# Osteolysis in total joint replacement – are patient factors important?

**Ben Ollivere**

MB BS, MA (Oxon), MRCS, FRCS (Tr &Orth)

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Thesis submitted for :

**Doctorate of Medicine**

**School of Medicine, Health Policy and Practice**

**University of East Anglia**

**Norwich, NR4 7TJ**

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Supervisors : Professor Ian Clark, Professor Simon Donell & Mr James Wimhurst

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# ABSTRACT

**INTRODUCTION**: Osteolysis is the most common cause for failure of total hip replacements (THR) and 1 in 10 of the 61,000 joint replacements performed each year will require revision surgery. The pathway of osteolysis is well studied in tissue and animal models, but there is little work examining differing responses between patients. Lack of effective diagnosis and a poor understanding of the epidemiological risk factors for osteolytic change compounds the problem.

**METHODOLOGY**: Samples of tissue from osteoarthritic hips, osteolytic prosthesis and well-fixed prosthesis collected intra-operatively. RNA was extracted from the tissue, and expression levels measured with a quantitative real time PCR technique. Expression of 96 genes was measured utilising specific primer and probe sets for 96 genes.

At twelve year follow-up a cohort of patients were identified and their risk for interval revision surgery based on potential co-morbid risk factors assessed. In addition studies evaluating the value of radiographs and patient reported outcome measures in the diagnosis of osteolysis were undertaken.

**RESULTS**: Significant changes were seen in many genes in study group as compared to the two control groups. In addition seven genes (BMP4, FRZB, FGF18, IL8, IRAK 3, OPG and PTGS2) were found to be significantly ($p<0.05$) predictive ($AUC>0.77$) of osteolytic change when compared to the well fixed prosthesis. Addtionally nine genes (VEGFB, SFRP, TLR3, TLR5, TP53, IGF1, CTSK, CHIT 1, CCL 18) were found to be predictive of osteolytic change ($AU>0.77$) but did not reach significance.

Patient co-morbidities were found to be predictive of eventual failure of THR, and improved sensitivity and specificities were demonstrated with iliac-oblique radiographs and the Harris Hip Score when compared to other outcome measures.

**CONCLUSIONS**: This study presents the first comprehensive expression profiling of osteolytic hips, and comparison to the two control groups allows conclusions to be drawn about those genes involved in osteolysis, and how patient responses may differ between individuals. In addition improvements in diagnosis and epidemiological risk factors have been identified that enable identification of individuals at risk of loosening, and improved follow up regimes.

# DECLARATION

I declare that no part of this thesis has been accepted, or is submitted for any degree or diploma or certificate or any other qualification in the University or elsewhere.

This thesis is the result of my own work unless otherwise stated.

All of the research presented here has been carried out in collaboration and discussion with my supervisors, Ian Clark, Simon Donell and James Wimhurst.

The work presented in Chapter 3 was carried out in collaboration with Richard Evans-Gowing and Rose Davidson. Some of work presented in Chapters 4 & 5 was also carried out in collaboration with Clare Darrah and Neil Walton.

Some of this work has been already published in peer reviewed publications or presented at meetings. The material in Chapter 3 has been published or presented in full or in part at the following meetings and journals :

1. Ollivere B, Darrah C, Evans-Gowing R, Wimhurst J, Donell S, Clark I Classification of osteoarthritis of the hip: matching the cytokines to the radiology
   British Orthopaedic Association Annual Meeting 2009 (Abstract in press JBJS(Br))

2. Ollivere B, Darrah C, Davidson R, Wimhurst J, Donell S, Clark I Variations in cytokine tissue expression in osteoarthritic hips: is it all the same disease?
   British Orthopaedic Association Annual Meeting 2009 (Abstract in press JBJS(Br))

3. Ollivere B Osteoarthritis – A diagnosis of exclusion or the Exclusion of Diagnosis?
   Cambridge Orthopaedic Club 2009 (Awarded 1st Prize)

The work presented in Chapter 4 has been presented at the following meetings and journals :

1. Ollivere B, Darrah C, Shepstone L, Wimhurst J, Walton N The longevity of total hip arthroplasty – choose the patient, not the surgeon. British Hip Society 2008

The work presented in Chapter 5 has been presented at or published in the following meetings and journals :

1. Ollivere B, Darrah C, Phadnis A, Donell S, Walton N. Missing something? The use of the modified iliac oblique view in evaluating total hip arthroplasty. British Hip Society 2008 (Abstracted in JBJS(Br))

2. Ollivere B, Darrah C, Brankin R, Donell S, Walton N The continued value of clinical and radiological surveillance: The Charnley Elite Plus at a minimum 12 years JBJS(Br) 2009;91-B:720-4

The work presented in Chapter 6 has been presented at or published in full or in part at the following meetings and in the following journals :

1. Ollivere B, Darrah C, Walton N The value of patient reported outcomes in long term hip surveillance
   British Orthopaedic Association Annual Meeting 2009 (Abstract in press JBJS(Br))

2. Ollivere B, Darrah C, Brankin R, Donell S, Walton N The continued value of clinical and radiological surveillance: The Charnley Elite Plus at a minimum 12 years JBJS(Br) 2009;91-B:720-4

3. Ollivere B, Duckett S, August A, Porteous M The Birmingham Hip Resurfacing independent results at 5 years from a District General Centre
   International Orthopaedics - Accepted

In addition for some of the work presented in Chapter 3 I was awarded the Cambridge Orthopaedic Research Prize 2009, and for some of the work presented in Chapter 6 I was awarded the East Anglian Orthopaedic Club Prize in 2008.

# ACKNOWLEDGEMENTS

I must acknowledge a large number of people for their help and support in the production of this thesis and the underlying scientific work associated with it. I would like particularly to acknowledge Ian Clark who has against the odds managed to coax, encourage, goad and cajole me in to producing something worth writing up over the past two years.

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Neil Walton did a fantastic job as surrogate supervisor for much of the clinical work associated with this project – and for that I am eternally grateful – I hope the subsequent publications make the hard work and patience worthwhile.

The other members of the Clark group, Rick, Rose (who has the highest standards on the planet) and Tracy. They all contributed with ideas and laboratory help. Without the help support and knowledge of Rick, this simply would never have been completed.

This work has been funded by various Charitable Organisations (British Hip Society, Gwen Fish Fund & Action Arthritis) all of whom deserve much thanks. Keith Tucker deserves credit for helping obtain funding from all three of these institutions and for his ongoing professional and personal support.

My various consultant bosses (Clare Marx, Ivan Hudson, David Sharp, John Powell, Matthew Porteous & Martin Wood) during the 'MD Years' who put up with multiple afternoons off, and the occasionally one man clinic. Without their support and tolerance I would actually have had to become real student again.

I would also like to extend my gratitude to my orthopaedic training programme director, Mark Bowditch who has not only encouraged and supported me throughout my SpR training, but was kind enough to ensure I was never placed anywhere too far from the lab. Without his support and encouragement this would likely never have happened.

Finally I would like to thank my parents for printing all 300 odd pages without charging me for the ink or paper!

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# SECTION 1 : BACKGROUND

Total hip replacement (THR) is one of the commonest procedures performed in the world. Over 61,000 were performed in the UK in 2007[1] (NJR 2007), and the year on year incidence is increasing. THR is a successful intervention and marked increases in quality of life can be expected with an improvement in pain and function[2-4]. This makes THR one of the most successful orthopaedic interventions[5]. Total joint arthroplasty is the single intervention performed for diseased hip joints irrespective of the primary diagnosis.

THR has been found to be successful for periods in excess of 30 years in patients with primary osteoarthritis[6]. The same prosthetic joints have been found to be equally successful in secondary arthritidies :

- Inflammatory arthropathies (rheumatoid arthritis (RA)[7-9], ankylosing spondylitis[10;11] and other autoimmune conditions)
- Avascular necrosis[12-14]
- Previous septic arthritis[15-17]
- Haemophiliac arthropathy[18]
- Developmental dysplasia of the hip (DDH)[19-21]
- Fracture[22-24]

However the rate of survival is not uniform across these groups (Fig 1.1) with significantly higher failure rates in some groups such as rheumatoid arthritis (RA) and avascular necrosis (AVN) when compared to osteoarthritis (OA). The increased rate of revision in fracture patients can be explained by higher dislocation rates[22]. However the reasons for increased revision rates in patients with rheumatoid arthritis and avascular necrosis remain unclear.

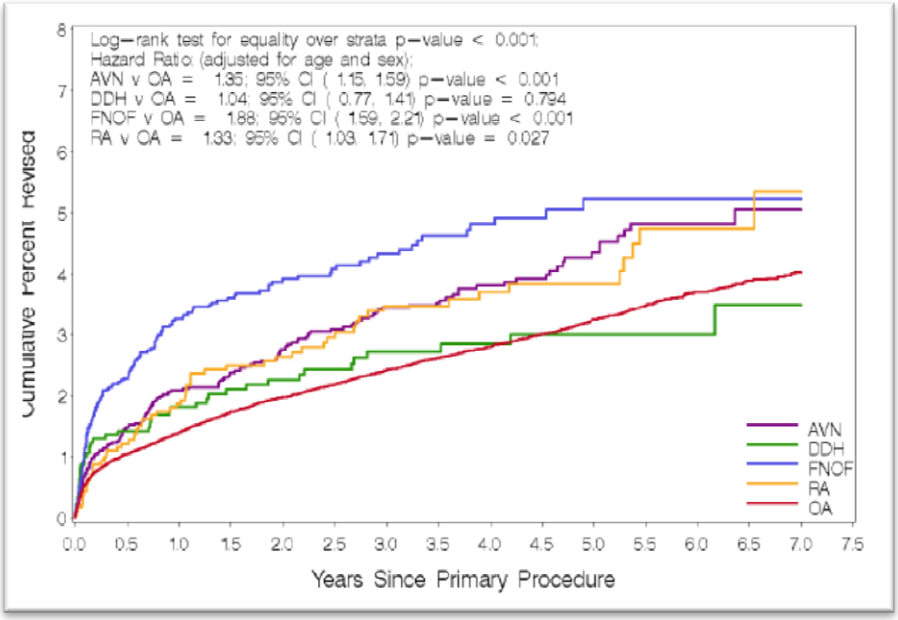



Figure 1.1.1: Graph showing survival outcomes (Kaplan Meier) for THR since primary procedure by diagnosis (Avascular Necrosis - AVN; Developmental Dysplasia of the Hip – DDH; Fractured Neck Of Femur - FNOF; Rhematoid Arthritis – RA; Osteoarthritis OA) . Reproduced from Australian NJR 2008 report[92]

## 1.2 Success of Arthroplasty

Defining success in THR is fraught with difficulty, and there is no uniform agreement as to what constitutes a well functioning arthroplasty. The success of a joint replacement can be difficult to define. Measures of success can be broadly categorised into :

- Patient reported or other outcome measures
- Measures of longevity
- Radiographic measures of success

There is little agreement amongst authors as to what the best measure is, and consequentially there are many different criteria and research instruments in each of these categories.

Patient reported outcome measures are questionnaire based outcome scores, they differ from other outcome measures in that they do not include review by the clinician. They rely on responses to a number of subjective questions, the results of which are summed to provide an eventual score. Although many of these scores have been validated as a measure of functional improvement following joint replacement surgery, little is known about the sensitivity of these measures in detection of survivorship or a failing arthroplasty in the longer term. The most commonly used outcome scores are the Harris Hip Score[25], Oxford Hip Score[26], Merle d'Aubigne[27] and the Hospital for Special Surgery Score[28]. Although these scores are in widespread use and many published articles rely on quality as assessed by these scores[1-3;29] very few have been independently validated and there are few data to suggest these scores are predictive of loosening or failure of a prosthesis. The majority of commercially available THRs have similar post operative scores when using any of these instruments with 90-95% good or excellent results.

Radiographic evaluation of THR allows for a measure of quality of the surgical procedure through evaluation of cement mantles[30], component position[31] and evaluation of any osteolytic lesions[2]. There are a number of radiographic criteria that have been developed to define failure the most commonly used being those of Hodgkinson[32], Harris[31;33] and Kobayashi[34]. The validity of these individual scores as a predictor of intraoperative findings of a 'loose component' probably varies with prosthesis type and criteria. Hodgkinson validated his own criteria against intraoperative findings of 200 acetabulae and found 94% to be loose when a complete radiolucent line was present when compared to only 7% with a radiolucent line in a single zone. Harris' criteria for radiological loosening when applied to a six year radiograph were found by Ollivere *et al*[2] to be predictive of revision by their 12 year follow up. Not all authors however agree on the validity of these scoring systems, and no national joint registries include this data.

Measures of longevity quantify the working life of a prosthesis. Whilst the majority of THRs all produce similarly good results in the short term, as reflected in their clinical outcome scores, there are wide variations in longevity of the prosthesis. Patients undergoing arthroplasty are becoming younger and the demands placed on their prosthesis and the likely working life have extended[4]. Consequentially the success of THR is increasingly being measured as survivorship at a fixed time point (usually 10 years). Whilst revision surgery (or

changing the prosthesis) is a definite end point it is also a coarse one, and there is increasing concern that the measure of revision surgery as failure may not reflect the true number of failing prosthesis[2].

This lack of general agreement surrounding what is defined as a 'failed' THR has led most authors to use the end point of revision surgery as their outcome measure when evaluating performance of a THR. Further study is required in this area to clarify the value of radiographic and patient reported outcomes not only in long term follow up, but also as tools to detect failing prostheses.

## 1.3 Long Term Results

The natural history of total joint arthroplasty is of an increasing rate of failure as the interval between implantation increases. The mode of failure however changes over time. During the first few years after implantation early infection and dislocation predominate. However as time progresses aseptic loosening (osteolysis) and periprosthetic fractures predominate as the primary modes of failure.

The results for each prosthesis differ and the success rates are measured either as failures per 1000 observed component years[35], absolute survival at a ten year bench mark, or most commonly using a Kaplan-Meier survival analysis model[1;2;36]. With modern joint replacements a survivorship of 90% at ten years for all causes of revision across all patients is considered by the National Institute for Clinical Excellence (NICE) a minimum standard.

## 1.4 Causes of Failure in Arthroplasty

Osteolysis (or loss of bone) is currently the commonest cause for revision surgery and late failure of any prosthetic joint. The rates of revision surgery are continuing to increase and in 2003, 36,000 revision hips were performed for 200,000 primary procedures[37], giving a current lifetime incidence of at least 18%. Revision surgery is undertaken for a variety of reasons including:

- Periprosthetic fracture
- Dislocation
- Bearing surface wear/fracture
- Aseptic loosening
- Infective loosening
- Implant failure
- Technical surgical error

Aseptic loosening is a process where the generation of particulate debris from a prosthesis results in an inflammatory response leading to bone resorption (osteolysis). Failure due to aseptic loosening of one or both of the components of a THR is the most common reason for revision surgery. The precise incidence of the individual indications for revision surgery varies between prosthesis and between published series. However

national registries give an indication of the overall incidence of failure (Table 1.1), and in every published registry aseptic loosening is the leading cause of failure[1;2;35;36].

| Number of reoperations per reason and year primary THRs performed 1979-2007 | | | | | | | |
|---|---|---|---|---|---|---|---|
| Reason for reoperation | 1979-2002 | 2003 | 2004 | 2005 | 2006 | 2007 | Share |
| Aseptic loosening | 14,869 | 1,105 | 988 | 996 | 1,018 | 952 | 58.30% |
| Dislocation | 2,584 | 255 | 320 | 265 | 256 | 290 | 11.60% |
| Deep infection | 2,185 | 240 | 288 | 281 | 286 | 305 | 10.50% |
| Fracture | 1,666 | 168 | 172 | 181 | 164 | 191 | 7.40% |
| 2-stage procedure | 993 | 107 | 99 | 98 | 78 | 80 | 4.30% |
| Technical error | 834 | 17 | 17 | 19 | 15 | 36 | 2.70% |
| Miscellaneous | 793 | 21 | 36 | 26 | 15 | 27 | 2.70% |
| Implant fracture | 338 | 35 | 33 | 23 | 23 | 23 | 1.40% |
| Pain only | 270 | 11 | 16 | 8 | 15 | 11 | 1.00% |
| Secondary infection | 0 | 0 | 1 | 1 | 0 | 3 | 0.00% |
| (missing) | 36 | 0 | 1 | 6 | 1 | 4 | 0.10% |
| Total | 24,568 | 1,959 | 1,971 | 1,904 | 1,871 | 1,922 | 100% |

Table 1.1 : Causes for reoperation in THR. Reproduced from the Swedish Arthroplasty Register[3]

Whilst there is general agreement that aseptic loosening is due to the production of wear debris from the joint articulation, this is where the consensus of opinion ends. There is no clear agreed definition of aseptic loosening[2]. Furthermore there is variability in the outcome of; and revision rates within published groups of patients implanted with the same prosthesis within the same hospital. Objective scientific study into the causative factors, natural history and incidence of this condition is therefore difficult.

It is however clear that the incidence of aseptic loosening increases with time[38], and that all varieties of total hip replacement suffer from aseptic loosening[2;4;29;33;39;40] and subsiquentosteolysis.

## 1.5 Implant Designs

Total joint arthroplasty has been in widespread successful clinical use in the UK for in excess of 40 years[41]. The continual evolution of hip replacement prosthesis has resulted in a multitude of different designs often changing in iterative steps. Designs vary in their method of fixation, either cemented or uncemented, bearing surfaces and method of lubrication. The most successful designs[41;42] involve replacement of both sides of the articulation with a stemmed femoral component articulating a stainless steel head against an ultrahigh molecular weight polyethylene (UHMWPE) acetabular cup.

The components are either cemented into place, or implanted with uncemented fixation techniques. The earliest designs of uncemented prosthesis such as the Austin-Moore relied on a ‘press fit’ technique where the component is forced into the bone and friction at the interface provides the stability. More modern designs have relied on either bone on-growth or bone in-growth to the bone prosthesis interface through use of

porous coatings which provides a 'biological fix'.. However despite the attractive prospect of a biological fix the long term registry survivorship analysis demonstrates inferior results when compared to the cemented designs (Fig 1.5.1)

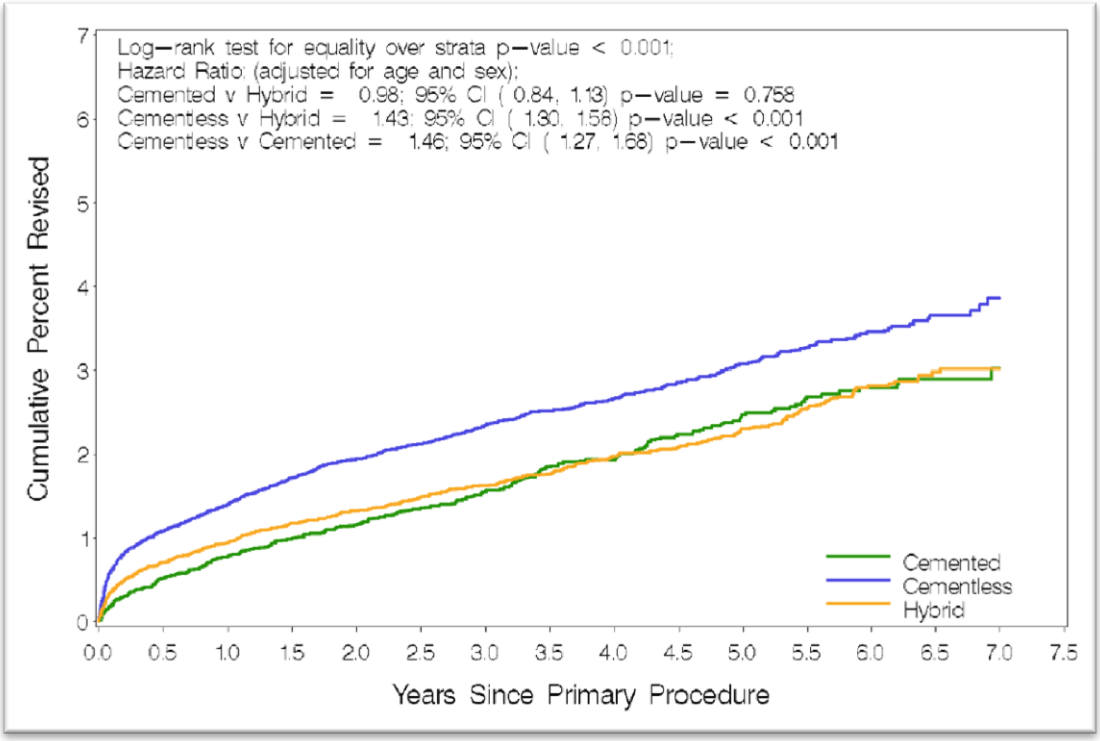



Figure 1.5.1 : Kaplan-Meier survivorship graph of total hip arthroplasty. A comparison of different fixation types. Reproduced from the Australian Joint Registry 2008 report[92]

Cemented designs rely on bone fixation using a layer of bone cement between the prosthesis and the bone. As cemented metal-on-plastic articulations are the most successful and commonly used design of THR's they will form the basis for study.

## 1.6 Aseptic Loosening

Aseptic osteolysis is now the single biggest limitation to joint longevity in the UK[2] and accounts for approximately 60% of all revision surgery[1;35;36]. The rate of aseptic loosening increases with time, and the process results in both loss of bone and loss of fixation of the prosthesis.

Aseptic loosening is characterised by loss of fixation at the bone/cement or prosthesis/cement interfaces and is often associated with large osteolytic bone defects (Fig 1.6.1). The American Academy of Orthopaedic surgeons classifies these as two distinct but potentially related entities[43].

Despite the wide spread use of THR as a treatment for osteoarthritis and the increasing health-economic and social burden of revision joint surgery there is little published literature concerning the likely risk factors for osteolytic failure. Reports are emerging of successful results in selected cases at in excess of 30 years following

implantation with no signs of loosening[6;41] whilst other patients from the same cohort have failed many years earlier.

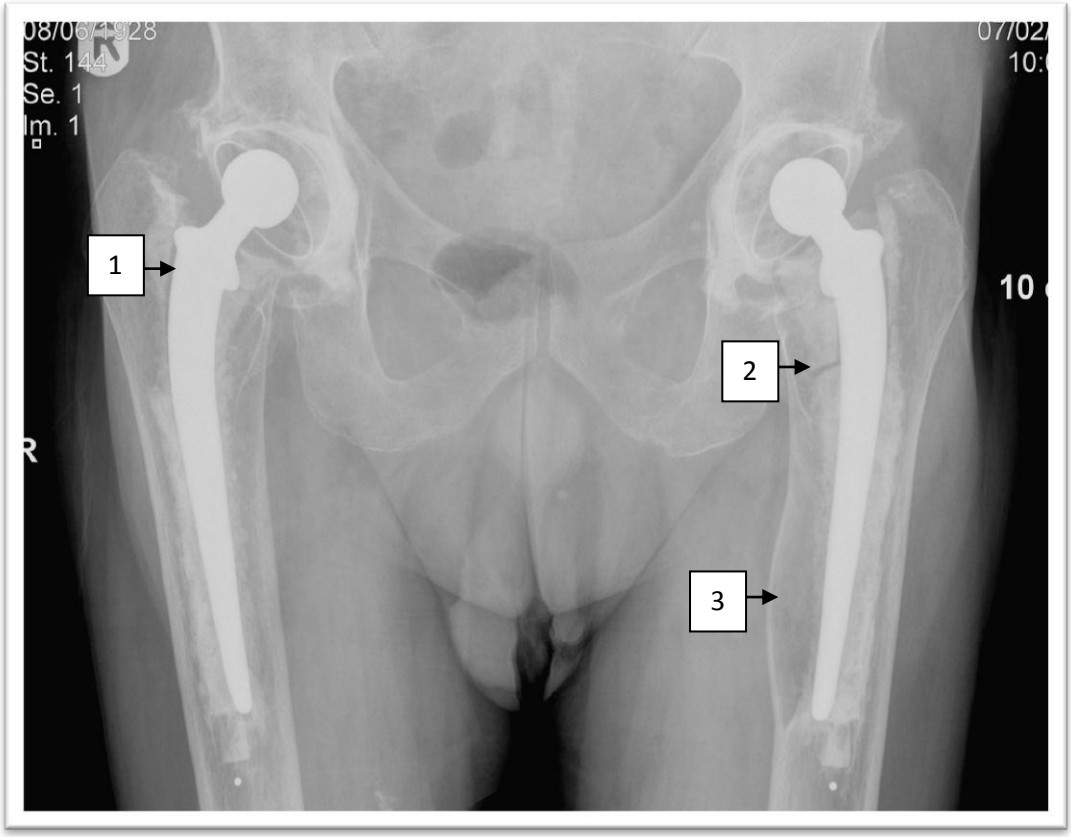



Figure 1.6.1 : Pelvic anteroposterior radiograph of two osteolytic total hip replacements showing typical appearance of an osteolytic total hip replacement: radiolucent lines (1), cement fracture (2) and cavitatory bone defects (3).

## 1.7 Mechanical Theories of Loosening

It was originally described by Wolff[44] that bone responds and remodels to mechanical forces. Mechanical loading of bone can cause remodelling and compensatory growth[45]. There are widespread reports in the literature of proximal femoral resorption with distally fixing prostheses. It is likely that these are in part responsible for the characteristic patterns of loosening described by Gruen[46] with the forces passing from the pelvis through the stem and into the distal femur bypassing the proximal femora. The subsequent stress shielding of the proximal femora results in bone loss and the characteristic failure pattern of 'cantilever bending'[46] associated with distally fixed prostheses.

It is important to distinguish the form of mechanical loosening, which is a function of failure to load the proximal femora, from the process of osteolysis which is an active process and is a biological response to wear debris. Both result in a common mode of failure through loss of fixation and stem stability but through very different processes.

1.8 Biologic Theories of Osteolysis

Osteolysis is an active biological process that results in the loss of bone as a direct response to stimulation of macrophages by biologically active particles. Aseptic loosening is also closely associated with osteolysis, an active process of bone destruction and loss which is cell mediated[47].

It is commonly accepted that mechanical wear of the articulating surface releases particulate debris. This is phagocytosed, activating the macrophages and osteoclasts resulting in bone resorption. This mechanism and the existence of an underlying biological process of aseptic osteolysis was first hypothesised by Willert & Semlitsch[48] in 1977.

Early in the development of total hip arthroplasty bone cement was adopted as the fixation method of choice for both the femoral and acetabular components. Bone cement is composed of polymethylmethacrolate (PMMA) and is similar to dental cement. The earliest descriptions of osteolysis were following revision of cemented total hip replacements. Areas of bone loss and osteolysis were found in association with large granulomas filled with PMMA particles[48;49] and the incorrect association was made between the cement and osteolysis.

Although originally described as "Cement Disease"[48;49] it is now understood that any particulate debris may result in an osteolytic reaction. Ultrahigh molecular weight polyethylene (UHMWPE), bone cement (polymethylmethacrylate - PMMA), Co-Cr, $Ti2O_3$, $ZrO_2$ particles have all been extensively shown to produce biologically active wear debris[47].

## 1.9 Generation of Particles

The generation of wear particles occurs primarily at the primary articulating surface of the femoral head against the acetabulum, mainly through adhesive wear [50]. These biologically active particles form the majority of the burden of wear debris, although metal ions and PMMA particles have also been identified from fretting at the head stem interface[51] and abrasion at the cement-prosthesis interface[52].

### 1.9.1 Morphology of Wear Particles

The size of wear particles has a profound effect on their biological activity. The majority of polyethylene wear particles are submicron (<1 micrometer) in size. This makes visualisation with light microscopy difficult due to the wavelength of visible light (0.4 – 0.7 micrometer)[47]. This led to an initial underestimation of the number and volume of particles in periprosthetic tissues. The use of proteolytic enzymes on periprosthetic tissues and subsequent density-gradient centrifugation[53-55] has allowed accurate characterization of smaller particle numbers and size by use of a particle analyser. More recently electron-microscopy has allowed accurate visualisation of these particles (Fig 1.9.1). Several electron microscopy studies have confirmed that the

majority of wear debris particles are submicron in size with 70-90% of polyethylene particles measuring approximately 0.5 μm [54-56].

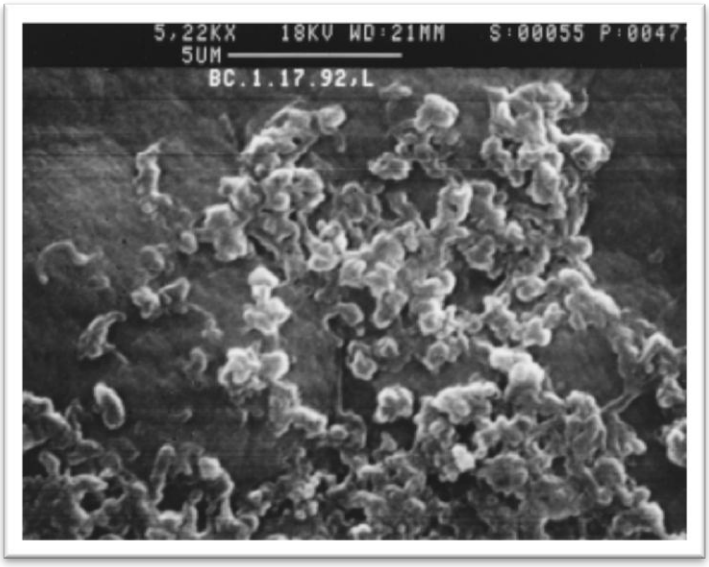



Figure 1.9.1 : Scanning electron micrograph of periprosthetic tissues. The section contains florid polyethylene debris (5-10μm) in the soft tissues. Reproduced from Shanbhag A. S. *et al*[55]

1.9.2 Biological Effects of Particulate Debris

The relationship between the intensity of the biological response, and the number, size and type of particles has been extensively investigated. Phagocytosable particles (<10 μm) appear to be more biologically active, although, at high concentrations, particulate debris becomes cytotoxic and provokes apoptosis in the macrophage.

The initial supposition that osteolysis was due to cement particles led to a large number of *in vitro* experiments with PMMA particles. These demonstrated PMMA particles to be phagocytosable by monoclonal macrophages at less than 7 microns in size, and a resultant release of TNF-α, whilst larger particles were not phagocytosable[57]. These findings were corroborated in a rat model by Gelb[58] who also demonstrated metalloproteinase and $PGE_2$ release in response to smaller PMMA particles.

Subsequent investigation with other particle types has established that all types of metal wear debris (stainless steel, cobolt-chromium-molybdenum, titanium-oxide)[59;60], polyethylene wear debris and ceramic debris all invoke a similar cell mediated response. Whilst some types of wear debris have been shown to be more cytotoxic than others[55;59], and the type of cytokine release has been found by some investigators to depend on the type of wear particles[60], the current body of evidence suggests that all wear particle provoke a similar immunologically mediated response[47].

Although all types of particles are capable of provoking a biological response in vitro the precise nature and type of the response depends greatly on the size and concentration of the particles studied. The size of particles has a profound effect on the nature of the biological response. Contrary to original thinking 90% of particles are small and fall in the <10μm size[54]. These particles are less likely to be cytotoxic[61] and are therefore likely responsible for a macrophage initiated and possibly mediated response. Kubo[62] demonstrated higher levels of biological activity to polyethylene particles of 11 μm than larger controls. The activity level is not linearly related to number of particles, or size, but most likely the biologically active surface area of particles. The activity of macrophages in response to polyethylene debris has been shown to be both size and concentration dependent[60;63] with the apoptotic rate reaching a plateau at concentrations of more than 150 particles per macrophage.

## 1.10 The Process of Osteolysis

Osteolysis is an active biological cascade which is a response to particulate wear debris. There are a huge number of studies investigating the process. There is no currently universally agreed consensus as to the precise mechanism, precipitating factors and regulatory factors of this cascade. Rather there are many studies examining the process from different perspectives. These can be divided into :

- Histopathological and *in vitro* studies examining the cellular interactions
- Tissue explants studies examining sampled tissue using a variety of protein and mRNA detection techniques
- Animal studies using simulated animal models

In addition to studies examining the "traditional" theory of macrophage driven osteolysis there are other newer studies examining the potential role of alternate macrophage activation pathways and extrinsic cascade regulation.

### 1.10.1 "Traditional" Pathways of Osteolysis

There are a complex and poorly understood series of interactions between multiple cell types which regulate the pathway of osteolysis. A summary of the recognised signalling pathways and cascade of osteolysis is given (Fig 1.10.1). The individual interactions are well understood, but the effect of changes on the whole pathway is poorly understood.

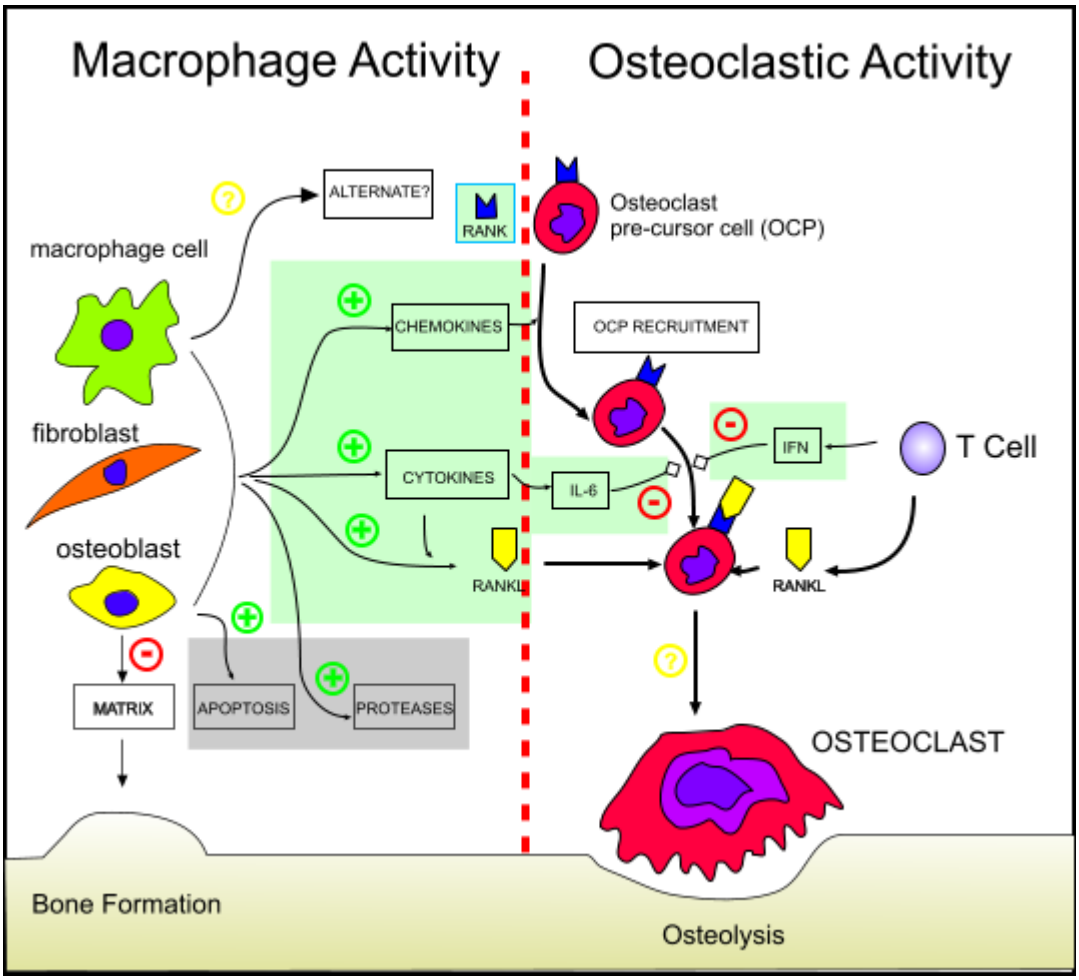



Figure 1.10.1 : A schematic summary of the cellular interactions occurring in the biological pathway of osteolysis. Modified from Purdue et al[64]

## 1.10.2 Cells involved

Histopathological studies have shown profuse macrophage infiltration into periprosthetic tissues and the interstitial membrane[48;65] in addition polarised light microscopy studies have identified large volumes of peri-prosthetic polyethylene debris undergoing phagocytosis[66]. There is histopathological evidence for involvement of macrophages, osteoblasts, osteoclasts, fibroblasts and other antigen presenting cells have all be implicated in osteolysis. The role of each is outlined below.

## 1.10.3 Macrophages

Activation of a macrophage mediated response is central to the initiation of an osteolytic response[65]. *In vitro* studies using cultured monoclonal macrophage cell lines challenged with a variety of wear particles demonstrate phagocytosis of the wear debris[67-69] and production of pro-inflammatory signal molecules.

Stimulation of cultured macrophages in this way has been found to modulate the activity of a range of inflammatory mediators including prostaglandin E2 (PGE2), tumour necrosis factor alpha (TNF-$\alpha$)[70], interleukin 1 beta (IL1-$\beta$)[71;72] and interleukin 6 (IL-6)[67]. In addition to regulation of immunomodulators there is accumulating evidence that matrix regulatory proteins are implicated in macrophage mediation of osteolysis.

Metalloproteinase expression has been demonstrated to be up regulated in response to wear debris in cultured monoclonal macrophages[73] and subsequently high rates of expression of a number of matrix metalloproteinases (MMP)[74;75] have been demonstrated in periprosthetic tissues.

Differing macrophage populations have been previously shown in vitro to respond to wear debris in different manners. Glant & Jacobs demonstrated a differing response between peritoneal macrophages and transformed cell lines (P388D and IC-21) and their response to controlled doses of titanium particles[76].

The results from these studies and animal and tissue models suggest the macrophage plays the key initiating role in osteolysis. In a recent review Purdue *et al*[64] identified the exposure of macrophages to particulate wear debris as the critical initiating event in osteolysis.

1.10.4 Osteoblasts

Osteoblasts differentiate from the common osteoprogenitor cell lineage. Osteoprogenitor cells in the periosteum are induced to mature into osteoblasts under the influence of BMPs, fibroblast growth factors (FGFs), platelet derived growth factors (PDGFs) and transforming growth factor β (TGF-β). Osteoblasts play a critical role in bone turnover, remodelling and metabolism in conjunction with osteoclasts. They are responsible for generation of and mineralisation of the bone matrix. Disorders in osteoblast and osteoclast function or balance can result in a range of metabolic bone diseases.

There are few studies exploring the role of osteoblasts in osteolysis despite the potential for inhibition of osteoblast function to result in bone loss[64]. Lohmann *et al*[77] established that not only can osteoblasts phagocytose wear debris, but that this can modulate differentiation and cytokine expression of cultured osteoblasts.

The composition of the wear particles the osteoblasts are exposed to has been shown to vary the subsequent response. Exposure to UHMWPE reduces production of bone matrix by down regulating both collagen type I and III production[78;79]. There is also some evidence from cell culture studies that certain types of particulate debris may also inhibit osteoblast differentiation[80].

Although the current data are inconclusive they do implicate regulation of osteoblast activity and differentiation in osteolysis, and for a complete understanding of the process this gap in knowledge should be addressed in future studies.

### 1.10.5 Osteoclasts

Osteoclasts are derived from a common cell lineage with macrophages and are multinucleated cells derived from osteoclast precursor cells in the general circulation. Not surprisingly as osteoclasts are the only cell lineage capable of active bone resorption[81] much research surrounding osteolysis has been directed at establishing the role and function of osteoclasts in aseptic loosening.

Histological studies have demonstrated extensive infiltration of osteoclasts and multinucleated giant cells into tissue surrounding loose implants[82;83]. Osteoclasts are recruited from precursor cells through expression of monocyte chemotactic protein (MCP-1) and macrophage inflammatory protein (MIP-1$\alpha$), the expression of both of these has been found to be up regulated in periprosthetic tissues[64;84;85].

Although there are some studies demonstrating a direct biological effect of wear debris on osteoclasts [86;87] it is likely that the majority of their modulation is via signal pathways from activated macrophages[64], chiefly through up regulation of IL1, TNF-$\alpha$ [88–90] and IL6[86], all of which play a part in regulation of osteoclastic activity.

Much emphasis has been placed on the importance of the Receptor Activator of Nuclear Factor κ B **(**RANK) and its ligand (RANK-L) in osteolysis[91;92]. The RANK receptor is a membrane receptor found predominantly on osteoclasts. Ligand binding at this receptor is the initiator for osteoclast differentiation. Osteoprotegerin (OPG) is the antagonist for this pathway[93;94]. It is secreted by osteoblasts and regulates osteoclast activity by providing an alternate binding site for RANK-L. The RANK pathway is a key modulator of bone turnover[93;94]. Alteration in ratio of RANK-L to OPG is reflected in changes to the rate and type of bone turnover and consequentially has been implicated in many metabolic diseases of bone[95].

Numerous studies have quantified the expression of RANK-L in periprosthetic membrane tissues. RANK-L expression is raised in tissue surrounding loose osteolytic components, that expression has been localised to macrophages, giant cells and fibroblasts[96-98]. This up regulation of RANK-L is therefore of likely significance in osteolysis. Modulation of RANK-L activity through increased expression of OPG[99;100] or use of knock-out mice with a deficient RANK receptor[101] results in down regulation of osteolysis in a mouse model. Further, exposure of fibroblasts[102], osteoblasts[103] and a murine calvarial model[101] to particulate wear debris resulted uniformly in an increase in the RANKL/OPG ratio.

## 1.11 Tissue Explant Studies

Tissue explant studies are a method for investigating the process of osteolysis through removal of tissue intra-operatively and analysis through:

- Light or electron microscopy to identify particulate debris and cells
- Immuno-histochemistry and *in-situ* hybridisation techniques
- Quantitative rt-PCR and protein expression analysis

The histopathological picture of periprosthetic osteolysis (Fig 1.11.1) are well characterised and the periprosthetic tissues surrounding a loose implant contain abundant macrophages, evidence of particulate debris and giant cells[82;83].

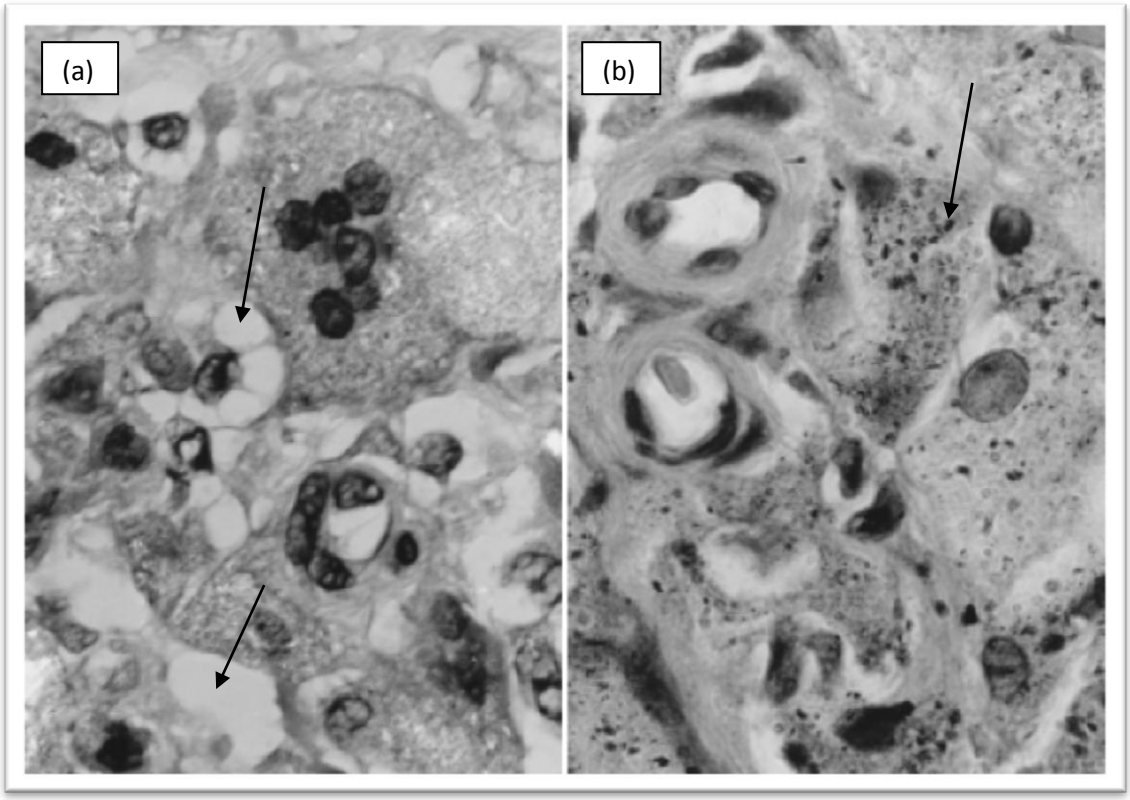



Figure 1.11.1 : Photomicrographs of periprosthetic interface tissue. Submicrometer birefringent polythelyne particles (a) and dark metal particles (b) are seen within the cells (Magnification, x50). (Reproduced from: Glant, T. T.; Jacobs, J. J.; Mikecz, K.; Yao, J.; Chubinskaja, S.; Williams, J. M.; Urban, R. L.; Shanbhag, A. S.; Lee, S.; and Sumner, D. R.: Particulate-induced prostaglandin- and cytokine-mediated bone resorption in an experimental system and in failed joint replacements. Am. J. Ther., 3: 33, 1996)

## 1.12 Animal models

These ex-vivo studies have been corroborated with selective animal studies. This allows the standardisation of most factors and the majority of studies are designed to quantify and characterise the response of living cells and tissues to a challenge of particulate wear debris.

In one of the earliest animal studies Goodman[104] observed a reactive fibrous-tissue layer in response to implantation of UHMPE, metals and PMMA when implanted as a single large implant. However when the same materials were implanted in particulate form a more aggressive inflammatory response was noted. Histological examination showed infiltration of macrophages, giant cells and a profuse inflammatory infiltrate. This was similar to that seen surrounding loose implants[61;104] confirming the findings of other tissue culture and ex-vivo studies[60;62;64;82;105].

More advanced animal models of osteolysis have also been developed. Experimental models exist in the rat[106], mouse[107], dog[108], rabbit[108;109] all of which have been able to replicate previous experimental findings in an animal model setting.

Aseptic osteolysis has been demonstrated in animal models as a response to particulate polyethylene debris[106]. Tissue culture from animal experimental models has confirmed up regulation of IL-1, $PGE_2$[108;109] and TNF-$\alpha$[107]. The more refined air-pouch model of Gelb *et al*[58] allowed them to conduct a series of experiments to quantify the in vivo effect of the size, morphology and surface area of PMMA particles when inducing an acute inflammatory response.

The development of mouse models specific for individual components of the osteolytic signal pathways has allowed the effects of various cytokines to be evaluated in animal models. Yang *et al*[110] conducted a series of experiments demonstrating decreased osteolytic function in a mouse model with induced expression of anti-inflammatory cytokines. A retroviral vector was used to induce expression of IL-1Ra and IL-10. In both cases the osteolytic pathway was significantly suppressed in the animal model.

These animal experiments confirm and add weight to the body of cell culture and ex-vivo experimental work. Although useful in providing corroborative evidence for the process of osteolysis by their very nature they cannot tell us about the likely variation between individual patients in response to the same particulate debris.

## 1.13 Pathway regulation

The events leading towards osteolysis have been better explained by cell and organ culture studies than biomaterials research. Particulate wear debris has been shown to be phagocytosed by macrophages and to release a plethora of mediators. It seems likely that IL-1, IL-6, TNF-$\alpha$ and $PGE_2$ are the most important factors in the promotion of osteolysis[47]. These mediators stimulate cell proliferation and have been shown to be capable of stimulating activity of osteoclasts and therefore resorbing adjacent bone. Catabolism of bone matrix has been linked to the production of matrix metalloproteinases such as collagenases and stromelysin[111].

TNF-$\alpha$ has been implicated as the primary mediator released by activated macrophages. Osteoblasts are stimulated to release GM-CSF and IL-6 as a response to TNF-$\alpha$, which in turn recruits more macrophages (GM-CSF) and increases their state of activation.

Matrix-metalloproteinases (MMPs) have also been implicated in osteolysis and MMP-1, MMP-2 and MMP-11 have been shown by Vidovszky[112] to be present in areas of osteolysis. This may be through either direct stimulation of fibroblasts by secondary actions of particulate debris (which has been demonstrated in explant studies) or through activation via macrophages and osteoclasts.

## 1.14 Alternate Activation Pathways

Macrophages are able to respond to a single stimulus through the activation of multiple signalling pathways. The classical or M1 macrophage activation pathway results predominantly in TNF $\alpha$ and IL1 production. It is seen in response to Th1 cytokines (typically IFN-$\gamma$) which are typically produced in response to microbial stimulation[113].

However macrophages are capable of a broad spectrum of responses and these alternate (or M2) responses are responsible for activation of alternate signally pathways such as NFkB, and the MAP kinases (p38, JNK and ERK) [86;87]. This M2 activation results predominantly in production of other inflammatory mediators cytokines such as PGE2. The alternate activation pathway is driven by response to the Th2 cytokines (predominantly IL4 and IL13).

There is accumulating evidence that these 'alternate' activation pathways are important in the host response to conditions such as Gaucher's disease[114-116], multiple sclerosis[117], atherosclerosis[118] and tuberculosis.

Whilst evidence surrounding the role of alternate macrophage activation in osteolysis is far from complete there is accumulating evidence that this process may have a role. Koulouvaris *et al*[105] demonstrated high levels of alternate macrophage activation markers chitotriosidase and CCL18 when comparing periprosthetic tissue from failed hips to primary controls[105]. These same markers have been implicated in alternate macrophage activation in Gaucher's disease[119-121] and sarcoidosis[122]. Macrophages are responsible for formation of giant cells in granulomatous diseases and both DC-STAMP [123] and TREM2[124] have been found to be elevated in periprosthetic tissue.

It seems likely that the M2 responses play as crucial a role in the osteolytic response as they do in other granulomatous and macrophage driven diseases. The implication of M2 responses may help to explain difficulties in reproducing the in-vitro cell culture experiments in-vivo or ex-vivo. Recent work has shown that in prolonged incubation with wear debris macrophages move through an M1 (TNF $\alpha$ & IL6) response which peaks at an hour and returns to normal within 24 hours[86]. Further incubation periods appear to result in a gradual increase in M2 cytokines with a slow increase in markers such as chitotriosidase. It seems possible that the balance between these two responses may play a role in the differential activation of the osteolytic cascade seen from patient to patient.

### 1.14.1 Differential Responses

The majority of in vitro experiments have been performed using monoclonal macrophages, and the observed response presumed to be generalisable to humans. Giant *et al* investigated the effects of different cell lines to the same wear debris and demonstrated markedly different responses with significantly different responses between macrophage populations when exposed to different wear debris particles[76].

# SECTION 1.15 CANDIDATE GENES

In addition to those genes previously identified in periprosthetic osteolysis a thorough literature search was undertaken to identify genes which previous work had identified in other musculoskeletal conditions as markers of :

- Arthritis disease activity
- Cellular function (macrophage, osteoblast and osteoclast)
- Bone and matrix turnover
- Apoptosis
- Macrophage differentiation and activation

This led to the inclusion of 92 candidate genes and 4 housekeeping genes in the current study. An overview of the function and reason for selection of each candidate gene is outlined below.

## 1.16 Housekeeping Genes

Housekeeping genes are expressed uniformly across tissues, and hence may be used to correct tissue expression studies for the inaccuracies associated with RNA extraction and expression analysis from tissue samples.

Use of multiple house-keeping genes allows for normalisation to the most stably expressed gene, and analysis with appropriate software such as *geNorm* or *NormFinder* allows normalisation to the most suitable housekeeping genes[125]. We identified four housekeeping genes previously demonstrated[125-127] to have stable expression in musculoskeletal tissues and therefore to be good candidates for housekeeping genes in this study.

18S – The 18S ribosomal subunit is the most commonly used housekeeping gene. It forms part of the translation apparatus and the ribosome. It is stably expressed in many mammalian tissues.

β Actin (βACT) – is a non-muscle isoform of actin which is found throughout the body. It is an important cytoskeleton protein and as such is found expressed at relatively stable levels in the majority of tissues.

GAPDH - glyceraldehyde-3-phosphate dehydrogenase is indirectly responsible for the catalysis of glucose in the glycolytic pathway. It is has relatively stable expression across all tissues as glycolysis is an essential pathway in most tissues.

SDHA - Succinate dehydrogenase forms a co-enzyme complex (Co-Q) and is expressed in the mitochondria. It has stable expression properties in tissues involved in aerobic respiration.

## 1.17 Bone Regulatory Proteins & Markers of Degradation

Bone is a tissue that undergoes continuous turnover and regulation, and is capable of healing without scar. The remodelling process originally described by Wolff and a variety of other mechanical[128], bioelectrical[129] and endocrine[130;131] factors have been implicated in bone turnover.

There is little current evidence to support a role of bone regulatory proteins such as bone morphogenic proteins (BMPs) in processes of osteoarthritis or osteolysis. However there is accumulating evidence that these are important factors in osseoinduction and fracture repair[132-135].

Bone Morphogenetic Protein 4 (BMP4)

The BMP family of proteins are a group of proteins that regulate bone formation and growth. Most are a subset of the TGF-$\beta$ family of proteins. Originally seven members of the family were identified, BMP 1-7. They are aside from BMP-1 (an astacin metalloproteinase)all TGF-$\beta$ super family proteins. There are now 20 identified BMP proteins[135].

BMPs bind to cell surface receptors BMPr's and induce the SMAD secondary messenger pathway. BMPs and the SMAD pathway have also been implicated in developmental pathways[133]. BMP-4 has been shown to be the most potent stimulator of bone growth and has clinical applications in induction of fracture healing.

Collagen Synthesis – COL

The COL group of genes encode for a variety of collagen chains. Most human collagen molecules contain two $\alpha 1$ and one $\alpha 2$ chain. Measurement of COL gene expression may be related to collagen synthesis rate. However the collagen molecule has considerable post-transcriptional and post-translational regulation with cleavage of pro-peptides, cross linking and glycosylation prior to eventual fibril formation. This should be remembered when interpreting COL gene expression as a surrogate marker for collagen synthesis.

Changes in expression of COL genes have been shown to be associated with osteoarthritic change in a horse model[136]. Measurement of collagen synthetic rate will give an indication of the formation rate of new bone and connective tissue[137]. Measurement of expression of COL1A1, COL1A2 and COL3A1 mRNA has been shown along with other markers of gene expression in cartilage to relate to the severity of osteoarthritic change[138].

COL1A1 & COL1A2

The COL 1A1 and COL 1A2 genes encode the components of type 1 collagen found as the major component in the extra cellular matrix of bone. COL1A1 encodes the major component of type 1 collagen and has been previously implicated in osteogenesis imperfecta[139]. COL1A2 encodes an additional copy of the alpha chain,

the two genes are not always co-regulated. Both COL1A1 and COL1A2 have been implicated in osteogenesis and in Ehlers-Danlos syndromes[140;141].

COL3A1

COL3A1 encodes for the alpha chain of type III collagen which is found in association with type I collagen in bone and connective tissues. Type III collagen is also associated with fibrocartilage formation and as such is implicated in the cartilaginous repair process[142].

## 1.18 Toll Like Receptors (TLR 1-5)

Toll like receptors (TLR) are named due to their genetic similarity to the *Drosophila* protein Toll. They are a group of cell membrane proteins responsible for co-activation of a number of immune responses and play a key part in mediation of innate immunity. They usually function as a co-signalling receptor responsible for recognition of microbial molecules (such as bacterial lipopolysaccharides) they have the poteintial activate a number of intracellular inflammatory pathways.

The exact function of each TLR is unclear and although traditionally associated with response to infection[143] there is some evidence that TLRs are associated with aseptic loosening[144-146]. TLR-3 expression has been demonstrated in inflammatory arthritides to be associated with activation of the RANK-L pathway and hence potentially macrophage activation and aseptic loosening[147]. The breakdown of cartilage by matrix metalloproteinases (MMPs) and glycosidases produces breakdown products that are known to activate the TLR pathway[148].

## 1.19 WNT Pathway (WNT10B, WNT5A, WNT7B)

The WNT signalling pathway is a well described and much studied[149-151] signalling pathway which has been implicated in tumour genesis[152], embryological skeletal development and in the disease process of osteoarthritis. WNT genes are related to the wingless gene originally characterised in *Drosophila spp.* The family of proteins codes for the WNT secreted signalling ligands and a wide range of effects have been associated with a variety of WNT ligands. The WNT pathway functions through activation of frizzled (FZD) cell surface receptors. The frizzled surface receptor is responsible for the signal transduction of WNT pathway. Frizzled and associated proteins have been implicated in bone turnover.

The WNT family of genes have been implicated in a huge range of cell regulatory and disease processes. However WNT5A[153], WNT7B[154] and WNT10B[151;153] have been particularly implicated in pathogenesis of arthritis and osteolysis.

Activation of the WNT/Frizzled pathway results in subsequent activation of three different downstream pathways : Wnt/β-catenin (canonical pathway); WNT/Ca2+ and WNT/polarity. It is not clear how the subsequent pathway activation is determined however it is likely that this is determined by a combination of WNT ligand and the Frizzled receptor present.

The WNT/β-catenin pathway is the most studied pathway as it results in regulation of gene expression. The WNT β-catenin pathway has been shown to modulate bone resorption and may play a key role in the pathogenesis of osteoarthritis and osteolysis. Activation of β-catenin results in up regulation of Dishevelled (DVL) and production of a non-phosphorylated form of β-catenin which localizes to the nucleus. This is thought to induce the expression of downstream target genes[152].

1.19.1 SFRP-1, SFRP-2 & SFRP-3 (secreted frizzled-related protein)

SFRPs are a family of secreted proteins that share the receptor binding domain for the Frizzled receptor with the WNT pathway. Hence SFRPs function as secreted ligands and are able to bind WNTs extracellularly and thus modulate their activity. The SFRPs are capable of down regulating the activity of the WNT pathway. Modulation of the WNT/β-catenin pathway has been implicated in the pathogenesis of osteoarthritis[149].

Frizzled related protein is a WNT antagonist and has been potentially implicated in periprosthetic osteolysis[155]. Genotype variations in FRZB have been shown to predispose patients to both osteolysis and heterotrophic ossification. However differences in gene inheritance rate were not significant[155].

There are two major antagonists that act on the WNT pathway, Dickkopf (DKK) and the secreted frizzled-related proteins (SFRPs) this regulatory function is critical to the activity of the WNT pathway. In order to adequately assess the activity of the WNT pathway the activity of these regulators must also be known.

1.19.2 Dickkopf (DKK1, DKK2, DKK3)

The Dickkopf (DKK) family of proteins are extracellular secreted proteins which act to inhibit the activity of the WNT signalling cascade. DKKs have been shown to be involved in embryonic tissue differentiation and also implicated in osteolytic lesions associated with myeloma. Suppression of DKK1 has been shown to profoundly affect the process of osteolysis in myeloma[156]. Inhibiting DKK1 prevents the suppression of bone formation and in doing so is effective in prevents the development of osteolytic bone disease in animal models of myeloma. DKK1 has additional roles in rheumatoid arthritis, where it regulates bone loss, and also in osteoarthritis where it is associated with osteophyte formation.

## 1.20 Interleukins & other Cytokines

Interleukins and other cytokines previously implicated in the osteolytic cascade (as outlined above) have been included as candidate genes. In addition to those factors previously identified some modulators of cytokine function and other family members have been included.

### 1.20.1 Interleukins

The role of interleukins is well established in both the osteolytic cascade and inflammatory arthropathies. In addition to the widely recognised and previously discussed IL1, IL4, IL6 and IL13 a number of other receptors and modulators have been included as candidate genes.

Beraudi and colleagues[157] performed a series ex-vivo studies of limited cytokine expression analysis in tissue surrounding osteolytic hip replacements. They examined and documented variations in the levels of IL1-$\alpha$, IL1-$\beta$, IL6, IL8 and IL10 using an ELISA technique. They did not evaluate any well fixed stems, but concluded that that cytokines had an important role to play in periprosthetic osteolysis. Interleukin 1 has been shown to be implicated in the osteolytic cascade and function of IL1 is modulated by both IL1RA (interleukin 1 receptor antagonist) and results in activation of IRAK3 (interleukin-1 receptor-associated kinase 3).

IL11 has been demonstrated to have a marked effect on bone turn over and has been implicated in metastatic[158] associated osteolysis, in regulation and differentiation of osteoclasts[159] and has been implicated in osteolysis[160]. Bone destruction (cyst formation) in rheumatoid arthritis has been associated with elevation in the expression of interleukins IL12$\alpha$, IL12$\beta$ and IL17[161]. It is likely that this occurs through an interaction with OPG and perhaps also through the actions of natural killer (NK) cells.

1.20.2 IFN$\gamma$ (interferon-gamma) – IFN-$\gamma$ is a soluble cytokine which is the sole member of the type II interferon family. IFN-$\gamma$ is critical in activation of macrophages, and as such is likely to be involved in any macrophage mediated response such as osteolysis.

### 1.20.3 TNF Family-(TNF-$\alpha$, CD120A, CD120B, FASLG, OPG, RANK, RANK-L)

TNF $\alpha$ has been previously implicated in the process of periprosthetic loosening. A range of TNF family receptors and modulators have been included as candidate genes in this study. A major TNF-$\alpha$ receptor subtype, TNFRSF1A (CD120A) is known to be a key mediator of the inflammatory response and is responsible for activation of the NF-$\kappa\beta$ pathway and consequentially plays a key role in the induction of apoptosis, a pathway which itself has been linked to osteolysis[162;163].

A number of key cellular receptors are responsible for apoptosis, and if apoptosis does indeed play a role in osteolysis then these receptors and ligands may be expected to play a pivotal role. The CD120A related receptor TNFRSF1B (CD120B) is thought to be involved in induction of the apoptotic cascade. Activation of

CD120B has been previously shown to up-regulate c-IAP1 and c-IAP2 which then function as anti-apoptotic signals[164] although not previously implicated in osteolysis its structural and functional similarity to CD120A make it an ideal candidate gene.

FAS Ligand (FASLG) is a TNF family cellular receptor which is responsible for mediation of apoptosis. Binding of FAS-Ligand to its receptor is the final common pathway for apoptosis, and as such FASLG forms a marker of overall apoptotic rate, and should apoptosis be responsible for cell death In osteolysis FASLG expression should be raised.

The OPG/RANKL/RANK pathway is the final common mediator of osteoclastogenesis. OPG is a soluble, decoy receptor belonging from the TNF receptor super family and blocks RANK-L, the key mediator of osteoclastogenesis. RANK-L is found endogenously as both a membrane-bound form and as a soluble form. The RANK-L, binds the RANK receptor found predominantly on osteoclast cells and is responsible for modulation of bone metabolism.

## 1.21 Chemokines (CCL2, CCL3, CCL18, CCR1, CXCL9, CXCL10, CHIT 1)

Chemokines are small secreted chemo-attractant molecules which play an essential role in directing the migration of a variety of inflammatory and immune cells. Chemokines have been shown to be involved in leukocyte migration in juvenile idiopathic arthritis (JIA) and facilitate the formation of an infiltrate of activated inflammatory T cells, as well as B cells, macrophages and dendritic cells[165].

Chemokine receptor expression is specific to subsets of leukocytes and hence the secreted chemokines and chemokine receptors are likely reflective of activated, effector and memory T cells[166-169]. Chemokine receptor expression can be used to distinguish Th-1 T cells (which typically express CXCR3 and CCR5) from Th-2 populations (typically CCR3 positive). CCL2 (monocyte chemotactic protein-1) has been implicated in the activation of the osteolytic cascade and regulation of osteoblastic function in the pathogenesis of periprosthetic osteolysis[166]. Previous RT-PCR analysis of periprosthetic soft tissue from osteolysis patients has demonstrated activation of chemokines associated with alternative macrophage activation markers (CHIT1, CCL18)[170].

Macrophages are responsible not only for the initiation and maintenance of the inflammatory process, but also for the inhibition and resolution of inflammation. Switching of activated macrophages from one state to the other is influenced by secreted chemokines (CCL3, CCL18)[171]. If macrophage switching were to occur in osteolytic tissues it might be central to suppression of the osteolytic cascade.

## 1.22 Cluster of Differentiation (CD2, CD58, CD14, CD28, CD80, CD86, CD36)

The cluster of differentiation (CD) protein family includes a wide range of cell surface markers expressed on T and B cells. The group is heterogeneous and includes molecules responsible for cell signalling, cellular adhesion and differentiation. CD proteins may act as either receptors or ligands and those included here have all been implicated in the inflammatory process associated with osteolysis.

CD 2 is a cell surface receptor found on T cells and also on NK cells. It functions as an adhesion molecule, but may also be used as a surrogate maker for T cell activation due to its secondary role as a co-stimulatory molecule[172;173]. CD 2 binds to CD58, another cellular adhesion molecule expressed on antigen presenting cells (APC), particularly macrophages.

Lymphocyte function-associated antigen (CD58) functions as a macrophage specific cell adhesion molecule and is expressed as part of the initial activation of T-cells by the antien presenting cell (APC). The CD58 binds to CD2 and plays and important role in strengthening cell adhesion between the macrophage and the T-cell it is working to activate. CD58 has not been implicated previously in osteolysis, but has been shown to have variable expression in rheumatoid patients, and specifically increased expression has been shown to be related to clinical outcome[174]. Previous studies demonstrating a variation in expression levels in patients undergoing primary joint replacements make this a candidate gene.

CD14 is a cell surface receptor expressed on macrophages and dendritic cells. It forms a co-receptor with Toll like receptor 4 (TLR4) for bacterial lipopolysaccharide[175]. CD14 is found both bound to the cell membrane (mCD14) and insoluble form (sCD14)[176]. Expression levels of CD14 are analogous to macrophage and dendritic cell function, and would be expected to mirror that of TLR4. Expression of CD14 is analogous to macrophage function and makes this a candidate gene.

The CD28-80-86 co-stimulatory pathway is responsible for T-cell activation. CD28 is the cell surface receptor for CD80 and CD86. Activation of antigen presenting cells (APCs) and B-cells results in increased expression of CD80 and CD86 and a resultant stimulation of T-cell activation via the CD28 pathway. Activation of this pathway results in IL2 and IL6 production. This pathway is likely the mediator for the protective effects of oestrogen on bone loss seen in a mouse model[177]. Up regulation of osteoprotegerin (OPG) in response to CD28 stimulation inhibits the activity of osteoclasts. Some authors have identified the CD28 pathway as involved in peri-acetabular osteolysis[178]. Given the previous implication of this pathway in osteolysis and the key role these proteins play in activation of the T and B cell response these candidate genes may give an indication of an individual's likelihood to activate the osteolytic cascade.

CD36 is a collagen binding receptor, and is a marker of collagen turnover. It has been implicated in a number of collagen disease processes including atherosclerosis and more recently as a marker of chondrocyte

activity[179]. CD36 has been implicated as an important anti-inflammatory factor critical to cartilage repair in inflammatory arthritidies[179]. Osteoarthritis involves the destruction of articular cartilage and it is possible that CD36 is implicated in this. Additionally given previous implication as providing a protective effect against degenerative joint disease it is possible that CD36 activation will also protect against the effects of osteolysis a process in which the destruction of bone and collagen matrix is key to the disease process.

## 1.23 MMPs, ADAMTSs (MMP1,12,13, ADAMTS 2,3,14, TIMP 1)

The matrix metalloproteinases (MMPs) are a family of 23 enzymes found in man that are the only enzymes able to degrade the collagen triple helix under physiologic conditions .As osteolysis involves lysis of collagen within bone it seems likely that collagen and matrix collagenases will have a role to play in the process. Several MMPs have been shown to have a collagenolytic effect. Initially MMP-1, -8, and -13 were identified as having this activity. More recently, MMP-2 and MMP-14 have also been shown to perform this function but with lower efficacy.

Extracellular matrix metabolism is also regulated by a further family of proteins, a disintegrin and metalloproteinase domain with thrombospondin motif (ADAMTS). There are 19 identified members of the ADAMTS family in humans. ADAMTS are known to function both as procollagen N-propeptidases (ADAMTS-2, -3, and -14) and aggrecanases (ADAMTS-1, -4, -5, -8, -9, and -15), the function of other members of the family are unclear.

There are four specific inhibitors for the MMP and ADAMTS family of collagenases. The tissue inhibitors of metalloproteinases (TIMPs). It appears that the 4 members of the TIMP family are able to inhibit the majority MMPs but only TIMP3 is capable of inhibiting ADAMTS 4 & 5.

## 1.24 Cell Growth Factors & Regulatory Proteins

Particle-challenged cells release cytokines and chemokines, which contribute to periprosthetic osteolysis. Released growth factors from particle challenged macrophages are responsible in part for recruitment and differentiation of macrophages (CSF1) osteoblasts and osteoclasts. Osteoblast growth and differentiation is dependent on fibroblast growth factor (FGF-18), platelet derived growth factor (PDGF $\alpha$ and $\beta$) and transforming growth factor $\beta$ (TGF-$\beta$ 1, 2 & 3). Other growth factors including vascular endothelial growth factors (VEGF $\alpha$, VEGF $\beta$) and insulin like growth factor (IGF 1) have also been demonstrated to be up regulated in murine models of osteolysis[180].

## 1.25 Other Potentially Involved Genes

There are a number of other gene regulators or families that have either been identified as involved in the osteolytic pathway, or given their known functions and sites of action are likely candidate genes.

1.25.1 Proteinases

Other than the matrix metalloproteinases there are also several other proteinases which are important in degradation of the organic portion of bone matrix. The cathepsins are proteases that have been implicated in bone turn over. Cathepsin G (CTSG) is secreted from activated mast cells and has been localised in the prosthetic bone interface in aseptically loose hips[181]. Cathepsin K (CTSK) is a related protease which can may degrade collagen and has been strongly associated with bone metastasis and osteolysis[182-184]. Cathepsin K also plays a role in and has been used as a marker of osteoclast differentiation[184]. Higher levels of expression of either of these proteins are likely to indicate increased breakdown of the bone matrix. In support of this, activation of macrophages has been shown in the presence of the cathepsins, to facilitate osteolysis by the macrophage population themselves[185]. SERPINA3 (serpin peptidase inhibitor 3) is antichymotrypsin that has been shown to inhibit the function of the cathepsins.

1.25.2 Apoptotic Factors

Apoptotic pathways probably play a major role in the process of osteolysis. Although a number of apoptotic molecules have been included as members of other families, the regulatory proteins BAK1 and BCL2 can modulate the apoptotic pathway and are important to interpret other results in the correct context. Additionally the caspase group of proteins (CASP3) play a central role in execution of apoptosis and are also included as candidate genes.

TP53 (p53) is a transcription factor and tumour suppressor gene that has been implicated in BAK/BCL2 associated apoptosis. P53 expression has been identified in association with BAK in peri-prosthetic tissues using immune-histochemical techniques[186].

1.25.3 Markers of cellular Function

Several genes have been included as markers of cellular function. ITGAM (CD-11$\beta$ integrin) is a marker of macrophage function and acts to activate the complement cascade. Integrin $\beta$2 (ITG-$\beta$2) is also a component of the complement system and forms the $\beta$ subunit of CD11$\alpha$, CD11$\beta$ and CD11c.

MARCO (macrophage receptor with collagenous structure) is rarely expressed in healthy tissues. It is a scavenger receptor and has been demonstrated to be up regulated in response to wear debris challenge in vivo[187]. CILP (cartilage intermediate layer protein) is seen as breakdown product in early cartilage degeneration. It is unclear if CILP is a marker of osteolytic change.

CTLA4 is expressed on the surface of T helper cells and functions in a similar way to the co-stimulatory protein CD28. Both molecules bind CD80 and CD86 on the surface of antigen-presenting cells, and this interaction has been demonstrated to have an immunomodulatory effect[188].

ACP5 (tartrate-resistant acid phosphatase 5 - TRAP) is essential for osteoclastic function and plays a key role in bone remodelling. Whilst TRAP is highly expressed in osteoclasts although it is also found in neurons and activated macrophages. TRAP has been implicated as a marker of osteoclastic function[189]. TM7SF4 (DC-STAMP) is also a marker of osteoclast differentiation and has been implicated in osteoclastic differentiation and the production of multinucleated cells which are key to periprosthetic osteolysis[189].

ERCC1 (excision repair cross-complementing, complementation group 1) is an endonuclease enzyme responsible for the 5' incision during DNA repair. It has been used as a surrogate marker for DNA repair potential in a range of settings including periprosthetic osteolysis. ERCC1 is one of the only markers that has been previously found to be sensitive and specific for early osteolytic change[190].

Sclerostin (SOST) is an inhibitor of the WNT signalling pathway and the BMPs. Sclerostin is able to inhibit BMP-induced bone formation a process mediated by WNT signalling, but not BMP signalling pathways[150].

β2M (β-2-microglobulin) forms part of the MHC-1 complex and as such is expressed on all antigen presenting cells. While it has been previously evaluated as a housekeeping gene, it is used in this context as a marker of overall APC activity. VDR (vitamin D receptor) is a nuclear hormone receptor for vitamin D3 and gives an indication of vitamin D function in bone.

PTGS2 (prostaglandin-endoperoxide synthase), known as cyclooxygenase (COX) catalyses a key step in the production of all prostanoids (including prostaglandins, prostacyclin and thromboxanes). There are two isoforms of PTGS: a constitutive PTGS1 and an inducible PTGS2, which differ in their regulation of expression and tissue distribution. The discovery of COX-2[191] and differing tissue distributions for COX-1 and COX-2[192] resulted in the development of COX-2 specific anti-inflammatories which preferentially target COX-2. Bone and joint tissues have been shown to have a higher proportion of COX-2 activity. COX-1 and COX-2 inhibition has been shown to inhibit bone healing and remodelling in fracture[193].

# SECTION 1.26 RISK FACTORS FOR OSTEOLYSIS

The natural history of total joint arthroplasty is well studied. There are a number of complete population studies in the form of joint arthroplasty registers[1;35;36] and many longitudinal cross sectional and cohort studies reporting the survivorship of individual prosthesis[4;6;40;194].

Failure due to aseptic loosening of one or both of the components used in this procedure is the most common long-term complication after surgery. The incidence of aseptic loosening is known to increase with time[38] and published reports suggest there is variability in outcomes and revision rates even within groups implanted with the same prosthesis and within the same hospital[29].

The ability to identify patients who have risk factors for osteolytic change at the time of their surgery would allow for more targeted and successful follow up programmes reducing the risk of catastrophic silent failure.

Published cohort based studies and the national joint registries have identified a number of variables which have an impact on survival: implant, cement, surgeon, procedure as well as patient related factors such as age, BMI, diagnosis, smoking and sex [195-197]. The completeness and size of the joint registries allows for ever more detailed analysis and the recent report from the Swedish National Hip Arthroplasty Register suggests an increased risk of revision surgery associated with stem size and neck offset[198]. However although increasingly complete, joint registries have a limited data set on the patients they include, are not able to access patients hospital records and no registry currently includes information relating to radiographic or clinical scoring. This limits the sensitivity with which they are able to detect association between risk factors for failure.

This gap is addressed with more complete cohort or cross sectional studies reviewing individual patients. Careful control for known confounders or case matching has allowed the possible impact of BMI, tobacco use and non-steroidal anti inflammatory (NSAID) use to be quantified. Malik reported in 2004 in a retrospective cohort review of 224 patients that tobacco and NSAID use had no association with aseptic loosening[196].

## 1.27 Smoking as a Risk Factor

Smoking is known to have a detrimental effect on fracture healing and bone remodelling[199-203]. Smokers are known to have a longer anaesthetic and surgical time as well as have an increased risk for heart attack, stroke and respiratory disease in the post operative period[204]. Smoking has also been demonstrated to interfere with bone metabolism, revascularisation and bone formation[205]. A single cadaveric retrieval study[206] identified vascular injury and subsequent compromise of the implant-bone interface as a risk factor for failure in THR. Smoking is known to affect blood supply, making this a potential risk factor for failure. However only a single published review of 147 patients at a minimum of five years post THR reported a greater risk of implant loosening in smokers[197].

## 1.28 Co-morbidities as a Risk Factor

Co-morbidities may be expected to affect patient reported outcomes where many scores contain general functional questions which may be affected by other co-morbidities. However the impact of co-morbidity on longevity of total joint replacement is still unknown.

In a case matched prospective series 219 patients undergoing THR were matched to a cohort from the population who were not[207]. At short term follow up (3.6 years after surgery) similar quality of life scores were demonstrated. They also noted patients with other musculoskeletal co-morbidities had less long term functional improvement after THR. This study also suggested that preoperative existing co-morbidities did not predict a worse functional outcome[207]. At such short follow up the impact of co-morbidities on survivorship cannot be estimated.

Charnley proposed that the involvement of other joints also influences the outcome[208] and this has been confirmed by other investigators[204;209]. However these studies again do not investigate the impact on long term outcomes, only on post operative outcome scores, where a lower score will be expected due to the nature of the questionnaires.

Charlson and colleagues proposed and validated[210;211] a co-morbidity score, which has been validated for use in oncology patients. Their score has been shown to be an independent predictor of survival in oncology patients, but has never been applied to joint arthroplasty. It is one of only a few co-morbidity indexes that have been validated.

## 1.29 Obesity

The effect of obesity on longevity of joint replacement is controversial. However objective review of the literature clearly shows no link between obesity and poorer functional result or longevity of the THR[212-214]. This lack of evidence surrounding the effects of obesity and longevity of total joint replacements may represent poor study methodology as there are no studies containing a representative normal range of BMIs which correct for other known confounding factors.

Several studies have shown that the rate of post-operative complications is linked to obesity. There are several studies indicating higher rates of infection following joint replacement in the obese[215;216]. Biomechanically there is concern that increased weight increases joint reaction forces hence increasing wear of the polyethylene. However since obese patients tend to have lower functional demands it is unlikely that obesity increases wear of polyethylene components[217]. The improvement in outcome following THR appears to be similar for the obese and the non obese as is the satisfaction and function[218;219].

Despite the large number of studies relating to outcomes in total joint arthroplasty it is still not clear if patient related risk factors are likely to have an outcome of periprosthetic loosening. This is due to the short term follow up of the majority of case series, and lack of radiographic data in the joint registries.

## 1.30 Surgeon Related Factors

The biggest predictor of outcome in total joint arthroplasty is the surgeon, surgical team and implants selected[1;35;36]. High hospital and high surgeon volume has been demonstrated to reduce early post operative complications[220], however this does not appear to affect functional outcomes at three years[221]. A recent systematic review of the literature found that higher hospital volume had the effect of lowering patient mortality and rates of hip dislocation; where higher surgeon volume only had the effect of lowering rates hip dislocation[222].

There are few follow up studies in the literature establishing if grade of surgeon has any effect on the longevity of the joint replacement.

# SECTION 1.31 DIAGNOSIS OF IMPLANT FAILURE

Establishing a definite diagnosis of implant failure or loosening relies on :

- Clinical history and examination
- Radiographic evaluation

Joint loosening is often asymptomatic, and although proposed criteria for radiographic failure[32-34] do exist there is no consensus of opinion as to when a joint replacement is loose, even less when an implant requires revision[2].

The decision to revise an implant due to loosening is rarely made at the patients request, but more commonly during a routine follow up appointment. The British Orthopaedic Association recommends regular radiographic and clinical follow up for all patients who have undergone a total joint replacement[223].

At these follow up appointments plain radiographic films are recommended to evaluate fixation of the prosthesis and clinical review. Increasingly clinical outcome scores are becoming part of this review, and are recommended by the Department of Health as a performance indicator. It is however unclear, and there is little literature surrounding, the best method for establishing the diagnosis of implant failure. There is no evidence surrounding the use of clinical scores[224], and there is little evidence as to which radiographic assessment is likely to be most sensitive for osteolysis.

Changes on x-ray may precede symptoms of loosening and therefore presentation of the patient for revision surgery; however, there is not always consensus as to what x-ray changes actually represent definite radiological loosening. Radiological findings as predictors of implant failure are rarely reported.

## 1.31 Radiographic Diagnosis of Implant Failure

Osteolysis is normally diagnosed on plain x-rays, the patients themselves may present with pain, dislocation or periprosthetic fracture, but most are asymptomatic. Osteolysis is difficult to establish on plain radiographs such as those taken in follow up clinics. Temerman & Raijmakers[225] evaluated the sensitivity and specificity of plain films, subtraction arthrography, nuclear arthrography, and bone scintigraphy in a retrospective study of 86 patients with aseptic acetabular loosening. Anteroposterior (AP) and lateral films were used and plain radiography had a sensitivity of 85% (95% confidence interval, 71 to 94) and a specificity of 85% (95% confidence interval, 66 to 96) however intra-observer reliability was poor ($\kappa$ value 0.37)

Pfahler & Schidlo[39] evaluated 326 serial patients revised for aseptic loosening of their hip arthroplasty. Patients did not have a standardised imaging protocol, but in those just receiving plain films accuracy of plain films was found to be 89% specific for aseptic loosening. In this series only 40% were evaluated on plain films alone, the

majority being supplemented with a combination of digital subtraction arthrography or bone scan if the diagnosis was uncertain.

The explanation for poor sensitivity and specificity is likely due to the shape of the pelvis. On an AP view there is much overlying bone, and although the posterior and anterior acetabular walls are distinguishable they are not clearly visible. The standard lateral hip radiograph views the superior acetabulum tangentially, but does not 'shoot through' at the ideal angle to view the acetabulum, or anterior/posterior columns.

Southwell & Bechtold[226] constructed a computer simulation to produce computer-generated images to assess the effectiveness of standard radiological views in depicting periacetabular osteolysis surrounding a metal backed acetabular component. They additionally undertook radiological analysis of simulated defects in a cadaveric pelvis. AP view alone showed only 38% of the simulated defects and failed to depict a 3 mm lesion over 83% of the area of the cup, however addition of the 45 degree obturator-oblique and iliac-oblique projections increased the detection rate, showing 81% of the defects. They did not analyse, or follow up any real patients.

Kawate *et al*[227] undertook the only investigation to compare different acetabular views to assess cement mantle, however they did not investigate patients with osteolysis. They performed AP, lateral, iliac- and obturator-oblique views in assessing adequacy of cement mantle.

| | |
|---|---|
| AP View Alone | 25% incidence thin cement |
| AP & Lateral | 37% incidence thin cement |
| Four views | 57% incidence |

They concluded that "Thin cement mantles were mainly seen on the iliac oblique radiographs". It is similarly likely that aseptic loosening is under diagnosed on plain radiographs, and that investigation of the use of further projections is warranted to increase detection of osteolysis.

Evaluation of the available plain radiographic films to establish the best projection for arthroplasty follow up would potentially increase the sensitivity and specificity of arthroplasty follow up and consequentially improve our ability to diagnose and hence understand this condition.

## 1.32 Patient Outcome Measures and Failure

The benefits of total joint arthroplasty have been quantified by a variety of scores. Over time some of these have been refined and validated, and some have fallen by the wayside. Most scores have not been designed and validated from the outset, but rather invented and then retrospectively validated.

Hip outcome scores come in two varieties; patient administered and surgeon administered. Patient administered scores are a series of questions designed to quantify the current status of the hip joint based on a series of questions. Although varying from score to score most rely on multiple questions across several domains, usually pain, function and occasionally some functional or psychosocial questions. Patient administered scores do not include range of movement or other objective outcome measures, but are not subject to administrator bias as the patient themselves completes the questionnaire. Most scores rely heavily on pain often scored as a descriptive variable, however this method has been shown to be inaccurate in comparison with a visual analogue scale[228].

Charnley recognised that these outcome measures often rely on function of the whole limb, and as such results could be reflective of a diseased knee or a lumbar stenosis, not the joint in question. He argued that the results of differing groups of arthroplasty patients could only be compared if the groups are similar in other respects. He defined three different categories[208]. Patients in the A group had monoarticular disease; B had bilateral disease and C other diseases such as rheumatoid arthritis. Systematic reviews of primary total hip replacement outcomes[229;230] have concluded that the quality of evidence in this field is poor and that use of objective validated scores is likely to improve the evidence base.

There are many validated scoring systems in current use for the evaluation of hip replacement. However all of these scores have been designed as a measure of clinical outcome, and none have been validated to monitor for failure. Whilst it seems to make sense that a failing hip replacement is likely to perform more poorly than a successful one and therefore have an appropriately lower hip score there is no data to assess the sensitivity or reliability of patient reported outcome scores (PROMS) in arthroplasty surveillance, a use for which most scores were not originally designed.

Some scores in common use are generic quality of life measures, and others have been specifically designed to assess the patient's health status before and after hip replacement. For an outcome measure to be fit for purpose it must be reliable, valid, sensitive and easy to administer[231].

The 12 question Oxford Hip Score (OHS)[232] was developed recognising the problems of measuring specific forms of pain and mobility in older people for reasons other than hip disease. It was specifically designed as an outcome measure for use in large studies. The questions assess the patient's perceptions of pain, mobility and function in relation to problems with their hip. The questionnaire has been shown to be reliable with most respondents completing all the questions and with an overall good response rate. Each question is scored out of a possible 5 with 5 being the worst case scenario thus a perfect health scenario would give a OHS of 12 and a worse case 60.

Ferguson and Howarth[233] designed the first widely accepted hip outcome measure or hip score for grading disability after a slipped upper femoral epiphysis. The Merle d'Aubigne and Postel[27] score (MDA) is a refinement of the Ferguson and Howarth score and is still in widespread use. The score is based on questions in pain, range of movement and walking domains. Scored 1-6 in each domain and the scores are summed to give a single score.

The Harris Hip Score[234] (HHS) is the most widely used hip outcome score. Although it has been subsequently validated it was initially reported as an unvalidated score. The initial cohort of patients the score was designed to assess were young males who had sustained a high energy acetabular fracture. The Harris hip score asks questions relating to pain, function, deformity and range of motion. Initially a surgeon administered score the HHS has since been modified to be a patient administered score.

The Hospital for Special Surgery Score[28] was designed to provide a linear score which was responsive for both pain and function. It has been widely used in assessment of function post surgery for fracture of the neck of femur[235], but also in reporting the results of arthroplasty[236].

Despite the widespread use of these scores in reporting the outcomes of total joint arthroplasty none of these scores has been demonstrated to be sensitive or specific for osteolysis or failure in total joint replacement, and as such cannot be relied upon as an indicator of success in the longer term. It has been shown that hip scores deteriorate with time[3;236], whilst this may be reflective of early failure it may also represent falling functional level as the studied population ages.

# SECTION 1 : SUMMARY

## Biology

The biological cascade initiated by particulate wear debris is well characterised in tissue culture, ex-vivo and animal model studies. Gene therapy studies have demonstrated inhibition in the cascade when specific cytokines are knocked out.

Whilst the pathway itself is well characterised in established osteolysis there is little work that looks at the differences in response between patients who undergo an aggressive osteolytic response and those who do not. Some small scale studies have demonstrated differences in the presence of certain SNPs in cohorts of patients with osteolytic and well fixed joint replacements.

There is however no extensive characterisation of the local biological environment in end stage osteoarthritis into which the particulate debris is released, and no extensive studies quantifying the differences between patients with aggressive osteolysis and those without.

## Diagnosis

The diagnosis of osteolysis is fraught with difficulty and no agreed radiographic or clinical diagnostic criteria exist. Whilst clinical outcomes are often reported using patient reported outcome scores the value of these scores in detection of prosthetic failure is unclear.

It is likely that the radiographic projections used to follow up joint arthroplasty have a profound impact on the sensitivity of the test. However there are no studies evaluating the use of different projections in diagnosis of osteolysis.

Hip outcome scores although in widespread use have not been validated for detection of early clinical failure, but rather as quality outcome measures. The value of these scores and the differences between the scores in the longer term setting has not been established.

In order to understand the natural history of the disease it is important to establish accurate agreed diagnostic criteria. The first step in this process is evaluation of the methods currently available.

## Risk Factors

Identification of patients who are at risk of arthroplasty failure is not an easy task, and although a number of factors have been identified through use of registries and case controlled series there is little high quality data on the way patient epidemiological factors affect the osteolytic process over a period of many years.

# SECTION 2 : STUDY QUESTIONS & PROPOSAL

The aim of this MD project is to explore the contribution of patient related factors to osteolysis, and the best measures both radiographic and clinical to identify osteolytic hip joints.

**Study Questions :**

1. Does the osteolytic cascade vary from patient to patient?
2. What are the patient characteristics most likely to predispose to joint loosening?
3. What is the best radiographic evaluation of osteolysis?
4. Is it possible to use a patient administered outcome scores to identify osteolytic arthroplasties?

In order to answer these questions four studies are to be undertaken exploring the cellular biology of osteolysis, the epidemiology of loosening and the value of radiographs and PROMs in detecting osteolysis

## Section 2.1 Laboratory Study

Hypothesis : "There is a difference in the activation of biological cascade between patients subjected to polyethylene wear debris as evidenced by cytokine expression"

A laboratory study has been designed to test the experimental hypothesis using RNA expression analysis. Initially gene RNA expression analysis will be undertaken used to quantify gene expression in human patients at the time of their hip surgery. In order to address the hypothesis a number of smaller research questions must first be answered :

1. Can we reliably measure gene expression in osteoarthritic human joints?
2. Does osteoarthritis differ in biological environment from patient to patient?
3. Is there any observed variation local or global within the joint?
4. Do joints with osteolytic change have a different gene expression pattern to native hip joints?
5. Are these changes due to the joint replacement in situ, or to loosening itself?
6. Are observed biological changes wear debris related or do they represent a tendency to loosen?

## 2.2 Patient Risk factors for Osteolysis

Hypothesis : "Patient characteristics such as age, BMI, smoking and co-morbidities will have a measurable effect on the survivorship of total hip prosthesis over long term study"

In order to test the hypothesis a long term cohort study with the end point of prosthesis failure was undertaken. Careful prospective follow up of a cohort of patients having undergone a similar procedure at a similar time in the same institution is required to reduce the likelihood of confounding factors. At the Norfolk & Norwich University Hospital all patients who have undergone a total joint arthroplasty procedure are

entered into a joint review programme. Prospective data has been carefully collected and institutional and ethical review of this process has already been undertaken.

A cohort of patients who had undergone their total joint arthroplasty in 1995 were due to have their 12 year follow-up appointments at which point they would all receive surveillance radiographs and clinical scores and a decision on the survival of the prosthesis or need for revision would be made.

The data from these patients was used in conjunction with some additional radiographs (after ethical consent, see below) to establish if any identifiable patient characteristic factors present at the time of their surgery that may contribute to the survival or failure of their arthroplasty. Survivorship analysis was used to establish any significant differences in survival based on epidemiological factors.

## 2.3 Radiological Study

Hypothesis : "The addition of iliac oblique radiographs to radiographic follow up films will increase the sensitivity of radiographic follow up for osteolytic lesions"

To test the hypothesis and establish the sensitivity of plain film radiographs for prosthesis loosening a complete set of all combinations of radiographs is required in a cohort of patients known to be likely to have a high rate of radiographic failure.

Ethics permission was sought, and given, to perform after consent additional radiographic examination of a cohort of patients undergoing their 12 year radiographic follow up appointments. At 12 years approximately 10% of THRs can be expected to have failed or be in the process of early failure.

The radiographs were evaluated by two independent observers and scored using recognised radiographic scoring systems. The contribution of each project was then assessed to establish the value of each projection and combination of projection.

## 2.4 Patient Outcome Scores Study

Hypothesis : "Although increasingly used for arthroplasty surveillance no single patient outcome score has been designed for that process. There will be differences in the ability of widely used scores to detect clinical failure of a THR"

In order to establish the sensitivity and reliability of each widely used outcome score several cohorts of patients would be required all being followed up with different arthroplasty scores. Use of multiple surgeons and different prosthesis is important to make the data as generalisable as possible.

Patients with a known outcome (i.e. surviving or failed prosthesis) undergoing their ten year arthroplasty follow up at two different institutions received one of five different outcome measures. Their resultant scores were analysed for sensitivity and specificity for outcome using receiver operator characteristic (ROC) statistics.

Using this method the value of scores in general, the different domains of each score and a comparative analysis between scores could be undertaken.

# SECTION 3 : GENE EXPRESSION PROFILING

## 3.1 Introduction

The aim is to establish what range of cellular interactions are occurring in human hip tissue under different conditions. In order to establish this, tissue was taken of capsule (C) and ligamentum teres (LT). TaqMan analysis aims to establish the range of gene expression between patients in these two tissues. Having established this, the study then needs identify which pathways of cellular interactions differ between loose and well fixed total hip replacements in order to answer the hypothesis. The aim to establish which pathways are variable between patients with end stage osteoarthritis, those with osteolysis, and which are modulated in response to long term exposure to wear debris, but not osteolysis. This study is not aiming to elaborate the details of any pathways, but to identify known pathways which may be differentially activated from patient to patient.

There are a range of techniques that can be used to establish what the activity of a gene, or rather the protein it encodes is. The activity of a protein or pathway can be measured at any point between the genomic DNA and measurement of the activity of the protein (eg measurement of breakdown or synthetic products). Commonly gene expression is measured at a DNA, RNA or Protein level. Each has its own strengths.

DNA Analysis – The most commonly used technique for DNA expression analysis is through evaluation of single nucleotide polymorphisms (SNPs). SNPs are individual base pairs at a known locus that vary between genotypes so act as markers for known genetic polymorphisms. A large number of SNPs are defined and the genotype of an individual can be ascertained. SNPs have the advantage that the genotype can be established from a peripheral blood test and that large numbers of genes can be tested from a single sample. However SNP analysis focuses on the genotype of the patient, not the activity of the gene. For this study SNPs would be inappropriate as this technique does not allow up or down regulation of gene expression in response to wear debris challenges to be characterised.

RNA Analysis – The most commonly used RNA expression analysis technique is the TaqMan technique (Figure 3.2.8.1). TaqMan relies on reverse transcription of mRNA into DNA and then use of a PCR reaction for the DNA with a fluorescently labelled probe. The process is quantitative in that the levels of gene expression can be measured and compared relative to each other. The process also allows multiple different genes to be analysed from the same tissue sample. However it does have a number of draw backs. There is some downstream regulation of protein activity during folding, and the site of expression cannot be determined. However despite these drawbacks TaqMan remains the most versatile technique for this kind of quantitative study.

Protein Analysis – Protein expression analysis is the most accurate way of determining how much of a protein is expressed. Use of specific assays or *in situ* immunofluorescence techniques allow not only for quantitative analysis of protein expression, but also to allow for localisation of that expression within the tissues. However these techniques are expensive and specific assays are required for each protein to be investigated. This limits greatly the number of studies that can be done. This type of analysis is therefore most suited to localise expression after an RNA or DNA study has been performed.

Protein products – Some of the specific assays for proteins do not assay the protein itself, but rather breakdown products. This allows for activity of the protein to be established. They have the same drawbacks as other protein based studies.

Animal models – A validated animal model provides the closest laboratory model possible to human tissue. They allow for manipulation of the environment through use of knock-out mice or addition of inhibitors to the model. However animal models are best suited for elaborating pathway and establishing the likely biological mechanism for previous observations. They do not as a rule allow for variation between individuals to be characterised.

The study aims to establish what the activity in explanted hip tissue if of previously identified pathways and how this varies between patients. A TaqMan technique is best suited for this purpose. Levels of RNA expression in tissue samples obtained directly from the hip during surgery will be determined using a TaqMan technique. Messenger RNA expression is analogous to protein production, although there can be some modulation of transcription downstream from the mRNA production step. It is important to ensure that mRNA expression for known inhibitors and co-factors is also quantified to allow the results to be interpreted in context.

## 3.2 Materials & Methods

### 3.2.1 Ethics

Ethical approval for this section of the study was sought from the 'Cambridge 3' regional ethics committee, under the application "Osteolysis in total joint replacement – are patient factors important" (reference 07/H0306/80). Ethical approval was granted in December 2007. During the preparation of the ethics submission a number of consultant surgeons were approached and an informal peer review process undertaken. Approval was also sought from both the research and development committee and a site specific assessment was also carried out, as the study was to make use for the tissue bank facilities at the Norfolk & Norwich University Hospital (NNUH).

The full ethics submission, patient information sheets and consent forms are included in the Appendix.

### 3.2.2 Tissue Sampling & Storage

Tissue samples were taken intra-operatively from patients undergoing primary joint replacement for osteoarthritis (primary group – P), patients undergoing revision for aseptic loosening (loose group – L), or patients undergoing revision for other reasons (well fixed group – F).

Tissue was taken according to a standardised protocol (Appendix 2) by a consultant orthopaedic surgeon during the procedure. The primary group had samples of ligamentum teres (LT), hip capsule or both excised during surgery. The patients undergoing revision surgery had samples of capsule (both groups) and samples of femoral or acetabular membrane tissue (loose group) as appropriate. All samples were stored at room temperature submerged in an RNA stabilising agent (RNALater; Qiagen Ltd, Crawley, UK) for an hour prior to storage at -80ºC in the tissue bank.

The NNUH has an existing tissue bank based in the Histopathology department that has full ethical and Research & Development approval. Tissue stored in the tissue bank are anonymised to a tissue bank number. There is a separate consent procedure required for storage of human tissue and previously approved tissue bank consent forms and information sheets were used for this purpose. The tissue bank itself is overseen by a consultant histopathologist and rigorous procedures are in place to safeguard the tissue and confidentiality.

Sample collection took place over a six month period, and all samples were collated prior to eventual analysis to reduce errors associated with laboratory technique and RNA degradation over time.

### 3.2.3 Consent Process

Informed consent is vital when undertaking any research in which human tissue is to be used. In order for informed consent to be obtained patients must be fully informed about the study, risks and potential benefits to either themselves or the wider community. Patients must be approached at an appropriate time so that they have time to read the information sheet, ask any appropriate questions and make a decision having fully understood the study. Patients were approached by a specialist nurse and supplied with an information sheet and a copy of a study consent form to review at their pre-operative assessment visit. All of the study materials had been approved by the ethics committee.

Patients were identified by their operating surgeon at their pre-operative assessment visit. Written information was supplied to the patient, and a nurse specialist met with the patient, explained the study and invited the patients to take part. Although study materials were provided at this point patients were not asked to make a decision until their admission for surgery six weeks later.

Patients were visited on the morning of the surgery by both the operating surgeon and a nurse specialist. They were invited to ask any questions regarding the study, and if they wished to proceed they consented for study

participation on both the tissue bank and study consent forms. All patients approached about participation in the study agreed to take part. A copy of both completed consent forms is retained by both the tissue bank at the NNUH and in the Academic Department of Orthopaedics.

Copies of the consent and information forms are included in the Appendix.

### 3.2.4 Patient Identification and Data Collection

Potential study participants were identified from the waiting lists of six consultant Trauma and Orthopaedic surgeons with an interest in hip arthroplasty. The patients were initially identified by the consultant surgeon as they were placed on the waiting list as potential participants, and approached at their pre-assessment clinic. Patients were considered suitable for inclusion in the study if were :

Primary Group : Undergoing primary hip arthroplasty for osteoarthritis

Osteolytic Group : Undergoing revision hip arthroplasty for an osteolytic metal-on-polyethylene cemented THR

Well Fixed Group : Undergoing revision surgery of a well fixed metal-on-polyethylene cemented THR for other reasons (e.g. dislocation)

Patients were excluded from the study if they had evidence of autoimmune arthropathy, infection, alternate bearing surfaces, fracture round a loose stem, were receiving immune-modulating drugs (e.g. steroids) or had any known immunosuppressive condition.

Patients were anonymised at recruitment to a study number, and all data thereafter was referenced only by study number. For patients in the primary arthroplasty group data were collected concerning the patient's demographics (age and sex), pre-operative diagnosis and concomitant medical conditions. In addition preoperative radiographs were reviewed and scored for osteoarthritic change. In addition patients underwent an Oxford Hip Score and EQ4D quality of life index.

Patients in the two revision groups (osteolytic and well fixed) had the same demographic data and preoperative scores collected, but in addition details of the previous surgery and reason for revision surgery was recorded. Following surgery the results of routine intra-operative tissue samples for infection were also reviewed to ensure no patient had subclinical infection.

### 3.2.5 RNA Extraction & Purification

RNA extraction was performed in an identical stepwise process from the tissue. The full protocol is detailed in the Appendix. Initially the tissue is homogenised, cells lysed and the RNA dissolved in solution. Phase separation by chloroform separates the RNA containing fraction from the residue and this is further purified in spin columns.

Tissue samples were defrosted to 4ºC and the excess RNA stabiliser removed. Tissue was kept at 4ºC on ice to reduce the rate of RNA degradation which is unstable at room temperature.

Samples were weighed and 3g of tissue homogenised in 2mls phenol reagent (TRIzol; Invitrogen Paisley, UK) using an UltraTurrax homogeniser (UltraTurrax, IKA, Staufen, Germany). The phenol lyses the cells and the RNA passes into solution. The resultant particulates were centrifuged at 14,000 $g$ for 10 minutes at 4°C and the supernatant was recovered.

A total of 400μL chloroform was added per 1 mL TRIzol and vortexed for 15 seconds. The TRIzol/chloroform solution was then centrifuged at 14,000 $g$ for 10 minutes at 4°C and the aqueous layer was placed into a fresh Eppendorf tube.

The resultant supernatant was mixed with 100% ethanol at 1:1. Samples were applied to spin columns (RNeasy Mini Kit; Qiagen, Crawley, West Sussex, UK), centrifuged at full speed for 15 seconds, and the flow through was discarded. Columns then were washed and eluted according to the manufacturer's instructions.

### 3.2.6 Nanodrop

RNA samples were quantified using a spectrophotometer (NanoDrop; NanoDrop Technologies, Wilmington, DE) and subsequently stored at -80°C. The NanoDrop allows quantification of RNA, DNA and protein contamination by use of absorption spectrometry. Quantification of RNA was used to calculate concentration of RNA and standardisation of RNA quantity for the reverse transcription phase.

The NanoDrop also allows for a quality control step to ensure the RNA purification is acceptable. Good quality RNA has a typical characteristic curve (Fig 3.2.6.1). The absorbance curve starts at 220 nm with a "reverse tick" climbing to a peak at 260 nm then diminishing to zero at around 310 nm.

Calculation of the 260:280 absorbance ratio gives an indication of sample contamination eg with DNA. Samples were only included in subsequent analysis if >100 ng/μl mRNA was present, and the 260:280 ratio was between 1.9 and 2.1 indicating low contamination

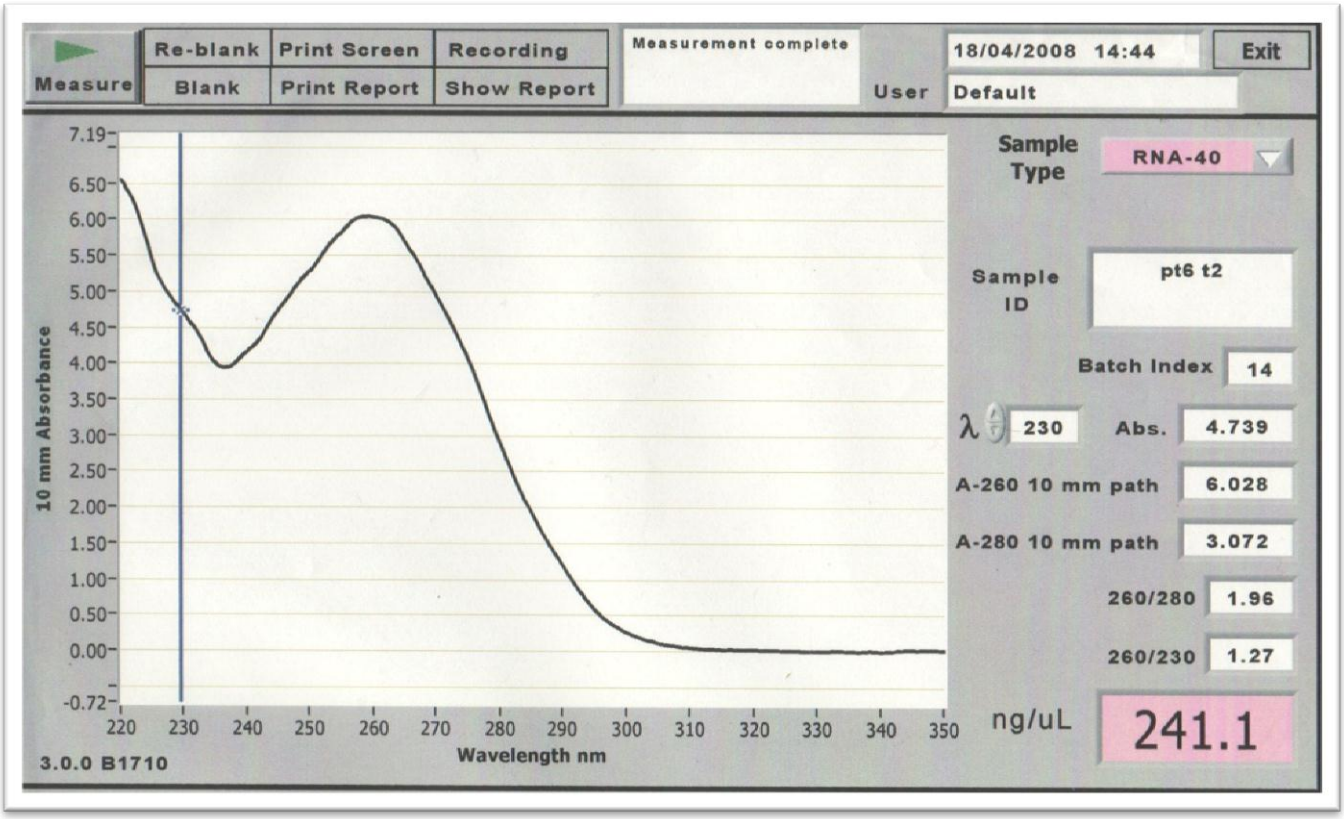



Figure 3.2.6.1 : Sample Nanodrop Spectrometer curve for Patient 6 demonstrating the classical 'double tick' spectroscopy. The spectrograph measures absorbance (y axis) against wavelength (x axis).

### 3.2.7 Reverse Transcription

Synthesis of complementary DNA was performed from 1μg of total RNA through a reverse transcription (RT) reaction. This was added to an appropriate volume of RNAase free $H_2O$ to give a final 9μL volume. To this 2μL $pd(N)_6$ random hexamers (1 μg $\mu l^{-1}$) were added and incubated for 10 minutes at 70ºC. This anneals the random hexamers to the RNA chains and which then act as a primer.

To each sample was added :

- 1 μL (200U) SuperScript II reverse transcriptase
- 1 μL RNase inihibitor (RNaseOUT Ribonuclease Inhibitor 40U)
- 4 μL 5x Buffer
- 1 μL dNTPs (10 mM of each deoxyribonucleotide)
- 2 μL 0.1M DTT (dithiothreitol)

The resultant mixture was incubated at 42ºC for an hour and then inactivated by incubating at 70ºC for 10 minutes. Superscript II performs the reverse transcription step using the dNTPs to produce the cDNA chains. The 5x buffer gives optimum conditions for the RT enzyme and holds the pH at 8.3. The DTT acts as a reducing agent improving the efficiency of the reaction. Complementary DNA was subsequently stored at -20ºC until used in downstream PCR. The RT reaction is assumed to be 100% efficient and subsequent dilutions performed on this assumption.

Due to the potential to introduce error at any stage in the RT process, all samples underwent RT in the same batch, using the same Superscript II and reagent mix. This increases the reliability of the results and allows for comparison between groups.

### 3.2.8 TaqMan Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

After RNA extraction and reverse transcription initial analysis was undertaken with manual TaqMan qRT-PCR to quantify housekeeping gene expression, and ensure the reverse transcription process had produced uniform cDNA libraries across each sample.

The principle behind quantitative PCR is the repeated amplification of cDNA and during each cycle a fluorescent marker is released that is detected by the machine. During each low temperature cycle the primer anneals to the gene of interest (Fig 3.2.8) and the quenched fluoroscopic probe binds to a downstream sequence. The TaqMan polymerase enzyme then produces the complementary DNA chain and in the process degrades the probe to release the fluorescent molecule. This fluorescence is detected by the analyser. During the higher temperature cycle the DNA chain is denatured and single stranded DNA is ready for the next cycle. The number of cycles taken for the signal to rise above the background noise equates to the quantity of cDNA present at the beginning of the process.

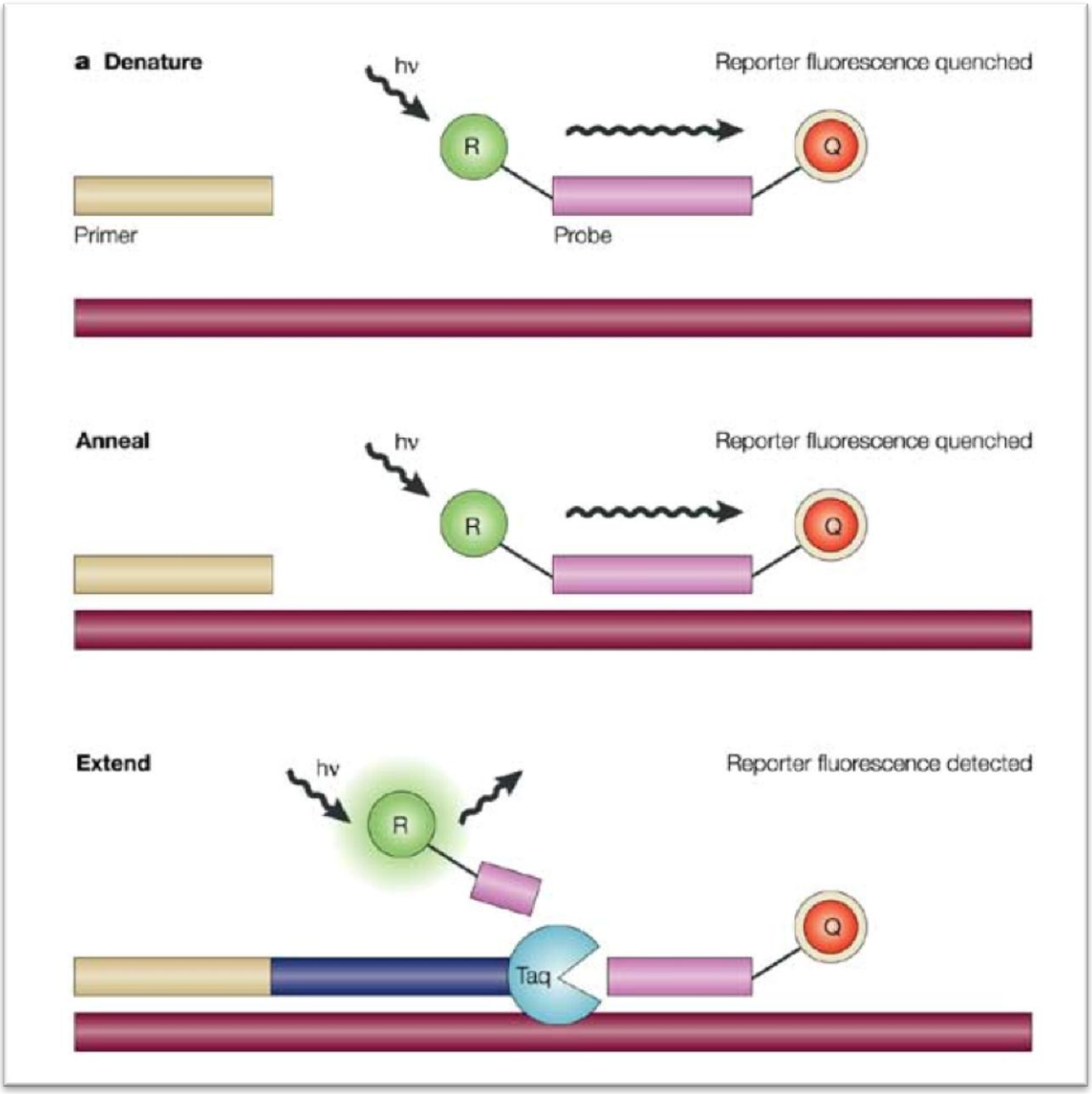



Figure 3.2.8.1 : The TaqMan process. During the annealing phase the primer and probe bind to the DNA chain. During the extending phase the Taq polymerase polymerises the chain from the primer and in so doing cleaves the reporter from the probe. The fluorescence molecule is released and detected by the machine. Reproduced from Nature Reviews Drug Discovery 3, 749–761 (1 September 2004) Koch. W: Technology platforms for pharmacogenomic diagnostic assays

Initially TaqMan analysis was performed using primer probe sets designed using the universal human probe library. Oligonucleotide primers were designed (Primer Express 1.0 software; Applied Biosystems, Warrington, UK) using known sequences for candidate genes TNF-$\alpha$, IFN-$\gamma$, OPG, RANK, RANK-L and IL-6. In addition housekeeping genes 18S, GAPDH and $\beta$-actin were also measured.

Although the RNA extraction process and reverse transcription is designed to remove the majority of genomic DNA and precautions are taken to reduce DNA contamination a potential for error is inadvertent amplification of genomic DNA. To reduce this risk primers were designed placed within different exons and close to an

intron/exon boundary, with the probe spanning two neighbouring exons where possible. This makes inadvertent amplification of a gene present on genomic DNA impossible.

The 18S ribosomal RNA (18S) gene, GAPDH, succinate dehydrogenase (SDHA) and β-actin were used as endogenous control, or housekeeping genes, to normalise for differences in the amount of total RNA present in each sample. All four of these genes have been previously shown to have a uniform expression in a range of musculoskeletal tissues and therefore to be suitable for use as housekeeping genes. Custom primer probe sets were purchased from Applied Biosystems. The relative quantification of genes was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems) in accordance with the manufacturer's protocol.

The ABI Prism 7700 can analyse 96 samples concurrently on a 96 well plate. The TaqMan plates were prepared with either candidate gene, standard curve, negative control or housekeeping gene preparation prior to PCR. To candidate gene wells 5ng of cDNA was added with 15μl of reagents. The reagents consisted of 8.33 μl of TaqMan master mix, 5 pmol forward primer, 5 pmol reverse primer and 5 pmol of probe made up to 15μl with nuclease free $H_2O$. The housekeeping (18S) gene wells contained 1ng of cDNA as 18S is so highly expressed only 1ng is required. The negative control wells contained no cDNA, and as such no amplification should occur.

In addition standard curves for each gene were constructed to correct for the efficiency of the primer/probe sets across different RNA concentrations. Serial dilutions were performed which can then be used to calculate a relative concentration of RNA (ng/dL).

The PCR reaction was performed over 40 cycles. The plates were initially incubated for 2 minutes at 50°C, 10 minutes at 95°C and then 40 cycles each consisting of 15 seconds at 95°C and 1 minute at 60°C. The qRT-PCR method outputs fluorescence values against time (Fig 3.2.8.2). After adjustment for noise a threshold value is selected that intersects the most linear portion of the sigmoid curve the number of cycles to threshold ($C_T$) is proportional to the quantity of cDNA present in the initial sample.

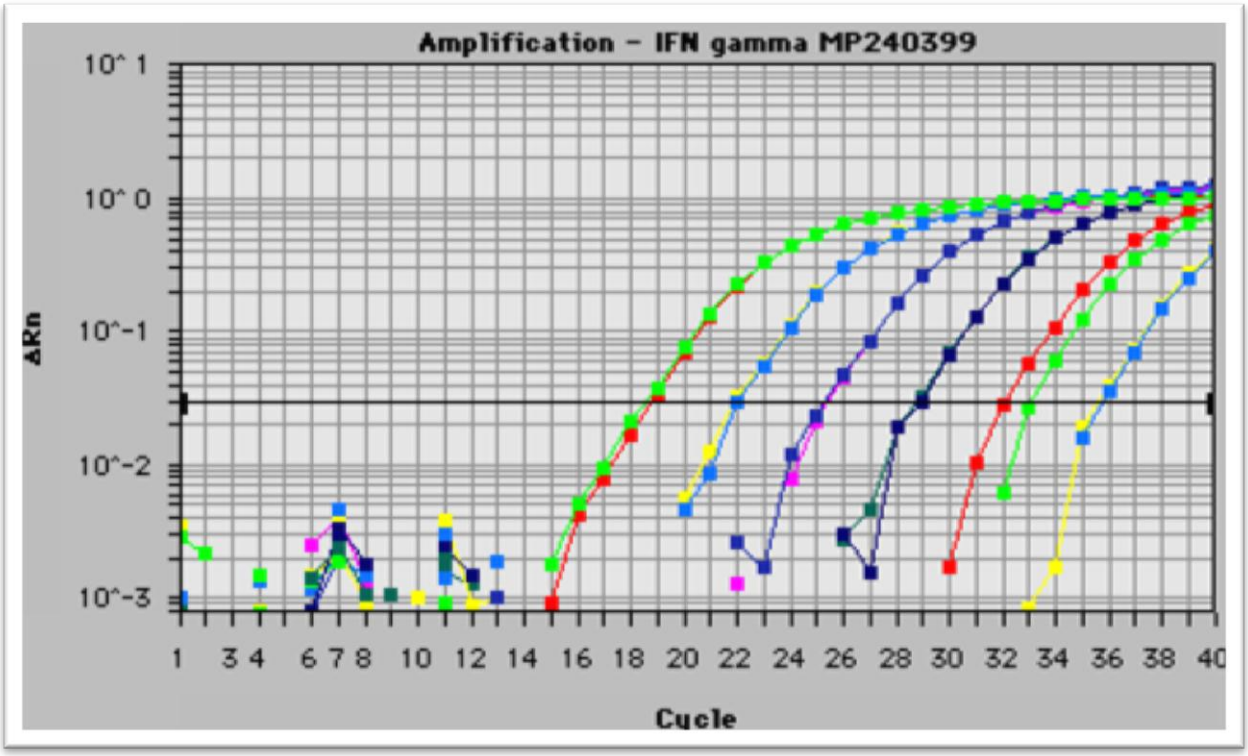



Figure 3.2.8.2 : Sample TaqMan mRNA amplification curves for IFN gamma.

An approximate comparison across all genes measured can be obtained assuming the amplification reaction efficiency was identical across all primer sets. The raw $C_T$ values measured by the analyser are normalized to the housekeeping gene expression using a transformation proportional to normalized copy number ($2^{-\Delta C_T}$), where $\Delta C_T$ is the change in $C_T$ (target gene) – $C_T$ (housekeeper).

### 3.2.9 Housekeeping Genes

Housekeeping genes are genes that are known to have very similar expression of mRNA across all samples, and their expression therefore correlates closely with the total mRNA present in the sample. Deviation from the median expression value likely represents errors introduced in the preparation stage due to poor laboratory technique. For this reason median expressions for housekeeping genes are calculated and samples included only if they fall within +/- 2 $C_T$ of the median values to minimise the effects of laboratory errors.

### 3.2.10 Standard Curves

When comparing the expression of a single gene across sample groups a further potential error is introduced as the binding characteristics of the primer/probe sets are not linear with DNA concentration and vary between both genes and primer/probe sets. This problem can be overcome with production of standard curves for each gene. A standard curve is generated using the known concentrations of complementary DNA (cDNA) from one sample. Serial 2-fold dilutions across an appropriate range are used to construct the standard curve. The standard curve can then be used to allow for the variable efficiency of the reaction and calculate ng/dL of mRNA.

### 3.2.11 Universal library Primer/Probe sets

The universal probe library is a library of predetermined downstream probes that provides probe sites for the whole of the human genome. When paired with a custom primer set designed for use with the Universal Library gene expression can be measured for almost any gene. Whilst this adds flexibility the position of the probe cannot be as tightly controlled as with a custom primer/probe set and as such may not sit in an ideal position on the gene of interest.

For initial analysis after RNA extraction primers were designed for the Universal Library probes to quantify expression of two housekeeping genes (18S, $\beta$ Actin) and six candidate genes (TNF $\alpha$; IFN $\gamma$; IL1 $\beta$; IL6; OPG & RANK-L). TaqMan plates were run including standard curves for all samples for housekeeping genes, and for a selection of 16 patients (10 primaries and 3 revision from each group).

The quality of RNA extraction was ascertained from the expression of the housekeeping genes, and the protocol fine tuned until all samples were within +/- 2 $C_T$ of the median value.

### 3.2.12 Repeated Analysis

To assess for intra- and inter- observer error repeated analysis was performed. Different portions of the same tissue sample were used for parallel extraction and RNA quantification to assess intra-observer error. This also allowed measurement of gene expression variation within the same tissue. A third party evaluated the inter-observer error using RNA extracted by the investigator and the same reverse transcription and TaqMan protocols. Results were analysed for correlation with 18S expression and for $C_T$ values of the target genes.

### 3.2.13 Custom Primer/Probe Sets

One final quality control step was introduced prior to moving analysis to the custom low density arrays. Specific primer probe sets were designed for three genes IL6, IFN $\gamma$ and $\beta$ Actin. These were used to prepare TaqMan plates using the technique outlined above to corroborate the results from the Universal Probe Library. Once this had been performed and the initial results confirmed further analysis was performed using a TaqMan Low Density Array (TLDA) technique.

### 3.2.14 TaqMan Low Density Array (TLDA)

The TaqMan low density arrays (TLDA) are custom manufactured microfluidic arrays. Each TLDA array is a 384-well micro fluidic card that enables 384 simultaneous real-time PCR reactions. A custom designed TLDA array card allowed 96 genes from 4 samples. The TaqMan low density array cards are pre-loaded with custom primer probe sets and all reagents required for the reaction.

Patient samples are loaded into the array through ports, and centrifugation used to distribute the samples across the wells. The PCR reaction is performed in an identical manner to the manual TaqMan using an Applied

Biosystems 7900HT Fast Real-Time PCR System. The TLDA array is optimised such that the custom designed primer-probe sets have as near uniform efficiency across cDNA concentrations. Although no independent standard curve analysis is performed the system is designed not to require this further analytic step.

# 3.3 Laboratory Results

## 3.3.1 Standard Curves

Standard curves were plotted from the known serial dilutions of mRNA (Fig 3.3.1). A best fit line was plotted for each. The raw $C_T$ values were converted to relative DNA concentrations in ng/µl using the standard curve. The intercept and slope of the best fit line can then be used to calculate the relative expression of initial mRNA. Using this method corrects for the differing primer-probe set affinities.

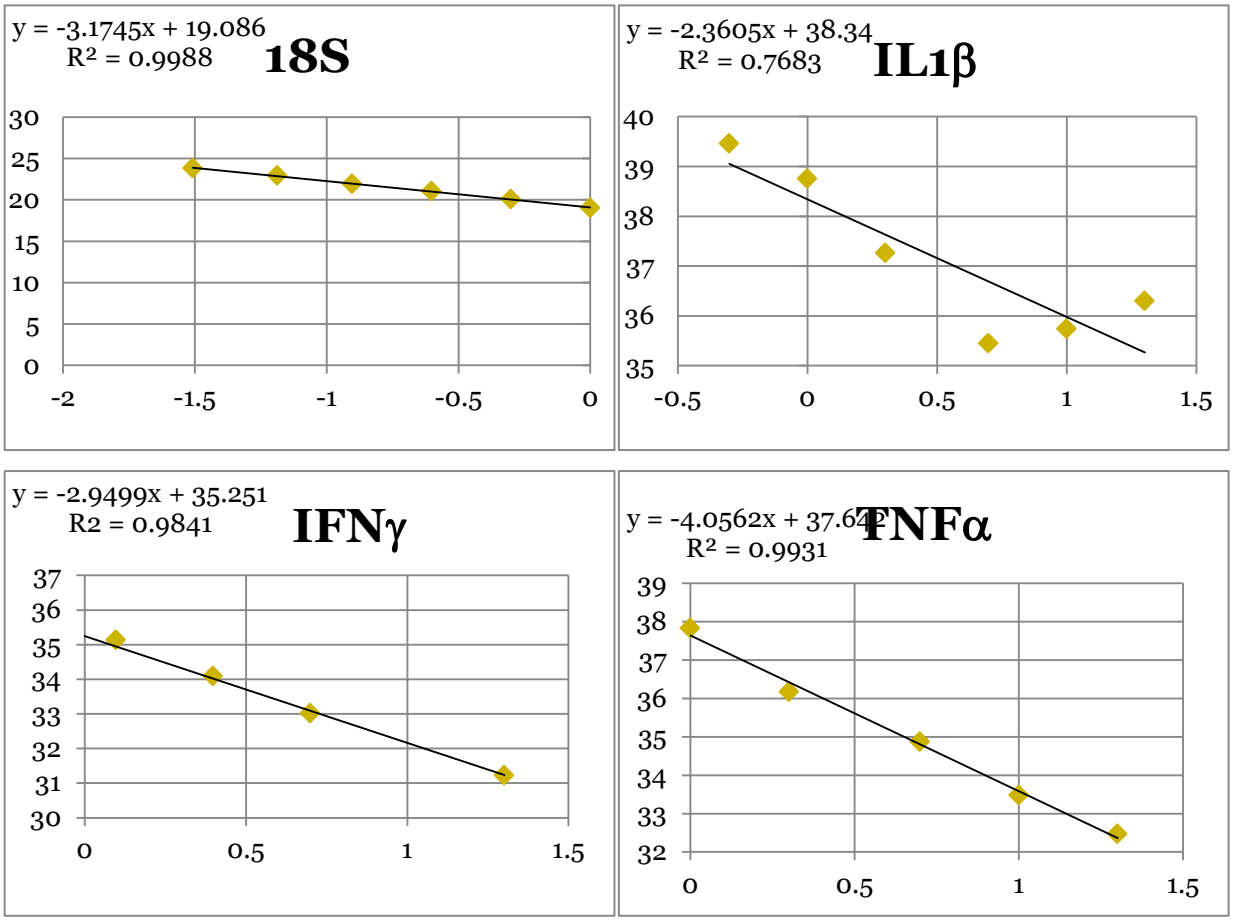



Figure 3.3.1 : Sample standard curves plotted for different genes. Serial dilutions of mRNA $C_T$ values are plotted on the y-axis against [$\log^{10}$ cDNA] and best fit lines calculated.

## 3.3.2 Tissue Gene Expression Results

The housekeeping gene 18S was used as a control to ensure comparable quantities of total RNA were processed from each sample, thereby allowing for comparison of results. 18S expression was comparable with all included samples falling between +/- 1.5 $C_T$ values of the median value (Fig 3.3.2.1). No samples had to excluded at this stage due to variation in the 18S $C_T$ values.

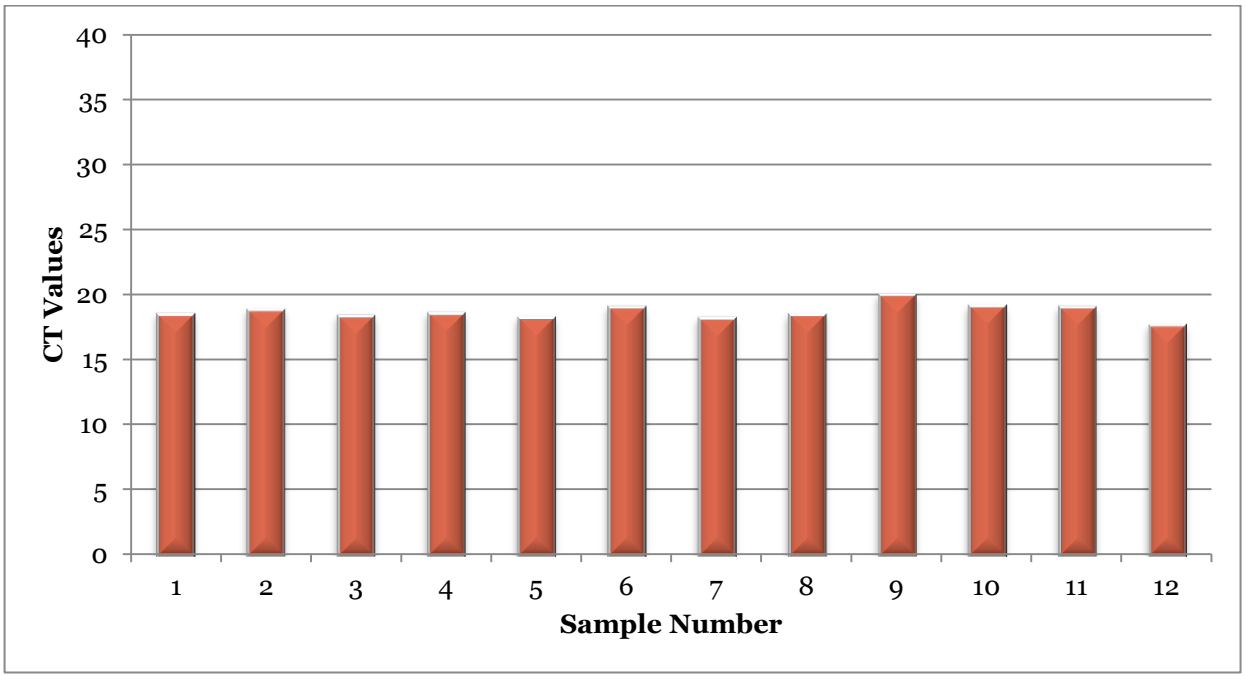



Figure 3.3.2.1 : Plot of 18S Expression ($C_T$ values) for each sample tested, demonstrating acceptably uniform expression.

### 3.3.2.1 Comparison of Tissue Gene Expression

Tissue expression ranges, medians and quartile ranges are given for each tissue type (Fig 3.3.2.2).As can be seen the tissue expression ranges of the capsule (light bars) and ligamentum teres (hatched bars) is closely related between the two tissues, with similar medians, ranges and spread of the data about the median for all five genes.

In all cases the gene expression is more tightly clustered about the median in the ligamentum than it is in the capsular samples. The closely mirrored distributions of each gene gives an indication that tissue expression is similar in each tissue within the joint. However the number of samples included in this part of the study (12) are too low for formal statistical analysis.

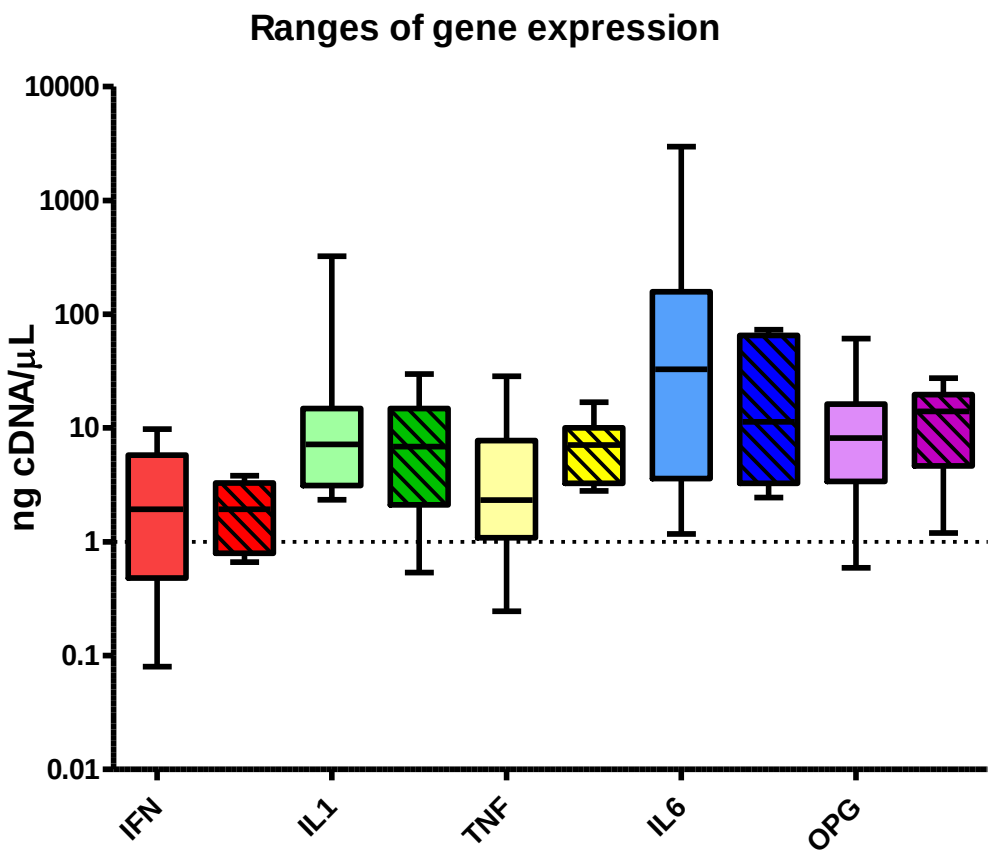



Figure 3.3.2.2 : Gene expression ranges, quartiles and medians capsule (solid bars) and ligamentum teres (shaded bars) for all 12 samples.

### 3.3.2.3 Variability of gene expression

The variation in expression between each gene across both tissues is given (Fig 3.3.2.3). All genes tested for expression demonstrated high levels of variation between tissues, with all tested genes demonstrating at least 100 fold variation in tissue expression levels. This supports the original hypothesis that gene expression is variable within osteoarthritic hips.

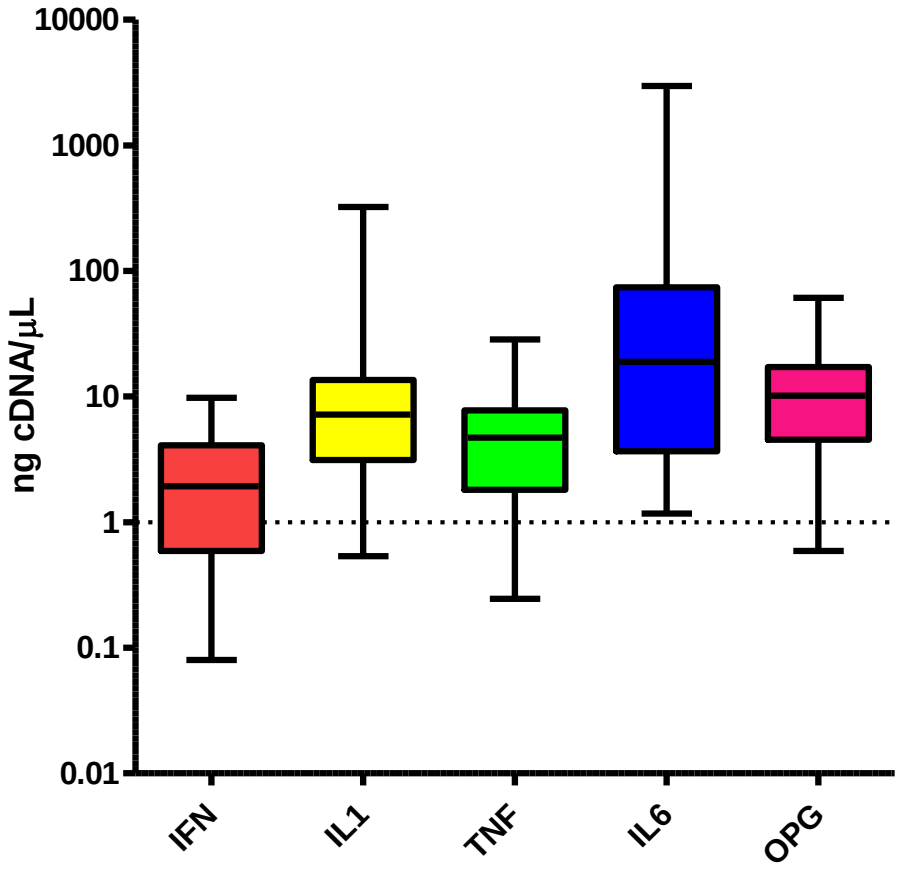



Figure 3.3.2.3 : Gene expression ranges (median, range and quartiles) by gene for the whole group

### 3.3.2.4 Range of Variation

When sampling from a population of an unknown size it is difficult to know how many samples are required to capture adequately the complete range of expressed variation. The nature of a cohort study makes it impossible to capture every possible variation in expression without performing a population study. However if gene expression is assumed to be a Gaussian distribution, and mean expression is being measured it is possible to assess the number of observations required to obtain a stable estimate of the mean through measuring changes in mean expression and standard deviation as additional observations are performed. This is in essence measuring the observed stability of the mean.

Plotting mean expression of IL-1β and standard deviation against number of observations (Fig 3.3.2.4) shows stabilisation of both the mean and the SD after 10 observations. It is therefore likely that with the planned sample size of 30 observations that enough variation will be observed to be representative of variation in the wider patient population.

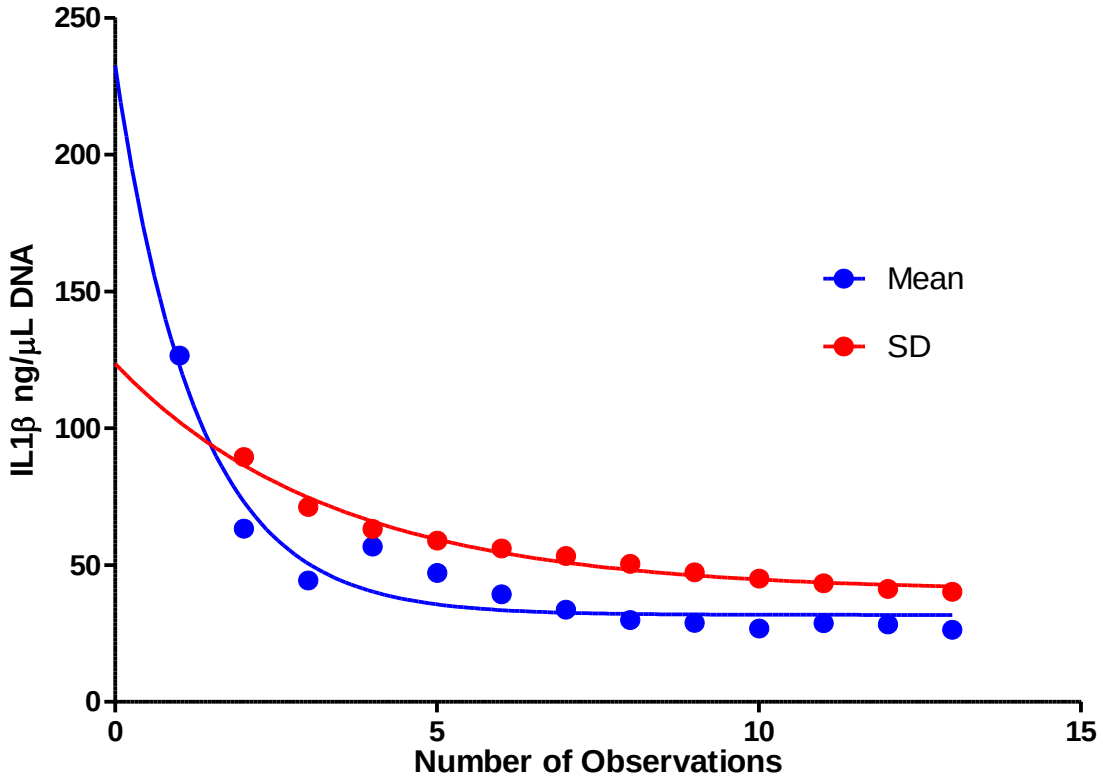



Figure 3.3.2.4 : Variation in mean and standard deviation of IL1β expression shown for increasing population size

3.3.2.5 Reliability and Repeatability

The nature of PCR related analysis is one in which errors in technique are multiplied many hundred fold. Due to the exponential nature of the PCR reaction any errors in pipetting or other aspects of experimental technique will be multiplied on each cycle of PCR. The sensitivity of this method necessitates meticulous laboratory technique, and errors introduced in this manner may outweigh any observed differences between samples.

In order to assess the accuracy of the process and quantify the errors introduced in the laboratory two validation exercises were undertaken at this stage. Two of the samples (4 and 5) were divided in half and the whole process from RNA extraction to TaqMan expression analysis repeated independently on different days by the author. The expression of three candidate genes (IL1β, IFN γ and TNF α) was then quantified (Fig 3.3.2.5). This intra-observer reliability test yielded a correlation co-efficient of κ=0.89 (Pearson's test).

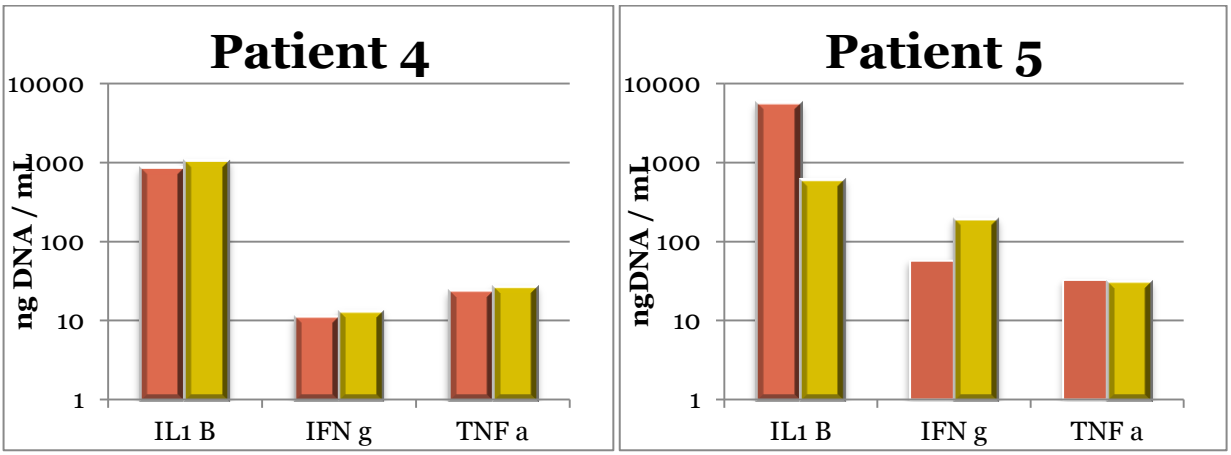



Figure 3.3.2.5 : Gene expression with repeated sample analysis by the same observer.

Correlation co-efficient κ=0.89

A second validation exercise was then undertaken to assess the inter-observer reliability. A second experienced independent investigator repeated the reverse transcription and TaqMan analysis of TNF α using RNA extracted by the author from the tissue. Expression rates of IL1β were calculated using the method described above (Fig 3.3.2.6). The correlation co-efficient (Pearson's method) was 0.93 representing excellent agreement. The improved agreement over repeated samples probably represents elimination of errors introduced during the RNA extraction stage or from minor variations in expression between the two sampling sites.

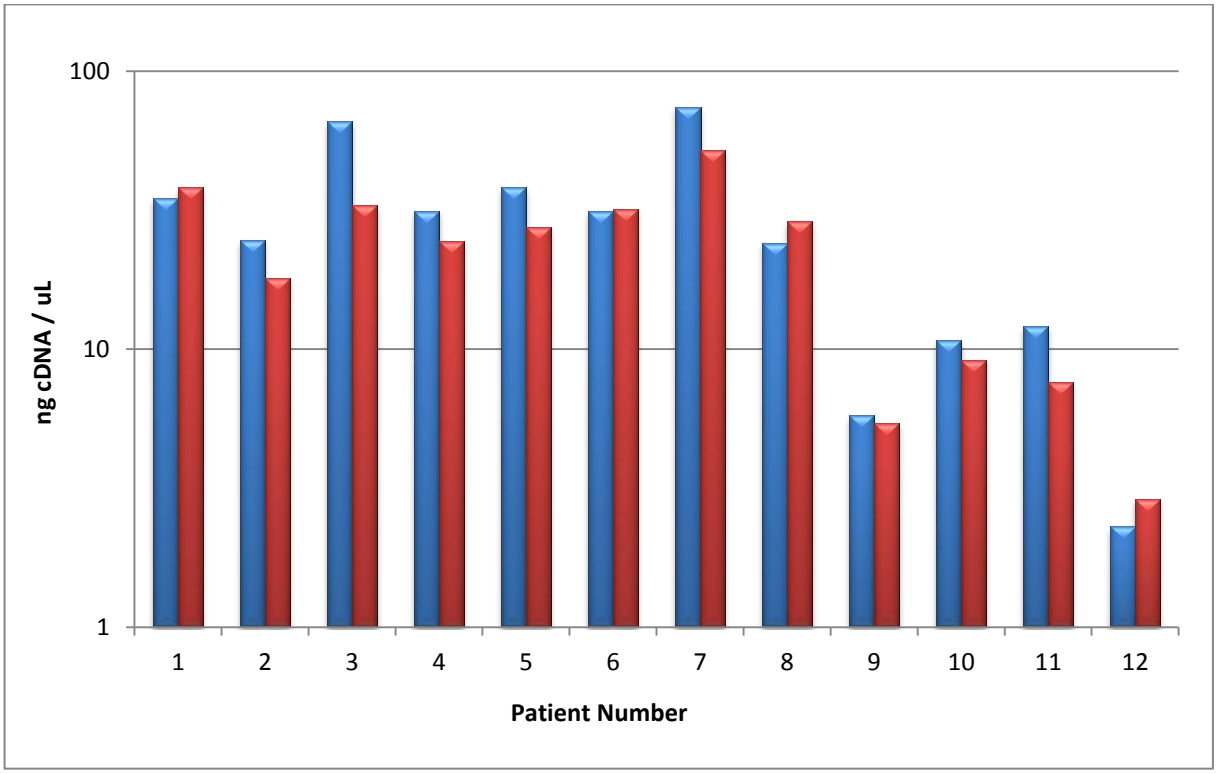



Figure 3.3.2.6 : Repeated samples of different tissues for TNF α expression by a different observer performed independently from the initial experiments. Correlation co-efficient κ=0.93

### 3.3.3 TLDA Results

#### 3.3.3.1 Housekeeping Genes

40 revisions and 27 primary joints were successfully analysed and expression characterised for all four housekeeping genes using the TLDA method. The primary and revision samples were analysed on the software as separate studies (due to the number of samples), so comparison of housekeeping genes is necessary, not just between genes, but also between studies to ensure accurate calibration of the analyser.

The distribution of raw $C_T$ values is given as a scatter plot (Fig 3.3.3.1) with the median. The median $C_T$ value and standard deviation is given (Table 3.3.3.1). The expression for succinate dehydrogenase (mean 35.7 SD 3.08) was significantly higher than the other genes, and the low levels of expression make it unsuitable for use as a housekeeping gene in this case. Standard deviations, coefficient of variation and standard errors were similar for each of the remaining three housekeeping genes (Table 3.3.1).

| | Revisions | | | | Primaries | | | |
|---|---|---|---|---|---|---|---|---|
| | 18S | ACTB | GAPDH | SDHA | 18S | ACTB | GAPDH | SDHA |
| **Values** | 40 | 40 | 40 | 40 | 27 | 27 | 27 | 27 |
| **Minimum** | 16.04 | 26.1 | 27.64 | 31.37 | 17.2 | 27.58 | 28.36 | 32.92 |
| **Maximum** | 30.71 | 40 | 40 | 40 | 30.96 | 40 | 40 | 40 |
| | | | | | | | | |
| **Median** | 20.87 | 31.5 | 32.71 | 35.7 | 19.73 | 30.39 | 31.73 | 35.7 |
| **Std. Deviation** | 4.508 | 4.69 | 4.218 | 3.082 | 2.626 | 2.611 | 2.177 | 2.091 |
| **Std. Error** | 0.7127 | 0.7416 | 0.667 | 0.4873 | 0.5054 | 0.5025 | 0.419 | 0.4024 |
| **Coefficient of variation** | 21.60% | 14.89% | 12.90% | 8.63% | 13.31% | 8.59% | 6.86% | 5.86% |

Table 3.3.3.1 : Housekeeping gene expression ($C_T$ values) for the whole sample from each tested cohort

All housekeeping genes can be seen to have tightly clustered expression (Fig 3.3.3.1). The Pearson's product moment correlation co-efficient for each housekeeping pair is given (Table 3.3.3.2). There was a high rate of concordance between 18S, GAPDH and ACTB with r values of 0.94 to 0.98 (Table 3.3.3.2). However there were poorer correlations between SDHA and all other housekeepers.

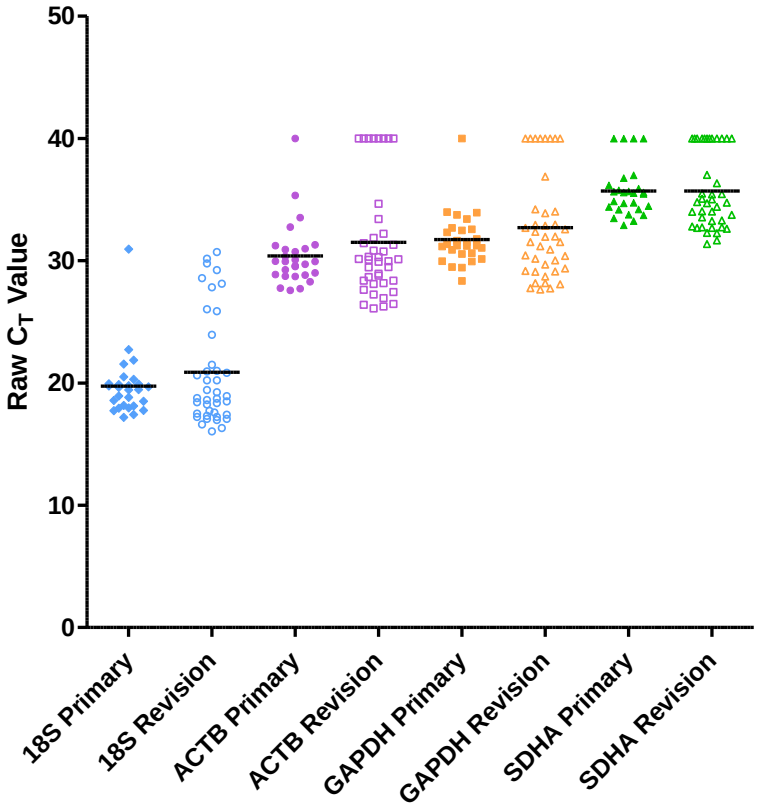



Figure 3.3.3.1: Scatter plot of $C_T$ values for housekeeping genes showing medians. All tested samples are included

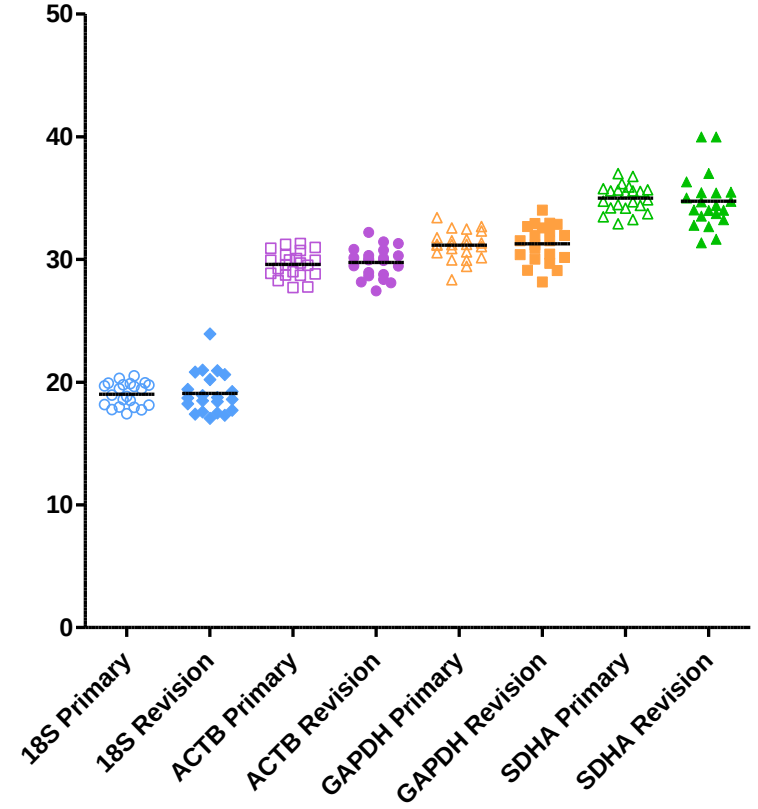



Figure 3.3.3.2 : Scatter plot with medians. $C_T$ values for all housekeeping genes for samples included in eventual analysis are shown. Narrow spread about the median is seen in all 4 housekeeping genes.

| | 18S | ACTB | GAPDH | SDHA |
|---|---|---|---|---|
| 18S | | 0.96 | 0.94 | 0.87 |
| ACTB | 0.96 | | 0.98 | 0.85 |
| GAPDH | 0.94 | 0.98 | | 0.84 |
| SDHA | 0.87 | 0.85 | 0.84 | |

Table 3.3.3.2 : Calculated Pearson's product moment correlation co-efficents of housekeeping gene expression.

Uniform expression of housekeeping genes is required for adequate comparison of samples across each group. As there are some errors introduced in the laboratory protocols, the standardisation of housekeeping gene RNA expression as a measure of quality across the groups is essential if comparative analysis is to be undertaken.

Samples were excluded from analysis if more than one housekeeping gene was found to deviate more than $2C_T$ values from the median value. There were nineteen revision samples that did not fulfil these criteria and so where excluded, and a further five primaries were excluded. Thus samples from 22 revisions (16 aseptic, 6 well fixed) and 22 primary native hips were suitable for final analysis.

This improved the co-efficient of variance and standard deviations for all housekeeping genes in all groups (Table 3.3.3.1). In addition the distribution of $C_T$ values for each housekeeping gene can be seen to be more tightly clustered around the median value (Fig 3.3.3.2). There were no significant differences (student's t-test) between expression of 18S (p=0.85), β-Actin (p=0.68), GAP-DH (p=0.83) or SDHA (p=0.63) between the primary and revision study groups.

| | Primaries | | | | Revisions | | | |
|---|---|---|---|---|---|---|---|---|
| | 18S | ACTB | GAPDH | SDHA | 18S | ACTB | GAPDH | SDHA |
| Values | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 |
| Minimum | 17.42 | 27.72 | 28.36 | 32.92 | 17.07 | 27.45 | 28.17 | 31.37 |
| Maximum | 20.52 | 31.3 | 33.41 | 37 | 23.95 | 32.2 | 34.03 | 40 |
| Median | 19.02 | 29.61 | 31.16 | 35.01 | 19.1 | 29.75 | 31.27 | 34.75 |
| Std. Deviation | 0.94 | 1.06 | 1.17 | 1.1 | 1.68 | 1.22 | 1.54 | 2.23 |
| Std. Error | 0.2 | 0.23 | 0.25 | 0.23 | 0.37 | 0.27 | 0.34 | 0.49 |
| Coefficient of variation | 4.96% | 3.57% | 3.75% | 3.15% | 8.79% | 4.11% | 4.93% | 6.43% |

Table 3.3.3.3 : Housekeeping gene expressions for the included sample cohort

Gene expression was normalised to β-Actin (ACTB) as this candidate has the lowest co-efficient of variation (3.75% primaries; 4.11% revisions) and standard deviation across both groups. β-Actin is therefore more uniformly expressed across the cohort (Table 3.3.3.3).

### 3.3.3.2 Selecting Genes to Include

Of the 91 candidate genes there was no measurable gene expression in 9 genes (BAK1, DKK1, DKK4, IL12A, IL12A, IL13, IL17A, IL4, NOS2), and less than ten samples amplified cDNA in 12 further genes (ADAMTS 3, CD80, CTLA4, DKK2, FASLG, IFN-$\gamma$, IL11, IL12B, IL1A, IL1B, SOST, TM7SF4). There was measurable DNA amplification in the remaining 70 candidate genes. There were seven genes (ADAMTS 14, CXCL10, FRZB, MMP12, PTGS2, TNF$\alpha$, TNFRSF1B) where less than fifteen samples amplified DNA and the results should be interpreted in this light. The median expressions, range, number of successfully run samples and standard deviations for raw CT values for all candidate genes are given in Appendix 3.

It is impossible to determine if those genes in which only a few samples amplified DNA indicates there is no gene expression, or if it represents a failure of the primer-probe set to bind to the sample or effectively measure expression. It is however likely that in genes where expression is measured in some samples and low or no measurable expression is seen in others that this is an accurate observation. As a single sample is used in the TLDA technique for all genes and an automated primer-probe set, error in the preparation of the samples or array is likely to affect whole samples or genes.

### 3.3.3.3 Multiple Sample Testing

A further quality control measure was undertaken by analysis of two separate samples of the same tissue from two patients for all candidate genes. Each sample was collected and processed separately and thus not only gives an indication of local variation in gene expression, but also gives a measure of the total error in the process. Variations between the observations include sampling, tissue extraction, reverse transcription and the Taqman process. Observations were normalised to the housekeeping gene ($\beta$-Actin) in each case and thus are a reflection of the overall accuracy of the method as well as local tissue variations is given. The two sets of observations for patient 4 are given in figure 3.3.3.3, and those for patient 5 in figure 3.3.3.4.

Spearman's correlation co-efficient and 95% confidence intervals were calculated for each pair of observations, and a best fit line calculated using linear regression. Spearman's correlation co-efficient was 0.72 (95% CI 0.58-0.81) for patient 4 and 0.84 (95% CI 0.75 - 0.90) for patient 5. Both these sets of observations represent excellent intra-observation reliability, and in both cases the correlations were statistically significant.

In both cases relatively poorer correlations can be seen when one sample has borderline expression (i.e. a very high $C_T$ value). These observations represent genes in which very low levels of cDNA were detected. They have been included for completeness, but probably represents a breakdown in the reliability of the method where very high $C_T$ values are recorded.

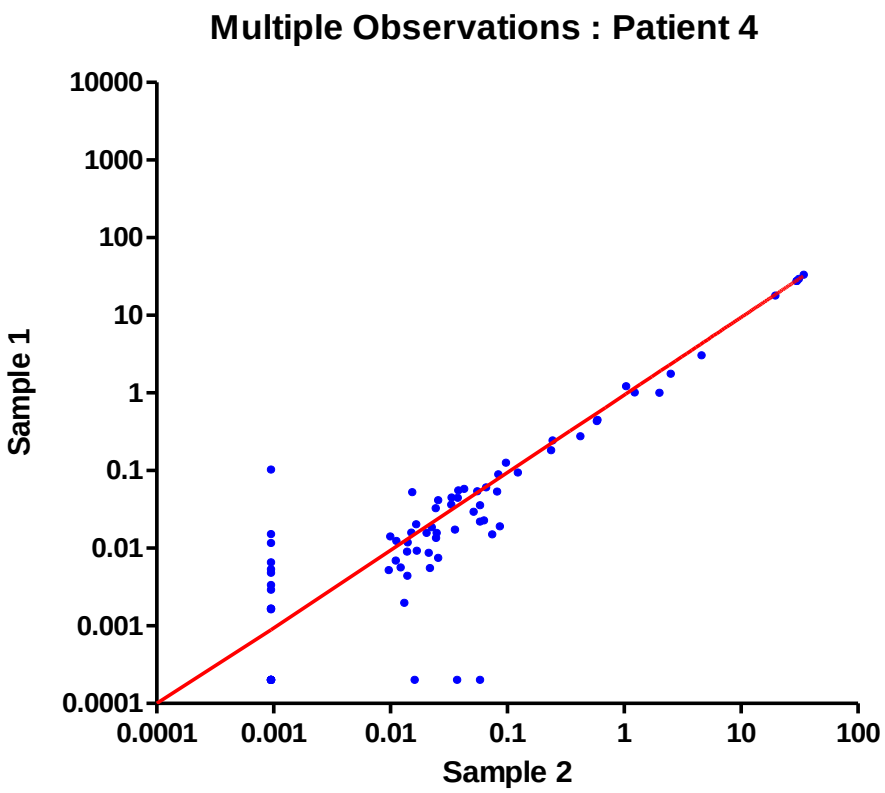



Figure 3.3.3.3 : Scatter plot – Multiple tissue samples Patient 4

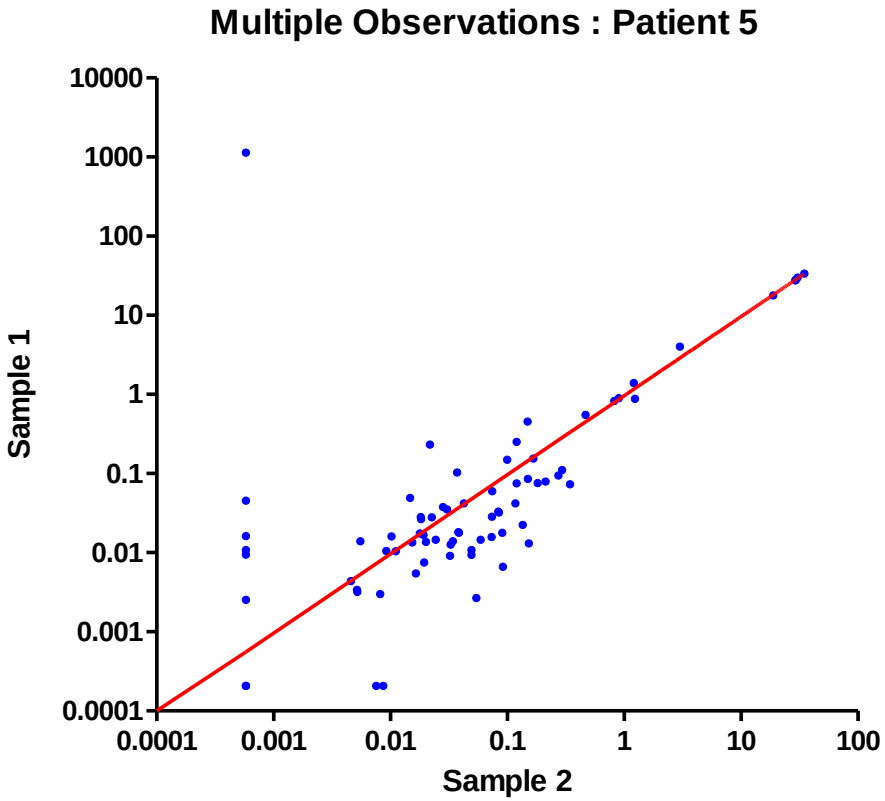



Figure 3.3.3.4 : Scatter plot – Multiple tissue samples Patient 5

### 3.3.3.4 Analysis of Results

The data have been analysed for statistical significance using non-parametric analysis techniques. As the data are all a series of experiments it is important to analyse the data in the same way for comparison of significance levels. Whilst some of the data conform to a normal distribution (d'Agostino and Pearson omnibus normality test) the majority does not. It would be possible to transform the data to a normal distribution or to utilise a statistical correction for skew (e.g. Welch's correction). However due to the size of the data sets different transformations would be needed for each group making comparison difficult. It was therefore decided to use non-parametric testing for significance. These tests have less power, and are therefore more likely to lead to a Type II error, but allow for non-parametric data making a type I error less likely.

Significance testing was performed using a stepwise approach. The three cohorts were tested for significant difference in median values using a Kruskal-Wallis test with an $\alpha$ error (p value) of 0.05. If this were significant then Dunn's multiple comparison test (as post-hoc testing to the Kruskal-Wallis) was used to determine significance between the two groups. The Dunn's test was performed for significance levels of <0.05 (defined as significant) and <0.10 (defined as borderline significant). Dunn's test makes an adjustment to allow for the likelihood of false positives seen when undertaking multiple tests.

Genes where no statistical significant variances were found between groups were excluded from further analysis. Further analyses were performed according to the differences pattern seen between each cohort the possible differences and their likely causes (Table 3.3.3.7).

For any given gene it is possible that differences in observed mRNA expression between the cohorts may represent either up regulation in gene expression, or that natural variation between individuals may result in a predisposition to loosening. In the latter case it is likely that expression in the osteolytic or well fixed cohorts will be a subset of the gene expression in primary osteoarthritis.

It is likely that changes in observed gene expression will represent up-regulation or down-regulation of the individual protein expression, and as such there will be a statistically significant difference between the primary osteoarthritis cohort and one or more of the replaced cohorts. This could represent either changes due to the effects of joint replacement or changes due to osteolysis.

Alteration in regulation of gene expression (up or down regulation) can be assessed through significance testing as previously described. However a tendency towards one or other outcome is poorly quantified in this manner. Genes tend to work as families, and high or low expression of one cytokine, receptor or modulator might not reach significant difference as they work together with other factors. More information can often be gained through either multi-variant analysis (looking at several factors which may be associated) or through quantifying association.

Multi-variant analysis is a powerful statistical technique in which complex relationships can be explored between multiple factors and known outcomes. Whilst multi-variant analysis increases the power of the analysis and allows modelling of contributory factors the analysis requires high numbers of observations in order to be valid. For each factor to be analysed roughly 20 observations are required. There are simply not enough observations in this study to use multi-variant techniques.

Receiver operator curves (ROC) is an alternative method, and quantifies association. ROC curves may be calculated for any continuous variable which may be predictive of a known outcome. ROC analysis is a well recognised and widely used technique and can be applied to a variety of data including scoring systems and observed characteristics. A curve is plotted of sensitivity vs. (1 – specificity) whilst the discrimination threshold is varied. The area under the curve (AUC) is analogous with the association with the strength of association with a value of 1 equating to perfect association and 0.5 to random chance. ROC statistics have been used in this study to assess the association between gene expression and outcome.

#### 3.3.3.5 Variation in Gene Expression in Osteoarthritis

Osteoarthritis is was historically considered as due to "wear and tear" and therefore different from inflammatory arthropathies and a biologically "inactive" disease. Although there are many studies looking at gene expression in early and late cartilage degeneration and the role that proteases and other proteins may play there are few studies looking at variability of expression in end stage disease.

If there is little variation in a gene's expression from patient to patient then it is unlikely that any potential tendency towards eventual loosening is due to that individual gene. Distribution of gene expression is given as a box and whisker plot (Figure 3.3.3.5) for primary osteoarthritis in our cohort. The co-efficient of variation was calculated for each gene (Table 3.3.3.4). Co-efficient of variation was used in preference to standard deviations as this allows for better comparison between groups as co-efficient of variation is a dimensionless number and therefore can be used to make comparison across groups with different scales, and does not require parametric data.

Co-efficient of variation ranged from 3 to 202 with different genes (Table 3.3.3.4). The box and whisker diagram is colour coded to indicate the co-efficient of variation. It is clear that whilst some genes vary greatly (MMP13, CXCL9, MMP9, CCL3, TNF, MMP1, CHIT1, FGF18, CTSG, IL1RN, BCL2A1, IL6, CXCL10, CD2, FRZB, MMP12) others have a much more linear expression (TGFB1, VEGFB, TNFRSF1A, B2M, ERCC1, COL1A2, ITGB2, CD58, CD14, CSF1, PDGFA, CTSK, COL3A1, COL1A1, ADAMTS 2). This initial variation must be taken into consideration when interpreting the results. A gene which demonstrates little pre-operative variation in expression, but greater post operative variation is likely to have been regulated. Similarly a gene with large pre-operative variation and a smaller significantly different variation in expression between osteolytic and well-fixed cohorts is likely to indicate a tendency towards loosening.

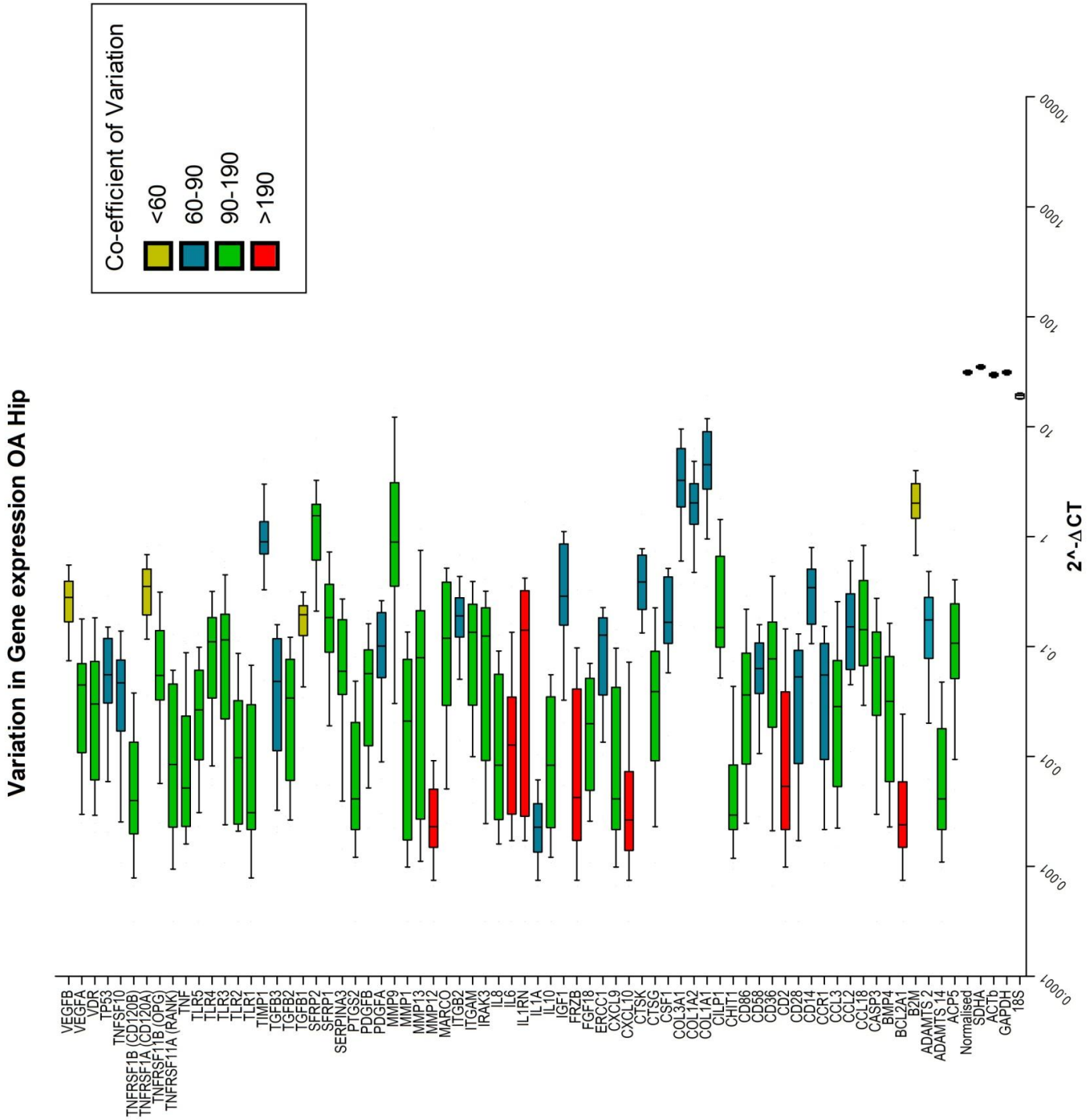



Figure 3.3.3.5 : Box and whisker plot giving an overview of all gene expression ranges for the whole co-hort. Coded for co-efficient of variation within the group.

| | Min | 25% Percentile | Median | 75% Percentile | Maximum | Coefficient Variation |
|---|---|---|---|---|---|---|
| 18S | 17.42 | 18.1 | 19.2 | 19.81 | 20.52 | 4.96 |
| ACP5 | 0.002207 | 0.05116 | 0.1107 | 0.2454 | 1.15 | 134.37 |
| ACTb | 27.72 | 28.8 | 29.64 | 30.45 | 31.3 | 3.57 |
| ADAMTS 14 | 0.0003123 | 0.002164 | 0.004121 | 0.01792 | 0.05239 | 138.14 |
| ADAMTS 2 | 0.00625 | 0.07795 | 0.1743 | 0.2801 | 0.5651 | 79.27 |
| B2M | 0.2217 | 1.469 | 2.012 | 3.036 | 5.965 | 56.93 |
| BCL2A1 | 0.0003123 | 0.001491 | 0.002387 | 0.005893 | 0.06802 | 192.61 |
| BMP4 | 0.002034 | 0.005915 | 0.03177 | 0.08138 | 0.163 | 110.81 |
| CASP3 | 0.0003123 | 0.02366 | 0.0792 | 0.1355 | 0.3837 | 99.63 |
| CCL18 | 0.005433 | 0.06702 | 0.1423 | 0.3988 | 1.291 | 112.65 |
| CCL2 | 0.01103 | 0.06193 | 0.1511 | 0.3022 | 0.7287 | 90.29 |
| CCL3 | 0.001508 | 0.005336 | 0.02843 | 0.07465 | 0.4705 | 162.86 |
| CCR1 | 0.001079 | 0.009392 | 0.0552 | 0.107 | 0.2055 | 88.74 |
| CD14 | 0.03068 | 0.1596 | 0.3432 | 0.5066 | 1.049 | 69.33 |
| CD2 | 0.0003123 | 0.002164 | 0.005343 | 0.03875 | 0.3049 | 207.64 |
| CD28 | 0.0003123 | 0.008612 | 0.05295 | 0.09263 | 0.2019 | 88.9 |
| CD36 | 0.0006615 | 0.01849 | 0.07742 | 0.1672 | 0.5777 | 123.56 |
| CD58 | 0.002828 | 0.03744 | 0.06297 | 0.1079 | 0.1804 | 69 |
| CD86 | 0.002207 | 0.008559 | 0.03633 | 0.08713 | 0.2892 | 123.02 |
| CHIT1 | 0.000945 | 0.002164 | 0.002933 | 0.008368 | 0.07392 | 164.96 |
| CILP1 | 0.04443 | 0.09882 | 0.1486 | 0.6625 | 2.188 | 130.67 |
| COL1A1 | 0.3513 | 2.703 | 4.512 | 8.987 | 19.15 | 77.13 |
| COL1A2 | 0.1494 | 1.295 | 2.029 | 3.035 | 6.367 | 65.47 |
| COL3A1 | 0.1531 | 1.863 | 3.245 | 6.322 | 11.5 | 74.89 |
| CSF1 | 0.01529 | 0.1065 | 0.1664 | 0.4296 | 0.5287 | 70.23 |
| CTSG | 0.001079 | 0.009144 | 0.03886 | 0.0908 | 0.7331 | 177.29 |
| CTSK | 0.08026 | 0.2171 | 0.387 | 0.6796 | 1.609 | 73.87 |
| CXCL10 | 0.0003123 | 0.001401 | 0.002647 | 0.007281 | 0.09342 | 202.66 |
| CXCL9 | 0.0006615 | 0.002164 | 0.004121 | 0.04256 | 0.1422 | 158.3 |
| ERCC1 | 0.002828 | 0.03629 | 0.1268 | 0.1824 | 0.2405 | 65.24 |
| FGF18 | 0.001079 | 0.004907 | 0.01984 | 0.0515 | 0.3258 | 169.17 |
| FRZB | 0.0003123 | 0.001723 | 0.004235 | 0.04105 | 0.3263 | 208.1 |
| GAPDH | 28.36 | 30.46 | 31.22 | 31.91 | 33.41 | 3.75 |
| IGF1 | 0.005433 | 0.1566 | 0.2865 | 0.8556 | 1.273 | 90.45 |
| IL10 | 0.0003123 | 0.002252 | 0.008311 | 0.03494 | 0.06183 | 105.9 |
| IL1A | 0.0003123 | 0.001349 | 0.00228 | 0.003726 | 0.01037 | 80.67 |
| IL1RN | 0.0003123 | 0.002855 | 0.1411 | 0.3223 | 2.057 | 186.33 |
| IL6 | 0.0003123 | 0.002985 | 0.01272 | 0.03462 | 0.3523 | 201.26 |
| IL8 | 0.0003123 | 0.002673 | 0.008311 | 0.05579 | 0.1271 | 119.32 |
| IRAK3 | 0.0003123 | 0.009223 | 0.1247 | 0.2255 | 0.4097 | 90.13 |
| ITGAM | 0.003753 | 0.0293 | 0.1349 | 0.2427 | 0.6324 | 94.16 |
| ITGB2 | 0.01383 | 0.1222 | 0.1904 | 0.2768 | 0.678 | 67.63 |
| MARCO | 0.002466 | 0.0292 | 0.1192 | 0.3858 | 0.557 | 102.35 |
| MMP1 | 0.0003123 | 0.001741 | 0.02095 | 0.07639 | 0.3649 | 164.73 |
| MMP12 | 0.0003123 | 0.001491 | 0.002301 | 0.005041 | 5.152 | 469.04 |
| MMP13 | 0.0003123 | 0.002694 | 0.07939 | 0.2118 | 1.166 | 151.83 |
| MMP9 | 0.002207 | 0.3536 | 0.8924 | 3.093 | 14.67 | 159.14 |
| Normalised | 28.36 | 30.46 | 31.22 | 31.91 | 33.41 | 3.75 |
| PDGFA | 0.003038 | 0.05229 | 0.1012 | 0.2035 | 0.3366 | 73.16 |
| PDGFB | 0.002207 | 0.01254 | 0.05676 | 0.09339 | 0.2115 | 91.67 |
| PTGS2 | 0.0003123 | 0.002164 | 0.004121 | 0.02039 | 0.07977 | 147.99 |
| SDHA | 32.92 | 34.2 | 35.18 | 35.71 | 37 | 3.15 |
| SERPINA3 | 0.001439 | 0.03662 | 0.05961 | 0.1757 | 0.3608 | 95.02 |
| SFRP1 | 0.005433 | 0.08882 | 0.1839 | 0.3685 | 1.069 | 98.62 |
| SFRP2 | 0.005433 | 0.6119 | 1.547 | 1.97 | 7.924 | 101.44 |
| TGFB1 | 0.01197 | 0.126 | 0.1947 | 0.2357 | 0.3217 | 48.52 |
| TGFB2 | 0.002308 | 0.006046 | 0.03405 | 0.07654 | 0.3163 | 130.91 |
| TGFB3 | 0.002034 | 0.01129 | 0.04816 | 0.1176 | 0.1727 | 87.29 |
| TIMP1 | 0.04906 | 0.6947 | 0.8948 | 1.37 | 5.034 | 90.16 |
| TLR1 | 0.0003123 | 0.002164 | 0.00308 | 0.02967 | 0.07304 | 146.35 |
| TLR2 | 0.0003123 | 0.002427 | 0.009758 | 0.03215 | 0.1114 | 128.46 |
| TLR3 | 0.001079 | 0.02194 | 0.1153 | 0.1971 | 0.5923 | 111.31 |
| TLR4 | 0.005433 | 0.03395 | 0.1107 | 0.1832 | 0.7256 | 113.38 |
| TLR5 | 0.0003123 | 0.009342 | 0.02654 | 0.061 | 0.1294 | 92.92 |
| TNF | 0.001079 | 0.002305 | 0.005172 | 0.0233 | 0.1106 | 164.72 |
| TNFRSF11A | 0.0003123 | 0.002272 | 0.008441 | 0.04571 | 0.1285 | 131.48 |
| TNFRSF11B | 0.002466 | 0.03275 | 0.05462 | 0.1395 | 0.8375 | 145.87 |
| TNFRSF1A | 0.03566 | 0.1934 | 0.3521 | 0.5054 | 0.8031 | 55.76 |
| TNFRSF1B | 0.0003123 | 0.00198 | 0.003974 | 0.01348 | 0.04042 | 128.09 |
| TNFSF10 | 0.001079 | 0.01706 | 0.0468 | 0.07532 | 0.1588 | 84.03 |
| TP53 | 0.002034 | 0.03064 | 0.05543 | 0.1183 | 0.287 | 89.03 |
| VDR | 0.002034 | 0.006131 | 0.0301 | 0.0732 | 0.2103 | 113.41 |
| VEGFA | 0.002466 | 0.01086 | 0.04464 | 0.06995 | 0.2364 | 103.34 |
| VEGFB | 0.0691 | 0.1675 | 0.2803 | 0.3921 | 0.6478 | 54.52 |

Table 3.3.3.4 : Coefficients of Variation for primary cohort

3.3.3.6 Other potential causes of variation

It is possible that some of the variability seen in gene expression is related to variation in sampling method or laboratory technique. Whilst this seems an unlikely explanation it is important to consider. It has been established in this study by conducting intra- and inter- observer reliability that repeated sampling from the same tissue type leads to high correlations and little differences in expression across genes. Additionally during the manual TaqMan expression profiling stage multiple samples from the same tissue, and samples from different tissues from the same patient were evaluated all showing excellent correlations.

It is important to ensure that none of the observed variation is due to differential responsiveness of the primer probe sets to variation in differences in tissue sampling site. All patients had two different tissues sampled at the time of surgery (ligamentum teres and hip capsule). In six cases both of these samples were run on the TLDA microarray cards (Fig 3.3.3.6). Correlation plots were constructed for each paired set of samples and Spearman's test performed to determine the correlation co-efficient. Correlations were then tested for statistical significance. Spearman's r and significance values are given on each correlation plot. Correlation coefficients varied between r=0.77 and r=0.987. Correlations were all found to be excellent, and in all cases the correlation was statistically significant.

There is some clustering along the origin of the X and Y axis, which is likely due to genes which only just reached threshold in one sample. This is a technique dependent process, and this finding is not unexpected. The 'funnel' shape of the cluster plots, with more variation at lower expression levels is simply due to the logarithmic scale of the charts, the variation from best fit line is actually linear in each case.

It has been previously established that site of tissue sampling may be unimportant, and that similar results have be obtained from multiple sites within the same tissue in this study. This should be interpreted in combination with the finding that tissue sampling from different tissues within the joint yields highly concordant gene expressions. This raises the possibility that each osteoarthritic hip has a biological status that is similar across the whole joint.

When taken in combination with the initial finding of variability in gene expression between patients it can be concluded that there are differing biological environments from patient to patient, but that there is a similar biological status across each individual hip joint, irrespective of sampling site. This is a new observation, and suggests that osteoarthritis is an active process with a unique environment which is similar across the investigated joint tissues.

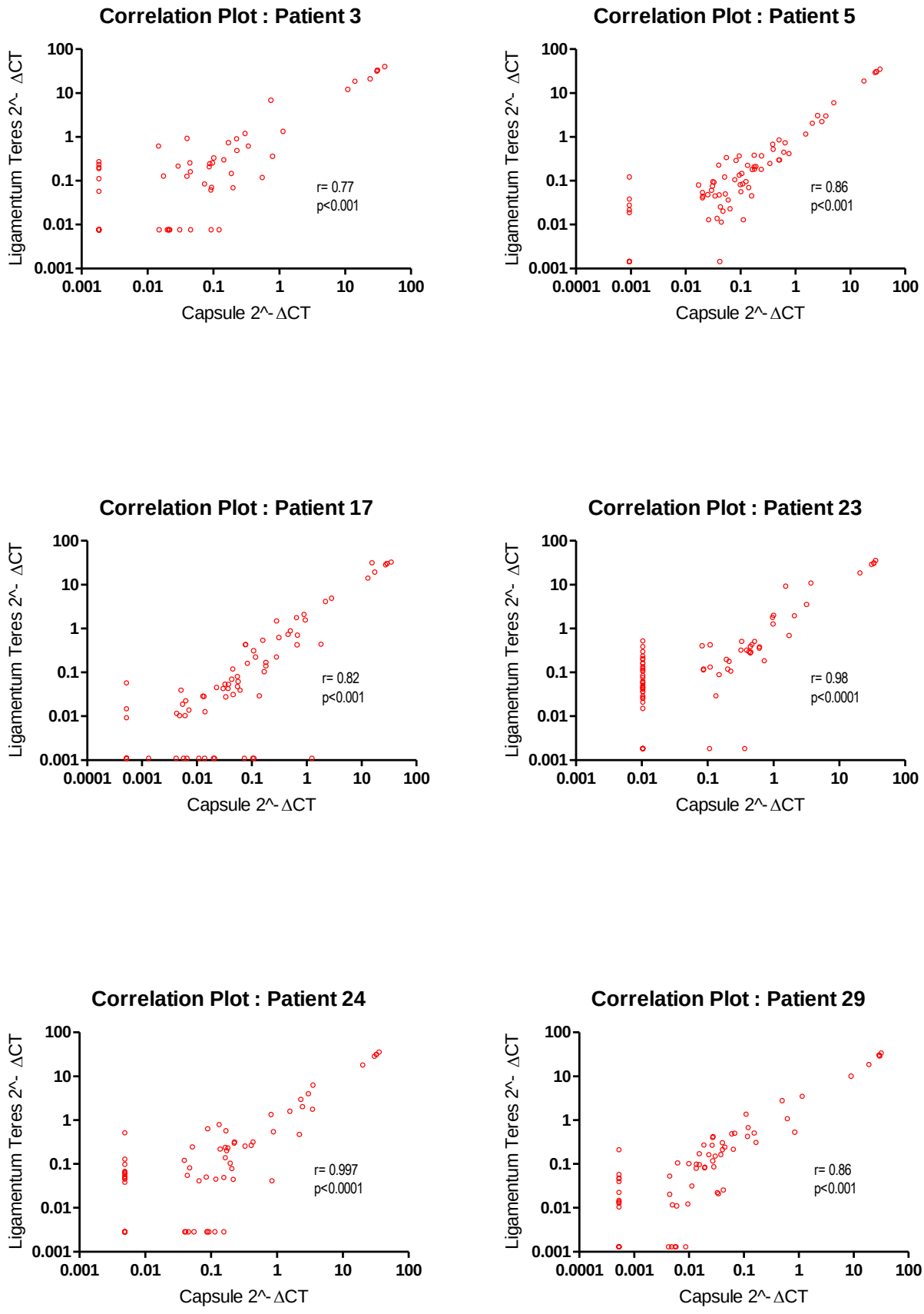



Figure 3.3.3.6 : Multiple tissue samples from the same patients. Correlation plots of $2^{\wedge -\Delta CT}$ expression. Spearman's correlation co-efficient (r values) and significance value (2 tailed T-test are shown)

### 3.3.3.7 Subgroup Gene Expression

Having established that gene expression does vary between patients with primary osteoarthritis the interpretation of expression results in the remaining two subgroups should be interpreted within this broader context.

The distribution of gene expression for each cohort has been plotted as a Box and Whisker diagrams (Figure 3.3.7 – Figure 3.3.12), and the median values marked. The median values for each cohort, standard deviation and Kruskal-Wallis test is given for each gene (Table 3.3.3.6), and the statistical analysis was performed as previously described. With visual inspection of the results in combination with interpretation of the statistical testing several patterns of gene expression emerge.

#### 3.3.3.7.1 Markers of Joint Replacement

In a number of cases both the well fixed and osteolytic groups had up or down regulation of gene expression. This is likely to represent a gene that is either up regulated in osteoarthritis and falls post joint replacement, or a gene where expression is increased due to the presence of wear debris. If there is no apparent or statistical difference between the well fixed and osteolytic groups these genes cannot be used as markers of loosening, but probably represent genes where activity is modulated in response to the presence of an arthroplasty and the associated wear debris, not the process of loosening.

The genes which fall into this category and where there was statistically significant change between subgroups (Kruskal-Wallis test) are MMP12, MMP9, COL3A1, COL1A2, TIMP1, CD120A, TRAIL, PDGFA, FGF18, CSF1, CD14, CD36, CD86, IL1RN, BCL2A1, ERCC1 & MARCO.

#### 3.3.3.7.2 Genes not involved in wear debris response

There were some genes in which no significant changes were seen between any subgroup. Although selection of genes after a careful literature review makes this finding less likely there were 29 genes in which there were no statistically significant differences between subgroups. These genes were CASP3, CCL2, CCR1, CD120B, CD14, CD2, CD28, CD86, COL1A1, CTSG, CXCL10, CXCL9, FRZB, GMCSF, IL1RN, IL10, IL6, ITGAM, MMP1, MMP13, PDGF$\beta$, PTGS2, RANK-L, TLR1, TLR2, TLR4, TNF$\alpha$, TP53, VDR & VEGFA.

The Kruskal-Wallis test determines difference between all the subgroups, due to the non-parametric nature of the test it is less powerful than ANOVA. With all statistical testing significance is more likely to be reached with larger groups, resulting in changes in the osteolytic cohort more readily detected than those in the well fixed group. In some cases there was no significant difference detected across the whole group, but the median values moved in a different direction between the two groups. This may represent a difference the study is underpowered to detect. The direction of median deflection from the primary group and corresponding significance levels is given (Table 3.3.3.7).

In the case of CD120B, FRZB, GMCSF-1, IL10, PDGFβ, PTGS2, TP53 and VDR the medians have moved in opposite directions, and it seems likely that the lack of significance is due to a type 2 error i.e. lack of power in the study and statistical testing.

### 3.3.3.8 Face Validity

An indication of the reliability of the results can be made by assessing face validity. As most of the candidate genes come from well studied pathways, and their interactions are known, it is possible to make an assessment of the value of the results by exploring some of the interactions between genes. For example specific ADAMTS are known to have different function to specific MMPs, so one would not expect up regulation of MMPs and ADAMTS in the same subgroup of patients. If a gene is a known synergist then the ratio of expression will be expected to remain relatively constant.

| Antagonistic Expression | | |
|---|---|---|
| | Primary | Osteolytic |
| MMP 9 | 0.26 | 8.5 |
| ADAMTS 2 | 0.174 | 0.00725 |
| Ratio | 1.494253 | 1172.4138 |

| Synergistic Expression | | |
|---|---|---|
| | Primary | Osteolytic |
| COL1A2 | 4.512 | 1024 |
| COL3A1 | 3.24 | 1294 |
| Ratio | 1.392593 | 0.7913447 |

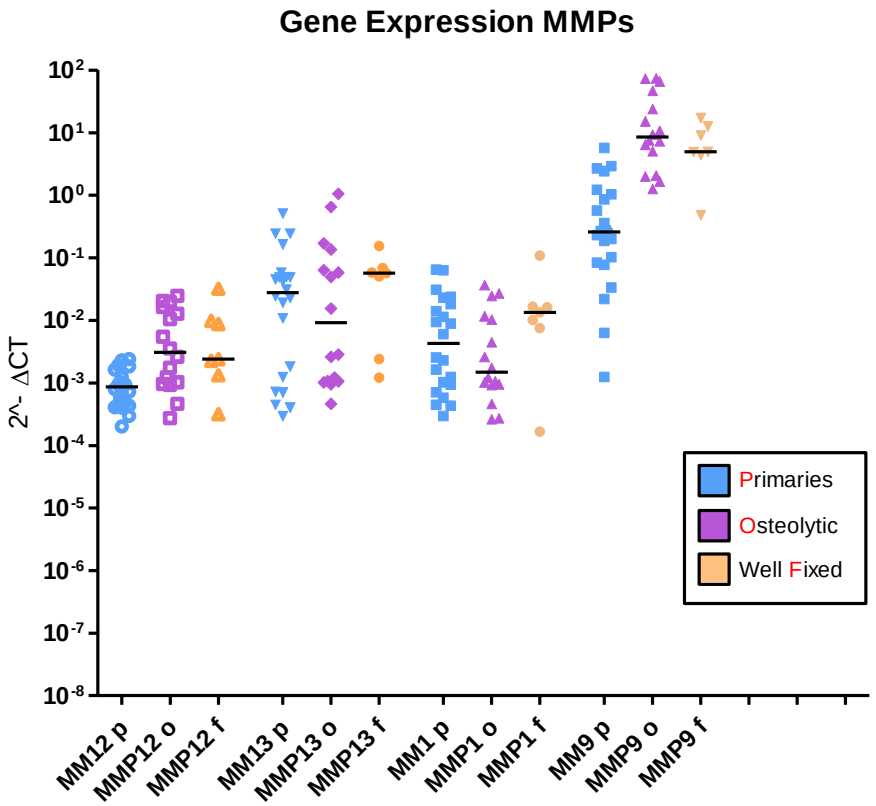
| | Primary | Osteolytic |
|---|---|---|
| OPG | 0.054 | 0.00725 |
| RANK-L | 0.0084 | 0.0165 |
| Ratio | 6.428571 | 0.4393939 |

| | Primary | Osteolytic |
|---|---|---|
| SFRP1 | 0.1839 | 0.017 |
| SFRP2 | 1.547 | 0.0807 |
| Ratio | 0.118875 | 0.2106568 |

Table 3.3.3.5 : Median Expression and ratios of selected genes

Table 3.3.3.5 gives median expressions and expression ratios for two known antagonistically regulated genes (MMP 9 & ADAMTS 2; OPG & RANK-L) and two known synergistically regulated genes (SFRP1 & SFRP2; COL1A2, COL3A2). As one antagonist is up regulated the other is down regulated, where the synergists move together. Similarly the ratio of median gene expression is relatively constant in the synergists, and markedly different in the antagonist pairs.

Whilst these observations remain simply observations the finding of face validity makes it more likely that the results of the study represent the true biological picture.



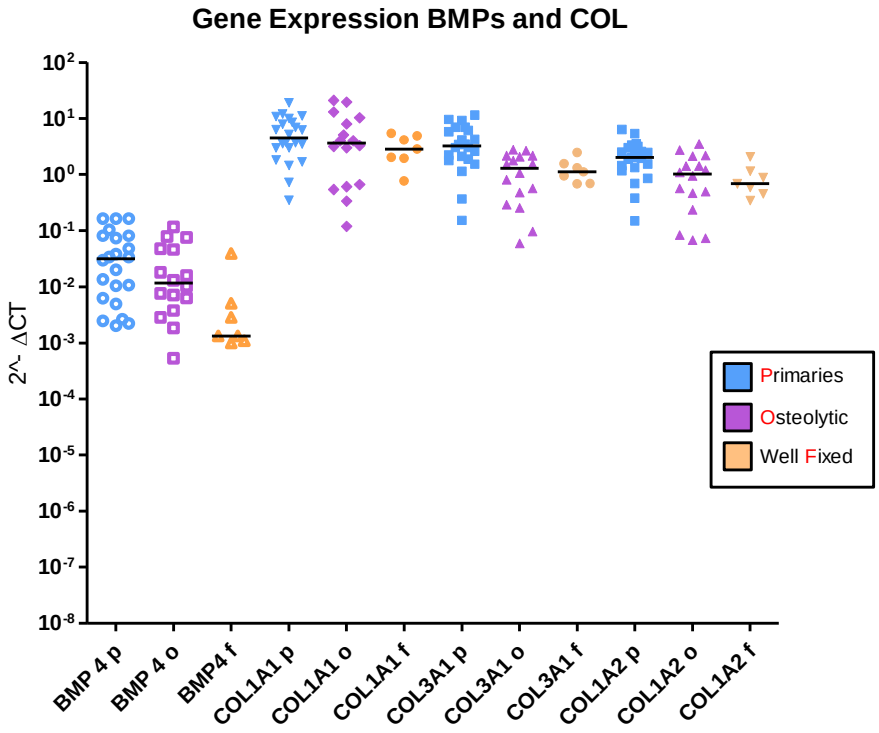
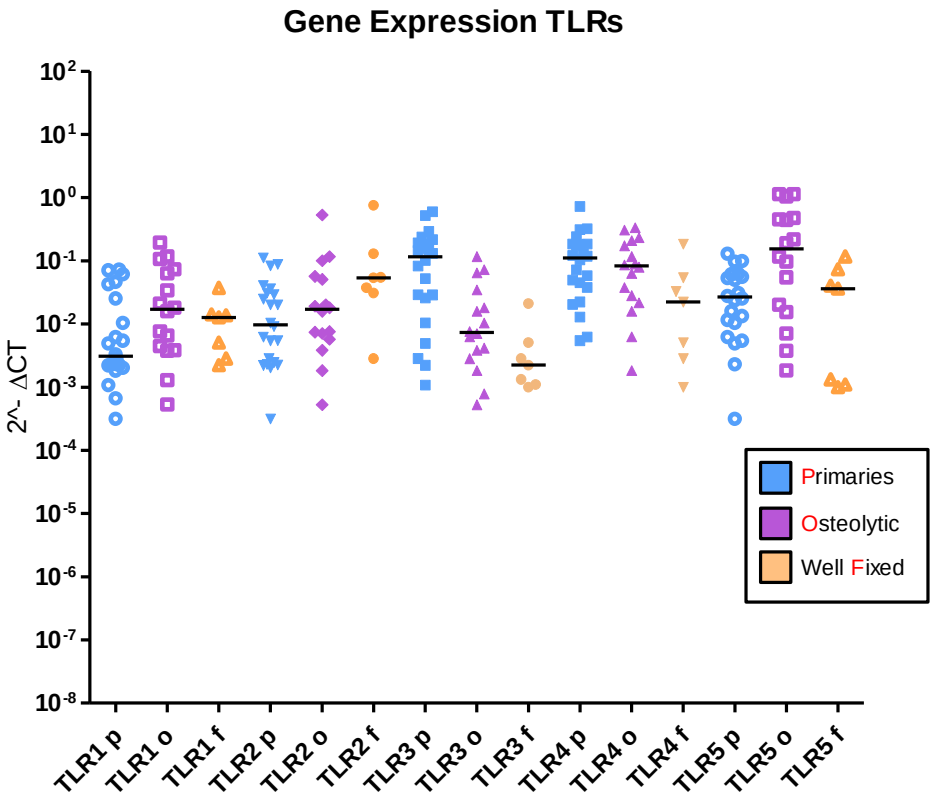




Figure 3.3.3.7 : Gene expression by subgroup (median marked) a) MMPs b) BMPs and collagen



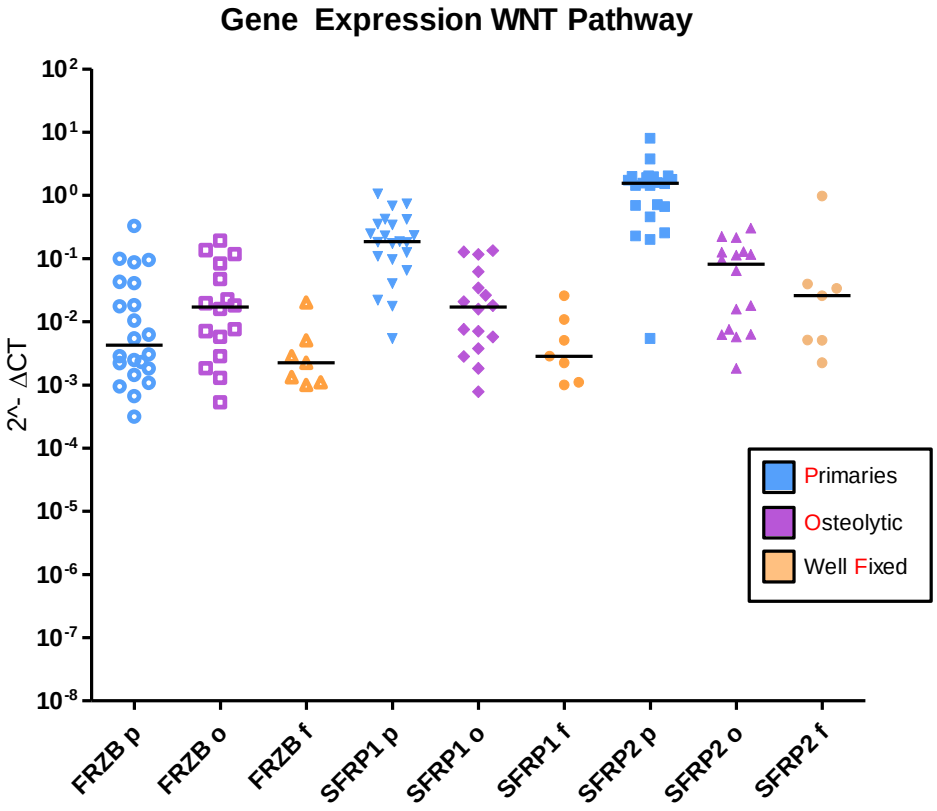
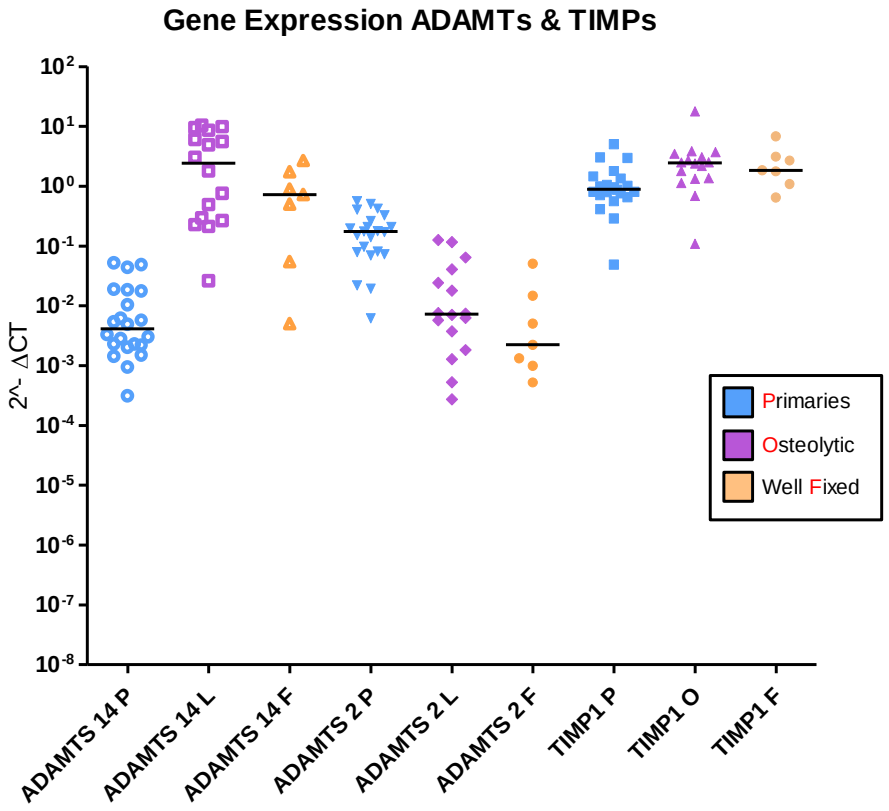




Figure 3.3.3.8 : Gene expression by subgroup (median marked) a) TLRs b) WNT pathway



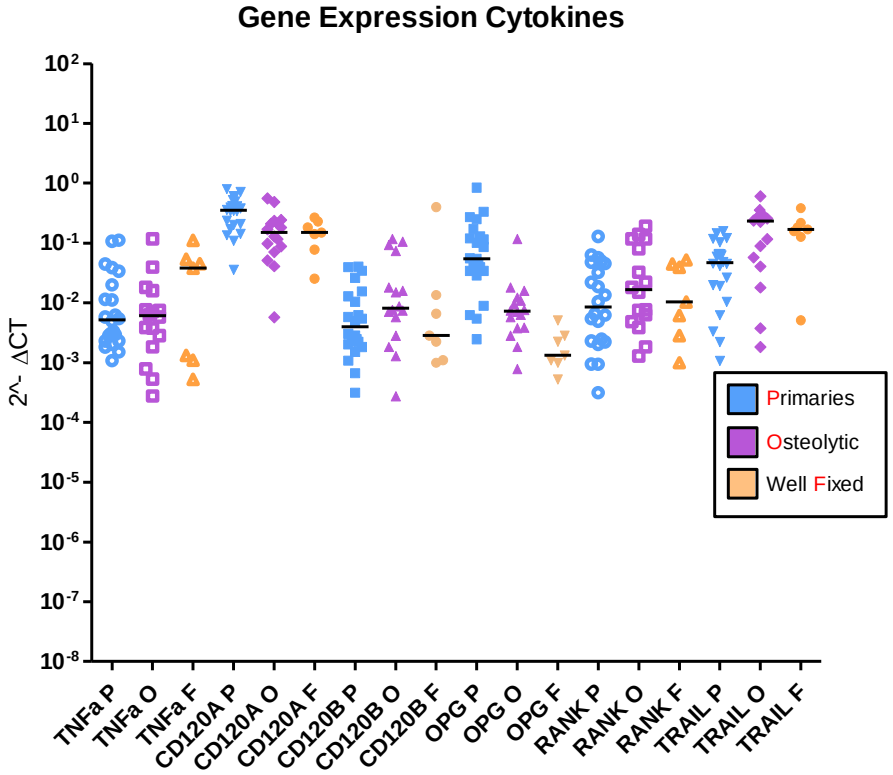




Figure 3.3.3.9 : Gene expression by subgroup (median marked) a) ADAMTs and TIMPs b) Cytokines

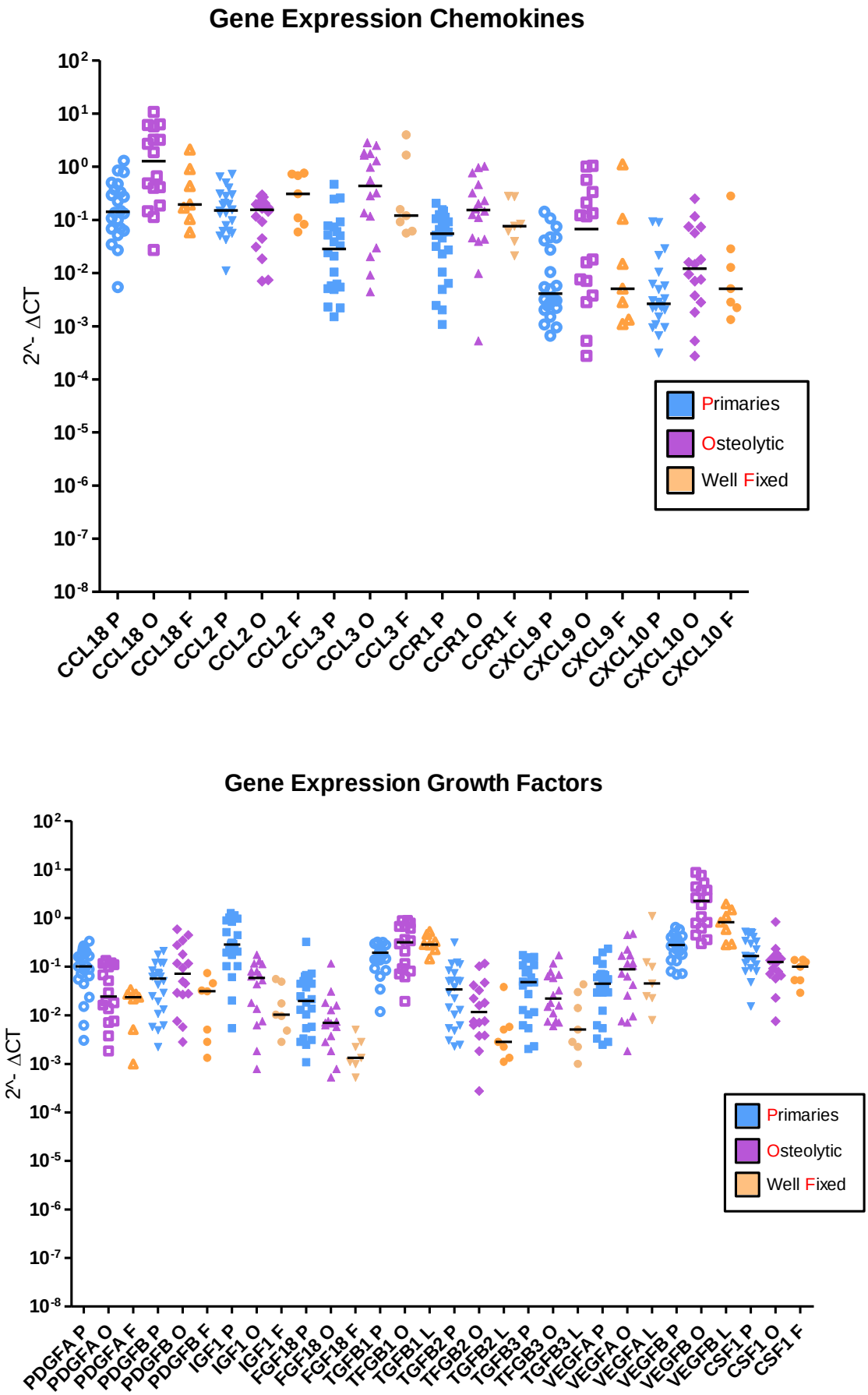
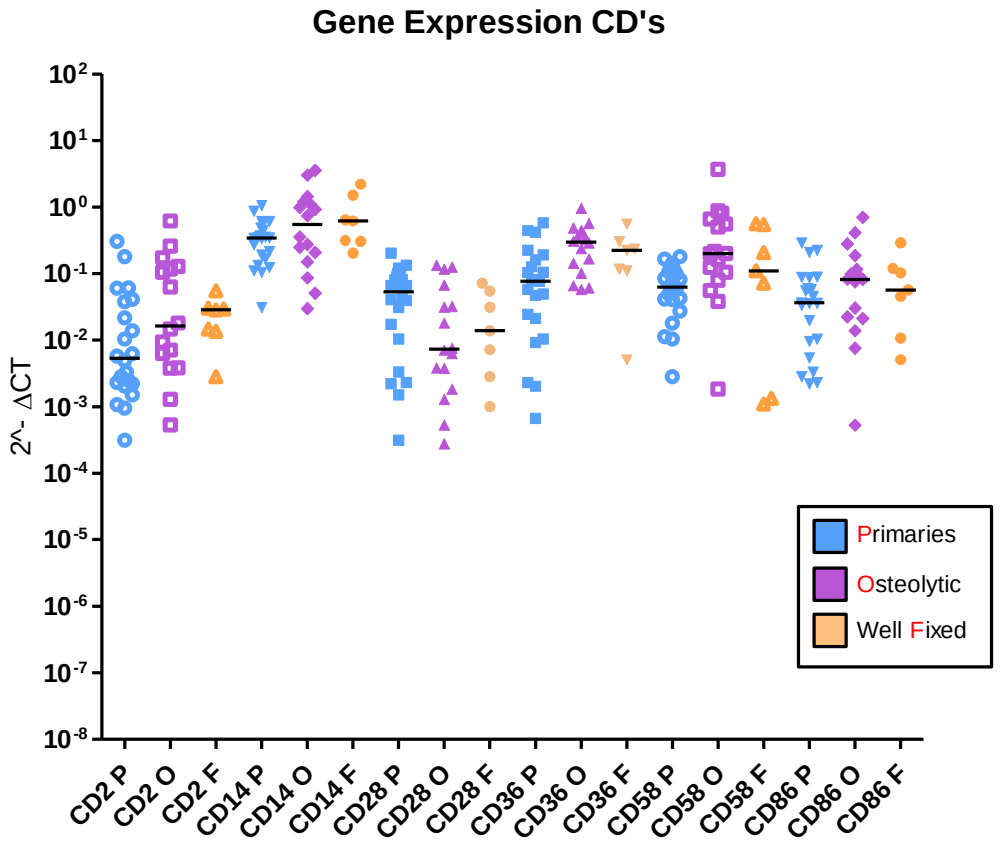



Figure 3.3.3.10 : Gene expression by subgroup (median marked) a) Chemokines b)Growth Factors



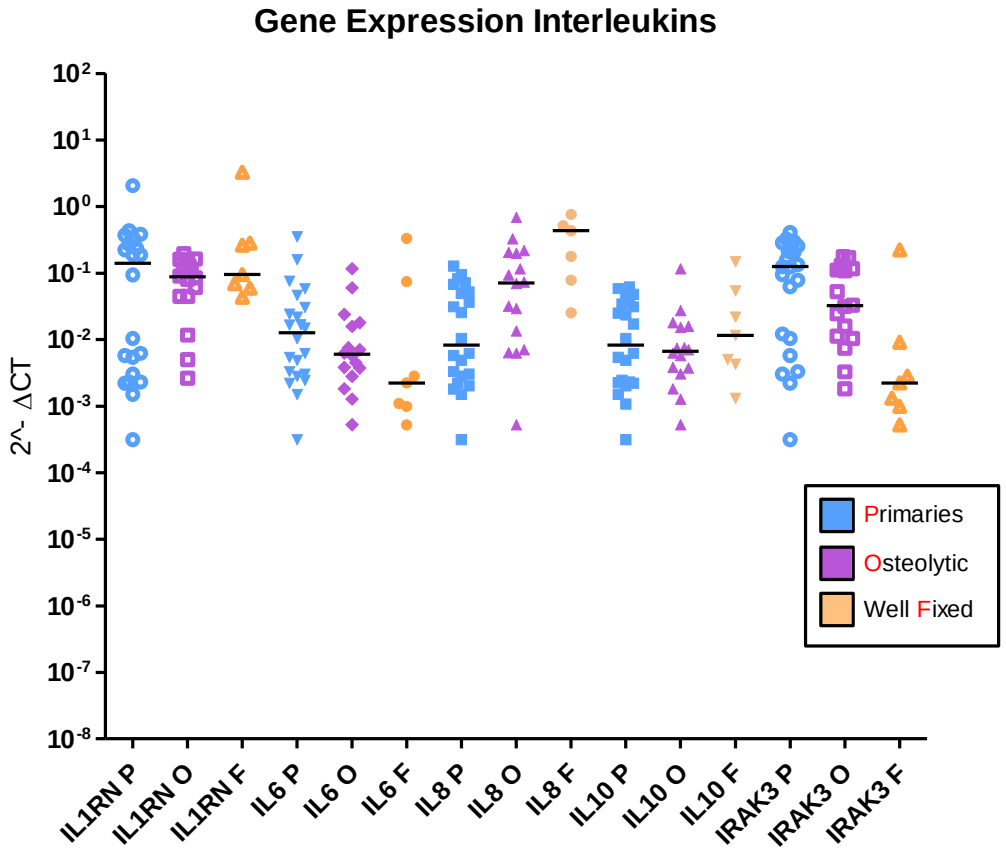




Figure 3.3.3.11 : Gene expression by subgroup (median marked) a) CD b) Interleukins

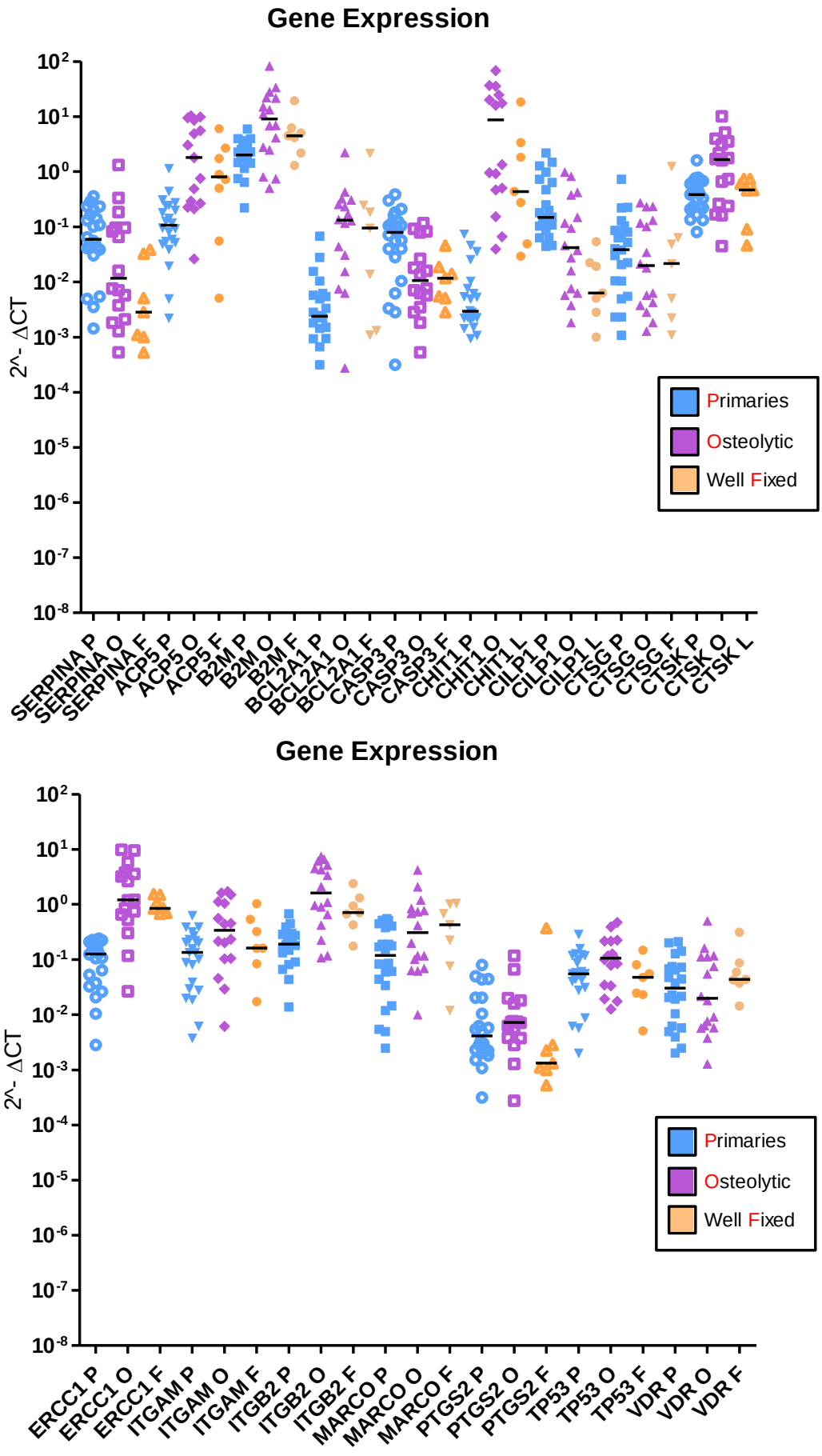



Figure 3.3.3.12 : Gene expression by subgroup (median marked) other genes

| | Primary (P) | | Osteolytic (O) | | | Well fixed revision (F) | | | | Kruskal-Wallis |
|---|---|---|---|---|---|---|---|---|---|---|
| | Median | St Dev | Median | St Dev | p vs (P) | Median | St Dev | p vs (P) | p vs (O) | Difference (p=) |
| MMP1 | 0.0043 | 0.019 | 0.0015 | 0.012 | >0.05 | 0.013 | 0.038 | >0.05 | >0.05 | 0.3674 |
| MMP9 | 0.26 | 141 | 8.5 | 26.88 | <0.05 | 4.9 | 5.72 | <0.05 | >0.10 | <0.0001 |
| MMP12 | 0.00086 | 241 | 0.0031 | 0.0085 | <0.05 | 0.0024 | 0.011 | <0.10 | >0.10 | 0.009 |
| MMP13 | 0.0092 | 0.1226 | 0.0092 | 0.30 | >0.05 | 0.057 | 0.051 | >0.05 | >0.05 | 0.5808 |
| ADAMTS 2 | 0.1743 | 0.158 | 0.007252 | 0.04069 | <0.05 | 0.002234 | 0.01814 | <0.05 | >0.10 | <0.0001 |
| ADAMTS 14 | 0.004121 | 0.01605 | 2.437 | 3.952 | <0.05 | 0.721 | 0.9646 | <0.05 | >0.10 | <0.0001 |
| TIMP1 | 0.8948 | 1.118 | 2.463 | 4.05 | <0.05 | 1.841 | 2.06 | >0.10 | >0.10 | 0.0058 |
| BMP4 | 0.03177 | 0.05477 | 0.01166 | 0.03472 | >0.10 | 0.001328 | 0.01404 | <0.05 | >0.10 | 0.0129 |
| COL1A1 | 4.512 | 4.608 | 3.665 | 6.744 | >0.05 | 2.845 | 1.721 | >0.05 | >0.05 | 0.488 |
| COL1A2 | 2.029 | 1.497 | 1.024 | 1.036 | <0.05 | 0.6877 | 0.5899 | <0.05 | >0.10 | 0.0085 |
| COL3A1 | 3.245 | 3.109 | 1.294 | 0.9377 | <0.05 | 1.122 | 0.6311 | <0.05 | >0.10 | 0.0005 |
| FRZB | 0.004235 | 0.07244 | 0.017 | 0.05802 | >0.05 | 0.002234 | 0.006911 | >0.05 | >0.05 | 0.1129 |
| SFRP1 | 0.1839 | 0.2663 | 0.017 | 0.04717 | >0.05 | 0.00283 | 0.008989 | <0.05 | >0.10 | <0.0001 |
| SFRP2 | 1.547 | 1.655 | 0.08074 | 0.09347 | <0.05 | 0.02587 | 0.36 | <0.05 | >0.10 | <0.0001 |
| TLR1 | 0.00308 | 0.02456 | 0.017 | 0.05532 | >0.05 | 0.01266 | 0.01207 | >0.05 | >0.05 | 0.1137 |
| TLR2 | 0.009758 | 0.03131 | 0.017 | 0.1306 | >0.05 | 0.0534 | 0.2682 | >0.05 | >0.05 | 0.0716 |
| TLR3 | 0.1153 | 0.1592 | 0.007327 | 0.03332 | <0.05 | 0.002234 | 0.007204 | <0.05 | >0.05 | 0.0004 |
| TLR4 | 0.1107 | 0.1607 | 0.08265 | 0.1076 | >0.05 | 0.02224 | 0.06471 | >0.05 | >0.05 | 0.0803 |
| TLR5 | 0.02654 | 0.03578 | 0.1532 | 0.4113 | <0.10 | 0.03608 | 0.04388 | >0.10 | <0.10 | 0.0268 |
| TNFa | 0.005172 | 0.03159 | 0.006026 | 0.02876 | >0.05 | 0.03803 | 0.04041 | >0.05 | >0.05 | 0.8696 |
| CD120A | 0.3521 | 0.2023 | 0.1491 | 0.1519 | <0.05 | 0.1487 | 0.08271 | <0.10 | >0.10 | 0.0047 |
| CD120B | 0.003974 | 0.01288 | 0.00816 | 0.04119 | >0.05 | 0.00283 | 0.1476 | >0.05 | >0.05 | 0.2328 |
| OPG | 0.05462 | 0.1832 | 0.007252 | 0.02759 | <0.05 | 0.001324 | 0.00156 | <0.05 | >0.10 | <0.001 |
| RANK-L | 0.008441 | 0.03116 | 0.01651 | 0.06068 | >0.05 | 0.01035 | 0.02194 | >0.05 | >0.05 | 0.4854 |
| TRAIL | 0.0468 | 0.04712 | 0.2321 | 0.1625 | <0.05 | 0.1683 | 0.1126 | <0.05 | >0.10 | 0.0057 |
| CCL18 | 0.1423 | 0.3251 | 1.273 | 3.131 | <0.05 | 0.1958 | 0.7341 | >0.10 | >0.10 | 0.0064 |
| CCL2 | 0.1511 | 0.1968 | 0.1549 | 0.09986 | >0.05 | 0.3104 | 0.3248 | >0.05 | >0.05 | 0.2067 |
| CCL3 | 0.02843 | 0.1142 | 0.4389 | 0.9693 | <0.05 | 0.1198 | 1.488 | <0.05 | >0.10 | 0.0009 |
| CCR1 | 0.0552 | 0.05793 | 0.1543 | 0.3397 | >0.05 | 0.07627 | 0.1094 | <0.05 | <0.05 | 0.332 |
| CXCL9 | 0.004121 | 0.03849 | 0.06714 | 0.3498 | >0.05 | 0.005067 | 0.4137 | >0.05 | >0.05 | 0.1045 |
| CXCL10 | 0.002647 | 0.02651 | 0.01212 | 0.06601 | >0.05 | 0.005067 | 0.103 | >0.05 | >0.05 | 0.1314 |
| PDGFα | 0.1012 | 0.09264 | 0.02438 | 0.04879 | <0.05 | 0.02368 | 0.01218 | <0.05 | >0.10 | 0.0034 |
| PDGFβ | 0.05676 | 0.05779 | 0.07192 | 0.1792 | >0.05 | 0.03111 | 0.02698 | >0.05 | >0.05 | 0.1206 |
| IGF-1 | 0.2865 | 0.3959 | 0.05834 | 0.05301 | <0.05 | 0.01029 | 0.02171 | <0.05 | >0.10 | 0.001 |
| FGF 18 | 0.01984 | 0.06799 | 0.006952 | 0.02802 | >0.10 | 0.001324 | 0.00156 | <0.05 | >0.10 | 0.001 |
| TGFβ-1 | 0.1947 | 0.08773 | 0.3186 | 0.3192 | >0.10 | 0.2865 | 0.1341 | >0.10 | >0.10 | 0.0706 |
| TGFβ-2 | 0.03405 | 0.07047 | 0.01177 | 0.03516 | >0.10 | 0.00283 | 0.01341 | <0.05 | >0.10 | 0.0125 |
| TGFβ-3 | 0.04816 | 0.05877 | 0.02195 | 0.04724 | >0.10 | 0.005067 | 0.01635 | <0.05 | >0.10 | 0.0317 |
| VEGFA | 0.04464 | 0.06186 | 0.08849 | 0.148 | >0.05 | 0.04514 | 0.3991 | >0.05 | >0.05 | 0.374 |
| VEGFB | 0.2803 | 0.1593 | 2.248 | 2.628 | <0.05 | 0.82 | 0.6305 | <0.10 | >0.10 | 0.0001 |
| GMCSF-1 | 0.1664 | 0.1744 | 0.1255 | 0.1923 | >0.10 | 0.1002 | 0.0457 | <0.05 | >0.10 | 0.0298 |
| CD2 | 0.005343 | 0.07242 | 0.01637 | 0.1592 | >0.05 | 0.02838 | 0.01685 | >0.05 | >0.05 | 0.1411 |
| CD14 | 0.3432 | 0.2554 | 0.5519 | 1.048 | >0.05 | 0.6213 | 0.7487 | >0.05 | >0.05 | 0.2253 |
| CD28 | 0.05295 | 0.0523 | 0.007327 | 0.04835 | >0.05 | 0.01384 | 0.0277 | >0.05 | >0.05 | 0.1997 |
| CD36 | 0.07742 | 0.1578 | 0.2983 | 0.2362 | <0.05 | 0.2236 | 0.1771 | >0.10 | >0.10 | 0.0093 |
| CD58 | 0.06297 | 0.05023 | 0.2004 | 0.8912 | <0.05 | 0.1095 | 0.242 | >0.10 | >0.10 | 0.0112 |
| CD86 | 0.03633 | 0.07832 | 0.08219 | 0.1864 | >0.05 | 0.05676 | 0.0979 | >0.05 | >0.05 | 0.2797 |
| IL1RN | 0.1411 | 0.4352 | 0.08791 | 0.06012 | >0.05 | 0.09495 | 1.191 | >0.05 | >0.05 | 0.5498 |
| IL6 | 0.01272 | 0.0788 | 0.006026 | 0.0302 | >0.05 | 0.002234 | 0.1235 | >0.05 | >0.05 | 0.3196 |
| IL8 | 0.008311 | 0.03701 | 0.07164 | 0.1777 | <0.10 | 0.4329 | 38.16 | <0.05 | >0.05 | 0.0008 |
| IL10 | 0.008311 | 0.02045 | 0.006682 | 0.02795 | >0.05 | 0.01164 | 0.053 | >0.05 | >0.05 | 0.6602 |
| IRAK 3 | 0.1247 | 0.1237 | 0.03232 | 0.06207 | >0.10 | 0.08354 | 0.002234 | <0.05 | >0.10 | 0.0156 |
| SERPINA 3 | 0.05961 | 0.1018 | 0.01177 | 0.3296 | >0.10 | 0.00283 | 0.01645 | <0.05 | >0.10 | 0.0064 |
| ACP5 | 0.107 | 0.244 | 1.813 | 4.045 | <0.05 | 0.8059 | 2.019 | <0.10 | >0.10 | 0.0003 |
| B2M | 2.012 | 1.311 | 9.011 | 20.63 | <0.05 | 4.479 | 6.057 | >0.10 | >0.10 | 0.0037 |
| BCL2A1 | 0.002387 | 0.01485 | 0.1322 | 0.5382 | <0.05 | 0.09534 | 0.7871 | <0.10 | >0.10 | 0.0002 |
| CASP 3 | 0.0792 | 0.09957 | 0.01064 | 0.03861 | >0.05 | 0.0117 | 0.01483 | <0.10 | >0.10 | 0.104 |
| CHIT 1 | 0.002933 | 0.01846 | 8.686 | 29.19 | <0.05 | 0.4379 | 6.72 | <0.05 | >0.10 | <0.0001 |
| CILP1 | 0.1486 | 0.5708 | 0.04197 | 0.3067 | <0.05 | 0.006287 | 0.0186 | <0.05 | >0.10 | 0.0002 |
| CTSG | 0.03886 | 0.1577 | 0.01969 | 0.09609 | >0.05 | 0.02163 | 0.4619 | >0.05 | >0.05 | 0.7653 |
| CTSK | 0.387 | 0.3404 | 1.663 | 2.608 | <0.05 | 0.4657 | 0.2824 | >0.10 | >0.10 | 0.0397 |
| ERCC1 | 0.1268 | 0.07787 | 1.211 | 3.152 | <0.05 | 0.8464 | 0.3581 | <0.05 | >0.10 | <0.001 |
| ITGAM | 0.1349 | 0.1592 | 0.3402 | 0.6097 | >0.05 | 0.1613 | 0.3537 | >0.05 | >0.05 | 0.0711 |
| ITGB2 | 0.1904 | 0.1486 | 1.603 | 2.579 | <0.05 | 0.7127 | 0.7301 | <0.05 | >0.10 | <0.0001 |
| MARCO | 0.1192 | 0.191 | 0.3081 | 1.082 | <0.10 | 0.4281 | 0.4291 | >0.10 | >0.10 | 0.0415 |
| PTGS2 | 0.004121 | 0.02114 | 0.007245 | 0.03056 | >0.10 | 0.001324 | 0.1393 | >0.10 | <0.10 | 0.0853 |
| TP53 | 0.05543 | 0.06541 | 0.1053 | 0.1347 | >0.05 | 0.04756 | 0.04778 | >0.05 | >0.05 | 0.1821 |
| VDR | 0.0301 | 0.06238 | 0.01987 | 0.1248 | >0.05 | 0.04363 | 0.1025 | >0.05 | >0.05 | 0.5854 |

Table 3.3.3.6 : Gene expression by subgroup, standard deviations and significance tests

| | Primary | | | | | Primary | | |
|---|---|---|---|---|---|---|---|---|
| Well Fixed | Gene | Osteolytic | (p=) | | Well Fixed | Gene | Osteolytic | (p=) |
| ↑ | ACP5 | ↑ | 0.0003 | | ↑ | IL10 | ↓ | 0.6602 |
| ↑ | ADAMTS 14 | ↑ | <0.0001 | | ↓ | IL6 | ↓ | 0.3196 |
| ↓ | ADAMTS 2 | ↓ | <0.0001 | | ↑ | IL8 | ↑ | 0.0008 |
| ↑ | B2M | ↑ | 0.0037 | | ↓ | IRAK 3 | ↓ | 0.0156 |
| ↑ | BCL2A1 | ↑ | 0.0002 | | ↑ | ITGAM | ↑ | 0.0711 |
| ↓ | BMP4 | ↓ | 0.0129 | | ↑ | ITGB2 | ↑ | <0.0001 |
| ↓ | CASP 3 | ↓ | 0.104 | | ↑ | MARCO | ↑ | 0.0415 |
| ↑ | CCL18 | ↑ | 0.0064 | | ↓ | MMP1 | ↓ | 0.3674 |
| ↑ | CCL2 | ↑ | 0.2067 | | ↑ | MMP12 | ↑ | 0.009 |
| ↑ | CCL3 | ↑ | 0.0009 | | ↑ | MMP13 | ↑ | 0.5808 |
| ↑ | CCR1 | ↑ | 0.332 | | ↑ | MMP9 | ↑ | <0.0001 |
| ↓ | CD120A | ↓ | 0.0047 | | ↓ | OPG | ↓ | <0.001 |
| ↓ | CD120B | ↑ | 0.2328 | | ↓ | PDGFα | ↓ | 0.0034 |
| ↑ | CD14 | ↑ | 0.2253 | | ↓ | PDGFb | ↑ | 0.1206 |
| ↑ | CD2 | ↑ | 0.1411 | | ↓ | PTGS2 | ↑ | 0.0853 |
| ↓ | CD28 | ↓ | 0.1997 | | ↑ | RANK-L | ↑ | 0.4854 |
| ↑ | CD36 | ↑ | 0.0093 | | ↓ | SERPINA 3 | ↓ | 0.0064 |
| ↑ | CD58 | ↑ | 0.0112 | | ↓ | SFRP1 | ↓ | <0.0001 |
| ↑ | CD86 | ↑ | 0.2797 | | ↓ | SFRP2 | ↓ | <0.0001 |
| ↑ | CHIT 1 | ↑ | <0.0001 | | ↑ | TGFβ-1 | ↑ | 0.0706 |
| ↓ | CILP1 | ↓ | 0.0002 | | ↓ | TGFβ-2 | ↓ | 0.0125 |
| ↓ | COL1A1 | ↓ | 0.488 | | ↓ | TGFβ-3 | ↓ | 0.0317 |
| ↓ | COL1A2 | ↓ | 0.0085 | | ↑ | TIMP1 | ↑ | 0.0058 |
| ↓ | COL3A1 | ↓ | 0.0005 | | ↑ | TLR1 | ↑ | 0.1137 |
| ↓ | CTSG | ↓ | 0.7653 | | ↑ | TLR2 | ↑ | 0.0716 |
| ↑ | CTSK | ↑ | 0.0397 | | ↓ | TLR3 | ↓ | 0.0004 |
| ↑ | CXCL10 | ↑ | 0.1314 | | ↓ | TLR4 | ↓ | 0.0803 |
| ↑ | CXCL9 | ↑ | 0.1045 | | ↑ | TLR5 | ↑ | 0.0268 |
| ↑ | ERCC1 | ↑ | <0.001 | | ↑ | TNFa | ↑ | 0.8696 |
| ↓ | FGF 18 | ↓ | 0.001 | | ↓ | TP53 | ↑ | 0.1821 |
| ↓ | FRZB | ↑ | 0.1129 | | ↑ | TRAIL | ↑ | 0.0057 |
| ↑ | GMCSF-1 | ↓ | 0.0298 | | ↑ | VDR | ↓ | 0.5854 |
| ↓ | IGF-1 | ↓ | 0.001 | | ↑ | VEGFA | ↑ | 0.374 |
| ↓ | IL1 RN | ↓ | 0.5498 | | ↑ | VEGFB | ↑ | 0.0001 |

Table 3.3.3.7 : Subgroup expression direction of change of median and significance levels

### 3.3.3.9 Genes Associated with Osteolysis (Significance Testing)

The Kruskal-Wallis test identifies any significant differences between the three groups. The majority of genes were found to be statistically significantly different (Figure 3.3.3.6). There were 37 genes that reached statistical significance. This is not an unexpected finding as all of these genes have been previously implicated in osteolysis.

There were however 28 genes (VDR, TP53, ITGAM, CASP 3, IL10, IL6, IL1RN, VEGFA, PDGFβ, CCR1, CXCL9, CXCL10, CCL2, RANK-L, TLR1, TLR2, TLR4, COL1A1, MMP1, MMP13) where no significant differences were

found between the subgroups. This may be attributed to either a type 2 error, or (given the large sample size) is most likely an accurate observation. It is therefore likely that these genes are up regulated as part of the osteoarthritic process, not in response to the presence of a joint arthroplasty.

Using Dunn's correction each subgroup was also tested for significance against each other (Figure 3.3.3.6) with the majority of genes being significantly different in the two arthroplasty groups as compared to the osteoarthritis group. There were however no genes in which a statistically significant difference were found between the well fixed and osteolytic groups (Table 3.3.3.6). There were two genes, TLR5 and PTGS2 in which border line significance was found ($p<0.10$). Statistical significance only evaluates the likelihood that any observed change in the mean (or median) value is due chance alone. It does not evaluate the magnitude of any difference, quantify the likely clinical effect of any change or demonstrate the strength of association between a factor and any given outcome.

As there were no genes reaching significant differences between the well fixed and osteolytic subgroups a further pairwise analysis was undertaken (Table 3.3.3.7) using multiple Mann-Whitney U tests. It must be remembered in interpreting these results that without a post hoc correction for multiple testing there is the potential to introduce a type 1 error. The test however has more statistical power and thus is able to detect significant changes in smaller sample sizes. Using this method 39 genes demonstrated statistically significant differences between the well fixed and osteolytic subgroups.

There are many reasons why an observed difference may not reach statistical significance including $\alpha$-error (as assessed by the p value), but other factors include inappropriate statistical testing and too small sample sizes ($\beta$-error). In this case it is likely that the eventual sample size of 7 well fixed patients was too small, so although patterns can be observed in median expression and distribution there were no significant differences when compared to the osteolytic group. In other words it is likely that the analysis suffers from a Type II or $\beta$ error.

Further analysis is warranted in genes where the medians diverge between the two groups (section 3.3.7.2) and in those where there is an apparent difference in gene distribution between groups. Genes falling into both of these categories are VEGFA, VEGFB, IGF 1, IGF18, CCL3, CCL18, CCR1, ADAMTS, CXCL9, CXCL10, TNF $\alpha$, CD120 $\beta$, OPG, RANK-L, TLR3, TLR5, FRZB, SFRP, SFRP2, BMP4, MMP1, MMP13, IL8, IRAK3, ACP5, BCL2A1, CHIT1, CTSK, TP53, PTGS2. A further analysis has been performed on all of these genes to measure the strength of association between known gene expression and known outcome (i.e. osteolytic or well fixed).

3.3.3.9.1 Measuring Strength of Association

Statistical testing of median gene expression may not be the most appropriate test when interpreting gene expression data between similar groups. Genes tend to work in families, and are often associated with other co-stimulatory or inhibitory pathways. A complex web of interactions is more usual than a simple cause and

effect model between a gene and an outcome. In this case measuring strength of association is the most appropriate statistical technique. This can be done at the most simple level with positive predictive values, negative predictive values, sensitivity and specificity where an arbitrary cut off for expression is taken.

Although positive and negative predictive values are a measure of strength of association they use an arbitrary threshold value. Use of this kind of threshold value does not allow quantification of strength of association across the full range of gene expression, only interprets values as “high” or “low” expression. Receiver Operator Characteristic (ROC) is a method of analysis that measures strength of association of a continuous variable with a known outcome. Originally developed during WWII for tuning radar to detect aircraft effectively it is a method for quantifying and characterising the strength of an association when an outcome is known. Use of ROC not only allows an association to be characterised, but also the strength of association across the full range of observed values.

The sensitivity and specificity are calculated for all possible threshold values across the range of observed gene expression for both groups. This data can then be used to plot a ROC curve of Sensitivity vs. 1-Specifity. The curve shows the association of gene expression with outcome (in this case osteolysis). Calculation of the area under the curve (AUC) can be used to compare association between genes and outcome. A maximum value of 1 is 100% sensitive and specific, where a value of 0.5 represents random chance.

The calculated ROC curve, and hence strength of association, can be tested for significant difference from chance. This is a more appropriate way to investigate associations in this data.

3.3.3.9.2 ROC Analysis

ROC analysis was carried out to explore the association between candidate genes and osteolytic loosening. Genes were included when median expression and distribution differed between the F and O groups, but no statistically significant difference had been found with Dunn’s test.

ROC analysis was performed with the intention of quantifying association between gene expression and loosening, hence genes were excluded if median expression of the F group was outside the range of expression seen in the primary (P) group. If both F and O groups appeared to differ in expression from the primary group a further separate ROC analysis was carried out. When comparing these two analyses it should be possible to determine the value of each candidate gene as a marker of osteolytic change as compared to response to wear debris from a total joint replacement.

ROC analysis was performed for 30 of the candidate genes (Table 3.3.3.8). Differences in gene expression was predictive of loosening in sixteen genes with an AUC>0.7. The complete ROC tables for all candidate genes are presented in the appendix.

Seven genes were seen to be predictive of osteolytic change with a statistically significant ROC curve and an AUC>0.77. These were BMP4, FRZB, FGF18, IL8, IRAK 3, OPG and PTGS2. All of these genes had a co-efficient of variation (CoEV) over 90% in the P subgroup. It seems likely that variation in expression of these genes has a role to play in development of osteolytic change. Nine genes were highly predictive of osteolytic change, but their ROC curves did not reach statistical significance. These genes were predictive of osteolytic change (AUC >0.7), but not statistically significantly different from chance : VEGFB, SFRP, TLR3, TLR5, TP53, IGF1, CTSK, CHIT 1, CCL 18.

There were two genes were the median significances (as tested with the Kruskal-Wallis test) and the ROC analysis did not agree. FRZB and PTGS2 were found to be statistically significantly different with ROC analysis, but not on comparison of the medians. This can be an expected finding with more complex statistical analysis. The ROC analysis tests the significance of the predictive value of gene expression for the known outcome (loose or not loose). This analysis includes values across the whole observed range. The Kruskal-Wallis test simply compares the median observed values.

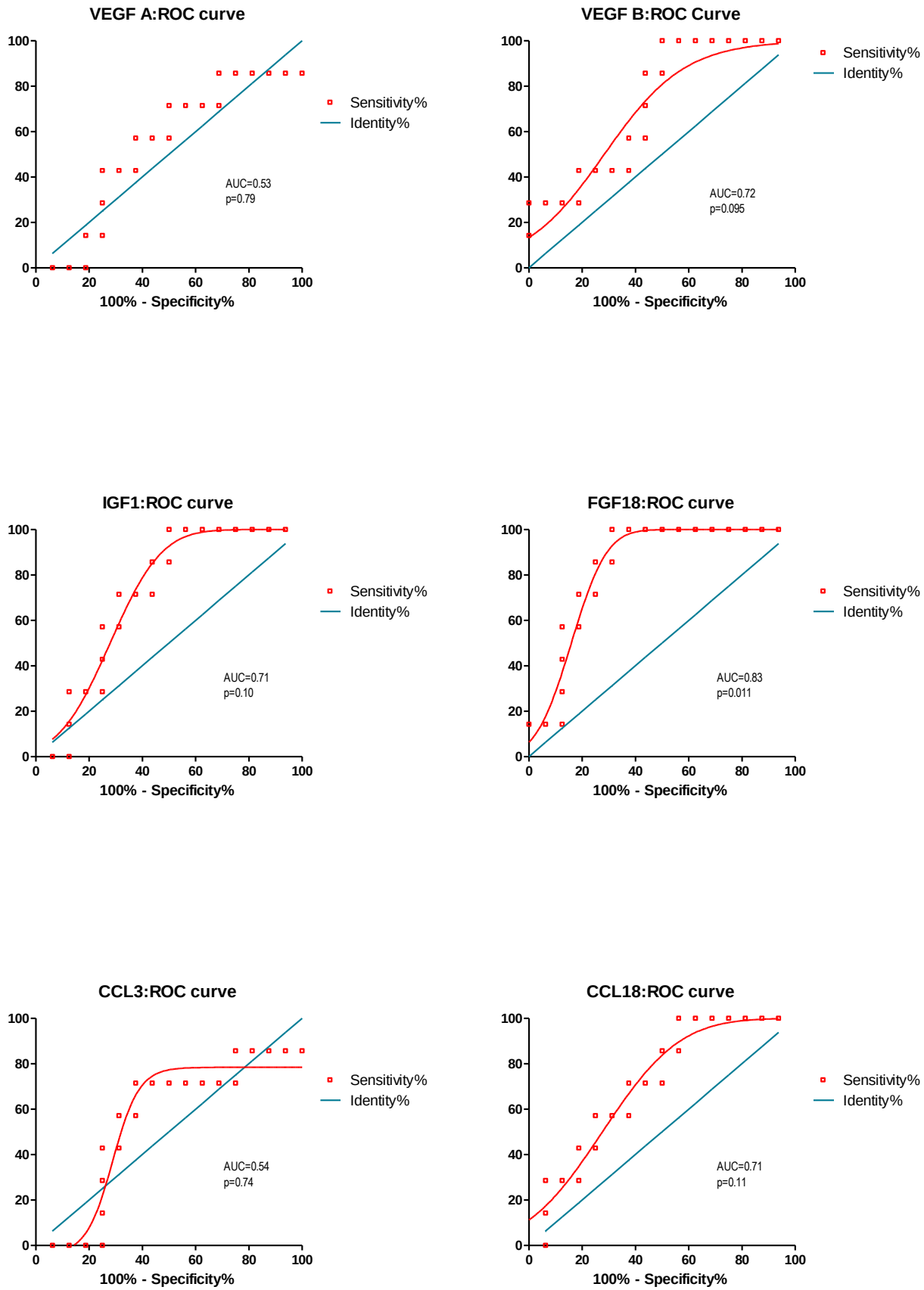



Figure 3.3.3.13 : ROC Curves with calculated AUC and p values for (a) VEGFα (b) VEGFβ (c) IGF1 (d) FGF-18 (e) CCL3 (f) CCL18

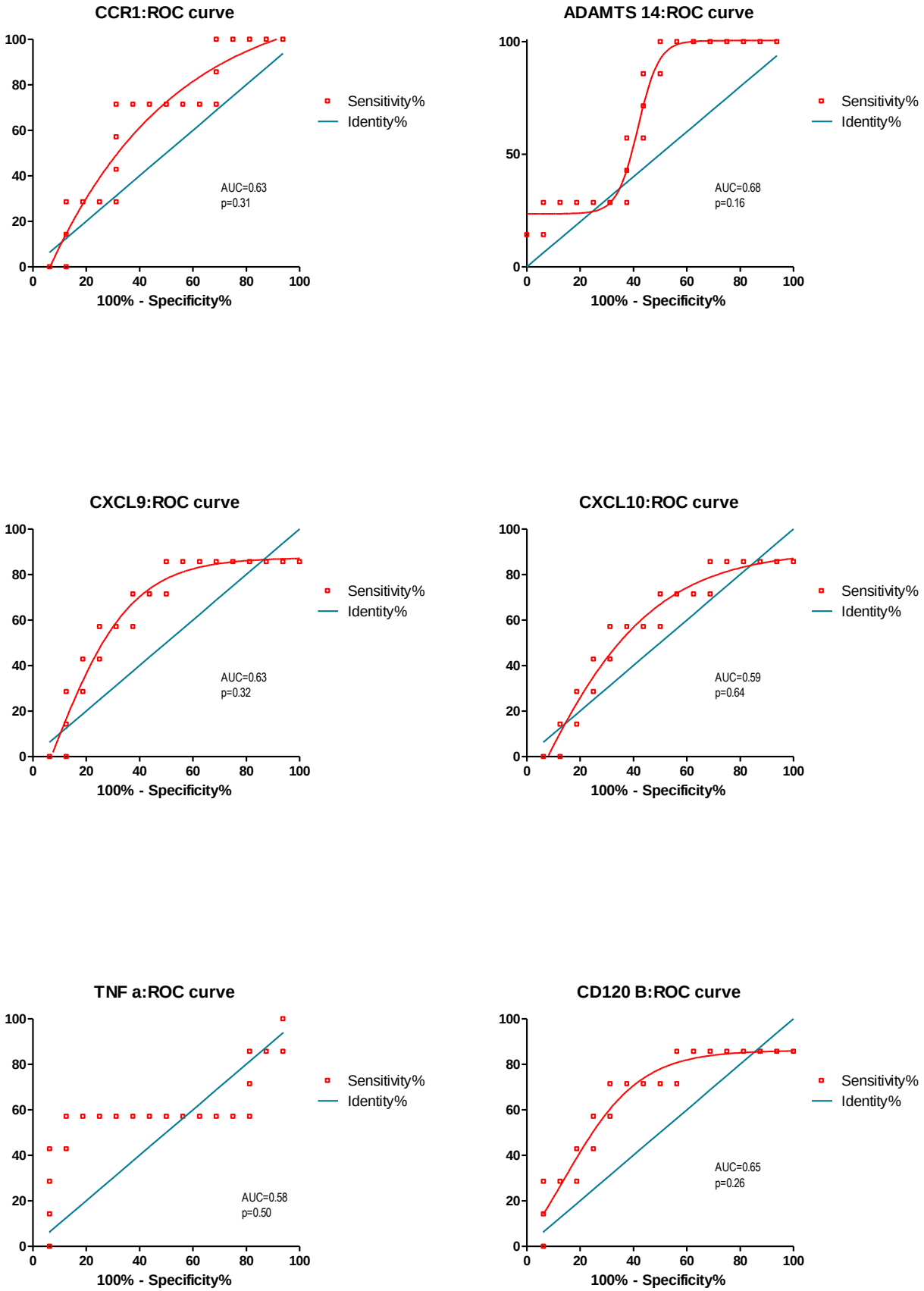



Figure 3.3.3.14 : ROC Curves with calculated AUC and p values for (a) CCR1 (b) ADAMTS 14 (c) CXCL9 (d) CXCL10 (e) TNFα (f) CD120β

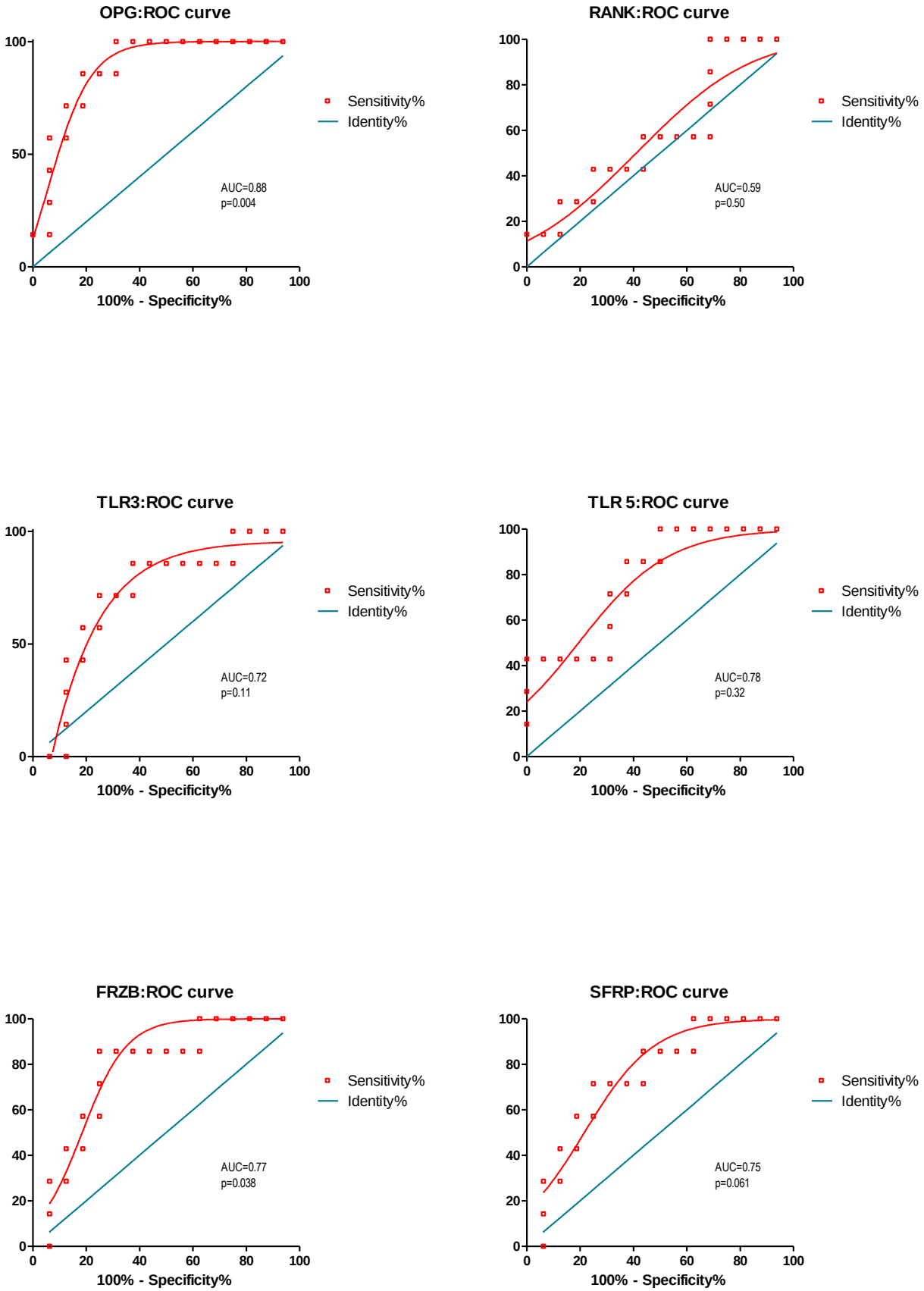



Figure 3.3.3.15 : ROC Curves with calculated AUC and p values for (a) OPG (b) RANK (c) TLR-3 (d) TLR-5 (e) FRZB (f) SFRP

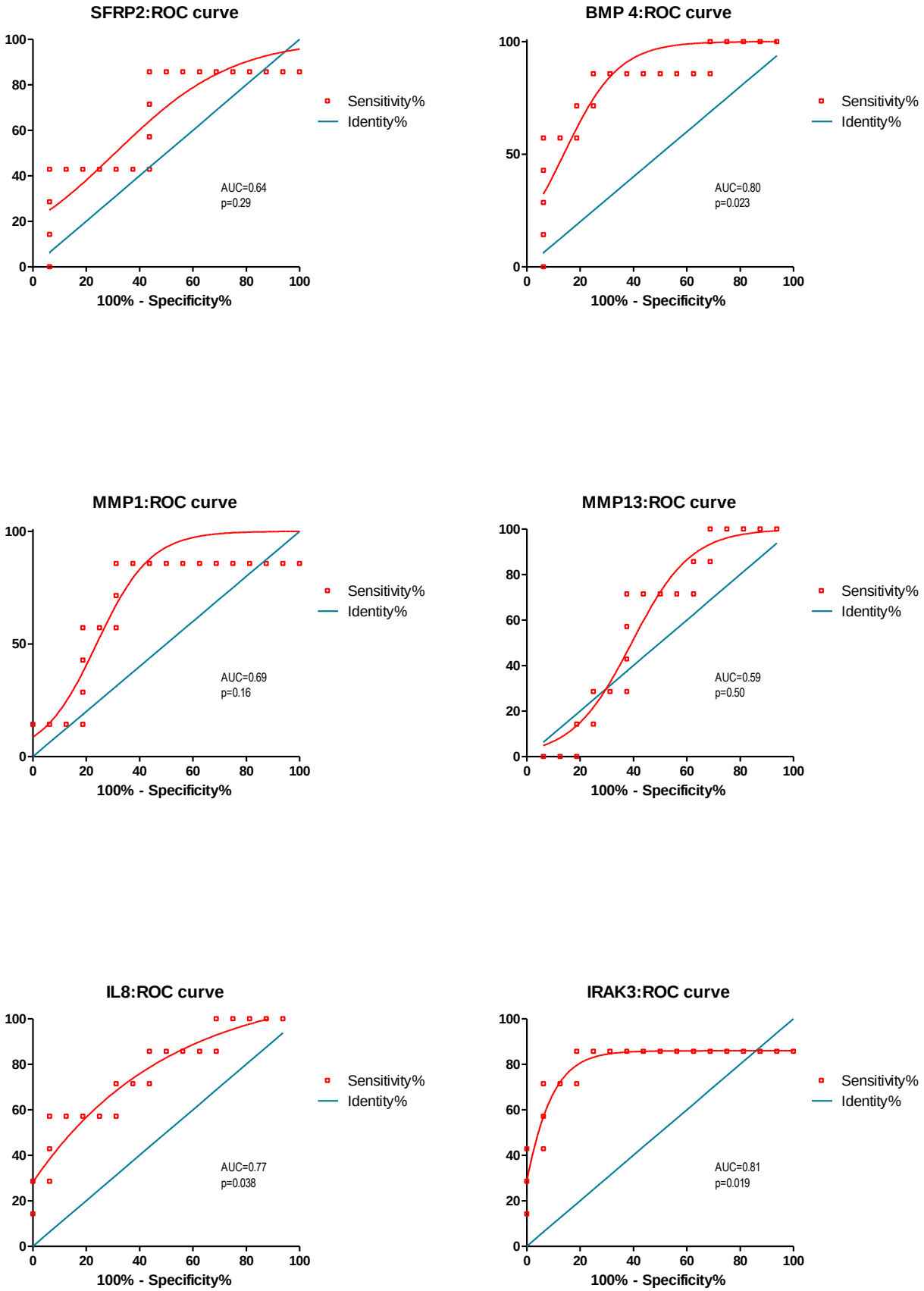



Figure 3.3.3.16 : ROC Curves with calculated AUC and p values for (a) SFRP-2 (b) BMP 4 (c) MMP 1 (d) MMP 13 (e) IL 8 (f) IRAK-3

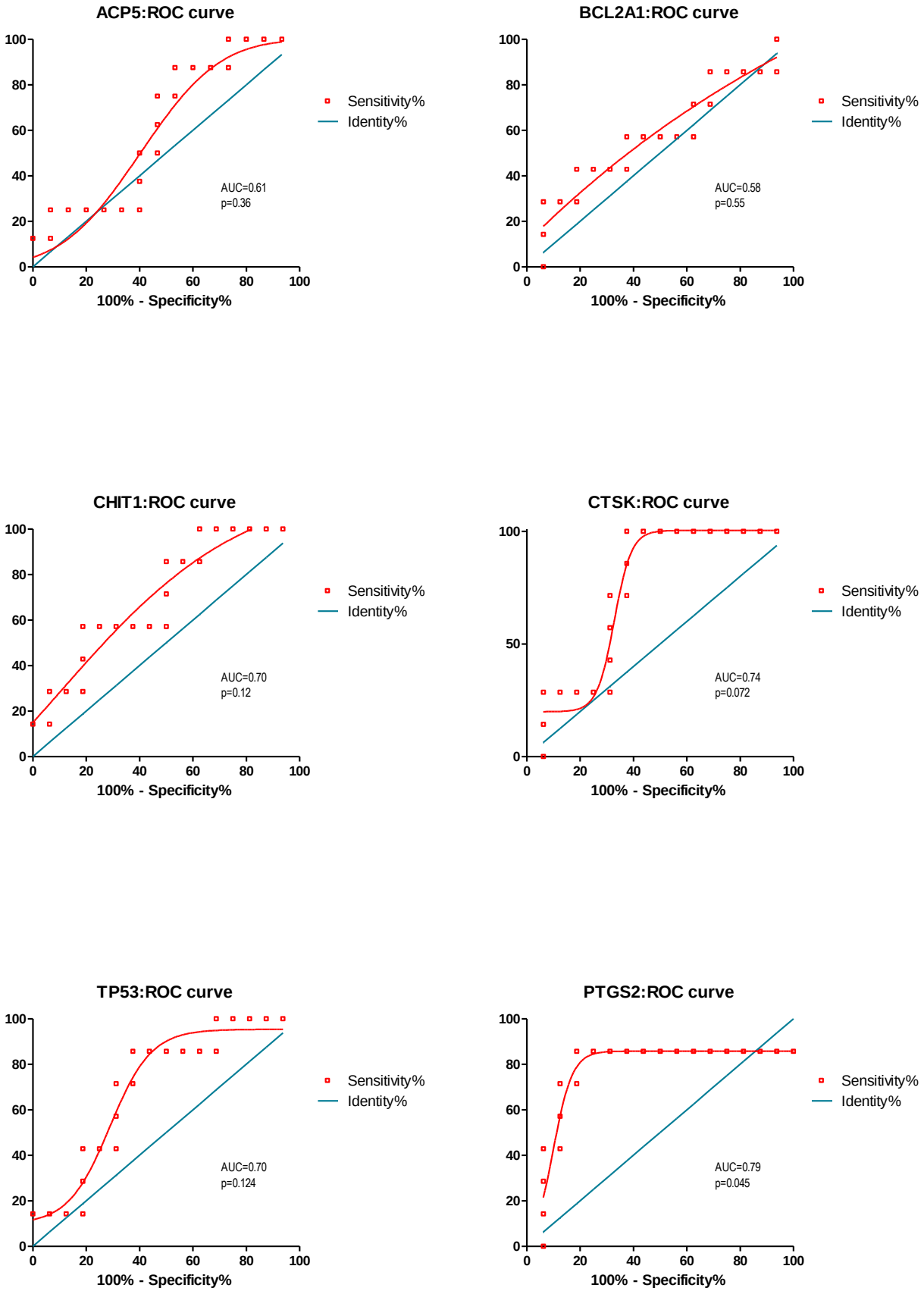



Figure 3.3.3.17 : ROC Curves with calculated AUC and p values for (a) APC5 (b) BCL2A1 (c) CHIT-1 (d) CTSK (e) TP53 (f) PTGS2

| Gene | AUC | p= |
|---|---|---|
| ACP 5 | 0.61 | 0.36 |
| ADAMTS 14 | 0.68 | 0.16 |
| BCL2A1 | 0.58 | 0.55 |
| BMP 4 | 0.8 | 0.023 |
| CCL 18 | 0.71 | 0.11 |
| CCL 3 | 0.54 | 0.74 |
| CCR1 | 0.63 | 0.31 |
| CD120B | 0.65 | 0.26 |
| CHIT 1 | 0.7 | 0.12 |
| CTSK | 0.74 | 0.072 |
| CXCL 10 | 0.59 | 0.64 |
| CXCL 9 | 0.63 | 0.32 |
| FRZB | 0.77 | 0.038 |
| IGF 1 | 0.71 | 0.1 |
| FGF 18 | 0.83 | 0.011 |
| IL8 | 0.77 | 0.038 |
| IRAK 3 | 0.81 | 0.019 |
| MMP1 | 0.69 | 0.16 |
| MMP13 | 0.59 | 0.5 |
| OPG | 0.88 | 0.004 |
| PTGS2 | 0.79 | 0.045 |
| RANK | 0.59 | 0.5 |
| SFRP | 0.75 | 0.061 |
| SFRP2 | 0.64 | 0.29 |
| TLR3 | 0.72 | 0.11 |
| TLR5 | 0.78 | 0.32 |
| TNF a | 0.58 | 0.5 |
| TP53 | 0.7 | 0.124 |
| VEGF A | 0.53 | 0.79 |
| VEGF B | 0.72 | 0.095 |

Table 3.3.3.8 : ROC Analysis summary

### 3.3.3.10 Genes Reaching Significance (ROC Testing)

The more detailed ROC analysis demonstrated that 16 genes were highly associated with osteolytic change (AUC >0.77), of these seven genes reached statistical significance for the strength of association. Each gene is discussed with an overview of how this observation may fit with what is currently known about the pathway.

Bone Morphogenic Protein 4 (BMP 4)

Expression levels of BMP 4 are lower in patients with a well fixed prosthesis than the two other groups. BMP 4 is known to be an active stimulator of bone growth and has been used in clinical trials to stimulate bone healing[237]. BMP 4 is also known to play a role in ectopic bone formation[238]. BMP-4 has been previously studied in animal models and shown to be up regulated in response to stimulation by particulate wear debris. The observation in this study of lower BMP4 expression in the well fixed group fits with previous work.

BMP-4 functions to induce differentiation of the osteoblast from the osteoprogenitor cell[239]. It has been demonstrated to up regulate osteocalcin expression and to induce bone formation, although not as aggressively as BMP2, 6 & 9[240]. BMP-4 is responsible in part for heterotrophic ossification, although this may be due to relative down regulation of the BMP inhibitor noggin[241] rather than over expression of BMP-4.

Given the regulatory role that BMP's are known to have on TGF-β in the bone remodelling pathway (Figure 3.3.3.18) the finding of similar expression patterns in TGFβ-2 and TGFβ-3 lend weight to the finding that BMP expression may be important in osteolytic change.

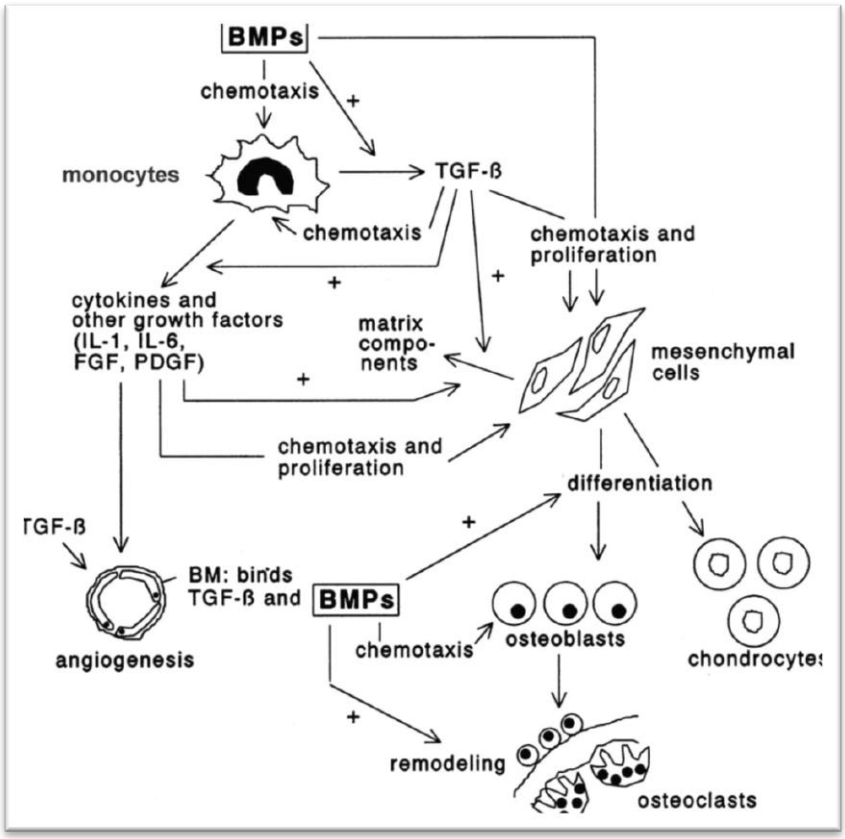



Figure 3.3.3.18 : An overview of the BMP pathway. Reproduced from Termaat & Den Boer[242]

Frizzled Related Protein (FRZB or sFRP3)

Frizzled related protein is highly expressed on chondrocytes. FRZB encodes the secreted frizzled-related protein 3 (SFRP3), which acts as an antagonist of extracellular Wnt ligands. Although Wnt signalling is implicated in multiple processes, like many cell surface receptors the interaction with intracellular pathways, and secreted signal proteins may significantly alter the eventual effect[243]. The Wnt pathway has the ability to modulate osteoblastic differentiation and is therefore potentially important in osteolytic change (Fig 3.3.3.19).

Frizzled related protein has been previously implicated in periprosthetic osteolysis[155]. Genotype variations in FRZB have been shown to predispose patients to both osteolysis and heterotopic ossification, however differences observed were not statistically significant[155]. The confirmation that higher expression of FRZB is associated with osteolytic prosthesis, but not with well fixed hip prosthesis, adds significantly to what is already known on this topic. As there is a spread of FRZB expression in the primary osteoarthritis group, and that both the osteolytic and well fixed groups are a subset of this expression it seems possible that preoperative FRZB expression levels may predispose a patient to osteolytic change. This theory is supported by similar although not conclusive findings in some genotyping studies[155]. Further support for this hypothesis can be found from the variations seen in SFRP1 (described below) which is also a modulator of the Wnt pathway.

It is not possible from the data collected in this study to determine which of the downstream pathways are responsible for osteolysis, as we did not characterise mRNA expression for all the pathways. However the WNT/β-catenin pathway seems the most likely candidate as it has been previously shown to modulate bone resorption and may play a key role in the pathogenesis of osteoarthritis and osteolysis. Activation of Wnt pathway results in up regulation of Dishevelled (Dvl) and production of a non-phosphorylated form of β-catenin which localises to the nucleus. This is thought to directly induce the expression of downstream target genes[152].

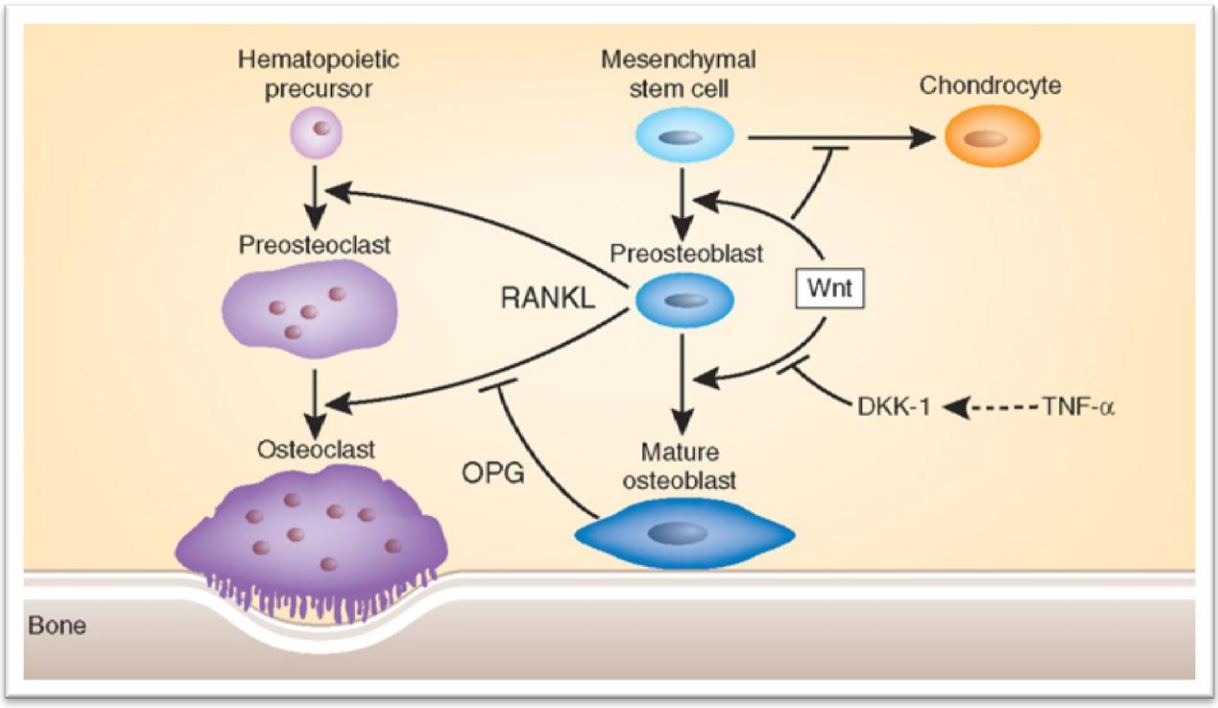



Figure 3.3.3.19 – The role of the Wnt signalling pathway in regulation of osteoblastic and osteoclastic differentiation. Reproduced from "Eating bone or adding it: the Wnt pathway decides" Steven R Goldring & Mary B Goldring Nature Medicine 13, 133 - 134 (2007)

Fibroblast Growth Factor 18 - FGF18

Fibroblast growth factor 18 functions in combination with BMPs and other growth factors to promote differentiation of osteoblasts from osteoprogenitor cells. The findings of significantly lower levels of FGF-18 which is partly responsible for osteoblast differentiation is at first puzzling. FGF-18 along with a number of other growth factors have been shown to be associated with increasing bone thickness (Fig 3.3.3.20). Whilst one might expect increased bone formation in the well fixed group, the contrary is true. An osteolytic loose stem will be undergoing a high rate of bone turn over and remodelling in response to the osteolytic process. In a stable joint replacement one would not expecting a higher than normal rate of bone turn over to be seen. The raised levels of FGF-18 are probably indicative of reduced bone turnover in well fixed prosthesis. This suggestion is supported by similar findings with VEGFB.

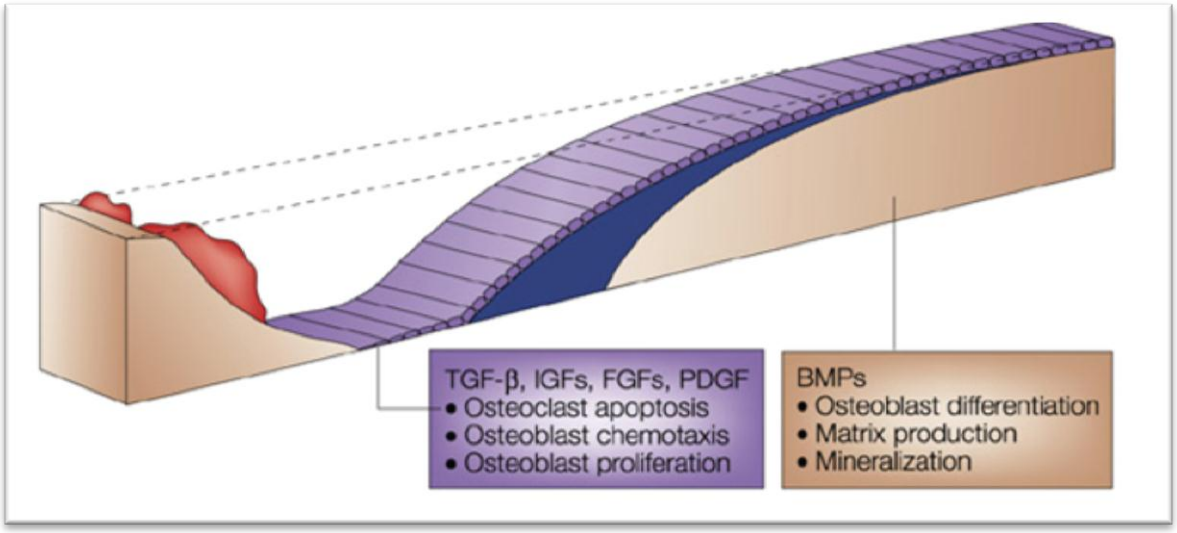



Figure 3.3.3.20 : Growth factors effect on bone thickness. Reproduced from Mundy, Metastasis: Metastasis to bone: causes, consequences and therapeutic opportunities. Nature Reviews Cancer (2), 2002:584-593

Interleukin 8 (IL 8)

The role of interleukin 8 has been mostly evaluated as part of the effect of tumour associated osteolysis. Interleukin 8 has been shown to stimulate both human osteoclast formation and bone resorption[244], although initially evaluated as part of the RANK/OPG pathway more recent work has demonstrated that IL 8 is able to stimulated osteoclast formation directly. The findings of raised IL8 expression associated with loose prosthesis fits with previous work.

Beraudi and colleagues[157] have performed a further series ex-vivo studies of limited cytokines and implicated IL-8 in osteolytic change through use of an ELISA technique. Although they did not evaluate any well fixed stems these findings suggest IL-8 may be implicated in periprosthetic as well as metastatic osteolysis through a similar mechanism. Our findings support this hypothesis.

Interleukin 1 Receptor Associated Kinase 3 – IRAK 3

IRAK 3 is an intracellular kinase which is associated with a range of intracellular pathways. IRAK is involved in signal transduction from the TLR and IL-1R receptors, and as such is a possible marker of the function of these pathways. The findings of lower expression of IRAK3 in the well fixed group suggests that IRAK3 mediated pathways may be key in the intracellular pathways responsible for the osteolytic response.

Although not specifically previously implicated in periprosthetic osteolysis IRAK has been extensively studied in it's role as an immune modulator. Activation of IRAK mediated pathways play a central role in reducing macrophage cytokine production[245] and have been implicated in induction of macrophage tolerance through down regulation of the TOLL pathway[246-248]. IRAK-3 has been shown to be up regulated in response to repeated toxin exposure[249], and the lower expression of IRAK-3 in the well fixed group likely corresponds to lower activity of the macrophages and antigen presenting cells when compared to the osteolytic subgroup.

Osteoprotegerin OPG

Osteoprotegerin levels were significantly lower in the well fixed group, and high levels of OPG were predictive of a loose implant in this study. As has been previously discussed OPG is key to activation of osteoclasts (Fig 3.3.3.21) and has been implicated in wear debris related osteolysis.

The finding of lower levels of OPG in well fixed hips when compared to the osteolytic cohort is to be expected however under expression of OPG in the osteolytic group when compared to the primary osteoarthritis group is difficult to explain. However OPG is rarely taken in isolation, and should be considered in combination with the RANK receptor. When found in combination with the RANK ligand and RANK receptor OPG is a potent stimulator of the osteolytic process. Lower levels of OPG in the osteolytic cohort when compared to the primaries may represent either low osteoclastic activity, or as seems more likely the OPG could be more effective in the osteolytic group due to up regulation of the RANK and RANK-L expression in the osteolytic patients. This would increase the OPG:RANK ratio and the subsequent effect of the OPG.

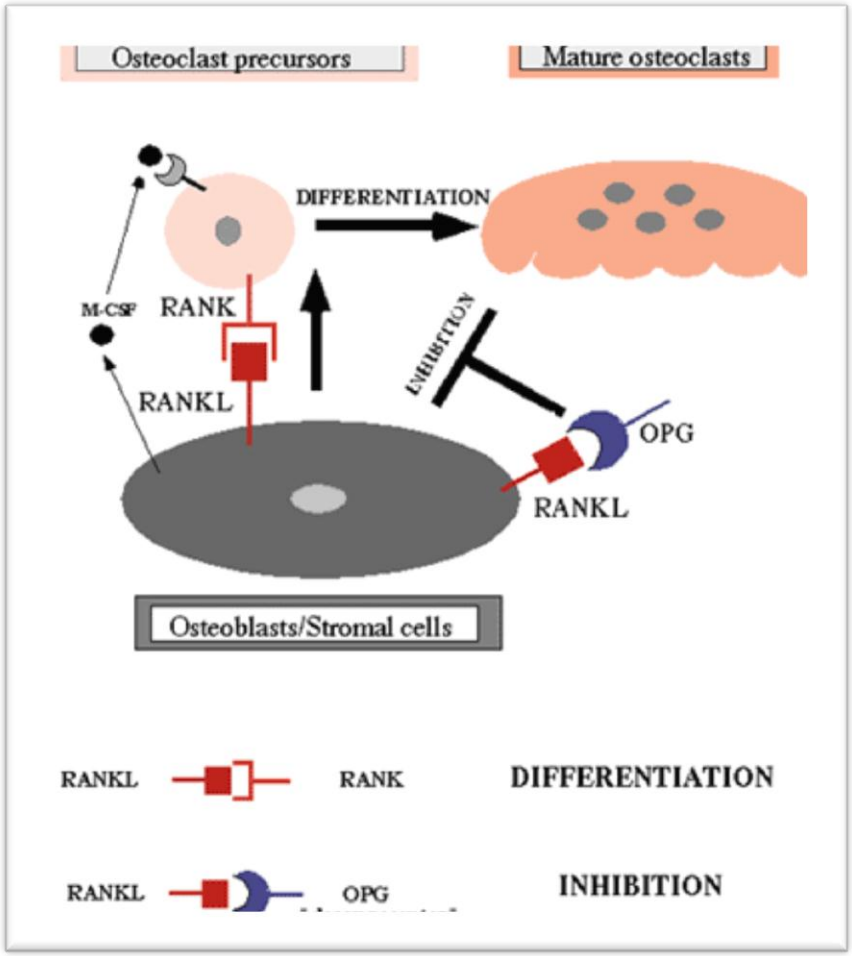



Figure 3.3.3.21 : OPG-RANK(L) interactions. Reproduced from http://www.Qiagen.com GeneGlobe Pathways view)

Prostaglandin-endoperoxide synthase 2 (PTGS2)

Expression of PTGS2 was significantly lower in the well fixed revision cohort than the osteolytic group, but still seen as a subset of the primary group. Although not statistically significant the osteolytic group had higher median gene expression than the well fixed group.

PTGS2 is the enzyme responsible for synthesis of the prostanoids, and PTGS2 is the inducible form (COX2). Previous studies have demonstrated that production of IL-6 and prostaglandin E2 in response to wear debris is mediated by PTGS2[250]. Further work has demonstrated that inhibition of PTGS2 has the potential to suppress periprosthetic osteolysis[251;252].

The finding of higher expression of PTGS2 in the osteolytic group as compared to the well fixed group, with both being subsets of the range of primary expression is interesting. Although it is possible that the observed raised PTGS2 in the osteolytic group is an induced effect it is also possible that this observation represents a tendency of patients with higher PTGS2 levels to undergo periprosthetic osteolysis in response to exposure to wear debris.

### 3.3.3.11 Genes Highly Predictive of Osteolytic Change

Some genes were identified in the ROC analysis where expression levels were predictive of osteolytic change, but the ROC curve was not statistically significantly different from chance. The genes discussed all had an AUC of >0.7, but a p value of >0.05.

Vascular Endothelial Growth Factor β (VEGFB)

VEGF is one of a range of growth factors that have been implicated in previous studies in osteolysis. Expression of VEGFB is not significantly altered in the well-fixed subgroup when compared to the primaries suggesting that the presence of an arthroplasty and wear debris is not responsible for its up regulation. However it is significantly raised in the osteolytic subgroup compared to the primaries, and borderline significance ($p<0.10$) when compared to the well-fixed group. This pattern likely associates the process of osteolysis with VEGFB. As the expression is up regulated this is likely an induced pattern.

VEGFB has been associated with neovascularisation in the periprosthetic tissue, which has been hypothesized to augment and sustain the inflammatory response to wear debris[253]. The induction of VEGF release from macrophages in response to wear debris likely contribute to neovascularisation subsequent periprosthetic osteolysis and implant loosening **(Miyanishi, Trindade 2003)**.

VEGF has also been shown to have a role in the regulation of the RANK/RANK-L osteoclastogenic pathway **(Ren, Markel, Zhang 2006)**, and in light of these previous findings the new data presented as part of this study confirms the importance VEGF signaling in the pathogenesis of prosthetic loosening. This data suggests that

VEGF is induced to high rates of expression, so although it may be useful as a surrogate marker for osteolytic change or a potential point for therapeutic intervention. VEGFB expression preoperatively is unlikely to indicate a tendency towards periprosthetic loosening.

Secreted frizzled-related protein 1 (SFRP-1)

Levels of SFRP-1 in the periprosthetic tissues are lower in the well-fixed group than in the osteolytic group. Whilst highly associated with outcome (AUC = 0.75) SFRP-1 was only borderline significance (p=0.075). SFRP-1 is a secreted inhibitor of the WNT pathway. SFRP-1 shares a binding domain for the Frizzled pathway and therefore is able to function as a competitive inhibitor of the WNT pathway. SFRPs function as secreted ligands and are able to bind WNTs at an extracellular level and hence modulate their activity. The SFRPs are therefore capable of down regulating the activity of the WNT pathway. Modulation of the WNT/β-catenin pathway has been implicated in the pathogenesis of osteoarthritis[149].

The WNT pathway functions to promote bone formation and mineralisation, therefore one might expect a reduction in an inhibitor to equate to increased function of the pathway. Other WNT inhibitors have been shown to promote bone resorption when levels are elevated[156]. Our findings would be consistent with this role, and although SFRP-1 has not been previously implicated in periprosthetic osteolysis. These findings taken in conjunction with those for FRZB (SFRP-3) provide strong evidence that the WNT pathway is strongly implicated in periprosthetic osteolysis.

Toll Like Receptors (TLR 3 & TLR 5)

Toll like receptors (TLR) are a group of trans-membrane receptor proteins which all act as a co-activation factors in a range of immune responses. TLR receptors are co-signalling receptor molecules usually activated in response to stimulation with microbial molecules (such as bacterial lipopolysaccharides) they modulate a broad range of intracellular inflammatory pathways (Fig 3.3.3.24).

There are 11 Toll like receptors, and the 5 most commonly studied were included, as although their role is not fully delineated there is accumulating evidence that they are associated with aseptic loosening[144-146]. Both TLR 3 and TLR 5 were shown to be highly associated with failure (AUC 0.72 and 0.78 respectively), where the others were not. The strength of association for both TLR 3 and TLR 5 was not statistically significant.

TLR-3 expression has been shown in inflammatory arthritidies to be associated with activation of the RANK-L pathway, and taken in association with the data for OPG also presented in this study it seems likely that TLR 3 activation is associated with macrophage activation and aseptic loosening[147].

The association of TLR 5 with aseptic loosening is a new finding. All of the TLR receptors function to activate subtly different pathways, although there is some overlap (Fig 3.3.3.25). TLR 3 is particularly associated with increased T cell stimulation through up regulation of CD40, CD80 and CD86, where TLR 5 activation lacks this function. TLR5 is thought to be stimulated by bacterial cell components and results in up regulation of cytokine expression (Fig 3.3.3.25). The finding that TLR 5 is up regulated in response to periprosthetic loosening warrents further study, as this may represent the pathway through which cytokine levels are increased in response to osteolytic change.

Protein 53 (p53)

The ROC analysis demonstrates an association between high levels of expression of p53 (AUC=0.70) and osteolytic hips. Interestingly both loose and well fixed group's expression is a subset of primary expression, which may indicate that p53 expression levels represent a tendency to loosen, not as a result of loosening.

p53 is a key signal protein situated at the crossroads of a network of signalling pathways that are essential for cell growth regulation[254-256]. In normal unstressed cells, the level of p53 protein is down regulated via the binding of proteins (eg MDM2, COP1 or PIRH2) that promote p53 degradation via the ubiquitin/proteasome pathway. As most of these genes are up regulated by p53, this leads to a regulation loop that will keep p53 levels very low in normal cells.

A large number of regulatory proteins are known to bind various regions of p53 in order to its activity. Regardless of the type of stress, the final outcome of p53 activation is either cell cycle arrest and DNA repair or apoptosis, but the mechanism leading to the choice between these fates has not yet been elucidated. P53 has been shown to be associated with apoptosis in osteolytic hip replacements[186;257].

This study demonstrates for the first time that p53 is likely to be significantly involved in the process of osteolysis, and that endogenous expression levels of p53 may be linked to a tendency towards aseptic loosening.

Insulin-like Growth Factor 1 (IGF 1)

IGF 1 has not been previously implicated in periprosthetic osteolysis. Growth factors are known to modulate osteoblast formation, and have been implicated in the age related changes which occur in bone marrow. IGF-1 has also been shown to have a modulatory function on the activity of osteoblastic function promoting collagen deposition[79].

IGF 1 was found to have a high association with aseptic loosening (AUC=0.71), although the ROC curve did not differ significantly from random chance (p=0.10). IGF-1 levels were suppressed in both the osteolytic and well fixed group when compared to the primaries, but where lower in the well fixed group.

This novel finding that IGF 1 is not only involved in the process of aseptic loosening, but may also be predictive of failure warrants further investigation. The role of IGF 1 in promoting bone turnover through osteoblastic function has already been established, and the findings of this study warrant further investigation.

Cathepsin K (CTSK)

Cathepsin K (CTSK) is a protease which plays a central role in osteoclast mediated bone resorption (Fig 3.3.3.26) and is also highly expressed by chondrocytes. CTSK function has been previously strongly associated with bone metastasis and osteolysis[183;184;258] and osteoclastic differentiation[184]. Activation of macrophages has been shown in the presence of the Cathepsins to facilitate osteolysis by activating the macrophage population themselves[259].

CTSK levels are higher in the osteolytic group, and there was a strong association between CTSK levels and loosening of the prosthesis (AUC=0.74 p=0.072). Interesting the ROC analysis demonstrated a sharp "tick" suggesting a tight threshold. Sharp threshold values have the potential to provide reliable predictive information, and in light of this finding CTSK may be a potential reliable biological marker for periprosthetic osteolysis.

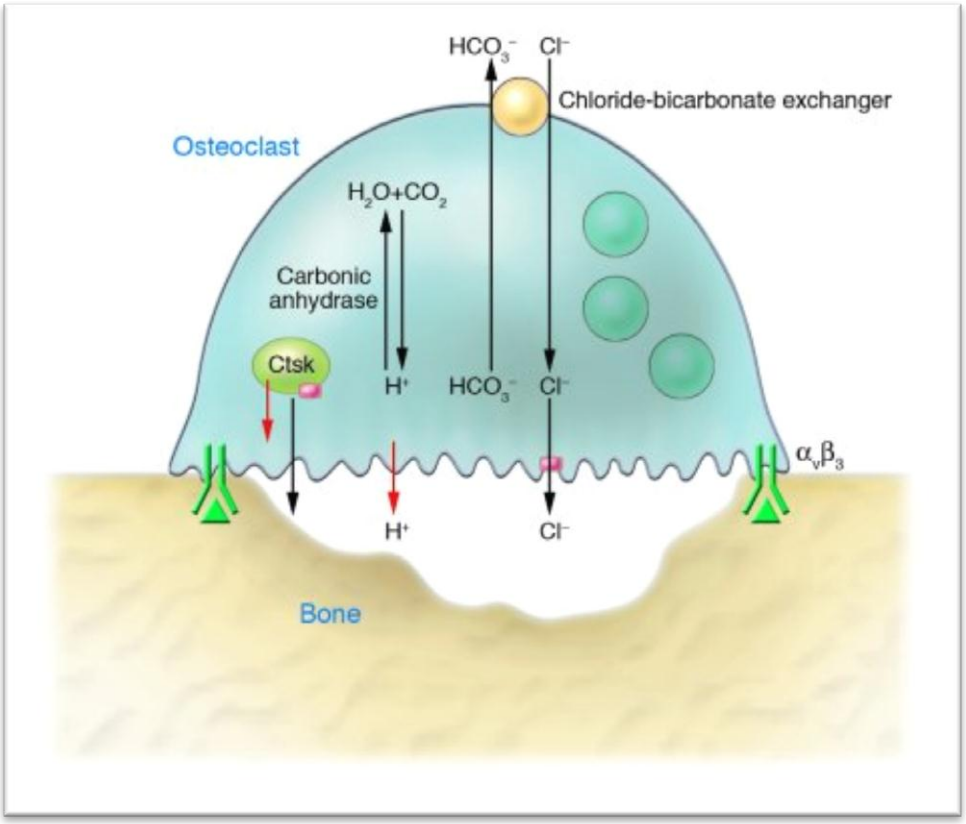



Figure 3.3.3.26 : CTSK mediated mechanism of osteoclastic bone resorption. Reproduced from J. Clin. Invest. 116:5 "Nothing but skin and bone"

Chitinase 1 (CHIT 1)

Chitotriosidase is an enzyme secreted by activated macrophages that is able to catalyze the hydrolysis of both chitin and chitin-like substrates. Chitotriosidase are responsible for defense against of chitin-containing pathogens such as fungi, nematodes, and insects. Chitotriosidase have been demonstrated to be elevated in the serum of patients with certain granulomatous conditions[260]. There have been no studies evaluating the role of CHIT-1 as a marker of periprosthetic osteolysis, despite its implication in other granulomatous conditions.

Although the ROC analysis did not find CHIT-1 to be a statistically significant predictor of outcome (p=0.12) there was a significant association (AUC=0.70). This in itself is an interesting finding as CHIT-1 is associated with alternative macrophage activation, a pathway in which there has been some significant interest in the recent osteolysis literature.

CCL 18

CCL-18 was found to be strongly predictive of a loose prosthesis (AUC=0.71, p=0.11) Tissue mRNA expression was significantly raised in the osteolytic group, with no change in the well fixed group when compared to the primary group. Given the ROC results demonstrating that CCL-18 may differentiate between a well fixed and loose prosthesis, and significantly raised median expression when compared to the primary group there it is extremely likely that CCL-18 is not only involved in the process of osteolysis, but may be used a marker for a loose joint replacement.

Macrophages are responsible for the initiation and maintenance of the inflammatory process. Macrophage "switching" is the process by which macrophages are turned off, and then function to inhibit and resolve inflammation. CCL-18 has been implicated in macrophage switching[171]. If as these results suggest macrophage switching occurs in osteolytic tissues it may be central to suppression of the osteolytic cascade.

# SECTION 3 : CONCLUSIONS

These results support and extend the current state of knowledge surrounding pathways involved in osteolysis. The design of the study with a native joint group for comparison with a well fixed and osteolytic study groups allows for speculation not only about which genes are responsible for the response to wear debris, but specifically those that are likely to be associated strongly with osteolysis.

It is unusual to find statistical significance between groups in biological studies, especially in those in which corrections for multiple testing have been made. The finding that seven genes were statistically significantly predictive of osteolytic change is an important finding and contributes to the current body of evidence surrounding this topic.

Genes working in similar ways and likely to be co-regulated (e.g. SFRP-1 and SFRP-3) have been found to have similar changes in expression patterns between the osteolytic and well fixed cohorts of patients, this adds to the face validity of the results, and makes the observations less likely to have occurred through 'random chance'.

The additional finding that a further nine genes were predictive of osteolytic change but did not reach significance levels is encouraging that with further study and larger groups of patients these genes are likely to reach significance levels.

It is not possible on the basis of these results to make a clear assertion if the expression of a particular gene makes osteolytic change more likely, or if it is the osteolytic change that causes a modulation in gene expression rates. This question is like the 'chicken and the egg' and difficult to prove either way. The finding that for some genes a subset of expression in the 'primary' patients is seen in either the 'well fixed' or 'osteolytic' groups makes it likely that regulation of gene expression causes osteolysis and not the other way around.

Without a long term follow up study from primary arthroplasty through to failure it is impossible to say conclusively that a variation in a particular gene expression is associated with risk of loosening. The data presented here however does imply, although not prove, that variation in gene expression is associated with osteolytic change.

# SECTION 4 : RISK FACTORS FOR OSTEOLYSIS

## 4.1 Study design

A prospective longitudinal cohort study was undertaken to establish patient related risk factors for osteolysis. The study was conducted using a previously identified and reported[29] cohort of patients. All patients were operated on at the Norfolk and Norwich Hospital in 1995 and received a cemented THR. The series consisted of 234 consecutive, non-selected primary total hip arthroplasties, performed in the calendar year 1995 on 217 patients at the Norfolk & Norwich University Hospital. Patients who had bilateral hip replacements either at the same operation or as another procedure in the same year were included.

All the patients received a cemented Elite Plus THR (De Puy Ltd, Leeds, UK) with a 28 mm Ortron 90 head (De Puy Ltd, Leeds, UK). The patients were recruited at the time of surgery and prospectively followed up in a post-operative clinical and radiological surveillance programme initiated at the time of surgery. This consisted of regular clinical and radiographic review. Final review took place at a minimum of 12 years (range 144 to 156 months) after surgery.

Following approval by the local Research Ethics Committee and the Research Governance Committee at the Norfolk and Norwich University Hospital patients were written to inviting them to take part in the study. Prior to the mailing of the letter the hospital information system and the National Tracing Service were used to confirm the patient was alive and their current address as many had relocated since their initial surgery. Patients were supplied with a patient information leaflet, approved consent form and a contact number to address any concerns.

The patients were identified from records held as part of the Joint Review Programme. This information is stored on a database, Medlog®, and contains patient demographic information, operative details and complete subsequent follow up data including details of radiograph and radiographic changes. Patients included were cross referenced with theatre log books to ensure all patients met the inclusion criteria. Patients were excluded if any of the following factors that may affect outcome were identified in any of their records:

- Revision surgery
- Prosthesis not Elite Plus
- Bone grafting required at primary THR

Data were gathered from patients notes, operative records and the joint review programme database. If data was incomplete the patients were contacted and asked to supply any missing records. Data collected for the purpose of this study included :

*Surgical Teams*

- Grade of primary Surgeon
- Surgical Approach
- Cement type
- Complications

*Demographics*

- Age
- Sex
- Diagnosis

*Co-morbidities*

- Smoking
- BMI
- Medications (NSAIDS)
- Charleson co-morbidity score

Data was anonymised by study number and entered onto an Excel (Microsoft Ltd, Richmond VA) database.

As part of their routine follow up patients were offered clinical and radiographic review at 12 years which coincided with the timings of this study. The review was undertaken by the hospital's independent arthroplasty review service. The review consisted of standardised AP and lateral hip radiographs, a 12 point Oxford Hip Score, and clinical examination. Review was undertaken jointly with an arthroplasty care practitioner. Patients in whom concerns about radiographic loosening or clinical concerns were raised were referred back to their operating consultant or their immediate replacement for a decision regarding revision surgery.

This study was undertaken after the 12 year review process was completed. For the purposes of this study the radiographs of all patients were reviewed by two independent observers using a PACS workstation (Centricity, GE Healthcare, Amersham, UK). The radiographs were assessed by two independent observers according to the criteria of Johnston *et al*[261]. Radiographs were reported in conjunction with post-operative and mid-term (mean 6.4 year) radiographs for the same patient. Agreement between both observers was necessary for data to be included and, when disagreement occurred, a consensus view was obtained with a third observer. A validation exercise was performed to determine intra-observer and inter-observer reliability of the data included.

The immediate post operative films were reviewed to establish any evidence of poor operative technique. Cement mantels were graded on the AP projection according to the criteria of Barak[30], stem varus/valgus alignment and leakage of cement below the restrictor was also assessed.

The radiographs were classified as well fixed, possibly loose or definitely loose according to recognised previously published criteria. Loosening of the femoral stem was defined according to the criteria of Harris[33]. The refined criteria of Kobayashi[34] for loosening of the femoral stem were separately analysed. Loosening of the acetabular component was defined according to the criteria of Hodgkinson[32]. The survival of the prosthesis was calculated using these radiographic definitions and separately for revision as an end point.

## 4.2 Statistical analysis

Statistical analysis was performed using GraphPad Prism V5.0 (GraphPad Software, La Jolla, CA) and SPSS v 16 (SPSS Software, Chicago, Illinois). Fisher's exact test was used to ascertain significance levels for categorical data and a two tailed Student's T-test for continuous data. Survivorship analysis was calculated using the Kaplan-Meier method, with Greenwoods method for calculation of confidence intervals. Survival curves were plotted and also tested for difference using the Mantel-Cox (log rank) test. A type 1 error level of 5% ($p<0.05$) was defined as significant in all cases.

Mean survival and significance levels were calculated for all variables included in the study and survivorship curves plotted for each. Charlson co-morbidity scores[210;211] were calculated both as raw and age adjusted values. These were used as a measure of overall medical co-morbidity.

## 4.3 Results

Of the 217 patients (234 THRs), 83 had died before our final review and nine were either lost to follow-up or no radiographs were available. Nineteen patients had been revised leaving 142 surviving hips in 133 patients available at the time of final follow up.

Mean age at surgery 69.3 years (range 24-93 years), bilateral procedures at separate operations were performed on 30 patients. One hundred and thirty two hips were replaced on the right and 82 on the left. The preoperative diagnosis included osteoarthritis 200 (90%), rheumatoid arthritis 16 (7%), avascular necrosis and sequelae of childhood disorders seven (3%). Complications in the immediate postoperative period included two (1%) dislocation, superficial wound infection two (1%) thrombosis seven (4%), proven infection one (0.5%), medical complications nine (5%).

### 4.3.1 Surgical Demographics

One hundred and thirty seven operations were performed by consultants and 97 by specialist registrars. All but three procedures had been undertaken through a modified anterolateral approach. Standardised second generation cementing technique was employed by all surgical teams.

A retrospective review of the immediate post-operative radiographs confirmed that 99.4% of THRs had a grading of A or B according to the criteria of Barrack, Mulroy and Harris[30] and 0.6% of C or D. The position of

the femoral stem was neutral in 111 (69.8%) of THRs, while 30 (18.9%) of stems were in > 4° of varus and 18 (11.3%) in > 4° of valgus.

4.3.2 Revision

Of the 234 arthroplasties, nineteen (8%) required revision during the 12 year follow up period (Table 4.3.1). One patient was revised for early deep infection and the remaining 18 for aseptic loosening. Kaplan-Meier survivorship analysis gives a 93.9% (CI 89.2 - 96.5%) survival at 10 years, and 88.0% survival at final follow up for all causes of revision (Fig 4.3.1).

A subset analysis excluding all patients with low viscosity cement, poor cement mantle or poor component position gives a 93.3% (CI 88.1 - 96.2%) survival at 10 years. There was no statistically significant difference between this subset and the whole group analysis.

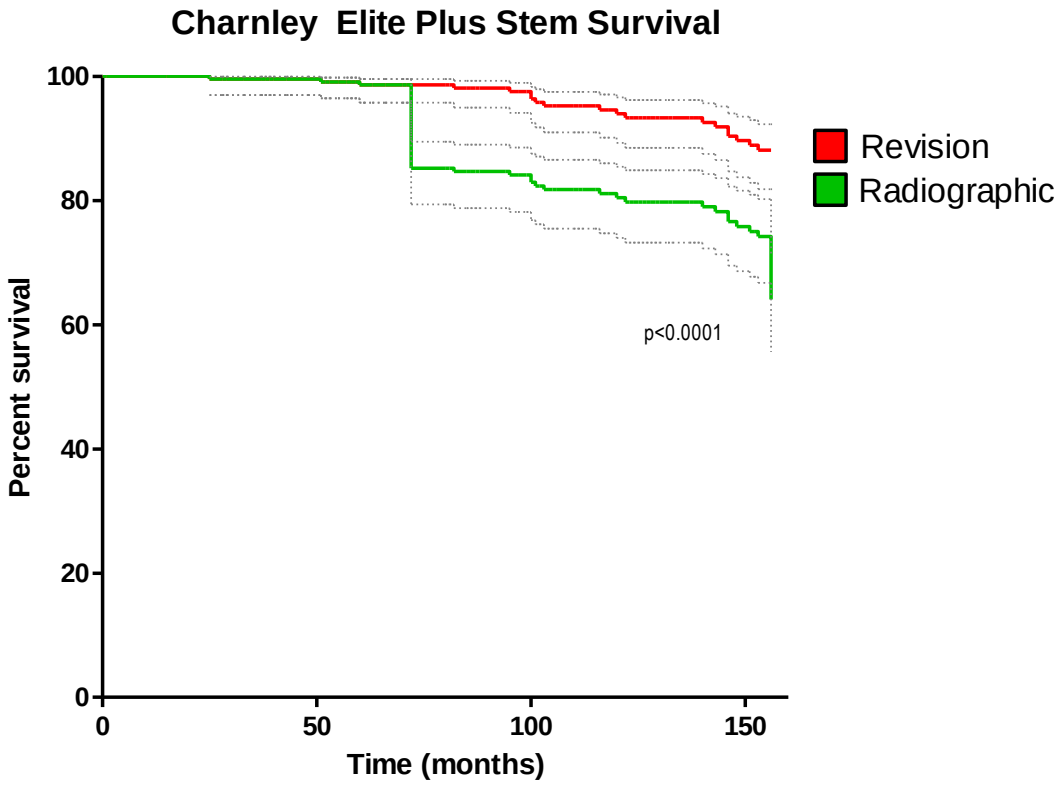



Figure 4.3.1 : Survival analysis at 12 years revision and radiographic failure

| Revisions | n = 19 |
|---|---|
| **Gender** | |
| Male | 7 |
| Female | 12 |
| **Side** | |
| Right | 12 |
| Left | 7 |
| **Diagnosis** | |
| OA | 18 |
| Post traumatic OA | 1 |
| **Reason for revision** | |
| Aseptic loosening | 18 |
| Infection | 1 |

Table 4.3.1 : Demographic details for revision patients

4.3.3 Radiographic Failure

Complete radiographic analysis was possible in 142 arthroplasties at 12 years, and in a further 19 who had previously undergone revision. According to Harris, definite evidence of femoral loosening was present in 28.8% of femora, and according to Hodgkinson, 46.5% of acetabulae were definitely loose. Fifty eight percent of patients had definite loosening of at least one component. At the time of final review 19 patients had undergone revision giving a combined failure rate of 63% (102 of 161) (Table 4.3.2). The validation exercise demonstrated the intra-observer reliability was excellent ($\kappa$=0.94) and inter-observer reliability was good ($\kappa$=0.89)

| **Component** | **Criteria** | **6 Year results (95% CI)** | **12 Year result (95% CI)** |
|---|---|---|---|
| Femoral | Harris[33] | 16.4% | 26.8% (n=38 of 142) (19.4% to 34.1%) |
| | Kobayashi[34] | 18.2% | 28.8% (n=41 of 142) (21.3% to 36.4%) |
| Acetabular | Hodgkinson[32] | 11.9% | 46.5% (n=66 of 142) (38.1% to 54.8%) |
| Either | Harris[33] and/or Hodgkinson[32] | 27.0% | 57.7% (n=82 of 142) (49.5% to 66.0%) |
| Either | Revision | 5.1% | 11.8% (19 of 161) (6.8% to 16.8%) |
| Either | Revision or Loosening | 31.0% | 63% (102 of 161) (56% to 71%) |

Table 4.3.2. Failure rate at 12 years follow up.

4.3.4 Patient Demographic Data

Of the surviving 161 arthroplasties with known outcome at final follow up complete data for all fields was available in 87 patients. Complete data bar smoking and NSAID use was available in all 161 patients.

Twenty percent of patients were smokers and continued to do so from the time of surgery and 67% of patients used NSAIDs. Obesity (BMI>30) was diagnosed in 16% of patients at their pre-operative assessment, and the mean Charlson score was 1.04 preoperatively across the whole cohort.

There were no statistically significant differences between any pre-operative co-morbid factor and post-operative outcome. Age at the time of surgery was found to be statistically significantly different in the failure and survival group (Table 4.3.3). There was no statistically significant link found between mean pre-operative Charlson scores and survival either using the raw or age adjusted scores (Table 4.3.4).

| | **Loose (%)** | **Not loose (%)** | **p value** |
|---|---|---|---|
| **BMI <30** | 51 (25.4) | 150 (74.6) | |
| **BMI>30** | 8 (32.0) | 17 (68.0) | p = 0.4768 |
| **Charnley Grade** | | | |
| **A** | 27(48.2) | 73 (43.7) | |
| **B** | 22 (39.3) | 70 (42.0) | |
| **C** | 7 (12.5) | 24 (14.4) | p = 0.8330 |

Table 4.3.3 Variables with potential relations to survival

| | **Mean** | **p value** |
|---|---|---|
| **Charlson index** | | |
| Loose n = 51 | 0.96 (SD0.7476) | |
| Not loose n = 129 | 1.08 (SD 0.8263} | p = 0.4134 |
| **Charlson age adjusted** | | |
| Loose n = 51 | 2.51 (SD1.8052) | |
| Not loose n = 129 | 2.96 (SD 1.9144) | p = 0.2062 |

Table 4.3.4 Charlson co-morbidity scores divided by patient groups

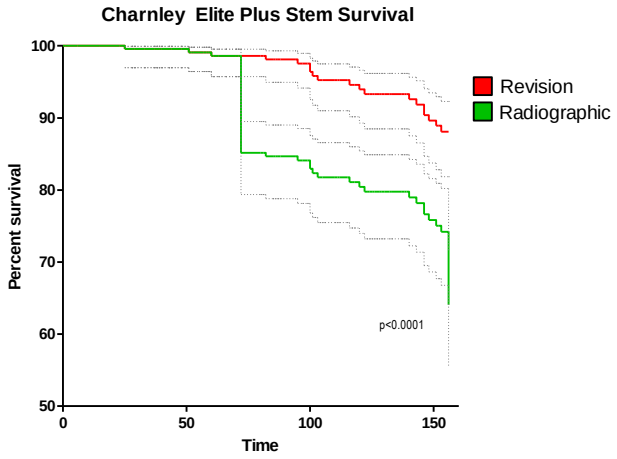
**4.3.5 Survivorship Analysis**

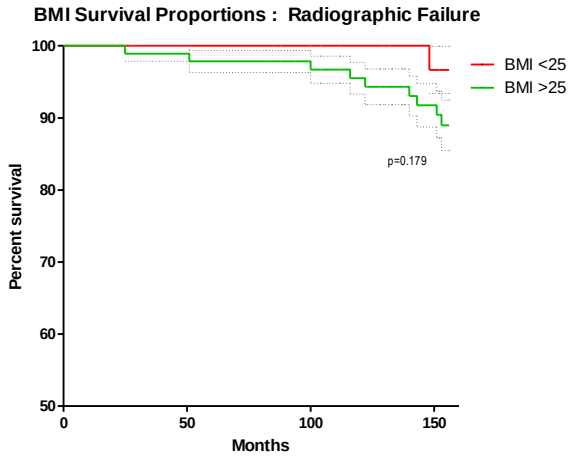
Kaplan-Meier survival analysis for the complete patient cohort is given in Figures 4.3.2 & 4.3.3. Survivorship is plotted with 95% CI. The two curves were tested for significant difference (Mantel-Cox test) and the p value is given. There was no significant difference between survivorship found when patients were stratified by their smoking status or use of NSAIDs.

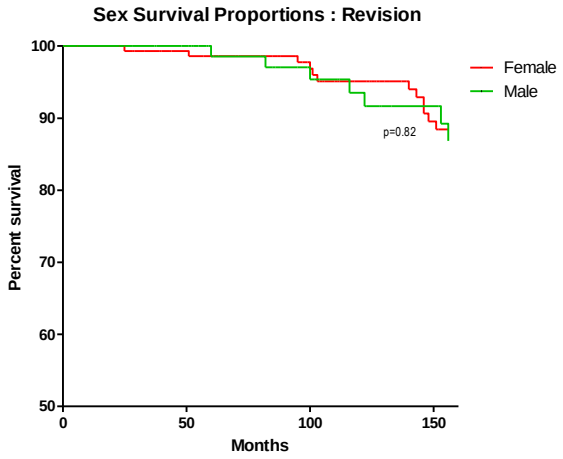
There were no significant differences between survivorship when patients were stratified by gender or initial diagnosis. However although not significant a higher failure rate was observed at ten years in patients with a high BMI (p=0.179). It is certainly possible that this trend towards significance will become a significant difference at further follow up.

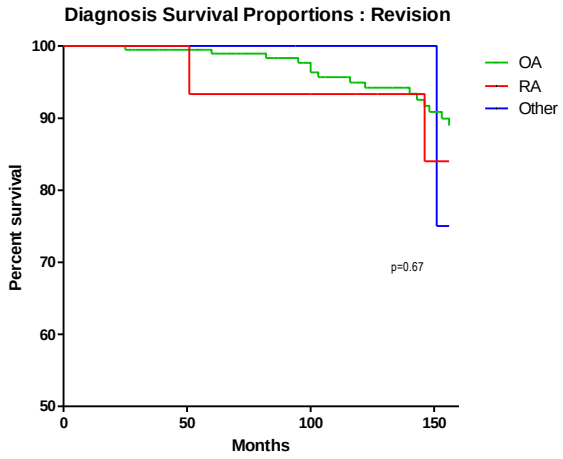
There was a significant difference (p<0.032) when patients were stratified into three age groups. Interestingly there were no differences observed between the 65-70 group and the 75+ group. This may be due to the small size of the 75+ group at final analysis.

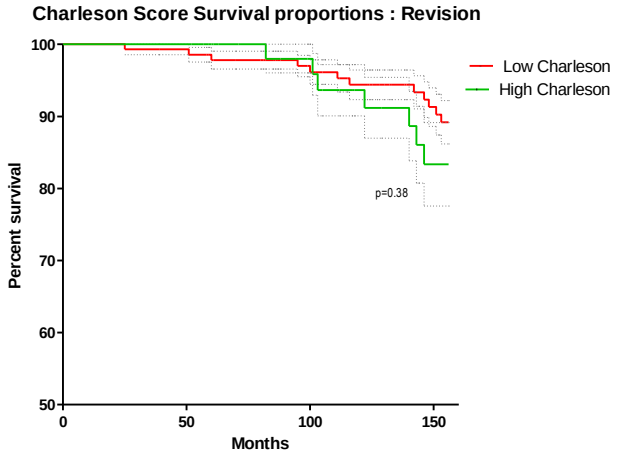
There were statistically significant differences between patients with a low Charlson score and high Charlson score (p=0.012) when radiographic loosening was taken as an end point. Although this trend was maintained when revision was taken as an end point the differences were no longer significant (p=0.38). This is likely explained by the use of revision as an outcome. Although patients with a high Charlson score appear to demonstrate evidence of radiographic loosening at an earlier stage the decision to perform a revision arthroplasty is not just a radiographic one. Patients with high Charlson co-morbidity scores have by definition higher risk associated with revision surgery and it may not therefore be in that patient's interest to undergo revision surgery.



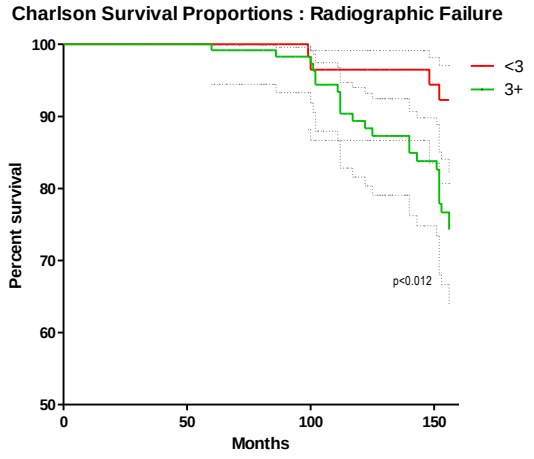




Figure 4.3.2 : Survival analysis for a) whole cohort and stratified by b) BMI c) Gender d) Diagnosis e) Charleson Score f) Charleson Score (radiographic failure)

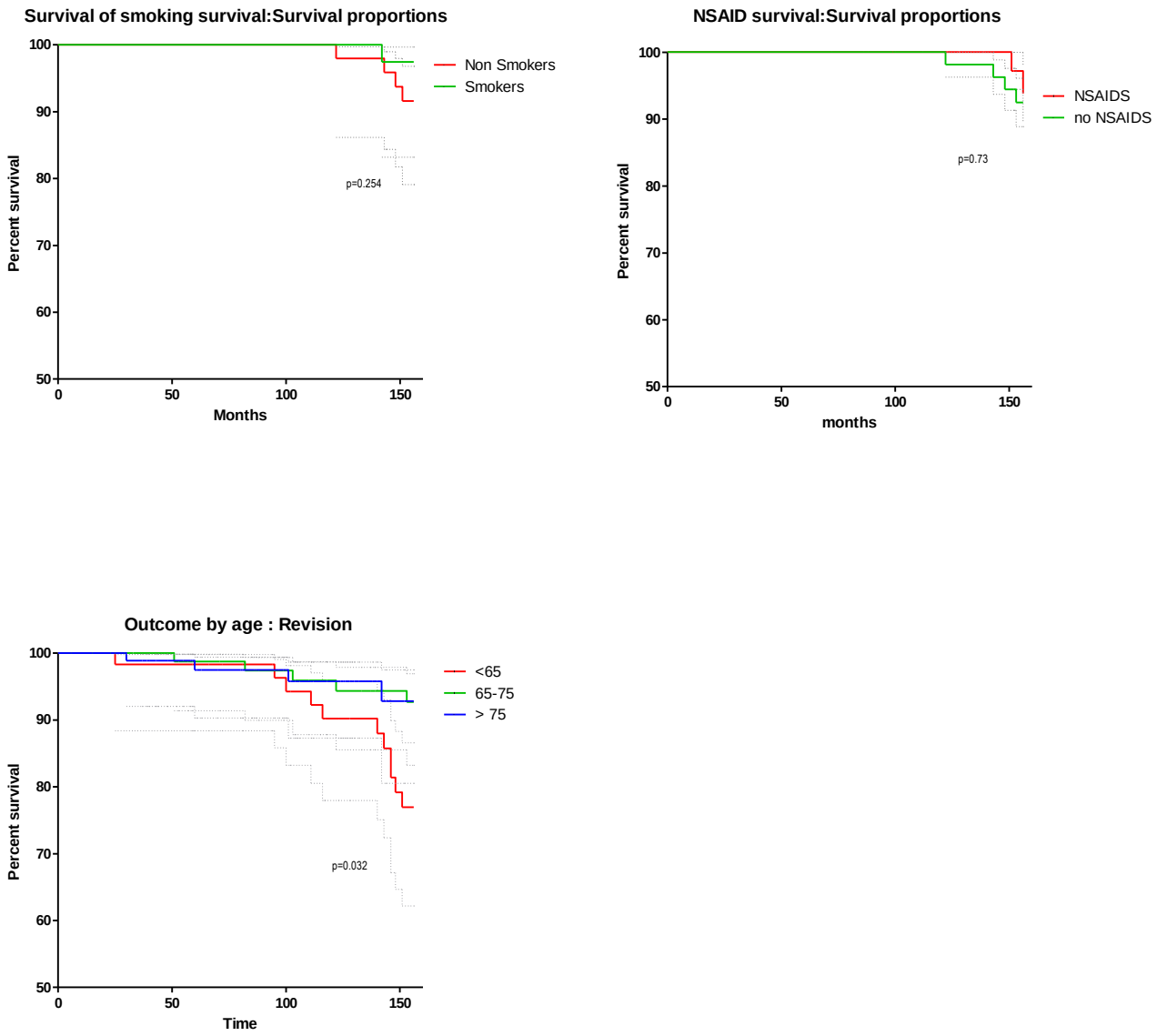



Figure 4.3.3 : Kaplan-Meier Survival analysis for stratified by a) Smoking status b) NSAID c) Age

## 4.4 Discussion

Patient related risk factors for failure of a total joint replacement are well studied. Although revision is a firm end point the number of patients who are revised is relatively low, and given the patient population age a number of patients will be deceased before their joint replacement fails, making determining the risk factors a tricky task. In addition to this the decision to revise a joint replacement is not based purely on the stability of the stem, but also patient wishes, co-morbidities and general health. As such revision is a coarse but firm end point.

Inclusion of radiographic definitions of loosening allows for a more sensitive definition of failure that is not subject to other patient factors such as fitness for surgery, however it does introduce some potential observer bias. Where appropriate both definitions of failure have been evaluated.

### 4.4.1 Co-morbidities

The Charleson co-morbidity score has been shown to be predictive of post operative radiographic survival. Patients with a high Charleson score (>3) have been shown to have a poorer survival at a ten year follow up with an outcome of revision, however the confidence intervals overlap (Fig 4.3.2) and Mantel-Cox testing did not show any significant differences.

When definite radiographic loosening or revision is taken as the end point then a statistically significant difference is seen between the survival curves. Patients with significant co-morbidities (Charlson scores >3) had a statistically significantly higher rate of radiographic failure in their THRs. This is a slightly surprising finding. One would expect the fitter patients to be more active and as such to have higher demands, increasing the likelihood of wear debris related failure. However the contrary appears to be true. All of the prostheses implanted were cemented Elite Plus prosthesis, so there can be no selection bias for prosthesis type, and observed failure rates are unlikely to be explained by any other factor. The patients with poorer co-morbidity scores tended to be older (although there was no significant difference), and as a young age is associated with failure this too is unlikely to explain the findings.

It is possible that the difference can be explained by changes to the bone itself. It has been established that as bone ages its morphology changes[262] and bone remodels with stress. Patients with high co-morbidity scores are likely to have had lower functional demands at the time of the surgery, putting them at risk of disuse osteoporosis[263-265].

The differences between the curves for radiographically defined failure and those hips that have been revised are also likely also due to the co-morbidity effects. Patients with a higher co-morbidity score are less likely to be suitable for revision surgery due to their co-morbidities. This likely explains the observed difference between patients potentially requiring revision and the numbers of revision procedures performed.

4.4.2 Smoking

An attempt to explore the possible relationship between cigarette smoking and hip implant survival was has been made. Of the study group 40 hips (17.8%) of the 226 hips were smokers. At the review 13 hips of the smoking group had been revised, however this difference was not statistically significant p =0.2838. Similarly the Kaplan-Meier survivorship analysis (Figure 4.3.3) does not demonstrate poorer survival outcomes in patients who smoke. This study supports previous work that smoking does not affect the longevity of cemented joint replacements[196].

4.4.3 BMI

The effect of BMI on the short and long-term outcome of THR is controversial in the literature. Whilst on the one hand the increased joint reaction forces caused by excessive load would be expected to result in increased abraded particles of polyethylene the lower functional demands, hence reduced number of wear cycles placed on the prosthesis by the more sedentary patient may more than counter this effect.

A recent publication by McLaughlin *et al*[212] concluded that obese patients had no worse clinical radiographic outcomes nor did they have any increased complications following THR in comparison to a group of normal weight patients. Previous authors have shown that operating on morbidly obese patients (BMI >40) has an adverse effect on the outcome of joint replacement surgery[218].

The effects of obesity on joint replacement longevity were not found to be significant in this study. There was a higher failure rate observed in the overweight and obese cohort when compared to the normal weight patients (90% vs 97% survivorship), but this difference was not found to be significant. Recently there have been efforts to limit the option of joint replacement to the obese patient. The benefit to the obese patient of joint replacement is the same as to the non-obese patient[218]. There is no evidence for this rationing, and whilst anaesthetic risk may be increased we have demonstrated that in this series there is no statistically significant effect on longevity associated with total joint arthroplasty in the obese.

4.4.4 Age

The effect of age on total joint replacement is well studied. The increased activity levels associated with a younger group of patients who have undergone implantation of traditional metal-on-polyethylene components has resulted in a much higher observed failure rates in young patients[1;36;206].
The reproduction of this finding in a reliable manner in this cohort adds weight to the strength of the data. Not only was there a statistically significant difference between mean age in the failure and surviving cohorts but failure rate was consistent higher in the young cohort throughout the period of the study in keeping with the observations of population based studies.

4.4.5 Non steroidal anti-inflammatory Drugs (NSAID) Use

The use of NSAID's is widely accepted for the treatment of pain in osteoarthritis, however NSAIDS are known to interfere with bone metabolism. Whilst NSAIDs do have different mechanisms of action all commonly used NSAIDs have been found to have similar effects on bone. NSAIDs have been shown to inhibit fracture healing[266-268], and COX-2 has been implicated in osteolysis[268;269]. Given this evidence, and coupled with some in vivo evidence that NSAIDs may inhibit important osteolytic pathways one might expect patients who are known to use NSAIDs to have a lower failure rate.

There was no statistically significant difference between the rate of NSAID use in the surviving and failure groups (p=0.2233). One quarter of all patients in the study had taken NSAID's at the time of operation, however it was not recorded if patients at their final review were continuing to take NSAID's, or if they had taken them throughout the intervening years. It would be important to have access to this data to determine any relationship with aseptic loosening.

Despite these limitations, and the lack of statistically significant difference there was a lower survivorship in the group not taking NSAIDs supporting, but not proving previous basic science studies and laboratory findings outlined elsewhere in this thesis.

## 4.5 Conclusion

The failure of THRs are predictable to certain extent from pre-operatively observed patient factors. We have demonstrated that in long term follow-up patients who are under 65 years of age and have a co-morbidity score >3 are at significantly higher risk of failure of their joint replacement. Overweight patients and those who smoke should be followed up more carefully as although not significant there was a higher failure rate in these groups. We have confirmed that there is no predisposition to failure of a joint replacement based on the patients age, sex, preoperative diagnosis or smoking status.

The follow-up patterns for total joint arthroplasty should be determined to a certain extent based round patient factors. An awareness of those factors likely to make a patient more at risk of failure will improve pre-operative decision making, providing an informed consent and will aid in designing post operative follow up protocols.

# SECTION 5 : RADIOGRAPHIC DETECTION OF OSTEOLYSIS

## 5.1 Background

Radiographic assessment of osteolysis and radiolucent lines is an essential part of the routine follow-up of total joint arthroplasty. Although highly specific, anteroposterior (AP) radiographs have been shown to be only 38%-41% sensitive for osteolysis [226;270]. In some centres it is therefore established practice to take an additional lateral radiograph to both improve sensitivity and for evaluation of prosthesis position. Both computer simulations [226] and cadaveric bone studies [270] have demonstrated that the Judet iliac oblique (IO) views may be more sensitive than the lateral views in detecting radiolucent lines. Despite these findings there are no clinical studies evaluating the use of differing radiographic views in the routine surveillance of total hip arthroplasty.

The aim of this part of the study was to assess the sensitivity of the iliac oblique, anteroposterior, and the lateral hip radiograph in detecting radiolucent lines in a cohort of patients undergoing 10 year follow up of their total joint arthroplasty (THR). Individual radiographs and possible combinations will be assessed against the "gold standard" of all three views to determine the most effective way to diagnose osteolysis on plain films.

## 5.2 Materials & Methods

The study was granted Ethics and Research Governance approval. The patient cohort had all undergone a cemented Charnley Elite Plus THR with a 28 mm Ortron 90 head and a cemented matched polyethylene cup in our institution in 1995. The patients were prospectively followed up in the local arthroplasty surveillance programme, and had had standardised AP and lateral films taken postoperatively, at 5 years and were then undergoing their 10-year follow-up. At their 10 year follow-up, in addition to their routine AP and lateral radiographs, a modified Judet iliac-oblique view was performed.

The radiographs were all taken in a standardised manner. The AP pelvic films (Fig 5.1) were taken with the patients positioned supine; the anterior superior iliac spines were equidistant from the table top, the femora internally rotated and the heels separated. The film and beam were centred 2.5 cm above the symphysis pubis with a focal film distance of 115 cm and the beam collimated to include the proximal femora. A standardised exposure of 81kVp and 16mAs was used. The lateral films (Fig 5.2) were taken with a horizontal beam. The patients were placed supine with their feet vertical. The cassette was positioned parallel to the femur and the film centred midway between the femoral pulse and greater trochanter. A horizontal beam at 80kVp and 70mAs exposure was used. The IO films (Fig 3) were taken with the patient supine, centred on the hip joint. The patient was positioned with the trunk rotated 45º towards the affected joint with the hip and knee in 30º

of flexion. A small pillow was placed under the contralateral buttock. The film was collimated to include the iliac crest and proximal femur and exposed at 81kVp and 20mAs.

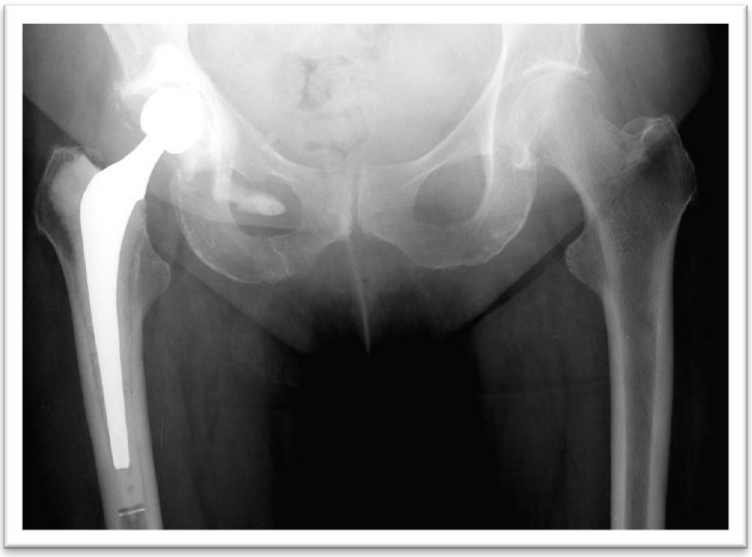


Figure 5.1 : Standardised AP Radiograph

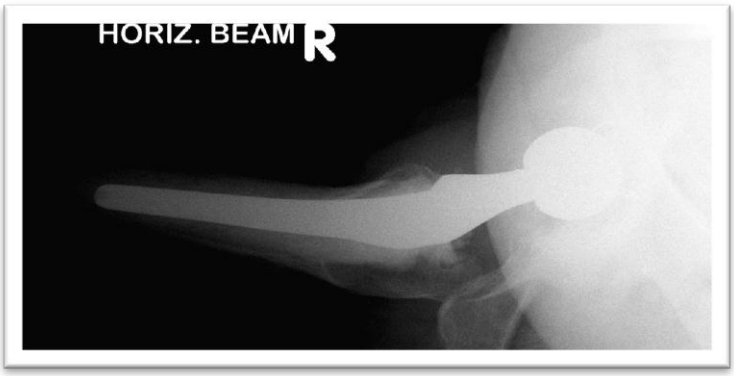



Figure 5.2 : Standardised Lateral Radiograph

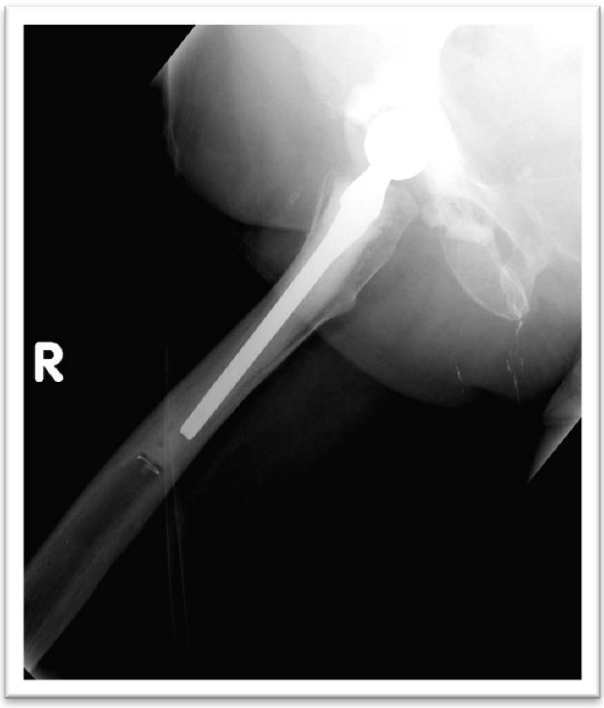



Figure 5.3 : Standardised Iliac Oblique Radiograph

All radiographs were assessed for signs of loosening separately by two independent observers according to the criteria of Johnston et al. Radiographs were reported in conjunction with post-operative and mid-term (mean 6.4 year) radiographs of the same view. Agreement between both observers was necessary for data to be included and, when disagreement occurred, a consensus view was obtained with a third observer. Each film was evaluated for evidence of radiographic loosening. The presence, location[46;271] and size of osteolytic lesions and radiolucent lines were recorded about both the acetabular and femoral components on each of three views. The sensitivity of each radiographic projection and each possible combination was then calculated with all three views in combination taken as a gold standard.

Radiographs of all patients in all three projections were available for review on the PACS system. Seven femoral and three acetabular zones were examined in the three radiographic projections. Ninety six patients were entered into the study giving 672 individual femoral zones in each projection, and 288 acetabular zones.

Statistical analysis was performed using GraphPad Prism (GraphPad Software Ltd, Chicago IL). Fisher's exact test was used for categorical data. A 'p' value of less than 0.05 was defined as significant in all cases. A validation exercise was performed with 10 radiographs and showed excellent inter-observer reliability ($\kappa$=0.94).

## 5.3 Results

Evaluation of all three views demonstrated radiographic evidence of radiolucent lines or osteolysis in one or more zones on any view in 97% of the patients. However only 2% fulfilled Harris' criteria for definite loosening[33].

Radiographic evidence of early loosening in either the femoral or acetabular component in a single zone or more was seen in 79% of AP, 18% of lateral and 87% of IO radiographs. The relative sensitivity of a single view showing evidence of loosening when compared to all three views was 0.81 for the AP, 0.18 for the lateral and 0.9 for the IO radiographs (Table 5.1). If two views were reported together the relative sensitivity (when compared to all three views) of an AP and lateral view was 0.81 compared to an AP and IO sensitivity of 1.0, this difference was statistically significant (p=0.03).

| | Views Taken | | | | | |
|---|---|---|---|---|---|---|
| | AP | Lateral | IO | 3 Views | AP & Lat | AP &IO |
| % Positive | 79% | 18% | 87% | 97% | 81% | 97% |
| Sensitivity | 0.81 | 0.18 | 0.90 | 1.00 | 0.83 | 1.00 |

Table 5.1: Sensitivity of each radiographic film and combination

### 5.3.1 Acetabular Components

The radiographic appearance of the acetabulae are summarised in Table 5.2. Radiolucent lines were visible in 43.4% (n=125/288) acetabular zones on the AP radiograph, 13.2% (n=38/288) on the lateral projection and 29.8% (n=86/288) on the IO The AP was significantly more sensitive than the other views ($p<0.0001$) and the IO significantly more sensitive than the lateral ($p<0.001$). Osteolytic lesions were seen in seven patients (7 zones) on the AP and lateral films, but in 16 patients (19 zones) on the IO films. The IO projection was therefore statistically more sensitive than the other views ($p=0.0018$).

| | Acetabulae (288 zones) | |
|---|---|---|
| Projection | Radiolucent line | Osteolysis |
| Antroposterior (AP) | 43.4% (n=125)* | 2.4% (n=7) |
| Lateral (Lat) | 13.2% (n=38) | 2.4% (n=7) |
| Iliac Oblique (IO) | 29.9% (n=86)# | 6.6% (n=19)£ |

Table 5.2 : Acetabular radiographic evidence of loosening detected on each projection. *Significantly better than any view $p<0.0001$. #Significantly better than lateral view $p<0.001$. £Significantly better than other views 0.0018

### 5.3.2 Femoral Components

The radiographic findings of the femora are shown in Table 5.3. The cement-bone and prosthesis-cement radiolucent lines were reported separately. Radiolucent lines were visible in 5.1% (n=34/672) of zones at the cement-bone and in 10.9% (73/672) of zones at the prosthesis-cement interface on the AP radiograph. The lateral film demonstrated radiolucent lines in 1.7% (n=12/672) of zones at both the cement-bone and prosthesis-cement interfaces. Evaluation of the IO films showed radiolucent lines at the cement-bone interface in 10.9% (n=73/672) of zones examined and 4.0% (n=27/672) at the prosthesis-cement interface. The IO was statistically significantly better than the other views for detecting radiolucent lines at the bone-cement interface ($p<0.001$) whilst the AP projection was significantly better at detection of prosthesis-cement radiolucencies ($p<0.001$).

| | Femoral (672 zones) | | |
|---|---|---|---|
| Projection | Radiolucent line | | Osteolysis |
| | Cement-bone | Prosthesis-cement | |
| Antroposterior (AP) | 5.1% (n=34) | 10.9% (n=73)# | 2.5% (n=17) |
| Lateral (Lat) | 1.8% (n=12) | 1.8% (n=12) | 1.0% (n=7) |
| Iliac Oblique (IO) | 10.9% (n=73)# | 4.0% (n=27) | 3.4% (n=23)* |

Table 5.3 : Femoral radiographic evidence of loosening detected on each projection. #Significantly better than lateral or IO $p<0.001$. * Significant difference compared to lateral $p=0.013$

Osteolytic lesions were seen in the femora of 17 patients on the AP, 7 on the lateral films and 23 of the IB films. The IB projections was statistically significantly better than the lateral film for detection of femoral osteolysis (p=0.013).

## 5.4 Discussion

Accurate radiographic assessment is a key part of effective long term arthroplasty follow up, not only as a diagnostic aid for asymptomatic loosening, but also to plan revision surgery. Despite widespread use of plain radiographs for THR follow-up, there is no accepted consensus as to which projections, and how many should be performed. When used in combination plain AP and lateral films are reported to be only 85%-89%[39;272] sensitive.

Whilst the rates of radiolucent lines and osteolysis in this study may appear high, the cohort of patients selected for this study has previously been reported to have a high rate of radiographic failure recognised at 6.4 years post THR and this has been confirmed at 12 years post THR[2]. To establish the effectiveness of plain films as a screening tool for radiographic failure it is essential to use a cohort of patients with a high rate of expected failure.

In this study use of a single AP radiograph demonstrated radiographic evidence of loosening in at least one zone in 79% of arthroplasties, as compared to 97% when all three views were utilised. The use of the IO film alone demonstrated radiographic change in 87% of arthroplasties, when compared to just 18% of lateral films. Addition of an IO film to an AP brings the positive rate to 97%, whilst combination of the AP and lateral demonstrated abnormalities in 81%. The addition of a lateral film to an AP projection does not significantly improve the sensitivity ($p>0.05$) of the AP alone. In this series the addition of the lateral film conveyed no additional information over a single AP film.

Assessment of the acetabulum for osteolytic defects demonstrated focal cavitatory lesions in 19 zones on the iliac oblique film, but only 7 on the AP or lateral projections. This data confirms in a clinical setting what previous studies with computer simulations[226] and simulated defects in a cadaveric pelvis[270] have demonstrated regarding the value of oblique films of the pelvis for detection of osteolytic lesions. Hodgkinson[32] has argued that acetabular radiolucent lines are predictive of acetabular component loosening. In this series radiolucent lines, although not osteolytic defects, around the acetabulum were seen significantly more commonly on the AP film. This finding is supported by a previous study Kawate & Yajima[227] examining radiographic assessment of cement mantle thickness in the acetabulum.

Adequate radiographic assessment of femoral component loosening requires assessment of radiolucent lines and osteolytic lesions[33;34]. The detection of these changes on plain AP radiographs is the commonly accepted practice[223]. In this study the AP view was significantly more sensitive for prosthesis-cement radiolucencies than

any other projection ($p<0.001$). The IO was the most sensitive view for cement-bone radiolucent lines ($p<0.001$). For the femoral component, the lateral view was statistically inferior to either the AP or IO views for all radiographic changes ($p>0.05$).

In this study, the lateral projection was the least sensitive view for both radiolucent lines and osteolysis in the femur and acetabulum. In evaluation of loosening the lateral view did not offer any additional information over the IO or AP projections alone. The lateral however does provide an orthogonal plane for assessment of implant position. Implant position has been associated with longevity of the hip replacement[273], and there may therefore be a role in a postoperative lateral film for prognostic value.

It is well established that addition of further imaging modalities can be used to increase the detection rate of early prosthetic loosening. Imaging modalities such as subtraction arthrography[272;274], nuclear arthrography[272;275] and bone scintigraphy[272;276] have all been used to increase the detection rate of early prosthetic loosening. Due to high dosage of radiation or the invasive nature of the procedure, these investigations are commonly used only when there is evidence of radiological change on plain x-ray.

Whilst it is a limitation to this study that we did not compare plain radiography with these other imaging modalities, we did not aim to establish the overall sensitivity of plain radiographs, rather to establish in the follow-up and screening setting the most effective combination of plain films to demonstrate radiographic evidence of loosening. There are no similar clinical studies evaluating this and the data presented demonstrates the combination of AP and IO films to be significantly more sensitive than the commonly used combination of AP and lateral films.

In plain radiographic surveillance of total hip arthroplasty substitution of the lateral film with a modified iliac oblique improves sensitivity for radiolucent lines and osteolysis. We would recommend this simple change to improve the sensitivity and therefore effectiveness of joint surveillance programmes for radiographic evidence of failure.

# SECTION 6 : PATIENT OUTCOME MEASURES AND FAILURE

## 6.1 Introduction

The long term follow up of post hip arthroplasty patients represents a significant challenge to all developed health care systems. Whilst the failure patterns of arthroplasty and the failure rates for individual prosthesis are well described by the joint registries it is important to identify individual patients with failing arthroplasties prior to catastrophic failure and fracture. Early identification of a failing arthroplasty allows for simpler revision surgery prior to catastrophic failure such as advanced osteolysis, peri-prosthetic fracture or component fracture.

In the early development of joint replacements it was appropriate for patients to be seen regularly by their operating surgeon however with an increasing proportion of the population having a joint replacement and ever increasing healthcare costs the follow up burden is immense. Increasingly NHS trusts are turning to 'joint review programmes' to provide clinical follow up without the health economic burden of unnecessary clinic visits and radiographs. Whilst for the majority of elderly patients it is unlikely that they will require revision surgery, in last year's British Joint Registry report revision surgery constituted 10% of all procedures performed. The most common mode of failure for total hip replacements reported on the registry was osteolysis, which is normally silent in the early stages.

Joint review programmes vary in their structure, but have a common theme of structured, often nurse led, review. The patients are not seen in the usual manner in clinic, but reviewed either by a joint review practitioner (normally a nurse or physiotherapist) with additional information in the form of radiographs and postal questionnaires. Patient outcome scores are becoming an important component of such review. In some centres Patient Reported Outcome Scores (PROMS) in conjunction with radiographs are being increasingly relied upon to guide the decision to review the patient clinically. In some centres a PROM score is being used as the sole outcome and surveillance measure.

Outcome scores in joint replacement vary in the structure, design, administration and validation. None of the commonly used outcome scores are validated for use as a surveillance tool for failing arthroplasties, and it is unclear if this is a valid use of measures designed for a different purpose.

The benefit of this approach has not been established and the sensitivity of these scores has yet to be determined for osteolytic change in total hip replacement. Whilst some outcome scores have been validated to be sensitive for post operative function following primary arthroplasty, no score has been validated as a long term surveillance tool, and it is unclear if any score is specific or sensitive enough detect failing hip replacements that potentially require revision. The aim is to establish the value of a variety of commonly used PROMs and other outcome measures in detecting early osteolysis without formal clinical patient review.

## 6.2 Materials & Methods

In order to establish the benefit of the 'Joint Review Programme' approach patients undergoing their 10 year post arthroplasty follow up were enrolled into this arm of the study. In addition to radiographic and clinical review patients completed one of a number of outcome questionnaires. Three cohorts of patients were evaluated, all being followed up with a different combination of questionnaires. The five questionnaires were the Harris Hip Score (HHS), Merle d'Aubigne Score (MDA), Oxford Hip Score (OHS), Hospital for Special Surgery Score (HSS) and a Visual Analogue Score for pain (VAS).

The patient cohorts were identified using the arthroplasty registers in two hospitals (The Norfolk and Norwich University Hospital, and the West Suffolk Hospital). Three cohorts of patients were identified who were already undergoing annual clinical, radiographic and patient reported scoring as part of their surveillance programme. These cohorts were identified as all patients had a similar cemented metal on polyethylene THR, and the follow-up protocol was identical in each case, bar the use of a different patient outcome score. These were non-selected cohorts of patients, and the whole cohort was included in each case, although each was operated on by a different surgical team.

Patients in these cohorts were reviewed clinically at 10 years prior to the start of this investigation, and the decision to revise their prosthesis or not had already been taken without access to the hip outcome scores. The patients were accordingly divided into a failed and surviving group based on the outcome of this 10 year review. Patients who were offered revision surgery were defined as failures. In all cases the indications for revision were similar; pain in the presence of radiographic evidence of loosening. Patients who were offered revision surgery, but declined, were also counted as failures for the purposes of this study.

Individual scores collected as outcome measures at the time of the 10 year review were identified from the arthroplasty database at each hospital, and the outcome of the clinic attendance through examination of the patients notes. The scores were collected in these cases as outcome measures, and not as part of the hip surveillance programme.

The mean scores in each group were calculated, and a two tailed Student's T test used to assess for significant differences between the failed and surviving cohorts. An alpha error of 0.05 was defined as significant in all cases. Data were further analysed with ROC analysis as previously described (Chapter 3) to assess the sensitivity and specificity of each score for detecting failure and to compare the usefulness of each score as a screening tool.

## 6.3 Results & Discussion

Four hundred and twelve patients were identified in two institutions who had been prospectively followed up for at least ten years, and had completed regular questionnaires. The patients were divided into four separate cohorts:

- Sixty six patients with Biometric stem/universal cup were identified who had been followed up with the hospital for special surgery score (HSS).
- Ninety two patients scored with the Oxford hip score (OHS) who underwent Elite Plus (DePuy, Warsaw Indina) total hip replacements were included at their 10 year follow up visit.
- A cohort of 149 patients who underwent Exeter Universal total arthroplasties were followed up with the Merle d'Aubigne (MDA) for a total of ten years.
- A further cohort of patients who received Exeter Total Hip replacements was included at their 12 year follow-up visit. These patients were scored with the Harris Hip score and also a visual analogue pain score (graded 0-10).

### 6.3.1 Scores Divided by Patient Outcome

The scores for each cohort are given (Table 6.3.1). There were statistically significant differences between the patients scored with the HSS (Surviving 55.7 vs Failed 50.6 $p<0.005$), Harris Hip Score (Surviving 78.0 vs Failed 46.9 $p<0.0001$) and VAS (Surviving 9.0 vs 5.2) between the surviving and failure groups. There was no statistically significant difference between the groups scored with the Oxford score, although the surviving arthroplasties scored 4 points better than the failed group (Surviving 22.2 vs 26.1 $p=0.1176$). There was no difference in the mean values between the subgroups scored with the MDA score.

| Score | Group | Number | Mean | St Dev | p= |
|---|---|---|---|---|---|
| HSS | Surviving | 46 | 55.7 | 5.06 | |
| | Failed | 16 | 50.6 | 5.01 | **0.0005** |
| Oxford | Surviving | 40 | 22.2 | 11.15 | |
| | Failed | 52 | 26.1 | 11.92 | 0.1176 |
| MDA | Surviving | 142 | 15.0 | 2.12 | |
| | Failed | 6 | 15.0 | 2.08 | 0.96 |
| Harris | Surviving | 95 | 78.0 | 15.59 | |
| | Failed | 10 | 46.9 | 18.34 | **<0.0001** |
| Pain | Surviving | 95 | 9.0 | 8.86 | |
| | Failed | 11 | 5.2 | 10.09 | **<0.0001** |

Table 6.3.1 : Mean Scores and Significance values

### 6.3.2 Scores Distribution

The distribution of each score is given (Figure 6.3.1). As can be seen the spread of data differs between subgroups in cohorts bar the MDA score. Interestingly the Harris score is associated with a large number of outliers in the surviving cohort, which is not seen with the other scores. This represents patients with a low score, who do not require revision surgery, a false positive result.

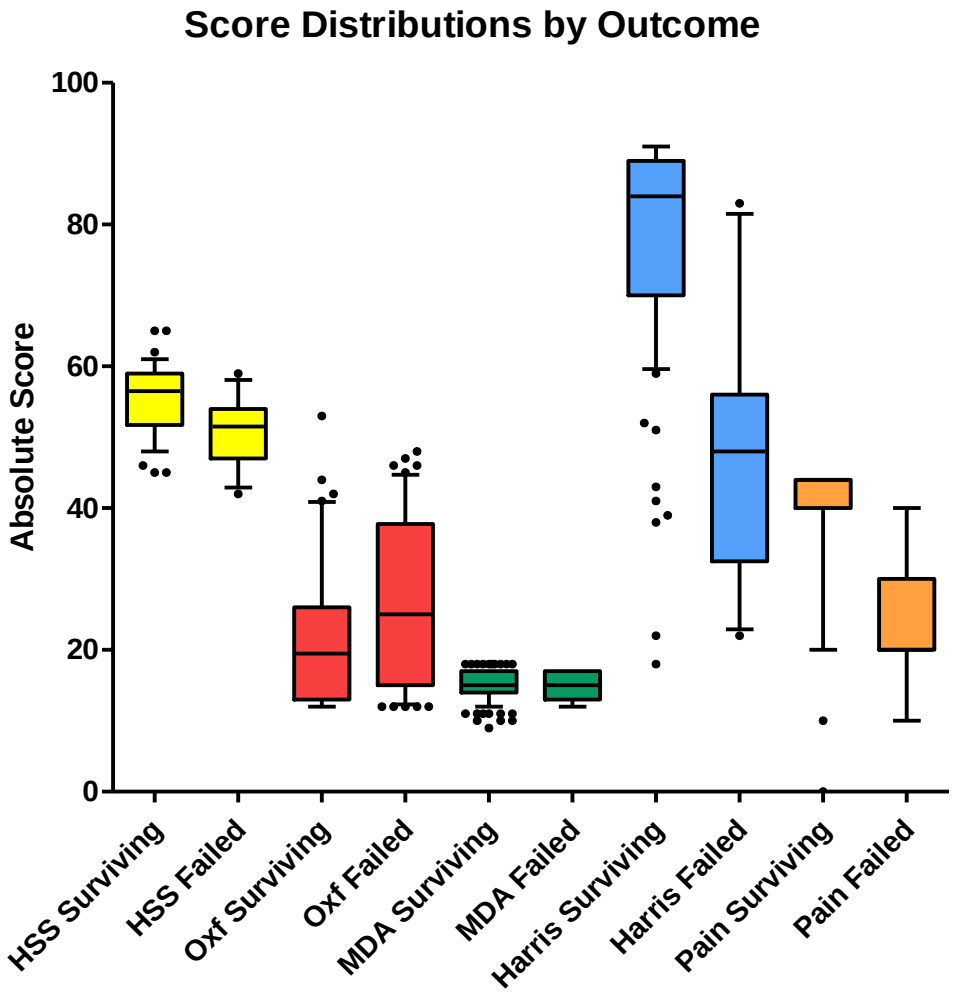



Figure 6.3.1 : Box & Whisker Plot. Distributions of scores, means and 95% confidence intervals per subgroup

### 6.3.3 ROC Analysis

The ROC analysis tables are given in full in the appendix and ROC curves have been plotted from this analysis (Figure 6.3.2). The area under the curve (AUC) is given and is analogous to the predictive value of the scoring system for arthroplasty failure (Table 6.3.2). All three scores (HSS, HHS, VAS) that showed statistically significant differences between the failed and surviving groups were highly predictive of loosening with an AUC of >0.70. The calculated ROC curve was significantly different from chance in each case.

Although all three scores were predictive of failure the HHS (AUC 0.89) and VAS (AUC 0.90) outperformed the HSS (VAS 0.77). Based on these results (see appendix for full results) screening patients with the HHS for loosening using a threshold value of <55.5 could be 80% (95% CI = 44.4 to 97.5 %) sensitive and 91% (95% CI = 84.1 to 96.3%) specific for aseptic loosening. The VAS marginally outperforms this and using a threshold of VAS >2 would be 82% sensitive (95% CI = 48.2 to 97.7%) and 87% Specific (95% CI = 79.0 to 93.1).

| Score | AUC | p= |
|---|---|---|
| MDA | 0.50 | 0.94 |
| OHS | 0.60 | 0.095 |
| HSS | 0.77 | 0.0009 |
| HHS | 0.89 | <0.0001 |
| VAS | 0.90 | <0.0001 |

Table 6.3.2 ROC Analysis scores

It appears likely that the discriminatory power of the Harris Hip Score is due to the large component of the total score that is derived from the pain score (50%) as is seen by the marginally improved scoring for the VAS. What is interesting is the wide disparity between accepted hip outcome scores as instruments to discriminate between failing and well performing hips at their follow up. While all the scores used have been validated as outcome measures for success of the arthroplasty it would appear that selection of score has a large bearing on the value of the score for hip arthroplasty surveillance. Encouragingly all scores showed some change between the two groups.

It is difficult to explain the failure of any score to improve on the VAS score, this may be due to the high specificity of the score. It is well recognised that outcome measure including a functional component suffer from the inability to discriminate between pathology in the same limb and else where[208]. The response to a question on walking distance for example could be reflective of limits imposed on the patient by conditions as diverse as congestive cardiac failure, vascular insufficiency, spinal stenosis and cerebrovascular accident. Whilst pain may radiate and may come from other sites it is more sensitive and specific to ask the patient to complete a VAS than any other measure[228]. An alternative explanation is that the decision to revise the prosthesis may be bias towards a pain score. Patients in pain are more likely to request or agree to revision surgery than those with 'silent' failure.

Although early revision may be desirable to prevent peri-prosthetic fracture it is often difficult to predict who will require surgery as most implant failures are painless in the initial stages. Annual clinical and radiological follow-up is the benchmark to detect early failure but is labour intensive and financially expensive. On this less resource intensive alternatives to follow-up are being implemented. There are many validated scoring systems in current use for the evaluation of hip replacement. However, all of these scores have been designed as a measure of clinical outcome and none are validated to monitor for failure. Whilst it seems to make sense that a failing hip replacement is likely to perform more poorly than a successful one and therefore have a lower score there is no previous data to assess the sensitivity or reliability of outcome measures in arthroplasty surveillance.

Whilst this study goes some way to attempting to validate the use of outcome measures for arthroplasty surveillance it is limited in that it does not evaluate serial measurements of the same patient. It is known that hip scores do change over time, and as such a single measurement may not be enough to answer the question "Is it loose?", and differing threshold values may be required at different follow up periods.

### 6.4 Methodology and Weaknesses

This study is a retrospective study, and assesses patients in whom the outcome has already been determined through clinical review. Whilst this has the potential benefit that the patients score has not influenced the decision to revise, and therefore minimises the potential for observer bias, it does potentially introduce other forms of bias.

The patients are all from different cohorts, and so as such there is no guarantee that there are the same selection of symptoms, or even that there outcomes are standardised. Whilst including patients who are being followed up in the same review programme reduces that potential for selection bias there is still that possibility.

A better study methodology would be to administer all questionnaires to each patient, and then blind the clinician performing the follow-up review to the results of the outcome score and assess which scores most closely reflect the clinical decision made. This would however require a prospective study undertaken over a ten year follow up period, making it impractical within the time constraints of this study.

Whilst this study is flawed in it's design it does provide some measure of the sensitivity and specificity of various scores for detecting aseptic loosening around the hip, and is the first study to do so. The use of prospectively collected scores which have not been used in the clinical decision making process reduces the likelihood of observer bias.

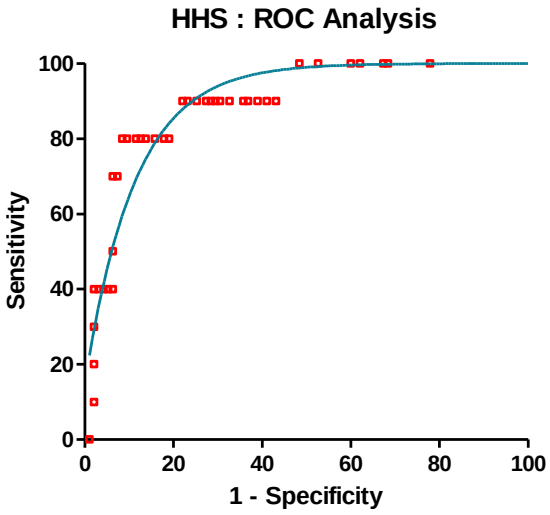
## 6.5 Conclusion

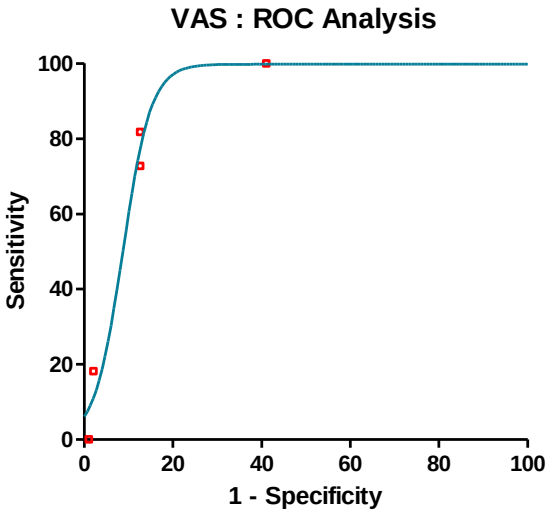
This data demonstrates that there is value in the use of patient reported outcome measures in the follow up of hip arthroplasty. This study demonstrates that PROMs may be used with acceptable sensitivity and specificity as a screening tool for arthroplasty follow up, and justifies the use of these instruments as screening tools for aseptic loosening in hip replacement.

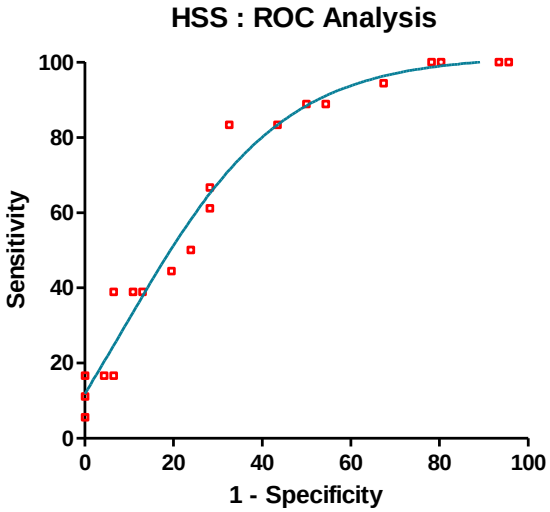
Despite the methodological flaws this study adds valuable information on a previously unstudied field. The wildly differing findings in the sensitivity and specificity of these scores for hip surveillance should be considered when hospitals are designing their arthroplasty follow up protocols.

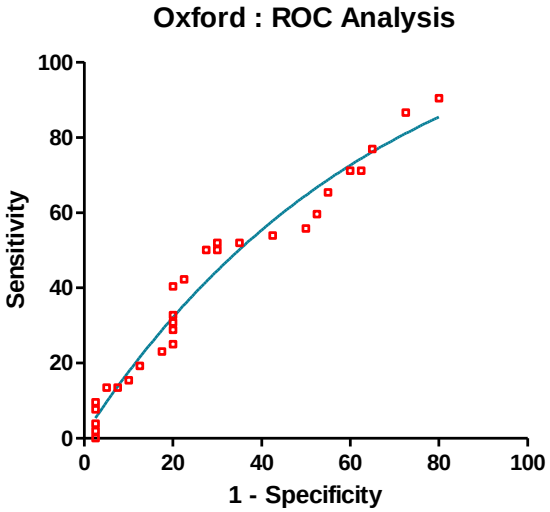
Use of the VAS for pain as a single follow up tool for hip arthroplasty compares favourably with the published results for radiographic review. Using a threshold of VAS >2 would be 82% sensitive and 87% specific, comparing well with standard AP and lateral radiographs reported to have a sensitivity for component loosening of between 85-89%.

The failure of any hip specific score to outperform a VAS for pain does raise some questions about the reliability of these measures. Are these scores failing on face validity – are we really measuring hip performance, or are they so influenced by co-morbidities that PROMs fail to add anything to the simple question “Does your hip hurt?”



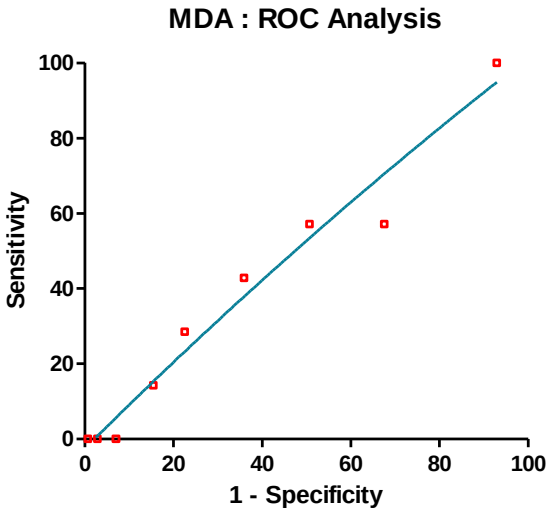




Figure 6.3.2 : Receiver Operator Curve. Sensitivity vs specificity for failure by score.

# SECTION 7 : CONCLUSION

The primary objective of this study was to evaluate if patient specific factors had any role to play in the process of periprosthetic osteolysis. The answer to this question is resoundingly "yes". The study has demonstrated that patient specific factors both biologically and epidemiologically have a key role to play in osteolysis. It has also established improved methods for radiographic and clinical follow up of patients with a total joint arthroplasty.

Returning to the original study questions (Chapter 2) we are able to answer the majority of these to a greater or lesser degree, and have taken some steps towards unpicking the process of osteolysis, and how it varies from patient to patient.

1. Does the osteolytic cascade vary from patient to patient?

   This question is difficult to answer has a whole, and as has been previously outlined is best answered as a series of smaller questions.

   a. Can we reliably measure gene expression in osteoarthritic human joints?

      Gene expression through both manual TaqMan and TLDA expression analysis was successfully performed on the majority of the tissue retrieved intra-operatively. Of the patients who were initially included in the study 67 had RNA extracted and reverse transcribed passing quality control for RNA extraction, lack of contamination and stability of housekeeping gene expression. Further quality control steps with assessment of intra- and inter- observer error demonstrated the technique to be reproducible. Despite the fibrous nature of hip tissue it has been possible to quantify gene expression through RNA extraction in a reliable and reproducible manner.

      Some patients failed the quality control steps, which is a usual finding in this type of study and is explained by the exacting laboratory technique for PCR studies, with any technique errors being multiplied many thousands of times. The addition of quality control steps and intra- observer reliability testing makes the results of this study robust and believable. It seems highly likely that the gene expression was reliably measured in the samples taken for this study.

   b. Does osteoarthritis differ in biological environment from patient to patient?

      Analysis of the gene expression ranges in the primary joint tissues tested demonstrated a mixture of variation in primary joint tissues. Coefficient of variation ranged between 3 and 202, showing some genes had uniform expression, whilst others had greatly varying expression. The results of this study were interpreted in light of this finding.

c. Is there any observed variation local or global within the joint?

Variation of gene expression was measured both with the manual TaqMan method, and the with the TLDA method. Although limited samples were taken, with only two tissues being sampled there was highly consistent gene expression rates between these two tissues, and between different control samples from the same tissue (correlation coefficient range 0.77-0.98).

This observation is interesting, and demonstrates a consistent intra-articular environment which is similar between tissues within the joint. This suggests that the disease process of osteoarthritis represent a global, rather than local change within the joint. Further study is required in this area to look at a more comprehensive set of tissue samples.

d. Do joints with osteolytic change have a different gene expression pattern to native hip joints?

In the majority of genes tested there were significant differences between the groups. This is likely a result of the selection of genes, which was based on a review of the literature and selection of genes previously implicated in animal models or human studies of osteolysis. Of the 68 genes eventually included in the analysis 35 reached statistically significant difference between the study groups and the primaries.

There was a marked difference in gene expression in this study between the osteolytic group and primary arthroplasties.

e. Are these changes due to the joint replacement in situ, or to loosening itself?

This has been a more difficult question to conclusively answer, however sixteen genes were identified where expression levels were highly predictive of the presence of a loose arthroplasty, rather than simply an arthroplasty being present.

This is the first study of this type to establish which of these factors are associated with an osteolytic response, rather than simply the response to a joint replacement in situ. It is possible that these factors could be used as tissue or serum markers for osteolytic change in the future.

f. Are observed biological changes wear debris related or do they represent a tendency to loosen?

It is impossible to conclusively answer this question with the current study design. Without following patients from the implantation of their arthroplasty through the failure of their joint replacement it is not possible to say if there is a biological tendency to loosen.

However this study has established that there are differences in baseline expression of genes between patients, and that those same genes are implicated in the process of osteolysis rather than simply in

the response to wear debris. It certainly seems plausible based on these results that osteolytic change may be in part due to a patient "tendency" towards osteolysis.

2. What are the patient characteristics most likely to predispose to joint loosening?
   There are a number of patient risk factors that have been identified as contributing to osteolytic change, and failure of THRs are predictable from pre-operatively observed patient factors. Over long term follow up patients under 65 years of age and with greater co-morbidities are at significantly higher risk of failure of their joint replacement. This study suggests that overweight patients and those who smoke should be followed up more carefully as there was a higher failure rate in these groups.

3. What is the best radiographic evaluation of osteolysis?
   Diagnosis of osteolysis is key to preventing the excess morbidity and health economic complications of a periprosthetic fracture. Careful evaluation of commonly used radiographic views demonstrated clearly that the currently used combination of anteroposterior and lateral films is inferior to an anteroposterior and iliac-oblique film.

4. Is it possible to use a patient administered outcome scores to identify osteolytic arthroplasties?
   Although in common use patient administered outcome scores have not been evaluated for their sensitivity and specificity. Unsurprisingly the questionnaires evaluated here all performed with different sensitivities and specificities. Surprisingly the best scores (visual analogue, and Harris) performed with similar specificity and sensitivity to traditional radiographs.

During the course of the study several incidental findings have been of interest. It was possible to establish that some additional pathways are likely to be involved in osteolysis that have not been previously identified. We were also able to establish that each osteoarthritic joint had a different local environment, and that while that varied between joints it did not vary appreciably throughout the joint tissues studied.

This study has made the first steps towards identifying which host pathways are responsible for variation between patients, and in addition opening up a new area for further research and understanding of the osteolytic pathway. We have also established some simple changes of practice that will benefit patients through earlier and more accurate diagnosis of their osteolytic joint.

There are a number of unanswered questions raised by this work, and a number of areas where further investigation could yield some interesting and clinically relevant results.

1. **Peripheral Blood Expression**. Having established that each primary OA joint is different, and that there are a number of traits that may be associated with eventual osteolytic loosening it would helpful to

establish if the tissue expression measured within the joint is reflected in the peripheral blood i.e. is the consistent biological environment found across the joint reflected in the peripheral blood. This could potentially allow identification of individuals at risk of loosening without the need for invasive tissue sampling. This study is currently being performed.

2. **In-situ Hybridisation and Histopathology** . Although the aim of this study was not to establish the mechanism of aseptic loosening, but rather to establish which pathways may be involved in variation between individuals a number of the pathways investigated have for the first time been implicated in the process of osteolysis. Further investigation with *in-situ* immunofluorescence would allow localisation of these factors within the tissue and potentially explain where these observations fit into what is already known about osteolysis

3. **Bilateral hip replacements**. Many patients suffer from osteoarthritis of many joints, and hence undergo several primary arthroplasty procedures. An extremely valuable extension to this study would be to take samples from different joints of the same patient. These samples could be used to establish if the 'biological' environment found in this study was common to the whole patient, or was a factor of the individual joint at the time of tissue sample.

This study has demonstrated that patient specific factors of a biological and epidemiological conclusively play a role in the process of osteolysis. On the basis of these results further research is justified in a number of areas.

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# SECTION 10 : APPENDICIES

**Contents :-**

# APPENDIX 1 : ETHICAL SUBMISSION

**The ethics submission is attached as a separate attachment**

Norfolk and Norwich University Hospital NHS
NHS Trust
DEPARTMENT OF TRAUMA & ORTHOPAEDICS

TELEPHONE: 01603 287850
FAX: 01603 287498

NORFOLK & NORWICH UNIVERSITY HOSPITAL
COLNEY
NORWICH NR4 7UY
01603 286286

# PATIENT CONSENT FORM

## Osteolysis in total joint arthroplasty – are patient factors important?

Investigators- Mr Ben Ollivere, Mr James Wimhurst, Miss Clare Darrah, Mr Simon Donell

**Patient Initials:** **Date of Birth:** **Hosp. No:**

**Initials**

**1)** I confirm that I have read and understood the attached information sheet and have had the opportunity to ask questions ☐

**2)** I understand that my participation is voluntary and I am free to withdraw at any time without giving any reason, without my medical care or rights being affected ☐

**3)** I agree that my GP will be informed about my participation in the study ☐

**4)** I agree to take part in the above study ☐

| ---------- | ---------- | ---------- |
|---|---|---|
| **Name of the patient** | **Date** | **Signature** |

| ---------- | ---------- | ---------- |
|---|---|---|
| **Name of the investigator** | **Date** | **Signature** |

Norfolk and Norwich University Hospital NHS
NHS Trust

TELEPHONE: 01603 287850
FAX: 01603 287498

DEPARTMENT OF TRAUMA & ORTHOPAEDICS
NORFOLK & NORWICH UNIVERSITY HOSPITAL
COLNEY
NORWICH NR4 7UY
01603 286286

# Patient Information Sheet

**Study Title :**
Osteolysis in total joint replacement – are patient factors important?

**Invitation :**
You are being invited to participate in this research project as you are about to undergo hip replacement or revision surgery. Before you decide if you wish to take part in the study it is important that you understand what the study is about and what it involves. Please take time to read the following and talk to others if you wish.

You may wish to discuss this with a member of PALS, the patient advice and liason service, who can be contacted on 01603 286 286 (extension 5306). Your orthopaedic consultant will also be happy to discuss this with you.

**What is the purpose of the study?**
This study is being conducted to study the way in which hip replacements wear out. All hip replacements fail, but they last different lengths of time in different people. This study aims to see if differences in immune cells between different people have an effect of the longevity of a hip replacement.

**Why have I been approached?**
You have been approached as you are either having a primary (first) hip replacement or revision (second) hip replacement at the Norfolk & Norwich University Hospital. There is no specific reason you have been approached other than that you are undergoing joint replacement surgery and your consultant has consented for your involvement in the study.

**Do I have to take part?**
You do not have to take part in the study, and your treatment will not change in anyway if you decide to take part or not. If you decide to take part you will be asked to sign a consent form and given this information sheet to take away with you.

**What will happen if I take part?**
If you decide to take part you will be asked to give a second blood sample in addition to the samples you will need prior to the operation. You will not have an additional venepuncture (needle), just slightly more blood will be taken when you have your pre-operative blood tests.

In addition some of the spare tissue from your hip that is normally incinerated after the operation will be kept, and tested for certain types of proteins that may give us a clue as to how people respond to hip replacements, and hence why they might fail. No additional tissue will be taken from you, and all tissue will be stored in a licensed tissue bank, anonymised by number.

**What do I have to do?**
You will be asked to sign a consent form at pre-assessment, but there is nothing else specific you must do. The extra blood sample will be taken at the same time as your pre-operative bloods, and the samples of tissue are from tissue normally wasted during joint replacement surgery.

**What are the benefits of taking part?**
The main benefits of taking part are in contributing to our knowledge of how joint replacement operations are affected by the immune system of the patient. A more complete understanding of this may help us treat patients in the future, but will not directly impact on your care.

**Will my information be kept confidential?**
All information which is collected during the study will be kept confidential. Although the overall results will be published no individual results that could be traced back to you will be released.

**What will happen to the results of the research?**
The results of the various laboratory tests will be collected and analysed statiscally. It is hoped that by comparing various groups of patients it will be possible to identify factors that may results in early failure of prosthetic joints. The duration of the study will be approximately 2 years. The results of study are likely to be published as scientific papers, and also as part of a doctoral thesis. You may obtain copies of the results by contacting us after the estimated completion date of the project.

**Who is organising and funding the research?**
The research is being organised by a partnership between the Institute of Orthopaedics at the Norfolk & Norwich University Hospital, and the School of Biological Sciences at the University of East Anglia. The work is supported by the Gwen Fish Trust, and Action Arthritis. Two charitable bodies who support research into musclo-skeletal disease.
The doctor undertaking the research is not being paid to do so, and you will not receive any payment for taking part in the research.

**Who has reviewed this study?**
Cambridge (3) REC have reviewed this study.

**Contact details**
For further information regarding the study please contact :

Mr B. Ollivere
C/O Clare Darrah
Research co-ordinator, Institute of Orthopaedics
Norfolk & Norwich University Hospital
Colney Lane
Norwich
NR4 7UY

# APPENDIX 2 : LABORATORY PROTOCOLS

# Specimen Collection Protocol
# Osteolysis Study

Ben Ollivere, Clare Darrah, Simon Donell

This study aims to examine the differences between patients immune response and how this affects their body's response to total hip replacement. We need to collect intra-operative samples from primary THR, revision THR and periprosthetic fracture patients.

**Primary Hip Replacement**

Surgeon's Instructions

- Please take samples of approximately 1cm x 1cm
- They should be washed in saline if possible
- They **do not** need to be taken with fresh instruments
- They may be in two pieces so long as from a similar anatomical location

Please take samples of tissue from :

- Hip joint capsule
- Ligamentum terres
- A bone chip from the femoral head

Scrub Nurse Instructions

- Please inform Clare on 2551 when the patient is in the anaesthetic room, and when the samples are taken
- Please place the sample in a sterile universal container (with a conical bottom), and label each pot with the origin of the tissue.
- Please call Clare on 2551 when the sample is ready
- Clare will cover the sample will be covered in RNAlater, and stored upright.
- Samples are sent to the tissue bank labelled 'FAO Ian Sherrifs Hip Tissue Study'

**Revision Hip replacement**

The sample technique and storage are the same, but please take samples of :

- Capsule
- Acetabular membrane
- Femoral membrane
- Bone

**Periprosthetic fracture or revision for dislocation**

The sample technique and storage are the same, but please take samples of :

- Capsule
- Acetabular membrane
- Femoral membrane
- Bone

Copies of the study protocol, patient information and consent forms are available from the Institute of Orthopaedics.

# Osteolysis Study Protocol

# Tissue Extraction

Suitable for :

Capsule, ligamentum teres, femoral and acetabular membranes

## Tissue Extraction

1. Thoroughly defrost tissue. Cut 1cm piece from tissue, and replace remainder in RNAlater and refreeze. Blot dry with sterile gauze and carefully slice on sterile petri dish to fine fragments
2. Mix with 2mls Trizol (in fume cupboard) and use UltraTurrox to 'blend' tissue. NB not all tissue will blend, and residual is expected. Falcon (wide) tubes are best.
3. Spin for 5 minutes.
4. Take supernatant mix with 400 μml/ml Chloroform so 800 μml and vortex
5. Spin down for 10 mins at 14,000. Take supernatant (**top layer only no solid!**)
6. Mix 1:1 with pure ethanol.

### RNA Extraction Column

For each run spin for 30 sec on desk centrifuge. Quagen RNeasy kits

1. Run the sample 700 μml at a time through tube until all collected. Discard run off
2. Run 700 μml RW1 through column. Discard excess
3. Run 500 μml RPE through column. Discard excess.
4. Repeat step 3.
5. Swap collector to fresh ependorf. Run 30 μml water through tube
6. Save sample and Nanodrop

### Nanodrop

Use the RNA syringe, and the RNA nanodrop.

1. Start the nanodrop software
2. Select nucleic acid measurement
3. Select RNA-40 from the coloured drop down box top right
4. Place 1.5 μml analystical grade water and select 'Blank'
5. Select 'Record'
6. For each sample :
    a. Type sample ID into white box on right hand side
    b. Wipe clean with tissue
    c. Place 1.5 μml sample on spectrometer
    d. Click Measure
7. Click 'print report' when done for summary tables.

Interpreting results :

- RNA levels above 100 ng/μml are ideal. Analysis is possible above 40 ng/μml
- The 260/280 measurements are purity measures and should be between 1.9 and 2.1

# APPENDIX 3: LABORATORY RESULTS

## Appendix 3.1 Candidate gene CT value summary

| | | CT Value | | Range | |
|---|---|---|---|---|---|
| Gene | Number Run | Median | St Dev | Min | Max |
| ACP5 | 43 | 33.5661 | 2.622386 | 29.05378 | 40 |
| ADAMTS 14 | 12 | 40 | 1.918017 | 34.30355 | 40 |
| ADAMTS 2 | 39 | 34.87006 | 2.323608 | 31.85571 | 40 |
| ADAMTS 3 | 6 | 40 | 1.078802 | 36.68633 | 40 |
| B2M | 45 | 29.73396 | 1.60453 | 26.19092 | 34.85827 |
| BAK1 | 0 | 40 | 0 | 40 | 40 |
| BCL2A1 | 20 | 40 | 2.971501 | 31.26563 | 40 |
| BMP4 | 24 | 37.00136 | 2.34139 | 33.51771 | 40 |
| CASP3 | 28 | 36.4507 | 2.659144 | 32.27854 | 40 |
| CCL18 | 44 | 32.7915 | 2.25033 | 29.44291 | 40 |
| CCL2 | 42 | 34.45749 | 2.315112 | 30.58138 | 40 |
| CCL3 | 37 | 34.90569 | 2.901999 | 30.38281 | 40 |
| CCR1 | 39 | 34.98052 | 2.146578 | 32.31565 | 40 |
| CD14 | 45 | 32.74352 | 1.37097 | 30.59909 | 37.00171 |
| CD2 | 23 | 37.41019 | 2.394313 | 32.92822 | 40 |
| CD28 | 26 | 36.44666 | 2.388612 | 33.34097 | 40 |
| CD36 | 40 | 34.02876 | 2.434159 | 31.33325 | 40 |
| CD58 | 40 | 34.94667 | 2.192413 | 31.84194 | 40 |
| CD80 | 7 | 40 | 1.450672 | 35.03566 | 40 |
| CD86 | 35 | 35.87174 | 2.423903 | 32.11194 | 40 |
| CHIT1 | 29 | 35.85667 | 4.983597 | 25.88196 | 40 |
| CILP1 | 35 | 34.30995 | 3.071921 | 30.4786 | 40 |
| COL1A1 | 45 | 29.83762 | 1.754838 | 26.1049 | 33.65847 |
| COL1A2 | 45 | 31.09769 | 1.8455 | 27.69495 | 36.5193 |
| COL3A1 | 45 | 30.78216 | 2.059671 | 26.89785 | 35.46236 |
| CSF1 | 43 | 34.39767 | 1.867974 | 30.95527 | 40 |
| CTLA4 | 3 | 40 | 0.798961 | 36.6002 | 40 |
| CTSG | 29 | 36.12246 | 2.788424 | 28.78508 | 40 |
| CTSK | 45 | 32.15099 | 1.666725 | 29.16348 | 36.81389 |
| CXCL10 | 13 | 40 | 2.159484 | 31.86969 | 40 |
| CXCL9 | 18 | 40 | 3.138086 | 29.88101 | 40 |
| DKK1 | 0 | 40 | 0 | 40 | 40 |
| DKK2 | 6 | 40 | 1.468108 | 33.15152 | 40 |

| | | | | | |
|---|---|---|---|---|---|
| DKK4 | 0 | 40 | 0 | 40 | 40 |
| ERCC1 | 42 | 32.67963 | 2.868466 | 28.58408 | 40 |
| FASLG | 2 | 40 | 0.646642 | 36.8782 | 40 |
| FGF18 | 18 | 40 | 2.207461 | 32.83255 | 40 |
| FRZB | 15 | 40 | 2.308359 | 33.63761 | 40 |
| IFNg | 1 | 40 | 0.446692 | 37.0035 | 40 |
| IGF1 | 37 | 34.81358 | 2.903377 | 29.7551 | 40 |
| IL10 | 18 | 40 | 2.118038 | 33.94907 | 40 |
| IL11 | 2 | 40 | 0.647567 | 36.77985 | 40 |
| IL12A | 0 | 40 | 0 | 40 | 40 |
| IL12B | 1 | 40 | 4.825464 | 7.629801 | 40 |
| IL13 | 0 | 40 | 0 | 40 | 40 |
| IL17A | 0 | 40 | 0 | 40 | 40 |
| IL1A | 1 | 40 | 0.604441 | 35.94529 | 40 |
| IL1B | 5 | 40 | 1.735695 | 30.89952 | 40 |
| IL1RN | 34 | 34.93176 | 3.060647 | 28.90755 | 40 |
| IL4 | 0 | 40 | 0 | 40 | 40 |
| IL6 | 16 | 40 | 2.451764 | 32.59131 | 40 |
| IL8 | 29 | 36.51638 | 3.35764 | 25.71296 | 40 |
| IRAK3 | 30 | 36.27755 | 2.715637 | 32.50203 | 40 |
| ITGAM | 44 | 33.66364 | 1.748641 | 31.77 | 40 |
| ITGB2 | 45 | 32.56638 | 1.813996 | 29.5571 | 36.94416 |
| MARCO | 41 | 33.23087 | 2.418824 | 31.12124 | 40 |
| MMP1 | 22 | 40 | 2.346803 | 33.16689 | 40 |
| MMP12 | 12 | 40 | 3.625256 | 17.61742 | 40 |
| MMP13 | 29 | 35.2654 | 3.350623 | 29.75378 | 40 |
| MMP9 | 44 | 28.73965 | 3.381699 | 23.25545 | 40 |
| NOS2 | 0 | 40 | 0 | 40 | 40 |
| PDGFA | 35 | 35.62775 | 2.485594 | 31.75851 | 40 |
| PDGFB | 34 | 35.37938 | 2.405701 | 33.10162 | 40 |
| PTGS2 | 11 | 40 | 2.037421 | 33.59637 | 40 |
| SERPINA3 | 29 | 36.28571 | 2.670849 | 32.22977 | 40 |
| SFRP1 | 29 | 35.64939 | 3.154021 | 30.50071 | 40 |
| SFRP2 | 35 | 33.03574 | 3.902975 | 28.03645 | 40 |
| SOST | 1 | 40 | 1.153686 | 32.26084 | 40 |
| TGFB1 | 44 | 33.49304 | 1.525177 | 30.93756 | 40 |
| TGFB2 | 23 | 36.97233 | 2.509282 | 33.10568 | 40 |
| TGFB3 | 30 | 36.34644 | 2.502236 | 32.65017 | 40 |
| TIMP1 | 45 | 31.02616 | 1.298979 | 28.54503 | 33.46099 |
| TLR1 | 18 | 40 | 2.152633 | 33.72356 | 40 |

| | | | | | |
|---|---|---|---|---|---|
| TLR2 | 24 | 36.91743 | 2.558957 | 32.78392 | 40 |
| TLR3 | 23 | 36.97402 | 2.966539 | 31.844 | 40 |
| TLR4 | 36 | 34.6432 | 2.49838 | 32.01769 | 40 |
| TLR5 | 32 | 35.51426 | 2.562497 | 32.50695 | 40 |
| TM7SF4 | 4 | 40 | 1.171767 | 34.5673 | 40 |
| TNF | 14 | 40 | 2.079828 | 34.39137 | 40 |
| TNFRSF11A | 24 | 36.89891 | 2.139238 | 34.22996 | 40 |
| TNFRSF11B | 22 | 40 | 2.781231 | 32.58606 | 40 |
| TNFRSF1A | 43 | 33.33953 | 1.979438 | 30.70426 | 40 |
| TNFRSF1B | 14 | 40 | 1.988424 | 33.71542 | 40 |
| TNFSF10 | 35 | 34.75537 | 2.746066 | 31.56017 | 40 |
| TNFSF11 | 5 | 40 | 1.042711 | 35.9604 | 40 |
| TP53 | 40 | 35.20268 | 1.902355 | 32.92422 | 40 |
| VDR | 33 | 36.07936 | 2.301988 | 33.21905 | 40 |
| VEGFA | 36 | 35.46609 | 2.311461 | 32.23267 | 40 |
| VEGFB | 45 | 31.92552 | 1.764105 | 29.3199 | 35.97604 |
| WNT10B | 1 | 40 | 0.456815 | 36.93559 | 40 |
| WNT5A | 9 | 40 | 1.685198 | 35.08881 | 40 |
| WNT7B | 3 | 40 | 0.819798 | 36.46114 | 40 |

## Appendix 3.2: ROC Tables

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.2870 | 14.29 | 0.3610% to 57.87% | 100.0 | 79.41% to 100.0% | |
| < 0.2942 | 28.57 | 3.669% to 70.96% | 100.0 | 79.41% to 100.0% | |
| < 0.3281 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |
| < 0.4047 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.5165 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.6013 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| < 0.7065 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.8022 | 42.86 | 9.899% to 81.59% | 68.75 | 41.34% to 88.98% | 1.37 |
| < 0.8158 | 42.86 | 9.899% to 81.59% | 62.50 | 35.43% to 84.80% | 1.14 |
| < 0.9458 | 57.14 | 18.41% to 90.10% | 62.50 | 35.43% to 84.80% | 1.52 |
| < 1.085 | 57.14 | 18.41% to 90.10% | 56.25 | 29.88% to 80.25% | 1.31 |
| < 1.294 | 71.43 | 29.04% to 96.33% | 56.25 | 29.88% to 80.25% | 1.63 |
| < 1.690 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| < 1.931 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 2.288 | 100.0 | 59.04% to 100.0% | 50.00 | 24.65% to 75.35% | 2.00 |
| < 2.627 | 100.0 | 59.04% to 100.0% | 43.75 | 19.75% to 70.12% | 1.78 |
| < 3.144 | 100.0 | 59.04% to 100.0% | 37.50 | 15.20% to 64.57% | 1.60 |
| < 3.713 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 4.116 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 4.872 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 6.463 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 8.186 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

VEGF β ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.004457 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.007327 | 0.0 | 0.0% to 40.96% | 87.50 | 61.65% to 98.45% | 0.00 |
| < 0.007763 | 0.0 | 0.0% to 40.96% | 81.25 | 54.35% to 95.95% | 0.00 |
| < 0.008743 | 14.29 | 0.3610% to 57.87% | 81.25 | 54.35% to 95.95% | 0.76 |
| < 0.01616 | 14.29 | 0.3610% to 57.87% | 75.00 | 47.62% to 92.73% | 0.57 |
| < 0.02376 | 28.57 | 3.669% to 70.96% | 75.00 | 47.62% to 92.73% | 1.14 |
| < 0.02514 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.03392 | 42.86 | 9.899% to 81.59% | 68.75 | 41.34% to 88.98% | 1.37 |
| < 0.04373 | 42.86 | 9.899% to 81.59% | 62.50 | 35.43% to 84.80% | 1.14 |
| < 0.05435 | 57.14 | 18.41% to 90.10% | 62.50 | 35.43% to 84.80% | 1.52 |
| < 0.06777 | 57.14 | 18.41% to 90.10% | 56.25 | 29.88% to 80.25% | 1.31 |
| < 0.08575 | 57.14 | 18.41% to 90.10% | 50.00 | 24.65% to 75.35% | 1.14 |
| < 0.1023 | 71.43 | 29.04% to 96.33% | 50.00 | 24.65% to 75.35% | 1.43 |
| < 0.1106 | 71.43 | 29.04% to 96.33% | 43.75 | 19.75% to 70.12% | 1.27 |
| < 0.1181 | 71.43 | 29.04% to 96.33% | 37.50 | 15.20% to 64.57% | 1.14 |

| | | | | | |
|---|---|---|---|---|---|
| < 0.1221 | 71.43 | 29.04% to 96.33% | 31.25 | 11.02% to 58.66% | 1.04 |
| < 0.1470 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.1713 | 85.71 | 42.13% to 99.64% | 25.00 | 7.266% to 52.38% | 1.14 |
| < 0.1994 | 85.71 | 42.13% to 99.64% | 18.75 | 4.047% to 45.65% | 1.05 |
| < 0.3412 | 85.71 | 42.13% to 99.64% | 12.50 | 1.551% to 38.35% | 0.98 |
| < 0.4672 | 85.71 | 42.13% to 99.64% | 6.250 | 0.1581% to 30.23% | 0.91 |
| < 0.7909 | 85.71 | 42.13% to 99.64% | 0.0 | 0.0% to 20.59% | 0.86 |

VEGF α ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.0005275 | 14.29 | 0.3610% to 57.87% | 100.0 | 79.41% to 100.0% | |
| < 0.0006589 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.0008929 | 14.29 | 0.3610% to 57.87% | 87.50 | 61.65% to 98.45% | 1.14 |
| < 0.001049 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.001211 | 42.86 | 9.899% to 81.59% | 87.50 | 61.65% to 98.45% | 3.43 |
| < 0.001578 | 57.14 | 18.41% to 90.10% | 87.50 | 61.65% to 98.45% | 4.57 |
| < 0.002033 | 57.14 | 18.41% to 90.10% | 81.25 | 54.35% to 95.95% | 3.05 |
| < 0.002529 | 71.43 | 29.04% to 96.33% | 81.25 | 54.35% to 95.95% | 3.81 |
| < 0.002827 | 71.43 | 29.04% to 96.33% | 75.00 | 47.62% to 92.73% | 2.86 |
| < 0.003304 | 85.71 | 42.13% to 99.64% | 75.00 | 47.62% to 92.73% | 3.43 |
| < 0.004422 | 85.71 | 42.13% to 99.64% | 68.75 | 41.34% to 88.98% | 2.74 |
| < 0.005418 | 100.0 | 59.04% to 100.0% | 68.75 | 41.34% to 88.98% | 3.20 |
| < 0.006026 | 100.0 | 59.04% to 100.0% | 62.50 | 35.43% to 84.80% | 2.67 |
| < 0.006553 | 100.0 | 59.04% to 100.0% | 56.25 | 29.88% to 80.25% | 2.29 |
| < 0.006952 | 100.0 | 59.04% to 100.0% | 50.00 | 24.65% to 75.35% | 2.00 |
| < 0.007252 | 100.0 | 59.04% to 100.0% | 43.75 | 19.75% to 70.12% | 1.78 |
| < 0.007497 | 100.0 | 59.04% to 100.0% | 37.50 | 15.20% to 64.57% | 1.60 |
| < 0.01017 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 0.01437 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 0.01700 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 0.02439 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 0.07351 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

IGF 18 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.001309 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.002331 | 0.0 | 0.0% to 40.96% | 87.50 | 61.65% to 98.45% | 0.00 |
| < 0.003837 | 14.29 | 0.3610% to 57.87% | 87.50 | 61.65% to 98.45% | 1.14 |
| < 0.005563 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.006927 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.008575 | 28.57 | 3.669% to 70.96% | 75.00 | 47.62% to 92.73% | 1.14 |

| | | | | | |
|---|---|---|---|---|---|
| < 0.009935 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.01194 | 57.14 | 18.41% to 90.10% | 75.00 | 47.62% to 92.73% | 2.29 |
| < 0.01560 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| < 0.01783 | 71.43 | 29.04% to 96.33% | 68.75 | 41.34% to 88.98% | 2.29 |
| < 0.03074 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| < 0.04626 | 71.43 | 29.04% to 96.33% | 56.25 | 29.88% to 80.25% | 1.63 |
| < 0.05109 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| < 0.05435 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 0.05956 | 100.0 | 59.04% to 100.0% | 50.00 | 24.65% to 75.35% | 2.00 |
| < 0.06570 | 100.0 | 59.04% to 100.0% | 43.75 | 19.75% to 70.12% | 1.78 |
| < 0.07394 | 100.0 | 59.04% to 100.0% | 37.50 | 15.20% to 64.57% | 1.60 |
| < 0.08027 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 0.09672 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 0.1146 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 0.1238 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 0.1536 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

IGF 1 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.04270 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.08088 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.1081 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |
| < 0.1292 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.1576 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.1790 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| < 0.1920 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.2986 | 57.14 | 18.41% to 90.10% | 75.00 | 47.62% to 92.73% | 2.29 |
| < 0.4075 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| < 0.4240 | 57.14 | 18.41% to 90.10% | 62.50 | 35.43% to 84.80% | 1.52 |
| < 0.4605 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| < 0.5748 | 71.43 | 29.04% to 96.33% | 56.25 | 29.88% to 80.25% | 1.63 |
| < 0.7849 | 71.43 | 29.04% to 96.33% | 50.00 | 24.65% to 75.35% | 1.43 |
| < 1.395 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 1.989 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| < 2.401 | 100.0 | 59.04% to 100.0% | 43.75 | 19.75% to 70.12% | 1.78 |
| < 2.966 | 100.0 | 59.04% to 100.0% | 37.50 | 15.20% to 64.57% | 1.60 |
| < 3.240 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 4.498 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 5.933 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 6.220 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 8.554 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

CCL 18 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.006838 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.01476 | 0.0 | 0.0% to 40.96% | 87.50 | 61.65% to 98.45% | 0.00 |
| < 0.02510 | 0.0 | 0.0% to 40.96% | 81.25 | 54.35% to 95.95% | 0.00 |
| < 0.04309 | 0.0 | 0.0% to 40.96% | 75.00 | 47.62% to 92.73% | 0.00 |
| < 0.05880 | 14.29 | 0.3610% to 57.87% | 75.00 | 47.62% to 92.73% | 0.57 |
| < 0.07665 | 28.57 | 3.669% to 70.96% | 75.00 | 47.62% to 92.73% | 1.14 |
| < 0.1051 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.1190 | 42.86 | 9.899% to 81.59% | 68.75 | 41.34% to 88.98% | 1.37 |
| < 0.1283 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| < 0.1468 | 57.14 | 18.41% to 90.10% | 62.50 | 35.43% to 84.80% | 1.52 |
| < 0.2205 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| < 0.3037 | 71.43 | 29.04% to 96.33% | 56.25 | 29.88% to 80.25% | 1.63 |
| < 0.4389 | 71.43 | 29.04% to 96.33% | 50.00 | 24.65% to 75.35% | 1.43 |
| < 0.7673 | 71.43 | 29.04% to 96.33% | 43.75 | 19.75% to 70.12% | 1.27 |
| < 1.138 | 71.43 | 29.04% to 96.33% | 37.50 | 15.20% to 64.57% | 1.14 |
| < 1.476 | 71.43 | 29.04% to 96.33% | 31.25 | 11.02% to 58.66% | 1.04 |
| < 1.658 | 71.43 | 29.04% to 96.33% | 25.00 | 7.266% to 52.38% | 0.95 |
| < 1.722 | 85.71 | 42.13% to 99.64% | 25.00 | 7.266% to 52.38% | 1.14 |
| < 1.804 | 85.71 | 42.13% to 99.64% | 18.75 | 4.047% to 45.65% | 1.05 |
| < 2.183 | 85.71 | 42.13% to 99.64% | 12.50 | 1.551% to 38.35% | 0.98 |
| < 2.705 | 85.71 | 42.13% to 99.64% | 6.250 | 0.1581% to 30.23% | 0.91 |
| < 3.423 | 85.71 | 42.13% to 99.64% | 0.0 | 0.0% to 20.59% | 0.86 |

CCL 3 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.005177 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.01553 | 0.0 | 0.0% to 40.96% | 87.50 | 61.65% to 98.45% | 0.00 |
| < 0.03027 | 14.29 | 0.3610% to 57.87% | 87.50 | 61.65% to 98.45% | 1.14 |
| < 0.03945 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.04159 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.04479 | 28.57 | 3.669% to 70.96% | 75.00 | 47.62% to 92.73% | 1.14 |
| < 0.05374 | 28.57 | 3.669% to 70.96% | 68.75 | 41.34% to 88.98% | 0.91 |
| < 0.06889 | 42.86 | 9.899% to 81.59% | 68.75 | 41.34% to 88.98% | 1.37 |
| < 0.08003 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| < 0.09740 | 71.43 | 29.04% to 96.33% | 68.75 | 41.34% to 88.98% | 2.29 |
| < 0.1188 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| < 0.1368 | 71.43 | 29.04% to 96.33% | 56.25 | 29.88% to 80.25% | 1.63 |
| < 0.1543 | 71.43 | 29.04% to 96.33% | 50.00 | 24.65% to 75.35% | 1.43 |

| | | | | | |
|---|---|---|---|---|---|
| < 0.1825 | 71.43 | 29.04% to 96.33% | 43.75 | 19.75% to 70.12% | 1.27 |
| < 0.2165 | 71.43 | 29.04% to 96.33% | 37.50 | 15.20% to 64.57% | 1.14 |
| < 0.2519 | 71.43 | 29.04% to 96.33% | 31.25 | 11.02% to 58.66% | 1.04 |
| < 0.2763 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.3008 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 0.3944 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 0.6185 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 0.8703 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 1.002 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

CCR1 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.0004031 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.0008152 | 0.0 | 0.0% to 40.96% | 87.50 | 61.65% to 98.45% | 0.00 |
| < 0.001211 | 14.29 | 0.3610% to 57.87% | 87.50 | 61.65% to 98.45% | 1.14 |
| < 0.002074 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.002827 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.003304 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| < 0.004422 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.006074 | 57.14 | 18.41% to 90.10% | 75.00 | 47.62% to 92.73% | 2.29 |
| < 0.007327 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| < 0.01119 | 57.14 | 18.41% to 90.10% | 62.50 | 35.43% to 84.80% | 1.52 |
| < 0.01539 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| < 0.01700 | 71.43 | 29.04% to 96.33% | 56.25 | 29.88% to 80.25% | 1.63 |
| < 0.06156 | 71.43 | 29.04% to 96.33% | 50.00 | 24.65% to 75.35% | 1.43 |
| < 0.1107 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 0.1193 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| < 0.1285 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| < 0.1730 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.2740 | 85.71 | 42.13% to 99.64% | 25.00 | 7.266% to 52.38% | 1.14 |
| < 0.4522 | 85.71 | 42.13% to 99.64% | 18.75 | 4.047% to 45.65% | 1.05 |
| < 0.7901 | 85.71 | 42.13% to 99.64% | 12.50 | 1.551% to 38.35% | 0.98 |
| < 1.029 | 85.71 | 42.13% to 99.64% | 6.250 | 0.1581% to 30.23% | 0.91 |
| < 1.079 | 85.71 | 42.13% to 99.64% | 0.0 | 0.0% to 20.59% | 0.86 |

CXCL9 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.0004031 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.0009279 | 0.0 | 0.0% to 40.96% | 87.50 | 61.65% to 98.45% | 0.00 |
| < 0.001578 | 14.29 | 0.3610% to 57.87% | 87.50 | 61.65% to 98.45% | 1.14 |
| < 0.002033 | 14.29 | 0.3610% to 57.87% | 81.25 | 54.35% to 95.95% | 0.76 |

| | | | | | |
|---|---|---|---|---|---|
| < 0.002529 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.002827 | 28.57 | 3.669% to 70.96% | 75.00 | 47.62% to 92.73% | 1.14 |
| < 0.003304 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.004422 | 42.86 | 9.899% to 81.59% | 68.75 | 41.34% to 88.98% | 1.37 |
| < 0.006074 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| < 0.007327 | 57.14 | 18.41% to 90.10% | 62.50 | 35.43% to 84.80% | 1.52 |
| < 0.008552 | 57.14 | 18.41% to 90.10% | 56.25 | 29.88% to 80.25% | 1.31 |
| < 0.01117 | 57.14 | 18.41% to 90.10% | 50.00 | 24.65% to 75.35% | 1.14 |
| < 0.01376 | 71.43 | 29.04% to 96.33% | 50.00 | 24.65% to 75.35% | 1.43 |
| < 0.01534 | 71.43 | 29.04% to 96.33% | 43.75 | 19.75% to 70.12% | 1.27 |
| < 0.01700 | 71.43 | 29.04% to 96.33% | 37.50 | 15.20% to 64.57% | 1.14 |
| < 0.02328 | 71.43 | 29.04% to 96.33% | 31.25 | 11.02% to 58.66% | 1.04 |
| < 0.04281 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.06547 | 85.71 | 42.13% to 99.64% | 25.00 | 7.266% to 52.38% | 1.14 |
| < 0.07425 | 85.71 | 42.13% to 99.64% | 18.75 | 4.047% to 45.65% | 1.05 |
| < 0.09545 | 85.71 | 42.13% to 99.64% | 12.50 | 1.551% to 38.35% | 0.98 |
| < 0.1840 | 85.71 | 42.13% to 99.64% | 6.250 | 0.1581% to 30.23% | 0.91 |
| < 0.2660 | 85.71 | 42.13% to 99.64% | 0.0 | 0.0% to 20.59% | 0.86 |

CXCL10 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| > 0.0003990 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |
| > 0.0005275 | 85.71 | 42.13% to 99.64% | 6.250 | 0.1581% to 30.23% | 0.91 |
| > 0.0006589 | 85.71 | 42.13% to 99.64% | 12.50 | 1.551% to 38.35% | 0.98 |
| > 0.0009425 | 85.71 | 42.13% to 99.64% | 18.75 | 4.047% to 45.65% | 1.05 |
| > 0.001211 | 71.43 | 29.04% to 96.33% | 18.75 | 4.047% to 45.65% | 0.88 |
| > 0.001578 | 57.14 | 18.41% to 90.10% | 18.75 | 4.047% to 45.65% | 0.70 |
| > 0.002329 | 57.14 | 18.41% to 90.10% | 25.00 | 7.266% to 52.38% | 0.76 |
| > 0.003301 | 57.14 | 18.41% to 90.10% | 31.25 | 11.02% to 58.66% | 0.83 |
| > 0.003810 | 57.14 | 18.41% to 90.10% | 37.50 | 15.20% to 64.57% | 0.91 |
| > 0.004806 | 57.14 | 18.41% to 90.10% | 43.75 | 19.75% to 70.12% | 1.02 |
| > 0.006026 | 57.14 | 18.41% to 90.10% | 50.00 | 24.65% to 75.35% | 1.14 |
| > 0.006682 | 57.14 | 18.41% to 90.10% | 56.25 | 29.88% to 80.25% | 1.31 |
| > 0.007252 | 57.14 | 18.41% to 90.10% | 62.50 | 35.43% to 84.80% | 1.52 |
| > 0.007497 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| > 0.01177 | 57.14 | 18.41% to 90.10% | 75.00 | 47.62% to 92.73% | 2.29 |
| > 0.01700 | 57.14 | 18.41% to 90.10% | 81.25 | 54.35% to 95.95% | 3.05 |
| > 0.02803 | 57.14 | 18.41% to 90.10% | 87.50 | 61.65% to 98.45% | 4.57 |
| > 0.03881 | 42.86 | 9.899% to 81.59% | 87.50 | 61.65% to 98.45% | 3.43 |
| > 0.04222 | 42.86 | 9.899% to 81.59% | 93.75 | 69.77% to 99.84% | 6.86 |
| > 0.04925 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |

| | | | | | |
|---|---|---|---|---|---|
| > 0.08267 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| > 0.1140 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |

TNF α ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.0006371 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.001049 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.001192 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |
| < 0.001559 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.002033 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.002529 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| < 0.002827 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.004300 | 57.14 | 18.41% to 90.10% | 75.00 | 47.62% to 92.73% | 2.29 |
| < 0.006169 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| < 0.006825 | 71.43 | 29.04% to 96.33% | 68.75 | 41.34% to 88.98% | 2.29 |
| < 0.007252 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| < 0.007497 | 71.43 | 29.04% to 96.33% | 56.25 | 29.88% to 80.25% | 1.63 |
| < 0.008160 | 71.43 | 29.04% to 96.33% | 50.00 | 24.65% to 75.35% | 1.43 |
| < 0.01111 | 71.43 | 29.04% to 96.33% | 43.75 | 19.75% to 70.12% | 1.27 |
| < 0.01427 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| < 0.01552 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| < 0.01700 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.04593 | 85.71 | 42.13% to 99.64% | 25.00 | 7.266% to 52.38% | 1.14 |
| < 0.08327 | 85.71 | 42.13% to 99.64% | 18.75 | 4.047% to 45.65% | 1.05 |
| < 0.09933 | 85.71 | 42.13% to 99.64% | 12.50 | 1.551% to 38.35% | 0.98 |
| < 0.1111 | 85.71 | 42.13% to 99.64% | 6.250 | 0.1581% to 30.23% | 0.91 |
| < 0.2556 | 85.71 | 42.13% to 99.64% | 0.0 | 0.0% to 20.59% | 0.86 |

CD120 β ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.0006548 | 14.29 | 0.3610% to 57.87% | 100.0 | 79.41% to 100.0% | |
| < 0.0008929 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.001049 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |
| < 0.001211 | 42.86 | 9.899% to 81.59% | 93.75 | 69.77% to 99.84% | 6.86 |
| < 0.001578 | 57.14 | 18.41% to 90.10% | 93.75 | 69.77% to 99.84% | 9.14 |
| < 0.002033 | 57.14 | 18.41% to 90.10% | 87.50 | 61.65% to 98.45% | 4.57 |
| < 0.002529 | 71.43 | 29.04% to 96.33% | 87.50 | 61.65% to 98.45% | 5.71 |
| < 0.002827 | 71.43 | 29.04% to 96.33% | 81.25 | 54.35% to 95.95% | 3.81 |
| < 0.003304 | 85.71 | 42.13% to 99.64% | 81.25 | 54.35% to 95.95% | 4.57 |
| < 0.003810 | 85.71 | 42.13% to 99.64% | 75.00 | 47.62% to 92.73% | 3.43 |
| < 0.004454 | 85.71 | 42.13% to 99.64% | 68.75 | 41.34% to 88.98% | 2.74 |

| | | | | | |
|---|---|---|---|---|---|
| < 0.005418 | 100.0 | 59.04% to 100.0% | 68.75 | 41.34% to 88.98% | 3.20 |
| < 0.006026 | 100.0 | 59.04% to 100.0% | 62.50 | 35.43% to 84.80% | 2.67 |
| < 0.006682 | 100.0 | 59.04% to 100.0% | 56.25 | 29.88% to 80.25% | 2.29 |
| < 0.007252 | 100.0 | 59.04% to 100.0% | 50.00 | 24.65% to 75.35% | 2.00 |
| < 0.007497 | 100.0 | 59.04% to 100.0% | 43.75 | 19.75% to 70.12% | 1.78 |
| < 0.008541 | 100.0 | 59.04% to 100.0% | 37.50 | 15.20% to 64.57% | 1.60 |
| < 0.01025 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 0.01167 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 0.01416 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 0.01700 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 0.06714 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

OPG ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.001142 | 14.29 | 0.3610% to 57.87% | 100.0 | 79.41% to 100.0% | |
| < 0.001559 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.002331 | 14.29 | 0.3610% to 57.87% | 87.50 | 61.65% to 98.45% | 1.14 |
| < 0.003336 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.004327 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.005463 | 28.57 | 3.669% to 70.96% | 75.00 | 47.62% to 92.73% | 1.14 |
| < 0.006198 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.006852 | 42.86 | 9.899% to 81.59% | 68.75 | 41.34% to 88.98% | 1.37 |
| < 0.007497 | 42.86 | 9.899% to 81.59% | 62.50 | 35.43% to 84.80% | 1.14 |
| < 0.008963 | 42.86 | 9.899% to 81.59% | 56.25 | 29.88% to 80.25% | 0.98 |
| < 0.01267 | 57.14 | 18.41% to 90.10% | 56.25 | 29.88% to 80.25% | 1.31 |
| < 0.01651 | 57.14 | 18.41% to 90.10% | 50.00 | 24.65% to 75.35% | 1.14 |
| < 0.02007 | 57.14 | 18.41% to 90.10% | 43.75 | 19.75% to 70.12% | 1.02 |
| < 0.02726 | 57.14 | 18.41% to 90.10% | 37.50 | 15.20% to 64.57% | 0.91 |
| < 0.03576 | 57.14 | 18.41% to 90.10% | 31.25 | 11.02% to 58.66% | 0.83 |
| < 0.04167 | 71.43 | 29.04% to 96.33% | 31.25 | 11.02% to 58.66% | 1.04 |
| < 0.04815 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.06603 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 0.09813 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 0.1164 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 0.1277 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 0.1651 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

RANK ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.0006589 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.0008929 | 0.0 | 0.0% to 40.96% | 87.50 | 61.65% to 98.45% | 0.00 |

| | | | | | |
|---|---|---|---|---|---|
| < 0.001049 | 14.29 | 0.3610% to 57.87% | 87.50 | 61.65% to 98.45% | 1.14 |
| < 0.001211 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.001578 | 42.86 | 9.899% to 81.59% | 87.50 | 61.65% to 98.45% | 3.43 |
| < 0.002033 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| < 0.002529 | 57.14 | 18.41% to 90.10% | 81.25 | 54.35% to 95.95% | 3.05 |
| < 0.002827 | 57.14 | 18.41% to 90.10% | 75.00 | 47.62% to 92.73% | 2.29 |
| < 0.003336 | 71.43 | 29.04% to 96.33% | 75.00 | 47.62% to 92.73% | 2.86 |
| < 0.004010 | 71.43 | 29.04% to 96.33% | 68.75 | 41.34% to 88.98% | 2.29 |
| < 0.004622 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| < 0.005675 | 85.71 | 42.13% to 99.64% | 62.50 | 35.43% to 84.80% | 2.29 |
| < 0.006682 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| < 0.007327 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 0.009017 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| < 0.01322 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| < 0.01700 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.01949 | 85.71 | 42.13% to 99.64% | 25.00 | 7.266% to 52.38% | 1.14 |
| < 0.02784 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 0.04973 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 0.06887 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 0.09464 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

TLR 3 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.001049 | 14.29 | 0.3610% to 57.87% | 100.0 | 79.41% to 100.0% | |
| < 0.001211 | 28.57 | 3.669% to 70.96% | 100.0 | 79.41% to 100.0% | |
| < 0.001578 | 42.86 | 9.899% to 81.59% | 100.0 | 79.41% to 100.0% | |
| < 0.002805 | 42.86 | 9.899% to 81.59% | 93.75 | 69.77% to 99.84% | 6.86 |
| < 0.005430 | 42.86 | 9.899% to 81.59% | 87.50 | 61.65% to 98.45% | 3.43 |
| < 0.01125 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| < 0.01783 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.02816 | 42.86 | 9.899% to 81.59% | 68.75 | 41.34% to 88.98% | 1.37 |
| < 0.03812 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| < 0.04731 | 71.43 | 29.04% to 96.33% | 68.75 | 41.34% to 88.98% | 2.29 |
| < 0.06379 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| < 0.08476 | 85.71 | 42.13% to 99.64% | 62.50 | 35.43% to 84.80% | 2.29 |
| < 0.1063 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| < 0.1166 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 0.1535 | 100.0 | 59.04% to 100.0% | 50.00 | 24.65% to 75.35% | 2.00 |
| < 0.2041 | 100.0 | 59.04% to 100.0% | 43.75 | 19.75% to 70.12% | 1.78 |
| < 0.3292 | 100.0 | 59.04% to 100.0% | 37.50 | 15.20% to 64.57% | 1.60 |
| < 0.4462 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |

| | | | | | |
|---|---|---|---|---|---|
| < 0.4621 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 0.7557 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 1.082 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 1.126 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

TLR 5 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.0007656 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.001049 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.001192 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |
| < 0.001304 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.001578 | 42.86 | 9.899% to 81.59% | 87.50 | 61.65% to 98.45% | 3.43 |
| < 0.002033 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| < 0.002529 | 57.14 | 18.41% to 90.10% | 81.25 | 54.35% to 95.95% | 3.05 |
| < 0.002827 | 57.14 | 18.41% to 90.10% | 75.00 | 47.62% to 92.73% | 2.29 |
| < 0.003948 | 71.43 | 29.04% to 96.33% | 75.00 | 47.62% to 92.73% | 2.86 |
| < 0.005418 | 85.71 | 42.13% to 99.64% | 75.00 | 47.62% to 92.73% | 3.43 |
| < 0.006426 | 85.71 | 42.13% to 99.64% | 68.75 | 41.34% to 88.98% | 2.74 |
| < 0.007327 | 85.71 | 42.13% to 99.64% | 62.50 | 35.43% to 84.80% | 2.29 |
| < 0.01177 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| < 0.01700 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 0.01879 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| < 0.01986 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| < 0.02138 | 100.0 | 59.04% to 100.0% | 37.50 | 15.20% to 64.57% | 1.60 |
| < 0.03479 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 0.06542 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 0.1001 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 0.1256 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 0.1630 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

FRZB ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.0008929 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.001049 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.001466 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |
| < 0.002033 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.002529 | 42.86 | 9.899% to 81.59% | 87.50 | 61.65% to 98.45% | 3.43 |
| < 0.002827 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| < 0.003304 | 57.14 | 18.41% to 90.10% | 81.25 | 54.35% to 95.95% | 3.05 |
| < 0.004422 | 57.14 | 18.41% to 90.10% | 75.00 | 47.62% to 92.73% | 2.29 |
| < 0.005418 | 71.43 | 29.04% to 96.33% | 75.00 | 47.62% to 92.73% | 2.86 |

| | | | | | |
|---|---|---|---|---|---|
| < 0.006426 | 71.43 | 29.04% to 96.33% | 68.75 | 41.34% to 88.98% | 2.29 |
| < 0.007327 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| < 0.009214 | 71.43 | 29.04% to 96.33% | 56.25 | 29.88% to 80.25% | 1.63 |
| < 0.01341 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| < 0.01700 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 0.01941 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| < 0.02332 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| < 0.02614 | 100.0 | 59.04% to 100.0% | 37.50 | 15.20% to 64.57% | 1.60 |
| < 0.03031 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 0.04817 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 0.08920 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 0.1221 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 0.1311 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

SFRP ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.002033 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.003650 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.005103 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |
| < 0.005455 | 42.86 | 9.899% to 81.59% | 93.75 | 69.77% to 99.84% | 6.86 |
| < 0.006026 | 42.86 | 9.899% to 81.59% | 87.50 | 61.65% to 98.45% | 3.43 |
| < 0.006322 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| < 0.006967 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.01177 | 42.86 | 9.899% to 81.59% | 68.75 | 41.34% to 88.98% | 1.37 |
| < 0.01700 | 42.86 | 9.899% to 81.59% | 62.50 | 35.43% to 84.80% | 1.14 |
| < 0.02195 | 42.86 | 9.899% to 81.59% | 56.25 | 29.88% to 80.25% | 0.98 |
| < 0.02977 | 57.14 | 18.41% to 90.10% | 56.25 | 29.88% to 80.25% | 1.31 |
| < 0.03653 | 71.43 | 29.04% to 96.33% | 56.25 | 29.88% to 80.25% | 1.63 |
| < 0.05191 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| < 0.08074 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 0.1055 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| < 0.1151 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| < 0.1213 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.1274 | 85.71 | 42.13% to 99.64% | 25.00 | 7.266% to 52.38% | 1.14 |
| < 0.1721 | 85.71 | 42.13% to 99.64% | 18.75 | 4.047% to 45.65% | 1.05 |
| < 0.2196 | 85.71 | 42.13% to 99.64% | 12.50 | 1.551% to 38.35% | 0.98 |
| < 0.2644 | 85.71 | 42.13% to 99.64% | 6.250 | 0.1581% to 30.23% | 0.91 |
| < 0.6377 | 85.71 | 42.13% to 99.64% | 0.0 | 0.0% to 20.59% | 0.86 |

SFRP2 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| > 0.0007097 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |
| > 0.0009897 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| > 0.001047 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| > 0.001070 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| > 0.001141 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| > 0.001219 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| > 0.001817 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| > 0.002516 | 71.43 | 29.04% to 96.33% | 37.50 | 15.20% to 64.57% | 1.14 |
| > 0.002740 | 71.43 | 29.04% to 96.33% | 43.75 | 19.75% to 70.12% | 1.27 |
| > 0.009209 | 71.43 | 29.04% to 96.33% | 50.00 | 24.65% to 75.35% | 1.43 |
| > 0.03280 | 71.43 | 29.04% to 96.33% | 56.25 | 29.88% to 80.25% | 1.63 |
| > 0.05024 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| > 0.05377 | 57.14 | 18.41% to 90.10% | 62.50 | 35.43% to 84.80% | 1.52 |
| > 0.05773 | 42.86 | 9.899% to 81.59% | 62.50 | 35.43% to 84.80% | 1.14 |
| > 0.05855 | 28.57 | 3.669% to 70.96% | 62.50 | 35.43% to 84.80% | 0.76 |
| > 0.06125 | 28.57 | 3.669% to 70.96% | 68.75 | 41.34% to 88.98% | 0.91 |
| > 0.06605 | 28.57 | 3.669% to 70.96% | 75.00 | 47.62% to 92.73% | 1.14 |
| > 0.1024 | 14.29 | 0.3610% to 57.87% | 75.00 | 47.62% to 92.73% | 0.57 |
| > 0.1457 | 14.29 | 0.3610% to 57.87% | 81.25 | 54.35% to 95.95% | 0.76 |
| > 0.1629 | 0.0 | 0.0% to 40.96% | 81.25 | 54.35% to 95.95% | 0.00 |
| > 0.4147 | 0.0 | 0.0% to 40.96% | 87.50 | 61.65% to 98.45% | 0.00 |
| > 0.8605 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |

MMP13 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| > 0.0002140 | 85.71 | 42.13% to 99.64% | 0.0 | 0.0% to 20.59% | 0.86 |
| > 0.0002673 | 85.71 | 42.13% to 99.64% | 6.250 | 0.1581% to 30.23% | 0.91 |
| > 0.0003691 | 85.71 | 42.13% to 99.64% | 12.50 | 1.551% to 38.35% | 0.98 |
| > 0.0006958 | 85.71 | 42.13% to 99.64% | 18.75 | 4.047% to 45.65% | 1.05 |
| > 0.0009403 | 85.71 | 42.13% to 99.64% | 25.00 | 7.266% to 52.38% | 1.14 |
| > 0.0009897 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| > 0.001047 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| > 0.001149 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| > 0.001485 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| > 0.002183 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| > 0.003558 | 85.71 | 42.13% to 99.64% | 62.50 | 35.43% to 84.80% | 2.29 |
| > 0.005986 | 85.71 | 42.13% to 99.64% | 68.75 | 41.34% to 88.98% | 2.74 |
| > 0.008804 | 71.43 | 29.04% to 96.33% | 68.75 | 41.34% to 88.98% | 2.29 |
| > 0.01023 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| > 0.01100 | 57.14 | 18.41% to 90.10% | 75.00 | 47.62% to 92.73% | 2.29 |
| > 0.01251 | 57.14 | 18.41% to 90.10% | 81.25 | 54.35% to 95.95% | 3.05 |

| | | | | | |
|---|---|---|---|---|---|
| > 0.01467 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| > 0.01626 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| > 0.02063 | 14.29 | 0.3610% to 57.87% | 81.25 | 54.35% to 95.95% | 0.76 |
| > 0.02598 | 14.29 | 0.3610% to 57.87% | 87.50 | 61.65% to 98.45% | 1.14 |
| > 0.03207 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| > 0.07276 | 14.29 | 0.3610% to 57.87% | 100.0 | 79.41% to 100.0% | |

MMP1 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.0007656 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.001049 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.001211 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |
| < 0.001326 | 42.86 | 9.899% to 81.59% | 93.75 | 69.77% to 99.84% | 6.86 |
| < 0.001580 | 57.14 | 18.41% to 90.10% | 93.75 | 69.77% to 99.84% | 9.14 |
| < 0.002329 | 57.14 | 18.41% to 90.10% | 87.50 | 61.65% to 98.45% | 4.57 |
| < 0.002827 | 57.14 | 18.41% to 90.10% | 81.25 | 54.35% to 95.95% | 3.05 |
| < 0.003304 | 71.43 | 29.04% to 96.33% | 81.25 | 54.35% to 95.95% | 3.81 |
| < 0.004422 | 71.43 | 29.04% to 96.33% | 75.00 | 47.62% to 92.73% | 2.86 |
| < 0.005675 | 85.71 | 42.13% to 99.64% | 75.00 | 47.62% to 92.73% | 3.43 |
| < 0.006682 | 85.71 | 42.13% to 99.64% | 68.75 | 41.34% to 88.98% | 2.74 |
| < 0.007327 | 85.71 | 42.13% to 99.64% | 62.50 | 35.43% to 84.80% | 2.29 |
| < 0.008941 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| < 0.01166 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 0.01450 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| < 0.01700 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| < 0.02854 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.04259 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 0.04691 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 0.06168 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 0.07701 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 0.09731 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

BMP4 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| > 0.003407 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |
| > 0.006364 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| > 0.006764 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| > 0.01037 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| > 0.01946 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| > 0.02737 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| > 0.03078 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |

| | | | | | |
|---|---|---|---|---|---|
| > 0.05114 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| > 0.07164 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| > 0.07548 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| > 0.08606 | 71.43 | 29.04% to 96.33% | 56.25 | 29.88% to 80.25% | 1.63 |
| > 0.1052 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| > 0.1477 | 71.43 | 29.04% to 96.33% | 68.75 | 41.34% to 88.98% | 2.29 |
| > 0.1888 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| > 0.2014 | 57.14 | 18.41% to 90.10% | 75.00 | 47.62% to 92.73% | 2.29 |
| > 0.2124 | 57.14 | 18.41% to 90.10% | 81.25 | 54.35% to 95.95% | 3.05 |
| > 0.2741 | 57.14 | 18.41% to 90.10% | 87.50 | 61.65% to 98.45% | 4.57 |
| > 0.3803 | 57.14 | 18.41% to 90.10% | 93.75 | 69.77% to 99.84% | 9.14 |
| > 0.4737 | 42.86 | 9.899% to 81.59% | 93.75 | 69.77% to 99.84% | 6.86 |
| > 0.6015 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |
| > 0.7242 | 28.57 | 3.669% to 70.96% | 100.0 | 79.41% to 100.0% | |
| > 51.02 | 14.29 | 0.3610% to 57.87% | 100.0 | 79.41% to 100.0% | |

IL8 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.0007615 | 14.29 | 0.3610% to 57.87% | 100.0 | 79.41% to 100.0% | |
| < 0.001162 | 28.57 | 3.669% to 70.96% | 100.0 | 79.41% to 100.0% | |
| < 0.001578 | 42.86 | 9.899% to 81.59% | 100.0 | 79.41% to 100.0% | |
| < 0.002033 | 42.86 | 9.899% to 81.59% | 93.75 | 69.77% to 99.84% | 6.86 |
| < 0.002532 | 57.14 | 18.41% to 90.10% | 93.75 | 69.77% to 99.84% | 9.14 |
| < 0.003063 | 71.43 | 29.04% to 96.33% | 93.75 | 69.77% to 99.84% | 11.43 |
| < 0.005359 | 71.43 | 29.04% to 96.33% | 87.50 | 61.65% to 98.45% | 5.71 |
| < 0.008304 | 71.43 | 29.04% to 96.33% | 81.25 | 54.35% to 95.95% | 3.81 |
| < 0.009781 | 85.71 | 42.13% to 99.64% | 81.25 | 54.35% to 95.95% | 4.57 |
| < 0.01080 | 85.71 | 42.13% to 99.64% | 75.00 | 47.62% to 92.73% | 3.43 |
| < 0.01360 | 85.71 | 42.13% to 99.64% | 68.75 | 41.34% to 88.98% | 2.74 |
| < 0.02030 | 85.71 | 42.13% to 99.64% | 62.50 | 35.43% to 84.80% | 2.29 |
| < 0.02806 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| < 0.03232 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 0.04287 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| < 0.08010 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| < 0.1094 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.1137 | 85.71 | 42.13% to 99.64% | 25.00 | 7.266% to 52.38% | 1.14 |
| < 0.1232 | 85.71 | 42.13% to 99.64% | 18.75 | 4.047% to 45.65% | 1.05 |
| < 0.1507 | 85.71 | 42.13% to 99.64% | 12.50 | 1.551% to 38.35% | 0.98 |
| < 0.1739 | 85.71 | 42.13% to 99.64% | 6.250 | 0.1581% to 30.23% | 0.91 |
| < 0.2001 | 85.71 | 42.13% to 99.64% | 0.0 | 0.0% to 20.59% | 0.86 |

IRAK3 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.01569 | 12.50 | 0.3160% to 52.65% | 100.0 | 78.20% to 100.0% | |
| < 0.04074 | 12.50 | 0.3160% to 52.65% | 93.33 | 68.05% to 99.83% | 1.88 |
| < 0.1345 | 25.00 | 3.185% to 65.09% | 93.33 | 68.05% to 99.83% | 3.75 |
| < 0.2210 | 25.00 | 3.185% to 65.09% | 86.67 | 59.54% to 98.34% | 1.88 |
| < 0.2478 | 25.00 | 3.185% to 65.09% | 80.00 | 51.91% to 95.67% | 1.25 |
| < 0.2818 | 25.00 | 3.185% to 65.09% | 73.33 | 44.90% to 92.21% | 0.94 |
| < 0.3934 | 25.00 | 3.185% to 65.09% | 66.67 | 38.38% to 88.18% | 0.75 |
| < 0.4978 | 25.00 | 3.185% to 65.09% | 60.00 | 32.29% to 83.66% | 0.63 |
| < 0.6129 | 37.50 | 8.523% to 75.51% | 60.00 | 32.29% to 83.66% | 0.94 |
| < 0.7416 | 50.00 | 15.70% to 84.30% | 60.00 | 32.29% to 83.66% | 1.25 |
| < 0.8265 | 50.00 | 15.70% to 84.30% | 53.33 | 26.59% to 78.73% | 1.07 |
| < 1.316 | 62.50 | 24.49% to 91.48% | 53.33 | 26.59% to 78.73% | 1.34 |
| < 1.777 | 75.00 | 34.91% to 96.81% | 53.33 | 26.59% to 78.73% | 1.61 |
| < 2.248 | 75.00 | 34.91% to 96.81% | 46.67 | 21.27% to 73.41% | 1.41 |
| < 2.873 | 87.50 | 47.35% to 99.68% | 46.67 | 21.27% to 73.41% | 1.64 |
| < 3.983 | 87.50 | 47.35% to 99.68% | 40.00 | 16.34% to 67.71% | 1.46 |
| < 5.238 | 87.50 | 47.35% to 99.68% | 33.33 | 11.82% to 61.62% | 1.31 |
| < 5.819 | 87.50 | 47.35% to 99.68% | 26.67 | 7.787% to 55.10% | 1.19 |
| < 7.323 | 100.0 | 63.06% to 100.0% | 26.67 | 7.787% to 55.10% | 1.36 |
| < 9.032 | 100.0 | 63.06% to 100.0% | 20.00 | 4.331% to 48.09% | 1.25 |
| < 9.702 | 100.0 | 63.06% to 100.0% | 13.33 | 1.658% to 40.46% | 1.15 |
| < 10.14 | 100.0 | 63.06% to 100.0% | 6.667 | 0.1686% to 31.95% | 1.07 |

APC 5 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.0006867 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.001211 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.003803 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |
| < 0.006852 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.01064 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.01462 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| < 0.02304 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.03736 | 42.86 | 9.899% to 81.59% | 68.75 | 41.34% to 88.98% | 1.37 |
| < 0.06968 | 42.86 | 9.899% to 81.59% | 62.50 | 35.43% to 84.80% | 1.14 |
| < 0.1058 | 57.14 | 18.41% to 90.10% | 62.50 | 35.43% to 84.80% | 1.52 |
| < 0.1225 | 57.14 | 18.41% to 90.10% | 56.25 | 29.88% to 80.25% | 1.31 |
| < 0.1322 | 57.14 | 18.41% to 90.10% | 50.00 | 24.65% to 75.35% | 1.14 |
| < 0.1531 | 57.14 | 18.41% to 90.10% | 43.75 | 19.75% to 70.12% | 1.02 |
| < 0.1781 | 57.14 | 18.41% to 90.10% | 37.50 | 15.20% to 64.57% | 0.91 |

| | | | | | |
|---|---|---|---|---|---|
| < 0.2095 | 71.43 | 29.04% to 96.33% | 37.50 | 15.20% to 64.57% | 1.14 |
| < 0.2420 | 71.43 | 29.04% to 96.33% | 31.25 | 11.02% to 58.66% | 1.04 |
| < 0.2523 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.2803 | 85.71 | 42.13% to 99.64% | 25.00 | 7.266% to 52.38% | 1.14 |
| < 0.3144 | 85.71 | 42.13% to 99.64% | 18.75 | 4.047% to 45.65% | 1.05 |
| < 0.3755 | 85.71 | 42.13% to 99.64% | 12.50 | 1.551% to 38.35% | 0.98 |
| < 1.293 | 85.71 | 42.13% to 99.64% | 6.250 | 0.1581% to 30.23% | 0.91 |
| < 2.196 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

BCL2A1 RIC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.03469 | 14.29 | 0.3610% to 57.87% | 100.0 | 79.41% to 100.0% | |
| < 0.04467 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.05805 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |
| < 0.1106 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.2147 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.3565 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| < 0.4529 | 57.14 | 18.41% to 90.10% | 81.25 | 54.35% to 95.95% | 3.05 |
| < 0.4875 | 57.14 | 18.41% to 90.10% | 75.00 | 47.62% to 92.73% | 2.29 |
| < 0.7157 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| < 0.9420 | 57.14 | 18.41% to 90.10% | 62.50 | 35.43% to 84.80% | 1.52 |
| < 1.153 | 57.14 | 18.41% to 90.10% | 56.25 | 29.88% to 80.25% | 1.31 |
| < 1.590 | 57.14 | 18.41% to 90.10% | 50.00 | 24.65% to 75.35% | 1.14 |
| < 2.610 | 71.43 | 29.04% to 96.33% | 50.00 | 24.65% to 75.35% | 1.43 |
| < 9.706 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 16.79 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| < 18.02 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| < 19.33 | 100.0 | 59.04% to 100.0% | 37.50 | 15.20% to 64.57% | 1.60 |
| < 22.44 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 30.24 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 36.31 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 52.73 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 85.61 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

CHIT1 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.04555 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.06813 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.1263 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |

| | | | | | |
|---|---|---|---|---|---|
| < 0.1657 | 28.57 | 3.669% to 70.96% | 87.50 | 61.65% to 98.45% | 2.29 |
| < 0.2046 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.2500 | 28.57 | 3.669% to 70.96% | 75.00 | 47.62% to 92.73% | 1.14 |
| < 0.3593 | 28.57 | 3.669% to 70.96% | 68.75 | 41.34% to 88.98% | 0.91 |
| < 0.4623 | 42.86 | 9.899% to 81.59% | 68.75 | 41.34% to 88.98% | 1.37 |
| < 0.5332 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| < 0.6311 | 71.43 | 29.04% to 96.33% | 68.75 | 41.34% to 88.98% | 2.29 |
| < 0.6983 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| < 0.7352 | 85.71 | 42.13% to 99.64% | 62.50 | 35.43% to 84.80% | 2.29 |
| < 0.7358 | 100.0 | 59.04% to 100.0% | 62.50 | 35.43% to 84.80% | 2.67 |
| < 1.175 | 100.0 | 59.04% to 100.0% | 56.25 | 29.88% to 80.25% | 2.29 |
| < 1.663 | 100.0 | 59.04% to 100.0% | 50.00 | 24.65% to 75.35% | 2.00 |
| < 1.749 | 100.0 | 59.04% to 100.0% | 43.75 | 19.75% to 70.12% | 1.78 |
| < 1.962 | 100.0 | 59.04% to 100.0% | 37.50 | 15.20% to 64.57% | 1.60 |
| < 2.633 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 3.339 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 3.772 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 4.580 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 7.605 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

CTSK ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.01171 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.02325 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.03760 | 14.29 | 0.3610% to 57.87% | 87.50 | 61.65% to 98.45% | 1.14 |
| < 0.06481 | 14.29 | 0.3610% to 57.87% | 81.25 | 54.35% to 95.95% | 0.76 |
| < 0.09347 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.1044 | 28.57 | 3.669% to 70.96% | 75.00 | 47.62% to 92.73% | 1.14 |
| < 0.1328 | 28.57 | 3.669% to 70.96% | 68.75 | 41.34% to 88.98% | 0.91 |
| < 0.1606 | 42.86 | 9.899% to 81.59% | 68.75 | 41.34% to 88.98% | 1.37 |
| < 0.1824 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| < 0.2099 | 57.14 | 18.41% to 90.10% | 62.50 | 35.43% to 84.80% | 1.52 |
| < 0.2261 | 57.14 | 18.41% to 90.10% | 56.25 | 29.88% to 80.25% | 1.31 |
| < 0.2798 | 57.14 | 18.41% to 90.10% | 50.00 | 24.65% to 75.35% | 1.14 |
| < 0.3840 | 71.43 | 29.04% to 96.33% | 50.00 | 24.65% to 75.35% | 1.43 |
| < 0.4505 | 71.43 | 29.04% to 96.33% | 43.75 | 19.75% to 70.12% | 1.27 |
| < 0.4981 | 71.43 | 29.04% to 96.33% | 37.50 | 15.20% to 64.57% | 1.14 |
| < 0.5506 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| < 0.7960 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 1.041 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 1.091 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |

| | | | | | |
|---|---|---|---|---|---|
| < 1.316 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 1.561 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 1.676 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

ITGAM ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.0003990 | 0.0 | 0.0% to 40.96% | 93.75 | 69.77% to 99.84% | 0.00 |
| < 0.0007615 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.001049 | 28.57 | 3.669% to 70.96% | 93.75 | 69.77% to 99.84% | 4.57 |
| < 0.001192 | 42.86 | 9.899% to 81.59% | 93.75 | 69.77% to 99.84% | 6.86 |
| < 0.001304 | 42.86 | 9.899% to 81.59% | 87.50 | 61.65% to 98.45% | 3.43 |
| < 0.001779 | 57.14 | 18.41% to 90.10% | 87.50 | 61.65% to 98.45% | 4.57 |
| < 0.002529 | 71.43 | 29.04% to 96.33% | 87.50 | 61.65% to 98.45% | 5.71 |
| < 0.002827 | 71.43 | 29.04% to 96.33% | 81.25 | 54.35% to 95.95% | 3.81 |
| < 0.003304 | 85.71 | 42.13% to 99.64% | 81.25 | 54.35% to 95.95% | 4.57 |
| < 0.003810 | 85.71 | 42.13% to 99.64% | 75.00 | 47.62% to 92.73% | 3.43 |
| < 0.004729 | 85.71 | 42.13% to 99.64% | 68.75 | 41.34% to 88.98% | 2.74 |
| < 0.005949 | 85.71 | 42.13% to 99.64% | 62.50 | 35.43% to 84.80% | 2.29 |
| < 0.006682 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| < 0.007245 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 0.007416 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| < 0.007497 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| < 0.01177 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.01700 | 85.71 | 42.13% to 99.64% | 25.00 | 7.266% to 52.38% | 1.14 |
| < 0.01900 | 85.71 | 42.13% to 99.64% | 18.75 | 4.047% to 45.65% | 1.05 |
| < 0.04337 | 85.71 | 42.13% to 99.64% | 12.50 | 1.551% to 38.35% | 0.98 |
| < 0.09152 | 85.71 | 42.13% to 99.64% | 6.250 | 0.1581% to 30.23% | 0.91 |
| < 0.2432 | 85.71 | 42.13% to 99.64% | 0.0 | 0.0% to 20.59% | 0.86 |

PTGS2 ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 0.008882 | 14.29 | 0.3610% to 57.87% | 100.0 | 79.41% to 100.0% | |
| < 0.01509 | 14.29 | 0.3610% to 57.87% | 93.75 | 69.77% to 99.84% | 2.29 |
| < 0.01848 | 14.29 | 0.3610% to 57.87% | 87.50 | 61.65% to 98.45% | 1.14 |
| < 0.02128 | 14.29 | 0.3610% to 57.87% | 81.25 | 54.35% to 95.95% | 0.76 |
| < 0.02387 | 28.57 | 3.669% to 70.96% | 81.25 | 54.35% to 95.95% | 1.52 |
| < 0.02915 | 42.86 | 9.899% to 81.59% | 81.25 | 54.35% to 95.95% | 2.29 |
| < 0.03413 | 42.86 | 9.899% to 81.59% | 75.00 | 47.62% to 92.73% | 1.71 |
| < 0.04109 | 42.86 | 9.899% to 81.59% | 68.75 | 41.34% to 88.98% | 1.37 |
| < 0.05145 | 57.14 | 18.41% to 90.10% | 68.75 | 41.34% to 88.98% | 1.83 |
| < 0.06693 | 71.43 | 29.04% to 96.33% | 68.75 | 41.34% to 88.98% | 2.29 |

| | | | | | |
|---|---|---|---|---|---|
| < 0.07955 | 71.43 | 29.04% to 96.33% | 62.50 | 35.43% to 84.80% | 1.90 |
| < 0.08237 | 85.71 | 42.13% to 99.64% | 62.50 | 35.43% to 84.80% | 2.29 |
| < 0.09195 | 85.71 | 42.13% to 99.64% | 56.25 | 29.88% to 80.25% | 1.96 |
| < 0.1053 | 85.71 | 42.13% to 99.64% | 50.00 | 24.65% to 75.35% | 1.71 |
| < 0.1135 | 85.71 | 42.13% to 99.64% | 43.75 | 19.75% to 70.12% | 1.52 |
| < 0.1217 | 85.71 | 42.13% to 99.64% | 37.50 | 15.20% to 64.57% | 1.37 |
| < 0.1373 | 85.71 | 42.13% to 99.64% | 31.25 | 11.02% to 58.66% | 1.25 |
| < 0.1825 | 100.0 | 59.04% to 100.0% | 31.25 | 11.02% to 58.66% | 1.45 |
| < 0.2175 | 100.0 | 59.04% to 100.0% | 25.00 | 7.266% to 52.38% | 1.33 |
| < 0.2222 | 100.0 | 59.04% to 100.0% | 18.75 | 4.047% to 45.65% | 1.23 |
| < 0.3100 | 100.0 | 59.04% to 100.0% | 12.50 | 1.551% to 38.35% | 1.14 |
| < 0.4327 | 100.0 | 59.04% to 100.0% | 6.250 | 0.1581% to 30.23% | 1.07 |

TP53 ROC Table

Appendix F – ROC Tables for PROMS

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 42.50 | 5.556 | 0.1406% to 27.29% | 100.0 | 92.29% to 100.0% | |
| < 43.50 | 11.11 | 1.375% to 34.71% | 100.0 | 92.29% to 100.0% | |
| < 44.50 | 16.67 | 3.578% to 41.42% | 100.0 | 92.29% to 100.0% | |
| < 45.50 | 16.67 | 3.578% to 41.42% | 95.65 | 85.16% to 99.47% | 3.83 |
| < 46.50 | 16.67 | 3.578% to 41.42% | 93.48 | 82.10% to 98.63% | 2.56 |
| < 47.50 | 38.89 | 17.30% to 64.25% | 93.48 | 82.10% to 98.63% | 5.96 |
| < 48.50 | 38.89 | 17.30% to 64.25% | 89.13 | 76.43% to 96.38% | 3.58 |
| < 49.50 | 38.89 | 17.30% to 64.25% | 86.96 | 73.74% to 95.06% | 2.98 |
| < 50.50 | 44.44 | 21.53% to 69.24% | 80.43 | 66.09% to 90.64% | 2.27 |
| < 51.50 | 50.00 | 26.02% to 73.98% | 76.09 | 61.23% to 87.41% | 2.09 |
| < 52.50 | 61.11 | 35.75% to 82.70% | 71.74 | 56.54% to 84.01% | 2.16 |
| < 53.50 | 66.67 | 40.99% to 86.66% | 71.74 | 56.54% to 84.01% | 2.36 |
| < 54.50 | 83.33 | 58.58% to 96.42% | 67.39 | 51.98% to 80.47% | 2.56 |
| < 55.50 | 83.33 | 58.58% to 96.42% | 56.52 | 41.11% to 71.07% | 1.92 |
| < 56.50 | 88.89 | 65.29% to 98.62% | 50.00 | 34.90% to 65.10% | 1.78 |
| < 57.50 | 88.89 | 65.29% to 98.62% | 45.65 | 30.90% to 60.99% | 1.64 |
| < 58.50 | 94.44 | 72.71% to 99.86% | 32.61 | 19.53% to 48.02% | 1.40 |
| < 59.50 | 100.0 | 81.47% to 100.0% | 21.74 | 10.95% to 36.36% | 1.28 |
| < 60.50 | 100.0 | 81.47% to 100.0% | 19.57 | 9.358% to 33.91% | 1.24 |
| < 61.50 | 100.0 | 81.47% to 100.0% | 6.522 | 1.366% to 17.90% | 1.07 |
| < 63.50 | 100.0 | 81.47% to 100.0% | 4.348 | 0.5309% to 14.84% | 1.05 |

HSS Score ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| > 12.50 | 90.38 | 78.97% to 96.80% | 20.00 | 9.052% to 35.65% | 1.13 |
| > 13.50 | 86.54 | 74.21% to 94.41% | 27.50 | 14.60% to 43.89% | 1.19 |
| > 14.50 | 76.92 | 63.16% to 87.47% | 35.00 | 20.63% to 51.68% | 1.18 |
| > 15.50 | 71.15 | 56.92% to 82.87% | 37.50 | 22.73% to 54.20% | 1.14 |
| > 16.50 | 71.15 | 56.92% to 82.87% | 40.00 | 24.86% to 56.67% | 1.19 |
| > 17.50 | 65.38 | 50.91% to 78.03% | 45.00 | 29.26% to 61.51% | 1.19 |
| > 18.50 | 59.62 | 45.10% to 72.99% | 47.50 | 31.51% to 63.87% | 1.14 |
| > 19.50 | 55.77 | 41.33% to 69.53% | 50.00 | 33.80% to 66.20% | 1.12 |
| > 20.50 | 53.85 | 39.47% to 67.77% | 57.50 | 40.89% to 72.96% | 1.27 |
| > 22.00 | 51.92 | 37.63% to 65.99% | 65.00 | 48.32% to 79.37% | 1.48 |
| > 23.50 | 51.92 | 37.63% to 65.99% | 70.00 | 53.47% to 83.44% | 1.73 |
| > 24.50 | 50.00 | 35.81% to 64.19% | 70.00 | 53.47% to 83.44% | 1.67 |
| > 25.50 | 50.00 | 35.81% to 64.19% | 72.50 | 56.11% to 85.40% | 1.82 |
| > 27.00 | 42.31 | 28.73% to 56.80% | 77.50 | 61.55% to 89.16% | 1.88 |
| > 28.50 | 40.38 | 27.01% to 54.90% | 80.00 | 64.35% to 90.95% | 2.02 |
| > 31.00 | 32.69 | 20.33% to 47.10% | 80.00 | 64.35% to 90.95% | 1.63 |
| > 34.00 | 30.77 | 18.72% to 45.10% | 80.00 | 64.35% to 90.95% | 1.54 |
| > 36.00 | 28.85 | 17.13% to 43.08% | 80.00 | 64.35% to 90.95% | 1.44 |
| > 37.50 | 25.00 | 14.03% to 38.95% | 80.00 | 64.35% to 90.95% | 1.25 |
| > 38.50 | 23.08 | 12.53% to 36.84% | 82.50 | 67.22% to 92.66% | 1.32 |
| > 39.50 | 19.23 | 9.627% to 32.53% | 87.50 | 73.20% to 95.81% | 1.54 |
| > 40.50 | 15.38 | 6.884% to 28.08% | 90.00 | 76.34% to 97.21% | 1.54 |
| > 41.50 | 13.46 | 5.588% to 25.79% | 92.50 | 79.61% to 98.43% | 1.79 |
| > 43.00 | 13.46 | 5.588% to 25.79% | 95.00 | 83.08% to 99.39% | 2.69 |
| > 44.50 | 9.615 | 3.196% to 21.03% | 97.50 | 86.84% to 99.94% | 3.85 |
| > 45.50 | 7.692 | 2.136% to 18.54% | 97.50 | 86.84% to 99.94% | 3.08 |
| > 46.50 | 3.846 | 0.4692% to 13.21% | 97.50 | 86.84% to 99.94% | 1.54 |
| > 47.50 | 1.923 | 0.04868% to 10.26% | 97.50 | 86.84% to 99.94% | 0.77 |
| > 50.50 | 0.0 | 0.0% to 6.848% | 97.50 | 86.84% to 99.94% | 0.00 |

Oxford Score ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 9.500 | 0.0 | 0.0% to 40.96% | 99.30 | 96.14% to 99.98% | 0.00 |
| < 10.50 | 0.0 | 0.0% to 40.96% | 97.18 | 92.94% to 99.23% | 0.00 |
| < 11.50 | 0.0 | 0.0% to 40.96% | 92.96 | 87.43% to 96.57% | 0.00 |
| < 12.50 | 14.29 | 0.3610% to 57.87% | 84.51 | 77.49% to 90.03% | 0.92 |
| < 13.50 | 28.57 | 3.669% to 70.96% | 77.46 | 69.70% to 84.05% | 1.27 |
| < 14.50 | 42.86 | 9.899% to 81.59% | 64.08 | 55.61% to 71.96% | 1.19 |
| < 15.50 | 57.14 | 18.41% to 90.10% | 49.30 | 40.81% to 57.81% | 1.13 |
| < 16.50 | 57.14 | 18.41% to 90.10% | 32.39 | 24.79% to 40.75% | 0.85 |
| < 17.50 | 100.0 | 59.04% to 100.0% | 7.042 | 3.429% to 12.57% | 1.08 |

MDA Score ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| < 20.00 | 0.0 | 0.0% to 30.85% | 98.95 | 94.27% to 99.97% | 0.00 |
| < 26.50 | 10.00 | 0.2529% to 44.50% | 97.89 | 92.60% to 99.74% | 4.75 |
| < 32.00 | 20.00 | 2.521% to 55.61% | 97.89 | 92.60% to 99.74% | 9.50 |
| < 33.50 | 30.00 | 6.674% to 65.25% | 97.89 | 92.60% to 99.74% | 14.25 |
| < 36.00 | 40.00 | 12.16% to 73.76% | 97.89 | 92.60% to 99.74% | 19.00 |
| < 38.50 | 40.00 | 12.16% to 73.76% | 96.84 | 91.05% to 99.34% | 12.67 |
| < 40.00 | 40.00 | 12.16% to 73.76% | 95.79 | 89.57% to 98.84% | 9.50 |
| < 42.00 | 40.00 | 12.16% to 73.76% | 94.74 | 88.14% to 98.27% | 7.60 |
| < 44.50 | 40.00 | 12.16% to 73.76% | 93.68 | 86.76% to 97.65% | 6.33 |
| < 48.00 | 50.00 | 18.71% to 81.29% | 93.68 | 86.76% to 97.65% | 7.92 |
| < 50.50 | 70.00 | 34.75% to 93.33% | 93.68 | 86.76% to 97.65% | 11.08 |
| < 51.50 | 70.00 | 34.75% to 93.33% | 92.63 | 85.41% to 96.99% | 9.50 |
| < 55.50 | 80.00 | 44.39% to 97.48% | 91.58 | 84.08% to 96.29% | 9.50 |
| < 59.50 | 80.00 | 44.39% to 97.48% | 90.53 | 82.78% to 95.58% | 8.44 |
| < 61.00 | 80.00 | 44.39% to 97.48% | 88.42 | 80.23% to 94.08% | 6.91 |
| < 62.50 | 80.00 | 44.39% to 97.48% | 87.37 | 78.97% to 93.30% | 6.33 |
| < 63.50 | 80.00 | 44.39% to 97.48% | 86.32 | 77.74% to 92.51% | 5.85 |
| < 64.50 | 80.00 | 44.39% to 97.48% | 84.21 | 75.30% to 90.88% | 5.07 |
| < 65.50 | 80.00 | 44.39% to 97.48% | 82.11 | 72.90% to 89.22% | 4.47 |
| < 67.00 | 80.00 | 44.39% to 97.48% | 81.05 | 71.72% to 88.37% | 4.22 |
| < 68.50 | 90.00 | 55.50% to 99.75% | 77.89 | 68.22% to 85.77% | 4.07 |
| < 69.50 | 90.00 | 55.50% to 99.75% | 76.84 | 67.06% to 84.88% | 3.89 |
| < 70.50 | 90.00 | 55.50% to 99.75% | 74.74 | 64.78% to 83.09% | 3.56 |
| < 71.50 | 90.00 | 55.50% to 99.75% | 72.63 | 62.52% to 81.28% | 3.29 |
| < 72.50 | 90.00 | 55.50% to 99.75% | 71.58 | 61.40% to 80.36% | 3.17 |
| < 74.50 | 90.00 | 55.50% to 99.75% | 70.53 | 60.29% to 79.44% | 3.05 |
| < 76.50 | 90.00 | 55.50% to 99.75% | 69.47 | 59.18% to 78.51% | 2.95 |
| < 77.50 | 90.00 | 55.50% to 99.75% | 67.37 | 56.98% to 76.64% | 2.76 |
| < 78.50 | 90.00 | 55.50% to 99.75% | 64.21 | 53.72% to 73.79% | 2.51 |
| < 79.50 | 90.00 | 55.50% to 99.75% | 63.16 | 52.64% to 72.83% | 2.44 |
| < 80.50 | 90.00 | 55.50% to 99.75% | 61.05 | 50.50% to 70.89% | 2.31 |
| < 81.50 | 90.00 | 55.50% to 99.75% | 58.95 | 48.38% to 68.94% | 2.19 |
| < 82.50 | 90.00 | 55.50% to 99.75% | 56.84 | 46.28% to 66.97% | 2.09 |
| < 83.50 | 100.0 | 69.15% to 100.0% | 51.58 | 41.10% to 61.96% | 2.07 |
| < 84.50 | 100.0 | 69.15% to 100.0% | 47.37 | 37.03% to 57.88% | 1.90 |
| < 85.50 | 100.0 | 69.15% to 100.0% | 40.00 | 30.08% to 50.56% | 1.67 |
| < 86.50 | 100.0 | 69.15% to 100.0% | 37.89 | 28.14% to 48.43% | 1.61 |
| < 87.50 | 100.0 | 69.15% to 100.0% | 32.63 | 23.36% to 43.02% | 1.48 |
| < 88.50 | 100.0 | 69.15% to 100.0% | 31.58 | 22.42% to 41.92% | 1.46 |
| < 90.00 | 100.0 | 69.15% to 100.0% | 22.11 | 14.23% to 31.78% | 1.28 |

Harris Score ROC Table

| Cutoff | Sensitivity% | 95% CI | Specificity% | 95% CI | Likelihood ratio |
|---|---|---|---|---|---|
| > 8.00 | 0.0 | 0.0% to 28.49% | 98.95 | 94.27% to 99.97% | 0.00 |
| > 6.00 | 18.18 | 2.283% to 51.78% | 97.89 | 92.60% to 99.74% | 8.64 |

| | | | |
|---|---|---|---|
| > 4.00 | 72.73 39.03% to 93.98% | 87.37 78.97% to 93.30% | 5.76 |
| > 2.00 | 81.82 48.22% to 97.72% | 87.37 78.97% to 93.30% | 6.48 |
| > 0.00 | 100.0 71.51% to 100.0% | 58.95 48.38% to 68.94% | 2.44 |

# APPENDIX 4 : GLOSSARY

| | |
|---|---|
| AP | Anteroposterior. A radiographic projection showing the patient from the front on the radiograph |
| APC | Antigen Presenting Cell. A cell that initiates an immune response by presenting antigens on it's surface usually a macrophage or dendritic cell. |
| AUC | Area Under Curve. An indicator of the over all strength of association in ROC analysis. |
| AVN | Avascular Necrosis. Death of a bone and subsequent collapse. Usually due to a loss of blood supply. |
| BMP | Bone Morphogenic Protein. A family of proteins that act as signal molecules to promote and control bone healing and repair. |
| BMI | Body Mass Index. A measure of patient weight. Given in $kg/m^2$. |
| CD | Cluster of differentiation. |
| $C_T$ | Cycles to Threshold. The number of PCR cycles undergone until the RNA expression is measurable |
| cDNA | Chimeric DNA. A DNA library normally produced by reverse transcription. |
| CTSG | Cathepsin G. An intracellular signal molecule. |
| CTSK | Cathepsin K. An intracellular signal molecule. |
| COL | One of the family of Collagen genes. |
| DKK | Dickkopf family of proteins inhibit the WNT pathway. |
| FRZB | Frizzled Related Protein. An extracellular antagonist of the WNT signalling pathway. |
| FGF | Fibroblast Growth Factor. Promotes differentiation of osteoblasts. |
| HHS | Harris Hip Score. A surgeon reported outcome measure |
| Housekeeping gene | A gene which is known to be linearly expressed in the tissue of interest and so may be used to correct for error in laboratory technique. |
| HSS | Hospital for Special Surgery score. A surgeon reported outcome measure. |
| IB | Iliac Oblique. A radiographic projection most usually used to visualise the acetabulum. |
| IGF | Insulin like Growth Factor. A secreted local growth factor. |
| MDA | Merle d'Aubigne. A surgeon reported outcome score. |
| MMP | Matrix Metalloprotein. A proteinase capable of breaking down extra-cellular matrix. |
| Osteolysis | The process of bone loss in response to wear debris or other stimulus. |
| OPG | Osteoprotegerin. A secreted decoy receptor which binds the RANK Ligand. |
| OHS | Oxford Hip Score. A patient reported outcome measure. |
| PCR | Polymerase Chain Reaction. A method for replicating DNA sequences of interest. |
| RA | Rheumatoid Arthritis |
| RANK | Receptor Activator of Nuclear Factor κ B **(**RANK). The receptor for the RANK pathway which activates osteoclastic activity. |
| ROC | Receiver Operator Characteristic. A statistical method for quantifying strength of association. |
| TaqMan | An method utilising the Taq Polymerase to perform quantitative PCR. |
| TNF | Tumour Necrosis Factor. A group of extra-cellular signal molecules involved in modulation of the inflammatory response. |
| TLDA | TaqMan Low Density Array. An automated method for undertaking TaqMan analysis. |
| TLR | Toll Like Receptor. A family of receptors similar in structure to Toll. |
| TRAP | Tartrate Resistant Acid Phosphatase. The enzyme that metabolises bone matric TRAP is therefore a marker of bone breakdown by osteoclasts. |
| THR | Total Hip Replacement |
| NJR | National Joint Registry |
| NSAID | Non-steroidal Anti Inflammatory Drug. A family of drugs that inhibit the action of cyclo-oxygenase and have been shown to inhibit bone healing and remodelling |
| NICE | National Institute for Clinical Excellence |
| VEGF | Vascular Endothelial Growth Factor. A secreted local growth factor associated with neovascularisation. |
| WNT | Wingless. A family intracellular signalling molecules that are related to the Wingless protein in Drosophila |