitment of subjects using a national arthroplasty registry achieves adequate response rates for large scale epidemiological studies. This supports the current evidence that large scale recruitment can be achieved from medical registries (John et al., 2004, Beskow et al., 2006, Whiffin et al., 2014). Of all subjects invited to the study, 44% agreed to participate and 36.5% returned a saliva sample. This is consistent with similar studies where response rates ranged from 21–72% and sample return rates ranged from 12 – 41% (Bhutta et al., 2013).

We found that saliva samples were more likely to be returned by patients who had undergone revision surgery compared with those having undergone primary arthroplasty. It is unclear whether this is because they had undergone surgery more recently or whether they were more likely to participate in view of the increased medical contact received. We found that response rates were higher in men than women. This contradicts much of the literature which states that women are usually more likely to participate in scientific studies (Galea and Tracy, 2007). Nishista et al found no difference in response rates between men and women in their saliva collection for a large genetic study (Nishista, 2009).

We found that postal collection of saliva samples is an effective, non-invasive and convenient method of establishing a DNA biobank for an arthroplasty registry. Our findings echo those of other studies that DNA yields from saliva are adequate for high throughput genotyping (Ng, 2005, Rylander-Rudqvist et al., 2006, Rogers et al., 2007, Nishista, 2009). We found that the DNA yield varies greatly between saliva samples, ranging from 0.0µg to 1260µg. The median yield of 52.1µg from our samples were higher than that achieved by other epidemiological studies using the Oragene saliva kit (Rylander-Rudqvist et al., 2006, Quinque et al., 2006, Ng, 2005, Hansen et al., 2007). Although this DNA yield was comparable to other studies we found it was lower than that achieved in our Sheffield postal recruitment subjects. There are a number

of possible contributors: 1. Extraction for the majority of Norway samples was undertaken by another individual; 2. Extraction was undertaken in larger batches in the Norway samples and therefore the amount of time scraping excess DNA adherent to the inside Falcon tube walls may have been less; 3. The Sheffield samples were extracted as soon as they were received whereas the Norway samples were stored for a longer period prior to extraction; 4. Some of the Norway samples were stored at -20^{o}C prior to DNA extraction whereas all Sheffield samples were stored at room temperature. Twenty-five percent of saliva samples yielded a concentration of less than the 30ng/µL required for GWAS genotyping. This is a comparable percentage to that achieved by Rylander–Rudqvist et al (Rylander-Rudqvist et al., 2006).

One of the potential limitations of using saliva as a source of DNA is the bacterial DNA content. Although we did not evaluate the bacterial DNA concentration in our samples we found those darker in colour, and therefore assumed contaminated, had higher DNA yields. This may represent a higher contribution by bacterial DNA. We recently measured the bacterial DNA quantity in a separate cohort using the same Oragene collection kit. The median percentage of bacterial DNA was 17% which is comparable to other studies using the Oragene kit (Rylander-Rudqvist et al., 2006, Nishista, 2009) and within the range of 2 – 29% quoted by the manufacturer (Chartier and Birnboim, 2005).

We found that saliva volume positively correlated with an increase in DNA yield. Nishista et al also found that a larger saliva volumes provided larger DNA yields (Nishista, 2009). Patients who had initially provided low DNA yield samples were contacted to provide a repeat sample. We found that the mean DNA yield in these duplicate samples was higher with a resultant 65% adequate for high throughput genotyping. This suggests that re-contacting such patients with an explanation is worthwhile as they are likely to provide a better quality sample.

DNA Genotek Inc. recommends saliva samples should be stored at room temperature prior to extraction. We found that storage of saliva samples at -20^{o}C was associated

with a lower DNA yield. This was similar to findings by Ng et al who also found that freezer storage of saliva prior to processing was associated with reduced DNA yield (Ng, 2005). This may be due to DNA degradation as a result of freeze-thawing (Grecz et al., 1980). Following extraction long term storage of DNA was at -80ºC. Isolated DNA may be stored at -20ºC for several months and -80ºC for years (Holland et al., 2003). An EU workshop for Biobanks has recommended that DNA samples are frozen to prevent bacterial growth and reduce risk of sample loss through evaporation (EU_Workshop, 2003). The UK Biobank recommends storing samples at -20ºC to inhibit nucleases (UK Biobank 2004). A study by Smith et al comparing optimal storage conditions found that the highest quantity of DNA remained in the samples stored at -80ºC (Smith and Morin, 2005). DNA Genotek advise that Oragene DNA samples can be freeze-thawed 3 times without evidence of DNA degradation (www.dnagenotek.com).

In conclusion, this study highlights the possible extended role of arthroplasty registries for the large-scale recruitment of subjects that may have particular value in diseases that are rare, or where very large numbers are needed because of small gene effect sizes. Postal recruitment of subjects identified using a national joint registry achieves reasonable response rates. Self-collection saliva samples received by mail provide DNA samples with adequate yield for high throughput genotyping and are therefore suitable for the establishment of a registry biobank. Although the majority of DNA extracted from saliva is suitable for whole genome analysis, our study suggests that about a quarter of samples will not yield sufficient DNA for genome wide genotyping, but may be used for replication analyses using other genotyping technologies, such as KASPar. This should be considered when planning recruitment for epidemiological studies using saliva as a source of DNA.

CHAPTER 7

A GENOME-WIDE ASSOCIATION STUDY OF OSTEOLYSIS FOLLOWING TOTAL HIP ARTHROPLASTY

7.1 Overview

This chapter describes a GWAS of peri-prosthetic osteolysis susceptibility and time to prosthesis failure following THA followed by a replication study and meta-analysis. Individuals vary in their susceptibility to osteolysis, and it is thought that genetic polymorphism contributes to this variation. To date, our knowledge of this genetic contribution has been based on candidate gene studies. We carried out whole genome association analysis in a discovery cohort recruited form the UK. Osteolysis susceptibility case-control analysis and a QTL analysis for time to prosthesis failure was undertaken. Index SNPs from signals with $p<9x10^{-5}$, were taken forward for replication in a separate cohort recruited from the Norwegian Arthroplasty Registry. Association analyses were carried out and a meta-analysis of the 2 datasets was undertaken.

We identified a several associating signals in both discovery analyses. The most significant signal associated with osteolysis susceptibility lay within *CAMK4* (index SNP rs306105, $p=6.54x10^{-7}$) and approached genome wide significance. The most significant signal associated with time to prosthesis failure lay within *DEFB129* (rs6105394, $p=5.75x10^{-7}$) but this signal failed to replicate. Following meta-analysis, the most significant signal in the susceptibility analysis remained that within *CAMK4* (rs306105, $p = 3.79x10^{-4}$). We have identified some possible risk loci for susceptibility to osteolysis with nominal replication in genes that are involved in the regulation of immune signalling and bone turnover and forms.

7.2 Introduction

Association between genetic variation and susceptibility to periprosthetic osteolysis was first identified at the promoter region of the gene encoding tumour necrosis factor (TNF) (Wilkinson, 2003). Subsequently, several investigators have since identified associations between SNPs in pro-inflammatory cytokines and bone turnover pathways and osteolysis susceptibility (Ambruzova Z, 2006, Gordon A et al., 2008, Kolundzic et al., 2006, Gallo, 2009, Malik, 2007, Gordon A et al., 2007, Malik MH et al., 2007, Malik MH et al., 2006, MacInnes et al., 2014). To date, our knowledge of the genetics of osteolysis has been based entirely on these candidate gene studies, and the only association that has been independently replicated is that found at the *TNF* promoter (Wilkinson, 2003, Gallo and Petrek, 2009).

Candidate gene studies are dependent on *a priori* knowledge and our limited understanding of the pathogenesis of osteolysis has the potential to result in a bottleneck in investigation. Advances in high throughput genotyping together and haplotype datasets allows us to now undertake whole genome scanning using a hypothesis-free approach. There have been no systematic studies of the genetic architecture of osteolysis at genome-wide level. We conducted a genome-wide case-control association study (GWAS) in patients following THA with the aim of identifying genetic loci associated with osteolysis susceptibility and time to prosthesis failure. Association signals were followed up by replication in independent patient sets.

7.3 Methods

7.3.1 Study Populations

The discovery cohort comprised 894 men and women (317 with osteolysis) as described in chapter 3. Five hundred and fifty-three (217 with osteolysis) were

recruited locally between April 2000 and April 2006 as part of a series of case-control candidate gene studies, 108 (68 with osteolysis) were recruited locally from Sheffield Teaching Hospitals between July 2011 and January 2012, 114 (14 with osteolysis) were recruited from the UK between April 2008 and August 2010 as part of the arcOGEN study, and 119 (37 with osteolysis) were recruited from the North West of England between 2002 and 2004 as part of a separate osteolysis candidate gene study (Malik MH et al., 2006). The replication cohort comprised 2,660 men and women (783 with osteolysis) recruited from the Norwegian Arthroplasty Register, as described in the previous chapter. Note that this number is greater than that shown in figure 6.3. The reason for this is that the final number of 2,335 stated in the previous chapter describes samples suitable for GWAS genotyping. The 2,660 comprising the replication cohort in this study were from the same pool of 3,056 but were suitable for sequenom genotyping hence the discrepancy in numbers.

7.3.2 Phenotype Characterisation

Discovery cohort phenotype characterisation is described in section 2.5. Briefly, all subjects in the discovery cohort had undergone either cemented or hybrid THA with a metal on polyethylene bearing couple for primary osteoarthritis. Exclusions are outlined in section 3.2.2. Subjects who were of non-UK European ancestry were also excluded. Cases comprised subjects with radiographic evidence of either femoral or acetabular osteolysis, as described previously (Gordon A et al., 2007). The control group comprised subjects who had received a primary THA not less than 7 years previously, were asymptomatic and had no radiographic evidence of osteolysis at the time of recruitment. Polyethylene wear measurements were made using EBRA (University of Innsbruck, Austria) software in patients with available radiographs, as described in section 2.7, and used as a covariate in the association analyses. Six hundred and five subjects had available radiographs suitable for wear measurements.

Patients in the replication cohort had previously undergone primary cemented or hybrid (cemented femur) THA for idiopathic osteoarthritis. Phenotype characterisation

and inclusion criteria have been described in detail in section 6.4 The recruitment strategy, described in section 6.4, was planned to minimize confounders between the cases and controls.

7.3.3 Genotyping and Association Analyses

Genomic DNA from subjects in the discovery cohort was extracted from either whole blood or saliva and genotyped using the Illumina 610k beadchip (Illumina, San Diego, CA). We undertook quality control checks at sample and SNP level described earlier in chapter 2.8.3. Samples identified by genotype to be from individuals of non-UK European ancestry were excluded from association analyses. SNP QC was carried out on autosomal SNPs. SNPs were excluded if they had a minor allele frequency (MAF) ≥5% and a call rate <95%, or a MAF <5% and call rate <99%. SNPs with Hardy Weinberg Equilibrium (HWE) $p<0.0001$ were excluded. Following QC, we imputed the GWAS dataset using the European reference panel from the 1000 Genomes Project (Dec 2010 phase I interim release) (1000 Genomes consortium et al, 2010), and using IMPUTE2 (Marchini et al., 2007).

Osteolysis susceptibility case-control analysis and a quantitative trait locus (QTL) association analysis for time to prosthesis failure was undertaken on >10 million variants under the additive model using method score implemented in SNPTESTv2. Phenotype data was normalised where required and we performed a linear regression to adjust for age, gender, fixation method, osteolysis-free survival time and polyethylene wear. Residuals were then transformed to z-scores. Index SNPs from signals with $p<9x10^{-5}$ were prioritised for replication. Genotype-calling intensity plots were examined and SNPs with poorly clustering plots were not taken forward. Only associating SNPs with an imputation information score >0.4 and a minor allele frequency (MAF) >0.05 were progressed for the replication association analyses.

DNA from subjects in the replication cohort was extracted from saliva and genotyping was carried out using the Sequenom MassARRAY iPLEX assay (Agena Bioscience

Inc, Hamburg, Germany). Samples failing gender check or with call rate <80% were excluded. SNPs were removed if their call rate was <80% or with HWE $p<10^{-4}$. Association analyses were undertaken using logistic regression in PLINK v1.07. Z-standardized residuals were generated on phenotype data requiring normalization and regressed on age, gender, implant type, fixation method and osteolysis-free survival time. Summary statistics from both stages were combined and meta-analysed using a fixed-effects model implemented in GWAMA v2.1. (Magi and Morris, 2010).

7.3.4 Sensitivity analyses

The effect of including polyethylene wear as an analysis covariate on the findings in the discovery analyses was assessed by repeating these analyses for the prioritized SNPs and excluding polyethylene wear as an analysis covariate to observe its effect on the resultant P-value. Both the case-control susceptibility and time to failure analyses were repeated without using polyethylene wear as an analysis covariate, and the amount of overlap of SNP selection between the analysis approaches was compared.

7.4 Results

In both the discovery and replication cohorts, subjects in the osteolysis group were younger, and a greater proportion were males when compared with the control group, although the differences were smaller in the replication cohort because of the covariate minimisation strategy. In the discovery cohort, annual polyethylene wear rate was greater in the osteolysis group versus the control group (table 7.1). These findings are consistent with known risk factors associated with osteolysis, and were adjusted for in subsequent analyses. The distribution of fully cemented and hybrid prostheses in each cohort is outlined in table 7.2.

Discovery cohort			
Characteristics	**Control Group**	**Osteolysis Group**	**P value**
	n = 577	n = 317	
Age at THA (years±SD)	65.8±8	60.5±8	<0.0001
Sex (male/female)	228/349	174/143	0.01
Osteolysis free survival (years±SD)	10.8±6	9.8±5	0.05
Polyethylene wear (mm/year)	0.08 (0.04 to 0.13)	0.14 (0.08 to 0.24)	<0.001
Replication Cohort			
Characteristics	**Control Group**	**Osteolysis Group**	**P value**
	n = 1877	n = 783	
Age at THA (years±SD)	65.7±7.1	64.3±6.9	<0.0001
Sex (male/female)	739/1138	342/441	0.039
Osteolysis free survival (years±SD)	13.8±5.4	8.6±4.95	<0.0001

Table 7.1 Patient characteristics for discovery and replication cohorts

Discovery Cohort				
	Controls	Controls (%)	Cases	Cases (%)
Fully cemented THA	466	80.8	291	91.8
Hybrid THA	111	19.2	26	8.2
Replication Cohort				
	Controls	Controls (%)	Cases	Cases (%)
Fully cemented THA	1771	94.4	727	92.8
Hybrid THA	106	5.6	56	7.2

Table 7.2 Distribution of fully cemented and hybrid prostheses

7.4.1 Osteolysis Susceptibility

In the discovery analysis we identified 20 independent signals that were associated with osteolysis susceptibility (Figure 7.1A and 7.1B, and Table 7.3) at $p<9.9x10^{-5}$. One signal approached genome-wide significance: variant rs306105 lies within intron 1 of *CAMK4* (effect allele C, EAF 91%, OR 0.4, 95% CI 0.28 to 0.62, $p=6.54x10^{-7}$, Figure 7.2.

These 20 independent signals were prioritised for replication through *de-novo* genotyping in the Norwegian cohort. At replication, one variant, within *FGL1*, showed evidence for nominally significant association with the same direction of effect as the discovery cohort: rs28603021 (effect allele T, OR 0.86, 95% CI 0.73 to 1.00, p = 0.05).

Following meta-analysis no signals approached the genome-wide significance threshold of $p \leq 5 \times 10^{-8}$ (Table 7.4). The most significant signal remained that within *CAMK4* (rs306105, allele C, OR=0.7, 95% CI=0.59 to 0.86, $p = 3.75 \times 10^{-4}$) and was followed by the nominally replicating variant rs28603021 (allele T, OR 0.79, 95% CI 0.69 to 0.89, $p = 3.78 \times 10^{-4}$) in *FGL1*.

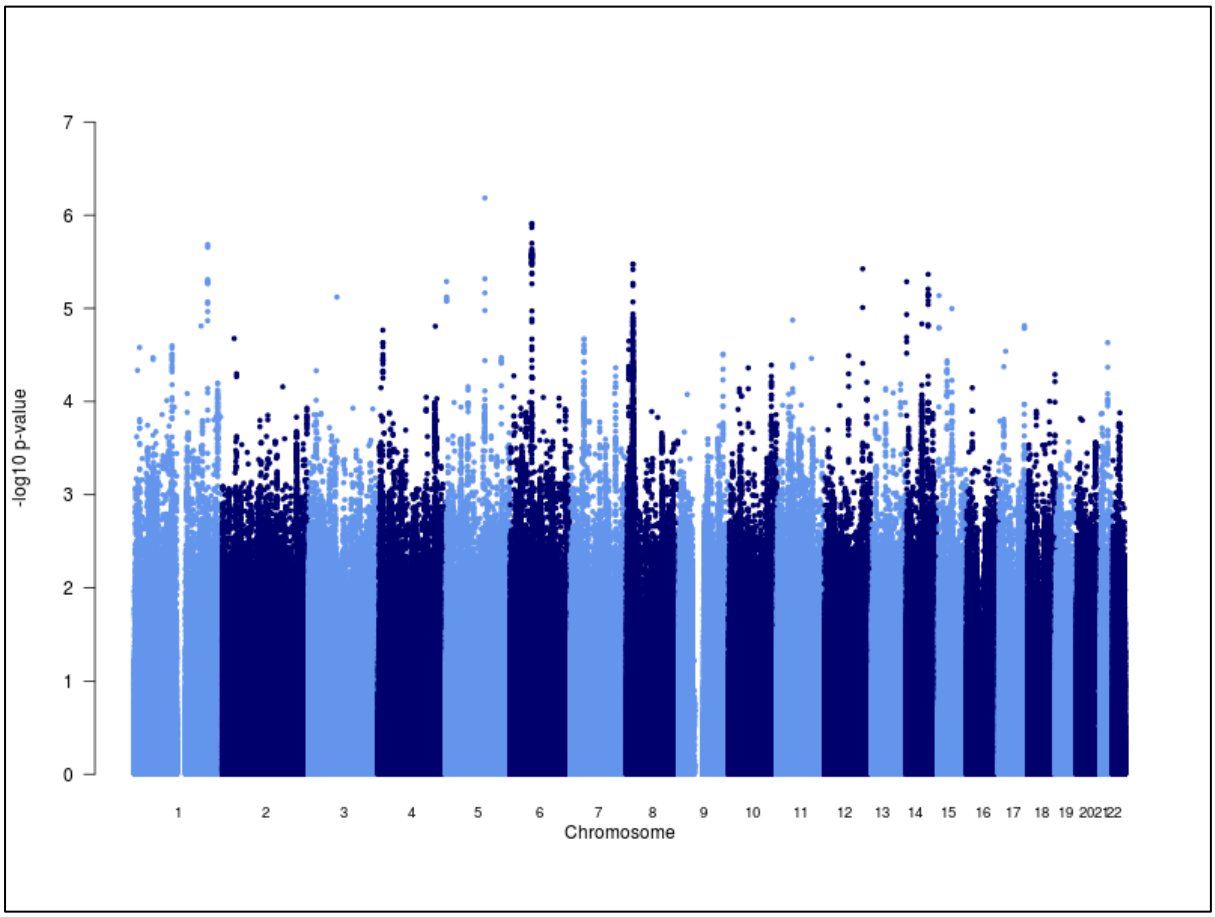


Figure 7.1A. Manhattan Plot for osteolysis susceptibility discovery analysis. The p-value is denoted on the y-axis and the chromosome position on the x-axis. Linear peaks within each chromosome represent signals consisting of clusters of SNPs.

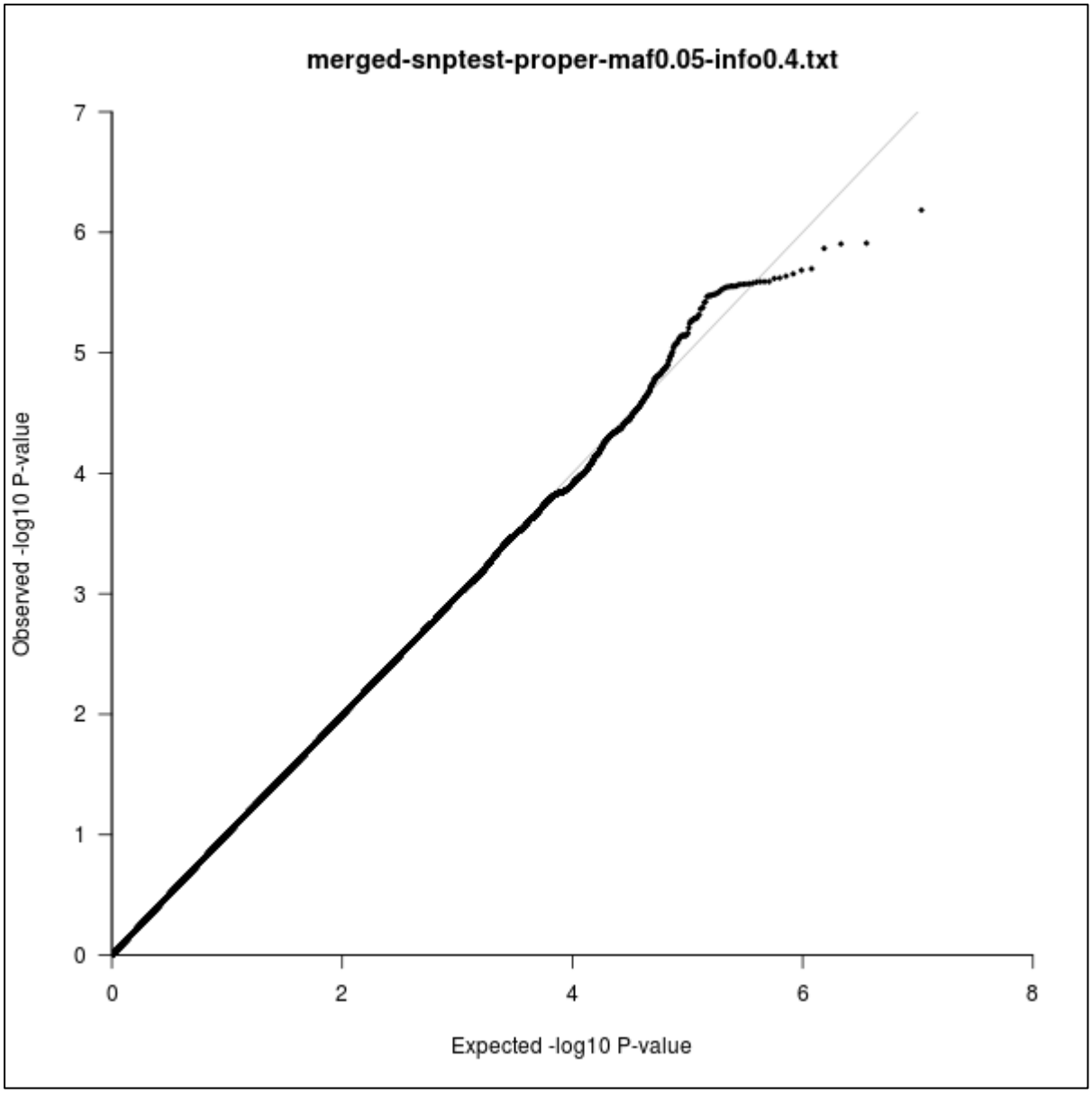


Figure 7.1B.

QQ plot for osteolysis susceptibility discovery analysis. The expected p-value is indicated by the grey line and the black dots indicate the observed p-values.

MARKER	EA	NEA	EAF	OR	95% CI	P-value
rs306105*	C	A	0.911	0.415	0.278 to 0.619	6.54E-07
rs11119057	A	G	0.278	0.541	0.419 to 0.699	2.06E-06
rs9445504	A	C	0.084	2.621	1.738 to 3.951	2.64E-06
rs28603021*	T	C	0.704	0.621	0.482 to 0.802	3.35E-06
rs10145023	T	C	0.202	1.869	1.405 to 2.488	5.18E-06
rs10055976	A	G	0.059	2.939	1.767 to 5.035	7.82E-06
rs7004096	A	C	0.080	0.351	0.203 to 0.609	2.25E-05
rs37476	A	G	0.913	0.445	0.297 to 0.666	2.44E-05
rs12810375	A	G	0.137	0.483	0.327 to 0.713	3.22E-05
rs9435429	G	A	0.647	1.597	1.236 to 2.063	3.38E-05
rs2160505	A	C	0.393	0.641	0.500 to 0.822	3.66E-05
rs3736279	C	T	0.146	1.699	1.227 to 2.343	4.34E-05
rs4143629	G	A	0.231	1.731	1.317 to 2.276	6.06E-05
rs1008727	T	G	0.396	1.612	1.267 to 2.051	6.58E-05
rs9527681	A	C	0.472	0.591	0.464 to 0.753	7.25E-05
rs4775378	G	A	0.637	1.513	1.175 to 1.948	7.94E-05
rs4301763	C	T	0.059	0.406	0.220 to 0.750	7.96E-05
rs7153101	T	G	0.705	1.444	1.105 to 1.887	8.64E-05
rs1891632	A	G	0.131	1.813	1.293 to 2.542	8.79E-05
rs2834401	T	G	0.621	0.638	0.500 to 0.814	9.38E-05

Table 7.3. Results of the osteolysis susceptibility discovery analysis for all independent signals with $p<9.9x10^{-5}$ taken forward for replication. EA = affect allele, NEA = non affect allele; OR = odds ratio, 95% CI = 95% confidence interval, *imputed variants, all other variants directly typed.

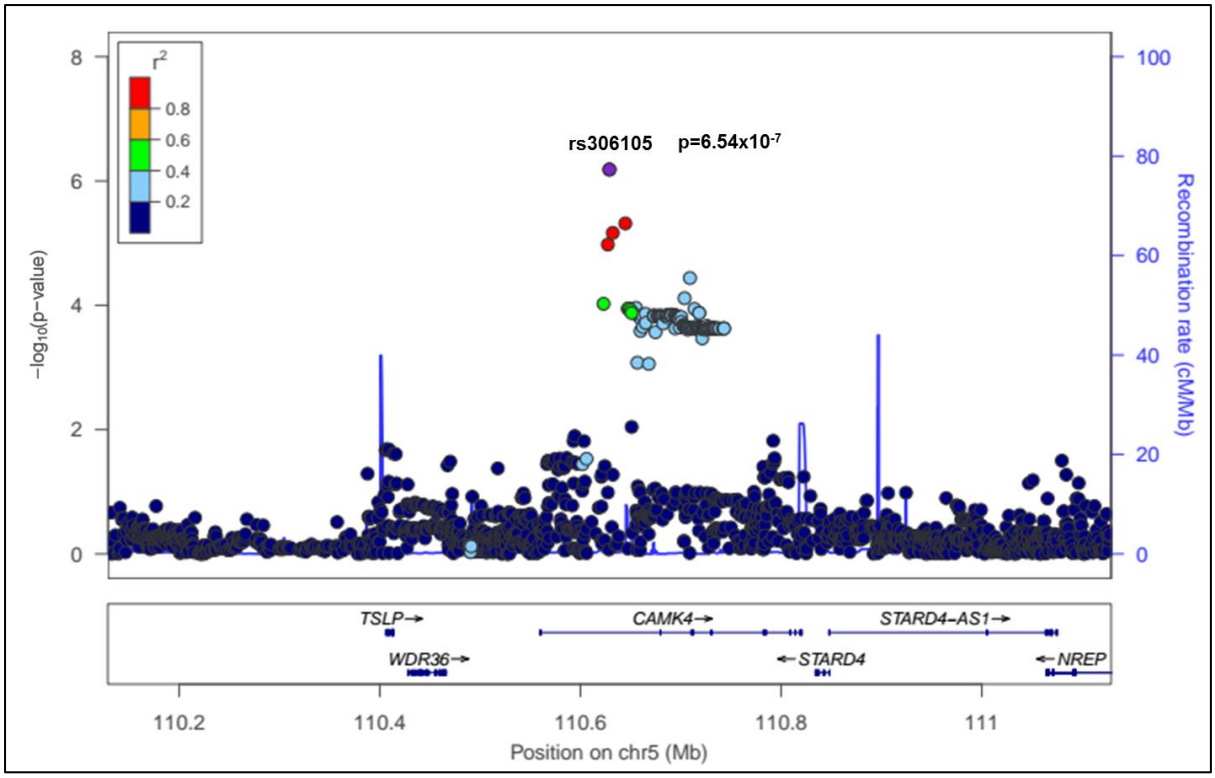


Figure 7. 2. Regional association plot for rs306105 (that lies within *CAMK4*) from the osteolysis susceptibility discovery analysis. The index SNP is denoted by the purple marker. The colour reflects the pairwise r^2 with index SNP. The region extends 500kb upstream and downstream of the index SNP.

Stage	rs_number	EA	NEA	EAF	OR	95% CI	p-value	Direction of effects	Gene
1	rs306105	C	A	0.911	0.414	0.278 to 0.619	6.54E-07		
2	rs306105	C	A	0.896	0.828	0.668 to 1.025	0.08		
MA	**rs306105**	**C**	**A**		**0.710**	**0.588 to 0.857**	**3.74E-04**	**- -**	***CAMK4***
1	rs28603021	T	C	0.704	0.622	0.482 to 0.802	3.35E-06		
2	rs28603021	T	C	0.693	0.859	0.739 to 1.000	0.05		
MA	**rs28603021**	**T**	**C**		**0.790**	**0.693 to 0.899**	**3.75E-04**	**- -**	***FGL1***
1	rs2834401	T	G	0.621	0.638	0.500 to 0.814	9.38E-05		
2	rs2834401	T	G	0.658	0.874	0.756 to 1.010	0.068		
MA	**rs2834401**	**T**	**G**		**0.805**	**0.711 to 0.912**	**6.42E-04**	**- -**	***LINC00310***

Table 7.4. Results of the osteolysis susceptibility meta-analysis showing signals with concordant direction of effect and $p<9.9x10^{-4}$. EA = affect allele, NEA = non affect allele, EAF = effect allele frequency, OR = odds ratio, 95% CI = 95% confidence interval, Direction of effects = allele effect direction.

7.4.2 Time to Prosthesis Failure

Genome-wide analysis in the discovery cohort identified 21 independent signals that were associated with time to prosthesis failure in the osteolysis cases at $p<9x10^{-5}$ (figure 7.3A and 7.3B, Table 7.5). The most significantly associated variant lay 2kb downstream from the *DEFB129* gene and approached genome wide significance (rs6105394, effect allele T, EAF 7%, beta 1.21, SE 0.24, $p=5.75x10^{-7}$, Figure 7. 4).

Twenty-one independent signals were prioritized for replication through *de-novo* genotyping in the Norwegian cohort. None of these variants showed evidence for nominally significant association in the replication cohort. Following meta-analysis, no signals approached the genome-wide significance threshold of $p\leq5x10^{-8}$ and no signals showed an increase in nominal significance.

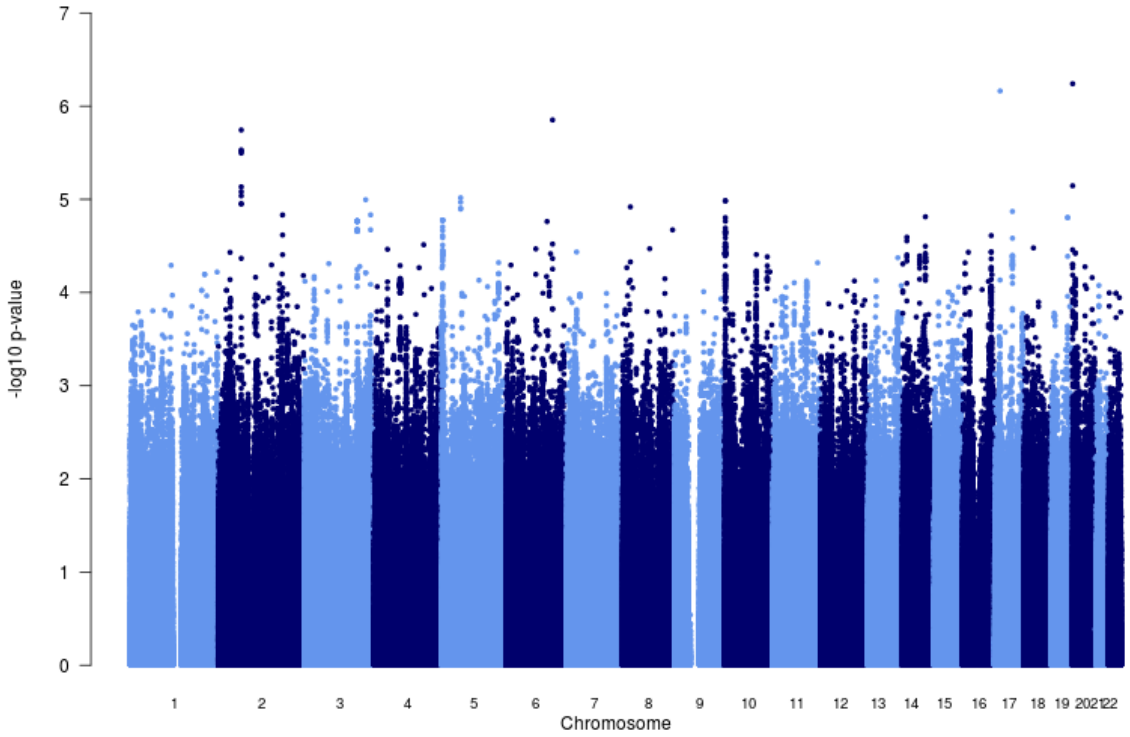


Figure 7.3A. Manhattan Plot for QTL time to prosthesis failure discovery analysis. The p-value is denoted on the y-axis and the chromosome position on the x-axis. Linear peaks within each chromosome represent signals consisting of clusters of SNPs.

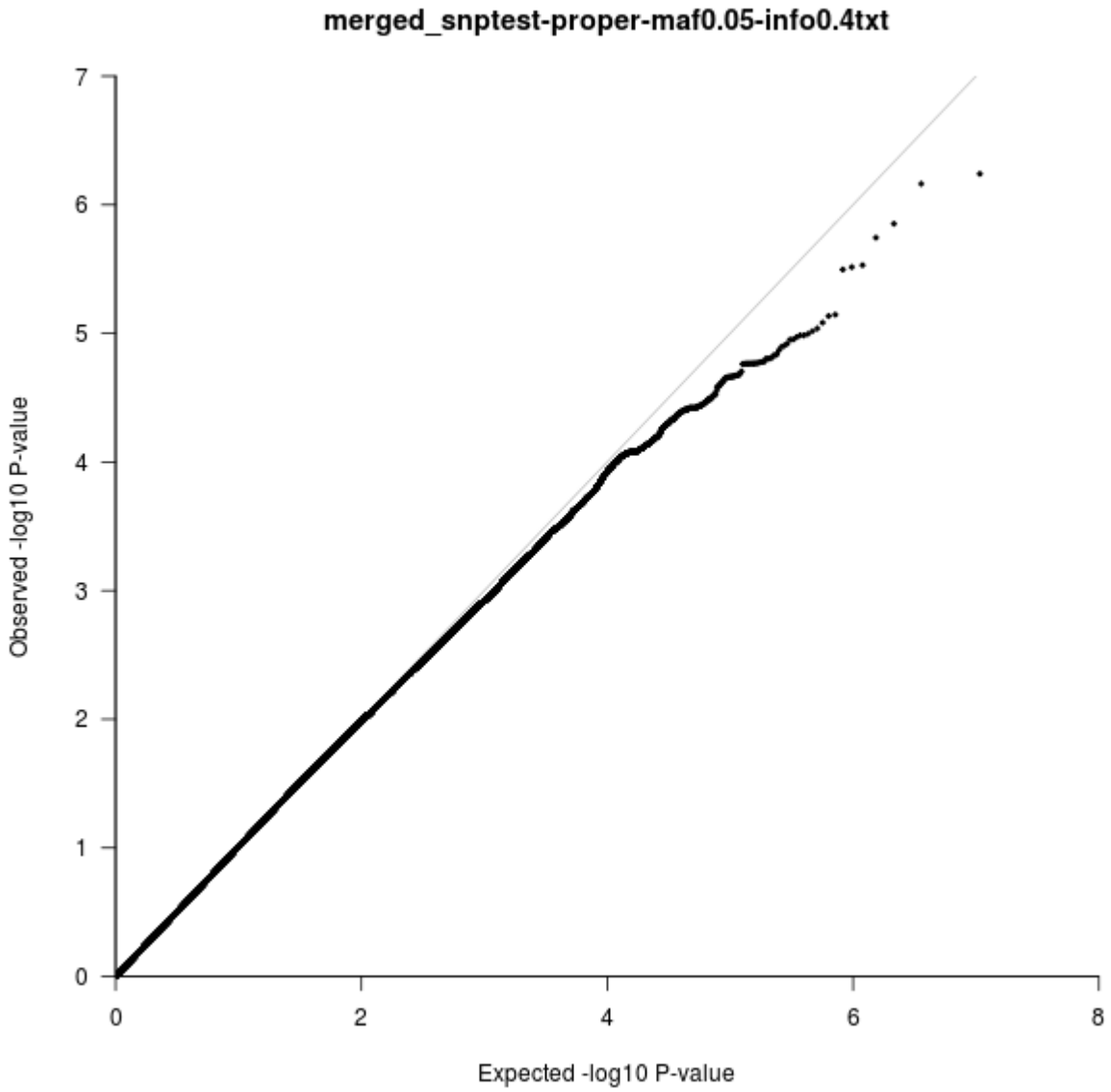


Figure 7.3B. QQ plot for QTL time to prosthesis failure discovery analysis. The expected p-value is indicated by the grey line and the black dots indicate the observed p-values.

MARKER	EA	NEA	EAF	BETA	SE	P-value
rs6105394*	T	C	0.072	1.217	0.243	5.75E-07
rs6754629	C	T	0.402	0.494	0.106	2.95E-06
rs755423*	G	C	0.909	-0.770	0.176	1.20E-05
rs702620	T	C	0.768	-0.528	0.121	1.27E-05
rs155109*	G	A	0.312	0.465	0.107	1.47E-05
rs1449413	G	T	0.356	-0.428	0.099	1.54E-05
rs4493711	A	G	0.153	-0.522	0.122	1.97E-05
rs9903651	T	C	0.773	0.476	0.113	2.61E-05
rs2029288	A	C	0.675	-0.421	0.101	3.07E-05
rs1499232	C	T	0.726	-0.461	0.112	3.67E-05
rs11904654	G	T	0.146	0.554	0.134	3.70E-05
rs6118046	T	C	0.180	0.520	0.126	3.78E-05
rs10430747	A	G	0.701	0.419	0.102	3.80E-05
rs17099320	G	A	0.115	0.657	0.160	3.94E-05
rs1887063	C	A	0.567	0.413	0.102	4.94E-05
rs6110749*	G	A	0.080	0.908	0.224	5.41E-05
rs9929066	A	G	0.094	-0.674	0.167	5.52E-05
rs10924771	A	G	0.639	0.417	0.104	6.02E-05
rs9313886	C	T	0.306	0.410	0.104	8.43E-05
rs2196565*	C	A	0.176	0.521	0.133	8.66E-05
rs11686724	A	G	0.196	0.471	0.121	9.94E-05

Table 7.5 Results of the QTL time to prosthesis failure discovery analysis. This table includes all SNPs with $p<9.9x10^{-5}$ taken forward for replication. EA = affect allele, NEA = non affect allele; OR = odds ratio; SE = standard error, *imputed variant, all other variants directly typed. rs6105394 was not taken forward for replication due to assay design issues.

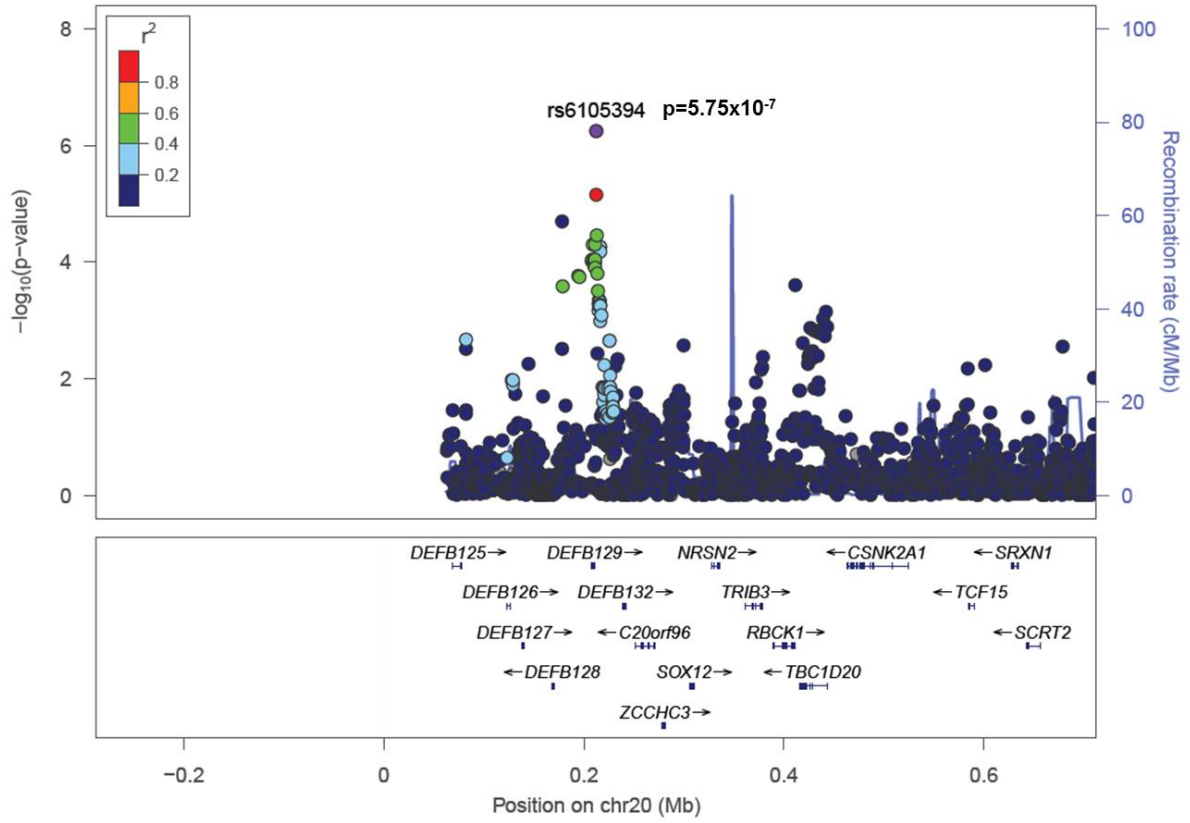


Figure 7.4. Regional association plot for rs6105394 from discovery QTL analysis time to prosthesis failure. The index SNP is denoted by the purple marker. The colour reflects the pairwise r^2 with index SNP. The region extends 500kb upstream and downstream of the index SNP.

7.4.3 Sensitivity analyses

When the case-control osteolysis susceptibility analysis for all signals with $p<9.9 \times 10^{-5}$ was repeated without wear as a covariate, all 20 SNPs showed the same direction of effect, albeit with varying ORs, and all showed a p-value that was at least nominally significant (Table 7.6). Only 4 signals retained a p-value $<9.9 \times 10^{-5}$. Conversely, when the same association analysis was repeated across all discovery SNPs without wear as an analysis covariate, 25 independent SNPs with $p<9.9 \times 10^{-5}$ were identified (Table 7.7), and all showed the same direction of effect and nominal significance when the

analysis was conducted using wear as an analysis covariate. Again, only 4 SNPs from this set showed significance at $P<9.9 \times 10^{-5}$ for both with and without wear analyses.

When the time to prosthesis failure QTL analysis for all signals with $p<9.9 \times 10^{-5}$ was repeated without wear as a covariate all 21 SNPs showed the same direction of effect, and all except one showed a p-value that was at least nominally significant (Table 7.8). Only 2 signals retained a p-value $<9.9 \times 10^{-5}$. Conversely, when the same association analysis was repeated across all discovery SNPs without wear as an analysis covariate, 20 independent SNPs with $p<9.9 \times 10^{-5}$ were identified (Table 7.9). All showed the same direction of effect and all except one showed nominal significance when the analysis was conducted using wear as an analysis covariate. Again, only 2 SNP from this set showed significance at $P<9.9 \times 10^{-5}$ for both with and without wear analyses.

In addition, we undertook replication and meta-analyses for those signals identified without wear as a covariate. We failed to replicate any signals in the case-control analysis. Two variants showed evidence for nominally significant association with the same direction of effect as the discovery cohort in the time to prosthesis failure analysis: rs12550574 (allele A, beta -0.16, SE 0.07, p = 0.019) which lies 49kbp downstream of *DOK2*; and rs1374879 (allele C, beta 0.175, SE 0.08, p = 0.035) within an intronic region of *CNTN3*. Following meta-analysis no signals approached the genome-wide significance threshold of $p \leq 5 \times 10^{-8}$. The strongest signal associated with time to failure was the replicating variant rs1374879 (allele C, beta 0.29, SE 0.07, $p=2.15 \times 10^{-5}$) within *CNTN3* and was followed by the replicating variant rs12550574 (allele A, beta - 0.24, SE 0.06, $p = 1.29 \times 10^{-4}$) which lies 49kbp downstream of *DOK2*.

			With wear analysis			Without wear analysis		
MARKER	**EA**	**NEA**	**OR**	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**
rs306105	C	A	0.415	0.278 to 0.619	6.54E-07	0.510	0.366 to 0.711	3.87E-06
rs11119057	A	G	0.541	0.419 to 0.699	2.06E-06	0.607	0.493 to 0.746	6.44E-07
rs9445504	A	C	2.621	1.738 to 3.952	2.64E-06	2.063	1.492 to 2.852	1.13E-05
rs28603021	T	C	0.622	0.482 to 0.802	3.35E-06	0.650	0.528 to 0.800	4.47E-06
rs10145023	T	C	1.869	1.405 to 2.488	5.18E-06	1.498	1.179 to 1.904	0.0007
rs10055976	A	G	2.939	1.767 to 5.034	7.82E-06	1.843	1.241 to 2.737	0.004
rs7004096	A	C	0.351	0.203 to 0.609	2.25E-05	0.587	0.395 to 0.873	0.002
rs37476	A	G	0.445	0.297 to 0.665	2.44E-05	0.592	0.425 to 0.825	0.01
rs12810375	A	G	0.483	0.327 to 0.713	3.22E-05	0.630	0.467 to 0.852	0.003
rs9435429	G	A	1.597	1.236 to 2.063	3.38E-05	1.351	1.099 to 1.661	0.0006
rs2160505	A	C	0.641	0.500 to 0.822	3.66E-05	0.718	0.588 to 0.876	0.0004
rs3736279	C	T	1.696	1.227 to 2.343	4.34E-05	1.377	1.055 to 1.798	0.002
rs4143629	G	A	1.731	1.317 to 2.276	6.06E-05	1.438	1.151 to 1.798	0.0002
rs1008727	T	G	1.612	1.267 to 2.051	6.58E-05	1.331	1.092 to 1.622	0.005
rs9527681	A	C	0.591	0.464 to 0.753	7.25E-05	0.696	0.572 to 0.846	0.001
rs4775378	G	A	1.513	1.175 to 1.948	7.94E-05	1.306	1.066 to 1.599	0.006
rs4301763	C	T	0.406	0.220 to 0.750	7.96E-05	0.570	0.359 to 0.905	0.002
rs7153101	T	G	1.444	1.105 to 1.887	8.64E-05	1.311	1.055 to 1.629	0.001
rs1891632	A	G	1.813	1.293 to 2.542	8.79E-05	1.613	1.217 to 2.139	0.0005
rs2834401	T	G	0.638	0.500 to 0.814	9.38E-05	0.708	0.579 to 0.865	0.0002

Table 7.6 This table outlines the SNPs identified in the case-control analysis with $p<9.9x10^{-5}$ comparing results when analysis with versus without wear rate as a covariate. EA = affect allele, NEA = non affect allele; OR = odds ratio; 95% CI = 95% confidence interval.

			Without wear analysis			With wear analysis		
MARKER	**EA**	**NEA**	**OR**	**95% CI**	**P-value**	**OR**	**95% CI**	**P-value**
rs11119057	A	G	0.607	0.493 to 0.746	6.44E-07	0.541	0.416 to 0.699	2.06E-06
rs306105	C	A	0.510	0.366 to 0.711	3.87E-06	0.415	0.278 to 0.619	6.54E-07
rs28603021	T	C	0.650	0.528 to 0.800	4.47E-06	0.622	0.482 to 0.802	3.35E-06
rs7973861	C	A	1.847	1.414 to 2.413	4.59E-06	1.612	1.170 to 2.222	6.88E-03
rs4361192	C	T	2.119	1.540 to 2.915	5.45E-06	1.892	1.291 to 2.774	0.008
rs9445504	A	C	2.063	1.492 to 2.852	1.13E-05	2.621	1.738 to 3.952	2.64E-06
rs11600347	A	C	0.491	0.327 to 0.736	1.60E-05	0.424	0.256 to 0.700	0.0005
rs1945691	A	G	1.524	1.197 to 1.942	2.43E-05	1.572	1.174 to 2.106	0.0008
rs6435067	C	T	1.584	1.253 to 2.003	2.69E-05	1.507	1.130 to 2.010	0.008
rs11686261	T	C	0.631	0.518 to 0.769	2.87E-05	0.631	0.496 to 0.802	0.002
rs12640141	G	A	1.617	1.256 to 2.080	2.93E-05	1.739	1.278 to 2.366	0.001
rs10495929	A	G	0.592	0.443 to 0.789	3.79E-05	0.610	0.433 to 0.860	0.003
rs4659764	G	A	1.503	1.228 to 1.839	3.92E-05	1.419	1.108 to 1.817	0.003
rs595298	A	G	1.368	1.125 to 1.662	3.99E-05	1.492	1.175 to 1.894	0.0001
rs2481952	T	C	0.698	0.574 to 0.848	4.53E-05	0.611	0.481 to 0.775	0.0002
rs4680029	T	G	0.425	0.271 to 0.666	4.57E-05	0.406	0.240 to 0.687	0.003
rs822872	A	G	0.729	0.592 to 0.897	4.63E-05	0.689	0.535 to 0.888	0.001
rs10272623	G	A	1.729	1.265 to 2.364	4.71E-05	1.616	1.098 to 2.379	0.03
rs644396	C	T	0.668	0.549 to 0.813	6.25E-05	0.699	0.550 to 0.888	0.004
rs11595566	A	G	1.675	1.255 to 2.236	6.71E-05	1.825	1.282 to 2.598	0.001
rs6065414	G	T	0.683	0.552 to 0.844	6.86E-05	0.674	0.518 to 0.875	0.002
rs10779685	C	T	1.521	1.170 to 1.978	7.28E-05	1.589	1.164 to 2.168	0.0006
rs6441583	G	A	0.597	0.454 to 0.783	7.79E-05	0.591	0.426 to 0.820	0.01
rs11039359	C	T	0.426	0.267 to 0.680	8.47E-05	0.445	0.254 to 0.780	0.007
rs6487867	A	G	0.658	0.535 to 0.809	9.14E-05	0.689	0.536 to 0.885	0.002

Table 7.7 This table outlines the SNPs identified ($p<9.9x10^{-5}$) in the case-control analysis without wear as a covariate and compares results with analysis with wear rate included as a covariate. EA = affect allele, NEA = non affect allele; OR = odds ratio; 95% CI = 95% confidence interval.

			With wear analysis			Without wear analysis		
MARKER	**EA**	**NEA**	**BETA**	**SE**	**P-value**	**BETA**	**SE**	**P-value**
rs6105394	T	C	1.217	0.243	5.75E-07	0.554	0.179	0.002
rs6754629	C	T	0.494	0.106	2.95E-06	0.275	0.085	0.001
rs755423	G	C	-0.770	0.176	1.20E-05	-0.404	0.132	0.002
rs702620	T	C	-0.528	0.121	1.27E-05	-0.388	0.101	0.0001
rs155109	G	A	0.465	0.107	1.47E-05	0.193	0.084	0.02
rs1449413	G	T	-0.428	0.099	1.54E-05	-0.290	0.083	0.0005
rs4493711	A	G	-0.522	0.122	1.97E-05	-0.255	0.100	0.01
rs9903651	T	C	0.476	0.113	2.61E-05	0.302	0.091	0.001
rs2029288	A	C	-0.421	0.101	3.07E-05	-0.367	0.085	1.67E-05
rs1499232	C	T	-0.461	0.112	3.67E-05	-0.225	0.087	0.01
rs11904654	G	T	0.554	0.134	3.70E-05	0.305	0.112	0.006
rs6118046	T	C	0.520	0.126	3.78E-05	0.456	0.111	4.09E-05
rs10430747	A	G	0.419	0.102	3.80E-05	0.257	0.083	0.002
rs17099320	G	A	0.657	0.160	3.94E-05	0.486	0.131	0.0002
rs1887063	C	A	0.413	0.102	4.94E-05	0.070	0.082	0.4
rs6110749	G	A	0.905	0.224	5.41E-05	0.468	0.162	0.004
rs9929066	A	G	-0.674	0.167	5.52E-05	-0.457	0.137	0.0008
rs10924771	A	G	0.417	0.104	6.02E-05	0.258	0.084	0.002
rs9313886	C	T	0.410	0.104	8.43E-05	0.229	0.080	0.004
rs2196565	C	A	0.521	0.133	8.66E-05	0.269	0.109	0.01
rs11686724	A	G	0.471	0.121	9.94E-05	0.219	0.096	0.02

Table 7.8 This table outlines the SNPs identified in the time to failure QTL analysis with $p<9.9x10^{-5}$ and compares results when analysis with versus without wear rate as a covariate. EA = affect allele, NEA = non affect allele; OR = odds ratio; SE = standard error.

			Without wear analysis			With wear analysis		
MARKER	**EA**	**NEA**	**BETA**	**SE**	**P-value**	**BETA**	**SE**	**P-value**
rs4394754	C	T	0.438	0.094	3.08E-06	0.278	0.119	0.02
rs284486	A	G	-0.383	0.083	4.28E-06	-0.363	0.103	0.0004
rs10764837	A	G	0.348	0.078	8.41E-06	0.228	0.097	0.02
rs1374879	C	T	0.533	0.120	9.50E-06	0.331	0.144	0.02
rs6808795	A	G	-0.351	0.080	1.18E-05	-0.280	0.096	0.003
rs2029288	A	C	-0.367	0.085	1.67E-05	-0.421	0.101	3.07E-05
rs2144456	A	G	0.321	0.075	2.03E-05	0.235	0.0907	0.01
rs2113590	C	T	0.773	0.187	3.48E-05	0.595	0.207	0.004
rs7931608	T	C	0.326	0.0791	3.83E-05	0.343	0.098	0.0005
rs6118046	T	C	0.456	0.111	4.09E-05	0.520	0.126	3.78E-05
rs9783205	C	A	-0.405	0.099	4.49E-05	-0.338	0.124	0.006
rs9371581	A	G	0.315	0.078	5.90E-05	0.333	0.097	0.0006
rs304343	C	T	0.581	0.145	6.14E-05	0.158	0.179	0.4
rs11790630	T	C	-0.373	0.093	6.71E-05	-0.269	0.120	0.03
rs9870957	C	A	0.329	0.083	7.16E-05	0.250	0.102	0.01
rs749763	G	A	0.324	0.082	7.25E-05	0.310	0.099	0.002
rs7985101	C	A	0.341	0.086	7.56E-05	0.329	0.104	0.002
rs11137472	G	A	0.413	0.105	8.90E-05	0.389	0.128	0.002
rs3766923	G	T	-0.345	0.088	9.31E-05	-0.336	0.110	0.002
rs12027243	T	C	-0.326	0.084	9.67E-05	-0.380	0.101	0.0002

Table 7.9 This table outlines the SNPs identified ($p<9.9x10^{-5}$) in the time to failure QTL analysis without wear as a covariate and compares results with analysis with wear rate included as a covariate. EA = affect allele, NEA = non affect allele; OR = odds ratio; SE = standard error.

7.5 Discussion

We conducted a genome-wide scan and replication study in an independent population in order to identify susceptibility loci for osteolysis after THA, and susceptibility loci for time to failure in osteolysis cases. We have identified possible associations for osteolysis susceptibility within *CAMK4* and *FGL1*, but no replicating evidence for association with time to prosthesis failure.

We undertook a sensitivity analysis to determine the impact of using polyethylene wear rates as an analysis covariate. We found that although all initially significant SNPs showed the same effect direction and almost all retained nominal significance only 14% of SNPs retained significance at $P<9.9 \times 10^{-5}$ when wear was excluded as an analysis covariate. When the association analyses were conducted de-novo without wear as a covariate we found that the majority of signals at $P<9.9 \times 10^{-5}$ were different to those identified when wear was included as a covariate. There was an overlap of only 5 signals in the case control analysis and 2 signals in the time to prosthesis analysis at $P<9.9 \times 10^{-5}$. For the SNPs which showed significance at $P<9.9 \times 10^{-5}$ for both with and without wear analyses the p-values were more commonly stronger in the with wear analyses. Interestingly, when we carried out replication analyses in the signals identified without using wear as a covariate we found 3 replicating signals. Two of these were within or in the flanking region of plausible gene candidates. From these analyses it remains unclear whether adjustment for polyethylene wear, when available, is the most effective strategy for genomic discovery. Polyethylene wear rate is an important risk factor for osteolysis as demonstrated by stronger association of overlapping discovery signals, however the increase in study numbers achieved by not using wear rate as a covariate may be equally important. Further study will be required to clarify this.

CAMK4 resides on chromosome 5, and encodes the calmodulin-dependent protein kinase 4 enzyme, which a member of the serine/threonine protein kinase family. This

enzyme has been implicated in transcriptional regulation of mature T cells and neutrophils (Lawson et al., 1999, Wang et al., 2001, Kitsos et al., 2005). Calmodulin signalling has been strongly implicated in osteoclastogenesis, both as an activator of receptor activator of nuclear factor-κb ligand RANK-L through AP-1 transcription factors and as a downstream regulator following RANK-L stimulation through nuclear factor of activated T cells (NFAT) transcription factors (Seales et al., 2006). Sato et al showed that the CAMK4-CREB pathway was crucial in osteoclastic bone resorption through NFAT induction and regulation (Sato et al., 2006). They also showed that *CAMK4*$^{-/-}$ mice have increased bone density compared to wild types and reduced bone erosion when subjected to a lipopolysaccharide (LPS) model of bone destruction.

FGL1 on chromosome 8, encodes fibrinogen-like protein 1 (FGL-1), a hepatocyte derived protein member of the fibrinogen family of proteins. FGL-1 is structurally similar to fibrinogen related proteins (FReP) (Yamamoto et al., 1993). The FRePs are upregulated in tissue injury and play a role in innate immunity by activating toll like receptor 4 (TLR4) (Zuliani-Alvarez and Midwood, 2015), which has recently been shown to play a role in periprosthetic osteolysis (Greenfield et al., 2010, MacInnes et al., 2014). They have also been shown to induce a number of inflammatory cytokines including interleukins 4, 6, 10, 17, tumour necrosis factor alpha (TNFα) and vascular endothelial growth factor (VEGF) (Zuliani-Alvarez and Midwood, 2015).

The most statistically significant variant in our discovery time to failure analysis, rs6105394, lies 2kb downstream of *DEFB129*. We were unable to replicate in the Norwegian population using the Sequenom MassARRAY iPLEX assay as primers for this variant could not be constructed. As there were no proxy SNPs we performed the replication analysis using the next most significant directly typed SNP, rs6110474 (beta 0.68, SE 0.17, $p=4.97 \times 10^{-5}$) in the signal using the KASPar assay. This SNP failed to replicate (beta 0.02, SE 0.04, p=0.62). Human β-defensins (hBD) are antimicrobial peptides which play an important role in innate immunity, and are thought to play a role in inflammatory disorders of bone (Warnke et al., 2006) (Kraus et al., 2012).

We also undertook replication and meta-analyses in the variants identified when not using wear as a covariate. This identified 2 further independent replicating signals within or in the flanking region of *CNTN3, DOK2* which associated with time to prosthesis failure. *CNTN3* encodes the protein contactin 3, which mediates cell adhesion (Mock et al., 1996). It's role in bone turnover is previously undescribed. *DOK2*, which encodes docking protein 2 (Dok2) is involved in the regulation of TLR 2 and 4 (Shinohara et al., 2005). More recently Dok1 and Dok2 were found to be downstream adaptors of TLR-2 inflammatory signalling through regulation of Erk and NF-κB signalling (Downer et al., 2013) Kawamata et al demonstrated that Dok1/2 deficient mice have pre-osteoclast hypersensitivity to macrophage colony stimulating factor (M-CSF) and increased numbers of TRAP positive osteoclasts and enhanced bone resorption (Kawamata et al., 2011).

Our discovery analysis had ≥80% power to detect variants with a risk allele frequency of 0.35 and an allelic OR of 1.65. The replication analysis had ≥80% power to detect variants with a risk allele frequency of 0.45 and an allelic OR of 1.45. We attempted to reduce the impact of modest power by looking only at common variants (MAF ≥0.05) but consequently the study was insufficiently powered to detect modest effects associated with rarer variants.

In summary, we have carried out the first GWA study for osteolysis and time to prosthesis failure. We have identified some possible risk loci for susceptibility to osteolysis with nominal replication in an independent population. However, a larger study with substantially more cases and controls is required to better identify genetic association signals with osteolysis. In order to achieve 80% power for our observed most significant variants (risk allele frequency 0.1, OR 0.7) we would require 750 cases and 2000 controls. Table 7.10 shows sample size required to achieve 80% power for an osteolysis GWAS for varying allele frequency and effect size.

MAF	OR	Cases (n)	Controls (n)
0.3	1.5	600	1650
0.3	1.4	1000	1750
0.2	1.4	1350	2000
0.1	1.4	2000	5000
0.05	1.35	5000	10000

Table 7.10 Sample size required for osteolysis GWAS at 80% power

CHAPTER 8

THESIS DISCUSSION

8.1 Overview

This chapter provides a summary of the studies presented in this thesis. A synopsis of the findings and significance in keeping with primary and secondary study aims will be discussed as well as study strengths, limitations and proposed future work.

8.2 Summary

In this work I have explored the genetic contribution to two common conditions associated with adverse outcome in total hip arthroplasty: osteolysis and heterotopic ossification. As an investigative team, we have followed a systematic approach starting with a candidate gene study using previously recruited subjects and progressed to whole genome association studies which required large scale prospective recruitment. We have identified several putative novel associated loci which have the potential to further our understanding of the complex pathological processes involved. Throughout these processes we have demonstrated the feasibility of recruitment for large genetic epidemiological studies using a national joint register. We have demonstrated that postal collection of saliva provides adequate DNA suitable for these studies and can also be stored establishing a biological repository with linkage to the registry. This has significant potential for the study of a number of orthopaedic conditions and is especially useful when studying low incidence phenotypes where local recruitment is likely to yield insufficient subject numbers to achieve adequate power.

In the candidate gene study presented in chapter 4 we identified 4 SNPs within *TNFRSF11A*, (rs4524033, rs9960450, rs7226991, and rs4485469), and one each within *KREMEN2* (rs4786361), *SFRP1* (rs921142), *TIRAP* (rs8177375), and *TNFRSF11B* (rs11573847) that were associated with osteolysis susceptibility. Our finding of possible association of variation in KREMEN2 with osteolysis is novel. Kremen2 modulates Wnt signalling, and mice deficient in Kremen2 have increased bone formation. (Schulze et al., 2010) The variant rs8177375 lies in the 3' UTR of TIRAP associated with osteolysis susceptibility and showed a trend toward association with time to prosthesis failure. This variant has previously been associated with risk of sepsis-induced lung injury. (Song et al., 2010) Functional studies subsequent to this thesis within our group have shown that carriage of the ‘G’ allele may result in mRNA degradation and likely inhibition of translation (MacInnes et al., 2014).

Despite this being the largest candidate gene study for osteolysis to date, the main limitation was that the sample size was still small in genetic association study terms. We aimed to mitigate against this in part by using a 2-stage analysis, followed by meta-analysis. We also attempted to reduce confounding by studying a single ethnic group and using strict exclusion criteria. Candidate gene selection, however, relied on *a-priori* knowledge of osteolysis pathogenesis and this hypothesis-driven approach may have led to potentially important genes not being investigated. This, therefore led to the hypothesis-free whole genome association analysis described in chapter 7.

We undertook the first GWAS of HO susceptibility and severity following THA, and identified promising novel loci within or in the flanking regions of the *ARHGAP18*, *BMP2*, *LGI1* and *KIF26B* genes. GWAS are hypothesis-free studies, which reduces the bias afforded by candidate gene selection. As with the candidate gene study, the main limitation of this study was that sample size was very small in GWAS terms, with consequent limited power. We aimed to mitigate this to some degree by imposing strict phenotyping criteria, ensuring that all controls were free from HO using radiographs taken no earlier than 1year post THA, by which time any HO development would have occurred. This created a group of “super-controls” which increased power compared to using a background population control group that is commonly used in GWAS. Because of the small sample size, we included only common variants with MAF>0.05. Although this method increases confidence in identified common variants, it reduces the chances of identifying associating rarer variants. As this was a single stage GWAS study some of the associated variants may represent false positives and recruitment is currently underway for a replication analysis. The use of the Brooker grading did not provide volumetric quantitation of HO but remains the accepted standard used in clinical practice. Although we did not undertake a repeatability study looking at inter and intra-observer reliability in our HO measurements, the Brooker grading has previously been shown to have good inter and intra-observer reliability with 77% and 86% agreement respectively (Wright et al., 1994).

The Norwegian Arthroplasty Register cohort study was the first demonstration of the possible extended role of national arthroplasty registries for large scale recruitment of subjects. We also showed that postal recruitment of subjects using this approach achieves reasonable response rates and postal self-collection saliva samples provide adequate DNA yield for high throughput genotyping and are therefore suitable for the establishment of a registry biobank. This strategy is scalable and potentially applicable across a range of common complex diseases where large-scale epidemiological datasets are held. The savings achieved due to lower personnel costs using saliva as a source of DNA compared to whole blood makes large scale postal collection of saliva a more cost-effective approach. Although most saliva samples provided adequate DNA concentration for whole genome analysis, we found that about a quarter of samples did not. These samples could either be amplified or used for replication analyses using other genotyping technologies. Compliance with providing adequate samples should be strongly encouraged in the information provided to patients to improve sample quality. Another limitation of postal collection of saliva was that, although unlikely, we were unable to confirm that the sample was provided by the study subject.

We conducted the first GWAS of osteolysis susceptibility and time to prosthesis failure following THA. Although a number of candidate genes studies for osteolysis have been carried out previously, the lack of a hypothesis-free whole genome scan led to the inception of this study with the aim of identifying novel genes not previously implicated in the pathogenesis. We identified possible associating signals within *CAMK4*, *FGL1* and *DEFB129*. Unfortunately, we failed to identify any signals at genome-wide significance and this is likely due to small sample size in the discovery cohort. The genetic architecture of osteolysis is complex and the genetic contribution likely consists of variants with modest allele frequency and small effect size. A larger study with substantially more cases and controls is required to robustly identify associated signals. A whole genome analysis of the Norway cohort is planned to improve study power. Having repeated the discovery analyses without polyethylene wear rate as a covariate we undertook a sensitivity analysis which confirmed wear rate as an important covariate in the study of osteolysis following THA. The advantage of the inclusion of wear rate was offset by a loss in number of study subjects. Wear rate

measurements for all study subjects would therefore be the ideal, to prevent this reduction in study sample size. As with the HO GWAS study described earlier, this study was strengthened by all subjects sharing ethnicity and the imposition of a strict inclusion/exclusion criteria with the aim of reducing confounding. This population matching is evidenced by similar allele frequencies in the discovery and replication cohorts. All subjects had undergone THA for a primary diagnosis of OA and implant type was accounted for as a covariate in analyses. By ensuring that controls were asymptomatic following THA and with no evidence of osteolysis on radiographs we again created a group of "super-controls" increasing power compared to using a background population. We undertook strict pre and post genotyping quality control measures as outlined by the WTSI. We conducted a replication study and meta-analysis to weed out false positives. We attempted to reduce the impact of modest power by looking only at common variants (MAF ≥0.05). We did not carry out a repeatability study for identification of osteolysis radiologically. There are no published studies exploring the inter and intra-observer reliability of the Harris and McGann and Harris and Penenberg classification. We acknowledge that this may have introduced a degree of error into our case and control definition, however, these are the standard classifications accepted in clinical practice and most of the cases underwent revision surgery where the presence of osteolysis was further confirmed.

8.3 Future work

The proof of concept of using a national arthroplasty registry for recruitment and the establishment of a linked biorepository has already been implemented in a further GWAS in the UK examining the genetic architecture of developmental dysplasia of the hip using the National Joint Registry of England, Wales, Northern Ireland and the Isle of Man.

The strongest signals identified in the HO GWAS have been taken forward for a replication study in a separately recruited population of patients who have undergone THA. The results from this study will be meta-analysed with the study in this thesis to examine for replicated association. The lack of stronger association in the osteolysis GWAS meta-analysis has led to a GWAS within the replication cohort. It is hoped that meta-analysis of 2 whole genome datasets will confirm signals with stronger association and improved power.

Following the identification of associations in populations of European ancestry these variants should then be further examined across other ethnic populations. Trans-ethnic replicability of variants further reinforces the robustness of their association and reduces the problem of population stratification. This can also lead to larger trans-ethnic GWAS providing larger study populations with improved power.

The GWAS described in this thesis have identified a number of associations with osteolysis and HO. Although GWAS demonstrate association they do not explain causation. Functional studies are therefore required to further investigate how the risk loci affect gene function thereby influencing the development of these conditions. Proven functional associations may then provide the basis for therapeutic target development for treatment or prevention. Further investigation may involve the study

of gene expression within the membrane surrounding loose prostheses. Common studies employed to examine gene function include knockout models and the more recently introduced gene silencing approaches. Unlike gene knockout modes where expression is completely repressed, gene silencing methods such as clustered regularly interspaced short palindromic repeats (CRISPr) and small interfering RNA (siRNA) reduce gene expression without completely eliminating it. These techniques have been used to correct the development of heritable conditions including age related macular degeneration (Kaiser et al., 2010) and muscular dystrophy (Long et al., 2014).

8.4 Conclusion

THA remains an extremely successful orthopaedic procedure. Periprosthetic osteolysis remains the leading cause of THA failure and is the result of an accumulation of complex steps which results in prosthetic loosening. Advances in the design and materials used in prostheses including improved wear profiles of bearing surfaces have led to substantial amelioration of this problem. This has resulted in reduced incidence of osteolysis and aseptic loosening, however with increasing numbers of THAs performed annually and with more active patients now undergoing arthroplasty, osteolysis will likely remain a major complication of THA for the foreseeable future. The incidence of other causes of failure of THA, such as infection, is increasing and may be a possible avenue for further whole genome study in the future.

Furthering our understanding of the biological processes involved in the pathogenesis of diseases such as osteolysis and HO is essential for forming prevention strategies and treatments. The studies described in this thesis are novel and comprise the largest genetic studies to date investigating osteolysis and HO. They have followed advances in genomic and bioinformatics technology and have provided valuable insight into the pathogenesis of these disorders following THA. The approaches taken for these studies are scalable and applicable across a range of common complex diseases that have a heritable component. This has the potential to facilitate the development of biological markers for prediction and early detection and the development of selective therapeutic agents for treatment and prophylaxis.

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APPENDIX A

Oxfordshire REC C
Room 002
TEDCO Business Centre
Rolling Mill Road
Jarrow
NE32 3DT

Telephone: 0191 428 3564
Facsimile: 0191 428 3432

16 June 2010

Mr Mark Wilkinson
Metabolic Bone Centre
Sorby Wing
Northern General Hospital
Herries Road
Sheffield
S5 7AU

Dear Mr Wilkinson

Title of the Research Tissue Bank: **Sheffield Musculoskeletal BioBank**
REC reference: **10/H0606/20**
Designated Individual: **Prof Simon Heller**

The Research Ethics Committee reviewed the above application at the meeting held on 28 May 2010. Thank you for attending to discuss the application.

In discussion, the Committee noted the following ethical issues.

The Committee noted that this was a Research Tissue Bank application and that it had been very thoroughly prepared and showed good awareness of the Human Tissue Act legislation

Members noted that Question 14 of the IRAS Application Form states that researchers will use the BioBank for commercial work; however Question 30 of the IRAS Application Form and Point 15-8 in the protocol state that all results will be available in the database – it was therefore questioned whether this would include commercial studies and negative findings.

It was felt that the ideal composition of a steering group for a BioBank such as this one would include a patient representative, service user or researcher representative, and a member of the local HTA governance committee.

The Committee felt that information regarding how the participant can withdraw at a later stage, if they wish to, should be added to the Participant Information Sheet.

Members felt that 'research project' on the Participant Information Sheet should be amended to 'BioBank'; to make it clear that this is for multiple research studies.

It was noted that the questionnaires used were for different populations, and it was felt that it would be better if standard validated form of questionnaires could be used. It was also noted, for instance, that the demographic questions are different lengths for the different groups, and it was felt that terms such as 'housewife' should be not be used.

The Committee welcomed you to the meeting and thanked you for attending.

The Committee questioned whether the rights would be retained to use the data from all users including commercial companies and that it would be available on the database, as stated in the application.

You replied that, if companies were to use samples from the bank they would have to comply with the RTB policy.

Members informed you that if this was insisted upon then companies may not deal with the BioBank. It was suggested that the wording was changed to '...all results may be available in the database...' to allow for leeway for commercial studies.

You were asked to confirm whether or not Hepatitis A or HIV samples would be accepted.

You replied that these samples would not be accepted only were known to be positive for Hepatitis A or HIV, and that there would be no testing of the samples for these diseases.

The Committee then discussed your responses further and felt that the you had addressed many of the ethical concerns adequately.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research tissue bank on the basis described in the application form and supporting documentation.

The following points are recommended changes only and are not mandatory:

1. Amendment of the wording to '...all results may be available in the database...' to allow leeway for including commercial studies.

2. Composition of the steering group to include a patient representative, service user or researcher representative, and member of the local HTA governance committee.

3. Addition of information to the Participant Information Sheet regarding how the participant can withdraw at a later stage, if they wish to.

4. Amendment of the Participant Information Sheet so that 'BioBank' is used instead of 'Research Project' to make it clear this is for multiple research projects.

5. The use of Validated Questionnaires for the different participant populations - this would constitute a substantial amendment to the study.

The Committee has also confirmed that the favourable ethical opinion applies to all research projects conducted in the UK using tissue or data supplied by the tissue bank, provided that t he release of the tissue or data complies with the attached conditions. It will not be necessary for these researchers to make project-based applications for ethical approval. They will be deemed to have ethical approval from this committee. You should provide the researcher with a copy of this letter as confirmation of this. The Committee should be notified of all projects receiving tissue and data from the tissue bank by means of an annual report.

Duration of ethical opinion

The favourable opinion is given for a period of five years from the date of this letter and provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully. The opinion may be renewed for a further period of up to five years on receipt of a fresh application. It is suggested that the fresh application is made 3-6 months before the 5 years expires, to ensure continuous approval for the research tissue bank.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering Letter	Stacy Young (Research Coordinator)	23 April 2010
REC application	IRAS Version 2.2 20923/114909/3/848	23 April 2010
Protocol for Management of the Tissue Bank	V1.0	20 April 2010
Participant Information Sheet: Participant Information Sheet - Metabolic Bone Centre Collection	v1.0	20 April 2010
Participant Information Sheet: Participant Information Sheet - Orthopaedic Collection	v1.0	20 April 2010
Participant Consent Form: Consent Form - MBC Patients	v1.0	20 April 2010
Participant Consent Form: Consent Form - Orthopaedic Patients	v1.0	20 April 2010
Human Tissue Authority Licence	Licensing Number: 12182	05 January 2010
MBC Questionnaire	v1.0	20 April 2010
Orthopaedic Questionnaire	v1.0	20 April 2010
EQ-5D Questionnaire	v1.0	20 April 2010
Oxford Hip Score	v1.0	20 April 2010
Oxford Knee Score	v1.0	20 April 2010
Withdrawal of Consent Letter	v1.0	20 April 2010
Confirmation of Destroyed Samples Letter	v1.0	20 April 2010

Licence from the Human Tissue Authority

Thank you for providing a copy of the above licence.

Research governance

A copy of this letter is being sent to the R&D office responsible for Sheffield Teaching Hospitals NHS Foundation Trust.

Under the Research Governance Framework (RGF), there is no requirement for NHS research permission for the establishment of research tissue banks in the NHS. Applications to NHS R&D offices through IRAS are not required as all NHS organisations are expected to have included management review in the process of establishing the research tissue bank.

Research permission is also not required by collaborators at tissue collection centres (TCCs) who provide tissue or data under the terms of a supply agreement between the organisation and the research tissue bank. TCCs are not research sites for the purposes of the RGF.

Research tissue bank managers are advised to provide R&D offices at all TCCs with a copy of the REC application for information, together with a copy of the favourable opinion letter when available. All TCCs should be listed in Part C of the REC application.

NHS researchers undertaking specific research projects using tissue or data supplied by the research tissue bank must apply for permission to R&D offices at all organisations where the research is conducted, whether or not the research tissue bank has ethical approval.

Site-specific assessment (SSA) is not a requirement for ethical review of research tissue banks. There is no need to inform Local Research Ethics Committees.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

Here you will find links to the following:

a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.
b) Annual Reports. Please refer to the attached conditions of approval.
c) Amendments. Please refer to the attached conditions of approval.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk

10/H0606/20	Please quote this number on all correspondence

Yours sincerely

Mr Robert West
Chair

E-mail: laura.kirkbride@sotw.nhs.uk

APPENDIX B

NHS
Health Research Authority

NRES Committee South Central - Oxford C
Level 3, Block B
Whitefriars Building
Lewins Mead
Bristol
BS1 2NT
Telephone: 01173 421383

29 April 2015

Professor Mark Wilkinson
Sheffield Teaching Hospitals NHS Foundation Trust
Metabolic Bone Centre
Sorby Wing, Northern General Hospital
Herries Road, Sheffield
S5 7AU

Dear Professor Wilkinson

Title of the Research Tissue Bank:	**SYNDMB renewal 2015**
REC reference:	**15/SC/0132**
Designated Individual:	**Professor Simon Heller**
IRAS project ID:	**170670**

The Research Ethics Committee reviewed the above application at the meeting held on 24 April 2015. Thank you for attending to discuss the application.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Miss Lauren Allen, nrescommittee.southcentral-oxfordc@nhs.net.
Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research tissue bank on the basis described in the application form and supporting documentation, subject to the conditions specified below.

The Committee has also confirmed that the favourable ethical opinion applies to all research projects conducted in the UK using tissue or data supplied by the tissue bank, provided that the release of the tissue or data complies with the attached conditions. It will not be necessary for these researchers to make project-based applications for ethical approval. They will be deemed to have ethical approval from this committee. You should provide the researcher with a copy of this letter as confirmation of this. The Committee should be notified of all projects receiving tissue and data from the tissue bank by means of an annual report.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

1) Please include the following sentence in the Participant Information Sheet. 'Samples may be used for laboratory research, if you wish to know more about this research then please ask a member of the study team.'

2) Please insert the REC name 'South-Central Oxford C' in the section 'Who has reviewed the project?' in the PIS.

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Research governance

Under the Research Governance Framework (RGF), there is no requirement for NHS research permission for the establishment of research tissue banks in the NHS. Applications to NHS R&D offices through IRAS are not required as all NHS organisations are expected to have included management review in the process of establishing the research tissue bank.

Research permission is also not required by collaborators at tissue collection centres (TCCs) who provide tissue or data under the terms of a supply agreement between the organisation and the research tissue bank. TCCs are not research sites for the purposes of the RGF.

Research tissue bank managers are advised to provide R&D offices at all TCCs with a copy of the REC application for information, together with a copy of the favourable opinion letter when available. All TCCs should be listed in Part C of the REC application.

NHS researchers undertaking specific research projects using tissue or data supplied by the research tissue bank must apply for permission to R&D offices at all organisations where the research is conducted, whether or not the research tissue bank has ethical approval.

Site-specific assessment (SSA) is not a requirement for ethical review of research tissue banks.

Summary of discussion at the meeting

Social or scientific value; scientific design and conduct of the study

The Committee asked how useful the tissue bank had been so far.

You responded that there were currently 30 studies using the blood, serum and urine samples as well as prospective collections of waste tissue from joint replacement surgery. You considered that the resource was critical in enabling researchers to be able to carry out their studies.

Recruitment arrangements and access to health information, and fair participant selection

The Committee was unclear whether any paediatric samples would be collected for the tissue bank.

You clarified that it was an adult only biobank. If there was a study involving children's bone disease that had obtained consent for tissue to be stored in a biobank after the research had been completed, then these samples would be considered for entry. You confirmed that there would not be any primary collection of samples from children.

Care and protection of research participants; respect for potential and enrolled participants' welfare and dignity

The Committee noted that the samples could be used for animal research; however, this was not made clear in the Participant Information Sheet or consent form.

You explained that the samples would not be used for cloning, only standard animal model work. You acknowledged that the current wording was subtle and this was approved as part of a Substantial Amendment to the Protocol three years ago. You further explained that you had been encouraged not to use the words animal testing as some people had strong views against this.

The Committee explained that there had historically been attacks on researchers in Oxford from animal rights campaigners, which was the reason why researchers were encouraged at the time not to mention animal research in documents that were in the public domain. However, the HRA's position now was for greater openness so that those who did not wish for their samples to be used for animal research could opt-out. The Committee advised that it would suggest a form of words that could be used.

You stated that you would be happy to incorporate the new wording. You informed the Committee that no-one had declined to enter the research so far because of an objection to animal research.

Informed consent process and the adequacy and completeness of participant information

The Committee was unclear why so many different Participant Information Sheets were needed. It was suggested that these could be reduced to 1 or 2 information sheets.

You answered that because the saliva sample was collected remotely the information sheet for this needed to be kept separate. You also needed the wording of the waste tissue information sheet to be slightly different to the information sheets for collection of blood, serum and urine samples.

The Committee was satisfied with this response.

The Committee noted that the REC name needed to be included in the information sheets.

Suitability of supporting information

The Committee commented that the letter for withdrawal of samples was very well-written and clear. You were asked if this had ever been used.

You confirmed that it had never been used, but the mechanism was there if needed.

The Committee requested confirmation of the composition of the steering committee and whether there were any lay members.

You responded that every application to use the resource needed to have lay approval and applicants to the tissue bank were required to provide a lay summary which would be available for public viewing. There was one lay member on the steering committee who reviewed the applications.

The Committee explained that it usually required there to be at least two lay members on the steering committee. The Committee asked whether it would cause any problems if it requested for there to be another lay member.

You replied that there were other lay people on the advisory panel who could fill in if needed. You agreed that a suggestion to add another lay member rather than a requirement would be helpful.

Duration of ethical opinion

The favourable opinion is given for a period of five years from the date of this letter and provided that you comply with the standard conditions of ethical approval for Research Tissue Banks set out in the attached document. You are advised to study the conditions carefully. The opinion may be renewed for a further period of up to five years on receipt of a fresh application. It is suggested that the fresh application is made 3-6 months before the 5 years expires, to ensure continuous approval for the research tissue bank.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Human Tissue Authority licence		05 January 2010
IRAS Checklist XML [Checklist_19022015]		19 February 2015
Other [PIS Ortho]	1.0	31 January 2015
Other [PIS saliva donation]	1.0	31 January 2015
Other [Consent form Ortho]	1.0	31 January 2015
Other [Consent postal saliva]	1.0	31 January 2015
Other [EQ5D]	1.1	31 January 2015
Other [Oxford Hip Score]	1.0	31 January 2015

Other [Oxford Knee Score]	1.0	31 January 2015
Other [Generic questionnaire]	1.0	31 January 2015
Other [Invitation letter MBC]	1.0	31 January 2015
Other [Invitation letter orthopaedics]	1.0	31 January 2015
Other [Invitation letter postal saliva donation]	1.0	31 January 2015
Other [invitation letter prev pts or res part]	1.0	31 January 2015
Other [appointment letter]	1.0	31 January 2015
Other [Withdrawal of consent]	1.0	31 January 2015
Other [confirmation of destroyed samples]	1.0	31 January 2015
Other [Postal donation instructions letter]	1.0	31 January 2015
Participant consent form [Consent MBC]	1.0	31 January 2015
Participant information sheet (PIS) [PIS MBC]	1.0	31 January 2015
Protocol for management of the tissue bank	1.0	31 January 2015
REC Application Form [RTB_Form_19022015]		19 February 2015
Summary of research programme(s)	1.1	02 February 2015

Licence from the Human Tissue Authority

Thank you for providing a copy of the above licence.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.There were no declarations of interest.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached standard conditions give detailed guidance on reporting requirements for research tissue banks with a favourable opinion, including:

- Notifying substantial amendments
- Submitting Annual Progress reports

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

15/SC/0132	**Please quote this number on all correspondence**

Yours sincerely

PP

Professor Nigel Wellman
Chair

E-mail: nrescommittee.southcentral-oxfordc@nhs.net

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*

Standard approval conditions

Copy to: *Professor Simon Heller, Sheffield Teaching Hospitals NHS Foundation Trust*

NRES Committee South Central - Oxford C

Attendance at Committee meeting on 24 April 2015

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Leonard Brookes	Consultant to the Pharmaceutical Industry	Yes	
Miss Gemma Davison	Solicitor	Yes	
Dr Avinash Gupta	Clinical Research Fellow	No	
Mrs Rebekah Howe	Farmer	Yes	
Mrs Vivienne Laurie	Barrister	Yes	
Mrs Susan Lousada	Company Director (Property) & Non-legal member of first-tier tax tribunal	Yes	
Mr Barry Muir	Retired NHS Management Consultant	No	
Dr Lee Potiphar	Clinical Trials Manager	Yes	
Professor David Scott	Pharmacist	Yes	
Dr Sabeena Sharma	Consultant Anaesthetist	Yes	
Dr Surjeet Singh	Clinical Trials Coordinator	Yes	
Ms Kayleigh Stanbury	Trial Coordinator and Developmental Assessor	Yes	
Professor Nigel Wellman (Chair)	Professor of Health and Human Sciences	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Miss Lauren Allen	REC Manager
Miss Maria Ayeni	Observer
Dr Liesl Osman	Observer

APPENDIX C

NHS

National Research Ethics Service

Oxfordshire REC C
2nd Floor, Astral House
Chaucer Business Park
Granville Way
Bicester
OX26 4JT

Telephone: 01869 604082
Facsimile: 01869 604055
Email: scsha.OxfordRECC@nhs.net

03 January 2008

Dr John Loughlin
Institute of Musculoskeletal Science
Botnar Research Centre,
Nuffield Orthopaedic Centre
Windmill Road
Oxford
OX3 7LD

Dear Dr Loughlin

Full title of study: **The arcOGEN Study - A genome wide association study of osteoarthritis**
REC reference number: **07/H0606/150**

Thank you for your letter of 17 December 2007, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form. Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed they have no objection.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application		25 October 2007
Investigator CV	Dr J Loughlin	29 October 2007

Investigator CV	Dr A Carr	
Protocol	1	
Covering Letter		29 October 2007
Covering Letter		01 November 2007
Letter from Sponsor		25 October 2007
Peer Review		
Questionnaire: Data Collection Form	5	18 October 2007
Letter of invitation to participant	3	24 October 2007
GP/Consultant Information Sheets	2	24 October 2007
Participant Information Sheet	4	17 December 2007
Participant Consent Form	4	17 October 2007
Response to Request for Further Information		17 December 2007
Letter from Funder		01 October 2007

R&D approval

All researchers and research collaborators who will be participating in the research at NHS sites should apply for R&D approval from the relevant care organisation, if they have not yet done so. R&D approval is required, whether or not the study is exempt from SSA. You should advise researchers and local collaborators accordingly.

Guidance on applying for R&D approval is available from http://www.rdforum.nhs.uk/rdform.htm.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

Here you will find links to the following

a) Providing feedback. You are invited to give your view of the service that you have received from the National Research Ethics Service on the application procedure. If you wish to make your views known please use the feedback form available on the website.
b) Progress Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
c) Safety Reports. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
d) Amendments. Please refer to the attached Standard conditions of approval by Research Ethics Committees.
e) End of Study/Project. Please refer to the attached Standard conditions of approval by Research Ethics Committees.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nationalres.org.uk .

07/H0606/150	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project

Yours sincerely

Mrs Janet Burton
Chair

Enclosures: *Standard approval conditions*

Site approval form

Copy to: Ms Heather House, Clinical Trials Unit, Manor House, John Radcliffe Hospital

This Research Ethics Committee is an advisory committee to South Central Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England.

Oxfordshire REC C

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	07/H0606/150	**Issue number:**	1	**Date of issue:**	03 January 2008
Chief Investigator:	Dr John Loughlin				
Full title of study:	The arcOGEN Study - A genome wide association study of osteoarthritis				

This study was given a favourable ethical opinion by Oxfordshire REC C on 03 January 2008. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes* [1]
Professor Andrew Carr	Nuffield Professor of Orthopaedics	Nuffield Orthopaedic Centre NHS Trust	Oxfordshire REC C	03/01/2008	
Prof Andrew McCaskie	Professor of Orthopaedic Surgery	The Newcastle upon Tyne Hospitals NHS Foundation Trust	Newcastle & North Tyneside 2 Research Ethics Committee	03/01/2008	

Approved by the Chair on behalf of the REC:

..... (Signature of ~~Chair~~/Co-ordinator)

(delete as applicable)

Kathryn Lucas ..... (Name)

APPENDIX D

Office use only: Study ID: _____ Initials: | | | |

Musculoskeletal BioBank Ethnicity Questionnaire (STH15691)

Please note we are not asking about citizenship or nationality but about the ethnic group to which you feel you belong. All information received will be used and treated in the strictest confidence. The level of care you will be offered at this hospital will not be affected by your decision to complete this form. If you have any queries please speak to a staff member. Otherwise please complete the form below by ticking the box of the ethnic group you feel you belong to. If you feel you are descended from more than one group **please tick** the one that you feel you belong to, or choose the 'any other group' option.

White		
A	British	
B	Irish	
C	Any other white background	
Mixed		
D	White and Black Caribbean	
E	White and Black African	
F	White and Asian	
G	Any other mixed background	
Asian or British Asian		
H	Indian	
I	Pakistani	
J	Bangladeshi	
K	Any other Asian background	
Black or Black British		
L	Caribbean	
M	African	
N	Any other black background	
Other ethnic groups		
O	Chinese	
P	Any other ethnic group	

Thank you for completing this questionnaire

APPENDIX E

SPØRRESKJEMA FØR STUDIEDELTAKELSE

Pasientens initialer og fødselsår:

Pasient identitetsnummer:

Dato for utfylling: date .....

Fødeland: country of birth .....

Mors fødeland: mothers — " — .....

Fars fødeland: fathers — " — .....

Etnisk gruppe: ☐ Etnisk norsk Norwegian

ethnic group ☐ Annet (spesifiser) other. specify. .....

(Spørsmålene over er tatt med fordi arvematerialet som vi samler inn skal sammenholdes med DNA fra en kaukasisk befolkning)

Smoking *Røyker du?* ☐1 Ja ☐2 Nei ☐3 Har røkt tidligere Have been smoking.

Diabetes? ☐1 Ja ☐2 Nei

Din høyde? height ..... cm

Din vekt? weight ..... kg

S1 *Har du besvær fra den andre hoften?* difficulties with other hip? ☐1 Ja ☐2 Nei

S2 *Er det andre årsaker til at du har gangproblemer?* ☐1 Ja ☐2 Nei
(For eksempel smerter fra andre ledd, ryggsmerter, hjerte-karsykdom eller andre sykdommer som påvirker gang-evnen din) other reasons for difficulty walking

Nedenfor og på neste side er ytterligere spørsmål som angår din fornøydhet, ditt aktivitetsnivå og din arbeidssituasjon før og etter operasjonen(e). Spørsmålene i grått gjelder bare de som er operert mer enn én gang satisfaction?

S3 **Kryss av for hvordan du i dag vurderer nytten av proteseoperasjonene.** satisfied with the prostheses? operations.

VG Meget god ☐1

G God ☐2

F Hverken god eller dårlig ☐3

B Dårlig ☐4

VB Meget dårlig ☐5

Tilleggsspørsmål

Det har vært mange som har kommet med ekstra opplysninger i forbindelse med spørreskjemaet som ble sendt ut i den første forsendelsen. Dette setter vi stor pris på. Det ville være til stor nytte for oss om de to ekstra spørsmålene under kunne bli besvart av alle.

1) Vet du om du har søsken eller foreldre som har fått satt inn en primær totalprotese på grunn av artrose? Have family had THR for arthritis

P19 Mor ☐
Far ☐
P21 Søsken ☐
P23 Vet ikke ☐

P22 Oppgi antall hvis du har flere: .....
How many siblings

How you had pain/problems after revision

2) Har du hatt senplager etter revisjonen?

P24 Nei ☐
P25 Ja ☐

P26 Hvilke type senplager?.....
What is the problem

Kommentar.

APPENDIX F

UNIVERSITETET I BERGEN
Regional komité for medisinsk og helsefaglig forskningsetikk, Vest-Norge (REK Vest)

Ove Furnes
Ortopedisk avdeling
Haukeland universitetssykehus
5021 Bergen

Deres ref	Vår ref	Dato
	2008/10018-ØYSV	08.09.2008

Ad. prosjekt: Arvelighet ved løsning av hofteproteser (188.08).

Det vises til din søknad om godkjenning av forskningsprosjekt, datert 14.08.08 og søknad om opprettelse av forskningsbiobank, datert 14.08.08.

Komiteen behandlet søknaden i møte den 28.08.08.

De regionale komiteene for medisinsk og helsefaglig forskningsetikk foretar sin forskningsetiske vurdering med hjemmel i Forskningsetikklovens § 4. Saker vedrørende forskningsbiobanker behandles i samsvar med Biobankloven. Saksbehandlingen følger Forvaltningsloven.

Komiteen mener at dette er en uproblematisk studie. En har ingen merknader til forelagt protokoll.

Spørreskjemaet til deltakerne er ikke lagt ved i søknaden. Komiteen ber om å få tilsendt spørreskjemaet.

Vedtak:
Prosjektet godkjennes i samsvar forelagt søknad. REK Vest forutsetter at søknad om opprettelse av forskningsbiobank godkjennes av Helsedirektoratet.

REK Vest tilrår at forskningsbiobanken blir opprettet.

Komiteen ber om å få tilsendt sluttrapport evt. trykt publikasjon for studien.

Komiteens vurdering av søknad om opprettelses av forskningsbiobank videresendes Helsedirektoratet for endelig vedtak der.

Postadresse
Postboks 7804
5020 Bergen

rek-vest@uib.no
www.etikkom.no/REK
Org no. 874 789 542

Regional komité for medisinsk og helsefaglig forskningsetikk, Vest-Norge
Telefon 55 97 84 97 / 98 / 99

Besøksadresse
Haukeland Universitetssykehus

Vennlig hilsen

Arnold Berstad
nestleder

Øystein Svindland
førstekonsulent

Kopi: Hdir