THE ROLE OF METAL IONS IN METAL-ON-METAL TOTAL HIP REPLACEMENT

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I confirm that the work submitted is my own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

The research presented here has been carried out in collaboration and discussion with my supervisors, Ian Clark, Simon Donell, and John Nolan.

The work presented in Chapter 3 was carried out in collaboration with Dr Heather Felgate (UEA), Dr Martin Lott (UEA), Dr Fernando Martinez-Estrada (Oxford University) and Dr Sarah Snelling (Oxford University).

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"For our darling Eileanóir Róisín Flaviana Ebreo. Gone on her adventures too soon. We see you in the rainbows, we see you in the stars, we all hold you in our hearts and think about where you have gone and what you have seen today. We love you always dearest girl; never stop exploring.

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D.E.

Abstract

Role of Metal Ions in Metal on Metal Total Hip Replacement

Metal-on-metal (MoM) total hip replacement (THR) offered theoretical advantages of decreased wear and increased functional outcomes. MoM bearings have been associated with sterile inflammatory masses and significant soft tissue destruction with poorer outcomes following revision surgery. The natural history of adverse reactions to metal debris (ARMD) is unknown, but may represent contributions from bearing surface wear, taper junction wear, and corrosion. Immunological and genetic factors may influence susceptibility to development of ARMD.

Between 1997 and 2004, 652 Ultima TPS THRs (DePuy) were implanted in 545 patients who went on to experience a high rate of early implant failure (13.8%). Management of these patients has evolved beyond regular clinical follow up with plain radiographs to include surveillance monitoring of trends in levels of blood metal ions and staging of disease using metal artefact reduction (MAR) MRI.

This study investigated:

- The natural history of ARMD in 28mm MoM THR using MAR MRI.
- The possibility of diurnal variation of cobalt (Co) and chromium (Cr).
- Differences in gene expression profiles of patients undergoing revision of a metal-on-polyethylene (MoP) THR for aseptic loosening and those with ARMD arising from MoM THR.

In patients whose MARS MRI staging on initial scan is normal or where mild evidence of disease is demonstrated, further deterioration is unlikely. Where it does occur, the rate of deterioration is slow and there may be a latent period of many years.

There is no diurnal variation in levels of cobalt (Co) in plasma or urine. There is diurnal variation in levels of plasma chromium, but this is not clinically significant.

Patterns of gene expression in patients with aseptic loosening of a metal-on-polyethylene (MoP) THR, and patients with ARMD from a MoM THR investigated by a microarray experiment were found to be similar on a genome-wide level suggesting that they may share common factors in their aetiologies.

These studies have helped to describe the natural history of ARMD on a cellular and radiological level, and have provided a contribution to the evidence base that will inform as to the optimum strategy for monitoring these patients before they develop catastrophic complications.

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List of Abbreviations

ALTR	Adverse Long Term Reaction to Metal
ALVAL	Aseptic Lymphocytic Vasculitis Associated Lesion
ARMD	Adverse Reaction to Metal Debris
ASTM	American Society for Testing and Materials
C-X-C	C-X-C motif chemokine
cDNA	Complementary Deoxyribonucleic Acid
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
Co	Cobalt
CP	Canonical Pathway
Cr	Chromium
CV	Co-efficient of variation
DNA	Deoxyribonucleic Acid
EQ-5D	Euroquol Quality of Life index 5D
FDA	Food and Drug Administration
FDR	False Discovery Rate

HES	Hospital episode statistic
HIF	Hypoxia inducible factor
IL	Interleukin
IPA	Ingenuity Pathway Analysis™ software
MAPK	Mitogen-activated protein kinase
MAR	Metal Artefact Reduction
MARS	Metal Artefact Reduction Sequence
MHRA	Medicines and Healthcare Regulatory Authority
MIAME	Minimum Information About a Microarray Experiment
MMP	Matrixmetalloproteinase
Mo	Molybdenum
MRI	Magnetic Resonance Imaging
MyD88	Myeloid differentiation primary response gene
NJR	National Joint Registry
OA	Osteoarthritis
OHS	Oxford Hip Score
OPG	Osteoprotegrin
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PGE2	prostaglandin E2
PMMA	Polymethylmethacrylate
ppb	Parts per billion
PROMS	Patient recorded outcome measure
qRT-PCR	Quantitative Real-time Polymerase Chain Reaction
RANK	Receptor activator of NF-κB
RANK-L	Receptor activator of NF-κB ligand
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
THR	Total Hip Replacement
Ti	Titanium
TIMP	Tissue inhibitor of matrix metalloprotease
TLDA	Taqman Low Density Array
TLR	Toll-like receptor
TNF	Tumour Necrosis Factor
TPS	Tapered Polished Stem
TRIF	Toll/interleukin-1 receptor domain-containing adaptor-inducing interferon-beta
UHMWPE	Ultra-high-molecular-weight polyethylene
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

1 : Introduction

1.1 Background

Total Hip Replacement (THR) is a common surgical intervention to improve quality of life for patients with painful hip arthritis. It is one of most successful and commonly performed elective surgical interventions of the modern era and is considered by the World Health Organisation to be one of the most cost-effective interventions in medicine[1]. In 2012 there were over 90,000 hip replacement procedures recorded by the National Joint Registry of England and Wales. Of these 76,448 were primary procedures and 10,040 were revision procedures [2]. Increasing demand for arthroplasty procedures is driven by an ageing population who wish to remain active and an escalation in the number of obese patients[3].

The success of a THR design can be measured via a number of factors that may be used in isolation or in concert. These measures can be patient reported, radiographic, or be based on implant longevity.

Patients can give valuable insight as to the success of their operation using validated instruments known as patient reported outcome measures (PROMS). These involve answering a number of subjective questions whose responses are assigned a numerical score; these scores are then added together to give a final overall score. Some PROMS are specific to hip arthroplasty e.g. the Oxford Hip Score (OHS) [4] whilst

others incorporate aspects of general health, perceived well-being and quality of life e.g. EQ-5D[5,6].

Radiological methods can be used to evaluate a THR via evaluation of the quality of the fixation in terms of the quality of cementation[7], the component position[8] and evidence of loosening[9]. These methods are not universally adopted and may not apply to all implant types.

Longevity of an implant can be measured as the time from implantation to time to failure mandating revision surgery. There is evidence that conventional cemented metal-on-polyethylene bearing THRs have a survival rate greater than 90% at 12 years [10] whilst certain designs have demonstrated up 78% survival at 30 years[11].

The long-term survival of an implant is subject to a number of intricately related factors (Figure 1.1-1) and implant failure remains a leading cause of pain and loss of function. The causes of implant failure can be broadly classified into three categories:

1) Factors relating to implant design, materials, and fabrication
2) Factors which are related to the surgeon; poor patient or implant selection, poor surgical technique resulting in adverse component positioning and soft tissue balancing which adversely affect implant survival.
3) Patient related factors including body weight, level of physical activity, comorbidity predisposing to delayed bone healing, and compliance.

It remains one of the major challenges in orthopaedic engineering to develop an implant system with superior wear resistant properties with a high degree of biocompatibility.

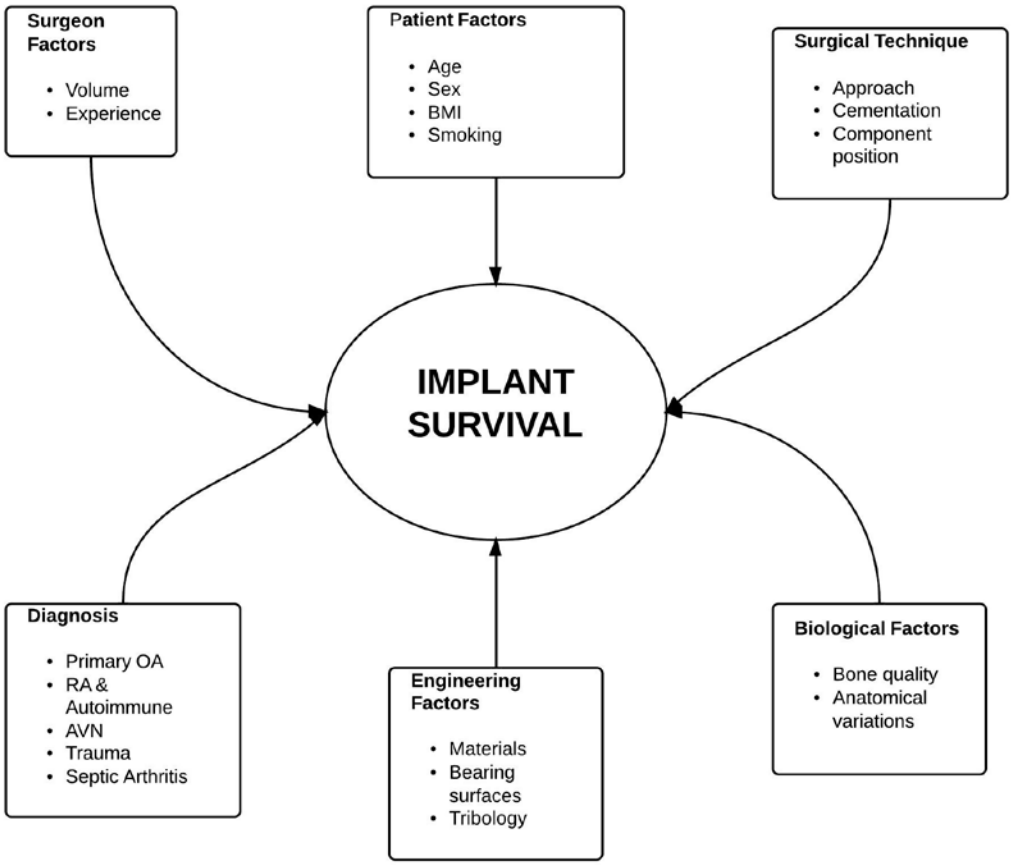

Figure 1.1-1 Parameters affecting the long-term survival of a Total Hip Arthroplasty (THA)

The ideal bearing solution for a THR should possess a number of important properties. The articulating surfaces should be resistant to wear and be made of biologically inert materials so as not to evoke a host response to the implant itself or wear debris generated. In terms of material composition all parts should be chemically stable and

not prone to corrosion, tough enough to resist fracture, and be hard and non-ductile to reduce susceptibility to scratching and third body wear[12].

Metal-on-metal bearing surfaces were thought to confer such advantages and in particular to be an ideal choice of bearing surface in younger or more active individuals. However, it could be argued that existing designs are excellent and that further attempt at improvement are ultimately not cost effective.

1.2 Periprosthetic Osteolysis & Aseptic Loosening

The process of periprosthetic osteolysis with accompanying aseptic loosening is a major factor affecting longevity of any device and is the result of wear debris generated at the bearing surface between the replacement femoral head and acetabular cup. Aseptic loosening is the most commonly recorded indication for revision surgery, accounting for 40% of all revisions[2].

All bearing surface combinations will wear in use and will generate particulate debris which can be responsible for adverse tissue reactions; this phenomenon is not exclusive to MoM bearings. Particles of ultra-high-molecular-weight polyethylene (UHMWPE), polymethylmethacrylate (PMMA) cement, titanium alloy, aluminium oxide (Al_2O_3) and zirconium dioxide (ZrO_2) have all been demonstrated to be biologically active when produced as wear debris[13]

Both the volume and morphology of wear debris are factors in determination of the host response and subsequent survival of the implant. Wear particles generate a complex inflammatory and foreign body reaction resulting in increased macrophage and foreign body giant cell activity with the net result of periprosthetic tissue destruction and implant loosening. Submicrometer particles stimulate periprosthetic macrophages to express proinflammatory and pro-osteoclastic cytokines, cell surface receptors and signalling molecules that promote formation, accumulation, activity and survival of osteoclasts and inhibit the osteogenic activity of osteoblasts with the result that bone resorption predominates over osteogenesis at the bone-implant interface.

Macrophage activation is central to the initiation of an osteolytic response. The reaction of macrophages depends on particle size. With particles of size <10μm, macrophages and foreign body giant cells are able to adhere to and phagocytose the particles[14]. This leads to the production of pro-inflammatory signalling molecules and production of an array of inflammatory mediators including prostaglandin E2 (PGE2), Tumour Necrosis Factor-alpha (TNFα), interleukin-1beta (IL-1β), interleukin-6 and matrix metalloproteinases (MMPs). For larger particles in the size range 20-100 μm that cannot be effectively phagocytosed by a single macrophage, foreign body granulomas are formed. These were first described by Harris et al in 1976[15] who observed aggressive granulomatous lesions in cemented MoP THR with localised tumour-like bone resorption in the definite absence of infection.

Osteoclasts are the only cell capable of active bone resorption and are recruited through up-regulated expression of monocyte chemotactic protein (MCP-1) and macrophage inflammatory protein (MIP-1alpha) in periprosthetic tissues in response

to wear debris[16]. Osteoclastic function is mediated via signal pathways from wear-debris-activated macrophages predominantly via up-regulation of the cytokines IL1, TNFalpha, and IL6. These cytokines induce expression of receptor activator of NF-κB (RANK) ligand (RANKL) which itself is a key regulator of osteoclast generation. The RANK receptor is found predominantly on the surface membrane of osteoclasts. Ligand binding at the RANK receptor is the initiator for osteoclast differentiation. This process is antagonised by the soluble decoy receptor osteoprotegrin (OPG) which is secreted by osteoblasts and regulates osteoclastic activity by providing an alternate binding site for RANKL. Hence the RANK pathway is the key modulator of bone turnover and can be considered the "on-switch" for bone loss

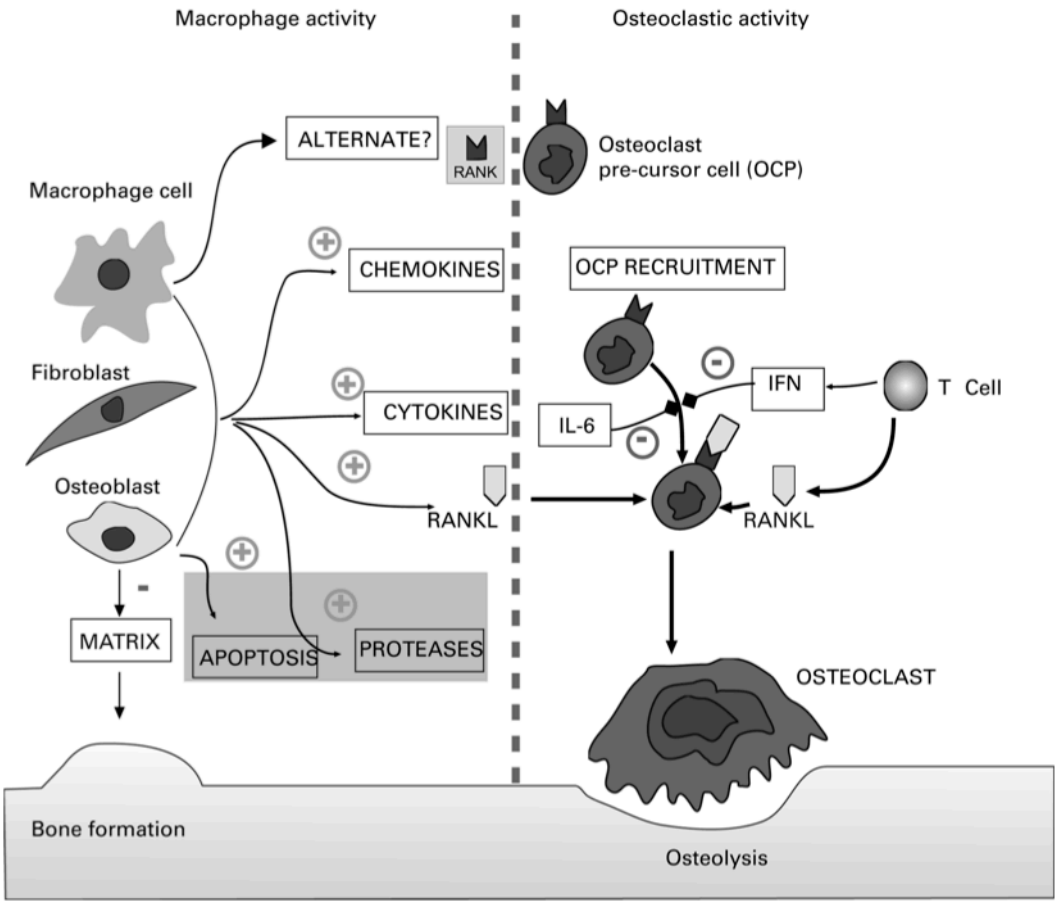

Figure 1.2-1 Simplified summary of cellular interactions occurring in periprosthetic osteolysis[13]

The volume of particulate debris generated is an important factor in biological clearance. Particles that are of a size small enough to be phagocytosed by macrophages are transported away from the joint via lymphatic drainage, hence the volume of debris that can be transported is finite. This either results in the attainment of a state of equilibrium whereby the volume of debris generated is transported and eliminated at the rate at which it is formed or a state whereby particles are allowed to accumulate in the synovium where they may produce a biological reaction[17].

As hip replacement arthroplasty evolved it became apparent that to improve longevity, both wear and particle generation would have to be minimised.

1.3 Development of Metal-on-Metal Total Hip Arthroplasty

The first generation of metal-on-metal total hip replacements was designed by Philip Wiles, who in 1938 implanted his stainless steel acetabular and femoral prosthesis at the Middlesex Hospital in London[18]. The acetabular cup gained fixation by 2 screws and the femoral component was secured by a bolt through the neck of the femur. The operation was performed in six patients for the indication of Still's disease. All patients were previously bed-ridden and the operation restored their mobility. Thirteen years later only one patient was alive and despite the disintegration of her acetabular component and limited overall mobility, her operated hip caused her no pain[19].

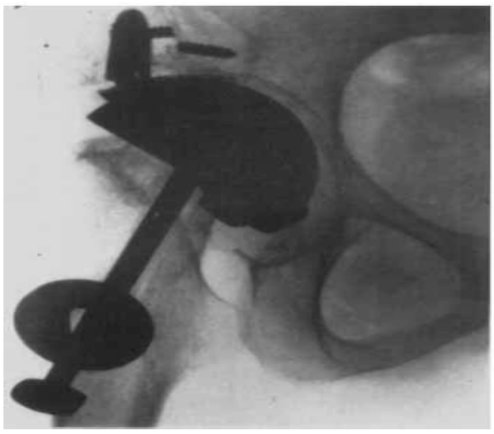

Figure 1.3-1 "Ball & Cup" arthroplasty radiograph taken with implant in situ at 13 years. Originally implanted by Wiles in 1938. The retaining lug and screws for the metal acetabular component have disintegrated, there is femoral resorption of bone and the bolt has broken away from the head. Despite all this the patient was pain free. [19]

It was Wiles' one-time registrar, Kenneth McKee, a Consultant at the Norfolk & Norwich Hospital who envisaged in the 1940s that:

"*If one could replace the bearing of a motor car then it must be possible in human joints"*[20]

The prevailing wisdom of that time was to use osteotomy in early arthritis and arthrodesis for advanced and painful arthritis. Inspired by a visit to the United States in 1953, McKee began work on developing a total hip replacement system using Cobalt Chromium Molybdenum (CoCrMo) "vitallium" alloys. His earlier designs had used stainless steel, but these had become loose in less than one year whereas using CoCr he found that prostheses remained held tight. McKee believed CoCr alloy to be superior due to lower frictional wear and a belief that there would be a "complete lack of any reaction by the body tissues to this metal"[21]. Cobalt alloys are particularly suitable as a bearing surface due to their "self-healing" properties in that they are able to polish out visible surface scratches with continued wear cycles rather than allowing a progressive deterioration of surface topography leading to accelerated wear[22]; it was the use of CoCr alloys which characterised the first generation of metal-on-metal THRs.

McKee also believed the metal-on-metal combination to be superior to the evolving Charnley metal-on-polyethylene bearing combination since he thought metal on plastic may give rise to trouble because of fine particles rubbed off the plastic would be liable to cause tissue irritation and bone absorption. Despite this, McKee does acknowledge that in his earlier series wear took place due to components not having been "lapped-in" sufficiently since at that time the Thompson head was not deemed to be perfectly spherical and had to be manufactured with a perfectly matching acetabular component.

Tribiology is the science and engineering of interacting surfaces in relative motion. It is a branch of mechanical engineering and material sciences incorporating the study and application of principles of friction, lubrication, and wear. At the time of manufacture important tribological factors which we now consider to be important such as sphericity, clearance, and surface roughness were yet to be fully understood and so the success or failure of a McKee-Farrar bearing coupling is now thought to have been due to the random chance occurrence of a compatible couple[23].

As well as being a pioneer of the MoM articulation, McKee unwittingly became one of the first heralds of what was to prove the Achilles' heel of his creations and its progeny; the process that has now been described as either aseptic lymphocytic vasculitis associated lesion (ALVAL), metal-on-metal pseudotumour or adverse reaction to metal debris (ARMD). In revising his earlier series of MoM implants McKee noted the presence of "sludge" although since he believed the CoCr alloy to be inert it was his overall conclusion that there were no undesirable effects from the sludge acting on soft tissues or bone.

McKee in conjunction with Watson-Farrar went onto further refine MoM prosthesis design by recessing the neck of the femoral component to minimise impingement, introducing smaller component diameters in order to improve the bony cover of the acetabular cup and reducing femoral head sizes to convert a more equatorial bearing on earlier models with associated increased wear, to a polar bearing where the head was smaller than the socket (Figure 1.3-2). Upon subsequent revision of later generation (post 1968) McKee-Farrar prostheses, no significant biological reaction

was apparent from the metallic staining that was encountered intraoperatively[24]. McKee ceased using the MoM THR in 1972, but his prosthesis went on to be the first MoM THR to be used in North America.

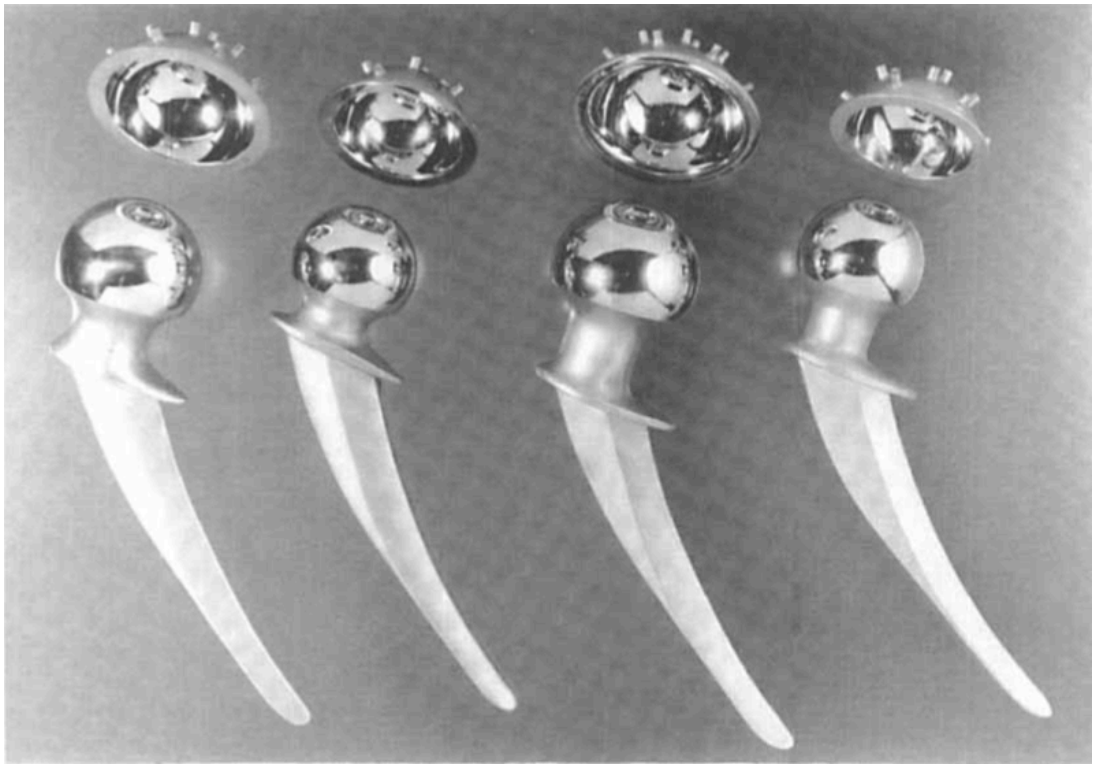

Figure 1.3-2 Final designs of the McKee-Farrar Total Hip Replacement system. 2 head sizes ($1\frac{5}{8}$ and $1\frac{3}{8}$ inch with the option of either a short or a long neck. [24]

The prosthesis fell out of favour due to reports of early failures [25] and fell victim to the success of Charnley's low friction arthroplasty, however, the longer term survival of the McKee-Farrar is comparable to those of the Charnley with one series demonstrating a 28 year survival rate of 74%[26].

The other major first generation CoCr MoM prosthesis was created by Peter Ring at Redhill, Surrey in 1964. It was a cementless design featuring a hemispherical cup with a long threaded stem that inserted into the iliopubic bar. This was paired with a standard 40mm fenestrated Moore prosthesis. The original design underwent a series of revisions in response to an initially disappointing 56% satisfactory outcome at 11-14 years [27]. The eventual final iteration comprised of a paired polar bearing with a femoral component with a neck-shaft angle of 135 degrees and a choice of 3 lengths. Although survivorship did increase with design modifications, the further use of the system was abandoned once the metal-on-metal coupling was discontinued in favour of using a polyethylene press-fit cup which resulted in granulomatous lesions as the result of polyethylene wear debris[28,29]. As far as the original metal-on-metal designs are concerned little wear of the CoCr bearing surface was observed at revision with minimal osteolysis of surrounding bone[30] and with a lower revision rate than the McKee-Farrar of 8.7% at a follow up of 5-20 years[31].

1.4 Abandonment of First Generation Metal-on-Metal Prostheses

The use of the MoM articulation was abandoned in favour of MoP due to a number of factors.

1.4.1 Manufacturing Problems

Cast cobalt chrome is much harder to manufacture than polyethylene which is easily mass produced. Contemporary machine tools were unable to retain the high degree of accuracy required in the manufacturing process since they required frequent sharpening and replacement. Creation of a CoCr alloy prosthesis was a labour intensive process which required skilled machinists, hence mass manufacture was difficult and more expensive compared to Charnley's alternative bearing surface[32].

1.4.2 Frictional Torque Issues

Further concerns regarding the longevity of MoM articulations arose from the problem of frictional torque. Charnley performed a series of experiments using a "pendulum comparator" in which he demonstrated the superiority of his 22mm MoP bearing couple above all others[33]. Using the McKee-Farrar MoM prosthesis he illustrated that when a pendular force was applied the MoP articulation moved freely whereas after a short period with only a minimal load the MoM prosthesis intermittently jammed. Charnley believed the McKee-Farrar to be a suboptimal annular bearing and as such prone to having high frictional torque under load and so intermittent seizure would occur due to engagement of a "clutch" mechanism.

Simulator experiments performed by Freeman, Andersson and Swanson [34,35]confirmed higher frictional torques from MoM articulations versus MoP. They recommended MoP on the basis that heat generated from the process of cementation weakened the fixation of the cup in the acetabulum and hence the lower frictional torque seen with MoP bearings would promote survival.

1.4.3 Wear Particle Generation

It is now known that all bearing surfaces generate wear particles which elicit an immune response with subsequent periprosthetic osteolysis. In the 1970s advocates of the second generation MoM agreed with McKee that CoCr wear particles were inert. At

the time it was not known which bearing couple would eventually triumph and the concern seemed to be more about the possible deleterious effects of polyethylene wear particles. The designers of the Stanmore MoM THR prosthesis claimed[36]

"…if the wear products of polyethylene do not produce an undesirable tissue response; neither adjacent nor in tissues remote form the implant then metal on metal bearings will be discarded"

The hip simulator study by Swanson et al[37] comparing the McKee-Farrar MoM THR and the Charnley MoP articulation demonstrated that over 4 million cycles Charnley's design did not release any wear polyethylene wear debris particles in contrast to the McKee which produced a visible quantity of debris.

1.4.4 Biological Concerns

The potential for metal particles generated by MoM articulations to induce adverse reactions local and distant to the site of implantation was a major factor in the original abandonment of MoM prostheses and the success of MoP. The work of Swanson, Freeman, and Heath [37,38] demonstrated the carcinogenic properties of CoCrMo wear particles by injecting particles obtained by running a MoM THR on a wear simulator into the thigh of a number of rats. The rats developed histological evidence of malignancy including localised rhabdomyosarcoma and lymphosarcoma. Metal particles had also spread to the para-aortic nodes and were noted to be intracellular. Their conclusion that there may be a risk of carcinogenesis from CoCrMo particles in humans, but that the induction period was likely to be longer than the life expectancy of most patients who might benefit from a THR was not reassuring. Colman, Herrington, and Scales (1973)[39] were the first to demonstrate raised levels of Co and

Cr in the hair, blood, and urine and the first to propose that after a period of establishing equilibrium the concentration of cobalt and chromium in urine might be equal to the rate of wear of the implant.

It was Evans et al in 1974[40] who discovered that not only did CoCr implants release both cobalt and chromium to the tissues adjacent to the prosthesis but also that these metals were carried in the bloodstream and were presented to the tissues of the body as a whole. Their study demonstrated positive skin sensitivity to metal in 9 out of 14 patients who had a loose MoM implant, thus implying that implantation of a MoM prosthesis led to a cascade of events resulting in a delayed hypersensitivity reaction to metal ions that led to vascular occlusion, bone necrosis and implant loosening. The overall conclusion being that MoP was the preferred bearing combination.

1.5 Development of Next Generation MoM THR

Interest in the use of MoM bearings was re-kindled in the 1980s and 1990s due concerns about polyethylene cup wear in conventional MoP THR designs. A review of the literature by Dumbleton et al [41]in 2002 has suggested the concept of an osteolysis threshold whereby osteolysis is rarely observed below a wear rate of 0.1mm/year and that a practical osteolysis threshold of below 0.05mm/year would greatly reduce if not eliminate the problem.

The perception that MoM bearings would be more resistant to wear was supported by good long-term results obtained by some series of first-generation MoM implants; it was reported that in a series of Ring THRs where these implants were revised there was very little wear of the Co-Cr interface and minimal observed osteolysis of surrounding bone [31]. This was aided by publication of favourable long-term survivorship rates of up to 77% at 20 years for first generation MoM McKee-Farrar THRs which is comparable to those of Charnley devices which had a 73% aseptic probability of survival in the same period [26].

Weber designed the Metasul prosthesis in 1988. This was a forged, high carbon prosthesis which was made possible by improvements in metallurgy and manufacturing leading to consistently reproducible and harder bearing surfaces. This MoM prosthesis had improved clearance (the space between the femoral head and acetabular surface permitting fluid film lubrication and clearance of debris from within the joint), but despite early good results with this system it was discovered that use of a low carbon acetabular component and a higher carbon stem possibly contributed to early osteolysis[42] and that cam impingement from the skirted version of the 28mm head may have led to biological reactions to wear debris[43].

Simulator studies and retrieval analyses by Sieber et al [44]revealed that second generation MoM had a volumetric wear rate 60 times lower than that of conventional MoP, with a typical linear wear rate of 5µm/ year; approximately 20 times lower than a MoP bearing. Less osteolysis was observed with MoM implants which suggested that the biological response may be governed more by wear particle size as opposed to the total volumetric amount of debris. It was proposed that metal solubility of wear

particles may predispose to an improved tissue clearing capacity hence reduced induction of the osteolysis cascade by MoM implants. The authors noted that an increase in serum and urine Co was observed in patients with MoM implants, but that in their series no clinical abnormality could be attributed to this even in patients with an implantation time of ten years. It was believed that second generation MoM implants may increase survival of THRs which would make them more suitable for younger patients with a higher level of activity.

Further developments in MoM THR were aided by a greater understanding of tribology and material science and was accelerated by the successful development of hip resurfacing arthroplasty, popularised by the Birmingham Hip resurfacing developed by McMinn. Larger diameter bearings allow for a better replication of normal hip joint biomechanics whilst preserving bone stock and reducing risk of dislocation by increasing the jump-distance to subluxation (the distance the prosthetic femoral head needs to travel in order for subluxation to occur). This led to the emergence of three distinct categories of MoM bearing coming into popular usage: 1) MoM resurfacing arthroplasty, 2) Large diameter THR (>36mm femoral head) and 3) Small diameter MoM THR (<36mm femoral head).

1.6 Failure of Current Generation MoM THR

An estimated 1 million MoM hip bearings have been implanted worldwide since 1996 with approximately 35% of all hip implants in the USA involving a MoM bearing. In the

UK 31,171 stemmed MoM prostheses were implanted between 2003 and 2011 representing 8% of THRs performed in that time[45].

According to the NJR, in 2012 over 10,000 hip revisions were performed which equates to 12% of all hip arthroplasties. Whilst aseptic loosening is the most common cause for revision surgery (40%), revision for ARMD associated with a MoM arthroplasty is now the second most common (13%), with infection (12%) as a cause in third place. The revision rate following primary MoM hip arthroplasty between 2003 and 2010 was 13.61% which is three times higher than any other bearing combination at 7 year follow up[46]. This contrasts with the UK National Institute for Health and Clinical Excellence (NICE) recommendation of an acceptable 10% revision rate for hip arthroplasty at 10 years. Between 2006 and 2012 use of MoM articulations declined dramatically from 10.8% to just 1.3% in response to concerns about necrosis of periprosthetic soft tissue and bone.

In 2010 the UK Medicines and Healthcare Regulatory Authority (MHRA) issued a medical device alert for all MoM articulations[47] with the advice that all patients with such a bearing be followed up by an orthopaedic surgeon with measurement of Co and Cr levels in blood and cross sectional imaging with either ultrasound or metal artefact reduction sequence (MARS) MRI. Further guidance was issued in 2012[48] which recommended:

1) Annual clinical review for at least five years in the case of MoM hip resurfacing arthroplasty (HRA) and for the life of the implant in the case of both large (>36mm head) and small (<36mm) MoM THRs.

2) Measurement of Co & Cr metal ion levels with a level of >7ppb being suggestive of soft tissue reaction.
3) Cross sectional imaging with MARS MRI or ultrasound for all symptomatic patients or in asymptomatic patients with rising metal ion levels.
4) Consideration for revision surgery if imaging abnormal or metal ion levels demonstrate a rising trend.

The revision burden for MoM bearings has significant financial implications with the full economic cost of a revision estimated at up to £30,000[49] this is compounded by the fact that these implants were marketed towards younger and more active patients who now face revision surgery complicated by soft tissue and bone loss at an early age.

The United States Food & Drug Administration (FDA), the European Federation of Orthopaedics & Traumatology (EFORT), The Australian Therapeutic Goods Adminstration and Health Canada have all issued their own guidance and risk stratification algorithms for long term follow up of patients with a MoM hip arthroplasty, but whilst there is some consensus between them, their recommendations are not uniform, are not wholly evidence based and may not be cost-effective[50].

1.6.1 Biology of Adverse Reactions to Metal Debris

As MoM resurfacing and THR became more commonplace, an apparently new and severe complication of adverse tissue reaction to metal debris became recognised. The nomenclature surrounding the biological processes that occur around a failing MoM articulation has not been uniform. However, it is becoming clear that the unique

reaction to MoM implants is due to the simultaneous presence of metal wear nanoparticulates, corrosion products, and metal ion species. The biologically active load is increased further as wear nanoparticles can themselves undergo corrosion thus increasing the total level of dissolved ions.

Co and Cr are transition metals and are the major constituents of orthopaedic alloys commonly used in MoM arthroplasty surgery. Both are present in the natural water supply and food and are essential trace elements required by the body for normal cellular metabolic function.

Cobalt is a constituent of vitamin B12 (cyanocobalomin). Chronic industrial exposure to cobalt containing fumes or dust can give rise to "hard metal lung disease", a fibrosis characterised by desquamative and giant cell interstitial pneumonitis[51]. Cobalt poisoning has been associated with cardiomyopathy, neurotoxicity, thyroid disorders, and dermatitis[52]. It is mainly present in vivo as Co(II) oxidation state.

Chromium is required for normal energy metabolism where it may enhance the activity of insulin via glucose tolerance factor (GTF)[53]. High doses of Cr are potentially harmful since Cr distributes to virtually all tissues with highest concentrations being found in the liver, kidney, and bone. The toxicity profile is dependent upon the oxidation state. Cr (VI) uptake into cells is an active process mediated via non-specific ion channels. In the intracellular environment Cr(VI) is unstable and reduces in a stepwise manner to Cr(III) with the release of free radicles. This reduction of Cr(VI) to Cr(III) gives rise to reactive intermediates which contributes to cytoxicity, genotoxicity, and carcinogenicity of Cr(VI) containing compounds; there is an

increased incidence of bronchial and nasal carcinoma in those exposed to industrial levels [54]. Cr(VI) is a class 1 human carcinogen by the World Health Organisation (WHO) and is considered to be 1000 times more toxic than trivalent Cr. Cr(III) has been shown to have antigenic properties with the ability to induce inflammatory and allergic reactions [55].

Whilst links between metal ions and carcinogenesis have been established for industrial levels of exposure, no demonstrable link between raised metal ion levels associated with failing MoM arthroplasty has been demonstrated in humans. A linkage study between the NJR and hospital episode statistic (HES) databases has failed to demonstrate any increased risk of malignancy in MoM arthroplasty patients at up to seven years[56].

The term "metallosis" has come to be the macroscopic descriptive term for the staining of periprosthetic soft tissues by metal particulate debris. In recent times the umbrella term of "adverse reaction to metal debris" or ARMD has been coined by Langton et al [57] to describe joint failure secondary to surface wear of the bearing surface or corrosion debris in the absence of any other obvious explanation. It encompasses metallosis, pseudotumour, and ALVAL. The United States Food and Drug Administration (FDA) has popularised the term "adverse local tissue reaction" (ALTR) to describe the same phenomenon (http://www.fda.gov/MedicalDevices/Safety/AlertsandNotices/ucm335775.htm)

In 2008 Pandit et al reported on 17 patients (20 hips) with a MoM resurfacing arthroplasty who had suffered a soft tissue reaction characterised by extensive tissue necrosis and bone loss termed and gave these lesions the term "pseudotumour"[58].

The term "pseudotumour" has been used to describe a granulomatous mass or destructive cystic lesion that is neither infective or neoplastic. There is formation of a synovial-like biomembrane which can lead to bone absorption via production of PGE2, collagenase, interleukin-1, and tumor necrosis factor[59] . Where pseudotumor is associated with a MoM hip arthroplasty there is a spectrum of damage ranging from a small indolent cyst to local invasion with substantial periarticular soft tissue destruction and at worst extensive bone destruction and pathological fracture[60]. Patients may present with pain, discomfort, or a palpable swelling.

MoM periprosthetic pseudotumour has been described histologically by Willert[61] as an aseptic lymphocytic vasculitis associated lesion (ALVAL) characterised by areas of tissue necrosis and dense perivascular lymphocytic infiltrate and lymphocytic cuffing in the absence of infection[62] (Figure 1.6-1). After hip arthroplasty a periprosthetic neocapsule is formed. The neocapsule is contained at the deep surface associated with a pseudosynovial membrane. Studies on explanted surgical tissue from MoM revision arthroplasty demonstrated dense lymphocytic infiltration in perivascular tissue with surface ulceration noted to be more extensive when compared to well fixed MoP implants which raises the question as to whether the mechanism of MoM implant failure is immune mediated. Metal particles are phagocytosed by histiocytes leading to release of inflammatory and osteolytic mediators which induce aseptic loosening and periprosthetic osteolysis [63]. Inflammation and localised oedema can lead to pain, however, this may be a late presenting sign and silent soft tissue pathology is common in patients with a MoM THR [64]

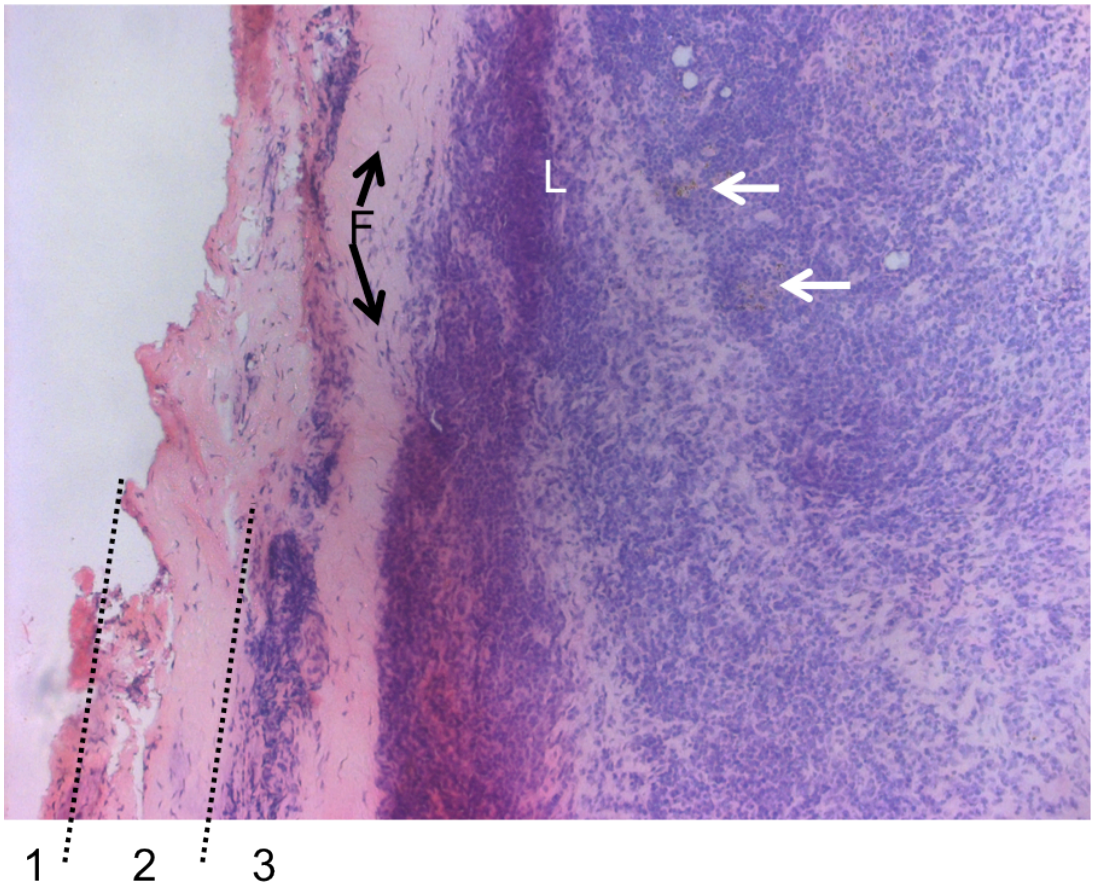

Figure 1.6-1 A representative micrograph of tissue from a failed 28mm MoM Ultima THR. The section is stained with haematoxylin and eosin (x100 magnification). There are 3 distinct layers: an outer layer (1) showing signs of ulceration and areas of fibrinoid necrosis, a middle layer (2) demonstrating features of necrosis, with few tissue features and typical "ghost" nuclei, and a third inner layer (3) demonstrating typical areas of dense perivascular lymphocytic infiltration (L), fibroblasts (F), vessels, and macrophages (white arrows)

In 2007, Goodman [65] proposed that there is a non-specific macrophage-mediated immune response to arthroplasty-generated particulate debris and that prolonged exposure to metal debris may be associated with a generalised immune reaction initially involving monocytes and granulocytes which has a variable course and is generally self-resolving, but that in some patients there is activation of the specific T lymphocyte immune system to metal particles complexed with specific serum protein. This metal-protein complex serves as a hapten[66,67]. This would correlate with the

perivascular lymphocytic cuffing, granulomatous response, and inflammatory infiltrate observed in periprosthetic tissues from revised MoM which would be suggestive of a T-cell mediated Type IV delayed hypersensitivity reaction[68 69].

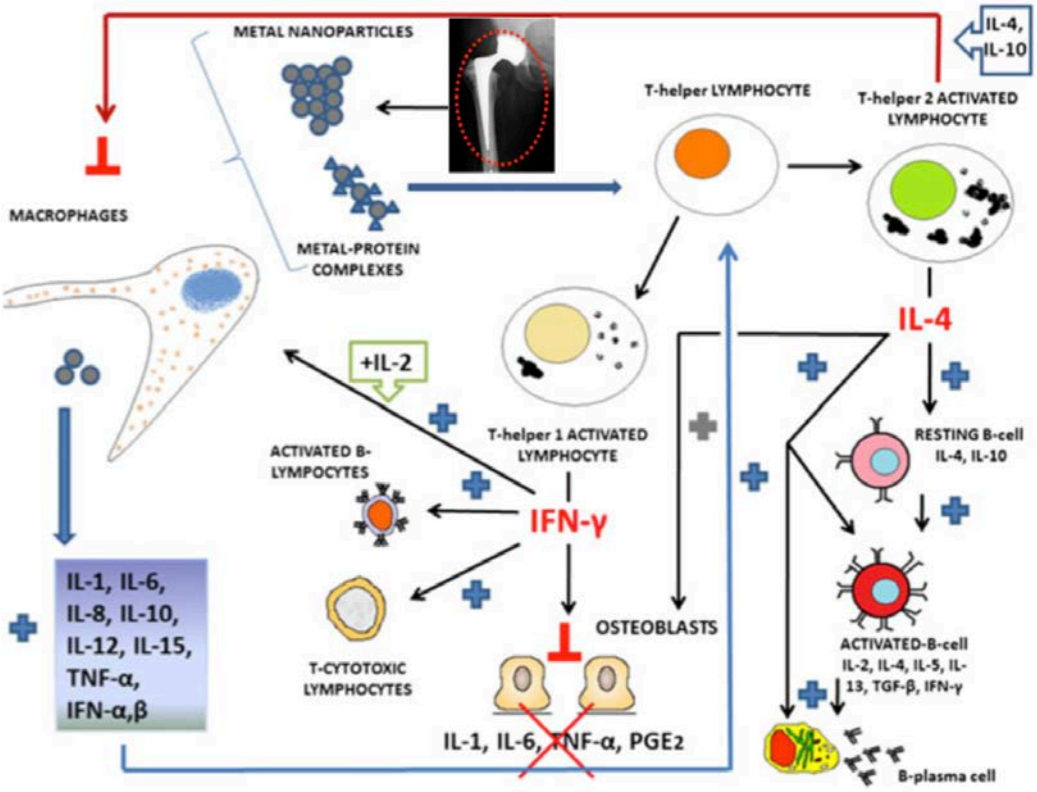

Figure 1.6-2 Simplified schematic overview of potential interaction between innate macrophage mediated immune and adaptive T-cell mediated response to metal debris from a MoM THR. Adapted from Polyzois et al 2012[70]

It is still a source of controversy as to whether the local effects seen in ARMD are the result of an innate hypersensitivity to metals or if it is an adaptive immune response. In 2009 Caicedo et al proposed that cobalt containing particles and the ions produced as the result of their corrosion activate a response arising from an "Inflammasome Danger-Signalling Pathway" which is dose-dependent. The inflammasome is a multiprotein intracellular complex that detects both pathogenic micro-organisms and sterile stressors and through a series of intermediate steps activates pro-inflammatory cytokines such as IL-1b, IL-18, TNFα, and NFκβ leading to an array of inflammatory

responses including osteoblast inhibition and maturation of osteoclastic precursors leading ultimately to osteolysis. Inflammasomes can also induce a form of cell death termed pyroptosis and dysregulation of inflammasomes may be associated with a number of autoimmune conditions. Further in vitro studies on human macrophages by Caicedo et al 2013 [71] further underlines the importance of particle size and morphology. It was found that increasing both CoCrMo-alloy particle size and surface irregularity induces increased macrophage inflammasome activation via potential lysosomal destabilization mechanisms. Small 1μm round particles were phagocytosed by macrophages without inducing a measurable response in terms of IL-1β whereas larger 6-7μm particles induced a greater than ten-fold increase in IL-1β. Large, irregularly-shaped 6μm particles induced lysosomal destabilisation with release of Cathepsin B which acts as a danger signal to activate a NALP-ASC (NACHT, LRR and PYD domains-containing protein 3) – (Apoptosis-associated speck-like protein containing a C-terminal caspase-recruitment domain) inflammasome complex of proteins leading to caspase-1 activation and cleavage of pro-IL-1β to activated IL-1β which is released from the cell to exert further inflammatory effects.

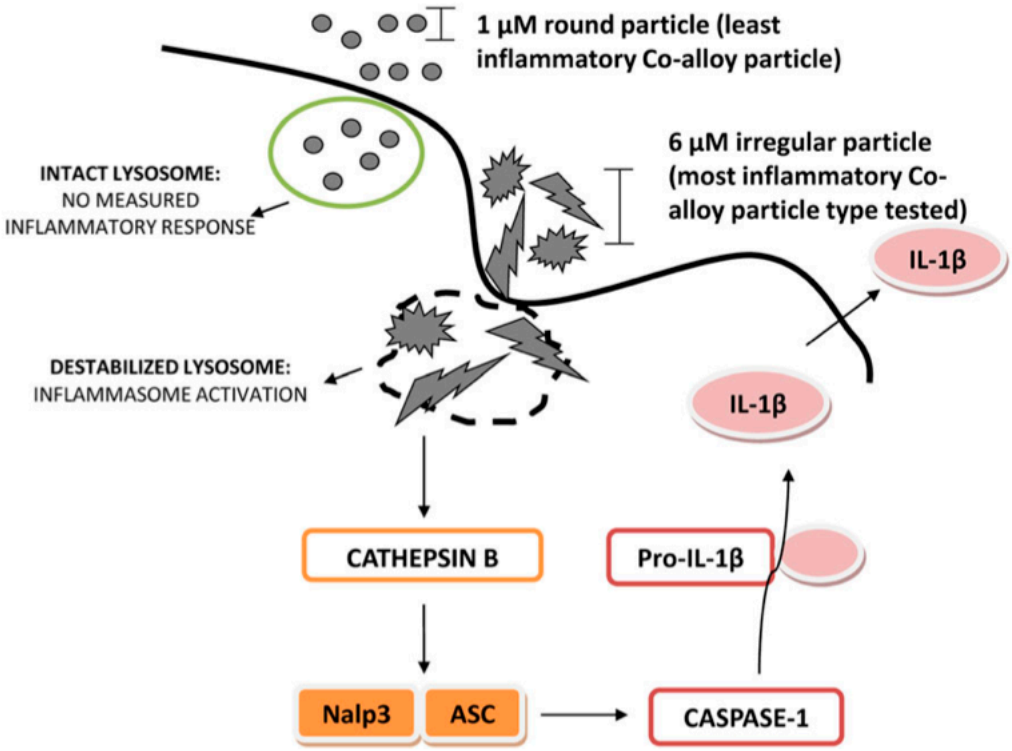

Figure 1.6-3 Effect of particle size and surface morphology upon Nalp3-ASC inflammasome complex activation. Reproduced from Caicedo et al 2013[71]

Flow cytometric experiments using peripheral blood mononuclear cells (PBMCs) cultured with laser-ablated particles from an Ultima TPS stem have shown that the volume of nanoparticulate sized (6nm) debris has an effect on cellular proliferation. Higher volumes of nanoparticles resulted in fewer observed cell cycles after six days incubation (Figure 1.6-4).

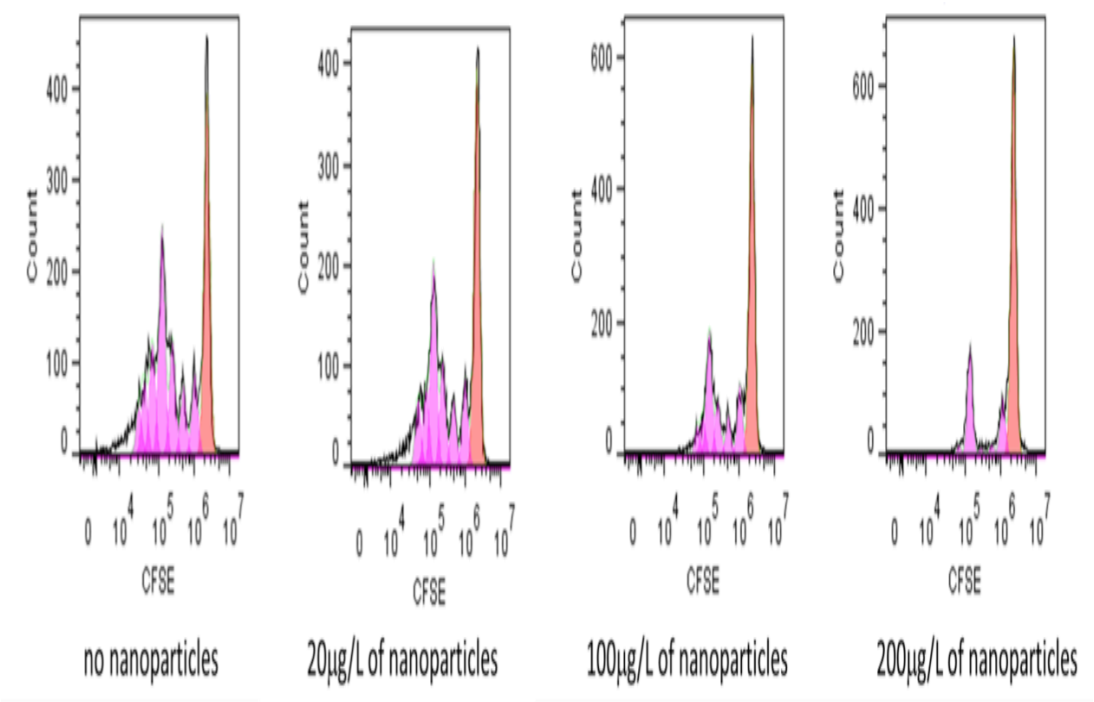

Figure 1.6-4 PBMCs incubated for sixe days with different concentration of metal particles from a laser ablated Ultima TPS stem. CFSE (carboxyfluorescein diacetate succinimidyl ester) staining on day 1 and day 6 reveals live cells. Tall peak represents day 1, shorter peak represents day 6. Data presented at EFORT 2013, Istanbul[72]. Reproduced with permission of Dr P Court.

At sites of inflammation there is generally a decreased availability of oxygen leading to a state of hypoxia which in turn is proinflammatory and promotes further tissue damage[73]. A hypoxic microenvironment can act as an "inflammatogen" to activate macrophages which infiltrate hypoxic tissues. Hypoxia inducible factors 1 and 2 (HIFs) are transcription factors involved in the adaptation of cells in a hypoxic and or inflammatory state. HIF-1 is a heterodimeric protein comprising an alpha and beta subunits of which the alpha subunit is oxygen sensitive. In a hypoxic environment the HIF1-alpha subunit stabilizes, translocates to the nucleus and dimerizes with HIF1-beta and begins the transcription of hypoxia-inducible genes including vascular endothelial growth factor (VEGF) which has been linked to aseptic loosening and bone resorption[74]. Samelko et al in 2013 demonstrated that cobalt alloy and its degradation

products in preference to other metal alloys can effect local innate immune responses. Increasing concentrations of Co ions caused significant up-regulation of HIF1-alpha with a maximal response at 0.3mM. Cobalt alloy particles also induced significantly elevated levels of HIF1-α, TNFα, VEGF, and ROS (reactive oxygen species) expression in human macrophages where Ti alloy particles did not. Elevated HIF1-α expression was also found in the periprosthetic tissues of patients being revised for ARMD compared to those being revised for aseptic loosening of a MoP implant.

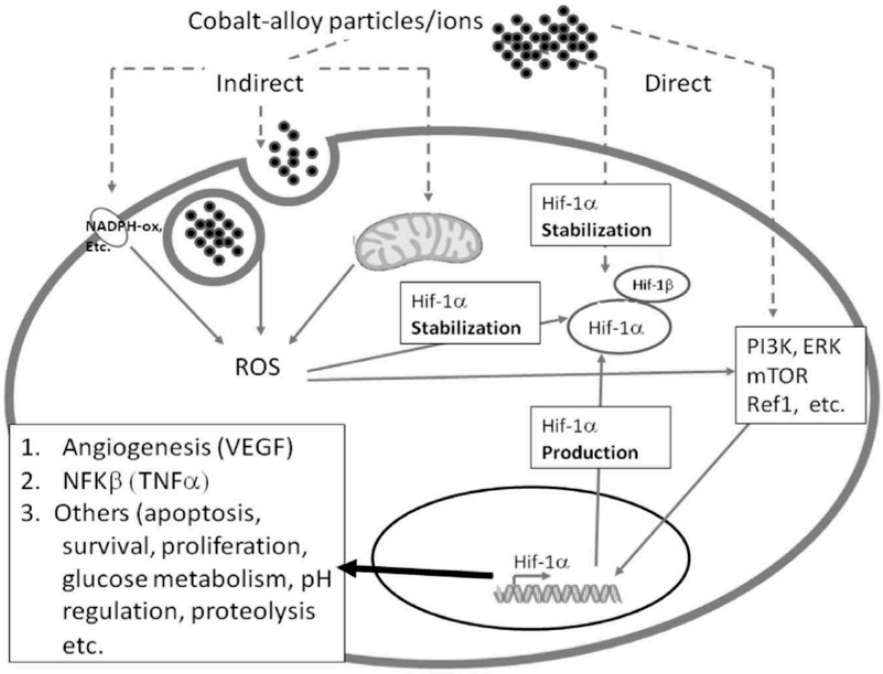

Figure 1.6-5 Schematic of HIF-1α induction in response to Co alloy particulate debris and ions. Reproduced from Samelko et al 2013[74]

Further studies on human macrophages by Nyga et al have confirmed that cobalt nanoparticles and Co ions are "hypoxia mimetic" which facilitates dimerization of HIF1-alpha to the beta subunit with subsequent upstream HIF-1 transcriptional

activity even in the presence of oxygen via a ROS-independent ascorbate depleting mechanism[75].

Toll-like receptors (TLRs) are a family of transmembrane receptors expressed mainly in immune cells including monocytes, macrophages, dendritic, and B cells and have critical roles in regulation of immune responses and the inflammatory cascade. Of the known TLR subtypes 1 to 10 are functional in humans. These receptors play a role in host immunity via recognition of various microbial components or pathogen-associated molecular patterns (PAMPs). Activation of TLRs by ligands initiates downstream signalling pathways such as NF-KB, mitogen-activated protein kinase (MAPK) and type-1 interferon pathways that are important in inflammatory responses and may ultimately lead to rapid cellular differentiation and apoptosis[76].

In examining if hypoxic stress modulates the TLR activity of macrophages, Kim et al [73] demonstrated that TLR4 expression in macrophages is upregulated via HIF-1 in response to both hypoxic stress and to the hypoxia mimetic $CoCl_2$. TLR4 is associated with a risk of hypoxia related disease, for example its expression is upregulated in the tissues of patients with myocardial ischaemic-reperfusion injury. In TLR4-deficient mice, tissue inflammatory responses and pathological changes are significantly decreased in response to ischaemic injury.

TLR4 requires adaptor proteins for activation including myeloid differentiation primary response gene (MyD88) and toll/interleukin-1 receptor domain-containing adaptor-inducing interferon-beta (TRIF). Although activated by the TLR4 receptor, stimulation of the MyD88 and TRIF pathways leads to the secretion of a diverse array

of cytokines. In the MyD88 pathway there is ultimate secretion of interleukin-6 (IL-6) and interleukin-8 (IL-8) which are both involved in the innate immune response e.g. neutrophil recruitment to sites of inflammation[77]. Additionally IL-8 has been shown to cause peripheral blood mononuclear cells (PBMCs) to differentiate into osteoclasts. The TRIF pathway results in the secretion of type 1 interferon as well as pro-inflammatory C-X-C motif chemokine (CXCL10) which is involved in the adaptive immune response where its function is to recruit activated T-lymphocytes which prolong the immune response. Lawrence et al in 2014[78] have demonstrated using a human monocytic cell line (Monomac 6) that Co^{2+} ions at clinically relevant concentrations specifically activate human TLR4 resulting in increased IL-8 and CXCL10 secretion and gene expression via activation of both the MyD88 and TRIF arms of the TLR4 signalling pathway. Overall this suggests Co^{2+} can initiate both innate and adaptive immune responses and that Co ions may cause a prolonged inflammatory response in periprosthetic tissues. However, even accounting for implant related factors which may contribute to a higher periprosthetic Co load, not all patients develop ARMD and in those that do, ARMD does not appear at a uniform rate post implantation which suggests that genetic variability and other patient factors may have a role to play.

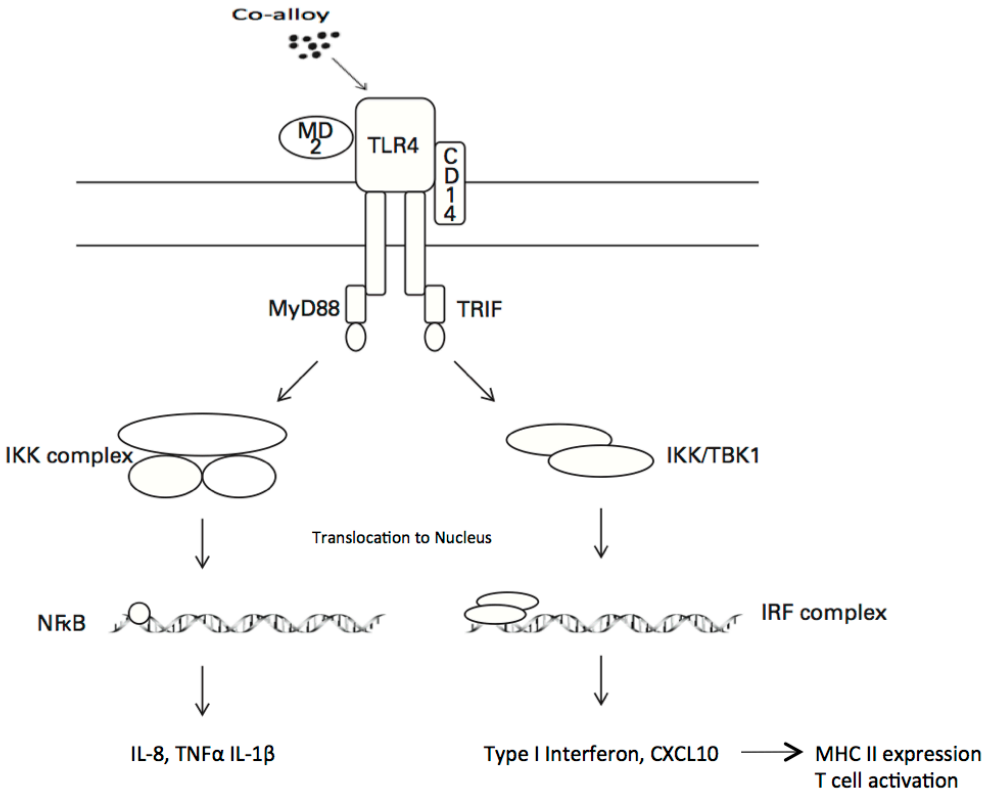

Figure 1.6-6 Cobalt alloy particle mediated TLR4 activation via the MyD88 and TRIF pathways. TLR4 with co-receptors for myeloid differentiationfactor-2 (MD-2) and cluster of differentiation-14 (CD-14) which are required for activation which initiates an intracellular signalling cascade, via 2 main mechanisms. The MyD88 pathway leads to activation of the enzyme complex inhibitor of kappa B Kinase (IKK) and the transcription factor Nuclear Factor Kappa-B (NFκB) with upregulation of proinflammatory cytokines IL-6 and IL-8. Activation of the TRIF pathway increases the expression of Type I interferon, CXCL10 and subsequent MHC II expression and T cell activation in the alternative pathway. Modified from Lawrence et al 2014[78]

Despite this recent expansion in knowledge of possible cellular mechanisms by which metal particles and ions may effect an inflammatory response, the precise mechanism of how ARMD develops in vivo and the roles and interplay of Inflammasome danger signalling pathway, HIF-1 pathway, and TLR4 mediated IL-8 and CXCL10 secretion has yet to be conclusively established.

1.6.2 Implant Factors

MoM hip implants are manufactured from Cobalt-Chromium-Molybdenum (Co-Cr-Mo) alloys which conform to the standards set by The American Society for Testing and Materials (ASTM). Alloys are composed of 58.9-69.5% Co, 27-30% Cr, 5-7% Mo and smaller amounts of other elements (Manganese, Silicone, Nickel, Iron, and Carbon) in order to comply with ASTM F-75 for cast alloys or F-1537 for wrought alloys. Surgical grade cobalt based alloys are generally well suited to use as a bearing couple due to their hardness and corrosion resistance. They have a capacity to "self-heal" that is to polish out visible surface scratches with continuous wear cycles as opposed to undergoing progressive deterioration of the surface leading to accelerated wear[79].

Based on their carbon contents CoCrMo alloys can be divided into either high-carbon alloy (0.05-0.35wt%) and low-carbon alloys (<0.05%wt%). High-carbon alloys are superior in terms of wear-resistance owing to strengthening effects of carbides and the fact that low-carbon bearings initially experience a higher wear rate in the "bedding-in" phase whilst steady state wear between high and low carbon alloys is indistinguishable[80]. Second and third generation MoM implants have a higher wear resistance secondary to their higher carbide content.

MoM bearings generate greater numbers of particles of a smaller diameter than MoP bearings. In the retrieval study by Doorn et al [81], MoM bearings were estimated to generate approximately $5x10^{11}$ particles per annum in comparison to MoP implants which produce approximately in the order of $6.7x10^{12}$ to $2.5x10^{14}$ particles per annum with the majority of wear particles being less than 50nm in diameter; ten times

smaller than polyethylene wear particles. Due to their smaller size, metal wear debris and their degradation products are bioactive and may enter the cell and affect cellular function.

Metal wear particles are insoluble and are principally composed of Co-Cr-Mo nanoparticles. Particles of 100nm or greater in size remain trapped in the joint since the synovial membrane acts as a porous barrier. These particles are subject to corrosion in the biological environment of the hip joint, its surrounding fluid, and tissues. Corrosion leads to the presence of the ionic species Co(II), Cr(III) and Cr(VI) in the extracellular and intracellular solutions. Since these larger particles remain trapped in the joint space their concentration builds up over the course of time and the concentration of metal ions in joint fluid is therefore typically higher than that found in the bloodstream or in urine[82].

Examination of bearings from simulator studies and from explanted articulations have led to the observation of corrosion at both the articulating surface and the taper at the neck of modular THRs. Both Co and Cr are highly reactive and are rapidly oxidized at an implant surface to form Cr_2O_3. The oxide layer provides a barrier between a solution and the implant surface. This process is known as passivation and reduces corrosion. Orthopaedic implants are artificially over-passivated as part of the manufacturing process to create a build up of protective oxide film to prevent in-vivo corrosion[83]. During the lifetime of the implant this passivation film can be mechanically removed leaving the reactive substrate exposed to the environment and free to oxidise or corrode. This is known as tribocorrosion.

It is generally acknowledged that MoM hip articulations are subject to sliding tribocorrosion at the articulation as well as fretting-corrosion at the modular taper and stem-cement interfaces. Fretting-corrosion is defined as a wear phenomenon occurring between two surfaces having oscillatory relative motion of small amplitude in which there is a chemical dissolution of a metallic substrate[84]. Additionally depending upon the nature and composition of the articulation a galvanic cell may be established with resultant galvanic corrosion as seen in the 28mm Ultima TPS MoM THR[85]. Galvanic interactions occur when a potential difference is established between two electrically connected metals immersed in a corrosive or conductive solution as well as between areas of passive and depassivated alloy.

The tribological interaction of an exposed solid surface with an interfacing material and the environment leads to material loss or "wear". From an engineering viewpoint the failure of MoM bearing surfaces is thought to be a combination of wear and corrosion.

The correct function of a MoM implant is dependent upon the presence of lubrication and where the fluid film lubrication of a MoM articulation is disturbed, such as may occur due to the phenomenon of edge loading, there is increased susceptibility to wear. Edge loading can be described as either primary, whereby the acetabular component is positioned at a steep orientation (typically >55 degrees) or secondary edge loading where there is impingement at the neck-cup junction leading to contrecoup edge loading upon weight bearing[86]. Both of these lead to material loss and characteristic "stripe wear" observable on explants. Certain designs, typified by the

ASR system by Depuy are particularly prone to edge loading, especially with respect to smaller head sizes and in females due to their acetabular morphology.

In MoM THRs the presence of a tapered femoral neck connecting the femoral head to the stem adds a further source of wear since frictional torque at the bearing surface is thought to be translated from the head to the neck with resultant damage to the taper[87]. This is compounded further by the use of modular implants[88], laterally engaging taper systems and larger head diameters which increase the lever arm acting on the head neck junction.

1.6.3 The Ultima Tapered Polished Stem (TPS) MoM THR

This thesis was developed to contribute to the understanding of the failure associated with a specific MoM THR as well as the failure mechanisms which may be common to all MoM THRs.

The Ultima TPS™ (Tapered Polished Stem) developed by Depuy International, Leeds is a second generation hybrid MoM THR introduced in 1997. It consists of a collarless, triple tapered, highly polished wrought low carbon (LC) CoCrMo cemented femoral stem with a 12/14 modular taper. The articulation itself comprises of a 28mm 10/12 LC CoCrMo Ultima femoral head and a 28mm high carbon (HC) CoCrMo Ultima acetabular liner. The outer acetabular shell was porous coated Ti-6Al-4V ranging from 48mm to 68mm in size with 3 holes available for supplementary fixation (**Figure 1.6-7**). The femoral component was cemented with either a plain PMMA bone cement

or antibiotic cement containing either Gentamicin (Palacos, Heraeus Inc) or Erythromycin and Colistin (Simplex, Stryker)

Figure 1.6-7 The Ultima TPS™ TPS THR (Depuy, Leeds) used in the Norwich cohort

Despite earlier studies by Wilert which claimed that the mechanism of crevice corrosion was not applicable to cobalt and iron based implant alloys [89] it became evident that this was not the case with the Ultima TPS. In 2007 the MHRA released a Medical Device Alert for the Ultima TPS in response to 43 early revisions in which extensive soft tissue necrosis was encountered with extensive corrosion of the femoral stem component[90]. In 2010, Donell et al[91] reported on a series of 545 patients with 652 Ultima TPS THRs implanted between 1997 and 2004. There was an unacceptably high early failure rate of 13.8% at five years in which 90 hips had undergone early revision;

17(18.9%) for periprosthetic fracture, early dislocation in 3 hips (3.3%), late dislocation in 16 (17.8%) cases, and infection in 9 hips(10%). 44 hips (48.9%) required revision for pain arising from extensive, periarticular soft tissue necrosis (Figure 1.6-8) of these 35 patients had normal radiographs. Talroth et al [92] demonstrated that the first clinical sign of a pseudotumour was often pain prompting radiological examination and detection of lesions around the stem of the prosthesis which can sometimes be recognised by the presence of calcar resorption or osteolysis [93 94].

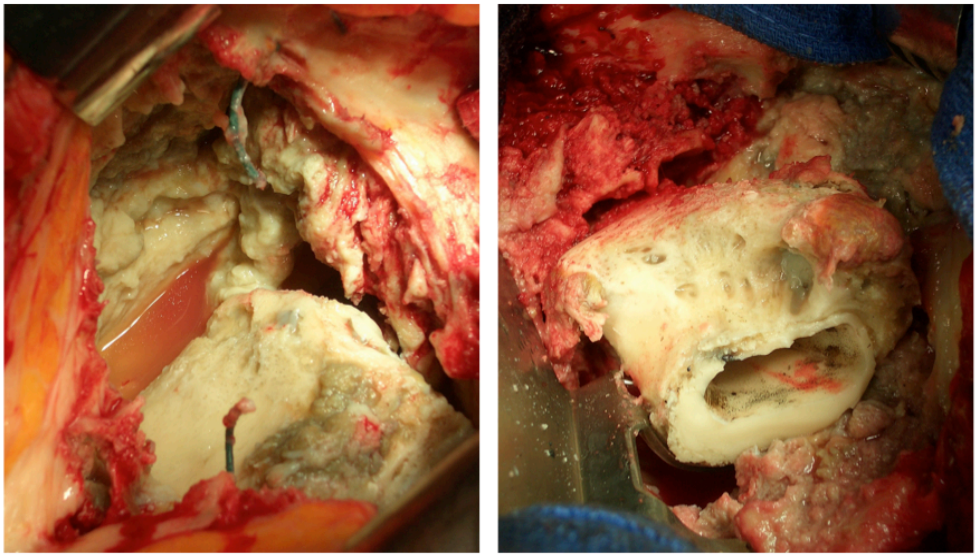

Figure 1.6-8 Severe macroscopic periarticular necrosis characteristic of soft tissue reaction found at revision of an Ultima TPS THR.

By early 2014, 178 patients (M:F 88:90) had been revised (27.4%) at a mean time to revision of 6.65 years (unpublished data)

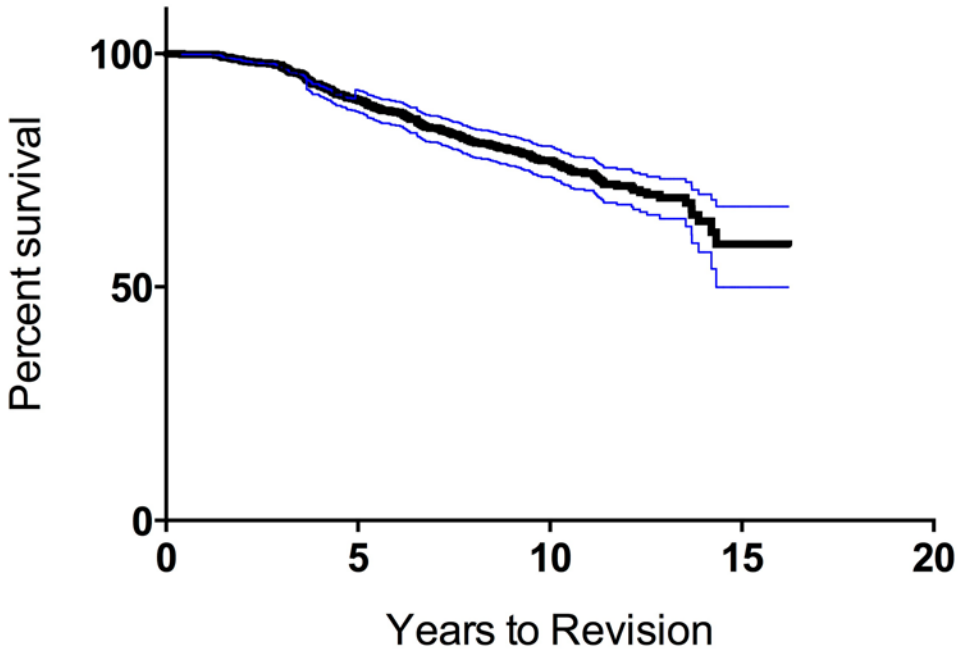

Figure 1.6-9 Kaplan-Meier survival curve for the Ultima TPS THR

The surveillance of the Norwich cohort has been an evolving process which now incorporates annual clinical follow-up, plain radiographs, measurement of blood metal ion levels and local staging of soft tissue reactions using the MARS MRI[95] [96]protocols and staging systems[97] developed at Norwich. In common with other cohorts of ASR MoM THRs [98] [99] it is known that blood metal ion levels in Ultima TPS patients on their own are insufficient as a screening tool for ARMD and do not correlate with MAR MRI scan findings (Figure 1.6-10).

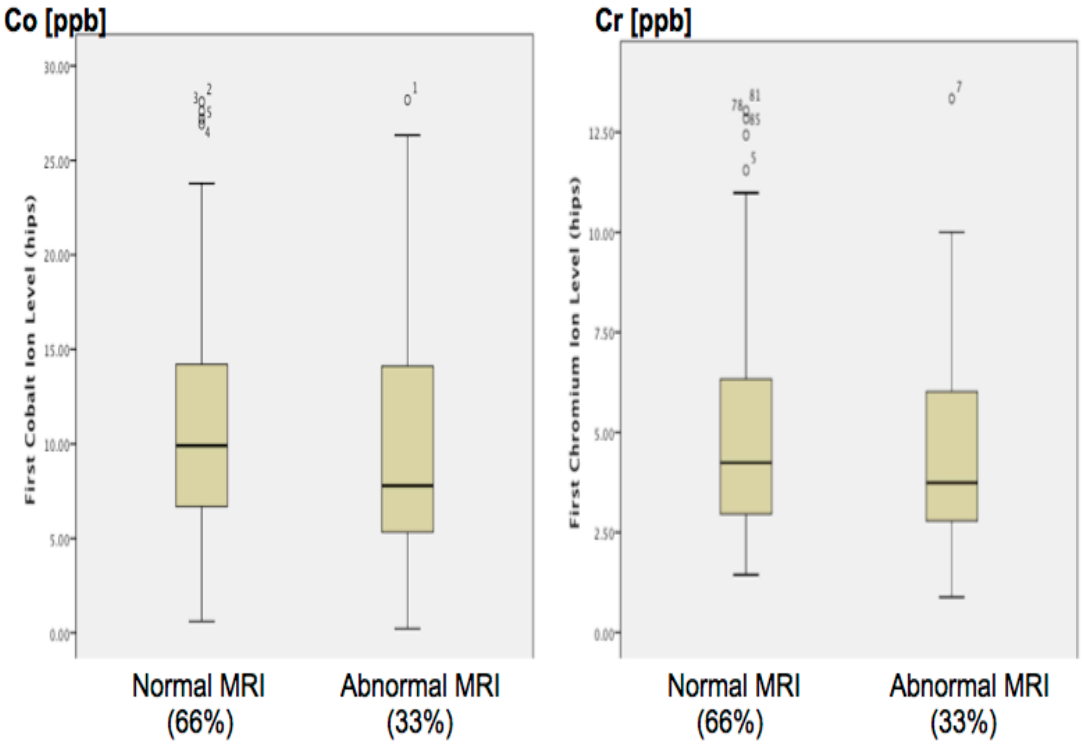

Figure 1.6-10 Box and whisker plots demonstrating Co and Cr levels in 152 Ultima TPS MoM THR with normal (Anderson Grade A) and Abnormal (Anderson Grade C1-C3) MARS MRI. The box represents the median and interquartile range and the whiskers represent the range of data excluding numbered outliers (Ebreo et al, BHS 2012).

CoCrMo polished stems have been demonstrated to have a good survivorship with revision rates of 4.1% at 10 years post implantation in the case of the collarless polished tapered CPT stem[100] (Zimmer, Warsaw, Indiana). However, it has come to light that localised tribocorrosion at the stem-cement interface has the ability to increase metal ion release and may lead to ARMD associated with marked macroscopic corrosion of well-fixed femoral stems as is commonly seen on the Ultima femoral stem component on explantation (Figure 1.6-11). Such corrosive phenomenon has also been observed with the CPT stem when used with a large diameter hybrid MoM head articulation [101].

Figure 1.6-11 Ultima TPS femoral stem explant exhibiting characteristic macroscopic corrosion.

Shetty et al[102] compared the performance of the Ultima TPS CoCrMo femoral stem and the established benchmark stainless steel Exeter design with both using a MoP Charnley cup and found at two years there was no difference in clinical scores and there was no evidence of osteolysis on radiographs. This suggests that the ARMD seen with the Ultima TPS is a product of the cemented femoral stem used in conjunction with the Ti backed MoM articulation; this was later confirmed by the manufacturer in a Field Safety Notice in May 2012 after extensive laboratory testing (personal communication Depuy Int).

The Ultima TPS THR system differs from large diameter systems in that the stem-cement interface appears to be the source of release of potentially toxic metal ions since the bearing surfaces of explants were found to be macroscopically clean of any evidence of wear or abrasion[103]

The mode of failure of the Ultima TPS THR MoM system is thought to be via galvanically-enhanced fretting crevice corrosion of the cemented femoral stem[104].

Using an in-vitro model where Ultima stems were cemented in PMMA cement, Bryant et al[105] have demonstrated that at the metal-cement interface fretting corrosion does occur and that this corrosion is accelerated by the presence of sulphates associated with antibiotics and radio-opaque agents contained within cement. The wear mechanisms present at the stem-cement interface are a complex combination of mechanical wear accompanied by electrochemical dissolution of the metallic substrate with a characteristic electrochemical response to cyclic loading. The CoCrMo surface is in a constant state of depassivation and repassivation due to cyclic micromotion at the stem-cement interface. With each loading cycle the Cr rich passive film is fractured exposing the base alloy to the aqueous solution. Oxidation is thought to occur at the point of contact between the stem and the cement with accompanying liberation of free electrons. This results in the formation of a thick Cr_2O_3 rich film and debris on the CoCrMo femoral stem and the counterpart PMMA bone cement. Cr is the favoured species to undergo reaction due to the lower activation energy required for its oxidation to Cr_2O_3, which then leaves Co^{2+} which is extremely soluble to migrate[85]. Plastic and elastic deformation of the surfaces may also result in formation of 3rd body

particulates which may also contribute to the overall quantity of metallic ions and PMMA debris released into the biological environment to contribute to the osteolytic and inflammatory response[106]. Bryant et al have further examined the role of galvanic corrosion in bio-tribocorrosion systems via electrochemical experiments where they have confirmed that galvanic coupling can occur between depassivated and still passive areas of the stem alloy. Further increase in the corrosion rate and release of metal ions into bulk solution also occurs in the Ultima system since the Ti alloy in the acetabular shell is electrically connected to the stem-cement interface thus forming a galvanic couple. These findings are in support of the findings and hypothesis presented by Hart et al (2012)[107] who demonstrated that in periprosthetic tissue retrieved from the Norwich cohort Co was found to be 10-fold more abundant than Cr and may be the clinically relevant active agent for periprosthetic tissue reactions due to the ability of Co to stimulate inflammatory pathways in macrophages.

2 : Study Questions & Proposals

The aim of this MD project is to evaluate adverse reactions to metal debris (ARMD) and its cellular mechanisms in a local cohort of patients with a MoM THR.

2.1 Overall Study Questions

1. What is the natural history of ARMD using MARS MRI?
2. What is the relevance of blood and urine metal ion levels as a predictor of disease activity and does diurnal variation exist in cobalt and chromium ion levels in patients with a MoM THR
3. Is there a difference in the activation of biological cascades between patients revised for periprosthetic osteolysis arising from polyethylene wear debris versus patients with a MoM THR undergoing revision arthroplasty for adverse reaction to metal debris?

2.2 Radiology Study

Hypothesis:

"In those patients whose staging on initial MARS MRI is normal or where only mild evidence of disease is demonstrated, further deterioration over time is unlikely. Where it does occur the rate of deterioration is slow and there may be a latent period of many years."

To test the hypothesis a retrospective review of MARS MRI scans of a cohort of patients with a MoM THR was undertaken with the inclusion criteria being that patients had to have at least two MRI scans and that they had not undergone revision surgery.

The MARS MRIs were evaluated by an experienced musculoskeletal radiologist and staged according to a recognised radiological method.

2.3 Diurnal Variation Study

Hypothesis:

"Circulating levels of metal ion levels fluctuate with activity in a fashion analogous to blood glucose level and that this variation is diurnal in nature"

In order to test the hypothesis a feasibility study was undertaken. At the Norfolk & Norwich University Hospital (NNUH) all patients who have received an Ultima TPS THR (Depuy) are recorded in a database. Prospective data has been collected and institutional and ethical approval for this has previously been secured.

Ethical approval was secured to sample the blood and urine of 12 of these patients to determine the following:

1. If there exists variation in metal ion levels detectable in blood and urine over the course of a day and over longer periods.

2. Whether data supporting the existence of diurnal variation of blood and urine metal ion levels exists in this population and if so how variable are the data?
3. What further study needs to be undertaken should a variation exist.
4. Determination of an appropriate scale for future study dependent on the magnitude of variation observed.

2.4 Laboratory Study

Hypothesis:

"There is a difference in the activation of biological cascades between patients revised for periprosthetic osteolysis arising from polyethylene wear debris versus patients with a MoM THR undergoing revision arthroplasty for adverse reaction to metal debris"

RNA expression analysis was used to quantify gene expression at the time of revision surgery. In order to address the hypothesis several preliminary questions needed to be addressed:

1. Can gene expression in joints affected by ARMD be reliably measured?

2. Do joints affected by ARMD have a different gene expression profile compared with native joints and joints with osteolytic change arising from metal-on-polyethylene bearings.

3 Serial Magnetic Resonance Imaging of Metal-on-Metal Total Hip Replacements

3.1 Background

Magnetic resonance imaging (MRI) is now widely used to assess pathological changes associated with metal-on-metal (MoM) total hip replacements (THRs) [95,96,108,109] . Metal artefact reduction (MAR) MRI allows assessment of the extent of soft-tissue pathology pre-operatively and the planning of revision surgery in cases where, for instance, neurovascular structures are at risk. MAR MRI may also demonstrate pathology outside the hip that may account for symptoms. An advantage over ultrasound is the potential to diagnose smaller, deeper foci of adverse reactions to metal debris (ARMD),[110] but it may miss lesions in the zone of the metal artefact, which ultrasound may detect more readily.

MoM bearings offer the theoretical advantages of decreased wear and increased functional outcome for younger and more active patients, but have been associated with the development of sterile inflammatory masses [58,111-113]. These may include soft-tissue destruction with poorer outcomes following revision surgery[114]. These lesions have also been described as aseptic lymphocytic vasculitis-associated lesions (ALVAL)[115], adverse local tissue reactions (ALTR)[116] or pseudotumours[58]. The mechanism by which they arise is uncertain, but may be associated with wear at the bearing surface, [61,117] taper junction wear [93,118] and corrosion [119,120].

MAR MRI has the potential to facilitate a classification system for ARMD that characterises and provides comparison of soft-tissue and bony pathology over time.

Various systems of scoring the severity of ARMD have been described[97,121,122],but only that of Anderson et al [97]has been shown to have interobserver reliability. This system assesses not only the size and cystic or solid nature of a lesion, but also any involvement of muscle, tendon and bone.

Although pain is a presenting symptom in a poorly functioning THR, silent soft-tissue pathology is common[64]and may occur with an asymptomatic well-functioning MoM THR[110,123]. The Medicines and Healthcare Products Regulatory Authority (MHRA) has issued renewed guidance recommending cross-sectional imaging for all symptomatic MoM THRs[48]. Annual clinical follow-up is recommended as well as measurement of metal ion levels in the blood. There is no recommendation as to the optimal timing of an initial investigation with cross-sectional imaging, nor at what intervals patients should be scanned as part of the surveillance. This cross-sectional study aimed to describe the natural history of MoM ARMD using MAR MRI in patients whose initial scans were normal or showed low-grade ARMD.

3.2 Patients & Methods

Cases were identified from an original cohort of 545 patients (650 THRs) who underwent 28 mm hybrid MoM THR (Ultima TPS; Depuy, Leeds, United Kingdom) between May 1997 and August 2004 performed by three surgeons including the senior author (JFN), as previously reported[91]. Inclusion criteria were the availability of at least two MRI scans and no revision surgery before any scans included in this review. A total of 37 THRs in 38 patients were excluded from the study as they had been revised without a prior MAR MRI. Also, 81 THRs in 72 patients were revised after a single MAR MRI scan and were therefore excluded. Of the original cohort, 80 patients were found to fulfil the inclusion criteria, 35 of whom were men (44%). Of these

patients, 57 (71%) had unilateral THRs and the rest were bilateral, resulting in a total of 103 THRs.

MRI was undertaken using MAR sequences with a 1.5T MR scanner (Siemens Symphony; Siemens, Ehrlingen, Germany). The MAR sequence used the following specification: coronal T_1-weighted (T1W) turbo spin-echo, echo time (TE) 23 ms, repetition time (TR) 669 ms and short τ inversion recovery (STIR); TE 37 ms, TR 3840 ms, axial T1W (TE 23 ms, TR 534 ms) and T_2-weighted (T2W) turbo spin-echo (TE 69 ms, TR 5600 ms) of the whole pelvis and a sagittal T2W turbo spin-echo of the hip (TE 69 ms, TR 2900 ms). The section thickness used was 5 mm, with a 340 × 340 mm field of view, matrix size up to 448 × 336 and pixel bandwidth 620 MHz

All scans were reported and graded by the same radiologist (AT). Scans were classified as A (Normal), B (Infection), C1 (Mild MoM ARMD), C2 (Moderate) or C3 (Severe) according to the classification of Anderson et al[97].

3.3 Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) PASW Statistics version 18.0 (SPSS Inc., Chicago, Illinois). Parametric tests were used for normal data and non-parametric tests for non-normally distributed data, and significance was set at $p \leq 0.05$.

3.4 Results

The mean age of the 80 patients was 57.9 years (25 to 69). There were 35 men (mean age 58.1 years (25 to 68)) and 45 women (mean age 58.2 years (29 to 69)). A total of

17 patients(17 THRs) who had already had at least two scans underwent revision at a mean of seven years (2 to 11) post-operatively.

Between 2002 and 2011 a total of 239 MRIs were performed on 103 THRs (two to four per THR). All patients had two MRIs, 29 had a third and four a fourth (Figure 3.4-1)

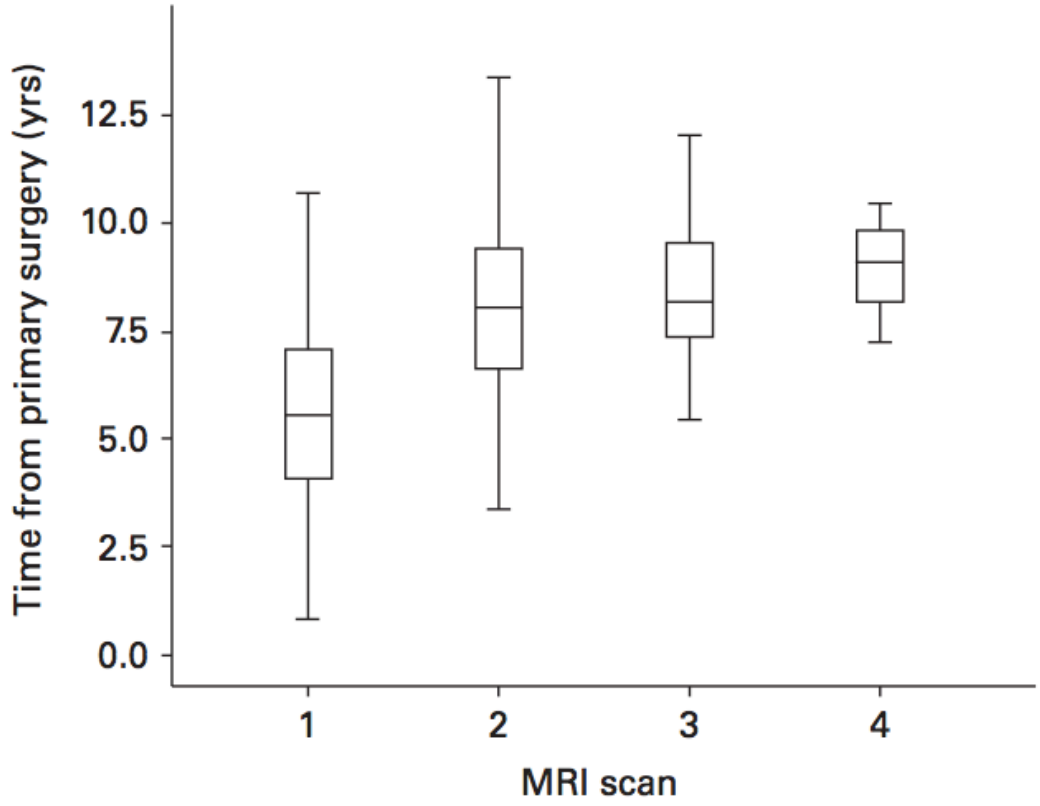

Figure 3.4-1 Box and whisker plot showing the time from primary surgery until the first, second, third, and fourth metal-artefact-reduction (MAR) MRI scans. The boxes represent the median and interquartile range and the whiskers denote the range of data.

When the grades of all 239 MRIs were considered together, there was a significant increase in grade as the post-operative time increased, with a Spearman's ρ of 0.235 ($p < 0.001$). The first scan was then considered in isolation, analysing the relationship between all first scans and the time from the operation, and again a positive

correlation existed between increasing post-operative time and increasing severity of grade (Spearman's ρ 0.257 ($p < 0.009$ for 103 first scans).

Overall, 140 of 239 MAR MRI scans were normal (Table 3.4-1), and of these, 63 had a normal (Grade A) first scan. Of those patients with a normal initial scan, only four advanced in grade between the first and second scans. In total, six patients (9.5%) went on to develop MR changes consistent with ARMD. In those whose stage became more advanced, they did so between seven and 11 years post-operatively, with most developing mild (C1) or moderate (C2) changes (Figure 3.4-2) . Only 15 (15%) of 103 THRs with sequential scans showed worsening of disease on subsequent imaging.

One patient with a normal initial scan progressed to a Grade B appearance suggestive of infection, which was confirmed on aspiration and a revision was subsequently undertaken. Four scans were initially reported as suggestive of infection. Repeat scans in one patient led to downgrading to a normal appearance owing to spontaneous regression of a peri-prosthetic collection. For the remaining three patients, no evidence of infection was found on blood tests. Subsequent scans indicated the presence of ARMD. Two patients developed radiological appearances of Grade C3 ARMD and underwent revision. One patient developed asymptomatic C2 ARMD and is under surveillance.

Table 3.4-1 Total numbers of patients undergoing revision surgery per grade of metal-artefact reduction (MAR) MRI scan

Grade	Hips (n, %)	Revisions (n, %)
A	140 (*59*)	4 (*2.86*)
B	6 (*2*)	1 (*16.7*)
C1	33 (*14*)	1 (*3.0*)
C2	41 (*17*)	4 (*9.76*)
C3	19 (*8*)	7 (*36.84*)
Total	239	17

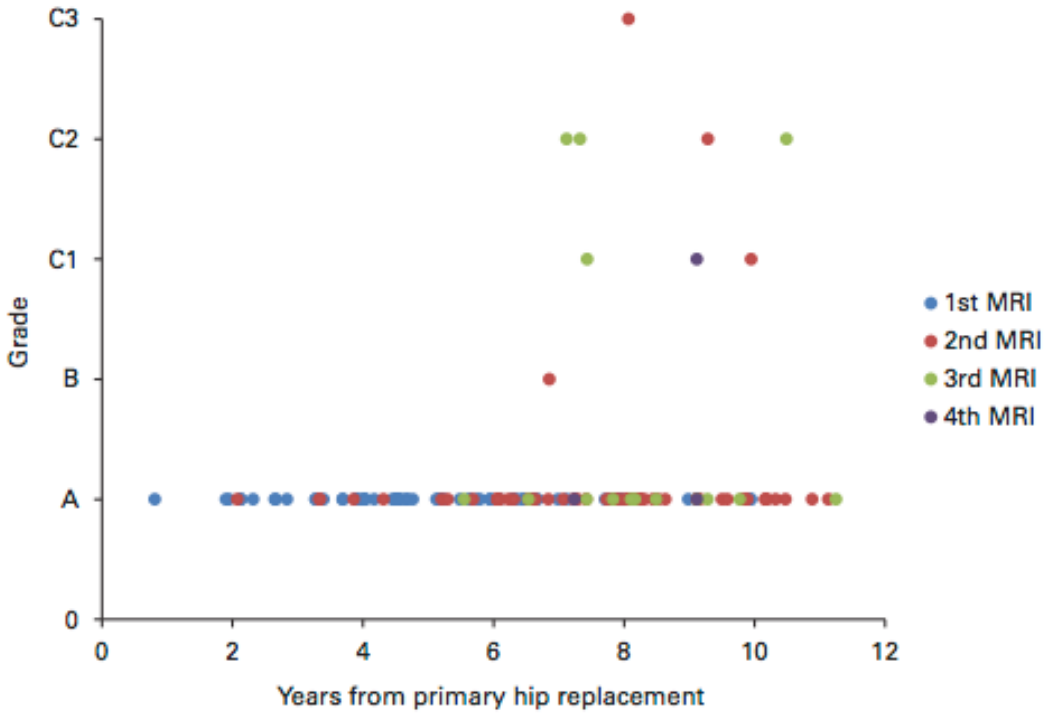

Figure 3.4-2 Scatter plot showing the change in metal-artefact-reduction (MAR) MRI grading with subsequent scans in patients whose initial scan is normal (Grade A)

On the initial scan 16 hips had the appearances of mild ARMD (Grade C1), but between five and seven years post-operatively three were downgraded to a normal Grade A appearance on repeat scans. A further two patients progressed to higher grades between 11 and 12 years post-operatively (Figure 3.4-3). The relationships between the grade and revision are illustrated in Figure 3.4-4.

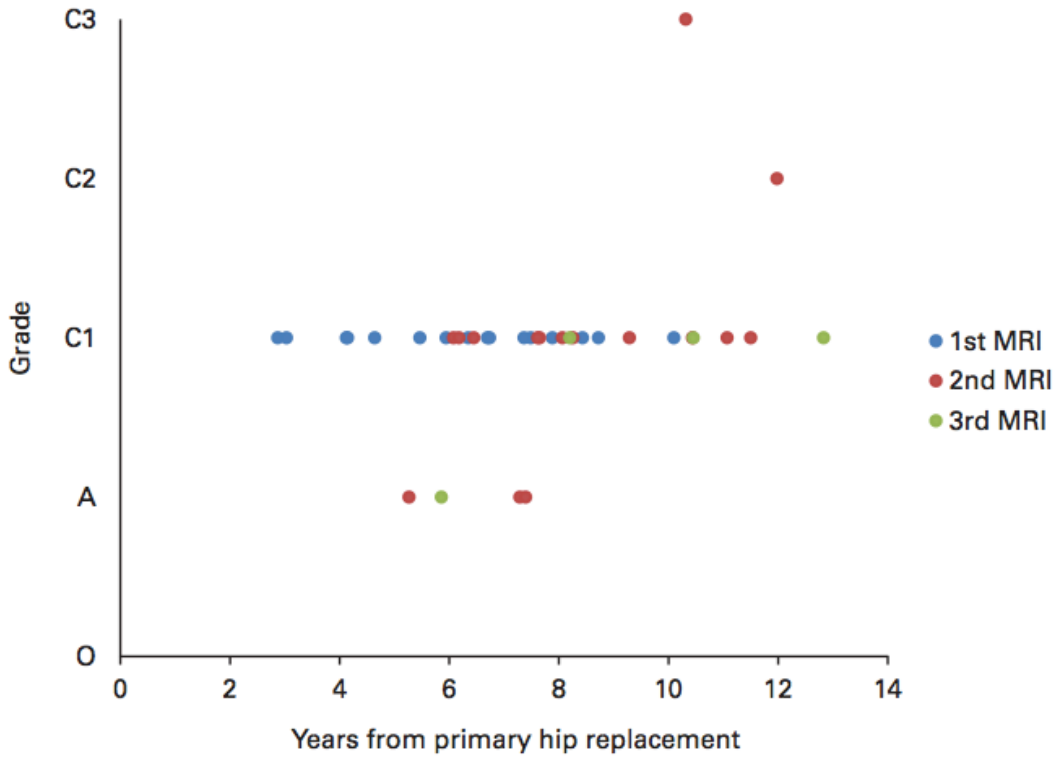

Figure 3.4-3 Scatter plot showing the change in metal-artefact-reduction (MAR) MRI grading with subsequent scans in patients whose initial scan showed Grade C1 (mild disease)

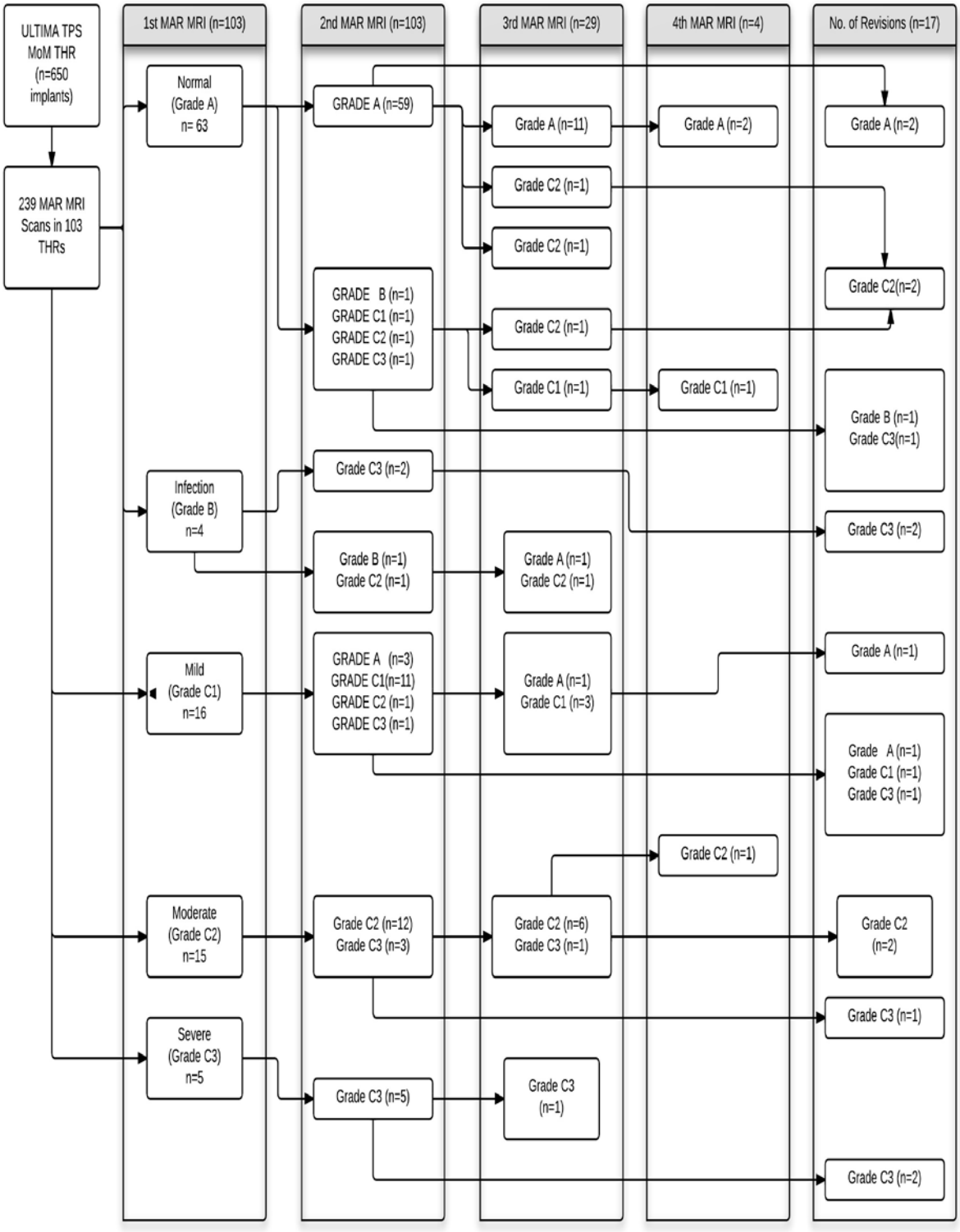

Figure 3.4-4 Flow diagram illustrating changes in metal-artefact-reduction (MAR) MRI grade with serial scans and progress to revision surgery

3.5 Discussion

Of the cohort of 103 THRs, 63 (61%) had a normal initial scan, and of these, six (9.5%) went on to require revision surgery. Where the initial scans were normal, progressive changes occurred unpredictably between seven and 11 years after operation and at a rate of 11%. Of the four cases initially reported as suggestive of infection, three progressed to more severe stages on subsequent scans, with the remainder being downgraded to normal on a follow-up scan. Previous evidence suggests that differentiating a small degree of ARMD from infection may not be reliable using MRI alone,[97] and therefore greater emphasis should be placed on other clinical and serological markers of infection in these patients.

The natural history of patients with a C1 staging is unknown. Most patients who were initially classified as having mild (C1) disease retained their original classification on a second scan at a mean of one year and nine months later. Only one patient went on to develop severe (C3) disease requiring revision surgery. From our observations it is not a certainty that the disease will progress, and indeed progression does not appear to be commonplace and where it does occur the rate of change is unclear. Where regression to a normal post-operative appearance has been observed on MRI, the mechanism for this phenomenon can at present only be speculated upon, and is a potential avenue for further research. Most THRs in this study had normal MRI scans and did not have any progression of disease. Where there is a change in grade it is more likely to indicate progression, and in turn this significantly increases with time since surgery.

Our results suggest that there may be a latent period of many years prior to any evidence of disease being detectable radiologically. There appears to be a bimodal distribution, where a larger proportion of patients present early with severe disease and are revised and a smaller proportion present later, between seven and 11 years, with mild or moderate changes.

The MHRA guidelines now recommend imaging in the form of MAR MRI or ultrasound for small-diameter MOM THRs in symptomatic patients, or 'if concern exists for the cohort'. We have previously reported a 13.8% failure rate of our entire series of Ultima TPS THRs at five years[91]. This has now increased to 20.3%, with a mean time to revision of 5.9 years (range 1 to 14 years). The MHRA guidance has emphasised a follow-up period of five years or more for small-diameter MOM THRs. The guidance refers to the presence of symptoms as suggestive of the need for longer follow-up. However, it is our experience that radiological evidence of disease can be demonstrated in asymptomatic patients in both small [109]and large-diameter MoM THRs[64].

The clinical picture in the management of these patients has been evolving. Significant disease can occur in the absence of symptoms [123,124].Our findings suggest that long-term surveillance of MoM THRs for the life of the implant is essential.

Patients in our cohort are routinely invited for review annually. An MRI scan has been offered to all our patients, although some have declined. No patient has been discharged.

The assessment of these patients is complex and extends beyond the categorisation of imaging, symptoms and serum metal ion levels. In isolation it is difficult, and perhaps not useful, to assign a negative predictive value to a normal MRI scan.

We have an increasing number of patients with more than one scan. It seems likely that patients presenting with a normal scan initially may be safely observed with annual clinical review, and a subsequent scan should there be any change in symptoms. This reflects current practice at our institution.

The study is limited by its retrospective design. There is a selection bias in that we have not included patients who were revised prior to having MAR MRI scans, or those in whom revision was undertaken after a single scan. The cohort of patients with two or more scans may not be representative of the whole, but in practice many cohorts of patients with MoM THRs will include some who have required early revision and some with initially low-grade problems that are observed over time. The timing of the sequential scans has not been at defined intervals according to any given protocol. This leaves us unable to comment on precisely when a normal MRI or a Grade C1 may progress in radiological staging. We have not accounted for variability in symptoms or serum metal ion levels, which are factors that frequently contribute to the referral for MAR MRI scans. Our findings may not be directly applicable to larger-diameter implants, as a small-diameter 28 mm MoM THR has a smaller zone of artefact around the prosthesis. This means that smaller significant lesions are picked up earlier in these patients, and hence may be revised before a second scan is undertaken.

We would recommend follow-up with MAR MRI scans as determined by a detailed clinical assessment at intervals no more frequently than annually. On the basis of the evidence presented, we are unable to recommend a specific optimal interval between scans. Further study is needed to correlate MRI grading with intra-operative findings, histological staging and patient-reported outcome measures. This would offer a better understanding of the status of patients with normal scans and mild disease.

4 Diurnal Variation of Metal Ions

4.1 Introduction

Elevated levels of metal ions in blood, urine, and synovial fluid following MoM THR or resurfacing arthroplasty are commonly reported [125] [126] [127] with systemic levels of Cobalt (Co) and Chromium (Cr) reaching a steady state approximately 1 year following implantation [128,129].

Metal ion levels have been proposed as a monitoring tool for detection of adverse reaction to metal debris (ARMD) [130] with the goal of early intervention before significant soft tissue damage has occurred[114]. Raised metal ion levels are known to arise via a number of mechanisms which may work in concert including wear at the bearing surfaces secondary to malposition or poor implant design[57,115], corrosion, either at the head-neck or taper junction[131] [87], corrosion of particulate wear debris[70] and in the case of the Ultima TPS, mechanically-assisted crevice corrosion at the cement-implant interface[103]

Whilst there has been an association between poorly functioning implants and high levels of metal ions in the blood stream [123] [132], [133], there is evidence that high levels do not correlate with findings at revision surgery. Griffin et al[134] in their study of 90 patients demonstrated that there was no correlation between average preoperative Co and Cr levels and the level of soft tissue damage observed intraoperatively, however a very significant correlation was noted between implant time *in situ* and tissue damage, suggesting that the biological reaction to metal debris in some patients may be more a time-dependent than dose-dependent phenomenon. Although high levels of metal ions

have been shown to be associated with abnormal MAR MRI [98,99], the sensitivity and specificity of such observations at the UK Medicines and Healthcare products Regulatory Agency (MHRA) cut-off level of 7ppb is poor. There remains no consensus as to an appropriate threshold level which would mandate revision surgery.

There is evidence that physiological exercise by fit patients with a MoM bearing results in a measurable rise in plasma Co and Cr levels[135]. It is not known if metal ion levels in blood and urine fluctuate during the course of normal daily activity in a fashion analogous to blood glucose. The aim of this study was to establish if such diurnal variation in metal ion levels exists and if there was any correlation to symptoms, MAR MRI scan, or functional scores.

4.2 Study Protocol

4.2.1 Ethical Approval

The National Research Ethics Service (NRES) facilitates ethical review in the UK via local Regional Ethics Committees (RECs). Application was made via the Integrated Research Application System (IRAS) which acts as an online portal and repository for dissemination of required documentation comprising: Study Protocols, Patient Invitation Letters, GP Information Letter, Investigator CVs and GCP certification, confirmation of indemnity information and sponsor details. An appointment was made to appear before a local REC in Norwich having submitted via IRAS (IRAS project ID 105822) and a "favourable opinion" was granted on March 4th 2013 (Norwich REC reference: 13/EE/0037).

4.2.2 Patients & Methods

Patients were also asked to provide informed consent for storage of a sample of blood and urine in the Human Tissue Bank. No details were passed to anyone outside of the clinical team or involved in the patient's usual care until the patient has agreed to enter the study. The full study protocol can be found in the appendices.

Cases were identified from an original cohort of 545 patients (650 THRs) who underwent 28mm hybrid MoM THR (Ultima TPS; DePuy, Leeds, United Kingdom) between May 1997 and August 2004 performed by three surgeons (including the senior author JFN), as previously reported[91]. Ethical permission was granted by the National Research Ethics Service Committee – East of England [REF 13/EE/0037] and our institutional review board. All patients who had an Ultima TPS MoM THR *in situ* were eligible to participate. Patients who had undergone revision of their Ultima prosthesis, those that were taking medications or supplements known to increase excretion of cobalt or chromium, those on immunosuppressant medication, and those who were unable to provide informed consent at the time of recruitment were excluded. Patients with bilateral MoM THRs *in situ* were also excluded as it is well recognised that these patients will have significantly higher levels of detectable Co and Cr in blood and urine[132 136]. No patients with evidence of infection were recruited.

Patients were seen in an outpatient clinic in the course of their normal follow-up as recommended by the MHRA. Patient selection was performed on the basis of reported symptoms (pain, limp, or mass) and whether their MAR MRI was normal (Grade A) or abnormal (Grade C1-C3) according to the classification of Anderson et al[97] . Since no

prior publication on the magnitude of effect of diurnal variation upon metal ion concentration in either blood or urine exists, ethical approval was granted to recruit a maximum of 12 patients as being adequate for this feasibility study. Patients were stratified into four categories: asymptomatic with normal MAR MRI (AN), asymptomatic with abnormal MAR MRI (AA), symptomatic with normal MAR MRI (SN) and symptomatic with abnormal MAR MRI (SA).

4.2.3 Trial Schedule

Patients were invited to attend a Clinical Trials Research Unit on four occasions and were asked to donate blood and urine specimens for analysis of Cobalt and Chromium levels at a specialist (TEQAS accredited) reference laboratory for trace metal analysis via Inductively Coupled Plasma Mass Spectrometry. Protocols for specimen collection, transport, and analysis were already well established.

Patients attended on 4 days with at least a week between visits. On the first day patients completed an Oxford Hip Score and had blood and urine samples collected in the morning between 08:00 to 09:30 (T1), at lunch-time between 11:45 to 13:30 (T2), and later in the afternoon between 15:30 to 16:40 (T3). Participants were encouraged to engage in their usual level of activity in between visits. To establish if there was significant longitudinal variation in Co or Cr levels patients had a corresponding repeat sample of blood and urine taken on Week A in the morning (TA), Week B at lunchtime (TB) and Week C in the late afternoon (TC).

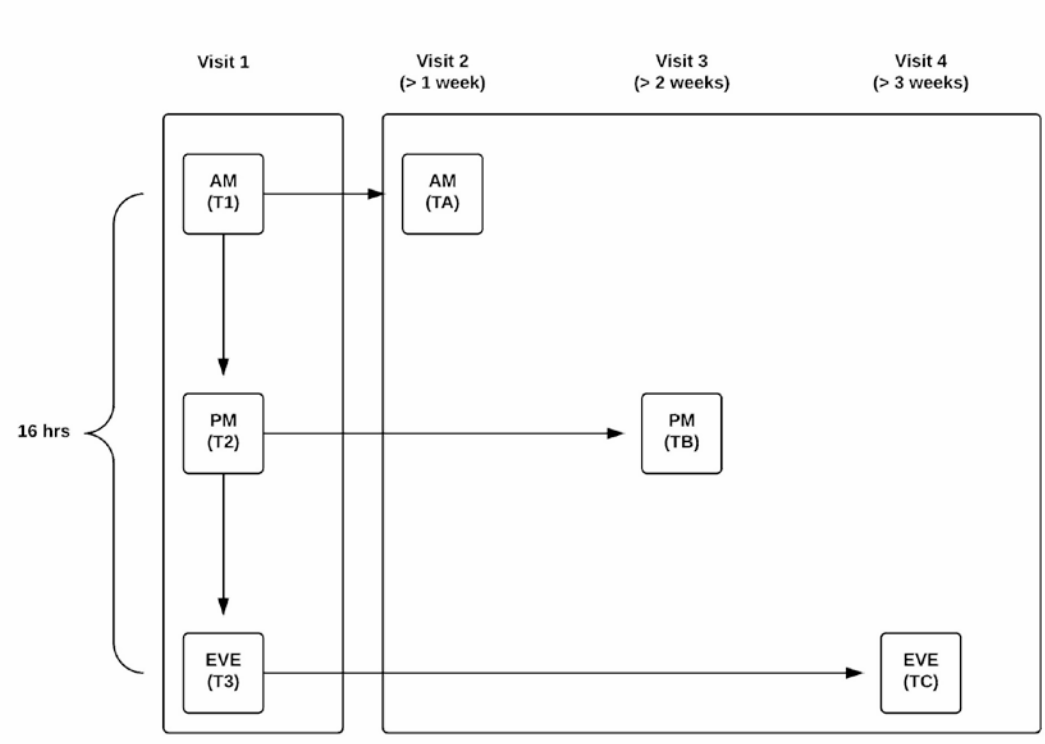

Figure 4.2-1 Study design. Each participant visited on 4 separate days. Upon first visit each participant provided blood and urine samples for Co & Cr analysis as well as blood sample for measurement of urea & electrolytes. Participants completed an Oxford Hip Score on this first visit. At all subsequent visits only samples of blood and urine for Co & Cr analysis were taken.

4.2.3.1 Study Endpoint

The study was concluded at the last visit of the last patient and once results of all investigations had been completed and verified.

4.2.4 Trace Metal Analysis

Plasma metal ion levels were measured using venous blood samples (5 ml) obtained using the vacutainer system (Vacuette; Greiner Bio-One GmbH, Kremsmünster, Austria) and directly collected in K2EDTA trace element tubes with no purging of samples[137]. The plasma metal ion analysis was carried out by the Department of Biochemistry at the University Hospital Wales, Cardiff, which is a participating laboratory in the Trace Elements External Quality Assessment Scheme (TEQAS) in accordance with MHRA guidance. The levels of cobalt and chromium were measured

by an inductively-coupled plasma mass spectrometer (Agilent 7700x, Agilent Technologies, Berkshire, UK). Plasma samples, standards and quality control material were diluted 1 in 15 with diluent containing 0.01% triton (Romil, Cambridge, UK), 0.01%EDTA (AnalaR, VWR, Lutterworth, UK), 0.2% ammonia (Romil, Cambridge, UK) and 20 ppb Gallium as an internal standard (Inorganic Ventures, Madrid, Spain). For urine samples, standards and quality control material were diluted 1 in 10 with diluent containing 0.05% nitric acid (BDH Aristar, VWR, Lutterworth, UK) 1% isopropanol (Romil Super Pure, Cambridge, UK) 0.1% triton and 20 ppb Gallium as an internal standard. Isotopes 59 and 52 were measured for Co and Cr respectively using Helium gas for interference correction for both plasma and urine samples.

4.2.5 Radiological Analysis

MRI was undertaken using MAR sequences with a 1.5 T MR machine (Siemens Symphony; Siemens, Ehrlingen, Germany). The MAR sequence used the following specification: coronal T1 weighted (W) turbo spin-echo (echo time (TE 23ms, repetition time (TR) 669ms) and short tau inversion recovery (STIR; TE 37ms, TR 3840ms), axial T1W (TE 23ms, TR 534ms) and T2W turbo spin-echo (TE 69ms, TR 5600ms) of the whole pelvis and a sagittal T2W turbo spin-echo of the hip (TE 69, TR 2900ms). The section thickness used was 5mm with a 340 x 340mm field of view, matrix size up to 448 x 336 and pixel bandwidth 620MHz.

All scans were reported and graded by a musculoskeletal radiologist. Scans were

classified using as either normal (Grade A) or abnormal (Grades C1-C3) using the classification of Anderson et al[97]. No patients with an infected (Grade B) arthroplasty were included.

4.2.6 Statistical Methods

Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, Sand Diego, CA) and Stata (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). The median and interquartile range (IQR) of Co and Cr in plasma and urine for each time point were calculated. Metal ion concentrations were also normalised to more clearly assess diurnal variation; normalised metal ion concentrations were derived for each patient by dividing metal ion concentration by the mean concentration at the three time points observed for that species. Results were analysed using nonparametric Friedman's test with Dunn's post hoc multiple comparison test to assess diurnal variability across the three time points. Wilcoxon matched pairs signed rank test was used to compare any differences in metal ion level between baseline values obtained on the first day and metal ion levels taken at matched time points at intervals of a week and greater. P value <0.05 was considered significant.

4.3 Results

A total of 11 patients with a mean age of 68 years (range 51 to 77 years) were recruited. There were five men (mean age 71 years; range 65 to 77 years) and six women (mean age 66 years; range 51 to 72 years) at an overall mean of 11 years from original surgery (range 9 to 14 years).

Only two patients were recruited into the category "symptomatic with normal MRI" (SN). During the course of investigation, it was found that their pain was likely to be referred from a site extrinsic to the hip. Given that this particular category represents a rare presentation, further recruitment was not possible.

Over the course of a single day there was no diurnal variation in levels of plasma Co, plasma Cr and urinary Cr, however, significant variations in urinary Co levels were noted, with the median level peaking at lunchtime ($p<0.03$) (**Figure 4.3-1**).

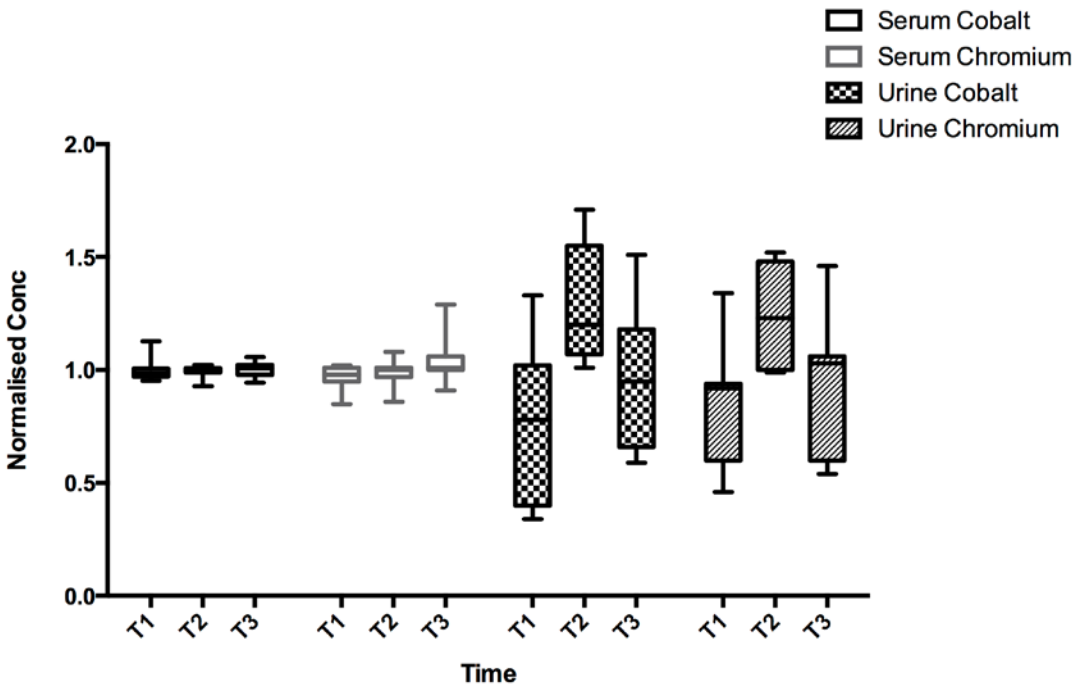

Figure 4.3-1 Normalised biomarker concentration over the course of a single day (T1= morning, T2= lunchtime, T3 = late afternoon). Within a patient, the biomarker value for a specific time point was normalised by dividing by the mean of the 3 time points. Each box represents the 25th and 75th percentiles. Lines outside the boxes represent the 10th and 90th percentiles (minimum and maximum limits respectively). Lines inside boxes represent the median.

Pairwise comparison between metal ion levels taken during the course of a single day with repeated measurements demonstrated significant differences in morning levels of urine Co (p=0.003*) and urine Cr (p=0.007*) only. Using the standard deviation for the change between T1 and T2 for urine biomarkers, the sample size needed for 80% power was found to be 371 patients (s.d. 24.28) for a difference in means of 5ppb.

At intervals of a week or greater, only plasma Cr demonstrated any significant degree of variability over the three time points (p=0.04*) (**Figure 4.3-2**).

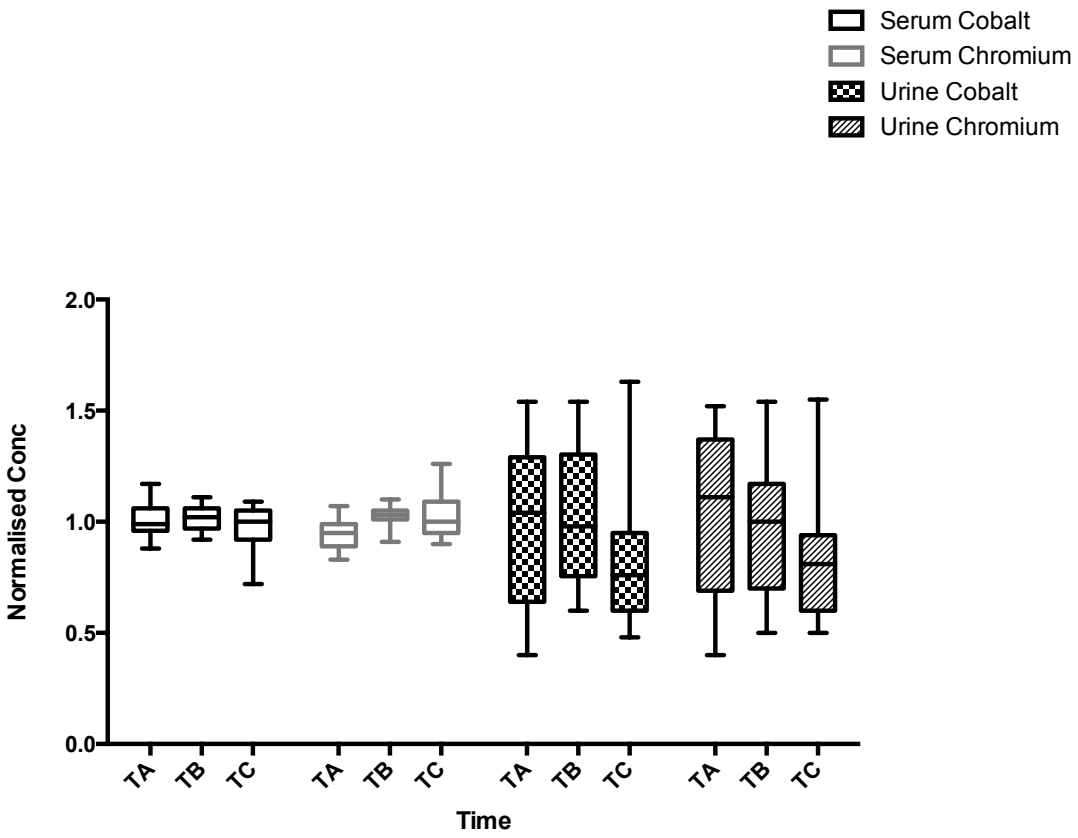

Figure 4.3-2 Normalised biomarker concentrations over time at intervals of one week or greater (TA = Week A morning, TB = Week B lunchtime, TC = Week C evening). Within a patient, the biomarker value for a specific time point was normalised by dividing by the mean of the 3 time points. Each box represents the 25th and 75th percentiles. Lines outside the boxes represent the 10th and 90th percentiles (minimum and maximum limits respectively). Lines inside boxes represent the median.

When examining diurnal variation of metal ion levels using values across all time points only plasma Cr was observed to show any significant variation ($p<0.03$*) with the highest median concentration (2.3 ppb) being in the evening (Figure 4.3-3) (Figure 4.3-3).

Table 4.3-1 Diurnal variation of metal ion levels combining values obtained from patients in a single day (T1, T2, T3) and at intervals of a week or greater (TA,TB, TC). Results were analysed using non-parametric Friedman's test with Dunn's post hoc multiple comparison test (*p<0.05)

	Median, IQR (range) ppb			
(n=22)	AM	PM	EVE	p
Plasma Co	10, 11.3 (4.8-20)	10, 11.4 (4.7-22)	10, 9(4.7-22)	0.66
Plasma Cr	2, 1.7 (0.69-7.1)	2.1, 1.7 (0.75-7.1)	2.3, 1.9 (0.93-7.2)	**0.03***
Urine Co	41, 47 (6.1-175)	53, 38 (5.6-209)	35, 31 (9-149)	0.19
Urine Cr	3.1, 3.4 (0.78-16)	4.6, 3.9 (1.4-13)	3.5, 2.1 (0.84-11)	0.19

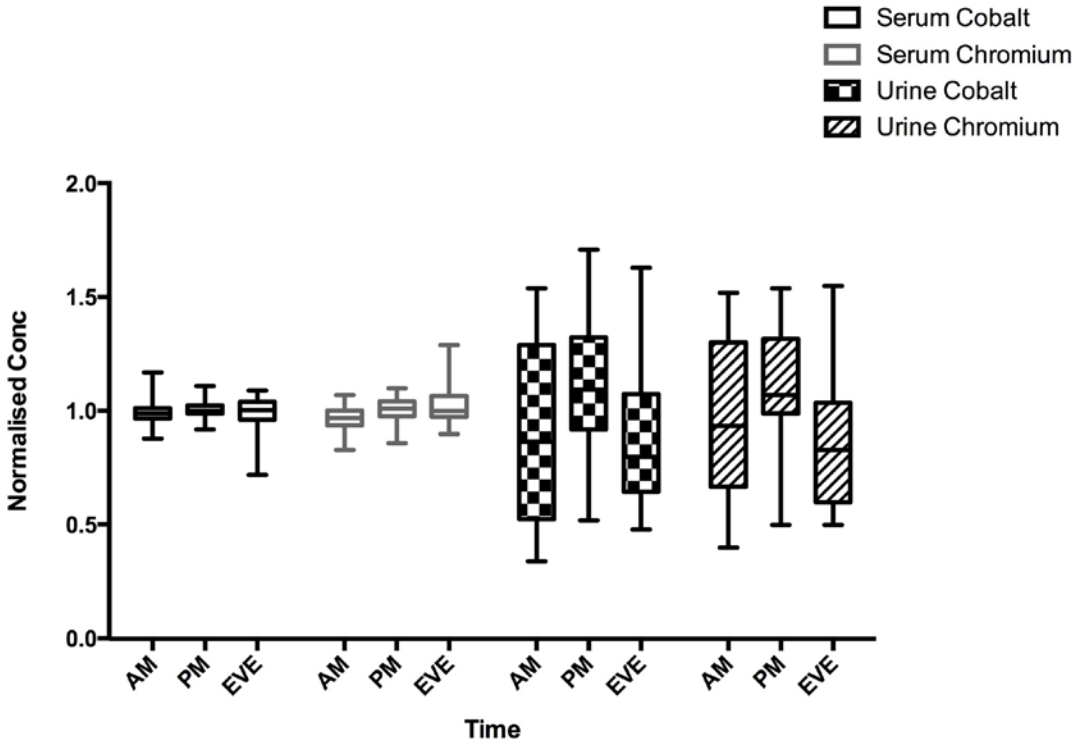

Figure 4.3-3 Overall normalised biomarker concentrations. Data for each time point was combined from the two data sets (variation within one day and variation at intervals of a week and greater). Within a patient, the biomarker value for a specific time point was normalised by dividing by the mean of the 3 time points. Each box represents 25th & 75th percentiles. Lines outside boxes represent the 10th & 90th percentiles (minimum & maximum limits respectively). Lines inside boxes represent the median.

Post-hoc analysis revealed this to be due to inter-patient variability and the presence of outlier values as opposed to an actual difference in medians (Figure 4.3-4).

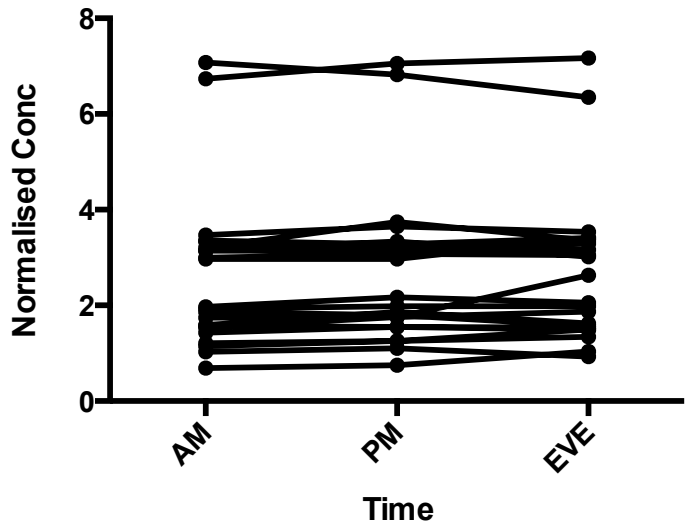

Figure 4.3-4 Overall within-patient diurnal variation of plasma Cr levels (ppb)

There were no differences by gender, symptoms, or MAR MRI scan (**Table 4.3-2**). There was no evidence of a correlation between any of the biomarkers and the Oxford Hip Score (r = 0.20, p=0.5601;r=0.04, p=0.9038; r=-0.15, p=0.6561; r=-0.29, p=0.3876 for Plasma Co, Plasma Cr, Urine Co, and Urine Cr respectively).

Table 4.3-2 Overall median biomarker levels (ppb) by sex, symptoms, and MAR MRI scan. Mann-Whitney test, (P<0.05*)

	Plasma Co	p	Plasma Cr	p	Urine Co	p	Urine Cr	p
Median(range)								
Sex								
Female	11.04 (6.56-17.21)	0.86	3.28 (2.02-3.41)	0.1	43.19 (17.61-63.58)	0.86	3.42 (2.95-5.11)	0.36
Male	10.20 (8.38-13.02)		1.89 (1.62-1.97)		47.20 (35.14-58.05)		4.74 (3.46-5.27)	
Symptoms								
No	12.98 (9.14-20.55)	0.2	3.24 (1.62-3.41)	0.36	47.51 (17.61-63.58)	0.86	3.36 (2.95-5.11)	0.1
Yes	8.38 (6.56-10.20)		1.97 (1.89-2.02)		47.20 (35.14-49.40)		4.74 (3.58-5.73)	
MAR MRI								
Abnormal	8.76 (6.56-12.94)	0.36	3.28 (1.97-3.41)	0.1	36.06 (32.10-49.40)	0.27	4.29 (3.26-5.73)	0.58
Normal	13.02 (10.20-17.21)		1.89 (1.62-2.02)		58.05 (47.20-91.00)		3.58 (3.46-4.74)	

4.4 Discussion

Over the course of 10 hours there was no diurnal variation in plasma metal ion levels, however, significant diurnal variation in urinary Co levels was observed with peak concentration in the afternoon (p<0.03*). Including all measurements taken during a single day and repeated measurements at intervals of at least a week, only a significant variation in plasma Cr was detected (p<0.03*). Whilst these results suggest that diurnal variation may exist for plasma Cr and urinary Co, such fluctuations may well be accounted for within the sphere of acceptable laboratory variation. For the low plasma Co and Cr concentrations observed in this study the co-efficient of variation (CV%) from our laboratory's internal quality control is 6.6% for Cr and 4.5% for Co and for urine this rises to 10% for Cr and 20% for Co. Our laboratory participates in the Trace Element External Quality Assessment Scheme (TEQAS) and as such these variations are comparable to other laboratories within the scheme.

Measurement of metal ion levels in either in whole blood, serum, or plasma remains a useful tool as part of the long-term monitoring of patients with a MoM THR. Urine levels of Co and Cr are more prone to fluctuation across the course of the day and may be affected by renal impairment and by an individual's level of hydration, making them an unsuitable method for monitoring.

No significant fluctuation with time was observed with measurement of plasma Co, hence it may be a more reliable marker than plasma Cr for ARMD. This raises the question as to whether it is necessary to measure both ions in the course of normal practice. Significant inter-laboratory variation has been noted in levels of plasma Co than Cr at the MHRA cut-off of 7ppb. It is not known if measuring a single species might affect the detection rates of ARMD[138]. It can be a source of confusion when there is a large difference between the Co and Cr values and where only one value exceeds the designated threshold level. In our study higher levels of Co were observed at all time points in comparison to Cr. It is known that changes in levels of Co and Cr correlate with each other[139]. In the Ultima TPS cohort Co may be the clinically relevant active agent responsible for periprosthetic tissue reactions[107], but this may not be true for all MoM implants.

There is some debate as to whether metal ion levels in whole blood, serum, or plasma should be used for monitoring. Since there can be a significant degree of inter-laboratory variation[138] it is essential to in the context of long term clinical follow up to consistently examine the same fraction and use the same laboratory. It has been demonstrated that Co concentrations are similar in red blood cells (RBCs) and plasma[140] and it is believed that a state of equilibrium between Co in RBCs and

plasma exists. Co is mainly present in the plasma fraction in vivo[141]. Cr levels in plasma for this cohort are consistently lower than Co which in part is due to alloy composition in which the Co:Cr is typically in the order of 60%:30% as well as differing rates of tribocorrosion and solubility[142].

The differences in Co and Cr concentration in urine may be explained by differences in binding to plasma proteins. Cr binds to both transferrin and albumin in plasma and hence is not filtered by the kidney and excreted into urine and hence has an extended half life in the body[140].

At our institution it is our practice to consider the trend in blood Co levels over time as an adjunct to serial clinical examination and MAR MRI to avoid any such confusion in the decision making process. Metal ion levels considered in isolation remain an inadequate screening tool for ARMD.

This study is limited by the small sample size and by our exclusion of patients with bilateral implants. The main mechanism of failure of the Ultima TPS is corrosion. The results of this study may not be generalisable to systems where more particulate metal is generated secondary to bearing wear.

We would recommend that blood metal ion levels continue to be used for long term monitoring and that for diagnostic purposes the time of day at which the sample is taken is not relevant.

5 Gene Expression Profiling

5.1 Introduction

The aim is to establish if a difference exists in the activation of biological cascades in human hip tissue of patients with end-stage osteoarthritis, those revised for periprosthetic osteolysis arising from polyethylene wear debris and those with a MoM THR undergoing revision arthroplasty for ARMD. A gene expression microarray experiment was used to identify the range of differences in gene expression between these groups. This was used to determine the different pathways of cellular interactions, how they differed between each group and were further explored using pathway analysis software. Quantitative real time polymerase chain reaction (qRT PCR) analysis was used to validate the findings of the microarray.

The biological pathways responsible for the changes seen in ARMD are not known. There may be a spectrum of necrotic and inflammatory changes in response to Co-Cr wear particles in periprosthetic tissues. Coagulative necrosis and a macrophage and T lymphocyte response occur in implant failure and pseudotumors, in which there is also granuloma formation. The pathogenesis of these changes is uncertain, but it may involve both a cytotoxic response and a type IV delayed hypersensitivity response to Co-Cr nanoparticles[111].

The discipline of genomics incorporates the study of all features of the genome and individual genes at the level of DNA. Genomics encompasses the study of the pattern of

transcription (gene expression) as a function of clinical conditions in response to natural or toxic agents or at different times during defined biological processes e.g. at specific stages in the cell cycle. The aim of the study of gene expression is to discover which genes are up- or down-regulated under specific conditions[143] . In doing so we may be able to find specific disease biomarkers that may be important in genetic epidemiology. This study does not aim to identify any novel pathway, but to clarify which pathways may be differentially activated in those patients undergoing revision surgery for ARMD associated with a MoM THR and those being revised for aseptic loosening or periprosthetic osteolysis of a MoP THR using the gene expression in patients with advanced osteoarthritis undergoing primary joint replacement as a control. No studies prior to these have been conducted using microarray experiments in this context.

5.2 Microarray

Quantitative real-time polymerase chain reaction (qRT-PCR) and gene expression microarrays are complementary approaches used to determine quantitative or relative levels of gene expression.

Previously gene expression analysis was performed in a low-throughput manner by examining handfuls of genes at a time by techniques such as Northern blot analysis or semi-quantitative end point RT-PCR. This limitation of volume has been overcome by the development of high-throughput technologies such as microarray. Whilst allowing rapid quantification of the expression of a large number of genes, the measurement of

the expression of each individual gene depends on the probe used on the array and may not be quantitative [144]. qRT-PCR can also be done in other ways than using a Taqman® probe (i.e. via incorporating the dye SyBr green), although our belief is that Taqman® is superior since it uses a probe which can cross exon-exon boundaries for specificity. For these reasons quantitative RT-PCR has been used to validate the results of these microarray experiments.

Microarrays allow the identification of genes that are expressed in different cell types and give the opportunity to examine how gene expression levels may change in disease states. They may also be used to identify which cellular processes genes may participate in.

A microarray can generate data in order to fulfil the following general experimental objectives[145]:

1. *Class comparison.* This is where one looks for differential gene expression between two or more conditions e.g. healthy versus tumour cells.
2. *Class prediction.* If we wish to develop a statistical model that might be used to predict which class an individual might belong to. Examples of this include prediction of the response to treatment (response vs. non-response) and disease progression (relapse vs. cured)
3. *Class discovery.* Here the objective is to identify novel subtypes of individuals within a given population. In some conditions it may be hard to differentiate between different subtypes based on cell morphology alone, hence definitive classification may be possible using gene expression.

4. *Pathway analysis.* These studies attempt to find genes whose co-regulation suggests that they may participate in the same or related biochemical processes.

The basic aim of the microarray experiment is to quantify concentration of a gene(s) mRNA transcript in a given cell type at a given time. The number of molecules of mRNA arising from the transcription of a given gene is used as an approximation to the level of expression of that gene[143].

Whilst there are cohorts of genes known to be involved in the process of aseptic loosening, the cellular mechanisms underlying the process of ARMD are not conclusively known. The use of a microarray enables an unbiased approach which will enable examination of not only gene families known to be affected in aseptic loosening across both the MoP and MoM phenotypes, but may also highlight pathways that are differentially activated in ARMD that would be more suggestive of the adaptive immune responses which are not present in aseptic loosening.

Microarray technology is based on the complementarity principle of nucleic acid base pairing. In microarray experiments thousands of DNA probes are fixed to a solid surface. RNA samples (targets) are labelled with fluorescent dyes for hybridization. After hybridization laser light is used to excite the fluorescent dye; the hybridization intensity is represented by the amount of fluorescence emitted from which an estimate of the relative amounts of the different transcripts that are represented within a sample can be made.

There are different platforms for whole-genome microarray can be carried out on cDNA or mRNA and may utilise either single colour or two colours. Earlier systems used two-colour cDNA microarray where both probes and targets are cDNAs. Sample mRNA undergoes reverse transcription and at the same time is labelled with the fluorescent cyanidine dyes Cy3 and Cy5. Post hybridization the fluorescence of each is measured separately and a composite image is formed which needs further processing prior to analysis[146].

High-density oligonucleotide microarray use pairs of probes which each consist of 25-mer oligonucleotides with each probe pair containing a perfect match (PM) probe and a mismatch probe (MM). The MM probe is identical to the PM probe except for the central base and functions as an internal control[143]. Unlike cDNA microarray the mRNA sample is converted to biotynlated cRNA with only one target hybridized and only a single fluorescent colour is used. This is the basis of the widely used Affymetrix Gene Chip.

Illumina Inc (San Diego, CA) produces a whole-genome microarray platform, the Human HT12 v4 Expression Beadchip. Sample RNA undergoes first and second strand reverse transcription followed by a single in vitro amplification that incorporates biotin-labelled nucleotides. Subsequent steps include array hybridization, washing, blocking and streptavidin-Cy3 staining. Fluorescence emission by Cy3 is quantitatively detected for downstream analysis (Figure 5.2-1). This platform was selected since early quality control experiments highlighted very low yields of high quality RNA from fresh and frozen periprosthetic tissue from both osteolytic MoP THR and ARMD MoM

THR revisions. The Illumina platform has been shown to maintain a low false discovery rate (FDR) even with small amounts of RNA (10ng). Lynch et al[147] have demonstrated that a bias in expression levels obtained when using lower amounts of RNA can be compensated by using more replicates to achieve the same levels of precision, however, any decrease in sensitivity cannot be compensated for by increasing sample size. The net effect of this strategy is that an experiment with few biological replicates and a low starting concentration of RNA may fail to detect subtle changes, but using the Illumina Beadchip system one can be reasonably confident in any changes that are reported.

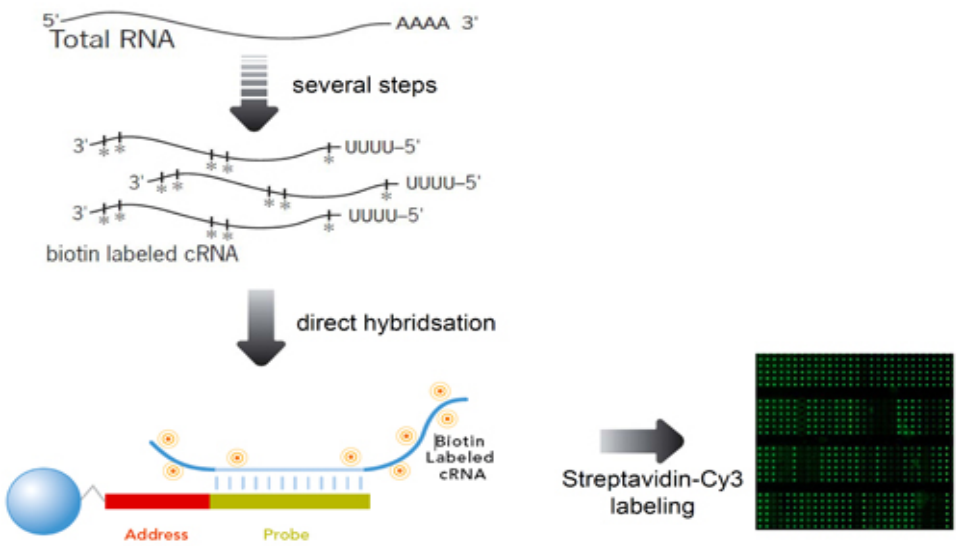

Figure 5.2-1 Schematic overview of microarray experiment using Illumina HT12 v4 Beadchip platform. Reproduced from Illumina HT12v4 Human Beadchip technical note (2012) [148]

5.3 Real-time Quantitative Polymerase Chain Reaction.

Quantitative real-time polymerase chain reaction (qRT-PCR) involves the repeated amplification of cDNA using sequence specific oligonucleotides, heat stable DNA polymerase, and thermal cycling. In traditional "end point" PCR, detection and quantification of the amplified sequence are preformed at the end of the reaction after the last PCR cycle and typically involve post PCR analysis via gel electrophoresis and image analysis.

qRT-PCR uses a double stranded template during low temperature cycles a primer anneals to the gene of interest and a probe binds to a downstream sequence. The Taqman® probes are covalently joined to two other molecules; a reporter and a quencher. At the 5' end is a fluorescent molecule termed a "reporter", so termed as it gives off or reports a detectable signal as more product is generated. At the 3'end is a molecule termed the quencher that quenches the fluorescent signal from the reporter as long as the probe itself remains intact. When an intact probe is exposed to light the fluorescent reporter molecule does not give off a detectable signal as there is energy transfer between the reporter and the quencher via a process termed fluorescent resonance energy transfer (FRET)[149]. Each time a new PCR amplicon is produced the reporter and quencher are permanently split such that the reporter produces a detectable signal in the presence of light and fluorescence will increase proportionally with product. The first step in qRT PCR involves denaturing the double stranded template at a high temperature. As that temperature is lowered, the probe and primers bind, the Taqman® polymerase enzymes then enter and begin the extension phase of PCR by creating new complementary strands of DNA. When the Taq polymerase encounters the reporter end of the probe its inherent exonuclease activity causes the probe to be destroyed, a new amplicon is created, and the reporter and quencher molecules are physically separated creating a permanent increase in fluorescence

which perfectly accords with the doubling of product[150](Figure 5.3-1). The real-time instrument can monitor and record this increase in fluorescence after each cycle to generate an amplification plot for interpretation.

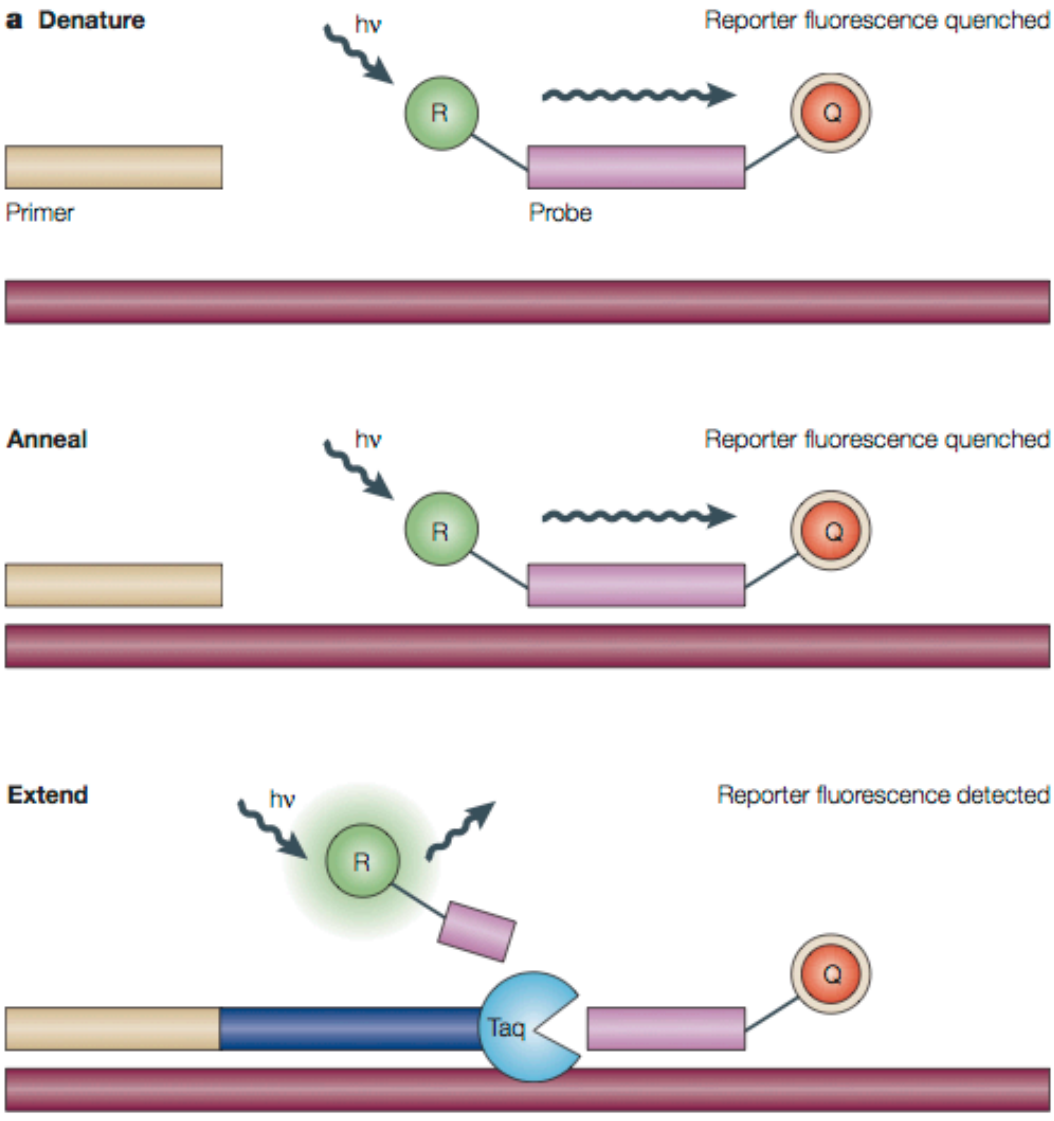

Figure 5.3-1 Principles of Taqman®. 5' nuclease activity of thermostable polymerases used in the PCR cleaves hydrolysis probes during the amplicon extension step that separates the detectable reporter fluorophore (R) from the quencher (Q). Fluorescence emitted when excited by an external light source (hv) at each PCR cycle is proportional to the amount of product formed. Reproduced from Koch (2004) [150]

5.4 Materials and Methods

5.4.1 Ethics

Ethical approval for analysis of tissue held in the Norwich Human Tissue Bank (REC Reference 08/H0304/85) was sought from the Faculty of Medicine & Health Sciences Research Ethics Committee at the University of East Anglia (Reference 2011/2012-62) after an informal peer review process amongst consultant orthopaedic surgeons. Ethical approval was granted on September 19th 2012.

The ethics submission inclusive of patient information materials and consent forms are included in the Appendices.

5.4.2 Tissue Sampling & Storage

Tissue samples were taken intraoperatively from patients undergoing primary hip arthroplasty for the indication of osteoarthritis, patients with a MoP primary arthroplasty in situ undergoing revision arthroplasty for aseptic loosening, or patients with a MoM primary arthroplasty undergoing revision arthroplasty for ARMD.

Samples of hip capsule or acetabular membrane were excised during the procedure. All samples were stored at room temperature submerged in an RNA stabilising agent (RNALater, Qiagen, Crawley, UK) and transferred to a -80^{0}C freezer within an hour.

5.4.3 Consent Process

Informed consent is essential for research undertaken on human tissue. Patients were fully informed about the study including potential risks and benefits. Ethical approval for use of human tissue collected as part of existing Human Tissue Bank infrastructure was granted by the University of East Anglia Faculty of Medicine and Health Sciences Research Ethics Committee on 19th September 2012 (REF: 2011/2012-62). Patients were approached prior to surgery by the Primary Investigator who explained the study, invited the patient to take part, and answered any questions that arose during the consultation. Patients were given written information and provided their consent using the Human Tissue Bank consent forms. All patients approached to take part in the study agreed to participate. A copy of the completed consent forms is retained at the NNUH and in the Academic Orthopaedic Department.

5.4.4 Determination of Sample Size

Traditional approaches to determining statistical power are not routinely used for microarray experiments since due to their nature microarrays can be used to test multiple hypotheses, use FDR estimates for inference, and can use classification techniques with thousands of transcripts.

For experimental designs where groups of cases are evaluated for differential expression a minimum of 5 biological cases per group are seen as acceptable[146]. The Illumina HT12v4 BeadChip has capacity for 12 samples per array. With 8 samples per

category across 2 separate chips this experiment overall is cost-effective and adequately powered to detect differential gene expression as supported by the method outlined in by Pan et al[151] where a Type 1 error rate $\alpha = 0.05/ 10,000$ genes for a 2-fold magnitude of expression change has 80% power.

5.4.5 Patient Identification and Data Collection

Potential patients were identified via the waiting lists of consultant hip arthroplasty surgeons at the NNUH. Patients were approached pre operatively.

Patients were considered eligible for inclusion via the following criteria:

Primary Hip Arthroplasty:

Undergoing primary total hip arthroplasty for osteoarthritis

MoP Revision Arthroplasty:

Undergoing revision arthroplasty for aseptic loosening of a MoP cemented THR

MoM Revision Arthroplasty:

Undergoing revision arthroplasty of a primary MoM THR for ARMD.

Exclusion criteria were:

1. Infection
2. Alternate bearing surfaces i.e. ceramics
3. Fracture
4. Autoimmune disease
5. Immunosuppressant medication or immunocompromised state

6. Inability to provide informed consent

Patient demographic data were stored in an anonymised form.

5.4.6 RNA Extraction & Purification

RNA extraction has been optimised by adaption of the tissue homogenisation and cell lysing method described for RNA extraction from hip tissue previously described by our laboratory[152]. The full protocol is detailed in the Appendix. In order to extract RNA, the source tissue must first be homogenised, the cells lysed, and the RNA dissolved into solution. The RNA fraction is isolated from the residue via phase separation using chloroform and then passed through spin columns to further purify. The previously described method involved use of an UltraTurrax homogeniser. The blade assembly of this instrument could not be completely disassembled to adequately sterilise in between samples to avoid cross contamination. In addition initial yields from tissues affected by ARMD were low using this equipment. We substituted the use of an UltraTurrax homogeniser with a TissueLyser LT (Qiagen, Manchester) which uses a cooled centrifugal system and an agitating ball to homogenise the sample. This optimisation delivered an approximate 40% increase in RNA yield (Appendix A).

Tissues samples taken from patients were stored in RNALater® (Ambion) at 4 ^{o}C. The samples were divided using sterile scissors and tweezers and placed in to RNAse free Safe Lock Eppendorf® tubes and kept on ice. Where archival tissue from the Human Tissue Bank or frozen tissue was used a cryotome was used to section tissue as required. For 0.5 g of tissue 500 µl TRIzol® (Invitrogen) was added to a tube along

with a stainless steal ball bearing cooled to -80 °C. The tubes were then placed into a TissueLyserLT (Qiagen) and then the machine set to 50 Hz for 2 minutes. The tubes were then cooled on ice for 2 minutes and returned to the machine for a further 2 minutes. This cycle was repeated a total of 5 times. The TRIzol® was removed from the Eppendorf and placed into a new 1.5 ml Eppendorf and 200 µl chloroform was added, vortexed for 30 s and spun at 13,000 rpm (~19,000 rcf) at 4 °C for 15 minutes.

5.4.7 RNA Quality Control

RNA quality plays a major role in the generation of accurate quantitative results from gene expression analysis experiments. Isolation of high quality and intact RNA from human tissue can be problematic due in part to the need for expedient handling, transport and storage of samples in an RNAse free environment. RNA is more labile than DNA and is susceptible to degradation by endogenous cellular RNases as well as by chemicals or heat such as is generated via surgical diathermy. Where the original tissue sample has been grossly affected by a pathological process and is of poor overall quality in the first instance, as seen in ARMD, optimal RNA yields for downstream processing can be difficult to obtain. The determination of RNA quality is therefore a crucial step in the workflow of quantitative gene expression.

5.4.7.1 Nanodrop RNA Quantification

Quantification of RNA yield was performed via the use of a spectrophotometer (Nanodrop: Nanodrop Technologies, Wilmington, DE). Nanodrop allows quantification

of proteins and nucleic acids by use of absorption spectrometry. Quantification of RNA is necessary prior to reverse transcription.

The Nanodrop is an essential quality control step to ensure an acceptable amount of RNA of an adequate quality has been extracted. Good quality RNA gives rise to a characteristic absorption curve Figure 5.4-1:

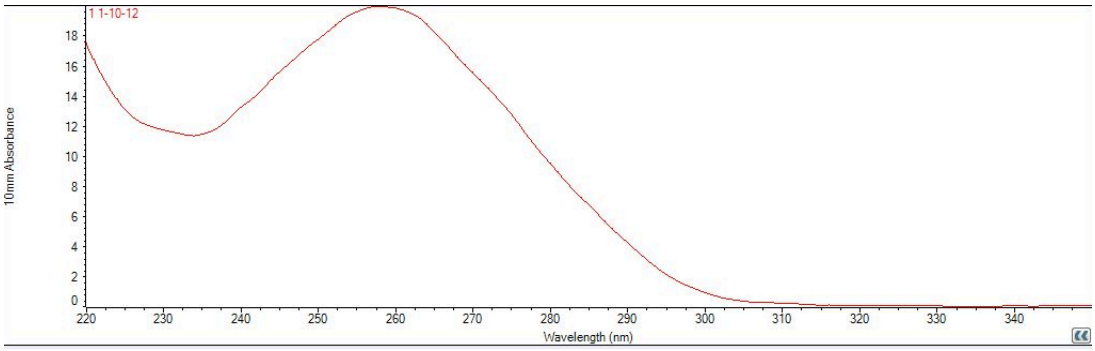

Figure 5.4-1 Sample Nanodrop spectrophotometer curve for RNA extracted from human hip tissue demonstrating classical "double tick" morphology. Absorbance is plotted on the y-axis against wavelength (mm) on the x-axis

DNA and phenol are common sources of sample contamination that can be screened for at this stage. Sample contamination can be determined with reference to the 260:280 absorption ratio. Proteins are absorbed at 280; a 260:280 ratio between 1.9 and 2.1 is indicative of low levels of protein contamination.

The 260:230 ratio is used as a secondary measure of nucleic acid purity with expected values commonly in the range 2.0-2.2, if the ratio is lower then the presence of contaminants which are absorbed at 230nm such as carbohydrate, phenol or TRIzol phenolic reagent.

5.4.7.2 RNA Degradation Assessment

RNA was extracted using RNEasy Mini-kit (Qiagen). Isolation of RNA was carried out as per the manufacturer's instructions. The RNA was checked for degradation using the Experion™ RNA StdSens system (Bio-Rad Laboratories Inc). The Experion™ automated electrophoresis system (Bio-Rad Laboratories Inc) provides an effective method for both confirming the results from the Nanodrop RNA quantification, and of determining the RNA quality prior to any further gene expression experiment. The integrity of a sample can be measured using as little as 200pg total RNA, much less than the 5-10ug of RNA needed to determine RNA quality via the traditional method of agarose gel electrophoresis. This was of particular benefit in the context of performing microarray experiments that typically require relatively large amounts (5-10ug) of RNA.

The RNA StdSens chips were loaded with 1 µl of RNA samples, which had been denatured at 90°C for 2 min. The chips were loaded on the Experion™ and run according to manufacturer instructions. Degraded RNA shows a smear, RNA that has not been degraded shows 2 distinct bands on the gel, one representing 18S RNA and the other 28S RNA.

RNA samples with RNA Integrity Number (RIN) or RNA Quality Index (RQI) ≥ 7 were selected for whole-genome expression array experiments. The RIN (Agilent Technologies) and RQI (Bio-Rad Inc) represent different methods of using software algorithms to standardize and quantitate RNA[153]. Both methods return a number between 1 (highly degraded RNA) to 10 (intact RNA).

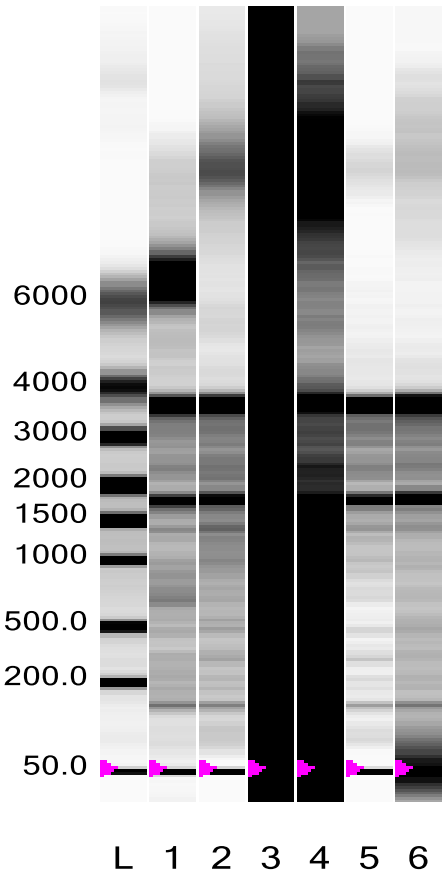

Figure 5.4-2 Example of an Experion™ quality control experiment. Column L represents the calibration RNA reference ladder. Visual inspection reveals two well-defined bands qualitatively indicating good quality RNA has been obtained in samples 1, 2, 5, and 6. This is quantitatively supported by the RQI (RNA Quality Index). The experiment has failed in columns 3 and 4 either due to contamination or human error filling the wells.

5.4.8 Microarray Experiment & Analysis

Microarray experiments are complex and require expensive specialised equipment, leading-edge computer hardware and software as well as specialist expertise and a high level of technical skill. For these reasons it is common practice to prepare samples to an agreed standard according to the MIAME (Minimum information about a microarray experiment) standard [154] and to outsource microarray hybridization to a

third party. These industry partners perform further quality control, perform the experiment and generate gene-expression values ready for statistical analysis.

The microarrays were carried out using an Illumina whole-genome expression array Human HT-12 v4 (Illumina Inc., Illumina United Kingdom, Saffron Walden, UK) to profile gene expression of RNA samples according to the manufacturer's protocol (www.illumina.com/technology/direct_hybridization_assay.ilmn.).

The first 12-sample microarray was performed by Source Bioscience PLC, the second 12-sample microarray by High Throughput Genomics, Wellcome Trust Centre for Genomics, Oxford University. Across the two microarrays experiments there were 8 biological replicates from 3 groups (8 x Primary osteoarthritis, 8 x metal-on-polyethylene revision for aseptic loosening and 8 x metal – on-metal revision for ARMD). Of the biological replicates for MoM revision for ARMD, six were from patients with an Ultima TPS 28mm THR, and two from patients with a large diameter (>36mm) ASR THR. Raw expression data were analysed using R [155] and processed with Lumi package Raw data were normalised with a quantile algorithm.

RNA was isolated using the RNeasy Mini Kit-Qiagen. Total RNA from 24 patients was labeled and hybridized onto Illumina HumanHT-12 v4 Expression BeadChips covering 31,000 annotated genes with over 47,000 probes incorporating microRNAs and splice variants(Illumina, San Diego, CA).

Microarray raw data were analysed using Lumi[156] with quantile normalization in R[155] (Bioconductor). Post normalisation, the data was annotated using the HumanHT-12_v4_0_R1_15002873_b.bgx package (www.switchtoi.com/annotationfiles.ilmn) Multiple Experiment Viewer (MEV)[157] was used for further analysis. Genes differentially expressed were selected using ANOVA, with a p value, based on permutations, <0.05. Genes were filtered for a fold ≥2 in at least one condition. Hierarchical clustering was done using Euclidian distance. Pathways analysis was done in Ingenuity Pathway Analysis (IPA). Data sets were deposited as Gene Expression Omnibus (GEO) datasets (accession number pending)

5.4.9 Reverse Transcription Polymerase Chain Reaction (RT PCR)

Synthesis of complementary DNA (cDNA) via reverse transcription was performed using 900ng of RNA (100ng/µL) added to a sterile and RNAse free Eppendorf tube with 200ng random hexamers (Invitrogen) and incubated at 70°C for 10 minutes. This anneals the random hexamers to the RNA chains which then act as a primer.

The samples were incubated on ice and to each the following were added:

- *4µL 5x Superscript buffer*
    - Provides optimum conditions for RT enzyme and stabilises pH at 8.3
- *2µL 0.1M DTT*
    - Reducing agent to improve reaction efficiency
- *1µL Superscript (Invitrogen)*
    - Performs reverse transcription using dNTPs to produce cDNA
- *1µL dNTPs (Roche)*
    - 10mM of each deoxyribonucleotide

The samples were incubated at 42°C for one hour and then inactivated via incubation at 70°C for 10 minutes.

The final concentration of cDNA after the reaction is 47.4 ng/µl. Samples were stored at -20°C until required for Taqman® assays.

5.4.10 Determination of Housekeeping Genes via Taqman®

To assess what housekeeping genes would be appropriate in Taqman® assays, a PerfectProbe GeNorm 12 gene kit was used (Primerdesign; Housekeeping gene panel: CYC1, RPL, B2M, GAPDH, YWH, EIF, ATP, TOP1, UBC, ACTB, SCHA). The kit was used according to manufacturer's instructions and RNA isolated and converted to cDNA from 3 Primary, 3 MoP and 3 MoM samples and probes were loaded onto a Micro Amp® optical 96 well plate (Applied Bioscience), controls were also carried out. The plate was loaded onto a 7500 Real Time PCR system (Applied Biosciences) and the run cycle was started (50 °C for 2 min, 95 °C for 10 min, 40 x (95 °C for 15 s, 60 °C for 1 min)) and fluorescence from the reporter dye, TAMRA™ was monitored. The primary analysis and labelling was carried out using Taqman® 7500 software.

For running a Taqman assay a standard curve was run for each set of primers. RNA was diluted to 20 ng, 10 ng, 5 ng, 1.25 ng and 0.625 ng samples, these were then loaded in a 96 well plate. The working stock of cDNA was diluted to 5 ng/µl. For each PCR reaction 12.5 µl of Precision 2 x qPCR Master Mix with low ROX (Primer design), 1.25 µl primers (designed by Primer Design, quencher dye is TAMRA™) 1.25 µl H_2O

and 10 µl cDNA sample were added to a well on a Micro Amp® fast optical 96 well plate (Applied Bioscience).

Once all the wells were filled the plates was placed into a 7500 Real Time PCR system (Applied Biosciences) and the run cycle was started (50 °C for 2 min, 95 °C for 10 min, 40 x (95 °C for 15 s, 60 °C for 1 min)) and the fluorescence from the report dye, TAMRA™ was monitored.

To analyse the data obtained standard curves were constructed using the cycle number (Ct) plotted against the log input (Log (µg RNA in sample)). The *x* and *y* intercepts of the standard curve, obtained from the line of best fit (R^2= > 0.98) are also used in calculating the gene expression (in arbitrary units). The following calculation was used to identify the input:

$$input = 10 \text{ ^ } \frac{(Ct - x)}{y}$$

The input of the gene of interest, was normalised by dividing the gene of interest by the geometric mean of Top1 and Cyc1 since Normfinder software (http://moma.dk/normfinder-software) analysis revealed that these two genes were contamination free and demonstrated the least degree of change. The geometric mean is defined as the n^{th} square root (n is equal to the number of samples) of the total values of each sample multiplied by each other i.e.

$$Geometric\ mean = \sqrt[n]{s1.s2.s3 \ldots}$$

n=number of samples

s = gene expression of housekeeping gene

Top1 and Cyc1 which were then used as housekeeping genes in future Taqman® low-density arrays (TLDAs).

5.4.11 Taqman® Low Density Array (TLDA)

The TLDA (Life Technologies, Paisley, UK) was designed to assess genes identified from the 36 genes from the microarray experiment which demonstrated the highest degree of significance on ANOVA ($p<0.05$) across all three phenotypes and which also had a FC>2 irrespective of the direction of change in expression. Where a gene was not readily available on the commercial platform used or was not financially viable to design the probe for, the next gene in the hierarchy was selected.

TLDA functions as an array of reaction vessels for the PCR step. The wells of the TLDA contain Taqman® gene expression assays that detect the real-time amplification of user specified targets. The relative levels of gene expression are determined from the fluorescence data generated during PCR. Custom designed TLDA array cards allowed a total selection of 48 genes (inclusive of 12 housekeeping genes) for 33 samples (10 x Primary THR for OA, 11 MoP THR revisions for aseptic loosening, and 12 MoM THR for ARMD). TLDA cards are preloaded with specified custom primer probe sets and all reagents required for the reaction.

Patient cDNA is loaded into the array via ports and centrifuged to distribute samples across the wells. The PCR reaction is identical to the process for manual Taqman® via

use of the Biosystems 7900HT Fast Real-Time PCR system. The TLDA is optimised so that the custom primer-probe sets have near uniform efficiency across cDNA concentrations. No independent standard curve analysis is performed since the system is designed not to need this step.

cDNA was loaded into the fill reservoirs and the plate run according to manufacturer's instructions using the Applied Biosystems 7900HT Real-Time PCR System and Applied Biosystems Sequence Detection Systems (SDS) software (Life Technologies, Paisley, UK). Undetected samples were discarded. Relative expression levels in each gene of interest were analysed by normalising to endogenous control genes *TOP1* or *Cyc1* (ΔCt [endogenous control gene Ct-gene of interest Ct]) and linearising the data by expressing it as $2^{\Delta Ct}$. Alternatively data was expressed as a fold change from the control ($2^{\Delta\Delta Ct}$ [treatment condition $2^{\Delta Ct}$ / Control $2^{\Delta Ct}$). Data shown was normalised to *TOP1,* as GeNorm analysis demonstrated *TOP1* to be the most stable housekeeping gene (normalising to Cyc1 yielded similar results).

5.5 Laboratory Results

5.5.1 Whole-Genome Microarray

Post processing via variance stabilization and quantile normalisation found that no genes were found to be statistically significantly differentially expressed between the MoP and MoM phenotypes via Student's t-test ($p<0.05$). Since the p-value is not corrected for multiple testing a false discovery rate (FDR) using a q-value of 0.2 was used as a threshold to control the number of false positives. The q-value represents the smallest value at which a gene becomes significant using a false-discovery rate (FDR) method using the Benjamini and Hochberg multiple testing correction[158]. The q-value is an adjusted p-value, taking in to account the false discovery rate (FDR). Applying a FDR becomes necessary when thousands of variables are measured (in this case gene expression levels from tens of thousands of transcripts) from a small sample set. A p-value of 0.05 implies that we are willing to accept that 5% of all tests will be false positives. An FDR-adjusted p-value (aka a q-value) of 0.05 implies acceptance that 5% of the tests found to be statistically significant (e.g. by p-value) will be false positives.

There was a high degree of noise in the analysis and it was noted that for multiple genes there was a large order of inter-sample variation in samples of the same phenotype. For example the gene CSN1S1 is the most highly expressed gene in the MoM phenotype samples but has a high q-value (0.89) hence is not considered to be significant despite having a high fold change (FC=4.5 MoP vs. MoM); the q value is high since expression level of that gene is highly variable amongst individual MoP samples.

Examination of the sample relations between the 3 phenotypes across all 33,777 genes demonstrates that whilst primary OA phenotypes tend to cluster separately from the other two phenotypes there is a high degree of clustering of individuals of the MoP and MoM phenotypes (Figure 5.5-1).

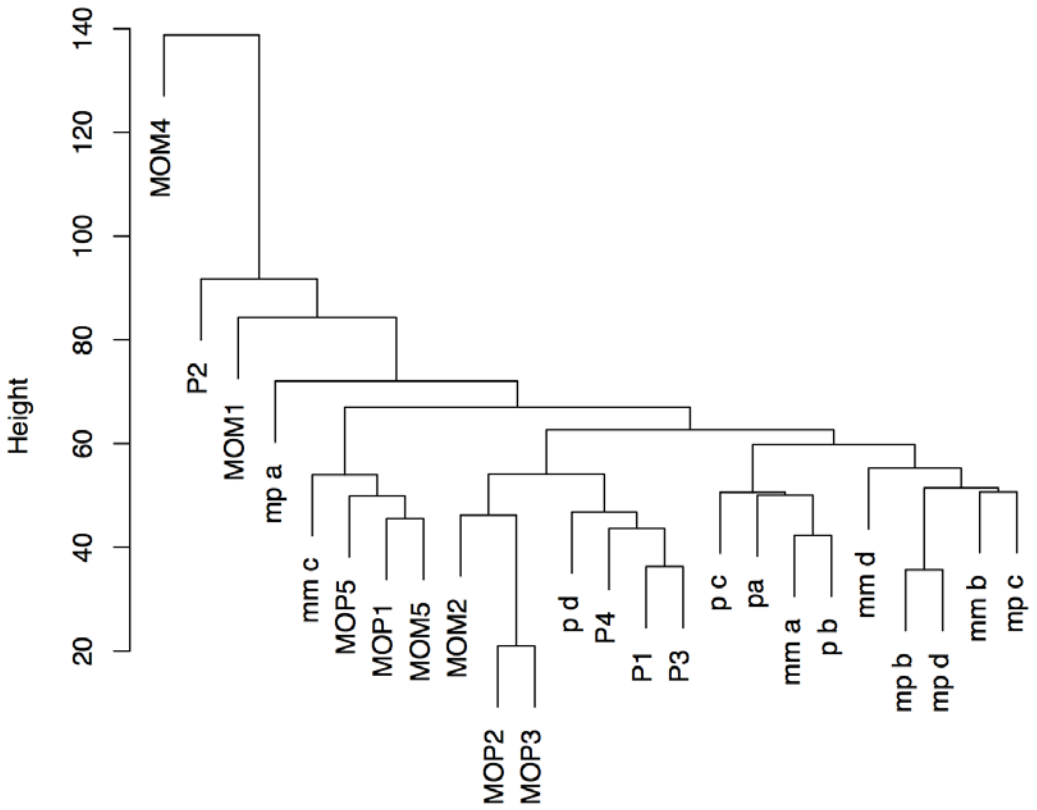

Figure 5.5-1 Sample relations based on 33,777 genes with sd/mean >0.1. P (primary OA phenotype), MOP or mp (metal-on-polyethylene phenotype) and MOM or mm (metal-on-metal phenotype). ALL CAPS + number (biological replicates in the first microarray experiment), lowercase + letters a to d (biological replicates in the second microarray experiment)

Since this approach, whilst being reasonable to avoid a high false positive rate, did not yield any genes for further examination, it was decided to apply an absolute fold-change (FC) cut-off >1.5 with a q-value of 0.2 across all 3 phenotypes. 56 genes were identified which fulfilled these criteria across the 3 phenotypes (Figure 5.5-2)

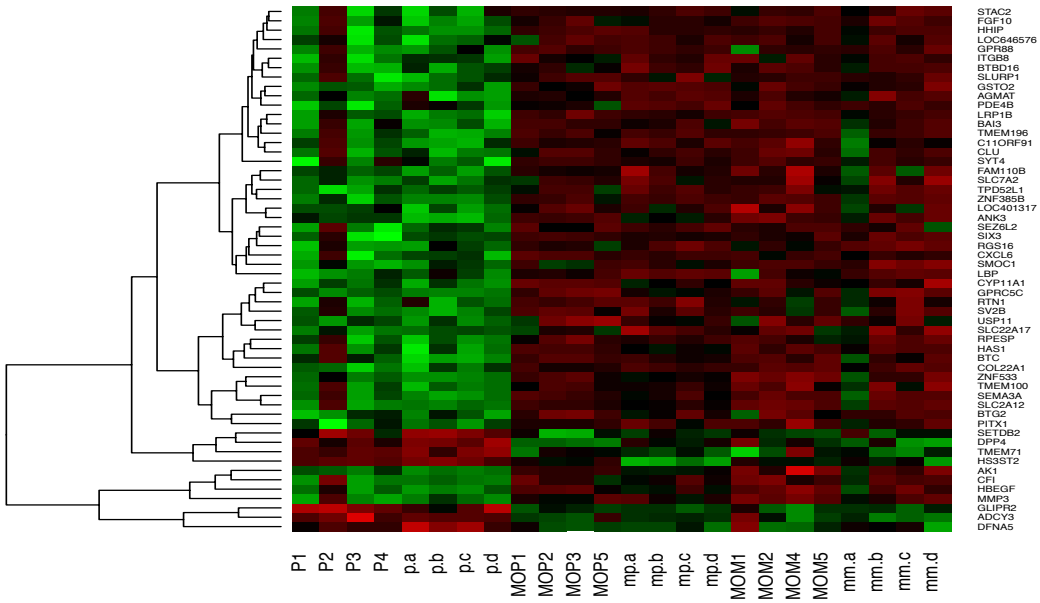

Figure 5.5-2 Heatmap of supervised hierarchical clustering by phenotype of 56 genes differentially expressed between 3 phenotypes (FC>1.5, q=0.2); primary osteoarthritis (p or P), MoP THR revision for aseptic loosening (MOP or mp) and MoM THR revision for ARMD (MoM or mm). Columns represent individual samples, rows represent relative expression values where green represents over-expression, red represents under-expression and black represents no change.

At this level MoP and MoM phenotypes demonstrate very similar patterns of relative gene expression in contrast to Primary OA phenotype. However, 56 genes is a small sample size for which to perform pathway analysis and so mathematical constraints

were redefined such that genes differentially expressed were selected using ANOVA, with a p value, based on permutations, <0.05. Genes were filtered for a fold change ≥2 in at least one condition. Hierarchical clustering was done using Euclidian distance. This approach yielded 130 transcripts (Figure 5.5-3)

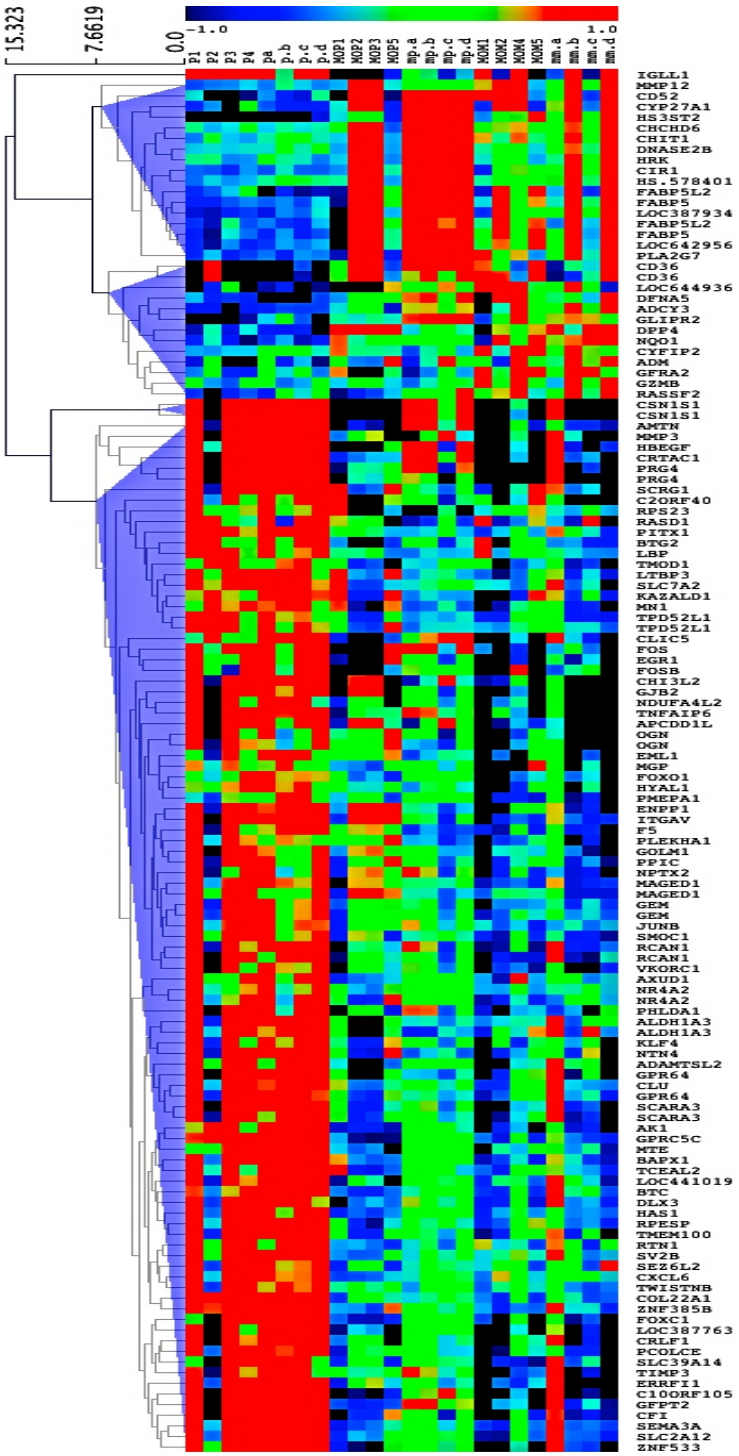

Figure 5.5-3 Heatmap of supervised hierarchical clustering by phenotype of 130 genes differentially expressed between 3 phenotypes, $p<0.05$ and $FC>2$. Phenotypes are denoted by primary osteoarthritis (p or P), MoP THR revision for aseptic loosening (MOP or mp) and MoM THR revision for ARMD (MoM or mm). Columns represent individual samples; rows represent relative expression values scaled according to the colour key at the top of the diagram.

For network generation, the 130 transcripts and Log ratio values were uploaded into IPA application. Each identifier was mapped to its corresponding object in the Ingenuity® Knowledge Base. All molecules were overlaid onto a global molecular network developed from information contained in the Ingenuity® Knowledge Base. Networks of network eligible molecules were then algorithmically generated based on their connectivity.

The functional analysis identified the biological functions and/or diseases that were most significantly associated to the data set. The 130 transcripts were associated with biological functions and/or diseases in the Ingenuity® Knowledge Base. The analysis of the networks identified the biological functions and/or diseases that were most significant to the molecules in the network. For every instance, right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that network is due to chance.

Canonical pathway (CP) analysis identified the pathways from the IPA library of canonical pathways that were most significantly associated to the data set. The significance of the association between the data set and the canonical pathway was measured in 2 ways[159]: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. 2) Right Tailed Fisher's exact test was used to

calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone (, Table 5.5-2, Table 5.5-3).

Table 5.5-1 Associated pathways of the differentially expressed genes (MoP vs. MoM phenotypes). Fifteen pathways in total were identified as associated with the differentially expressed genes using Ingenuity Canonical Pathway analysis. The ratio column is the proportion of the differentially expressed genes divided by the total number of genes associated with a pathway.

Ingenuity Canonical Pathway	p value	Ratio	Molecules
Inhibition of Matrix Metalloproteases	0.0004***	0.05	TIMP3,MMP12
Leukocyte Extravasation Signalling	0.0100**	0.01	TIMP3,MMP12
Oncostatin M Signalling	0.0280*	0.03	TIMP3
Primary Immunodeficiency Signalling	0.0398*	0.02	IGLL1/IGLL5
Glioma Invasiveness Signalling	0.0436*	0.02	TIMP3
GDNF Family Ligand-Receptor Interactions	0.0562	0.01	GFRA2
Bladder Cancer Signalling	0.0660	0.01	MMP12
HIF1 Signalling	0.0776	0.01	MMP12
Rac Signalling	0.0776	0.01	CYFIP2
IL-6 Signalling	0.0870	0.01	TNFAIP6
Mitochondrial Dysfunction	0.1250	0.01	NDUFA4L2
Granulocyte Adhesion and Diapedesis	0.1300	0.01	MMP12
Agranulocyte Adhesion and Diapedesis	0.1380	0.01	MMP12
Actin Cytoskeleton Signalling	0.1570	0.00	CYFIP2
Colorectal Cancer Metastasis Signalling	0.1690	0.00	MMP12

The p-value associated with a pathway analysis is a measure of the likelihood that the association between a set of focus genes in the experiment and a given process or pathway is due to random chance. The smaller the p-value the less likely that the association is random and the more significant the association.

In this method, the p-value for a given process annotation is calculated by considering (1) the number of focus genes that participate in that process and (2) the total number of genes that are known to be associated with that process in the selected reference set. The more focus genes involved, the more likely the association is not due to random chance, and thus the more significant the p-value. Similarly, the larger the total number of genes known to be associated with the process, the greater the likelihood that a association is due to random chance, and the p-value accordingly becomes less significant. In short, the p-value identifies statistically significant over-representation of focus genes in a given process. Over-represented functional or pathway processes are processes which have more focus genes than expected by chance (right-tailed)

Table 5.5-2 Associated pathways of the differentially expressed genes (MoM vs. Primary OA phenotypes). 36 pathways in total were identified as associated with the differentially expressed genes using Ingenuity Canonical Pathway analysis. The ratio column is the proportion of the differentially expressed genes divided by the total number of genes associated with a pathway.

Ingenuity Canonical Pathways	p value	Ratio	Molecules
ErbB Signaling	0.0007762	0.0465	FOS,FOXO1,BTC,HBEGF
Atherosclerosis Signaling	0.0029512	0.0325	MMP3,CD36,PLA2G7,CLU
Neuregulin Signaling	0.0087096	0.0341	BTC,HBEGF,ERRFI1
Oncostatin M Signaling	0.0114815	0.0588	TIMP3,MMP3
CDK5 Signaling	0.0120226	0.0303	FOSB,EGR1,ADCY3
Role of Tissue Factor in Cancer	0.0158489	0.0273	EGR1,ITGAV,HBEGF
FXR/RXR Activation	0.0234423	0.0236	FOXO1,CYP27A1,CLU
GNRH Signaling	0.0239883	0.0233	FOS,EGR1,ADCY3
UDP-N-acetyl-D-glucosamine Biosynthesis II	0.0281838	0.167	GFPT2
CXCR4 Signaling	0.0371535	0.0197	FOS,EGR1,ADCY3
Endothelin-1 Signaling	0.0501187	0.0174	FOS,ADCY3,PLA2G7
Granulocyte Adhesion and Diapedesis	0.0537032	0.0169	MMP3,MMP12,CXCL6
Role of IL-17A in Psoriasis	0.060256	0.0769	CXCL6
Bile Acid Biosynthesis, Neutral Pathway	0.060256	0.0769	CYP27A1
Pyrimidine Deoxyribonucleotides De Novo Biosynthesis	0.1004616	0.0455	AK1
IL-6 Signaling	0.1071519	0.0172	FOS,TNFAIP6
IL-17A Signaling in Gastric Cells	0.1135011	0.04	FOS
LXR/RXR Activation	0.1148154	0.0165	CD36,CLU
Pyrimidine Ribonucleotides Interconversion	0.121899	0.037	AK1
TNFR2 Signaling	0.1303167	0.0345	FOS
Pyrimidine Ribonucleotides De Novo Biosynthesis	0.1303167	0.0345	AK1
IL-12 Signaling and Production in Macrophages	0.1370882	0.0148	FOS,CLU
Aryl Hydrocarbon Receptor Signaling	0.1455459	0.0143	FOS,NQO1
Complement System	0.1468926	0.0303	CFI
Inhibition of Angiogenesis by TSP1	0.151008	0.0294	CD36
Cell Cycle Regulation by BTG Family Proteins	0.1552387	0.0286	BTG2
IL-17A Signaling in Fibroblasts	0.1552387	0.0286	FOS
Hepatic Cholestasis	0.18281	0.0123	CYP27A1,ADCY3
iNOS Signaling	0.1909853	0.0227	FOS
Role of NFAT in Regulation of the Immune Response	0.1981527	0.0117	RCAN1,FOS
RAR Activation	0.2070141	0.0114	FOS,ADCY3
IL-10 Signaling	0.2792544	0.0147	FOS
LPS/IL-1 Mediated Inhibition of RXR Function	0.2831392	0.00913	HS3ST2,FABP5
LPS-stimulated MAPK Signaling	0.2964831	0.0137	FOS
Toll-like Receptor Signaling	0.2999163	0.0135	FOS
Acute Phase Response Signaling	0.5584702	0.00592	FOS

Table 5.5-3 Associated pathways of the differentially expressed genes (MoP vs. Primary OA phenotypes). 149 pathways in total were identified as associated with the differentially expressed genes using Ingenuity Canonical Pathway analysis. The 34 statistically significant processes ($p<0.05$) are displayed above. The ratio column is the proportion of the differentially expressed genes divided by the total number of genes associated with a pathway.

Ingenuity Canonical Pathways	p value	Ratio	Molecules
Atherosclerosis Signaling	0.00081283	0.0325	MMP3,CD36,PLA2G7,CLU
ErbB Signaling	0.0030903	0.0349	FOS,BTC,HBEGF
Neuregulin Signaling	0.00331131	0.0341	BTC,HBEGF,ERRFI1
LPS/IL-1 Mediated Inhibition of RXR Function	0.00645654	0.0183	ALDH1A3,HS3ST2,FABP5,LBP
LXR/RXR Activation	0.00812831	0.0248	CD36,LBP,CLU
iNOS Signaling	0.00977237	0.0455	FOS,LBP
UDP-N-acetyl-D-glucosamine Biosynthesis II	0.01995262	0.167	GFPT2
IL-10 Signaling	0.02238721	0.0294	FOS,LBP
LPS-stimulated MAPK Signaling	0.02570396	0.0274	FOS,LBP
Toll-like Receptor Signaling	0.02630268	0.027	FOS,LBP
Role of IL-17A in Psoriasis	0.04365158	0.0769	CXCL6
Bile Acid Biosynthesis, Neutral Pathway	0.04365158	0.0769	CYP27A1
Histamine Degradation	0.04365158	0.0769	ALDH1A3
CDK5 Signaling	0.04466836	0.0202	FOSB,EGR1
Fatty Acid Î±-oxidation	0.05248075	0.0625	ALDH1A3
Oxidative Ethanol Degradation III	0.05248075	0.0625	ALDH1A3
Role of Tissue Factor in Cancer	0.05370318	0.0182	EGR1,HBEGF

5.5.2 Verification of Microarray using TLDA

Genes for TLDA validation of the microarray were selected by assessing the top 36 genes in terms of fold change (FC) >2 and with a significant difference ($p<0.05$) on ANOVA. After normalisation to Top1 the TLDA data was clustered to form a heatmap using Cluster 3.0 (Eisen) and Treeview software.

Qualitatively there is a mirroring of the pattern of expression between the heatmaps obtained from the microarray and the TLDA. Close associated between the expression patterns are seen between MoP and MoM phenotypes which are clearly different from the expression pattern associated with the primary OA phenotype in concordance with the microarray. Whilst overall the microarray and TLDA demonstrate an agreement in pattern of gene expression, a 2-way ANOVA comparing absolute fold change in expression values between microarray data and TLDA data demonstrated 7 genes out of 36 which had a significantly different level of expression; CHIT1, MMP12, APCDD1L, CSN1S1, TIMP3, CRTAC1 and OGN (see Tables in Appendix G), however this must be interpreted with caution since the microarray data is a qualitative estimation of gene expression whereas the TLDA is a wholly quantitative method. The direction of change in expression for individual genes was consistent between array and TLDA.

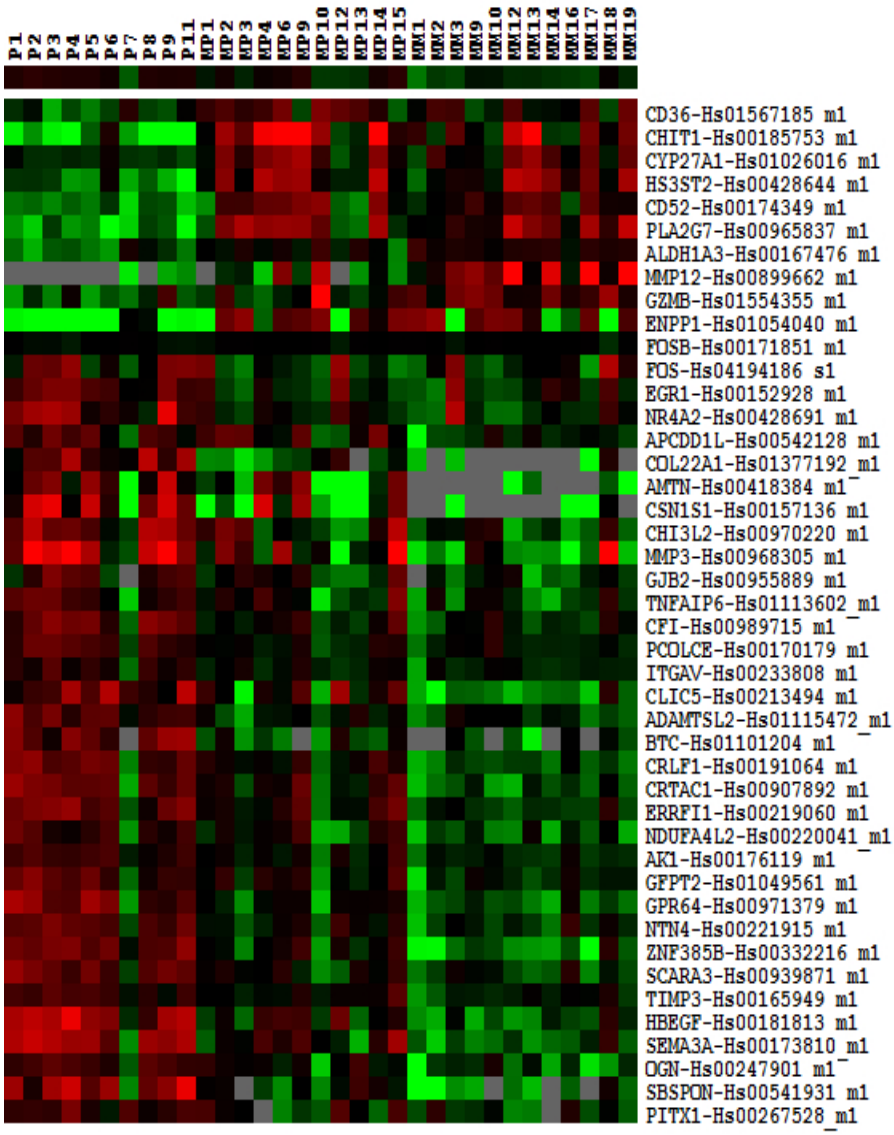

Figure 5.5-4 Heatmap displaying differential gene expression across 3 phenotypes via TLDA. Hierarchical clustering is by phenotype. Red represents relative over-expression, green represents relative under-expression and black indicates no change. For gene functions and 2-way ANOVA data please see Appendix.

5.6 Discussion

This is the first study to comprehensively investigate gene expression changes in periprosthetic tissue associated with revisions due to aseptic loosening of a MoP implant and ARMD from MoM THRs.

Extraction of RNA of sufficient quantity and quality for microarray analysis from either fresh or frozen ARMD tissue which has undergone extensive inflammatory and necrotic change is highly technically challenging and involved significant time and resources to develop a reliable method. Extraction of high quality and quantity RNA from fresh or frozen tissue associated with osteolytic lesions was also difficult; this may be due to the fact that some patients with long-term osteolysis may also have a degree of necrosis present. Other groups performing gene expression profiling of osteolytic lesions around THRs[160] have found similar issues even with immediate flash freezing to prevent RNA degradation. In retrospect examining tissue at the watershed between tissue affected by ARMD and healthy tissue may have provided a better insight into the active biological processes propagating ARMD, but would have significantly affected the sampling methodology since type of tissue being compared would not have been consistent. Further optimisation of tissue quality may be achieved by interventions such as the avoidance of cutting diathermy when obtaining samples to minimise thermal necrosis of tissue and immediate snap freezing in liquid nitrogen to prevent RNA degradation.

Of the fifteen pathways that were associated with the differentially expressed genes between the MoP and MoM phenotypes, no more than two significant differentially expressed genes per pathway were identified. This implies that overall there are no

signature pathways in which genes are up or down-regulated between these two conditions and as such any differentiation between the contributions of innate macrophage and adaptive T-cell immune responses cannot be made. From these findings no specific inference can be made regarding activation of pathways known or suspected to be important in the pathogenesis of ARMD such as the inflammasome danger signalling pathway, HIF, or TLR4 mediated pathways. However, microarray experiments do not account for any post-transcriptional modification, and under-representation of a given pathway in any dataset is not indicative that flux through that pathway is increased. Factors such as post-translational modification or control-coefficients for each step in a pathway are not taken into account and these may have a significant impact on the how the overall biological activity of a pathway is represented.

In the comparison of MoM and MoP phenotypes, where there is significant expression in a given pathway, typically this involves only one or two genes (**Table 5.5-1**). The most frequently involved genes common to the highlighted pathways are MMP12 and TIMP3. The relative levels and patterns of expression of both these genes in the microarray were found to be replicated in the TLDA validation. The matrix metalloproteinases and their endogenous regulators, the tissue metalloproteinases (TIMPs) are responsible for the physiological remodelling of the extracellular matrix. TIMP3 is a secreted protein which is tightly bound to the extracellular matrix and has been shown to be able to induce apoptosis through ligand-independent activation of death receptor signalling and blockade of survival pathways[161]. TIMP3 is under-expressed in MoM compared to MoP, perhaps reflecting the more aggressive nature of destruction in ARMD where extensive tissue damage may have already occurred by the time of revision surgery. The gene encoding MMP12 gives rise to an enzyme which

is known to degrade elastin and is moderately over-expressed in osteolytic periprosthetic tissue[162]. MMP12 expression is relatively even across MoP and MoM phenotypes which may suggest some commonality between the processes of osteolysis and ARMD in terms of the activation of periprosthetic osteolytic cascades giving rise to bone resorption and implant failure. It is not possible on the basis of these results to make a clear assertion if the expression of a given gene or set of genes within any given pathway makes ARMD or periprosthetic osteolysis more likely.

Individual genes which could be of significance have been highlighted as showing significant differential expression between MoM and MoP phenotypes and may represent avenues for further research. For example gene expression of PRG4 (proteoglycan 4 or lubricin) which plays a role in joint lubrication, synovial homeostasis and tendon gliding[163] [164]is consistently overexpressed in MoM phenotypes (Figure 5.6-1). Further investigation may reveal if over-expression this gene may contribute to the formation of the fluid-filled cystic masses associated commonly seen in ARMD.

Figure 5.6-1 Heatmap demonstrating relative overexpression of PGR4 in MoM phenotypes in comparison to primary OA and MoP phenotypes

Whilst the pattern of relative gene expression across MoM and MoP phenotypes appears to be in general agreement, there is some suggestion that there are sets of genes and pathways which differentiate the two when compared to the control primary OA phenotype. Using canonical pathway analysis to compare the MoM phenotype with primary OA phenotype (Table 5.5-2) there are ten significant pathways with at least 3 focus genes per pathway found. In the comparison of MoP

phenotype with primary OA phenotype there are 4 significant pathways with at least focus genes per pathway (Table 5.5-3). The high differential gene expression along with the higher number of focus molecules identified within these pathways has identified a starting point for further *in silico* modelling to examine any potential interaction which might provide a basis for a more focused series of experiments to examine gene expression using qRT-PCR and further our understanding of cellular mechanisms in ARMD or in aseptic loosening and periprosthetic osteolysis.

One of the limitations of the microarray approach is that it is only apt to detect large scale changes in levels of gene expression and so even taking into account an imperfect correspondence between RNA and protein level changes, a small change in one protein can be of much more biological significance in a given context than a massive change in another.

The gene expression profile of osteoarthritic, osteolytic, and ARMD tissues includes not only what a single cell type is expressing, but the integral expression of all genes of all cells and cell types present at the moment of harvesting. This is in contrast to *in vitro* studies which benefit from using single cell types and where gene expression profiling can be undertaken and the specific cell responses can be linked more readily. In clinical studies the number of variables (patient factors, implant factors, surgical and technical factors, length of implantation) offers an increased layer of complexity. Using the experimental techniques and analytical methods above we would have been in a good position to confirm any complex interactions discovered in the datasets were actually occurring in the patient at the moment that tissue was extracted. The fact that we have been unable to do so may reflect that tissue was harvested from patients who, irrespective of their phenotype, have an end-stage condition for which surgery has been mandated. Gene expression profiling from tissues at an earlier stage in the

disease process may have yielded more insights, but would have had to have been done via invasive biopsy with little diagnostic or therapeutic benefit to the patient.

6 General Conclusions

The primary objective of this study was to provide evidence to aid in the evaluation of ARMD and its cellular mechanisms in a local cohort of patients with a MoM THR. This has been accomplished. The study has contributed to the understanding of the natural history of ARMD on MAR MRI at up to 11 years post implantation and has provided evidence which supports the validity and accuracy of using blood metal ion levels as a means of surveillance of MoM THR patients. It has also demonstrated that gene expression profiles in periprosthetic tissues from MoP THR revisions for periprosthetic osteolysis and MoM THR revisions for ARMD are similar.

Returning to the original study questions in Chapter 2 we are now able to provide some answers and have an overall greater understanding of the biology and natural history of ARMD in the context of MoM THR.

1. **What is the natural history of ARMD using MARS MRI?**

   The minimal frequency of MR imaging required to adequately screen populations of relatively asymptomatic patients with MOM THR was not known because the natural history of the disease was not understood.

   For those patients whose first MRI was classified using the Anderson criteria as stage A or "normal postoperative appearances", 9.5% developed ARMD. This occurred at 7 to 11 years after the initial operation. For those patients

with ARMD of any grade, on any MR, 15% deteriorated on a subsequent MR. Nineteen percent of patients staged C1 were reclassified as normal on a subsequent MR at 5 to 7 years. This either reflects a reduced specificity for MR in detecting mild ARMD or true resolution of the disease

This study did not include a substantial number of patients in whom their MOM THR failed and were revised early, and so the conclusion is that for this cohort, ARMD appears to develop in the early post-operative phase. Those patients with severe disease present early on. Those with mild disease (C1) are often stable for many years, and only a small proportion of those with normal MRIs will develop ARMD but when they do it is usually 7 to 10 years post implantation. Our conclusion was that annual assessment of asymptomatic patients with MOM THR with MAR MRI, as recommended by the MHRA, would seem to be an adequate frequency for screening, but our evidence does not necessarily apply to all types of prostheses. Depending on prosthesis type, a patient with a normal post operative MRI may have a 10% chance of developing ARMD at 7 to 11 years after surgery.

Further study to conclusively establish a correlation between severity of changes on MAR MRI staging, blood metal ion levels, and clinical outcomes is required to develop a robust and economical surveillance method for patients with a MoM hip arthroplasty.

2. **What is the relevance of blood and urine metal ion levels as a predictor of disease activity and does diurnal variation exist in cobalt and chromium ion levels in patients with a MoM THR?**

Measurement of metal ion levels in either in whole blood, serum, or plasma remains a useful tool as part of the long-term monitoring of patients with a MoM THR.

Levels of plasma Co demonstrated no evidence of diurnal variation hence it may be a more reliable marker than plasma Cr for ARMD. This raises the question as to whether it is necessary or economical to measure both ions in the course of normal practice.

Where a diurnal variation in levels of plasma Cr was demonstrated, although statistically significant, it was found to be within the normal acceptable tolerances for trace metal analysis and as such is clinically irrelevant. Previous literature does indicate an exercise related cobalt rise with large diameter MoM bearings, but in our study we have demonstrated that in patients with a normal level of day-to-day activity there appears to be no discernable rise in plasma metal ion levels. The time of day at which a sample of blood for Co or Cr analysis is taken is clinically irrelevant. Further studies will be needed to validate these findings for systems in where more particulate debris is generated secondary to wear such as is seen in large diameter implants.

Urine levels of Co and Cr are more prone to fluctuation across the course of the day and may be affected by renal impairment and by an individual's level of hydration, making them an unsuitable method for monitoring.

3. **Is there a difference in the activation of biological cascades between patients being revised for periprosthetic osteolysis arising from a MoP THR versus patients with a MoM THR being revised for ARMD?**

Gene expression in joints affected by ARMD, periprosthetic osteolysis, and osteoarthritis can be reliably measured by microarray experiment. Our findings of the microarray were validated using TLDA which suggests that the method is robust. Significant technical challenges in terms of RNA quality control were overcome in order to achieve this.

Overall there is a great degree of similarity between osteolysis and ARMD in terms of gene expression. No definitive biological pathways were identified that significantly differentiated the two pathologies using canonical pathway analysis. This confirms that ARMD does share common pathways in the inflammatory response to particulate wear debris induced by secreted proinflammatory and osteoclastogenic cytokines and the periprosthetic osteolytic cascades governed by actions of MMPs. Genes of biological importance in pathways suspected in the aetiology of ARMD (HIF, TLR4, Inflammasome danger signalling) may be only moderately differentially expressed and so not readily identified using the mathematical constraints applied in this microarray experiment. Furthermore factors such as post-translational modification or control-coefficients for each step in a pathway are not taken into account in a microarray experiment and these may have a

significant impact on the how the overall biological activity of a pathway is represented.

Further research in this area may well be better served by further reduction of mathematical constraints when examining the microarray data and creation of an appropriately selected set of genes of interest for analysis by a qRT-PCR method which requires less RNA to deliver a result.

This work has added to the body of knowledge concerning ARMD, its natural history on a cellular and radiological level, and has provided a contribution to the evidence base which informs us as the optimum strategy for monitoring these patients before they develop catastrophic complications. MoM devices now comprise less than 1% of all hip arthroplasties performed in the UK and MoM THR has now largely been confined to history having taught us important lessons on implant design, implantation, regulation, and engineering. Periprosthetic osteolysis and aseptic loosening remains the most common reason for implant failure and need for revision arthroplasty. These studies have provided a potential starting point for further investigation of biological cascades involved in periprosthetic osteolysis using microarray techniques.

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Appendix A: Funding Grants

Grant Application Form

Full Proposal

Section 1: General Information		
Name of applicant	Mr Darren Ebreo	
Name of researcher (if recruited)		
Name of supervisor(s)	Professor S Donell (Academic Supervisor) Professor I Clark (Laboratory Supervisor) Mr JF Nolan (Clinical Supervisor)	
Name of Institution(s)	The Norfolk & Norwich University Hospital, University of East Anglia	
Proposed research title	Role of Metal Ions in Metal on Metal Hip Arthroplasty	
Study stream	☐ PhD ☒ MD ☐ Post-Doc ☐ Clinical Fellowship	
Duration (years)	☐ 1 ☒ 2 ☐ 3 ☐ Other	
	If other, please specify:	
Proposed start date	01/08/2012	
Cost breakdown	University fees	**£ 1,866 per annum** ☒ Home/EU ☐ International **TOTAL = £3,732**
	Salary	**£ 19,260 (20% FTE over 2 years)**
	Consumables	• General consumables, biochemical, plasticware • Molecular Biology reagents, RNA/DNA prep, enzymes • Histology reagents, microscopy • Cell culture reagents, Taqman probes, Master mix **TOTAL = £ 10,241**
	Technical Support	**£ 8,840.50 per annum** Laboratory technical support 30% FTE of Technician **TOTAL = £17,681**
	Conferences	• **£ 195** – British Hip Society meeting • **£ 210** – British Orthopaedic Research Society meeting **TOTAL = £ 405**
	Feasibility Study	**n = 12 PATIENTS with Ultima TPS THR** • **4 asymptomatic with normal MRI** • **4 symptomatic with normal MRI**

	Feasibility Study (cont'd)	• **4 symptomatic with abnormal MRI awaiting revision** • **£ 763.68 (£15.91 per patient per day x 4 days)** – Clinical Trials Research Unit overheads **<u>For each patient initial visit:</u>** • **£ 200** = 2 hourly blood cobalt and chromium analysis per patient (£50 per sample inclusive of consumables) • **£ 150** = morning, afternoon, and evening cobalt and chromium urine analysis (£50 per sample inclusive of consumables) • **£ 5.15** = food and beverage allowance • **£ 15** = travel costs • **£ 40.56** = fixed nursing costs per patient (cannulation, drawing blood, collection of urine specimens) **INITIAL VISIT = £4,928.52 (£ 410.71 per patient)** **SUBSEQUENT VISITS x 3 (costs per patient)** • **£ 50** = cobalt and chromium blood analysis inclusive of consumables (taken at either 8am, 12pm, or 5pm) • **£ 15** = travel costs **TOTAL = £ 2,340** **TOTAL = 8,032.20**
	Other	• **£ 74.63** – SPSS statistics software annual student licence • **£ 190.80** – Endnote X5 bibliographic software • **£234.00** – Introduction to Statistics using SPSS **TOTAL = £ 573.46**
	TOTAL	**£ 59,924.33 over 2 years.** • £ 40,664.66 (Total Research Costs over 2 years) • £ 19,260 (Salary at 20% FTE over 2 years)

Does your research involve testing on animals?	☐ Yes ☒ No If Yes, has ethical approval been obtained?
What is your current position within your institution?	Orthopaedic Research Fellow
Please indicate your speciality and research interests?	Trauma & Orthopaedic Surgery, Primary & Revision Hip Arthroplasty
Are there any other funders involved in this project?	☐ Yes ☒ No If Yes, please provide details:
Is there a conflict of interest that we should know about?	☐ Yes ☒ No If Yes, please specify:
The principal investigator (or the respective institution) is obliged to agree to the terms and conditions of Orthopaedic Research UK standard academic contract and return a signed copy of the contract, no later than 3 months after stage 4. Contracts received after this period will not be approved for funding and subsequently the proposals will be treated as void. ☒ Agree Date: 10/12/11	

Section 2: Goal of the Study
Please indicate research aims, objectives and deliverables *(strictly maximum 250 words)*

The proposed MD project will review the literature concerning radiological evaluation of adverse reactions to metal hip debris (ARMD), the cellular mechanisms behind this, and will investigate:

1. Radiographic evaluation of ARMD using MARS sequence MRI scans
2. Use of biomarkers including levels of cobalt and chromium species in blood and urine as a predictor of severity of disease.
3. The use of Liquid Chromatography-Tandem Mass Spectrometry to demonstrate biomarkers in bone and tissue (in collaboration with Prof Bill Fraser, UEA)
4. Diurnal variation in cobalt and chromium ion release.
5. Use of clinical outcome measures in establishment of a diagnostic algorithm to aid decision making for revision hip arthroplasty
6. The presence or absence of variation in immune cells and cytokine expression within the hip joint at revision surgery for metallosis and compare this with archived tissue.
7. The cellular and cytokine/chemokine/growth factor expression patterns in patients undergoing secondary surgery with symptomatic and asymptomatic metal on metal hip bearings.

Section 3: Background to Investigation
Relevant literature and work already conducted by the applicant related to the topic of research interest *(strictly maximum 1000 words)*

BACKGROUND:

Our institution is actively following a cohort of 545 patients who have had a 28mm head hybrid metal on metal (MoM) total hip replacement (Ultima TPS, Depuy) in which there has been an unexpectedly high failure rate [91]. We have experienced high failure rates in a cohort of 96 patients with large diameter MoM total hip replacements (ASR, Depuy). Both implants have been recalled by the manufacturer and are the subjects of Medicines and Healthcare Regulatory Authority (MHRA) Alerts. We are a major centre for primary and revision hip surgery with significant experience of revisions for metallosis in a variety of implants.

Despite initial promise of decreased wear rates and increasing suitability for use in younger more active patients, concerns remain about potential adverse consequences of MoM bearing surfaces in terms of aseptic loosening and pain secondary to soft tissue reaction to metal debris.

Elevated circulating levels of metal ions following MoM THR or resurfacing arthroplasty are not uncommon [125] [126] [127] with metal ion levels reaching a steady state approximately one year following implantation [128]. The biological pathways leading to adverse periprosthetic soft tissue reactions associated with metal debris have yet to be conclusively demonstrated, but may represent a delayed type IV (ALVAL) hypersensitivity reaction with subsequent effect on local cell populations in susceptible individuals, giving rise to painful soft tissue mass or pseudotumour as well as osteolysis and loosening [68].

It is suggested that soft tissue reactions may be the result of increased wear that is directly correlated with elevation of cobalt (Co) and chromium (Cr) *in vivo* where the presence of extensive necrosis and macrophage infiltrate implies a metal debris cytotoxicity [165].

The Clark laboratory at UEA has extensive experience of gene expression profiling in orthopaedic tissues [166] [167] including a recent comparison of tissues from primary THR and revision surgery (osteolysis vs. well-fixed). Tissue is stored in RNA Later immediately post-surgery, prior to downstream RNA purification and analysis using qRT-PCR. Professor Bill Fraser has expertise in the measurement of circulating biomarkers using LC-MS/MS including markers of bone metabolism.

WORK ALREADY UNDERTAKEN:

I have updated a database of 650 Ultima TPS hips implanted in 545 patients between February 1997 and March 2005. We revised 135 (21%) in 82 males and 53 females between July 2001 and November 2011 at a mean of 6.2 years from primary surgery. Mean age at revision was 58 years. We continue to revise Ultima TPS hips as well as other MoM bearings based upon clinical presentation supplemented by evaluation of

metal ion levels and radiological findings using Metal Artefact Reduction Sequence (MARS) MRI scan.

MARS MRI, developed at our institution reduces artefact and increase the conspicuity of soft tissue. A grading system has been developed which demonstrates great interobserver reliability. It is the imaging modality of choice in the failing MoM THR.

There remains no clear consensus on the role of metal ion levels in MoM disease.

The focus of work to date has been to investigate associations between blood metal ion levels and abnormal MARS MRI.

Metal ion levels have been measured in 191 patients (35%) with serial metal ion levels measured in a number of patients. Analysis of this cohort has demonstrated that mean levels of cobalt (Co) were significantly higher (p=0.003)* in bilateral THRs (12.6ppb) than unilateral THRs (9.21ppb). Mean levels of chromium (Cr) were not significantly raised (p=0.09) in bilateral THRs (5.3ppb) than unilateral THRs (4.22ppb). In unilateral THRs there were higher mean values for females for both Co and Cr metal ion levels. The mean value of Cr for females was 4.95ppb versus 3.81ppb for males (p=0.008)*. The mean value for Co ions for females was 9.69ppb, and for males 8.66ppb (p=0.254).

There was no statistical difference between metal ion levels based on gender for bilateral THRs.

We have performed MARS MRI scan on a total of 403 hips (62%) of which 169 (42%) have had at least one abnormal scan.

Blood metal ion levels are higher in bilateral Ultima MoM THRs and in those with renal impairment. Levels of metal ions are higher in females with unilateral implants. No association between abnormal MRI and metal ion levels was demonstrated.

Timing and indications for revision remain challenging clinical problems since the natural history of metallosis remains unknown. Radiological appearances of MoM disease may not correlate with symptom severity thus revision surgery may be indicated in asymptomatic patients with advanced disease. The benefit of serial MRI in monitoring disease progression is uncertain.

We performed a retrospective study describing the medium to long-term follow up of patients with MOM THR with MARS MRI. Inclusion criteria were symptomatic patients with MOM THRs who had not undergone revision surgery and who had at least two serial MARS MRI examinations.

Patients were identified from a database and all MRI examinations were reported by an experienced observer and classified as: A (normal), B (Infection), C1 (Mild MOM disease), C2 (Moderate disease), and C3 (Severe disease).

A total of 223 MRIs were performed on 103 THRs between 2002 and 2007 (range 2-4 MRI per THR). The median time from surgery to first MRI was 5.2 years (Interquartile range: 3.9 to 6.7). The time between surgery and all MRIs ranged from 0.8 to 13.4 years. Grades for all MRIs were: A 59% (131), B 2% (5), C1 15% (34), C2 16% (35) and C3 8% (18).

64% (66) of first MRIs were normal. On a second MRI of 59 normal hips, 93% (55) remained normal while 4 cases (7%) progressed one each to grade B, C1, C2 and C3. 10

of these normal hips were scanned a third time, 70% remained normal. Three normal hips remained normal at fourth MRI.

On sequential scans of 103 hips, only 14% showed disease progression.

In this subset of our cohort most patients with MOM THR who do not undergo early revision have normal MRI examinations. Progression from normal to abnormal, or from mild to more severe MOM disease is uncommon and takes place over several years.

Section 4: Plan of Investigation

Materials & methods, research timetable *(strictly maximum 1000 words)*

WORK PACKAGE 1 (WP1): Literature Review & Database Analysis

I have performed a literature review concerning MoM THR development, postulated theories on cellular mechanisms in adverse reactions to metal debris, and the putative mechanisms behind MoM THR and hip resurfacing failure.

Ongoing surveillance of our cohort of 650 Ultima TPS THRs and 96 ASR THRs will be undertaken throughout the course of the MD. Data in terms of metal ion levels, radiological MRI gradings, patient outcome measures (Oxford scores and EQ-5D scores), and revisions undertaken will be continually added and analysed for publication and discussion at scientific meetings. Initial work has been submitted for consideration for presentation at British Hip Society meeting in March 2012.

WP1 DELIVERABLES:

- Knowledge of cellular mechanisms in periprosthetic osteolysis and metal on metal disease to inform laboratory studies
- Identification of patients for recruitment into further studies

WORK PACKAGE 2 (WP2): Laboratory Studies

The study will seek ethical approval, and take place at Norfolk and Norwich University Hospital (NNUH), with the laboratory work being carried out at UEA School of Biological Sciences. Laboratory studies will commence August 2012 and continue for a period of one year.

Informed consent will be obtained from patients attending specialist MoM follow up clinics. Patients in the Ultima TPS follow-up group will have a patient information sheet posted 2 to 3 weeks before their routine follow-up clinic. Those willing to participate will then consented and the sample taken.

Collection of samples of capsule, femoral and acetabular membrane, muscle and bone from primary joints, and revision hip arthroplasty in 28mm head (Ultima) and large head (ASR) MoM articulations for adverse reactions to metal debris will allow the investigator to compare and contrast cytokine and cellular components. Tissue will be stored in RNA Later immediately post-surgery, prior to downstream RNA purification and analysis using qRT-PCR. We have already established permission and infrastructure for a tissue bank of samples collected from MoM revisions, revision

surgery of other bearing surfaces and primary THRs to act as comparators for our work.

WP2 DELIVERABLE:

- Knowledge of cellular and cytokine/chemokine/growth factor expression patterns in patients undergoing secondary surgery with symptomatic and asymptomatic metal on metal hip bearings.

WORK PACKAGE 3 (WP3): Feasibility study into Diurnal Variation in Metal Ion Levels

There is no evidence in the literature examining the diurnal variation in metal ion levels detectable in blood and urine. There is no consensus of opinion as to whether an isolated elevated metal ion level is indicative or predictive of a pathological state, or if circulating levels of metal ion levels fluctuate with activity in a fashion analogous to blood glucose levels. I wish to establish a feasibility study recruiting participants from our cohort of 650 Ultima TPS hips with symptomatic hips with a normal MRI, symptomatic hip with abnormal MRI and asymptomatic hips with a normal MRI. These patients will be seen in our research trials unit and have blood and urine specimens taken at regular intervals from 8am to 5pm for cobalt and chromium levels. I would also wish to examine if blood levels of these ions significantly alter following a period of time. Ethical approval will need to be sought and recruitment is planned to start in the second quarter of 2012 with completion of data collection by the end of 2012.

WP3 DELIVERABLE:

- Knowledge of variance of blood ion level and how this correlates with MRI grading in order to inform MHRA guidance.

WORK PACKAGE 4 (WP4): Retrospective Analysis of Serial MARS MRI scans in MoM Hips

A retrospective study of MARS MRI examinations. The early failure and revision rates for MOM THR are well described. The prognosis for patients who do not need early revision is unknown. The study will describe the medium to long-term follow up of patients with MOM THR with MRI.

We shall analyse our cohort of 650 Ultima TPS hips with the inclusion criteria of symptomatic patients with MOM THRs who had not undergone revision surgery and who had at least two serial MARS MRI examinations. Patients will be identified from a database and all MR examinations are to be reported by an experienced observer and classified according to a grading system developed in Norwich as: A (normal), B (Infection), C1 (Mild MOM disease), C2 (Moderate disease), and C3 (Severe disease).

WP4 DELIVERABLE:

- Determining the value of MRI scan in monitoring patients with MoM hips which may inform future MHRA guidance

WORK PACKAGE 5 (WP5): Prospective study correlating intraoperative findings at MoM hip revision surgery with pre-operative MRI grading

A review of the literature has established that there is no validated system for macroscopic classification of metal on metal disease observed intraoperatively. We regularly undertake revisions of MoM hips in our cohort of 650 Ultima TPS as well as revising a significant number of other MoM implant types. We would seek to develop a simple grading tool for assessment of extent of soft tissue destruction, bony destruction, implant loosening, and macroscopic evidence of wear and corrosion in order to establish if severity of disease found intraoperatively correlates to predicted severity of disease on MARS MRI scan.

WP5 DELIVERABLE:

- Reliable classification system for grading severity of MoM disease

Task Name	Start Date	End Date	Duration
WP1 - Literature Review	**08/03/11**	**01/04/12**	**111**
Maintenance of MoM Databases	08/03/11	08/07/13	526
WP2 - Laboratory Studies	**01/04/12**	**02/05/14**	**546**
Ethical Approvals	01/04/12	03/28/12	61
Learning Laboratory Techniques & Protocols	01/11/12	08/01/12	146
Patient Recruitment & Tissue Collection	04/04/12	10/31/12	151
Definitive Laboratory Studies (qRT PCR & gene profiling)	08/01/12	07/31/13	261
Data Analysis	01/30/13	10/30/13	196
Writing Up of Results	10/02/13	02/05/14	91
WP3 - Diurnal Variation Study	**11/09/11**	**02/04/13**	**324**
Protocol Writing	12/01/11	01/04/12	25
Ethical Approvals	01/04/12	03/09/12	48
Subject Recruitment	03/11/12	09/28/12	146
Data Collection	04/23/12	12/03/12	161
Data Analysis	11/05/12	01/07/13	46
Writing Up of Results	01/07/13	02/04/13	21
WP4 - Retrospective MRI study	**11/09/11**	**04/23/12**	**119**
Data Analysis of Existing Database	11/09/11	03/07/12	86
Writing Up of Results	03/05/12	04/23/12	36
WP5 - Prospective Study: MRI grade correlation with operative findings	11/01/11	06/29/12	174
Protocol Writing	11/01/11	01/04/12	47
Data Collection	01/04/12	06/29/12	128
Data Analysis	07/04/12	08/31/12	43
Writing Up of Results	08/08/12	10/03/12	41

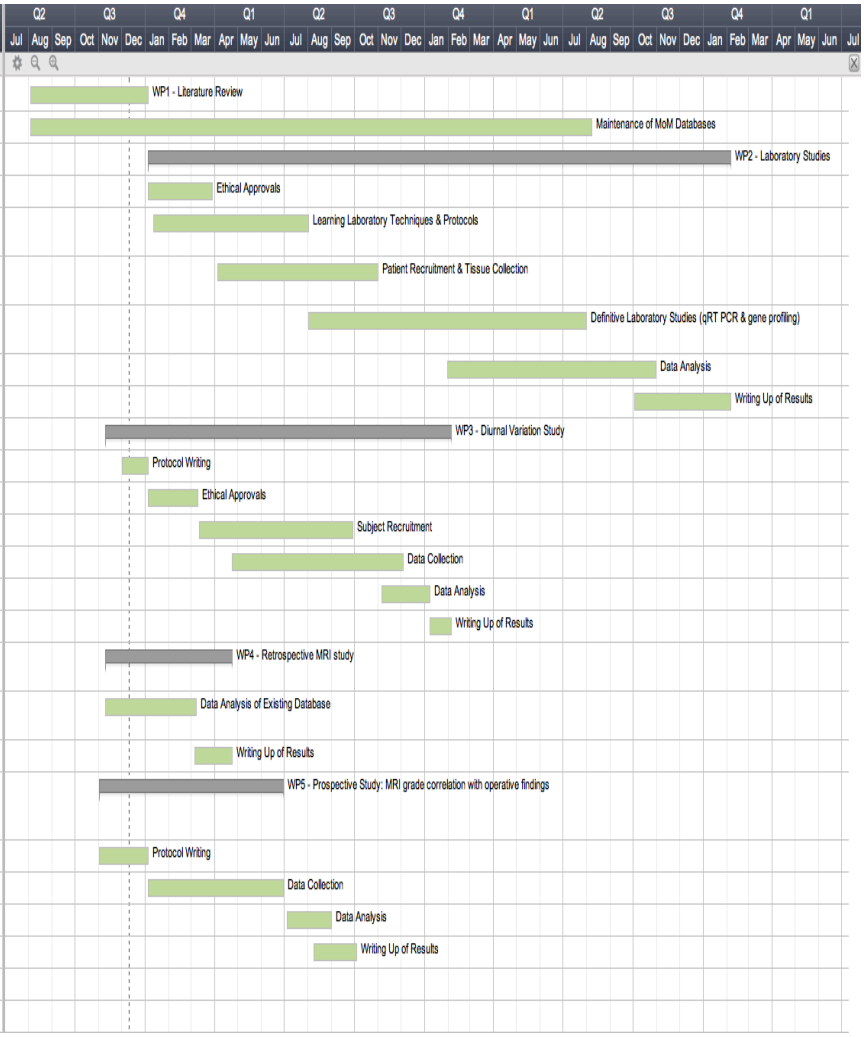

FIG 1. Gantt Chart: Research timetable for proposed MD "Role of Metal Ions in Metal on Metal Hip Arthroplasty" from August 2011 to February 2014 (date format mm:dd:yy)

Section 5: Facilities & Resources

Research/clinical/technical support, laboratory space, equipment and additional funding if any *(strictly maximum 250 words)*

Norwich Medical School provides a complete set of facilities and an educational programme for doctoral students including an 800,00 volume library of books and journals, a large range of electronic resources including major bibliographic and citation databases as well as full text journal archives.

The Institute of Orthopaedics at the Norfolk & Norwich University Hospital is a dedicated research facility with ample office space, full time research practitioners, and administrative support. We have IT support for multiple workstations running patient electronic medical records, clinical information systems, high-resolution Picture Archiving and Communication software, as well as standard office and statistical packages.

The NNUH in conjunction with UEA has a Clinical Research and Trials Unit staffed by full time research nurses. A dedicated 3T research MRI scanner is available which offers increased image clarity at higher speeds especially beneficial in demonstrating musculoskeletal pathology.

We have a fully approved Human Tissue Bank (08/H0304/85 Cambridgeshire 1 REC) at the Norwich Research Park in which to store tissue for this and future work.

The Clark laboratory is housed in the Biomedical Research Centre, a state-of-the-art facility for molecular research. All expertise and equipment is available to support the gene expression studies outlined above. There are excellent mammalian cell culture facilities and a wide range of multi-user equipment including ABI 7500 and 7700 Real-Time PCR machines. There is a Bioimaging facility with modern confocal, time-lapse and fluorescent microscopes, a fermentation suite for bulk culture and preparation of recombinant proteins, and a newly opened Disease Modelling Unit.

Section 6: Research Impact

Possible beneficial outcome, relevance to orthopaedics & the musculoskeletal system *(strictly maximum 250 words)*

A cohort of 545 patients had an Ultima MoM THR inserted up to 2004 when it became apparent that there was an unacceptable early failure rate. Failure may be due to high levels of corrosion from the implant stem. The reasons for this are unclear. Recent work indicates that unlike in large diameter head implants it is not the MoM bearing which is responsible. It could be galvanic corrosion, and work in Leeds has noted different levels of acidity from different bone cements. Greater knowledge has furthered the complexity.

Norwich was the dominant site for this implant and the problems have not been reported elsewhere. Furthermore the unusual feature is that a significant proportion

of these patients have pain but normal X-rays. New MRI techniques developed in Norwich allow us to see abnormalities otherwise not visible.

Analysis of cells, tissue, bodily fluids, and imaging may identify patients at risk, from those who are not. Although our population is unique, it transpires that all MoM implants risk the problems of metallosis. This research will provide valuable insights into the aetiology of metallosis in the Ultima cohort and in MoM hip articulations in general and enable us to offer those at risk a surgical option for revision before significant irreversible soft tissue damage is established.

Section 7: References

List of publications referred to by the applicant in this proposal *(strictly maximum 250 words)*

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Section 8: Suggested Reviewers

Names and contact details of 4 qualified reviewers who are able to critically review this subject. They must not be affiliated with your proposal or your institution.

Name of Reviewer	Institution	Speciality	E-mail Address
John Skinner	Royal National Orthopaedic Hospital	Hip arthroplasty including Metal on Metal revision surgery	john.skinner@ucl.ac.uk
Peter Kay	Wrightington, Wigan & Leigh NHS Trust	Hip arthroplasty including Metal on Metal revision surgery	peter.kay@wwl.nhs.uk
Phillip Johnston	Addenbrookes Hospital	Molecular biology & Orthopaedic Surgery	phillip.johnston@addenbrookes.nhs.uk
David Young	Newcastle University	Gene profiling	d.a.young@newcastle.ac.uk

Section 9: Layman's Summary

Provide lay answers to the following questions *(strictly maximum 500 words)*

1. **What is the basic problem that you are trying to address?**

Metal-on-metal (MOM) bearings in total hip replacement (THR) aim to improve clinical outcomes and survival by reducing formation of large polyethylene wear particles and the osteolysis (bone loss) that is associated with them. MoM implants include alloys of cobalt- chromium-molybdenum (CoCrMo) for their hardness. Wear particles generated by MOM hips in simulators are smaller than particles generated by metal-on-polyethylene (MoP) articulations, but MOM hips release at least three times more cobalt and chromium ions than MoP THRs. Wear particles and metallic by-products are readily distributed throughout the body by the blood and lymphatic vessels. Much attention is being given to the systemic distribution of metal particles and ions and potential risks of cytotoxicity, hypersensitivity responses, immune dysfunction and genotoxicity.

A cohort of 545 patients had an Ultima MoM THR inserted up to 2004 when it became apparent that there was an unacceptable early failure rate. Failure may be due to high levels of corrosion from the implant stem. The reasons for this are unclear. Recent work indicates that, unusually, it is not the metal-on-metal bearing. It could be galvanic corrosion, and work in Leeds has noted different levels of acidity from different bone cements. Greater knowledge has furthered the complexity.

Norwich was the dominant site for this implant and the problems have not been reported elsewhere. Furthermore the unusual feature was that a third had pain, but

normal X-rays. New MRI techniques developed in Norwich allow us to see abnormalities otherwise not visible.

Analysis of cells, tissue, bodily fluids, and imaging may identify patients at risk, from those who are not. Although our population is unique, it transpires that all MoM implants risk the problems of metallosis.

2. **What is the estimated incidence of this particular problem in the general population?**

The National Institute for Health and Clinical Excellence states a 10% revision rate for THR at 10 years. Analysis of our database of 650 Ultima THRs reveals that between July 2001 and November 2011 we revised 135 hips (21%).

3. **What are the aims and objectives of this research study?**

Timing of revision surgery remains a problem due to the unknown natural history of metallosis. We aim to characterise how these implants affect cells in surrounding tissues and which patients may have an increased susceptibility to metal reactions.

How we monitor disease using scans and metal ion levels along with symptom evaluation may help predict which patients will go on to develop severe disease and hence benefit from earlier revision.

4. **Is this research going to solve the problem?**

Yes. We will be better able to inform patients of this disease and offer them appropriate surgical options that will enhance their quality of life.

5. **What are the long-term benefits to patients with this problem?**

In patients with Ultima TPS hips and any with another type of MoM implant we may find those factors that make them at risk of a reaction and allow for closer follow-up. If they have had a reaction we may be able to tell them why.

6. **Provide a brief background about yourself and your interests.**

I am an orthopaedic trainee who has completed core surgical training. I am undertaking an MD to consolidate previous basic science research experience and apply this to future clinical practice. I wish to develop an interest in hip arthroplasty and pelvic trauma surgery.

Dear Mr Darren Ebreo, **Our New Ref: 489**
Title: Role of Metal Ions in Metal on Metal Hip Arthroplasty

Thank you for submitting your proposal to Orthopaedic Research UK (ORUK). In this call a total of 80 research proposals were received by ORUK and based on the budget that we were allocated for the 3rd call 2011, we were in a position to offer funding to the top 5 proposals which received the highest votes by our internal review committee.

I am pleased to inform you that the Trustees have agreed supporting the above **MD** research project by awarding **£59,924.33** over **24 months**.

A standard academic agreement will be sent to the **University of East Anglia** for countersigning. Please let me know the name of the person who deals with your research grants at the University.

On behalf of ORUK, I would like to take this opportunity to congratulate you and your team for achieving this grant award.

Kind regards,

Dr Arash Angadji
Project Manager

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www.oruk.org

orthopaedic research UK

ACADEMIC GRANT AGREEMENT

This Agreement is made on **24th April 2012**

Between

1. **Orthopaedic Research UK**, a registered charity number 111657 with its office at Furlong House, 10A Chandos Street, London W1G 9DQ; and

2. **University of East Anglia** whose administrative address is Norwich Research Park, Norwich NR4 7TJ **(hereinafter referred to as the "University")**

which are also referred to individually herein as **"a Party"** or collectively as **"the Parties"**.

It is hereby agreed that:

1. Orthopaedic Research UK shall contribute towards the cost of research at the University in the field of:

   1.1. **"Role of Metal Ions in Metal on Metal Hip Arthroplasty"**

   1.2. to be undertaken by **Mr Darren Ebreo**, a **MD Researcher**

   1.3. under the supervision of **Professor Ian Clark** and **Professor Simon Donell** in **Norfolk & Norwich University Hospital**, failing which the Research shall be supervised by such other member of the University's staff as is agreed between the Parties.

2. The effective period of the funding to which this Agreement relates shall be **2 years** from **1st August 2012**.

3. The University shall sign the contract by **1st July 2012**. If the signed contract is not returned by the mentioned date, Orthopaedic Research UK will reserve the right to terminate this contract and ask the University to resubmit the research proposal.

4. The University shall commence research by **1st August 2012**. If the research project has not commenced by the mentioned date, Orthopaedic Research UK will reserve the right to terminate this contract and ask the University to resubmit the research proposal at the appropriate call.

5. Orthopaedic Research UK's total contribution to the cost of the Research shall be for the firm fixed sum of **£59,924.33** which shall be payable in **three** equal annual instalments following receipt of official invoices from the University. The first such invoice may be raised on **1st August 2012** (subject to clause 3); the second and final invoices may be raised on **1st August 2013** and **1st August 2014**. Each invoice shall be for the sum of **£19,974.77** . Orthopaedic Research UK's contribution to the cost of the Research shall only be used for paying costs associated with the Research except with the prior written consent of Orthopaedic Research UK.

6. Orthopaedic Research UK shall settle all valid invoices submitted by the University under this Agreement within 30 working days of their submission.

7. The University shall provide Orthopaedic Research UK with brief interim reports on the Research at **4 month** intervals and a copy of a full final report on conclusion of the Research. There will also be progress meetings between the Parties at mutually agreed intervals, and at any other time reasonably requested by Orthopaedic Research UK. In the event that the University fails to comply with these reporting provisions, Orthopaedic Research UK shall be entitled to withhold up to 20% of payments due under this Agreement until the University has so complied.

Appendix B: Bone & Joint Publication on Serial MARS MRI Imaging

THE BONE & JOINT JOURNAL

■ HIP

Serial magnetic resonance imaging of metal-on-metal total hip replacements

FOLLOW-UP OF A COHORT OF 28 MM ULTIMA TPS THRS

D. Ebreo,
P. J. Bell,
H. Arshad,
S. T. Donell,
A. Toms,
J. F. Nolan

From Norfolk & Norwich University Hospital, Norwich, United Kingdom

Metal artefact reduction (MAR) MRI is now widely considered to be the standard for imaging metal-on-metal (MoM) hip implants. The Medicines and Healthcare Products Regulatory Agency (MHRA) has recommended cross-sectional imaging for all patients with symptomatic MoM bearings. This paper describes the natural history of MoM disease in a 28 mm MoM total hip replacement (THR) using MAR MRI. Inclusion criteria were patients with MoM THRs who had not been revised and had at least two serial MAR MRI scans. All examinations were reported by an experienced observer and classified as A (normal), B (infection) or C1–C3 (mild, moderate, severe MoM-related abnormalities). Between 2002 and 2011 a total of 239 MRIs were performed on 80 patients (two to four scans per THR); 63 initial MRIs (61%) were normal. On subsequent MRIs, six initially normal scans (9.5%) showed progression to a disease state; 15 (15%) of 103 THRs with sequential scans demonstrated worsening disease on subsequent imaging.

Most patients with a MoM THR who do not undergo early revision have normal MRI scans. Late progression (from normal to abnormal, or from mild to more severe MoM disease) is not common and takes place over several years.

Cite this article: *Bone Joint J* 2013;95-B:1035–9.

Magnetic resonance imaging (MRI) is now widely used to assess pathological changes associated with metal-on-metal (MoM) total hip replacements (THRs).[1-4] Metal artefact reduction (MAR) MRI allows assessment of the extent of soft-tissue pathology pre-operatively and the planning of revision surgery in cases where, for instance, neurovascular structures are at risk. MAR MRI may also demonstrate pathology outside the hip that may account for symptoms. An advantage over ultrasound is the potential to diagnose smaller, deeper foci of adverse reactions to metal debris (ARMD),[5] but it may miss lesions in the zone of the metal artefact, which ultrasound may detect more readily.

MoM bearings offer the theoretical advantages of decreased wear and increased functional outcome for younger and more active patients, but have been associated with the development of sterile inflammatory masses.[6-9] These may include soft-tissue destruction with poorer outcomes following revision surgery.[10] These lesions have also been described as aseptic lymphocytic vasculitis-associated lesions (ALVAL),[12] adverse local tissue reactions (ALTR)[13] or pseudotumours.[6] The mechanism by which they arise is uncertain, but may be associated with wear at the bearing surface,[11,14] taper junction wear[15-17] and corrosion.[18,19]

MAR MRI has the potential to facilitate a classification system for ARMD that characterises and provides comparison of soft-tissue and bony pathology over time. Various systems of scoring the severity of ARMD have been described,[20-22] but only that of Anderson et al[20] has been shown to have interobserver reliability. This system assesses not only the size and cystic or solid nature of a lesion, but also any involvement of muscle, tendon and bone.

Although pain is a presenting symptom in a poorly functioning THR, silent soft-tissue pathology is common[23] and may occur with an asymptomatic well-functioning MoM THR.[5,24] The Medicines and Healthcare Products Regulatory Authority (MHRA) has issued renewed guidance recommending cross-sectional imaging for all symptomatic MoM THRs.[25] Annual clinical follow-up is recommended as well as measurement of metal ion levels in the blood. There is no recommendation as to the optimal timing of an initial investigation with cross-sectional imaging, nor at what intervals patients should be scanned as part of the surveillance. This cross-sectional study aimed to describe the natural history of MoM ARMD using MAR MRI in patients whose initial scans were normal or showed low-grade ARMD.

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■ P. J. Bell, PhD, Research Practitioner
■ H. Arshad, MBBChir, MA, FRCS(Tr & Orth), Specialist Registrar
■ S. T. Donell, MBBS, MD, FRCS(Orth), Honorary Professor
■ A. Toms, PhD, FRCR, Consultant Radiologist
■ J. F. Nolan, MBBS, FRCS(Orth), Consultant Orthopaedic Surgeon
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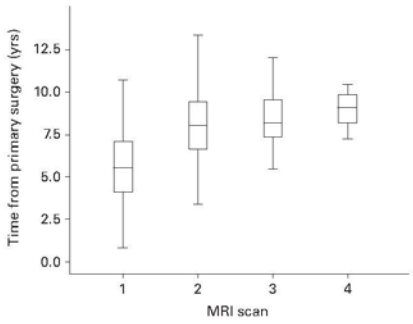

Fig. 1

Box and whisker plot showing the time from primary surgery until the first, second, third and fourth metal-artefact-reduction (MAR) MRI scans. The boxes represent the median and interquartile range and the whiskers denote the range of data.

Patients and Methods

Cases were identified from an original cohort of 545 patients (650 THRs) who underwent 28 mm hybrid MoM THR (Ultima TPS; DePuy, Leeds, United Kingdom) between May 1997 and August 2004 performed by three surgeons (including the senior author, JFN), as previously reported.[26] Inclusion criteria were the availability of at least two MRI scans and no revision surgery before any scans included in this review. A total of 37 THRs in 38 patients were excluded from the study as they had been revised without a prior MAR MRI. Also, 81 THRs in 72 patients were revised after a single MAR MRI scan and were therefore excluded. Of the original cohort, 80 patients were found to fulfil the inclusion criteria, 35 of whom were men (44%). Of these patients, 57 (71%) had unilateral THRs and the rest were bilateral, resulting in a total of 103 THRs.

Radiological analysis. MRI was undertaken using MAR sequences with a 1.5T MR scanner (Siemens Symphony; Siemens, Ehrlingen, Germany). The MAR sequence used the following specification: coronal T_1-weighted (T1W) turbo spin-echo, echo time (TE) 23 ms, repetition time (TR) 669 ms and short τ inversion recovery (STIR); TE 37 ms, TR 3840 ms, axial T1W (TE 23 ms, TR 534 ms) and T_2-weighted (T2W) turbo spin-echo (TE 69 ms, TR 5600 ms) of the whole pelvis and a sagittal T2W turbo spin-echo of the hip (TE 69 ms, TR 2900 ms). The section thickness used was 5 mm, with a 340 × 340 mm field of view, matrix size up to 448 × 336 and pixel bandwidth 620 MHz.

All scans were reported and graded by the same radiologist (AT). Scans were classified as A (Normal), B (Infection), C1 (Mild MoM ARMD), C2 (Moderate) or C3 (Severe) according to the classification of Anderson et al.[20]

Table I. Total numbers of patients undergoing revision surgery per grade of metal-artefact-reduction (MAR) MRI scan

Grade	Hips (n, %)	Revisions (n, %)
A	140 (59)	4 (2.86)
B	6 (2)	1 (16.7)
C1	33 (14)	1 (3.0)
C2	41 (17)	4 (9.76)
C3	19 (8)	7 (36.84)
Total	239	17

Statistical analysis. Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) PASW Statistics version 18.0 (SPSS Inc., Chicago, Illinois). Parametric tests were used for normal data and non-parametric tests for non-normally distributed data, and significance was set at $p \leq 0.05$.

Results

The mean age of the 80 patients was 57.9 years (25 to 69). There were 35 men (mean age 58.1 years (25 to 68)) and 45 women (mean age 58.2 years (29 to 69)). A total of 17 patients (17 THRs) who had already had at least two scans underwent revision at a mean of seven years (2 to 11) post-operatively.

Between 2002 and 2011 a total of 239 MRIs were performed on 103 THRs (two to four per THR). All patients had two MRIs, 29 had a third and four a fourth (Fig. 1).

When the grades of all 239 MRIs were considered together, there was a significant increase in grade as the post-operative time increased, with a Spearman's ρ of 0.235 ($p < 0.001$). The first scan was then considered in isolation, analysing the relationship between all first scans and the time from the operation, and again a positive correlation existed between increasing post-operative time and increasing severity of grade (Spearman's ρ 0.257 ($p < 0.009$) for 103 first scans).

Overall, 140 of 239 MAR MRI scans were normal (Table I), and of these, 63 had a normal (Grade A) first scan. Of those patients with a normal initial scan, only four advanced in grade between the first and second scans. In total, six patients (9.5%) went on to develop MR changes consistent with ARMD. In those whose stage became more advanced, they did so between seven and 11 years post-operatively, with most developing mild (C1) or moderate (C2) changes (Fig. 2). Only 15 (15%) of 103 THRs with sequential scans showed worsening of disease on subsequent imaging.

One patient with a normal initial scan progressed to a Grade B appearance suggestive of infection, which was confirmed on aspiration and a revision was subsequently undertaken. Four scans were initially reported as suggestive of infection. Repeat scans in one patient led to downgrading to a normal appearance owing to spontaneous

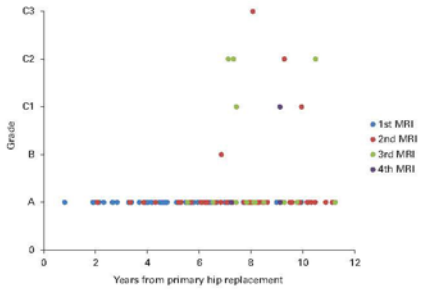

Fig. 2

Scatter plot showing the change in metal-artefact-reduction (MAR) MRI grading with subsequent scans in patients whose initial scan is normal (Grade A).

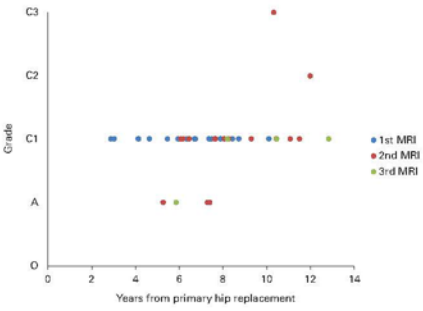

Fig. 3

Scatter plot showing the change in metal-artefact-reduction (MAR) MRI grading with subsequent scans in patients whose initial scan showed Grade C1 (mild disease).

regression of a peri-prosthetic collection. For the remaining three patients, no evidence of infection was found on blood tests. Subsequent scans indicated the presence of ARMD. Two patients developed radiological appearances of Grade C3 ARMD and underwent revision. One patient developed asymptomatic C2 ARMD and is under surveillance. On the initial scan 16 hips had the appearances of mild ARMD (Grade C1), but between five and seven years post-operatively three were downgraded to a normal Grade A appearance on repeat scans. A further two patients progressed to higher grades between 11 and 12 years post-operatively (Fig. 3). The relationships between the grade and revision are illustrated in Figure 4.

Discussion

Of the cohort of 103 THRs, 63 (61%) had a normal initial scan, and of these, six (9.5%) went on to require revision surgery. Where the initial scans were normal, progressive changes occurred unpredictably between seven and 11 years after operation and at a rate of 11%. Of the four cases initially reported as suggestive of infection, three progressed to more severe stages on subsequent scans, with the remainder being downgraded to normal on a follow-up scan. Previous evidence suggests that differentiating a small degree of ARMD from infection may not be reliable using MRI alone,[20] and therefore greater emphasis should be placed on other clinical and serological markers of infection in these patients.

The natural history of patients with a C1 staging is unknown. Most patients who were initially classified as having mild (C1) disease retained their original classification on a second scan at a mean of one year and nine months later. Only one patient went on to develop severe (C3) disease requiring revision surgery. From our observations it is not a certainty that the disease will progress, and indeed progression does not appear to be commonplace and where it does occur the rate of change is unclear. Where regression to a normal post-operative appearance has been observed on MRI, the mechanism for this phenomenon can at present only be speculated upon, and is a potential avenue for further research. Most THRs in this study had normal MRI scans and did not have any progression of disease. Where there is a change in grade it is more likely to indicate progression, and in turn this significantly increases with time since surgery.

Our results suggest that there may be a latent period of many years prior to any evidence of disease being detectable radiologically. There appears to be a bimodal distribution, where a larger proportion of patients present early with severe disease and are revised and a smaller proportion present later, between seven and 11 years, with mild or moderate changes.

The MHRA guidelines now recommend imaging in the form of MAR MRI or ultrasound for small-diameter MOM THRs in symptomatic patients, or 'if concern exists for the cohort'.[26] We have previously reported a 13.8% failure rate of our entire series of Ultima TPS THRs at five years.[27] This has now increased to 20.3%, with a mean time to revision of 5.9 years (1 to 14). The MHRA guidance has emphasised a follow-up period of five years or more for small-diameter MOM THRs. The guidance refers to the presence of symptoms as suggestive of the need for longer follow-up. However, it is our experience that radiological evidence of disease can be demonstrated in asymptomatic patients in both small-[4] and large-diameter MoM THRs.[23]

The clinical picture in the management of these patients has been evolving. Significant disease can occur in the absence of

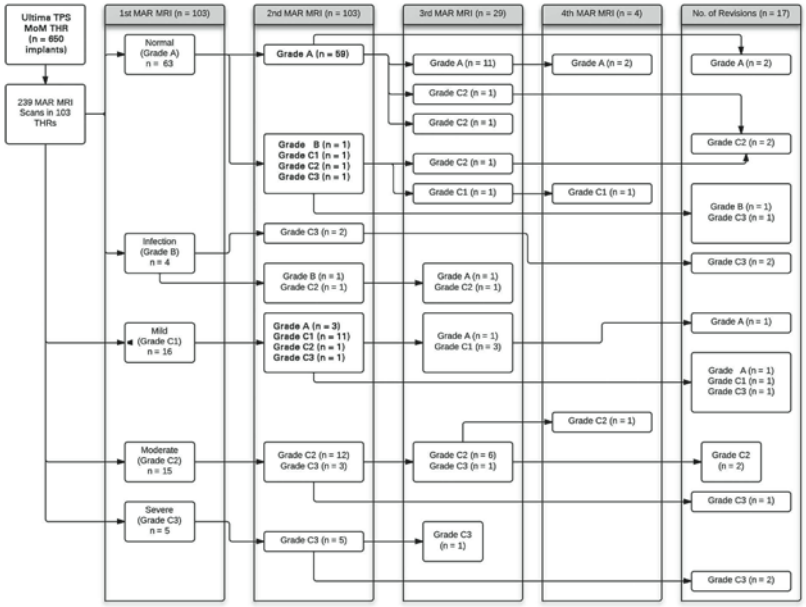

Fig. 4

Flow diagram illustrating changes in the metal-artefact-reduction (MAR) MRI grade with serial scans and progress to revision surgery.

symptoms.[24,28] Our findings suggest that long-term surveillance of MoM THRs for the life of the implant is essential.

Patients in our cohort are routinely invited for review annually. An MRI scan has been offered to all our patients, although some have declined. No patient has been discharged.

The assessment of these patients is complex and extends beyond the categorisation of imaging, symptoms and serum metal ion levels. In isolation it is difficult, and perhaps not useful, to assign a negative predictive value to a normal MRI scan.

We have an increasing number of patients with more than one scan. It seems likely that patients presenting with a normal scan initially may be safely observed with annual clinical review, and a subsequent scan should there be any change in symptoms. This reflects current practice at our institution.

The study is limited by its retrospective design. There is a selection bias in that we have not included patients who were revised prior to having MAR MRI scans, or those in whom revision was undertaken after a single scan. The cohort of patients with two or more scans may not be representative of the whole, but in practice many cohorts of patients with MoM THRs will include some who have required early revision and some with initially low-grade problems that are observed over time. The timing of the sequential scans has not been at defined intervals according to any given protocol. This leaves us unable to comment on precisely when a normal MRI or a Grade C1 may progress in radiological staging. We have not accounted for variability in symptoms or serum metal ion levels, which are factors that frequently contribute to the referral for MAR MRI scans. Our findings may not be directly applicable to larger-diameter implants, as a small-diameter 28 mm MoM THR has a smaller zone of artefact around the prosthesis. This means that smaller significant lesions are picked up earlier in these patients, and hence may be revised before a second scan is undertaken.

We would recommend follow-up with MAR MRI scans as determined by a detailed clinical assessment at intervals no more frequently than annually. On the basis of the

evidence presented, we are unable to recommend a specific optimal interval between scans. Further study is needed to correlate MRI grading with intra-operative findings, histological staging and patient-reported outcome measures. This would offer a better understanding of the status of patients with normal scans and mild disease.

The authors would like to thank Orthopaedic Research UK and the Gwen Fish Memorial Trust for funding this study.

Although none of the authors has received or will receive benefits for personal or professional use from a commercial party related directly or indirectly to the subject of this article, benefits have been or will be received but will be directed solely to a research fund, foundation, educational institution, or other non-profit organisation with which one or more of the authors are associated.

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Appendix C: Diurnal Variation Study Protocol

Lay Summary

We have a well-defined cohort of patients with an Ultima TPS metal on metal (MoM) total hip replacement (THR) with a catastrophic failure rate for which a series of ethically approved studies have been undertaken.

MoM THR is currently of national concern with the Medicines and Healthcare products Regulatory Agency (MHRA) guidelines recommending scans and measurements of metal levels in blood. However, it is unknown what variation there exists in levels of metals in blood on a daily or monthly basis.

This study aims to establish if there is any variation in levels of metal ions in blood or urine in patients with MoM THRs that are performing well, those that are failing, and those in which the picture is uncertain. We plan to provide data that will inform the MHRA in the recommended surveillance protocol of patients with MoM hip implants.

We plan to establish if there is an additional value in the use of blood measurements over the use of symptom-based questionnaires.

Research Team

Chief Investigator	Prof Simon Donell BSc, MB BS, MD FRCS(Orth)[1] Consultant Orthopaedic Surgeon
Principal Investigator	Mr Darren Ebreo BSc, MB BS, MRCS[1] Research Fellow in Orthopaedic Surgery

Research Team

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Clinical Trials Research Unit	
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Title

Feasibility Study: Diurnal Variation in Metal Ions in MoM THR

Background

The Department of Orthopaedics at the Norfolk & Norwich University Hospital (NNUH) is actively following a cohort of 545 patients who have had a 28mm head hybrid metal on metal (MoM) total hip replacement (Ultima TPS, Depuy) in which there has been an unexpectedly high failure rate [91]. The implant has been recalled by the manufacturer and is the subject of a Medicines and Healthcare products Regulatory Authority (MHRA) alert. The NNUH is a major centre for primary and revision hip surgery with significant experience of revisions for adverse reactions to metal debris (ARMD).

Despite initial promise of decreased wear rates and increasing suitability for use in younger active patients, concerns remain about potential consequences of MoM bearing surfaces in terms of aseptic loosening and pain secondary to soft tissue reaction to metal debris.

Elevated circulating levels of metal ions following MoM THR or resurfacing arthroplasty are not uncommon [125] [126] [127] with metal ion levels reaching a steady state approximately 1 year following implantation [128]. The biological pathways leading to adverse periprosthetic soft tissue reactions associated with metal debris have yet to be demonstrated, but may represent a delayed type IV (ALVAL) hypersensitivity reaction with subsequent effect on local cell populations in susceptible individuals, giving rise to painful soft tissue mass or pseudotumour as well as osteolysis and loosening [68].

Soft tissue reactions may be the result of increased wear that is directly correlated with elevation of cobalt (Co) and chromium (Cr) *in vivo* where the presence of extensive necrosis and macrophage infiltrate implies a metal debris cytotoxicity [165]. Failure may be due to high levels of corrosion from the implant stem. The reasons for this are unclear.

Metal ion levels have been used as a surrogate marker for wear of MoM THRs. The trigger level for Cobalt or Chromium detectable in the blood of a patient with a MoM THR that suggests a poor outcome and need for revision surgery, is unknown. Currently the figure of 7 parts per billion (ppb) suggested by the MHRA is the result

of the opinion of an expert panel informed by a single study(8). This study aims to provide more accurate and useful data as well as fostering genuine patient involvement in the research phase.

Aim

The purpose of the feasibility study is to establish:

1) Scientific Principles:

- If there exists variation in metal ion levels detectable in blood and urine over the course of a day and over longer periods.
- Whether data supporting the existence of diurnal variation of blood and urine metal ion levels exists in this population and if so how variable are the data?
- What further study needs to be undertaken should a variation exist.
- Determination of an appropriate scale for future study dependent on the magnitude of variation observed. Does the measurement of blood or urine metal ion concentration add anything extra to the accuracy of decision making over the use of Oxford Hip Score and EQ5D clinical outcome questionnaires?

2) Patient Acceptability

- Development of a focus group consisting of patients within the Norwich Metal on Metal hip cohort to encourage wider engagement in research activity and disseminate findings.
- Generate appropriate consents and patient information literature for this study.
- Refinement of logistics to inform ahead of a larger study.
- Evaluation of acceptability of multiple sampling of blood and urine.

Materials and Methods

Patients

For this feasibility study 12 patients will be recruited. There are no suitable data in the literature on which to base a sample size calculation.

Inclusion criteria

Patients who have undergone Ultima TPS THRs in Norwich.

Exclusion criteria

- Patients who have undergone Ultima TPS THR in whom the prosthesis has been revised.
- Patients taking medication or supplements known to increase cobalt or chromium excretion
- Patients who are unable to provide informed consent at time of recruitment.

Patient Recruitment

Suitable patients will be identified by the clinical team from a secured prospectively collated database of Ultima TPS MoM THR patients created for governance purposes. Participants will be stratified into the following categories.

- Asymptomatic with normal Metal Artefact Reduction Magnetic Resonance Imaging (MAR MRI) scan; n = 3
- Symptomatic with normal MAR MRI scan; n = 3
- Asymptomatic with abnormal MAR MRI scan; n = 3
- Symptomatic with abnormal MAR MRI scan; n = 3

Patients will be sent an information sheet at least 2 weeks in advance of their scheduled follow-up with their orthopaedic surgeon. At the scheduled follow-up appointment a member of the research team will be available to answer any questions and to invite patients to participate in the study. Patients will also be asked to provide informed consent for storage of a sample of blood and urine in the Human Tissue Bank. No details will be passed to anyone outside of the clinical team or involved in the patient's usual care until the patient has agreed to enter the study. At the first study visit a member of the research team will take informed consent.

Cobalt & Chromium Levels

MoM implants are associated with release of cobalt and chromium particles which are soluble in blood and are excreted in measurable quantities in urine. Analysis is via the technique of Inductively Coupled Plasma Mass Spectrometry. This can only be undertaken in an accredited reference laboratory for trace metal analysis. Samples from the Norfolk & Norwich Hospital are routinely sent to a laboratory in Cardiff which fulfils these obligations.

Informed Consent

Informed consent will be taken by the principal investigator Mr Darren Ebreo. Patients will be consented for blood and urine to be taken for this study and in addition will be consented for donation of blood and urine to be stored in the Human Tissue Bank for future research. Should a patient decline to consent for additional samples for storage in the Human Tissue Bank it does not preclude their inclusion in this study.

Schedule of Participant Visits

Patients will be invited to attend our dedicated Clinical Trials Research Unit on four occasions and be required to donate blood and urine specimens for analysis of Cobalt and Chromium levels at a specialist accredited reference laboratory for trace metal analysis via Inductively Coupled Plasma Mass Spectrometry. Protocols for specimen collection, transport, and analysis are already established. Patients will be reimbursed reasonable transport costs.

1st Visit:

Patients will be asked to complete an Oxford Hip Score and an EQ-5D form. Both of these are validated outcome measurement tools in hip arthroplasty. A medication

history will be taken on the first visit and patients will be asked to keep trial staff informed of any changes to their medication.

Patients will be free to leave the Clinical Trials Research Unit between appointments on this day. Refreshment will be provided.

	Blood	**Urine**
Early Morning (approx. 09:00)	U&Es (8.5ml Gold Vacutainer) Co & Cr (6ml Royal Blue Vacutainer) Tissue Bank (6ml Royal Blue Vacutainer) ***	Urinary Co & Cr
Lunchtime (approx.13:00)	Co & Cr (6ml Royal Blue Vacutainer)	Urinary Co & Cr
Early Evening (approx. 17:00)	Co & Cr (6ml Royal Blue Vacutainer)	Urinary Co & Cr

*** The Tissue Bank Sample is OPTIONAL. Patients will be asked to consent for a separate sample for storage in Human Tissue Bank for future research.

2nd Visit

	Blood	**Urine**
Early Morning (approx. 09:00)	**14.5 mls in total** U&Es (8.5ml Gold Vacutainer) Co & Cr (6ml Royal Blue Vacutainer)	Urinary Co & Cr

3rd Visit

	Blood	Urine
Lunchtime (approx.13:00)	**14.5 mls in total** U&Es (8.5ml Gold Vacutainer) Co & Cr (6ml Royal Blue Vacutainer)	Urinary Co & Cr

4th Visit

	Blood	Urine
Early Evening (approx. 17:00)	**14.5 mls in total** U&Es (8.5ml Gold Vacutainer) Co & Cr (6ml Royal Blue Vacutainer)	Urinary Co & Cr

Primary Endpoints

The study will conclude at the last visit of the last patient and once results of all tests have been completed and verified.

Data analysis

Frequencies and associations will be recorded. Complex statistical analysis is not expected from the data generated. These data will be used to perform a sample size calculation for an extended study using a larger sample.

Timeline

Stage	2013											
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
LREC												
NNUH Research Governance												
Recruitment												
Data Collection												
Data Analysis												
Manuscript Preparation												
Journal Submission												

GANNT chart outlining planned progress of study

Resources

The Clinical Trials and Research Unit at the UEA Campus is fully staffed and equipped to host this study.

Funding

This study is supported by a project grant from Orthopaedics Research UK. The research proposal constitutes part of an MD project which has been funded by a grant Orthopaedics Research UK who have undertaken an independent peer review process prior to the award.

Expertise

Professor Simon Donell and Mr John Nolan are consultant orthopaedic surgeons with expertise in the field of metal on metal hip research. Mr Darren Ebreo is an orthopaedic research trainee with responsibility for managing research projects, recruitment and obtaining informed consent. All have published in peer-reviewed journals and presented at national and international level on aspects of metal on metal hip disease.

Good Clinical Practice in Research

The researchers will adhere to the principles of the World Medical Association Declaration of Helsinki 2004 and the ICH Good Clinical Practice guidelines. All researchers listed in the protocol have up to date formal Good Clinical Practice training.

Patient Confidentiality

Standard operating procedures covering Information Governance are in place within the Trust and both clinical and research teams will adhere to those standards.

Biological samples will be dealt with in accordance with existing standards in clinical governance and standard operating procedures are in place for the processing, transport, analysis, and disposal of biological materials. Materials stored after the study has completed will be entered into the patient's clinical record and will be stored in an ethically approved human tissue bank. No access to these systems is permitted by non-authorised accounts.

Ethical Considerations

Blood aspiration is greater than is usual but total volume taken is significantly less than for blood transfusion donation. If required local anaesthetic gel can be applied to skin prior to venepuncture.

Reasonable transport costs for patients to attend will be met. Refreshments will be provided for participants

Risk

The study team have identified level of risk for this study as follows:

Risk to	**Severity**	**Frequency**
Patients	Mild discomfort	Moderate
	Severe adverse event	Rare
Study	Low	
Trust	Low	

Results

The results of these studies will be disseminated in the form of conference presentations and will be submitted for publication to peer reviewed journals.

Appendix D: Consent Forms

Our Vision
To provide every patient with the care we want for those we love the most

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

University of East Anglia

Department of Trauma and Orthopaedics
Norfolk & Norwich University Hospital
Colney Lane
Norwich
NR4 7UY

CONSENT FORM

Title of project: Diurnal Variation of Metal Levels in Patients with Metal on Metal Hips.

Participant Identification Number for this study: _____

Name of Researcher: _____

	Please initial box
1. I confirm that I have read and understood the information sheet dated February 18th 2013 (Information Sheet Version 5) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2. I confirm that I understand that with each visit a blood sample will be taken. The volume of that sample will be just over 2 teaspoons (11mls) EXCEPT on the very first visit when one more teaspoon of blood (approx. 6mls) may be taken if I consent for storage in a Human Tissue Bank.	☐
3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
4. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the Norfolk & Norwich University NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. The compensation arrangements have been discussed with me.	☐
5. I understand that the blood and urine specimens may be stored for education and research purposes until it is used. This will be anonymous (No name attached)	☐
5. I agree to take part in the study.	☐
6 I agree that my GP will be notified of my participation in the study	☐
7. I would like to be informed of the results of this study.	☐

_____ Name of participant	_____ Date	_____ Signature
_____ Name of person taking consent (If different from researcher)	_____ Date	_____ Signature
_____ Researcher	_____ Date	_____ Signature

When completed: 1 for patient; 1 for research file; 1 medical notes
Consent Form Version 2. (18th February 2013)

Norfolk and Norwich University Hospitals NHS

NHS Foundation Trust

Affix an addressograph label here or complete the following details:

Patient's name……..

Date of birth……

Hospital no. ……..

The Human Tissue Bank

Consent for the collection and storage of human tissue and/or fluids, and/or other materials for research

I agree (Please initial small box) that the following tissue or other material may be used for research, including genetic (DNA and/or RNA) studies and for the possible development of commercial products for the improvement of patient care, from which I would receive no financial benefit:

List samples for research:	

I also agree that (Please initial small boxes, as appropriate):

These samples become the property of the Norfolk & Norwich University Hospitals NHS Foundation Trust ("the Trust")	
The Trust may store these samples in a Tissue Bank	
The Trust may use these samples at its discretion in properly approved local research programmes	
The Trust may pass on these samples to other approved tissue banks and/or companies, which may be in this country or abroad, in properly approved research programmes	Yes / No
Information about my case may be kept on the Tissue Bank database	
Such information may be passed in an anonymous form to persons outside the Trust In connection with research and may be published with any research findings	
I agree that appropriately qualified staff employed by the Trust may review my hospital case notes, as appropriate, for the purposes of research using the donated samples	

I confirm that:

1) I have read and understand the Information Sheet for Patients, Version 10, dated 3 December 2008
2) The issues have been explained to me, and that I have had the opportunity to ask questions.

Signed _____ (Patient) Date _____

I have explained the request for tissue for research purposes and have answered such questions as the patient has asked.

Signed_____ Print name _____ Date _____

Medical / Nursing Practitioner

Continued……

Appendix E: Laboratory Protocols

Initial sample preparation

Suitable for: All soft tissue including:

- Capsule
- Ligamentum teres
- Femoral membrane
- Acetabular membrane
- Muscle

Intraoperative samples must be placed in a sterile universal specimen container by surgeon or scrub nurse to avoid RNAse contamination.

Samples must be kept chilled at 4^{o}C and transported to laboratory.

- The Tissue Lyser is incubated overnight at 4^{o}C
- Incubate cell lysis ball bearings at -80C - either on dry ice or in -80^{o}C freezer for 10-20 minutes.
- Cut tissue into approximately 2-3mm^{2} segments and place into eppendorfs. Do not place more than 0.5g of material per eppendorf
- Add 500 uL and a chilled ballbearing
- Place eppendorf in the cell tissue lyser and set to 50Hz for 20 minutes
- Remove approximately 500uL of the resultant supernatant
- Add 400uL chloroform per 1ml of supernatant recovered
- Vortex for 15 seconds
- Spin at 10,000 rpm for 20 minutes

- Remove the aqueous layer
- Add ethanol 1:1
- Continue with RNEasy kit as per manufacturer's instructions

RNA Extraction

- Incubate at 4°C for 5mins
- For 500ml trizol, 100μL chloroform was added (amount of trizol may vary with sample material)
- Vortexed 15 seconds
- Incubate at 25°C for 2 minutes
- Spin 12,000g for 10 minutes at 4°C
- Recover aqueous phase into RNAse free tubes
- Add 250μL isopropanol (propan-2-ol)
- Vortex 15 seconds
- Spin 12,000g, 10 minutes, 4°C
- Discard supernatant
- Air dry pellet
- Suspend pellet in 20μL DNA free water

OPTIONAL DNAse Treatment

- Add 1μL DNAase 1 (Roche) RNAse free
- Incubate at 37°C for 15 mins
- Heat at 70°C for 10 mins
- Freeze at -80°C until quantification

RNA QUANTIFICATION & QUALITY ASSESSMENT

All work to be done in Taqman® room with nuclease free eppendorf tubes and pipette tips.

- Keep RNA on ice and make sure thoroughly defrosted and mixed prior to use.
- Login to nanodrop computer (password nanodrop)
- Clean nanodrop w/ water prior to use and after each analysis
- Make sure software set to "RNA" on dropdown
- "Blank" the software w/ 1.5μL of nuclease free water
- Analyse 1.5μL of sample on nanodrop

Things to note:

[Nucleic Acid] (ng/μL) – generally the higher the better. Nanodrop is less accurate at >500

260nm/280nm – suspect contamination with values less than 1.8

260nm/230nm – suspect contamination with values less than 1.8

RNA = 260nm

Proteins = 280nm

Organic compounds (e.g. phenols etc) = 230nm

To prepare for RT reaction:

Need to derive volume of sample solution to contain 1μg RNA

i.e. 1/[RNA](ng/μL) from nanodrop reading obtained above

N.B. if this number is less than 2 then will need to dilute by adding 5μL of RNase free water to 5μL of sample solution in a new nuclease free tube and then running it through the nanodrop again.

Then need to derive column "μL up to 9 μL"

i.e. "9 – (1/[RNA](ng/μL))

Working table should looks something like this:

Sample ng/μL μL for 1μg μL up to 9μL

REVERSE TRANSCRIPTION (RT REACTION)

Randomly prime the RNA

- Add calculated volume of water
- Add 2μL of random primers (always to be kept on ice)
- Add calculated volume of RNA (1μg)
- Total volume per tube = 11μL
- Incubate at 70°C for 10mins

N.B. Don't forget to make up a "control" sample which is essentially a duplicate of one of your samples.

RT Reaction (Generation of cDNA)

- Make up a "Master Mix" (n.b. Reagents need to be defrosted and spun before use)
- 4μL 5 x buffer (first strand buffer)
- 2μL DTT (0.1M)

- 1μL dNTPs
- 1μL superscript II (replace w/H2O for the "control") NB always keep this one on ice.
- 1μL RNAsin or Water
- A separate "control" version of the above mix needs to be made for the control specimen
- Add 9μL of the relevant Master Mix to each sample, which should give you a total volume of 20μL
- Incubate at 42°C for 1 hour, then 70°C for 10 mins.
- Store at -20°C (long term – anything over a year), or 4°C (short term for up to a year)

RNA Isolation from Tissue Bound in OCT

Tissue specimens stored for histology purposes are typically snap frozen and bound to cork disks in Optimal Cutting Temperature Compound (OCT). RNAse later and other RNAse prevention treatments are not usually used as these specimens are typically sectioned and Hematoxylin and eosin (H&E) stained. The stock of various tissue samples are catalogued and a variety of specimens meet the requirements for the current research into Metal-on-Metal (MOM) hip replacements that have to be revised due to pain and or loosening.

Below is detailed the methods and optimization steps necessary for RNA extraction from tissue specimens bound in OCT and stored at -80°C.

Methods and Materials for RNA Extraction from Frozen Specimens

Binding tissue to cork

Transverse sections of tissue, approximately 1 cm in length, were cut using sterile and RNAse free scalpels and scissors, then stored on ice. Cool n-hexane (95 %) over dry ice for 20 minutes, then place a cork disk (cant remember the size) into the n-hexane and freeze for 2 minutes. Remove and place a drop of OCT onto the cork then the tissue. Place the sample in the n-hexane for a further 4 minutes until frozen. Remove and cover the sample in OCT and freeze again. Store in steralins at -80 ^{o}C.

Cryostat and Microtome

To section samples a … cryostat containing a microtome was used and the temperatures set between -27 ^{o}C and -30 ^{o}C. The cork bound samples are attached to a chuck using OCT and then the chuck is placed into the specimen holder. The angle of the microtome was set to 3 ^{o} and a feather S35 "fine" blade was inserted into the blade holder. Sections of 20 µm thick were used to cut into the tissue, the sample was then polished by taking 8µm sections. For histology sections the microtome is set to cut at 8 µm thick and sections were taken, then flattened and attached to glass slides. For RNA extraction the microtome was set to 20 µm sections were taken and the curls were removed and placed into sterile and cooled eppendorfs.

H&E Staining

Sections bound to glass slides were stained by following a standard H&E staining protocol, see table 1. Coverslips were attached using DPX mounting media and then observed with a microscope with a x10 and x40 objective lense and x10 optical lense.

Table 1 – H&E staining protocol

Solution	Time (minutes)
Ethanol 100 %	5
Ethanol 90%	5
Ethanol 70 %	5
Ethanol 50 %	5
Ethanol 30 %	5
dH_2O	0.25
Hematoxylin solution (Harris Modified; Sigma)	6
Bluing – running H_2O	3-6
Acid Alcohol (70 % ethanol, 1 % Hydrochloric Acid)	0.1
H_2O – running	Rinse
dH_2O	Rinse
1 % Eosin	0.1 - 0.2
H_2O – running	Rinse
dH_2O	Rinse
Ethanol 100 %	5
Ethanol 90%	5
Ethanol 70 %	5
Ethanol 50 %	5
Ethanol 30 %	5
Xylene	5
Xylene	5
DPX (BioScience)	

RNA extraction

The 20 µm sections, curls, taken from the tissue specimens were stored on ice and 500 µm TRIzol® Reagent (Invitrogen) was added and stored on ice. A Tissue LyserLT (Qiagen) system and 5mm stainless steal beads was used to lyse the tissue samples. The lyser was set at 50 Hz

and run for 10 minutes. The TRIzol® was removed and 200 µm of chloroform was added to every 500 µm TRIzol®, the samples were the vortexed for 15 s. The samples were then spun at 28,000 RCF 4 ^{o}C for 20 minutes. The aqueous phase was removed and mixed 1:1 with 100 % ethanol. This was then added to a Qiagen RNeasy Mini Kit column and then continued according to the manufactures guide.

RNA quality

RNA isolated was run on an Experion (BIORAD) using StdSense Chips. The methods were carried out according to the manufacturers guidelines.

Results

Optimising RNA extraction from tissue sections.

Sample 1278104 was used as a test tissue sample to optimize the RNA extraction from fixed tissue. Using the cryostat/microtome 20 µm curls were taken from the tissue sample, to optimize how many curls would be needed 2, 4 and 8 curls were taken and the RNA extracted. Table 2 show the RNA concentration obtained from the curls. From taking 8 curls 100 ng/µl was obtained, to gain 50 % more RNA, 12 curls of tissue will be used in further experiments. An increase in curls was decided as in MOM tissues some tissue may be necrotic and therefore the RNA yield will be reduced.

Table 2 RNA extracted from curls taken from sample 1278104

	ng/µl	260:280	260:230
2 curls	40	2.12	1.11
4 curls	65	2.05	1.4
8 curls	100	2.07	0.56

RNA extraction from MOM samples

Samples stored at the Cottman Centre (Norwich, Norfolk) contained various MOM samples from 2007 to 2012. One sample from each year was used (the year is identified in the first two digits of the sample ID [Table 3]). A total of 12 curls were taken from the samples and the RNA extraction was carried out as before. Table 3 shows that even in samples over 6 years old RNA was extracted from, however it is not known whether the RNA isolated is of a great quality. To investigate further the RNA was run on an Experion, see Fig 1 and Table 4.

Table 3 RNA isolated from MOM tissue samples

Sample ID	Nucleic Acid Conc. ng/µl	260:280	260:230
07TB0043	131.5	2.09	1.27
08TB0059	186.6	2.08	1.42
09TB0029	17.8	2.15	0.95
10TB0003	30.3	2.11	1.51
11TB0004	65.2	2.09	0.74
12TH0006	52.8	2.12	2.37

Out of the 6 samples of RNA , it was found that the RNA was of a good quality (RQI ≥7) and so this RNA could be used for Taqman® or other array techniques. The samples 09TB0029 and

10TB0003 showed no bands in the gel and so it could be that the RNA has been degraded, or that the RNA was not loaded correctly, either too little or too much. Therefore further tests will be carried out on these two samples.

Table 4 RNA quality and quantity for MOM hip tissue samples.

Lane	Sample	RNA ng/µl	Ratio (28S/18S)	RQI
L	Ladder	160		
1	07TB0043	136.25	1.19	7.6
2	08TB0059	143.19	1.41	7.9
3	09TB0029	8.41	1.04	N/A
4	10TB0003	1.95	1.74	N/A
5	11TB0004	73.67	1.24	8.8
6	12TH0006	47.34	1.57	8.1

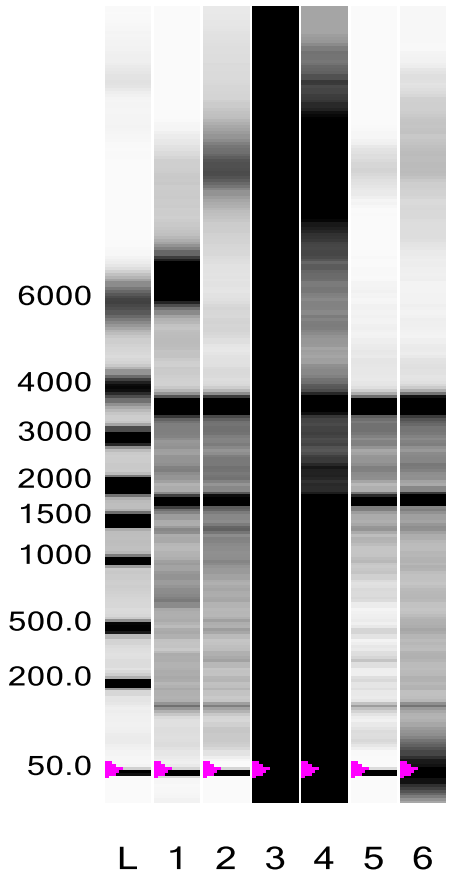

Fig 1 RNA quality, see table 4 for sample ID

Discussion

These results demonstrate that it is possible to isolate a good high quality yield of RNA from tissue samples bound in OCT. Low yields of RNA and low RQI numbers could be due to a variety of factors. The tissue from MOM patients can have large areas of necrosis, which yields little to no RNA. Other factors also include the tissue type, capsule tissue tends to yield more RNA than labrum, which is fibrocartilaginous and is harder to physically break down. The method developed above maybe able to over come RNA isolation from tougher tissues. Lysing fresh tissue can be problematic with regards to getting enough RNA out of more fibrous and cartilage tissues and so binding samples in OCT may over come this.

RNA Quality Control

Experion (Bio-Rad) Protocol

1) Bring following kit to room temp (15-20 mins)

- RNA stain (blue) – light sensitive
- Loading buffer (yellow)
- RNA gel (green)

Vortex all tubes and spin to bottom

2) **Clean electrodes before and after use**

- Zap cleaning chip, 2 mins (Experion electrode cleaner)
- Water cleaning chip, 2 mins (Experion DEPC treated water)
- Water cleaning chip, 1 min
- Air dry

3) **Prepare gel stain (18ul per chip)**

- Add 600 ul RNA gel to filter column and spin @ 1500 gx, 10 mins (use within 4/52 or re-filter)
- Add 65ul filtered gel to clean tube, add 1ul RNA stain (always at a 65:1 ratio)
- Briefly vortex

4) **Prepare RNA Ladder & samples (1ul per chip plus 1ul excess)**

- Denature at 70degC, 2 mins (only denature enough for 1 chip at a time)
- Place RNA ladder immediately on ice 5mins, spin down before use

- Transfer 1.2uL of RNA sample to new 0.5 tube and denature as above
- Keep on ice until needed

5) **Prime the Chip**

- Add 9ul gel-stain to highlighted GS well directly to the bottom of the well. Do not expel air, do not pipette down the side.
- Place chip in priming station
- Set to B1
- Start and wait for beep
- Check for air bubbles

6) **Load samples (use a new tip each time)**

- Add 9 ul gel stain to other GS well
- Add 9ul gel only to "G"
- Add 5ul loading buffer to all sample wells and well "L"
- Add 1ul denatured RNA ladder to "L"
- Add 1 ul to wells 1-12
- Vortex chip on specialised vortex platform for 1 min

7) **Launch software**

- Select "new run"
- Select "Eukaryote Total RNA StdSens"
- Select "start"
- Select number of samples, then "ok"
- Clean electrodes as before
- Export data via "print to PDF" option

TIPS

Check that all wells appear to have same diameter circles prior to running Experion

Will usually tell indicate within 20% of analysis if there is a problem with one of the wells

If there is a problem, visually inspect and if sample is adherent to the side of the well, push it down with a clean pipette tip taking care not to aspirate

If still an issue try uptaking well contents into a clean pipette and re-dispensing to expel air bubbles.

Appendix F: Minimum Information About a Microarray (MIAME) Requirements

Minimum information about a microarray experiment (MIAME)

Background information: A brief description of the experiment (e.g., the abstract from the related publication, links to the publication, any supplemental websites or database accession numbers:

Metal-on-metal (MoM) total hip replacements were thought to be more suitable for young patients with increased mobility on longevity compared to the metal-on- polyethylene (MoP) implants. However, there has been a high rate of failure, adverse reactions to the MoM bearing surfaces have resulted in implant failure, pain and the development of inflammatory masses. Adverse reactions to metal debris (ARMD) is an emerging cause of major morbidity in hip arthroplasty that significantly impacts upon quality of life, provides a major technical challenge, and contributes a significant workload in revision hip surgery.

The aim of our research is to characterise gene expression in tissue attained from MoM, MoP and Primary total hip replacements. With this information we can examine how reactions to polyethylene and metal debris might trigger the activation of biological cascades involved with osteolysis, loosening, and ARMD. If a characteristic gene expression signature is found, it is envisaged that this may lead to future work to reveal a reliable set of biomarkers of disease activity in these conditions.

Experiments purpose and justifications: The goal of the experiment – one line maximum (e.g., the title from the related publication):

Comparison of patterns of gene expression in patients with primary osteoarthritis, aseptic loosening following metal-on-polyethylene THR, and ARMD secondary to metal-on-metal THR.

Experimental factors and keywords: For example, time course, cell type comparison, the parameters or conditions tested, such as time, dose, or genetic variation:

Samples of hip capsule, pericapsular tissue and bursa from patients undergoing primary hip arthroplasty for osteoarthritis, MoP revision for aseptic loosening and MoM revision for ARMD.

Within the MoM group patients will have had either and Ultima MoM 28mm Hybrid THR primary arthroplasty or a large diameter ASR THR MoM arthroplasty.

Experimental design - relationships between samples, treatments, (e.g., a diagram or table showing experimental factors under study and biological replicates in each group/treatment):

Specimen ID	Tissue Type	Disease process
P A	Human Hip Capsule	Primary THR for Osteoarthritis
P B	Human Hip Capsule	Primary THR for Osteoarthritis
P C	Human Hip Capsule	Primary THR for Osteoarthritis
P D	Human Hip Capsule	Primary THR Osteoarthritis
MOP A	Human Hip Capsule	MoP Revision for Aseptic Loosening
MOP B	Human Hip Capsule	MoP Revision for Aseptic Loosening
MOP C	Human Hip Capsule	MoP Revision for Aseptic Loosening
MOP D	Human Hip Capsule	MoP Revision for Aseptic Loosening
MOM A	Human Hip Capsule	MoM Revision for ARMD [Ultima MoM THR]
MOM B	Human Hip Capsule	MoM Revision for ARMD [Ultima MoM THR]
MOM C	Human Hip Capsule	MoM Revision for ARMD [Ultima MoM THR]
MOM D	Human Hip Capsule	MoM Revision for ARMD [ASR MoM THR]

Type of chips required to use: Illumina Human HT-12 v4 Expression Bead Chip

The origin of each biological sample: (e.g., name of the organism, the provider of the sample) and its characteristics (e.g., gender, age, and developmental stage, strain, disease state):

Specimen ID	Tissue Type	Gender	Disease process
P A	Human Hip Capsule	Female	Primary THR for Osteoarthritis
P B	Human Hip Capsule	Female	Primary THR for Osteoarthritis
P C	Human Hip Capsule	Female	Primary THR for Osteoarthritis
P D	Human Hip Capsule	Female	Primary THR Osteoarthritis
MOP A	Human Hip Capsule	Male	MoP Revision for Aseptic Loosening
MOP B	Human Hip Capsule	Female	MoP Revision for Aseptic Loosening
MOP C	Human Hip Capsule	Male	MoP Revision for Aseptic Loosening
MOP D	Human Hip Capsule	Female	MoP Revision for Aseptic Loosening
MOM A	Human Hip Capsule	Male	MoM Revision for ARMD [Ultima MoM THR]
MOM B	Human Hip Capsule	Female	MoM Revision for ARMD [Ultima MoM THR]
MOM C	Human Hip Capsule	Male	MoM Revision for ARMD [Ultima MoM THR]
MOM D	Human Hip Capsule	Male	MoM Revision for ARMD [ASR MoM THR]

Cell line/tissue type/Source/ history/passage:

Human, Hip tissue, Capsule and peri capsular tissue

Genetic modifications/characteristics: N/A

Ref. to clinical data/information:

Developmental stage: N/A

Manipulation of biological samples and protocols used (e.g., growth conditions, treatments, separation techniques):

Hip Tissue was lysed at 4 ^{o}C.

In vivo/vitro treatments:

P (A, B, C, D) = 4 x Primary THR for clinical & radiological OA

MoP (A, B, C, D) = 4 x Revision arthroplasty for aseptic loosening of a Metal on Polyethylene THR

MoM (A, B, C, D) = 4 x Revision arthroplasty for ARMD in a patient with a Metal on Metal THR

Organism part: Human Hip Tissue [capsule and pericapsular tissue]

Tissue/cell harvesting methods/ Separation technique:

Intraoperative specimens were harvested and immediately placed in RNAlater (Life Technologies) and stored at -80 ^{o}C

Tissue was lysed using a Qiagen Tissue Lyser, at 50 Hz

Strain or line/ Genetic Variation: N/A

Growth condition: N/A

Technical protocols for preparing the hybridization extract (e.g., the RNA or DNA extraction and purification protocol), and labeling:

0.5g of Tissue was lysed in 500 µl TRIzol. RNA was extracted by chloroform (500 µl per 1 ml TRIzol) and precipitated in 100 % ethanol. RNA was then cleaned/purified using Qiagen RNeasy Kit (RNA isolation). To increase concentration RNA precipitation method: [1/10th vol, 3M sodium acetate, 2.5 x vol. 100 % ethanol], -80 ^{o}C 12 hr, spin [500 µl 75 % ethanol] spin, rehydrate.

RNA source and extraction methods:

RNA Amplification used if any: N/A

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We the above confirm that results obtained using the microarray facility will be fully and appropriately acknowledged within the paper. We will forward any publication citation upon receipt.

Appendix G: TLDA Gene Tables

These tables give details of the top 36 genes identified as being differentially expressed across 3 phenotypes with an absolute fold change FC>2 and $p<0.05$ used to verify the findings of the microarray experiment. A 2 way ANOVA was performed using the FC values from the microarray data versus those derived from TLDA. Information derived from gene ontology database at www.pubmed.com

Gene	Function	p value
CD36	**Thrombospondin receptor;cellular adhesion molecule** The protein encoded by this gene is the fourth major glycoprotein of the platelet surface and serves as a receptor for thrombospondin in platelets and various cell lines. Since thrombospondins are widely distributed proteins involved in a variety of adhesive processes, this protein may have important functions as a cell adhesion molecule. It binds to collagen, thrombospondin, anionic phospholipids and oxidized LDL. It directly mediates cytoadherence of Plasmodium falciparum parasitized erythrocytes and it binds long chain fatty acids and may function in the transport and/or as a regulator of fatty acid transport. Mutations in this gene cause platelet glycoprotein deficiency.	ns
CHIT1	**Chitotriosidase.** Secreted by activated human macrophages and is markedly elevated in plasma of Gaucher disease patients. The expression of chitotriosidase occurs only at a late stage of differentiation of monocytes to activated macrophages in culture	P<0.05*
CYP27A1	**Cytochrome P450, family 27, subfamily A, polypeptide 1** Encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids.	ns
HS3ST2	**Heparan sulfate (glucosamine) 3-O-sulfotransferase 2** The enzyme encoded by this gene is a member of the heparan sulfate biosynthetic enzyme family. It is a type II integral membrane protein and possesses heparan sulfate glucosaminyl 3-O-sulfotransferase activity. This gene is expressed predominantly in brain and may play a role in the nervous system	ns
CD52	**CAMPATH-1 Antigen/Cluster of Differentiation 52** CD52 is present on the surface of mature lymphocytes, but not on the stem cells from which these lymphocytes were derived. It also is found on monocytes[1] and dendritic cells	ns

Gene	Function	p value
PLA2G7	**Phospholipase A2.** The protein encoded by this gene is a secreted enzyme that catalyzes the degradation of platelet-activating factor to biologically inactive products. Defects in this gene are a cause of platelet-activating factor acetylhydrolase deficiency	ns
ALDH1A3	**Aldehyde dehydrogenase 1 family, member A3**. Gene encodes an aldehyde dehydrogenase enzyme that uses retinal as a substrate. Mutations in this gene have been associated with microphthalmia, isolated 8, and expression changes have also been detected in tumor cells.	ns
MMP12	**Matrix Metallopeptidase 12.** Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. It is thought that the protein encoded by this gene is cleaved at both ends to yield the active enzyme, but this processing has not been fully described. The enzyme degrades soluble and insoluble elastin. It may play a role in aneurysm formation and studies in mice suggest a role in the development of emphysema.	P<0.01**
GZMB	**Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1).** Cytolytic T lymphocytes (CTL) and natural killer (NK) cells share the remarkable ability to recognize, bind, and lyse specific target cells. They are thought to protect their host by lysing cells bearing on their surface 'nonself' antigens, usually peptides or proteins resulting from infection by intracellular pathogens. The protein encoded by this gene is crucial for the rapid induction of target cell apoptosis by CTL in cell-mediated immune response.	ns
ENPP1	**Ectonucleotide pyrophosphatase/phosphodiesterase 1.** The encoded protein is a type II transmembrane glycoprotein comprising two identical disulfide-bonded subunits. This protein has broad specificity and cleaves a variety of substrates, including phosphodiester bonds of nucleotides and nucleotide sugars and pyrophosphate bonds of nucleotides and nucleotide sugars. This protein may function to hydrolyze nucleoside 5' triphosphates to their corresponding monophosphates and may also hydrolyze diadenosine polyphosphates. Mutations in this gene have been associated with 'idiopathic' infantile arterial calcification, ossification of the posterior longitudinal ligament of the spine (OPLL), and insulin resistance.	ns

Gene	Function	p value
FOSB	**FBJ murine osteosarcoma viral oncogene homolog B** The Fos gene family consists of 4 members: FOS, FOSB, FOSL1, and FOSL2. These genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. Alternatively spliced transcript variants encoding different isoforms have been found for this gene.	ns
FOS	**FBJ murine osteosarcoma viral oncogene homolog.** Genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. Alternatively spliced transcript variants encoding different isoforms have been found for this gene.	ns
COL22A1	**Collagen, type XXII, alpha 1**. The COL22A1 gene on human chromosome 8q24.2 encodes a collagen that structurally belongs to the FACIT protein family (fibril-associated collagens with interrupted triple helices). Collagen XXII exhibits a striking restricted localization at tissue junctions such as the myotendinous junction in skeletal and heart muscle, the articular cartilage-synovial fluid junction, or the border between the anagen hair follicle and the dermis in the skin. It is deposited in the basement membrane zone of the myotendinous junction and the hair follicle and associated with the extrafibrillar matrix in cartilage. In situ hybridization of myotendinous junctions revealed that muscle cells produce collagen XXII, and functional tests demonstrated that collagen XXII acts as a cell adhesion ligand for skin epithelial cells and fibroblasts. This novel gene product, collagen XXII, is the first specific extracellular matrix protein present only at tissue junctions.	ns
EGR1	**Early growth response 1.** Protein encoded by this gene belongs to the EGR family of C2H2-type zinc-finger proteins. It is a nuclear protein and functions as a transcriptional regulator. The products of target genes it activates are required for differentitation and mitogenesis. Studies suggest this is a cancer suppressor gene.	ns

Gene	Function	p value
NR4A2	**Nuclear receptor subfamily 4, group A, member 2** Gene encodes a member of the steroid-thyroid hormone-retinoid receptor superfamily. The encoded protein may act as a transcription factor. Mutations in this gene have been associated with disorders related to dopaminergic dysfunction, including Parkinson disease, schizophernia, and manic depression. Misregulation of this gene may be associated with rheumatoid arthritis.	ns
APCDD1L	**Adenomatosis polyposis coli down-regulated 1-like**	p<0.05*
AMTN	**Amelotin** The mineralized portions of teeth, the dentin and enamel, are formed by mesenchyme-derived odontoblasts and epithelium-derived ameloblasts, respectively. Amelotin is specifically expressed in maturation-stage ameloblasts	ns
CHI3L2	**Chitinase 3-like 2** The protein encoded by this gene is similar to bacterial chitinases but lacks chitinase activity. The encoded protein is secreted and is involved in cartilage biogenesis.	ns
CSN1S1	**Casein alpha s1** Milk protein gene	P<0.001***
MMP3	**Matrix Metalloproteinase 3.** Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. This gene encodes an enzyme which degrades fibronectin, laminin, collagens III, IV, IX, and X, and cartilage proteoglycans. The enzyme is thought to be involved in wound repair, progression of atherosclerosis, and tumor initiation. The gene is part of a cluster of MMP genes which localize to chromosome 11q22.3	ns

Gene	Function	p value
GJB2	**Gap junction protein, beta 2, 26kDa** This gene encodes a member of the gap junction protein family. The gap junctions were first characterized by electron microscopy as regionally specialized structures on plasma membranes of contacting adherent cells. These structures were shown to consist of cell-to-cell channels that facilitate the transfer of ions and small molecules between cells.	ns
TNFALP6	**Tumor necrosis factor, alpha-induced protein 6** The protein encoded by this gene is a secretory protein that contains a hyaluronan-binding domain, and thus is a member of the hyaluronan-binding protein family. The hyaluronan-binding domain is known to be involved in extracellular matrix stability and cell migration. This protein has been shown to form a stable complex with inter-alpha-inhibitor (I alpha I), and thus enhance the serine protease inhibitory activity of I alpha I, which is important in the protease network associated with inflammation. This gene can be induced by proinflammatory cytokines such as tumor necrosis factor alpha and interleukin-1. Enhanced levels of this protein are found in the synovial fluid of patients with osteoarthritis and rheumatoid arthritis	ns
CFI	**Complement factor I** This gene encodes a serine proteinase that is essential for regulating the complement cascade. The encoded preproprotein is cleaved to produce both heavy and light chains, which are linked by disulfide bonds to form a heterodimeric glycoprotein. This heterodimer can cleave and inactivate the complement components C4b and C3b, and it prevents the assembly of the C3 and C5 convertase enzymes. Defects in this gene cause complement factor I deficiency, an autosomal recessive disease associated with a susceptibility to pyogenic infections. Mutations in this gene have been associated with a predisposition to atypical hemolytic uraemic syndrome, a disease characterized by acute renal failure, microangiopathic hemolytic anemia and thrombocytopenia. Primary glomerulonephritis with immmune deposits is another condition associated with mutation of this gene	ns
PCOLCE	**Procollagen C-endopeptidase enhancer** Fibrillar collagen types I-III are synthesized as precursor molecules known as procollagens. These precursors contain amino- and carboxyl-terminal peptide extensions known as N- and C-propeptides, respectively, which are cleaved, upon secretion of procollagen from the cell, to yield the mature triple helical, highly structured fibrils. This gene encodes a glycoprotein which binds and drives the enzymatic cleavage of type I procollagen and heightens C-proteinase activity.	ns

Gene	Function	p value
ITGAV	**Integrin, alpha V** This gene encodes a protein that is a member of the integrin superfamily. Integrins are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain. This protein undergoes post-translational cleavage to yield disulfide-linked heavy and light chains that combine with multiple integrin beta chains to form different integrins. This protein has been shown to heterodimerize with beta 1, beta 3, beta 5, beta 6, and beta 8; the heterodimer of alpha v and beta 3 is the Vitronectin receptor. This protein interacts with several extracellular matrix proteins to mediate cell adhesion and may play a role in cell migration. It is proposed that this protein may regulate angiogenesis and cancer progression. Alternative splicing results in multiple transcript variants that encode different protein isoforms. Note that the integrin alpha 5 and integrin alpha V chains are produced by distinct genes	ns
TIMP3	**TIMP metallopeptidase inhibitor 3** This gene belongs to the TIMP gene family. The proteins encoded by this gene family are inhibitors of the matrix metalloproteinases, a group of peptidases involved in degradation of the extracellular matrix (ECM). Expression of this gene is induced in response to mitogenic stimulation and this netrin domain-containing protein is localized to the ECM. Mutations in this gene have been associated with the autosomal dominant disorder Sorsby's fundus dystrophy	P<0.05*
CLIC5	**Chloride intracellular channel 5** This gene encodes a member of the chloride intracellular channel (CLIC) family of chloride ion channels. The encoded protein associates with actin-based cytoskeletal structures and may play a role in multiple processes including hair cell stereocilia formation, myoblast proliferation and glomerular podocyte and endothelial cell maintenance. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene.	ns
ADAMTSL2	**ADAMTS-like 2** This gene encodes a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) and ADAMTS-like protein family. Members of the family share several distinct protein modules, including a propeptide region, a metalloproteinase domain, a disintegrin-like domain, and a thrombospondin type 1 (TS) motif. Individual members of this family differ in the number of C-terminal TS motifs, and some have unique C-terminal domains. The protein encoded by this gene lacks the protease domain, and is therefore of a member of the the ADAMTS-like protein subfamily. It is a secreted glycoprotein that binds the cell surface and extracellular matrix; it also interacts with latent transforming growth factor beta binding protein 1. Mutations in this gene have been associated with geleophysic	ns

Gene	Function	p value
SEMA3A	**Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A** This secreted protein can function as either a chemorepulsive agent, inhibiting axonal outgrowth, or as a chemoattractive agent, stimulating the growth of apical dendrites. In both cases, the protein is vital for normal neuronal pattern development. Increased expression of this protein is associated with schizophrenia and is seen in a variety of human tumor cell lines. Also, aberrant release of this protein is associated with the progression of Alzheimer's disease	ns
BTC	**Betacellulin** The protein encoded by this gene is a member of the EGF family of growth factors. It is synthesized primarily as a transmembrane precursor, which is then processed to mature molecule by proteolytic events. This protein is a ligand for the EGF receptor	ns
CRLF1	**Cytokine receptor-like factor 1** This gene encodes a member of the cytokine type I receptor family. The protein forms a secreted complex with cardiotrophin-like cytokine factor 1 and acts on cells expressing ciliary neurotrophic factor receptors. The complex can promote survival of neuronal cells. Mutations in this gene result in Crisponi syndrome and cold-induced sweating syndrome	ns
CRTAC1	**Cartilage acidic protein 1** gene encodes a glycosylated extracellular matrix protein that is found in the interterritorial matrix of articular deep zone cartilage. This protein is used as a marker to distinguish chondrocytes from osteoblasts and mesenchymal stem cells in culture. The presence of FG-GAP motifs and an RGD integrin-binding motif suggests that this protein may be involved in cell-cell or cell-matrix interactions. Copy number alterations in this gene have been observed in neurofibromatosis type 1-associated glomus tumors. Alternative splicing results in multiple transcript variants	P<0.05*
ERRFI1	**ERBB receptor feedback inhibitor 1** ERRFI1 is a cytoplasmic protein whose expression is upregulated with cell growth. It shares significant homology with the protein product of rat gene-33, which is induced during cell stress and mediates cell signaling	ns

Gene	Function	p value
NDUFA4L2	**NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2** an enzyme of the respiratory chains of myriad organisms from bacteria to humans. It catalyzes the transfer ofelectrons from NADH to coenzyme Q10 (CoQ10) and, in eukaryotes, it is located in the inner mitochondrial membrane. It is one of the "entry enzymes" of cellular respiration or oxidative phosphorylation in the mitochondria	ns
AK1	**Adenylate kinase 1** Adenylate kinase is an enzyme involved in regulating the adenine nucleotide composition within a cell by catalyzing the reversible transfer of phosphate group among adinine nucleotides. Three isozymes of adenylate kinase have been identified in vertebrates, adenylate isozyme 1 (AK1), 2 (AK2) and 3 (AK3). AK1 is found in the cytosol of skeletal muscle, brain and erythrocytes, whereas AK2 and AK3 are found in the mitochondria of other tissues including liver and heart. AK1 was identified because of its association with a rare genetic disorder causing nonspherocytic hemolytic anemia where a mutation in the AK1 gene was found to reduce the catalytic activity of the enzyme	ns
GFPT2	**Glutamine-fructose-6-phosphate transaminase 2** is an enzyme that catalyzes the chemical reaction L-glutamine + D-fructose 6-phosphate $\rightleftharpoons$ L-glutamate + D-glucosamine 6-phosphate Thus, the two substrates of this enzyme are L-glutamine and D-fructose 6-phosphate, whereas its two products are L-glutamate and D-glucosamine 6-phosphate. This enzyme belongs to the family of transferases, specifically the transaminases, which transfer nitrogenous groups.	ns
GPR64	**G protein-coupled receptor 64** GPR64 is significantly overexpressed in the Wnt signaling-dependent subgroup of medulloblastomas as well as in ewing sarcomas and carcinomas derived from prostate, kidney or lung	ns

Gene	Function	p value
NTN4	**Netrin 4** Netrins are a class of proteins involved in axon guidance.	ns
ZNF385B	**Zinc finger protein 385B** Detected in germinal center of lymph node (at protein level). Expressed in spleen, lymph node and tonsil	ns
SCARA3	**Scavenger receptor class A, member 3** This gene encodes a macrophage scavenger receptor-like protein. This protein has been shown to deplete reactive oxygen species, and thus play an important role in protecting cells from oxidative stress. The expression of this gene is induced by oxidative stress	ns
HBEGF	**Heparin-binding EGF-like growth factor** HB-EGF-like growth factor is synthesized as a membrane-anchored mitogenic andchemotactic glycoprotein. An epidermal growth factor produced by monocytes and macrophages, due to an affinity for heparin is termed HB-EGF. It has been shown to play a role in wound healing, cardiac hypertrophy, and heart development and function	ns
OGN	**Osteoglycin** This gene encodes a protein which induces ectopic bone formation in conjunction with transforming growth factor beta. This protein is a small proteoglycan which contains tandem leucine-rich repeats (LRR).	P<0.05*
SBSPON	**Somatomedin B and thrombospondin, type 1 domain containing** O-glycosylation of TSR domain-containing proteins Receptor-mediated endocytosis	ns
PITX1	**Paired-like homeodomain 1** Transcriptional regulator involved in basal and hormone-regulated activity of prolactin	ns