

Morphology and Localisation of Patient Hip Replacement-Derived Debris and Hip Prostheses Materials in Macrophages

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Abstract

Whilst total hip replacement and hip resurfacing arthroplasties have been largely successful to relieve pain and restore mobility for patients, aseptic loosening is still the most common cause for revision surgery. It is thought there is a contribution from both the sensitivity to the debris produced by implant wear as well as the macrophage-mediated response, producing pro-inflammatory cytokines and increasing the risk of an adverse reaction, including osteolysis. Macrophages respond to the presence of wear debris and degrade the materials via phagocytosis, resulting in the production of toxic ions capable of migrating to other areas of the body. Both characterisation and localisation of wear debris from different implants were investigated as the level of reaction between patients varies and it is hypothesised that the size, shape and material contribute to the level of reaction. Macrophage response to implant material types found in patient samples was also explored, comparing material uptake and early phagocytosis. Using electron, confocal and light microscopy, results were gathered in the form of images for interpretation. Results confirmed macrophages responded to the presence of particles and inflammation varied with or without the presence of particles, characterised by numerous lymphocytes. Particle characterisation agreed with previous research, with both micron-sized and nano particles present. Elemental analysis revealed cobalt, chromium and titanium particles in patient tissues and patient-derived loose particles. Commercial particle uptake was confirmed for these materials *in vitro*, localising to phagosomes or in the cytoplasm, sometimes in gross quantities. Titanium formed needle-shaped particles which increased apoptosis and altered macrophage appearance. Surface characteristics varied for patient particles, further concluding the formation of aggregates. The findings suggest retention of large, irregular aggregates and cobalt ions from large quantities of readily phagocytosed nano particles promote inflammation. Future work would include assessing oxidative damage and cytokine production in repeated conditions.

Introduction

Implant failure

Prosthetic implants have been used since the 1960s to relieve symptoms in patients with conditions including osteoarthritis, improving patient quality of life and restoring normal joint function. Though largely successful, the long-term success of each generation of implant is hindered by the loosening of the joint. Aseptic loosening, the loosening of the joint without the presence of an infection, is considered the major cause of needing revision surgery. In the most recent report from the UK National Joint Registry, of 796,636 hip implants, 20,926 needed revision surgery. This was most commonly due to aseptic loosening and the presence of an adverse local tissue reaction (ALTR) to particulate debris, with metal on metal (MoM) bearings showing the highest incidence of having an adverse reaction. Notably, the failure rate increased between 2003 and 2008 when a more recent generation of MoM implants were used, followed by a reduction in failure rate as alternatives to MoM were used after 2008 due to this identification of higher than expected revision rates (National Joint Registry, 2016). Implant design, though improving, is still not without risk of failure after an unknown period of time, depending on both implant durability and independent patient response.

An adverse reaction encompasses a number of issues, including soft tissue reactions, tissue necrosis and osteolysis, occurring from the production of debris from the implant. Periprosthetic soft-tissue lesions have previously been described as metallosis (the presence of a large amount of metallic debris in tissues, as seen by black metallic deposits at revision surgery), pseudotumours, aseptic lymphocytic vasculitis-associated lesions (ALVAL) (Willert *et al*, 2005) and adverse reaction to metal debris (ARMD). Wear debris from the prostheses is associated with an adverse local tissue reaction, which is a significant problem for the lifetime of the implant. In another retrieval of data, 51% (of 77,438) of patients having revision surgery was due to aseptic loosening, another 14.1% due to implant wear and a further 10.8% due to adverse reactions to the implant debris, showing the highest causes of revision are associated with implant wear, wear products and the loosening of the joint (National Joint Registry, 2016). Early failure of implants can be due to the generation of increased early implant wear. Pain, lysis and dislocation of the joint further contribute to ~15% of the reasons for failure, also associated with implant wear. Tissue samples, taken at the time of revision surgery, reveal the extent of the local tissue reaction which may be responsible for implant failure. Perivascular infiltrates of lymphocytes, plasma cells and macrophages present, with or without metal debris also present, is common in histological examinations where there is a reaction (Willert *et al*, 2003). How ALTR associates with the known

genotoxicity, cytotoxicity and hypersensitivity to the materials used in the implants is still being speculated, as failure rates vary and are not dependent on the level of reaction seen in tissues. That is, some patients experience a more severe reaction than others but the failure rate does not necessarily coincide with the quantity of implant debris or inflammation present in surrounding tissues.

It is expected that the number of implants (and therefore failed implants) will increase, partly due to the aging population and an increase in population itself. Greater expectations for the implants means implants need to withstand higher activity levels in younger patients as well as provide relief for older patients living longer. Therefore the lifetime/longevity of the implant needs to compare to the increased time it may be needed by the patient, as revision surgeries incur increased additional costs, patient morbidity and an increased risk of complications. A number of factors can increase the risk of implant failure. Revision rates are increased by hospitals performing a low volume of surgeries (Jeschke *et al*, 2019) or initial surgeries are performed by inexperienced surgeons (<400 previous implant surgeries) (Bordini *et al*, 2007), possibly due to the positioning of the initial implant and therefore risk of early implant wear. Patient obesity, gender and age increases the risk of complications and revision rates (Haughom *et al*, 2015; Jeschke *et al*, 2018). The less expensive option of cemented arthroplasties has a higher risk of failure than the more expensive cementless arthroplasties (Bordini *et al*, 2007) due to cement debris and fractures, as well as the prostheses materials, contributing to the increase in failure rate. The UK National Joint Registry states the revision rate after 10 years should be less than 5% for future implants, as current cemented ceramic on polyethylene (CoP) have revision rates of 1.88-2.11% at 10 years, and cementless ceramic on ceramic (CoC) have revision rates of 3.93-4.33% at 10 years (Kandala *et al*, 2015).

Implant design

Different generations of hip implants are made of different materials, designed for durability and strength, however complications arise regardless of the type of implant produced over the years. Implants described as metal on metal are comprised of a metal femoral head surface articulating with a metal acetabular cup surface, introduced over 50 years ago. High revision rates led to the production of metal on polyethylene (MoP) bearings being designed, however even MoP was associated with higher rates of wear and early implant failure due to polyethylene wear debris-induced osteolysis. An improvement to MoP arthroplasties came with the development of crosslinked polyethylene, to reduce wear rates compared to standard polyethylene designs (McKellop *et al*, 1999) but even these suffered from aseptic loosening and implant failures. Adverse reactions to MoP led to a second generation of MoM implants being reintroduced, as

MoM bearings induced fewer inflammatory reactions and produced a lower wear rate (Tipper *et al*, 1999). This still led to an increase in unexpected early ALTR, secondary to corrosion of the prosthetic, seeing to further developments of MoM prostheses and other implants using alternate materials. More recently, CoP failure rates remain low in comparison and ceramic on metal (CoM) have significantly lower wear rates compared to MoM in simulated tests (Firkins *et al*, 2001).

Larger head MoM bearings increase stability and lower wear rates, which is beneficial to treat younger, active patients. Though these larger head arthroplasties offer greater stability, smaller diameter heads are equally not any less prone to the adverse reactions to wear debris (Lombardi *et al*, 2016). Polymethylmethacrylate (PMMA), bone cement, was used to fix older generations of hip prostheses in place. For non-active, elderly patients, using PMMA had satisfactory long-term results, but the failure of this fixative (fractures and de-bonding of the cement/implant) reduced the longevity of the implant (Willert *et al*, 1990). “Cement disease” prompted the development of porous metal prostheses designs, allowing bone growth to secure the implant in place. A generation of cementless implants were used from the early 1980s, lowering the failure rates caused by cement fixation failure. Hip resurfacing arthroplasties, developed in 1997, were designed to retain as much of the femoral head and acetabular cup as possible, assuming a more natural position with only mm of the articulating surfaces being replaced with a cast cobalt-chromium (CoCr) bearing.

Development of improved implant designs continues to reduce the failure rates due to cement fixation failure, stress shielding and dislocations. The persistence of aseptic loosening, inflammatory reactions and risk of osteolysis continues to be a problem for the development of implants, regardless of implant type, due to continued reactions to the materials used and the subsequent debris produced by the bearings. For all patients, there is a risk of developing complications and needing revision surgery. It has been argued prosthetic implants enter the market without thorough testing, as simulation tests do not reveal the *in situ* responses to wear debris produced by the implant to know if the implant is compatible longer term.

Implant wear

Implant wear results from friction between the articulating bearing surfaces, mediated by interfacial material, as well as the implant environmental conditions and surface corrosion. Wear debris can be produced from any articulating surface, including the head-cup, head-neck and attachment to the patient’s bone (cemented or cementless). Mechanical wear (abrasion, surface fatigue) and chemical-mechanical wear (adhesion/tribochemical reactions) are the main mechanisms leading to wear particles separating from the implant surface and being

disseminated into periprosthetic tissues. Tribocorrosion describes the material degradation caused by both mechanical loading at the bearing surface and the corrosive environment of the synovial fluid, transforming the surface to a tribolayer which differs to the base material and results in wear of the tribolayer. This tribolayer consists of a nanocrystalline structure, with characteristics similar to the wear debris found in surrounding tissue (Catelas *et al*, 2004; Wimmer *et al*, 2003).

The amount of implant wear can be affected by a number of factors, including age, activity level and implant type used. Implants used on younger patients receive a potentially higher particle burden due to the longer duration of having the implant and increased contact force between articulating surfaces with an increase in activity level. Prosthetic material influences the longevity of implants used in younger, more active patients; MoM bearings performed better than CoP types in young, active patients in a 12 year survival rate study (Migaud *et al*, 2011) and implants with a higher carbon content experienced a reduction of wear (Tipper *et al*, 1999). For older, less active patients, the wear characteristics can depend on the loading cycle. In a simulated study, particle analysis showed a decrease in particle size resulted from an increase in transitions (performing a sitting motion) whereas larger particles but a lower wear rate resulted from fewer transitions (Shorez *et al*, 2008). Cast/formed implants (melted into shape) are beneficial as they are more resistant to generating wear than shaped implants (re-shaped from a solid metal form), however for all types of implant, incorrect positioning can initially lead to additional wear. In standard circumstances, the 'bedding in' stage can see a high wear rate initially, until the articulating surfaces achieve an optimal position for the contact areas (Hu *et al*, 2004). Post initial wear, the implant wear rate is much lower for the remaining life of the implant.

As well as influencing the risk of wear, choices of material can affect the characteristics of wear, which in turn could affect the biological response to the wear debris. MoM and CoM hip simulations produced comparable particles of >50nm in size (mean value) with a round/oval/irregular morphology (Brown *et al*, 2007). CoM simulations also produced nano-sized particles which decreased in size the more cycles tested (Firkins *et al*, 2001). CoM prostheses had overall the lowest wear rate/size of particles compared to MoM and ultra-high-molecular-weight-polyethylene (UHMWPE) prostheses. In contrast, UHMWPE had the highest wear rate and produced the largest particles, which were a non-uniform shape (Tipper *et al*, 2001).

Volumetrically, MoM implants have a reduced wear rate compared to MoP implants, however the size of the metal particles (~50nm) means the number of particles are increased, as there are more of the nano-sized particles than in the same volume of larger (>50nm) polyethylene particles. Likewise in simulated situations, MoM implants generate more yet smaller particles

than MoP implants (MoP 5×10^{11} particles per year compared to MoM 6.7×10^{12} - 2.5×10^{14} particles per year) (Doorn *et al*, 1998). This production of an increased surface area (per equivalent mass of other types of material) may have an effect on nano particle reactivity and increase biological potential, compared to reactivity to micro particles. Smaller particles are taken up more quickly than larger particles (Chithrani *et al*, 2006) and agglomeration of nano particles (aggregates) affects cellular uptake as nano particles no longer remain nano-sized. Rod-like particles also rapidly internalise (Gratton *et al*, 2008), suggesting the morphology (size and shape) of particles does influence the uptake and therefore biological activity in response to wear debris. It is unknown which development is less biologically significant.

Materials and biocompatibility

Arthroplasties are still considered very successful, however the long term biological effects have been progressively studied. Metal alloys used to create implants consist of cobalt (Co), chromium (Cr), molybdenum (Mo) and possibly trace amounts of nickel (Ni), iron (Fe), carbon (C) and manganese (Mn) (Holzwarth *et al*, 2005). Chromium helps rapidly form a thermodynamically stable oxide layer, useful for the implants success as the cobalt-chromium-molybdenum (CoCrMo) alloy becomes more resistant to corrosion and abrasion (Milosev and Strehblow, 2003). In *in situ* exposure to biological fluids and mechanical wearing, this layer becomes damaged and Cr forms Cr oxide particles (Cr_2O_3). Whilst Cr is considered non-toxic, it is less soluble. Small Cr_2O_3 particles isolated from tissues also have a passive layer of organic material, inhibiting further oxidation (Pourzal *et al*, 2011). Cr-formed wear debris from the implant is released into the synovial fluid and then tissues, where larger particles remain. Larger metal particles have been found to promote tissue damage and inflammation (Caicedo *et al*, 2013) so the retained Cr particles may contribute to patient inflammatory responses due to size. Both Co and Mo particles also diffuse into the surrounding tissues, though Mo is only found in trace amounts. Co is more soluble and found as smaller particles, sometimes in abundance, easily up taken by phagocytic cells. High wear rates would result in a higher concentration of Co particles and therefore an inflammatory response could be in response to the high presence of Co particles being phagocytosed. Co nanoparticles are easily degraded in low pH conditions. At pH 4.0, comparable to lysosome conditions, ions were released from Co nanoparticles at a steady increase the longer the exposure, which did not occur in the extracellular environment equivalent of pH 7.2 (Wang *et al*, 2016). Tissue necrosis can occur after phagocytosis of implant-derived particles (specifically Co), recruiting multiple macrophages until secondary phagocytosis of local cells and particles increases the area of necrosis and results in aseptic loosening. Previously treated with Co, cells introduced with a second bought of CoCr saw more apoptosis, suggesting a second metal implant would be

likely to produce more adverse effects (Posada *et al*, 2014). Whilst there is a response to metallic debris, a less intense tissue reaction is seen towards MoM debris compared to MoP debris. Corrosion and dissemination of smaller metal particles, easily phagocytosed in the tissues, results in a less severe inflammatory reaction in comparison to longer-term retention of larger polyethylene particles (Tipper *et al*, 1999). Aseptic loosening may not be polyethylene particle induced however, as when looking at periprosthetic tissues with polyethylene particles, particles did not induce matrix metalloproteinases MMP-2 or MMP-9 activity, associated with loosening of cementless implants (De Jong *et al*, 2011). This is a good example of where an increase in inflammation did not result in a particle-mediated response associated with aseptic loosening. Some materials are even biocompatible, such as gold (Au). When introduced to cells, Au has no cytotoxic effect and no elevation in the production of pro inflammatory mediators despite being up taken by macrophages (Zhang *et al*, 2011). This all suggests particle material type and size is important, as inflammation differs between materials and can occur in response to both large and small particles.

Tissue inflammation, characterised by the presence of an increased lymphocyte population, can be present without a high wear rate of particles suggesting an alternative causation of the cellular response. Strong evidence for the size and shape of the particles being the inflammatory promoter is given in the Caicedo *et al* (2013) study, where it was found the phagocytosis of larger, more irregular CoCrMo particles led to lysosomal destabilisation and the production of Interleukin-1 β , crucial to mediating inflammation (Caicedo *et al*, 2013). Smaller, round-shaped particles did not induce this reaction, which is a significant point because Co particles are often observed in this form in tissues. Metal ions produced by phagocytosis of larger particles may be responsible for the level of inflammation. To degrade the foreign material, phagocytic cells uptake particles by endocytosis and store the particles in phagosomes (Thiele *et al*, 2003) ready for lysosome enzymes to degrade the material (Kinchen and Ravichandran, 2010). Electron dense, membrane-bound lysosomes fuse with phagosomes to form secondary lysosomes (also known as phagolysosomes) containing the ingested material. This process results in the production of ions, oxidative stress and DNA damage (El-Yamani *et al*, 2011). Oxidative stress activates specific pathways including nuclear factor of kappa B (NF- κ B) and produces pro-inflammatory cytokines, promoting osteoclast activity (and therefore osteolysis) and aseptic loosening. Though a larger amount of smaller particles may be phagocytosed at a faster rate, issues could be arising in response to the prolonged phagocytosis of larger, irregular particles. That said, Co ions produced are more toxic than Cr ions and are capable of decreasing cell viability and proliferation whilst

increasing apoptosis (Akbar *et al*, 2011; Catelas *et al*, 2005; Kwon *et al*, 2009), further supporting the argument that the impact on cells depends on the material as well as the size of the particles.

Overall, the impact of particles influences patient ALTR response in several ways (figure 1), including a non-specific foreign body response and adaptive immune response. Non-specific foreign body response is shown by macrophage presence and adaptive immune response by lymphocytic infiltrate in the periprosthetic tissue observed near to where the wear debris is generated.

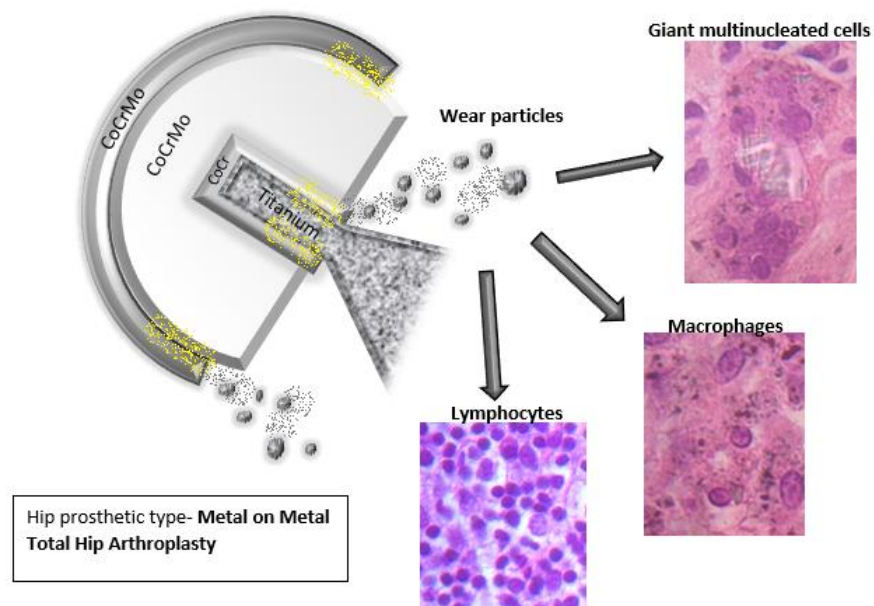


Figure 1. The production of particles occurs at articulating surfaces (indicated yellow regions) between CoCrMo socket and head, or CoCr metallic adapter sleeve and Ti neck. Large particles can be seen in giant multinucleated cells, macrophages frequently uptake debris and lymphocyte infiltrate occurs with or without the presence of larger particles, though lymphocytes do not appear to uptake the particles.

Hypersensitivity can also occur alongside ALTR. An overreaction of the immune system to the allergen, in this case metal particles, causes patient metal hypersensitivity after long term exposure to implant debris (wear particles, corrosion products and metal ions). Sensitisation to metal can occur via inhalation or dermal exposure to metallic nano particles, so with particulate matter within periprosthetic tissues it can also be assumed there may be metal hypersensitivity after a prolonged period of exposure, with a variation in patient sensitivity. Despite the reactivity, there does not seem to be any correlation between the risk of implant failure and the level of metal sensitivity (Thomas, 2013).

Higher concentrations of implant wear is also associated with an induced risk of pathogenesis of pseudotumours, though no record of implant wear has been associated with the development of cancerous lesions. Cobalt has the potential to be carcinogenic to animals (formation of sarcomas in treated animals), however there is no adequate evidence that it is the same for humans and metal alloys containing Co have little information on the risks of being carcinogenic despite Co toxicity at high levels (Toxicology Data Network, 2017). A cohort study of patients with metal-containing implants revealed there was no significant increase in cancer presence when compared to the general population (Visuri *et al*, 1996) and no sarcomas were observed at the site of implantation, so it is generally considered that there is little to no risk of cancer occurrences in patients with prosthetic implants. A further review on published material concluded cobalt-chromium (CoCr) implants are not associated with an increased risk of cancers (Christian *et al*, 2014) and a comparison of implant types also revealed there is no increased risk of cancer between MoM and non-MoM implant types (Makela *et al*, 2014). Mortality rate may even be reduced for patients with MoM resurfacing type bearings, compared to (cemented or cementless) total hip prostheses (Kendal *et al*, 2013).

Metal ions

Metal ions, positive atom compounds, are associated with failing implants. Metal ion release differs between implant types, as indicated by increased whole-blood metal ion levels for Co, Cr and Ti after implantation of arthroplasties. Whereas metal debris is found in local tissues, metal ions are found circulating throughout the body and can accumulate in the blood, liver, spleen and lymph nodes (Urban *et al*, 2000). In high concentrations, circulating Co can cause Co toxicity, with patients developing neurological damage, hypothyroidism and cardiomyopathy (Bradberry *et al*, 2014), however Co can be excreted in urine. Elevated blood Co and Cr levels can also correlate with the level of reaction seen in periprosthetic tissues, where functioning prostheses are fine in comparison, for both symptomatic and asymptomatic patients (Kwon *et al*, 2016). Determining which patients are at risk of an adverse reaction can be identified by the surveillance of ions in circulation, particularly for Co levels, with the threshold of what is deemed 'low risk' being more accurate if it is implant-specific (Matharu *et al*, 2017). Removal or revision of a failing implant lowers the whole-blood metal ion concentration (Bradberry *et al*, 2014). In implants where there is no development of an adverse reaction, whole-blood metal ions peak at 4-5 years and then fall after this time period (Bernstein *et al*, 2012). A cohort study compared metal ion concentrations of Co, Cr and Ti from patients with an implantation time of 10 years, to a control group of patients without implants and found all patients with a prostheses had levels significantly higher than the control group (Levine *et al*, 2013). Measured Co ion levels increase with a femoral head size of

50mm or greater, with no difference across implant types for Cr ion levels. The use of a femoral stem-adaptor sleeve junction also elevates Co ion production (Lavigne *et al*, 2011).

There is speculation surrounding the contributions of metallic ions, resulting from the wear particles, and how much this leads to the failure of the implant. Individual reactions are thought to be developed in response to metal-protein complexes (Goodman, 2007), Cr associated with transferrin, Co with albumin (Loeschner *et al*, 2016). Proteins complex with implant-derived metal debris, stimulating a lymphocytic immune response (Hallab *et al*, 2001). T-lymphocytes respond to the metallic debris-denatured proteins and hypersensitivity to such combinations with the materials has been reported in patients. The denatured carrier proteins, such as albumin, are seen across patients with a sensitivity to Nickel, and an increase in sensitivity to nickel has been seen in patients with MoM implants (Cohen, 1976). For a hypersensitivity reaction, the ions produced as a result of particles being phagocytosed would not be present without the production of particles in the first instance, supporting the thought that an immune response escalates from the production of particles.

Osteolysis

The production of wear debris, ions and an immune response in the local tissues stimulates osteolysis and joint loosening. This is a major concern for the life of the implant, as the loosening of the joint stimulates increased wear and further ALTR. In response to the presence of particles in the periprosthetic tissue, macrophages are recruited to mediate biodegradation of the materials via phagocytosis, whilst also being responsible for the regeneration of effected surrounding tissues by regulating target cells, including osteoblasts and osteoclasts. Normal bone turnover requires a balance of bone formation and resorption, so the wear debris-induced change in macrophage behaviour disrupts this equilibrium. As well as osteoclast activity being affected, osteoblast activity is affected by the presence of particles, and pro-inflammatory cytokines produced by macrophages results in a negative impact on bone matrix formation (Jonitz-Heincke *et al*, 2016). Osteoclast activity is mildly stimulated by either Co or Cr particles (Andrews *et al*, 2011). When osteoclasts and monocytes are co-cultured with Co, Cr and Ti particles, all materials are readily phagocytosed by the monocytes but long term exposure to Co or Cr leads to a reduction in cell numbers (Neale *et al*, 2000). Co, Cr and UHMWPE wear particles appear to stimulate monocyte-macrophage activation (Baumann *et al*, 2005), activating the signalling pathway NF- κ B, producing pro-inflammatory cytokines IL-1 β and tumor necrosis factor alpha (TNF α), seen in response to oxidative stress. As well as being material dependent, pro-inflammatory cytokine production may also be particle size dependent, as longer retaining materials like Cr and UHMWPE of 0.1-10 μ m were the most biologically active in another study

(Chikaura *et al*, 2016). Larger Ti particles (in the size ranges 0.2-1.2µm and 1.2-10µm) have also been found to affect osteoclastogenesis (Su *et al*, 2018). Osteolysis occurs where bone cement is worn. Foreign body giant cells (FBGC) uptake larger PMMA (cement) wear debris (Willert *et al*, 1990). Multi-nucleated giant cells (MNGC) and FBGC are fused morphologic variants of macrophages, capable of ingesting debris and phagocytosis. All phagocytic cells are capable of ingesting debris until toxic intracellular levels are reached, so whilst they play an important role in the biodegradation of implant debris, they also have a detrimental effect of stimulating pathways that lead to osteolysis. MNGC can resemble osteoclast morphology, are capable of phagocytosis of wear particles and may function as FBGC in the presence of debris. In tissues from sites of osteolysis, wear particles clearly activate a foreign body reaction and pro-inflammatory mediators are released by activated macrophages to stimulate osteoclast activity. It is yet unknown if osteoclast-like multinucleated giant cells also have an osteoclastic function when they have a phagocytic function (Anazawa *et al*, 2004). Overall, activated macrophages and fused macrophage variants, in response to wear debris, have a complex relationship with osteocytes leading to osteolysis, with particle characteristics and morphology seemingly altering the intensity of the reaction.

Project aim

If the level of reaction does not necessarily coincide with the amount of particles produced by the implant, there must be an alternative cause for the severity of the reaction seen between patients. The characterisation (size and shape) and composition (material type) of wear debris must contribute to the macrophage-mediated pro-inflammatory response. It is hypothesised that large, irregular particles are slow to uptake, which is also influenced by a difference in materials, affecting where and how quickly in the macrophage the particles localise and thus affecting a particle-induced response. To test this theory, the characterisation of particles present in periprosthetic tissues, taken at the time of revision surgery for failed implants where varying levels of reactions were seen, were analysed using light and electron microscopy for size, shape and composition within the tissue and within the cells (macrophages) that interact with the particles. Further analysis was done using three common materials used in implants, introducing manufactured particles to immune cells in culture to observe the uptake and early phagocytic response using confocal and electron microscopy. Exploring both the characteristics and composition of the particles and comparing the macrophage response *in situ* and *in vitro* will argue possible contributions from particle size, shape and material on the level of reaction.

Methods

Metal debris characterisation (failed implants)

Patient samples were provided by the Hospital for Special Surgery, New York, taken at the time of revision surgery and fixed on slides for histological examination via light microscopy or embedded in resin for transmission electron microscopy (TEM) analysis. A total of 84 samples (table 1) were used to characterise the presence of particles within the periprosthetic tissues for different implant types. Samples were from a group of patients (n=52) with failed implants, with a mean age of 61 years and a mean time from initial implantation of 48 months.

Table 1. Number of samples used for histological analysis per patient implant type.

Implant Type	Manufacturer	Number of Samples Observed
Metal on metal total hip arthroplasty (MoM THA)	DePuy Pinnacle	8
Metal on metal large head total hip arthroplasty (MoM LHTHA) with CoCrMo sleeve adapter	Birmingham Total Hip, Smith and Nephew	21
MoM THA with CoCr sleeve	ASR Total Hip	3
MoM LHTHA with CoCr metallic adapter sleeve (MAS)	Corin Cormet THA	2
Metal on metal hip resurfacing arthroplasty (MoM HRA)	Corin Cormet	2
Metal on polyethylene (MoP) with CoCr Dual Modular Neck THA	Rejuvenate, Stryker	26
MoM HRA	Birmingham Hip Resurfacing, Smith and Nephew	21
MoM LHTHA with Ti MAS	Biomet M2a THA	3

A total of 38 H&E stained or toluidine blue stained thin and semi-thin sections were imaged using a Zeiss AxioPlan light microscope with ProgRes C3 Jenoptik microscope camera system (Jenoptik, Germany). Images were taken using a 10x/0,30 magnification lens, alongside using a graticule

slide for scale (10mm scale with 100 divisions of 100µm intervals). Appearance of particles (size, shape, colour and localisation) was described.

One resin embedded sample per implant type (DePuy Pinnacle THA (MoM), Birmingham THA (MoM), Rejuvenate THA (MoP) and Birmingham Hip Resurfacing (MoM)) was selected. These were sectioned at a thickness of 100nm using a diamond knife and ultramicrotome (PowerTome, RMC Boeckeler, USA). Sections were mounted onto copper grids for TEM analysis. All patient samples were imaged using a Hitachi H-7650 transmission electron microscope (Hitachi) and AMT camera system. Particles were described for size, shape, electron density and localisation.

Characterisation of patient-derived wear debris was also done via scanning electron microscopy (SEM) to look at the particles in greater detail. Particles were loose on the removed implant surface and were retrieved by tapping the surface. These were directly stored and so represent unaltered aggregates of metallic nanoparticles from the implant surface, possibly admixed with blood products/necrotic cell debris. Particles were selected from one patient sample with histological tissue analysis (Birmingham THA (MoM)), sprinkled onto a 12mm diameter self-adhesive carbon disc (TAAB) coated stub for minimal interruption. Images were collected using a Hitachi S3400N scanning electron microscope operated at 5kV. Particle size, shape, surface texture and some surface brightness was described.

Energy-dispersive X-ray spectroscopy (EDS) elemental analysis for patient-derived loose particles and *in situ* analysis of materials was conducted by SEM using an Ultim Extreme detector (Oxford Instruments, High Wycombe, UK). Patient particles, loose from the surface of Rejuvenate THA (MoP), DePuy Pinnacle THA (MoM) and Birmingham THA (MoM) implants were analysed. The Birmingham THA (MoM) sample had been used for SEM analysis. Prepared grids with sections of patient tissue used for TEM analysis were reimaged for elemental analysis using a Tescan S8000 (Tescan, Czech Republic) microscope, Ultim Extreme EDS detector and Aztec (version 4.2, Oxford Instruments, High Wycombe, UK).

Metal debris characterisation (mouse tissues)

In situ characterisation of individual known materials (Co, Cr and Ti) and a combination of material (CoCrTi) was done using previously prepared copper grids with sections of fixed mouse tissue. These samples were imaged with a Hitachi H-7650 transmission electron microscope and AMT camera system. Particles were described for size, shape, quantity and localisation for each of the samples.

Cell population analysis

To consider the cell population and types of cells involved in the failure of implants, cells were analysed using the light microscopy images collected for particle characterisation. Cell types were identified with the use of a basic histology guide (Basic Histology, 1995, 8th Edition). Cells were described where an inflammatory response was present across the sample, representative of the sample in regions with or without the presence of particles.

Cellular response to commercial metal particles

To produce a timeline of particle uptake and early phagocytosis of the particles, cells identified to interact with particles *in situ* were co-cultured with implant materials Co, Cr and Ti, and also with dual-combinations of CoCr, CoTi and CrTi. All commercial particles were obtained from American Elements (99.8% purity, mixed sized particles with a range of 50nm-50µm). Particles were sterilised with 100% ethanol, left to evaporate. All chemicals and reagents were obtained from Sigma Aldrich unless otherwise stated. To make up a solution for each material/dual materials, 0.2g of each material/combination was stored in 2ml Dulbecco's Modified Eagle Medium (DMEM) culture media (concentration 0.1g/ml), stored at room temperature. RAW 264.7 macrophages (Thermo Fisher) were cultured using DMEM culture media with 4500mg/L glucose and sodium bicarbonate, supplemented with 10% foetal bovine serum, 200 mM L-glutamine and 10mg/ml Penicillin-Streptomycin.

Cell suspension was seeded onto a Falcon 6 well plate (5000 cells/ml, 2ml per well) containing sterile 1.8mm thick glass coverslips (1 per well) and incubated for 24 hours at 37°C. 10µl particle suspension was added to individual wells (1 particle suspension type per well) over the area of the cover slip with adhered cells. Cells were fixed after 120 minutes for 60 minutes using fresh 10% PFA. Cells were stained with 20mM Hoechst Blue (Thermo Fisher) and 5mg/ml CellMask Deep Red plasma membrane stain (Thermo Fisher) before cover slips were mounted onto microscope slides for confocal microscopy analysis. Longer-term fixes at 24, 48 and 72 hours were done using the same conditions, except cells were stained with 1mg/ml DAPI (Thermo Fisher) and 1mM LysoTracker Deep Red (Life Technologies) for 10 minutes before being fixed in 10% PFA and mounted onto slides. All washes between stains and fixes were done using 1x DPBS (Thermo Fisher). Images were collected using a Zeiss confocal microscope and Zen 2.3 System software.

A total of 30 randomly selected cells from confocal images were used to manually count the number of particles/aggregates inside those cells per fixture time for each of the materials/dual-combinations. Particles remaining outside of the cells in the local area were also counted in the

images containing the 30 selected cells. Percentages of extracellular particles in comparison to intracellular particles was used to show how time affects particle uptake across each time point.

SEM analysis of the commercial particles was done to reveal the particle morphology (size, shape, texture) before being introduced to the macrophages. Particles were sprinkled onto a 12mm diameter self-adhesive carbon disc (TAAB) coated stub for minimal interruption and imaged using a Hitachi S-3400N scanning electron microscope.

For intracellular localisation of the particles in more detail, one time point was selected, as influenced by confocal analysis. Cells with particles were fixed and embedded in resin, after 48 hours exposure, for TEM analysis. Cells were seeded onto a 6 well plate (5000 cells/ml, 2ml per well) with 10 μ l particle suspension (of each type for separate wells) and incubated for 48 hours at 37°C. A primary fix (2.5% glutaraldehyde solution (TAAB), fresh 10% PFA) was added to the wells for 60 minutes, then rinsed cells were scraped and suspended in a secondary fix (2% aqueous osmium) for a further 60 minutes. Cells were stained in pellet form with 2% uranyl acetate and stored at 4°C overnight. After dehydrating the cells in increasing concentrations of acetone, cells were embedded as pellets in TAAB 812 hard embedding resin and polymerised for 12 hours at 60°C. The resin-embedded samples were sectioned using an RMC PowerTome and mounted onto copper grids. Sections were 100nm in thickness using a diamond knife. Images were taken for each of the samples using a JEOL JEM-1400 Flash electron microscope. Particle characterisation and localisation was described for separate materials and dual-combinations of the materials.

EDS elemental analysis to confirm the uptake and location of materials after 24 hours was conducted by SEM (Oxford Instruments, Oxfordshire, UK). Cells were seeded onto sterile 13mm diameter Thermanox plastic coverslips (5000 cells/ml, 2 ml per well) with 10 μ l of particle suspension for Co, Cr and Ti, (in separate wells) and 3.3 μ l of each particle suspension for a mix of CoCrTi (in a further well). A control sample of cells with no particles was also made. Cells were incubated for 24 hours at 37°C. Cells were fixed in 2.5 % glutaraldehyde and fresh 10% PFA for 60 minutes and then dehydrated in increasing concentrations of ethanol. The plastic cover slips were critical point dried and carbon coated (10nm thickness). Elemental analysis was done using a Tescan S8000 scanning electron microscope with Ultim Extreme and Ultim 170 detectors (Oxford Instruments) and Aztec version 4.2 software.

Results

Metal debris characterisation (failed implants)

Results were collected firstly by observing histological sections of patient tissue using light microscopy, to identify the presence of debris from different types of failed implants. A total of 38 thin/semi-thin sections were analysed. These sections showed varying levels of wear debris within the patient tissue near to the replaced joint (figures 2 and 3).

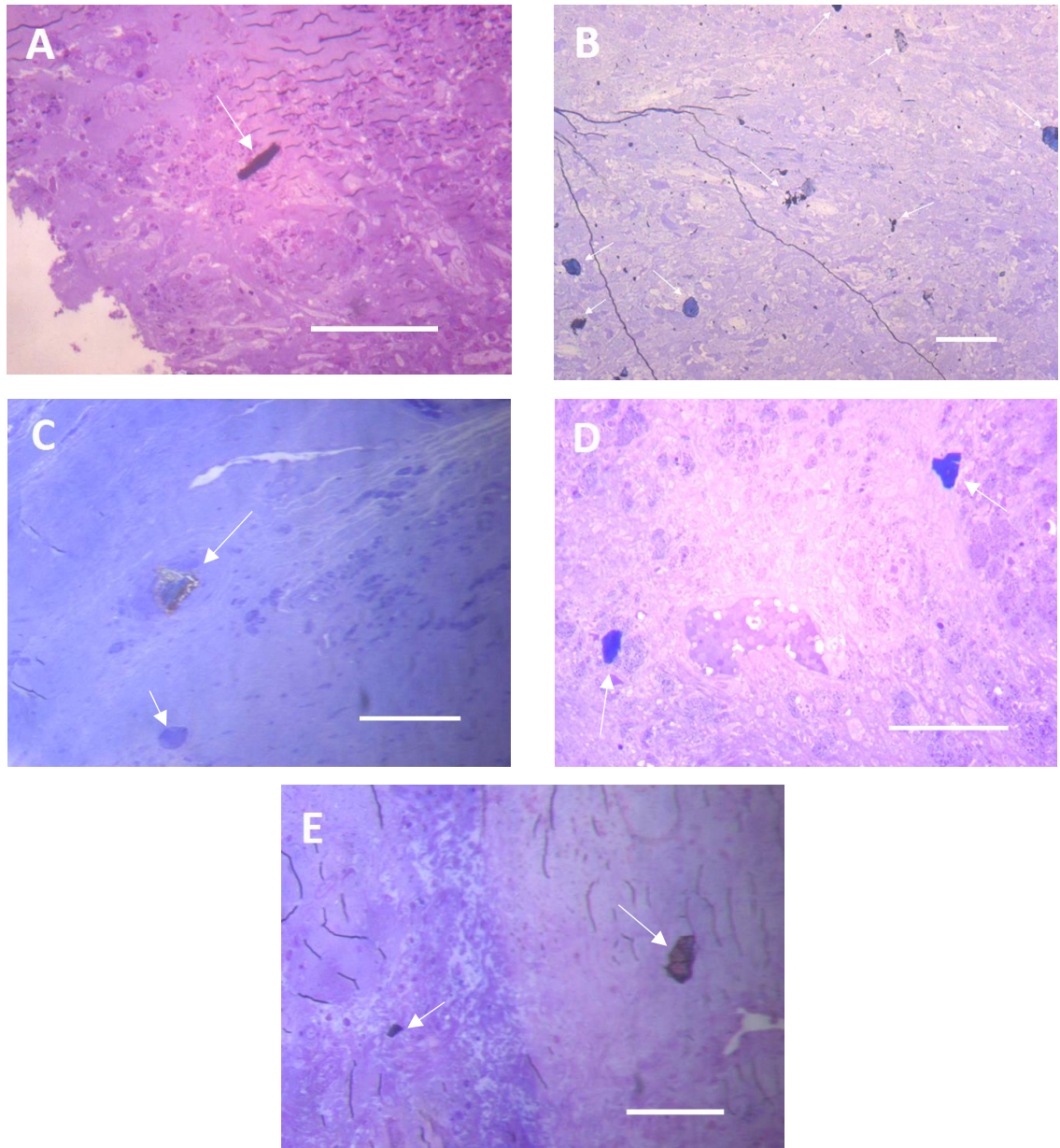


Figure 2. Thin tissue sections showing particles (white arrows) within periprosthetic tissues across different implant types: A- Birmingham THA (MoM); B- Birmingham Hip Resurfacing (MoM); C- Rejuvenate THA (MoP); D- ASR THA (MoM); E- Corin Comet THA (MoM). Scale bar represents 100µm.

In the thin sections of periprosthetic tissues, the particles were clearly defined within the tissue. Particles ranged from 10-100µm in size and all were irregularly shaped, with a few rod-like particles present for all implant types. Birmingham Hip Resurfacing (MoM) had the most particles present in all samples. Most particles across the samples were smaller and dark in colour, with some ranging in lighter colours as layered debris formed aggregates of smaller particles. Particles were lighter in colour for the Rejuvenate THA (MoP) implant type. It was not obvious if there was any presence of nano particles in these samples and identification of cells in relation to the particles was limited.

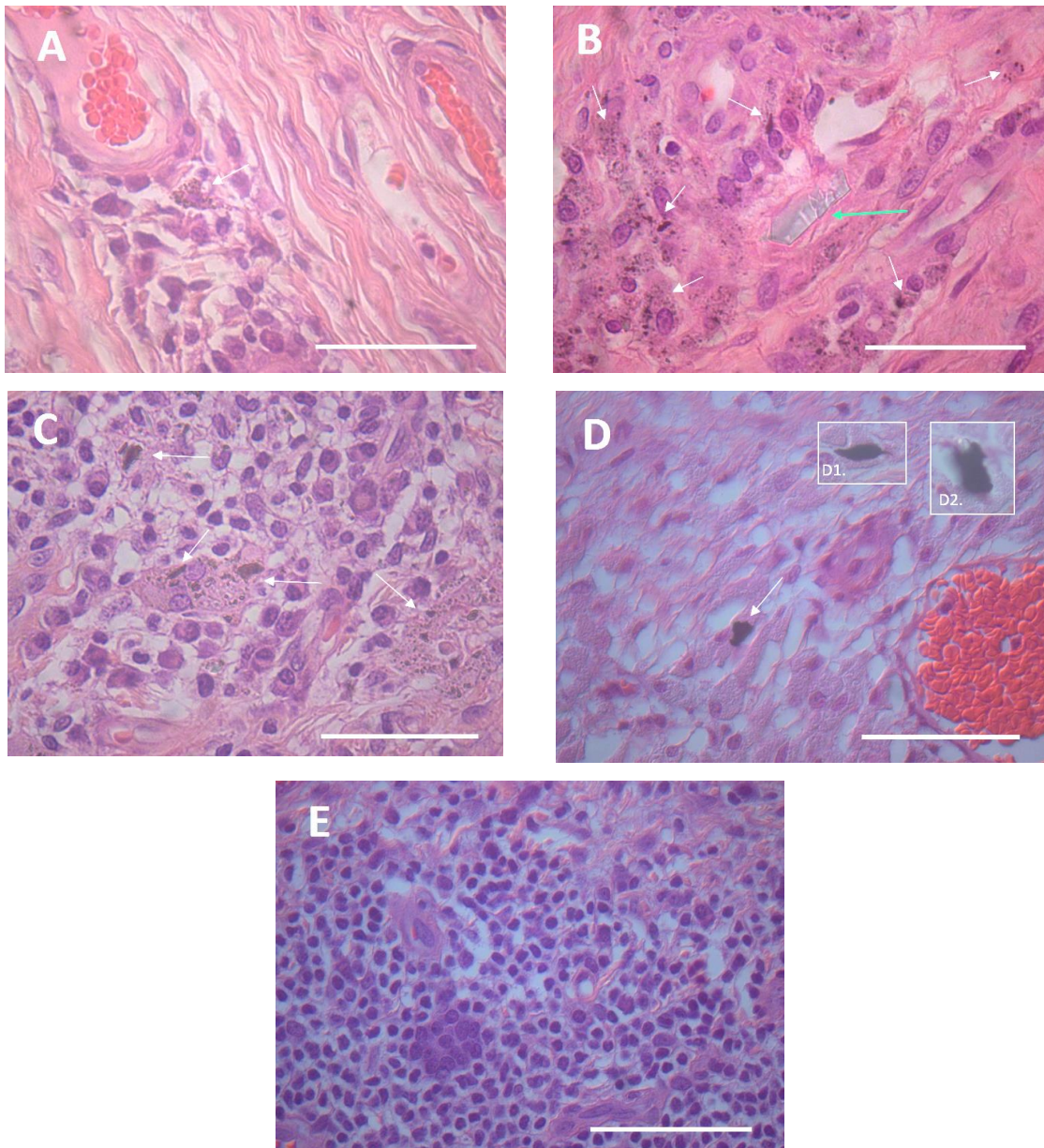


Figure 3. Semi-thin tissue sections showing particles (white and green arrows) within periprosthetic tissues across implant types: A- DePuy Pinnacle THA (MoM); B- Birmingham THA (MoM); C- Rejuvenate THA (MoP);

D- Birmingham Hip Resurfacing (MoM) with particles also shown in inset D1 and D2; E- ASR THA (MoM).

Scale bar represents 50 μ m.

Semi-thin sections revealed sometimes no particles were found where a reaction was clearly seen by the presence of numerous lymphocytes (figure 3, image E), and other sections revealed an abundance of, and sometimes large, particles embedded in the tissues or situated within cells, with a varied lymphocytic response. There were characteristic differences between implant types; three of the implants show micro (and possibly nano) particles within macrophages (figure 3, images A, B and C) with a particle size of <10 μ m. One MoM THA implant type also shows larger 'glass-looking' particles, ranging from <10 μ m to 30 μ m in size (figure 3, image B, green arrow).

TEM analysis was used to observe cell and tissue ultrastructure (figure 4), to further characterise wear debris in more detail and identify nano particles, if present. Patient tissue samples, fixed in resin, were prepared and imaged to reveal intracellular particles from failed implants *in situ*.

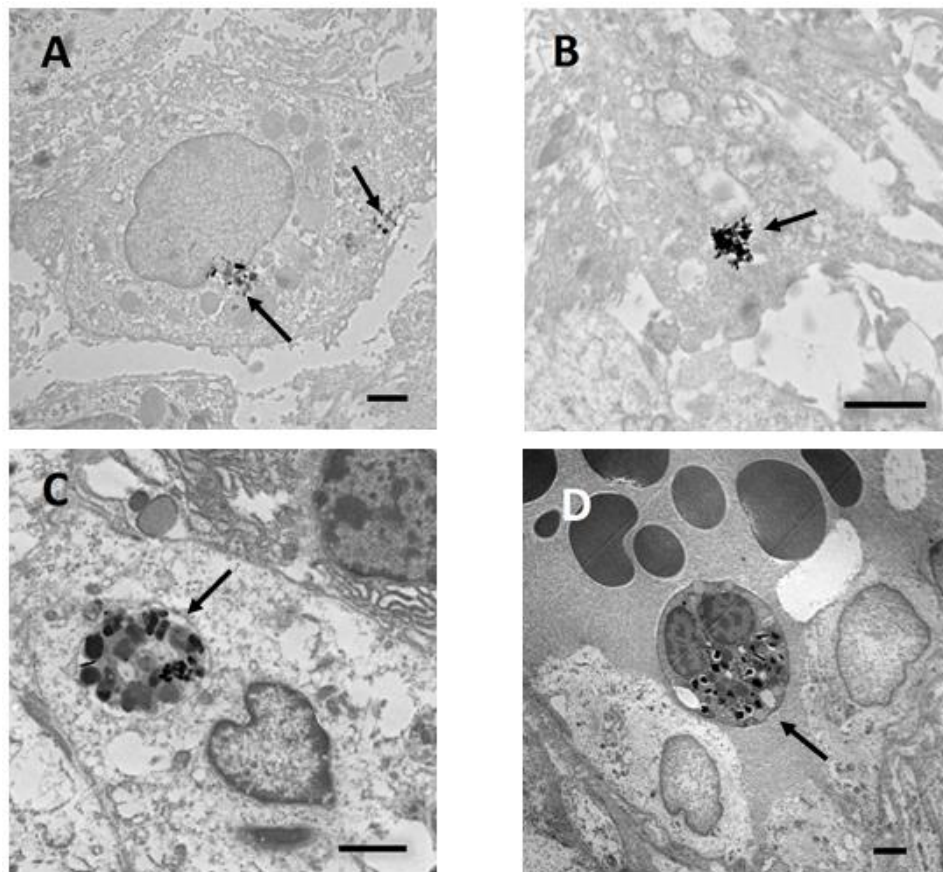


Figure 4. Patient tissues analysed by TEM showing particles (black arrows) within periprosthetic tissues across different implant types: A- Birmingham Hip Resurfacing (MoM); B- Rejuvenate THA (MoP); C- DePuy Pinnacle THA (MoM); D- Birmingham THA (MoM). Scale bar represents 2 μ m.

There were few particles across the four implant types observed, however the most wear debris seen was in Birmingham Hip Resurfacing (MoM) samples. The majority of debris observed across samples was in the form of nano particles (100-500nm), with some larger aggregates of particles. Particles appeared mostly as round to oval shaped, either scattered in the cytoplasm or packaged in phagosomes. The appearance of less electron dense particles suggests some may be secondary particles or there may be some variation in material (figure 4, image C). Little difference in particle characteristics was seen across implant types, and there seemed to be no complex particles that could contribute to reaction rates, affecting implantation times. The implants with the longest implantation time (C: 72 months and D: 66 months) did not correspond to the most particles seen in a sample. Birmingham Hip Resurfacing (MoM), with an implantation time of 47 months, had both the most and the largest particles. Rejuvenate THA (MoP) had the shortest implantation time of 38 months, yet had the least particles.

To explore particles that are released from an implant surface, characterisation of implant wear debris from a MoM LHTHA with CoCrMo adapter sleeve was analysed using SEM techniques (figure 5). Observed in more detail, particle size, shape and surface texture varied within the same sample.

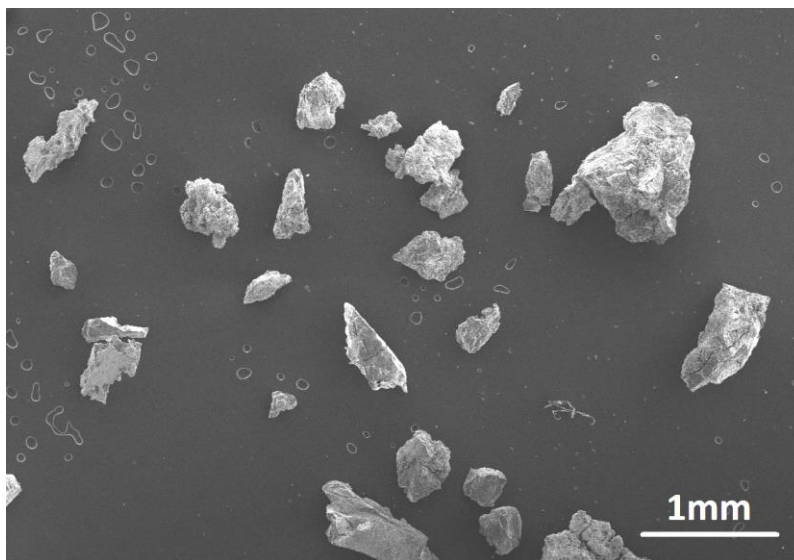


Figure 5. SEM analysis showing irregularity of implant surface particles, loose from a Birmingham THA (MoM) implant at the time of revision surgery. Size, shape and particle surface shows variation.

Micro scale morphological analysis, performed by SEM, revealed the examined particles from this failed implant were largely irregular shaped aggregates, with some biological material. Particle aggregates ranged from 250µm to 1.1mm, with an average of 500µm in diameter.

Surface characteristics varied between particles (figure 6) despite being from the same source, indicating both surface corrosion and aggregates forming from smaller particles on the implant surface. Particle surface characteristics could show solubility of the particles, with a rough, higher surface area leading to greater solubility.

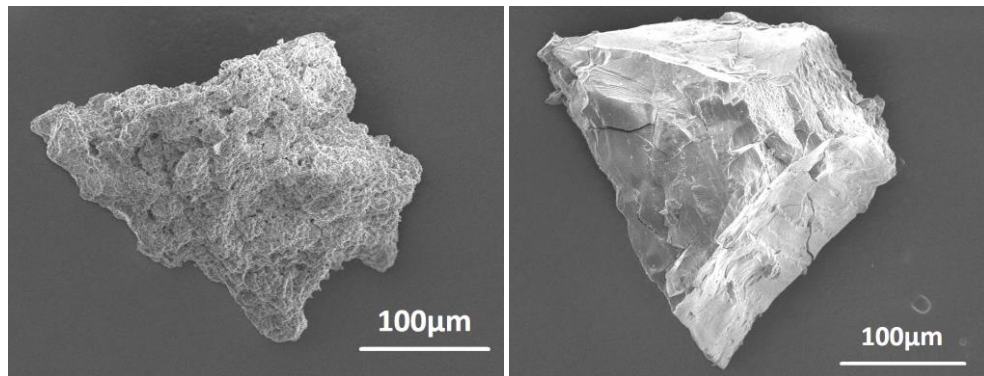


Figure 6. Particles of similar size appeared to have very different morphology. Some appear composed of much smaller particles, leading to a rough surface (left) and some remain larger, with a smoother but cracked surface, as if beginning to break down (right).

As well as the difference in surface appearance from the formation of aggregates or retained smooth surfaces, biological materials on the particle surface was sometimes observed (figure 7). Surface cracks were also common for these particles, though the relationship between the biological debris and surface cracks were not obvious.

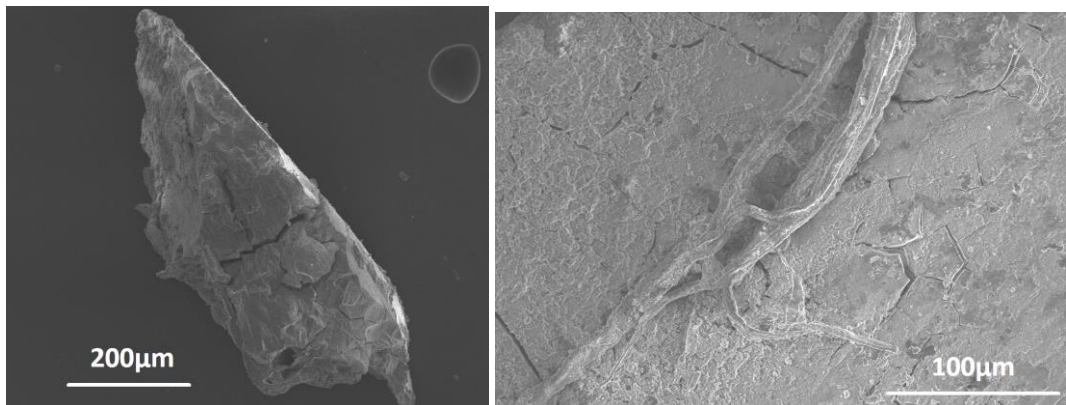


Figure 7. Biological material could be identified on the particle surfaces, either protein coating-like (left) or as long strands that sometimes joined particles together (right).

In some instances, a difference in surface brightness within the particles was seen, which suggests the presence of different materials forming an aggregate (figure 8). This irregularity of surface brightness is due to the different materials being more or less electron dense. Secondary electron

(SE) and backscatter electron (BSE) techniques reveal darker areas (SE) sometime became brighter areas (BSE).

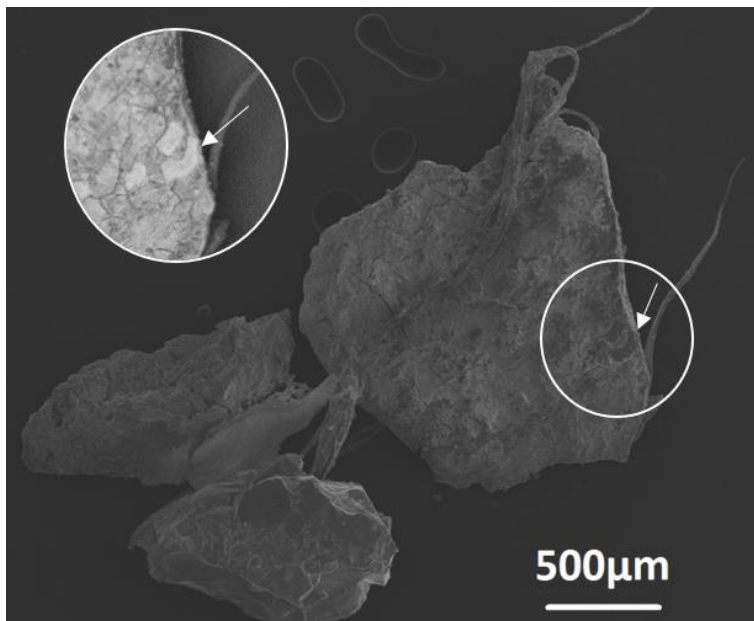


Figure 8. Using both SE and BSE (inset) techniques, the differences in electron density of the materials could be seen, appearing lightly contrasted to the surroundings.

To investigate this further, materials in loose particles from three different implant types (including the sample used for SEM analysis) were analysed for comparison. EDS elemental analysis revealed these particles (Birmingham THA (MoM)) contained mostly Cr, with some Mo and Ti and background amounts of Co present (figure 9).

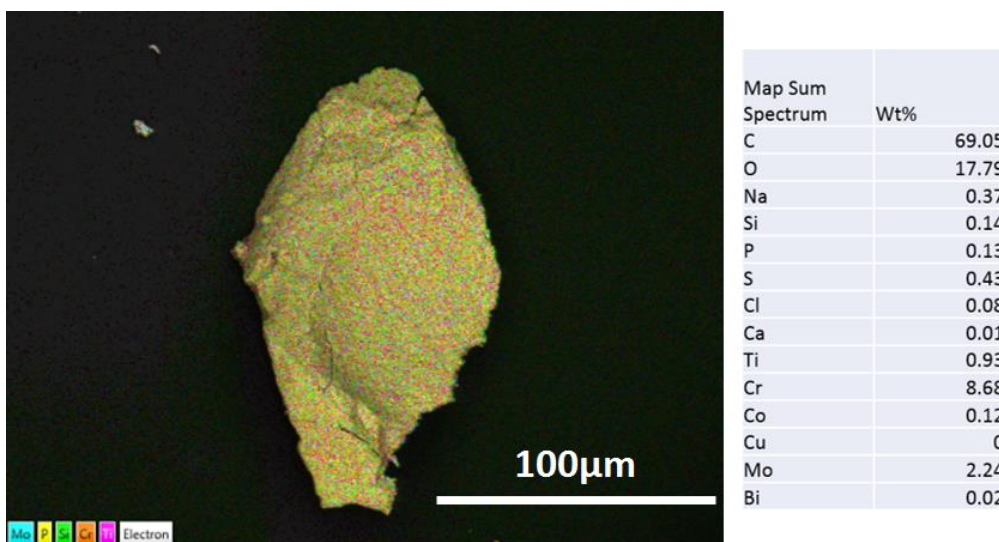


Figure 9. Birmingham THA (MoM) derived particle consisting of Cr, Mo, Ti and background presence of Co.

There was an obvious consistency to materials in this particle, appearing uniform in composition, with different material quantities contributing to the particle. The Rejuvenate THA (MoP) sample had two types of particles: Particles with a smooth appearance mostly contained Cr, with some Co and trace amounts of Mo. Particles with a rough surface mostly contained Co, still with high amounts of Cr content and trace amounts of Mo and Mn (figure 10).

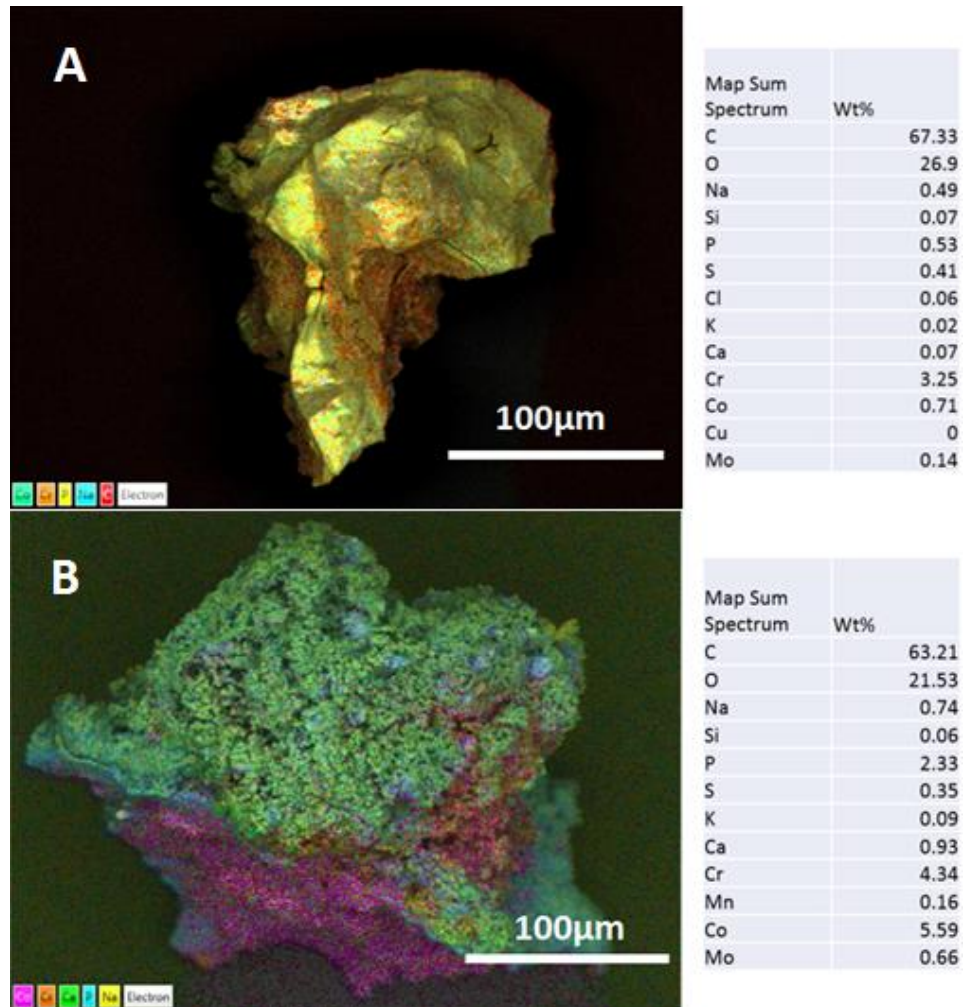


Figure 10. Rejuvenate THA (MoP): A- Smooth particle comprised of Cr, Co and trace amounts of Mo; B- Rough particle comprised of Co, Cr and trace amounts of Mo and Mn.

Some uniformity was seen in the smooth particles but clearly not in the particles with a rough surface. The DePuy Pinnacle THA (MoM) sample had particles containing mostly Cr, with some Mo and Ti and trace amounts of Co (figure 11).

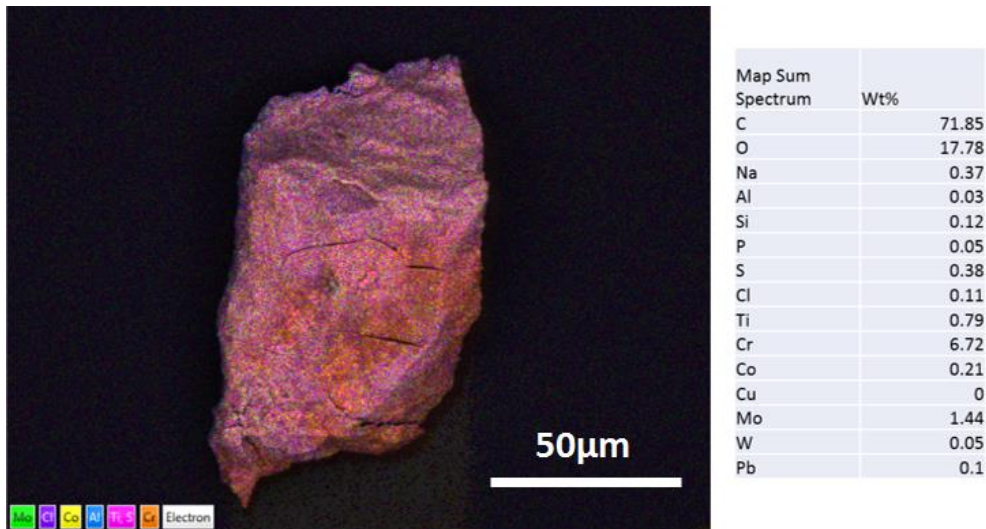


Figure 11. DePuy Pinnacle THA (MoM) derived particle consisting of Cr, Mo, Ti and trace amounts of Co.

These particles appeared consistently smooth on the surface but also had cracks. Though smooth, the particles were not as uniform as other particles for material composition. Overall, EDS elemental analysis of patient-derived particles revealed slight variations between the different implant types. The MoM THA samples were comparable, however the MoP THA sample showed internal variations depending on surface characteristics. The particles observed, when compared to the larger particles seen in tissue examined via light microscopy, are much larger in size but resemble the irregular shapes.

Tissues from a DePuy Pinnacle THA (MoM) were reimaged, using EDS analysis to confirm the materials of patient-derived metal wear debris *in situ* (figure 12).

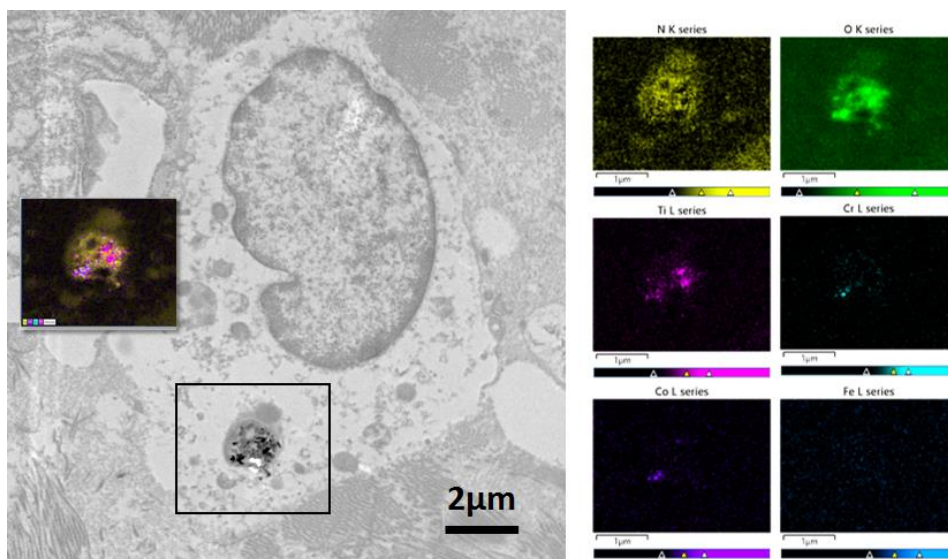


Figure 12. DePuy Pinnacle THA (MoM) elemental analysis showing a phagosome containing mostly Ti particles, with some Cr and Co.

This revealed the majority of metallic materials present were Ti, Cr and Co, supporting the use of the materials chosen for co-culturing with the immune cells. N, O and background Fe were also present.

Metal debris characterisation (mouse tissues)

As part of the characterisation process, different materials were introduced to mouse tissues to observe the cellular response to particle morphology and composition *in situ*. Prepared TEM samples with mouse tissue containing known materials were used to compare any characteristic differences between the materials commonly used in metal implants. Electron microscopy also revealed the localisation of the wear particles within the cells. The characteristic differences between known materials (Co, Cr and Ti) did not vary within the mouse tissues (figure 13).

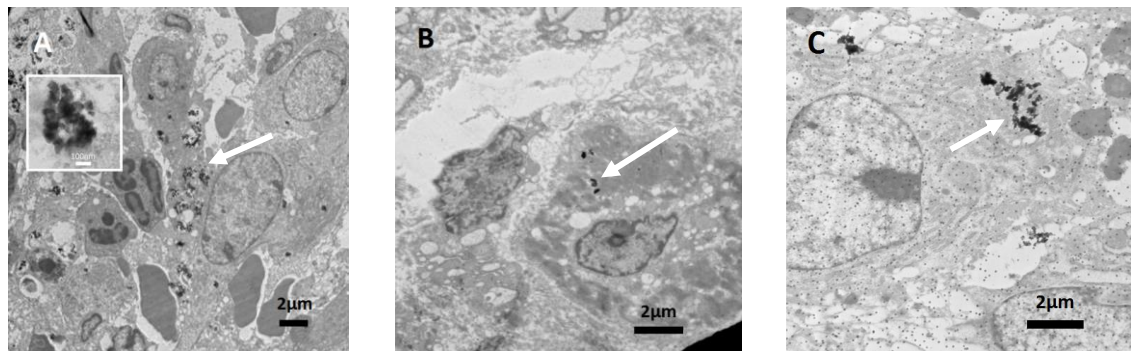


Figure 13. Mouse tissues containing known particles (white arrows): A- Co particles; B- Cr particles; C- Ti particles.

Particles were all electron dense and nano-sized, collected together to partially form aggregates. The localisation of these particles were either within the cytoplasm or within phagosomes. Co particles were more abundant whereas both Cr and Ti particles were less obvious in samples for TEM analysis. Mouse tissues containing all three materials however produced more diverse characteristics (figure 14).

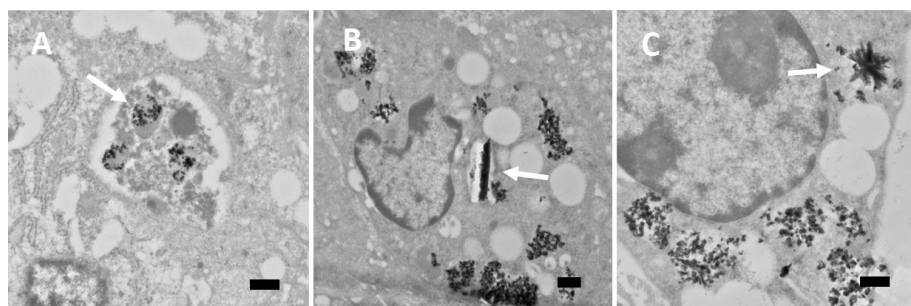


Figure 14. Mouse tissues containing all three materials (Co, Cr and Ti) with more characteristic variation (white arrows): A- particles within a phagosome; B- particles forming a needle shape; C- particles forming a crystalline structure. Scale bar represents 500nm.

Numerous granular particles of 50-100nm in size were collected in phagosomes. Larger, more complex aggregates were also seen in phagosomes. A large needle-shaped aggregate, 1.5µm in length, and a crystalline structure, 700nm in diameter were seen close to the nucleus. These cells also contained the smaller, granular particles in high numbers. Materials comprising the larger structures are unknown, however the presence in these samples suggests mixed materials are capable of resulting in more complex structures compared to individual materials.

Cell population analysis

An analysis of surrounding cells (to the debris) was done, identifying the cell types involved in the inflammatory response and which cell types interact and contain the wear debris. Cell types in semi-thin sections, observed via light microscopy, included lymphocytes, macrophages, giant multinucleated cells, plasma cells, mast cells and neutrophils, all involved in the immune response to the presence of particles (figure 15).

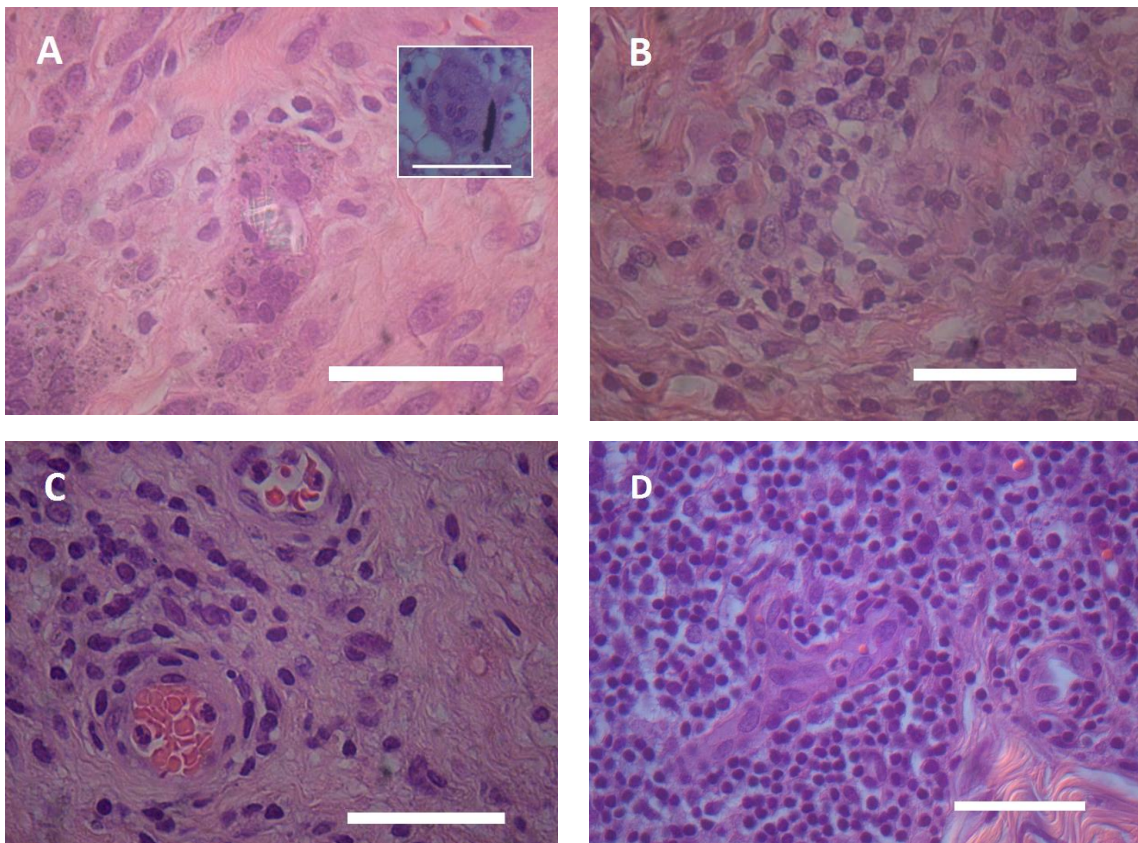
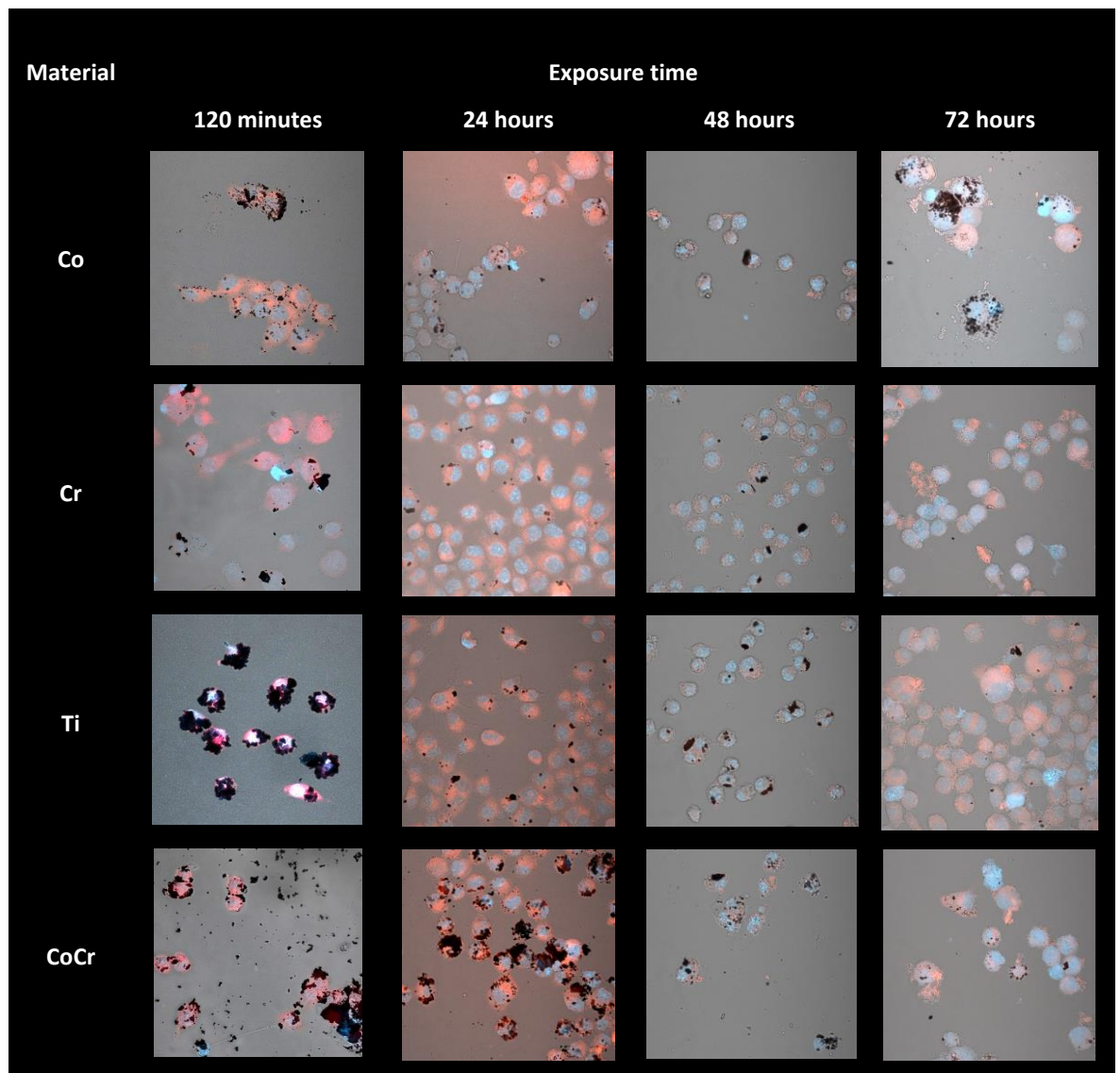


Figure 15. Light microscopy revealed patient tissue samples had the presence of numerous cell types across the implant types: A- Birmingham THA (MoM); B- DePuy Pinnacle THA (MoM); C- Rejuvenate THA (MoP); D- Birmingham Hip Resurfacing (MoM). Scale bar represents 50µm.

Of the cell types identified in tissues containing particles, only macrophages and occasionally giant multinucleated cells showed an uptake of the particles. Interestingly, small blood vessels were frequently seen near to the particles. This supports the notion that blood vessels are formed in tissues affected by the implant debris, particularly in cases where pseudotumours are formed.

Cellular response to commercial metal particles

By introducing commercial particles of known materials to the immune cell type (macrophages) identified to uptake particles in patient tissues, the response to the different materials could be partially observed *in vitro*. To create a timeline of particle-cell interaction, particles of the different material types (Co, Cr, Ti separately and Co/Cr, Co/Ti, Cr/Ti in combination) were co-cultured with macrophages and fixed after 120 minutes, 24, 48 and 72 hours for confocal analysis (figure 16).



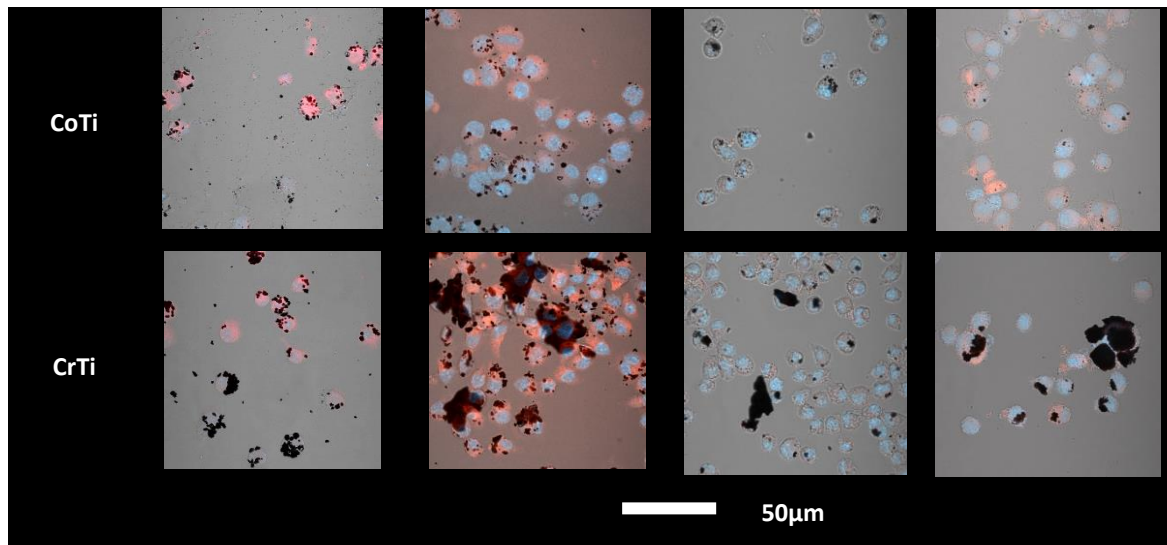


Figure 16. Timeline of particle-macrophage exposure: For each material or dual-combination, particle uptake after 120 minutes and longer term exposure at 24, 48 and 72 hours.

Initial uptake of the different materials showed Co was more soluble after 120 minutes, with internalised particles clearly seen. Ti particles smothered the macrophages, though particles appeared to be on the cell surface. Smaller Cr particles were beginning to internalise but mostly remained outside of the cells. Longer term exposure to particles revealed Co was mostly in small spherical intracellular areas, apart from a few larger particles, after 48 hours. 72 hour exposure showed even the larger Co particles were enveloped by the macrophages, however there was also an increase in smaller particles outside of the cells. Cr particles were slower to enter the cell, in part due to larger sizes, and again there was an increase in particles outside of the cells after 72 hours exposure. Ti particles of all sizes were readily up-taken by the cells. An increase in particles outside of the cells occurred after 72 hours exposure, which for Ti seemed a high increase due to the particles proximity to the cellular surface in shorter-term exposure. Dual-combinations of materials showed CoCr slowed for initial up-take of particles, were pretty consistent after 24 and 48 hour exposure and then were scarce for 72 hours exposure. CoTi particles were mostly up-taken until 72 hours exposure where a small increase outside of the cells was seen. CrTi had larger particles not up-taken but otherwise particles were seen inside cells, until 72 hour exposure showed larger particles still remained outside the cells. For all materials, after 48 hour exposure cells clearly had intracellular particles localised to small, spherical areas.

As the amount of particles varied in the samples for imaging, the number of particles within 30 randomly selected cells were counted to be compared to the number of particles remaining outside of the cells, creating a percentage of particles not up taken by the cells for each time point (figure 17).

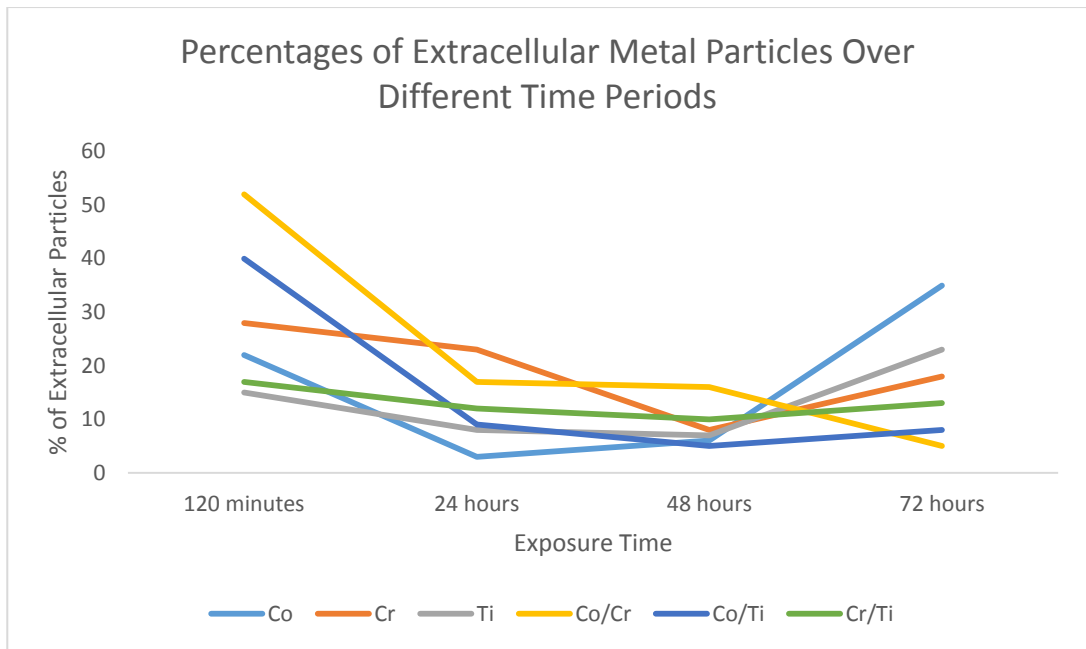


Figure 17. Percentages of particles remaining in the extracellular environment compared to particles within 30 cells, per material/dual-combination type, over different time periods.

The percentage of particles remaining outside the cells, for each of the materials, showed initial uptake of particles increased between 120 minutes and 24 hours, and for Cr a delayed uptake mostly occurred between 24 and 48 hours. After 24 hours, extracellular Co particle percentage increased. In contrast, CoCr particles outside the cells decreased over the whole timescale. For all other materials or combinations, an increase in particles seen outside of the cells occurred after 48 hours.

Manufactured particles used to co-culture with macrophages were of mixed sizes, confirmed via SEM analysis (figure 18).

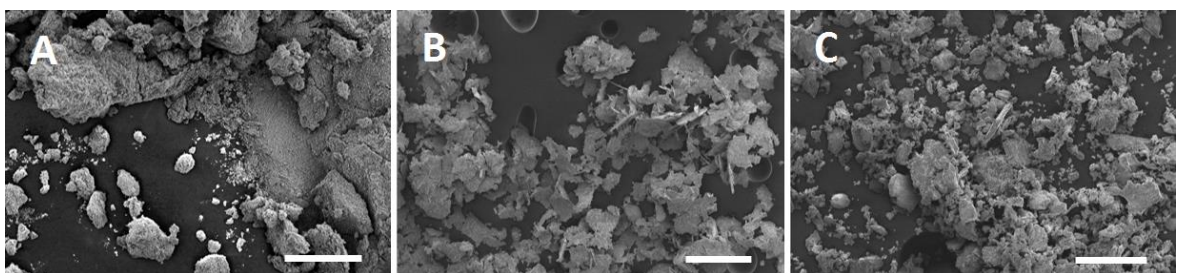


Figure 18. SEM micrographs of commercial particles used to co-culture with macrophages: A- Cobalt particles; B- Chromium particles; C-Titanium particles. Scale bar represents 100µm.

The particle size ranged slightly because the materials formed larger agglomerations (>10µm). Cobalt particles were <1µm but were capable of forming micron-sized aggregates. Chromium particles formed larger flakes and titanium appeared as nano particles, flakes and aggregates.

With mixed sized aggregates present, the commercial particles were smaller than the particles from an implant surface and similar in size to the particles seen in patient tissues.

Clearly defined intracellular localisation of particles across samples was apparent after 48-hour exposure, identified via confocal microscopy. 48 hours was therefore used for TEM fixation and analysis to observe the intracellular localisation and characterisation of particles in more detail (figures 19 to 24).

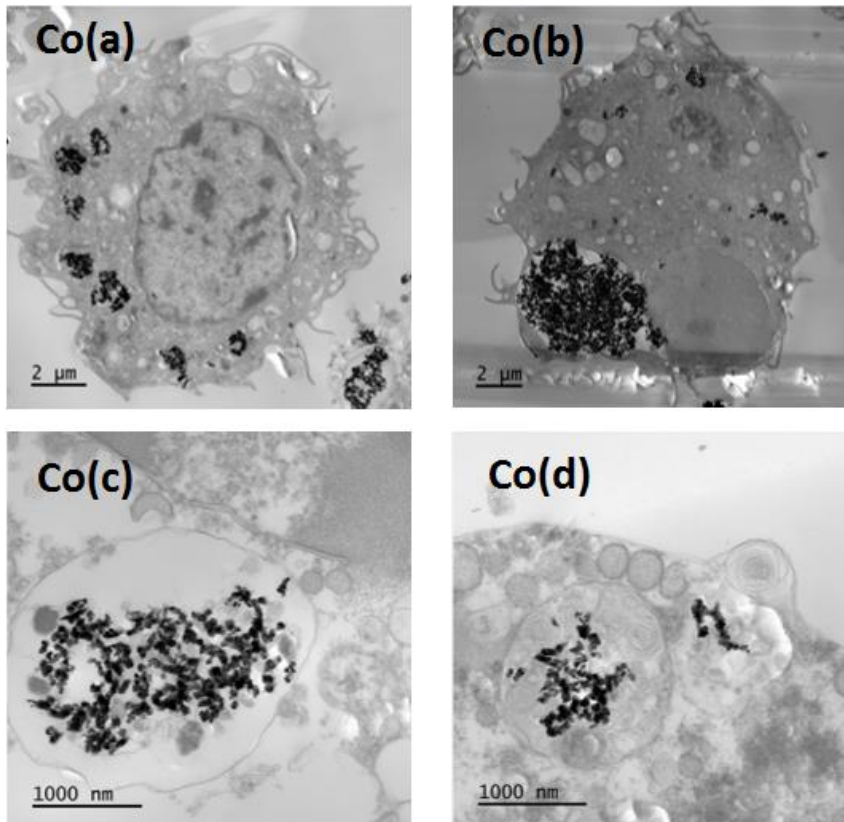


Figure 19. Intracellular characterisation and localisation of Co particles after 48 hour exposure.

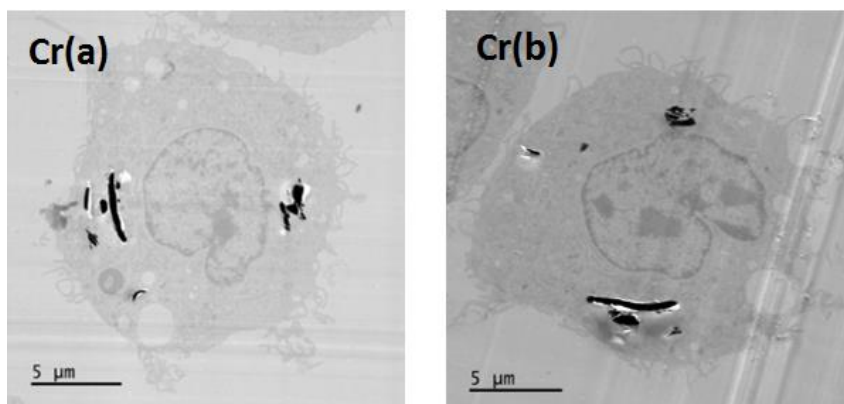


Figure 20. Intracellular characterisation and localisation of Cr particles after 48 hour exposure.

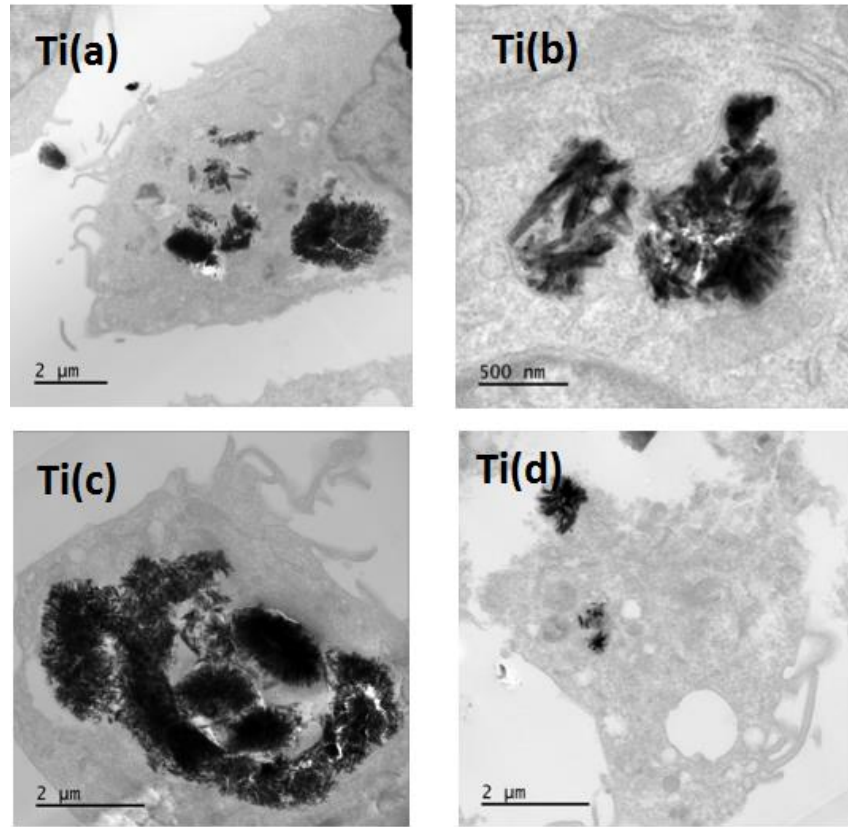


Figure 21. Intracellular characterisation and localisation of Ti particles after 48 hour exposure.

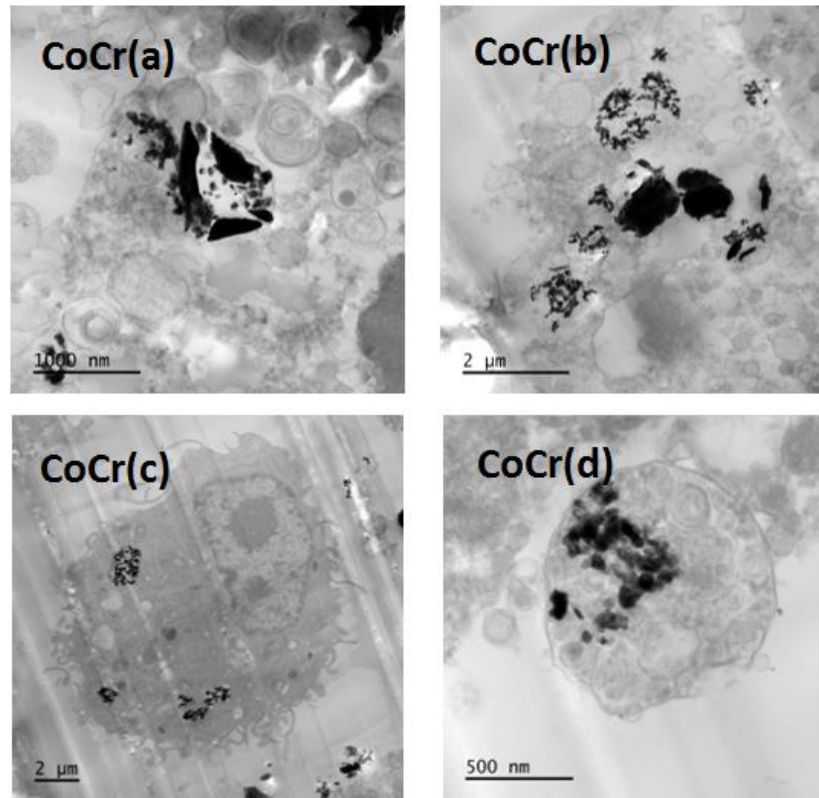


Figure 22. Intracellular characterisation and localisation of CoCr particles after 48 hour exposure.

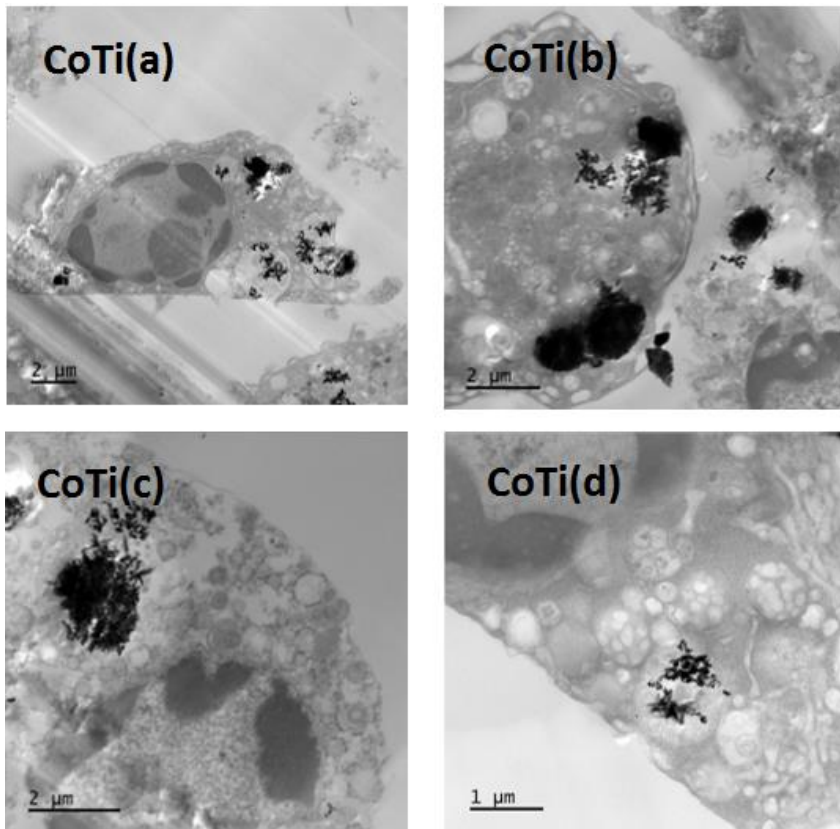


Figure 23. Intracellular characterisation and localisation of CoTi particles after 48 hour exposure.

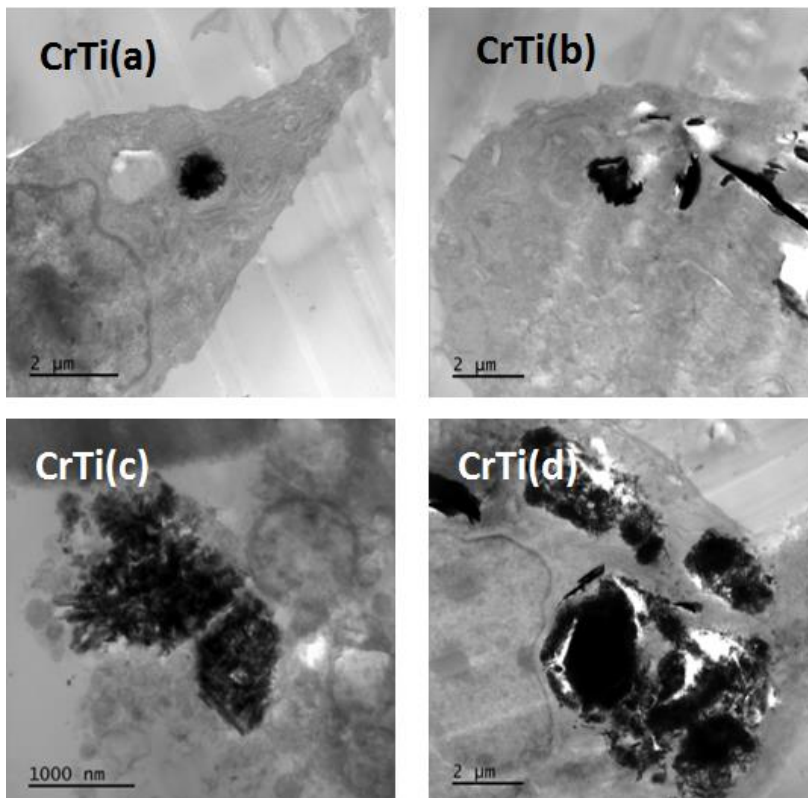


Figure 24. Intracellular characterisation and localisation of CrTi particles after 48 hour exposure.

Intracellular particle characterisation and localisation differed between materials. Co particles were collected in phagosomes, sometimes in larger quantities (figure 19, image Co(b)). All particles were of nano size (~50nm) and were electron dense. Cr particles remained in larger, elongated forms (200nm- 5µm) within the cytoplasm. Large phagosomes could be seen within the macrophages but not containing the particles. Ti particles were abundant when identified inside cells, forming agglomerations of smaller, needle-like particles (100nm- 700nm) until gross amounts were seen (figure 21, image Ti(c)). Crystalline structures formed, similar to those identified in mouse tissues with mixed particles. Not all particles appeared to be stored in phagosomes. Dual-combinations revealed the combined characteristics of particles and localisations comparable to the individual materials. Wherever Ti was present, so were needle-like particles.

EDS elemental analysis also confirmed the uptake of particles after 24 hours, for Co, Cr and Ti particles (figure 25), as well as a combination of all three materials (figure 26) as dual-combinations did not reveal any specific differences between combinations.

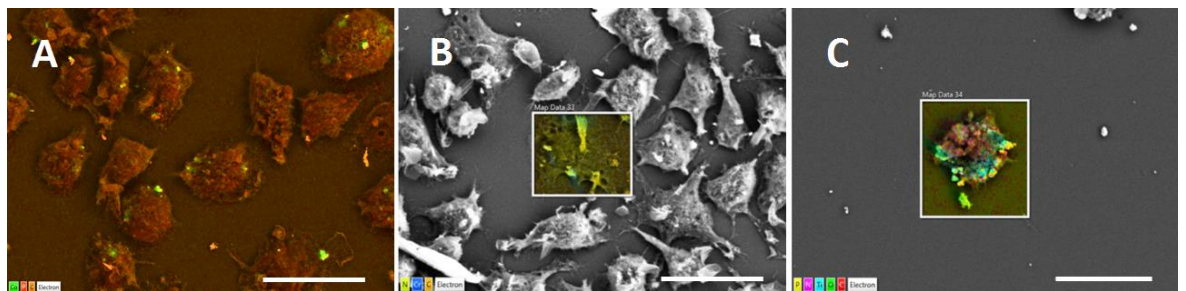


Figure 25. Cellular interaction between metals and macrophages: A- Co particles; B- Cr particles; C- Ti particles. Scale bar represents 25µm.

These showed that for each material there was a clear uptake by the macrophages. Co and Cr particles were mostly up-taken, with some surface particles and few extracellular particles. Cells appeared healthy compared to those seen in Ti samples, where very few cells were observed and cells were in their smaller, round state.

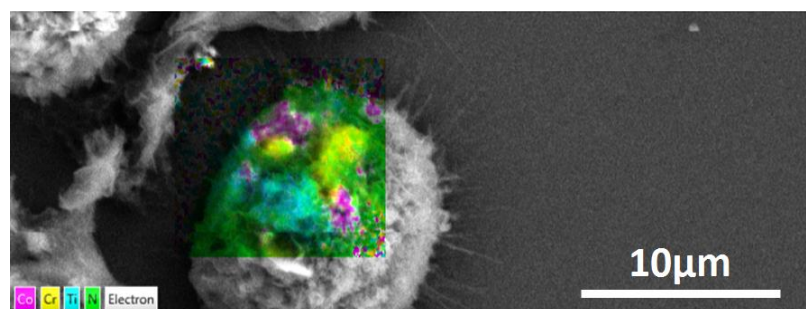


Figure 26. Cellular uptake of Co, Cr and Ti particles in macrophages.

Appearance of macrophages varied. Cells that showed some uptake of Ti were consistently in their smaller, spherical state. All had an irregular surface, surface ruffles, thin projections (filopodia and lamellipodia), and sometimes phagocytic craters (figure 27).

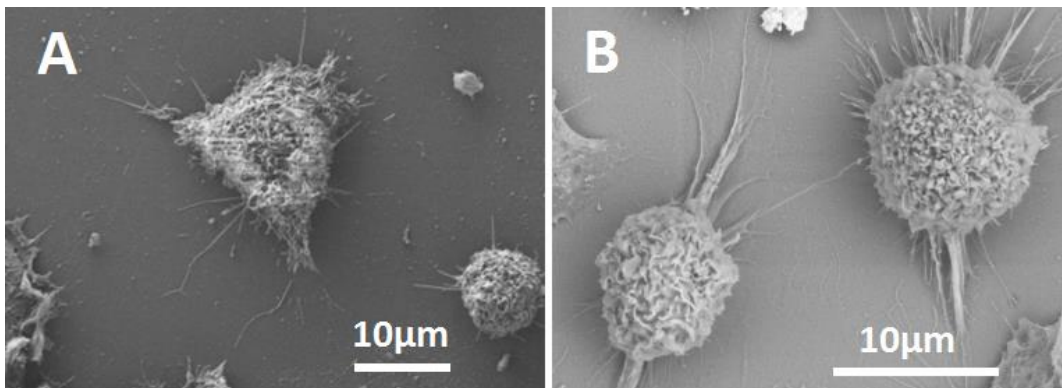


Figure 27. SEM macrophage characteristic variations: A- spread out macrophage (centre); B- small, round macrophages.

Discussion

It was hypothesised that both the characterisation and composition of wear debris contributes to the macrophage-mediated pro-inflammatory response seen in patients, with large, irregular particles being slow to uptake and internalisation of particles also being affected by material type. Overall, the results collected supported the theory, with complex, large particles being found in periprosthetic tissues across implant types and larger particles were shown to be slow to uptake *in vitro*. Analysis of particle presence in periprosthetic tissues via light microscopy revealed particles ranged in size across the implant types in thin tissue sections. All were irregularly shaped and were clearly defined in the tissues, with some rod-like particles. The majority of particles were 10-100µm in size and dark in colour. Birmingham Hip Resurfacing (MoM) had the most particles present and although few particles were seen, the Rejuvenate THA (MoP) had the largest particles, lighter in colour. Semi-thin sections showed noticeable characteristic differences between particles produced across the implant types, identified into two main categories. Micro and possibly nano particles in macrophages <10µm appeared in DePuy Pinnacle THA (MoM), Birmingham THA (MoM) and Rejuvenate THA (MoP) samples. Smaller, numerous particles are known to be comprised of cobalt-chromium-molybdenum, originating from the bearing surface (articulating surfaces), generated by adhesion or abrasion (Perino *et al*, 2018). Some of the debris may also be Ti particles that originate from non-articulating surfaces, forming micron-sized flakes (Topolovec *et al*, 2013). Birmingham THA (MoM) also had large 'glass-looking' particles, termed microplates, embedded in the tissue. Previous research suggests these are generated from corrosion of the cobalt-chromium metallic adapter sleeve between the ball and neck of the implant, producing less frequent corrosion particles comprised mainly of Cr (Perino *et al*, 2018). In the samples observed, microplates ranged from <10µm-30µm in size. Large, dark, irregular shaped particles were seen for Birmingham Hip Resurfacing (MoM) samples. Particle localisation, size and shape identified on a much smaller scale using electron microscopy revealed few particles were present across the samples from different implant types. The majority (and largest) of particles were seen in Birmingham Hip Resurfacing (MoM) samples. Across implant samples, round to oval shaped nano particles (100-500nm) were found either scattered in the cytoplasm or packaged in phagosomes. Secondary particles, or possibly different materials, were seen as less electron dense particles within the phagosomes. More complex particle characteristics were not seen in any of the samples for all implant types apart from Birmingham Hip Resurfacing (MoM). Of the samples analysed, the longest implantation time did not correspond to the most particles observed in tissues, whilst Rejuvenate THA (MoP) had the shortest implantation time and yet had the least particles in examined sections.

No particles were seen in ASR THA (MoM) samples, though there was a clear lymphocytic response. The absence of wear debris indicates hypersensitivity, though reactions with high wear, as seen in other implant types, suggests reactions could be multifactorial due to both the inflammation from the presence of wear debris and metal hypersensitivity. This is supported by histopathological findings in periprosthetic tissues, revealing both a non-specific foreign body response (macrophage presence) to the particles and an adaptive immune response (lymphocyte presence) otherwise described as a type IV hypersensitivity response. Birmingham Hip Resurfacing (MoM) had the highest lymphocytic infiltrate in response to particle presence. Cell population in semi-thin sections identified lymphocytes, macrophages, giant multinucleated cells, plasma cells, mast cells and neutrophils in response to the presence of debris. Neutrophils usually occur in septic loosening, and numbers were not significantly high, but the presence was noted in samples from Rejuvenate THA (MoP) implants. Macrophages and occasionally giant multinucleated cells contained the particles in all cases where particles were seen inside a cell. Birmingham THA (MoM) had the largest particles and so giant multinucleated cells up-took those particles as they were too large for single macrophages to uptake. The uptake of larger wear particles and formation of multinucleated giant cells has been thought to contribute to osteolysis (Santavirta *et al*, 1990). If this is the case, the formation of larger particles could increase the risk of aseptic loosening. SEM analysis of multiple particles (average of 500µm) from a Birmingham THA (MoM) implant showed surface characteristics varied between smooth, sharp surfaces and irregular, rough surfaces, indicating a difference in corrosion/origin or formation of the particles, and possibly a difference in solubility of the particles. Surface cracks appeared on smoother particles, characteristic of larger particles breaking into smaller particles. Biological material was also seen adhered to the surface of smoother particles, which may be indicative of surface proteins. Larger wear particles in patient tissues may be aggregates of smaller particles, admixed with biological substances, creating a protein corona referred to as 'corrosion products' instead of implant wear. The response to both implant wear (larger particles generated from the implant) and corrosion products may be the same, however material differences may alter the response. The size and shape of corrosion products have been known to influence the biological response, different to the nanoparticles in their original, smaller forms, where larger, complex corrosion products influence an increase in macrophage and giant multinucleated cell reaction (Caicedo *et al*, 2013). This formation of corrosion products includes extracellular serum proteins, forming a surface layer on the particles. This protein-nano particle complex can be recognised by receptors on the macrophage cell surface for cellular uptake. However, the protein surface structure can be altered, so the same protein corona can bind to a range of cellular receptors. Once inside the cell,

the protein coating remains, influencing the cells response to the particles (Fleischer and Payne, 2014). This would suggest formation of aggregates improves the uptake of larger particles, which in turn could increase the phagocytosis of particles or formation of multinucleated giant cells, influencing the negative effects associated with these factors.

The presence of lymphocytic infiltrations, with or without particles also present, suggests hypersensitivity to the implant wear products may also be towards phagocytosed materials. The appearance of no particles in tissues could be due to nano particles not being seen at a lower magnification, and the particle material found at higher magnification is most responsible for this reaction. EDS elemental analysis of patient tissues revealed Ti, Cr and Co nano particles in a phagosome for DePuy Pinnacle THA (MoM) sample. Considering the highest content found in the phagosome was Ti, in large particles from the implant surface there was little Ti. This suggests Ti is retained longer, as Cr and mostly Co are phagocytosed and so are less present. Elemental analysis of particles, loose on the implant surface, from a DePuy Pinnacle THA (MoM) implant showed large particles contained mostly Cr, Mo, some Ti and trace amounts of Co. Likewise, Birmingham THA (MoM) contained Cr, Mo, Ti and background Co in very uniform particles. Rejuvenate THA (MoP) particles comprised of Co, Cr, trace amounts of Mo and Mn for particles with a rough surface, and smooth particles comprised of Cr, Co and trace amounts of Mo. In future research, it would be valuable to compare the elemental composition of these loose particles with the composition in the intracellular environment, after particles are exposed to macrophages. *In situ* tissue analysis with elemental composition has been conducted by Xia *et al* (2017), using similar implant types to this study. Samples were analysed for particle size, shape, distribution and elemental composition. Needle particles were comprised of Cr₂O₃ and Co and large, irregular particles also contained Ti. Nano-sized Cr₂O₃ particles were the most present in tissues, further suggesting phagocytosis of Co and subsequent release of Co ions influences the adverse reaction (Xia *et al*, 2017). Loose particles for Birmingham Hip Resurfacing (MoM) weren't analysed but particle composition in tissues has been previously identified as mostly Cr (Hart *et al*, 2010). Perivascular lymphocytic infiltration has been identified where there are lower Cr levels in periprosthetic tissue, compared to those with no perivascular lymphocytic infiltrate, suggesting Cr is less bio reactive despite being retained as larger particles in the tissue, associated with more inflammation. In comparison, Co is easily phagocytosed and where produced ion concentrations are the highest so is the associated lymphocytic infiltration (Lehtovirta *et al*, 2018). CoCr wear debris and Co ions co-cultured with lymphocytes increases lymphocyte number and cytokine production (Posada *et al*, 2015). The influence of CoCr particles and Co ions could therefore contribute to both a macrophage-mediated pro-inflammatory response and a lymphocyte-

mediated pro-inflammatory response, though the influence of just Cr needs to be investigated. Blood vessels were also frequently seen near particles, with Rejuvenate THA (MoP) samples having the most blood vessels identified among the samples. The presence of blood vessels supports the idea that a hypoxic environment in the tissue samples is created in response to the presence of particles. In a study by Madathil *et al* (2010), fibroblast cultures exposed to CoCr particles, hypoxia regulated gene products are over-expressed and this creates a cellular oxidative stress response in conditions that would be normal without the presence of particles (Madathil *et al*, 2010). Co particles in particular are thought to be the main contributor to the increase in endothelial cells and vasculitis seen in tissues (Ninomiya *et al*, 2014). Overall, particle material, specifically Co, is capable of inducing a hypoxic response and inflammatory response, presented as increased vasculitis and lymphocyte infiltration.

Mouse tissue confirmed materials were either in the cytoplasm or phagosomes, comparable to patient tissues. Co was more abundant in tissues, whilst Cr and Ti particles were less present for characterisation. All three materials mixed together created complex structures, including needle and crystalline arrangements inside the cell. With little difference between the material types until there was a mixture of materials, there may be some influence between the materials on the formation of these larger, more complex particles, and therefore on macrophage response. Given the known carcinogenicity of Co in animal studies, there was little evidence of a severe reaction in these samples, though Co was more abundant and had the potential to produce high ion concentrations associated with tissue inflammation. To replicate these results *in vitro*, cellular response to commercial particles was analysed from creating a timeline of material uptake. SEM analysis confirmed the size and structure of commercial particles before being introduced to macrophages and then confocal analysis revealed the uptake of the different materials, or dual-combinations of materials, until possible phagocytosis and apoptosis was seen. Co was more soluble, indicated by nano particle formation within the acidic environment of phagosomes, but apoptosis occurred after 24 hours so extracellular particle number increased in longer-term samples. Ti particles initially smothered cells, which readily up-took all sizes by 24 hour exposure, whereas Cr was slow to uptake, with larger particles still remaining after 72 hours. For most of the materials, apoptosis occurred after 48 hour exposure, so extracellular particles after this time point may have been secondary particles from necrotic cells, or they may have remained as undigested particles. CoCr particle exposure was the only sample showing a consistent uptake over time. TEM analysis of 48 hour particle exposure for Co, Cr, Ti and dual-combinations revealed the intracellular localisation and characterisation of particles. This discovered cells could uptake particles to gross amounts. Within the cells, large aggregates of particles could be collected in

larger phagosomes, as opposed to a number of smaller phagosomes containing fewer particles. Macrophages are capable of enveloping particles to a toxic level, as identified by Xia *et al* (2011), which can affect other nearby cells (Xia *et al*, 2011). Similar to patient and mouse tissue samples analysed, not all particles were seen within a phagosome. Co particles were present as nano sized within phagosomes, however particle characteristics differed for other materials. Ti particles were dense aggregates of needle-like nano particles, not always in phagosomes, and Cr was largely elongated micron-sized particles in the cytoplasm. Dual-combinations of materials showed particle characteristics and locations were comparable to the individual materials, so there did not seem to be any influence between materials on the uptake, characterisation and intracellular localisation, as seen in mouse models. The same crystalline structures were observed in mouse tissues with mixed particles and titanium treated cells however, predicting that these structures form *in situ* from Ti particles. Bioreactivity to Ti is normally less when compared to CoCr. Nevertheless, the formation of needle-like particles in this pattern and the indicated retention of Ti in patient cells from elemental analysis suggests Ti may be able to increase reactivity. It may be the formation of this structure that causes the retention. This encourages the question of whether various morphological features (size, shape or material) have an impact on macrophage behaviour, particularly the pro-inflammatory cytokine release. Further investigation is needed, following the identification of these materials *in vitro*.

EDS elemental analysis confirmed the uptake of particles after 24 hour exposure for Co, Cr, Ti and CoCrTi. Morphology of macrophages were reported similarly to previous research, with an irregular surface and thin projections. SEM analysis after 24 hour exposure to nanoparticles disagreed with findings from Wang *et al* (2016), who found cells mostly in their smaller, round state in response to Co (Wang *et al*, 2016) but findings did agree Co is capable of reducing macrophage motility, seen by increased cell spreading, enhanced cell adhesion and the formation of podosomes (involved with extracellular matrix degradation) (Xu *et al*, 2018). Elemental analysis confirmed Ti particles effected the cells most. A combination of materials further showed cells that uptake Ti particles are consistently in their smaller, round state. It is therefore suggested further investigation is needed to discover Ti particle contributions to cytokine production. For a mix of materials, the formation of a protein corona could explain why initially the separate materials remained separate internally, as the cells had not yet digested the protein layer to begin degrading the particles. The particles were suspended in media with bovine serum, so there may be bovine serum proteins on the surface. A comparison between this response and a response to isolated particles from the implant surface could investigate protein contributions in the future.

It is thought the implant type influences particle characteristics over time, particularly quantity and size, however Catelas *et al* (2006) determined implant design had less of an impact on the particle characteristics than implantation time did. For MoM implants, Cr₂O₃ particles are produced short-term and larger CoCrMo, sometimes needle-shaped, particles are produced after a longer period of time (Catelas *et al*, 2006). Therefore, this difference in particle number and morphology may vary between the patient tissues due to initial implant wear and individual patient response over time. Complications arise when the association between metal debris in periprosthetic tissue, whole blood metal ion concentrations and histological characteristics correlate poorly, implying it is not just particle composition that is responsible for a reaction (Lehtovirta *et al*, 2018). The presence of a severe inflammatory reaction in histological findings, with varying degrees of metal debris/ion concentrations, suggests the variability is down to patient susceptibility. Poor correlation between findings could be implantation time-dependent, as over time the tissues may maintain some wear products, indicating longer term wear, whereas in blood the concentration levels indicate the amount of more recent wear (Lehtovirta *et al*, 2018). As well as looking at developing improved implant surfaces to produce either fewer larger particles or smaller particles, not knowing which is more biologically significant, for patients with implants prone to failure there are biological targets for treatment. These include preventing the drop of pH levels and thus reducing phagocytosis of the particles, interfering with the interaction between macrophages and wear debris (Haynes *et al*, 1996; Wang *et al*, 2016). In the presence of particle-induced osteolysis, the role of macrophages can be targeted for treatment to prolong the life of the implant. Targeting macrophages localising to the site of the implant, modifying macrophages to an alternate phenotype to encourage anti-inflammatory reactions and tissue healing, and inhibiting transcription factor NF-κB are all ways in which treatment can be used to stop the cascade that eventually leads to aseptic loosening (Goodman *et al*, 2014).

The analysis of these implant types is not unnecessary, as thousands of patients still have these implants and adverse reactions to implants presents an ongoing problem for patients. Research contributes to the development of future implants, noting the negatives of implant types (focusing on implant wear and bio reactivity). This is not just confined to hip implants, as knee implants also suffer from implant debris, producing fewer submicron particles than hip implants and producing larger irregular particles more frequently (Schmalzried *et al*, 2002). Future toxicology studies can be done for particles, such as those derived from patients, which are more relevant as are generated by implants and not commercially. Live cell imaging of particle uptake and phagocytosis may also be beneficial. Whilst imaging observes particle-cell response, the presence of inflammation can be further concluded by looking at the inflammatory cytokines

released in response to different particles to determine which is more biologically significant. When introducing CoCrMo and Ti particles to macrophages, osteoblasts and fibroblasts, the former was more toxic, decreasing cell viability and proliferation and also producing IL-6, IL-8 and TNF α across the cell types (Dalal *et al*, 2012). This provides some indication of pro-inflammatory reactions in response to particles, though does not explain why Ti was found to effect macrophage appearance the most. Nor do previous studies include results for implant-derived particles with specific characteristics identified in this study.

Limitations to the study were acknowledged. When counting the number of particles for graphical data, the samples varied. The total number was difficult to determine accurately due to the particles either overlapping or varying in size/becoming aggregates. All sizes were taken into consideration and weren't omitted from the count, and the randomly selected cells mostly contained particles across all samples. The standardisation of the distribution of particles across samples, despite attempting equal volumes, varied greatly overall. Some particles dispersed easily and some remained clustered in a smaller area on the cover slips with adhered cells. This may give a dose dependant affect. Catelas *et al* (2005) found macrophage mortality depended largely on the concentration of metal ions and the length of exposure to the metals (Co and Cr) (Catelas *et al*, 2005). Variations in particle size, shape and volume for each of the materials would need to be more controlled to have comparable results. Lastly, difficulty in sectioning patient samples for TEM analysis may have effected results, as few particles were found and may not be totally representative of patient tissues.

Conclusions

The results collected used a range of imaging techniques for both patient and cultured samples, exploring material-cell interaction to observe any trends that might influence an adverse reaction that leads to implant failure. It was hypothesised that both the particle composition and characterisation stimulate the macrophage-mediated response. Particle characterisation and localisation for patient periprosthetic tissues agreed with particles identified in previous research on a micron and nano scale. Cultured samples revealed the materials identified in patient tissues induced a varied response from macrophages, with titanium particles forming gross quantities of needle-shaped particles which increased apoptosis and altered macrophage appearance. Cobalt particles also induced earlier apoptosis, with gross quantities of readily up-taken nano particles identified. Larger particles were retained in all samples and electron microscopy revealed the surface texture of patient-derived particles could influence the solubility, with large aggregates forming. The results suggest that it is possible the size and composition of particles does influence macrophage response, as indicated by the rate of uptake, intracellular localisation, phagocytosis of different materials and apoptosis rate seen in cultures. Both titanium and cobalt showed the most negative effects on macrophages. Future research will evaluate the oxidative stress and production of cytokines in response to repeated conditions, evaluating the impact of internalisation of needle structures, aggregates and gross quantities of nano-sized oval-shaped particles seen in the current results.

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