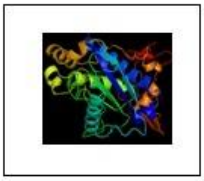
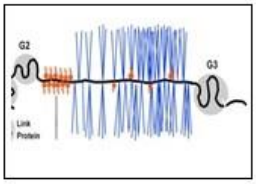
Evaluation of a novel biomarker as a predictor of response, stratification tool, and measure of pharmacology for a disease-modifying osteoarthritis therapeutic

Target  ADAMTS-5

Biomarker 

ARGS NEOEPITOPE

Therapeutic  GSK2394002 mAb

A DISSERTATION SUMITTED TO THE

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Abstract

Introduction

Osteoarthritis (OA) is a leading cause of disability in developed countries and is independently associated with increased mortality. Aggrecan degradation is an early process in OA-related cartilage degradation, principally attributed to activity of the aggrecanase ADAMTS-5. One of the products of this activity is ARGS neoepitope which is proposed as a biomarker of OA disease burden. Biomarker measurement and correlation could advance research into effective intervention in OA. In this study we aim to assess ARGS neoepitope as a marker of OA disease burden through correlation with MRI-derived imaging outcome measures. We also assess an ADAMTS5-specific monoclonal antibody for its viability as an OA pharmaceutical intervention.

Methodology

This study is a cross-sectional cohort study of 95 knee surgical patients to measure ARGS neoepitope using electrochemiluminescent assay on serum, urine and synovial fluid. Specific software was used to generate MRI-derived outcome measures such as volume and intensity measures, allowing comparison of these two outcome measures. This study also investigated ARGS neoepitope response to an ADAMTS-5 blocking monoclonal antibody *in vitro* in a human explant model.

Results

A significantly higher level of ARGS neoepitope was found in urine samples (1.58-fold, $P = 0.006$) of patients with late compared to early disease, whilst no significant differences in neoepitope values were detected in synovial fluid and serum samples. With regard to disease burden, ARGS neoepitope was correlated with medial compartment cartilage loss in medial unicompartmental knee replacement patients ($R^2 = 0.249$; $P = 0.012$) however in general it was not strongly related to imaging markers. In the *in vitro* work the ADAMTS-5 specific monoclonal antibody produced a dose-dependent reduction in ARGS neoepitope released from cartilage explants. Compared to the isotype control the mean reduction of ARGS neoepitope at 12 days was 43.6% ($P < 0.001$).

Discussion

The relationship between serological biomarkers of OA and disease burden remains complex. ARGS neoepitope may reflect enzymatic turnover of cartilage within the joint but the relationship with OA disease burden cannot be fully established by this study. Many factors including physical activity, disease status in other joints and patient muscle mass, could cause single time-point results to become less reliable and impact on the findings of both this and all OA biomarker studies.

Conclusion

This study demonstrates that ARGS neoepitope shows some promise as an OA biomarker and provides important information on conducting future studies assessing this and other biomarkers in OA.

Acknowledgments

On behalf of myself and other participants in the study I would like to express my gratitude to Innovate UK (formerly known as Technology Strategy board) and Glaxo Smithkline (GSK) for providing funding for this project which has benefited me enormously and provided me with an excellent experience in the research environment.

First and foremost I would like to thank my supervisors Professor Peter Taylor and Doctor Sarah Snelling for the opportunity to be involved in the project and for their overwhelming support in what has been a very intensive study. It has been a great privilege to conduct this study under the academic support and supervision of such accomplished and supportive academics, and I have also benefited from the wider exposure to other accomplished academic clinicians whilst conducting this project. This project has been quite a step into the unknown for a clinician with no prior research experience like myself and I have always felt very well supported both academically and professionally. The experience has also taught me about other aspects of life and being back at college has been a great experience for me.

Secondly saying this I would also like to convey my sincere gratitude to GSK in particular Doctor Jon Larkin and Doctor Katherine Sully who have been key to the success of the project and provided great support throughout. Alongside them, the team at GSK have had to put in a tremendous amount of work to process and analyse the results of nearly a thousand samples which now make up the results of this study.

Thirdly to Imageanalysis™ in particular Doctor Mark Hinton but also to the various members of the team who were required to develop the software in tandem with the analysis of the results in order to facilitate and translate MRI scans into reliable imaging outcome statistics. They made themselves constantly available and were very capable of remotely accessing and adjusting the software.

Furthermore the project just simply would not have been possible without the support of the Oxford Musculoskeletal Biobank who provided a framework for collecting the samples, including staff to retrieve and process the samples, and then store and catalogue the thousands of samples collected during this project.

I must also acknowledge and thank the clinical staff of the Nuffield Orthopaedic Centre who provided access to patients and in particular the surgeons who facilitated collection. Professor Andrew Price, Mr William Jackson, Mr Hemant Pandit and Mr Adrian Taylor all provided surgical samples without which the project could not have proceeded. I would also like to acknowledge extra contribution made by Professor David Murray and Mr Hemant Pandit in providing Oxford Knee Scores for patients preoperatively which added an extra dimension to the results.

I would just like to say in finishing that the University of Oxford and the Biomedical Research Centre have provided an invaluable life experience in research that I hope to expand on in my clinical career so that we can continue to improve the level of treatment we can offer to patients.

And finally a mention of my gratitude towards the patients and NHS staff who were able to help me complete this study.

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Chapter 1 Introduction

1.1 Osteoarthritis

1.1.1 Definition of OA

Osteoarthritis (OA) is a misnomer in as much as it implies an inherently inflammatory process. It has enjoyed many decades of use in the English-speaking world and will probably continue to do so because it has greater popular appeal than the more accurate term of degenerative joint disease. OA does in fact demonstrate a low level inflammatory process involved with degradation of cartilage, albeit not of the same magnitude as classically inflammatory arthritides such as rheumatoid arthritis[1].

OA is characterized by focal and progressive loss of the hyaline cartilage of joints with associated bony changes. The centre for disease control and prevention (CDC) defines OA as a disease of the entire joint involving the cartilage, joint lining, ligaments, and underlying bone[2]. OA can similarly be defined as a heterogeneous group of conditions that lead to joint symptoms and signs associated with defective integrity of articular cartilage, in addition to the related changes in the underlying bone and at the joint margins[3].

Diagnostic criteria for classification of knee OA were put forward by Altman *et al*. (Table 1)[3] and later updated and extended to cover a large array of joints[4].

Table 1 Criteria for classification of primary knee OA

Clinical and laboratory	Clinical and radiographic	Clinical§
Knee pain + at least 5 of the following 9: Age > 50 years Stiffness < 30 minutes Crepitus Bony tenderness Bony enlargement No palpable warmth ESR < 40 mm/hour RF < 1:40 SF OA	Knee pain + at least 1 of the following 3: Age > 50 years Stiffness < 30 minutes Crepitus + osteophytes	Knee pain + at least 3 of the following 6: Age > 50 years Stiffness < 30 minutes Crepitus Bony tenderness Bony enlargement No palpable warmth
92% sensitive	91% sensitive	95% sensitive
75% specific	86% specific	69% specific
*ESR = erythrocyte sedimentation rate; RF = rheumatoid factor; SF OA = synovial fluid signs of OA (clear, viscous, or white blood cell count < 2,000/mm^3).		
§ Alternative for clinical category would be 4 of 6, which is 84% sensitive and 89% specific.		

Essentially the definition of OA remains descriptive and significant issues persist over where symptomatic and diagnostic cut-offs should lie. Since quantifying the disease has proved technically difficult and there is no globally-accepted grading system of OA, the disease is often defined clinically in terms of treatment rationale; early (no intervention) or late (arthroplasty if required).

1.1.2 Origins of osteoarthritis

Fossil records demonstrate chronic arthritis existed six hundred million years ago making it one of the oldest diseases for which we have definite evidence and examination of mummies from ancient Egypt demonstrates lesions consistent with rheumatoid arthritis and OA[5]. The historical story of OA is somewhat imprecise due to the tradition of classifying the disease with the historical grouping of all deforming arthritides coming under the classification of rheumatism or gout. The word “rheumatism” first appeared in 1570 as a name for acute arthritis, whereas “gout” is believed to have been used by Alexander of Trailles in the 6th Century. The majority of historical references to arthritides refer to gout, a

disease traditionally associated with the affluent, which was therefore more frequently documented in times when writing skills and paper were a scarce resource. This classification was altered by the work by William Herberden who denounced the link with gout[6]. In continental Europe, Charcot and Virchow, the fathers of cellular pathology, used the term ‘‘arthritis deformans’’ for both rheumatoid and osteoarthritis[7]. A major advance in the appreciation of degenerative joint disease as a defined entity came with the description of malum coxae senilis, osteoarthritis of the hip, by Robert Smith in 1835 who distinguished OA from polyarticular rheumatoid arthritis by its localised character[8]. Soon after the introduction of X-rays in 1895 there was a shift in thinking, which was first documented in 1897 by Goldthwaite who distinguished chronic arthritides into two categories, rheumatoid arthritis and osteoarthritis. Clinically they appeared distinct in that rheumatoid arthritis was atrophic and polyarticular whilst osteoarthritis was often monoarticular with hypertrophic features around the joint[9].

1.1.3 Epidemiological and economic impact of OA

The epidemiological and economic figures for OA are complicated by the lack of clearly-defined criteria for diagnosing the disorder. In the UK, NICE has estimated the prevalence of OA to be 2.8 million and Arthritis Research UK estimated that 550,000 people have moderate to severe OA of the hip and/or knee. At least 33% of people over the age of 55 have radiographic evidence of knee OA[10-12] and in 2010/11 there were 181,350 admissions for hip and knee OA[13]. In the UK the lifetime risk of a total knee replacement is climbing and has been estimated at 10.8% in women and 8.1% in men[14].

Economically, direct costs of OA treatment (surgery, analgesia and the costs of managing the complications) are in excess of £1 billion annually in the UK[15]. An estimated further £3.2 billion is lost in indirect costs as a result of 36 million lost working days; furthermore in

2001 incapacity benefit of £2.4 billion was paid to those unable to work due to OA[15]. Conaghan *et al.* suggest that the overall burden in the EU is 0.5% of GDP annually, reflecting medical, social and loss of productivity costs[16].

1.1.4 Ageing in OA

Cartilage undergoes structural and physiological changes with ageing. Biologically, cartilage becomes more crosslinked and brittle with age[17]. There is increased matrix metalloproteinase- (MMP-) mediated cartilage damage with ageing and cathepsin K has also been implicated[18]. Ageing is also associated with a reduction in the number of chondrocytes[19, 20] and there is evidence of chondrocyte senescence[21]. Chondrocyte senescence may contribute towards the decline in the ability of chondrocytes to respond to growth factors, resulting in the anabolic/catabolic imbalance evident in OA[22]. Increasing levels of advanced glycation end products (AGE) have also been cited as contributors towards age-related degeneration[23, 24], enhancing IL-6 and MMP-13 levels whilst reducing expression of type II collagen, the major collagen of cartilage[25]. TNFα may also be increased in response to the higher levels of AGEs[26]. The connection between TNFα and OA remains unclear[27]; however a modest correlation between age and serum TNFα has been reported[28]. Furthermore there is evidence for the role of oxidative damage by reactive oxygen species (ROS)[29] preferentially disadvantaging aged cells[30]and leading to damage to chondrocyte DNA[31]. There is also evidence that expression of pro-inflammatory genes in meniscal cartilage is higher in younger patients than in older patients[32] which may result in increased extracellular matrix turnover. In animal models, age affects the pattern of gene expression in mice with surgically-induced OA, leading to an age-related decrease in matrix gene expression[33]. Findings in primates and equine models demonstrate a reduction in the sensitivity of chondrocytes to IGF-1[34] [35]. Given the

impact of age on chondrocyte activity and OA development, subject age must be taken into account when interpreting measures of OA.

1.1.5 Body composition and OA

Lean body mass, skeletal muscle mass and body composition are related to OA although the pattern of this is not clear. It is well established that female sex, increased age and increased body mass index (BMI) are major risk factors for knee OA[36-40] (both symptoms and radiographic changes). Risk for knee OA is increased almost 4-fold in obese women[41]and 4.8-fold in obese men[42]. There is a linear relationship between body weight and knee OA[43], with risk for knee OA increasing by approximately 15% for each additional unit of BMI increase in people with BMI > 27 kgm^2[42]. Although lean body mass increases with BMI[44, 45], it is sarcopenic obesity which has been more closely associated with knee OA than non-sarcopenic obesity[46]. Other work has shown that body composition is more reliable than BMI for explaining radiological evidence of OA (Kellgren Lawrence score)[47] and not as well correlated as BMI for osteophytogenesis in another[48].

Skeletal muscle mass has also been demonstrated to be protective against OA whilst fat mass is positively associated[47, 49]. The ratio of lean body mass to fat mass may be more important than absolute values of lean body mass[45, 50]. However although thigh muscle mass does not appear to protect against radiographic OA (ROA) extensor strength appears to[51] and OA has been associated with decreased muscle mass and alterations in muscle contractility[52]. On the other hand Eckstein *et al.* have shown no significant evidence that isometric muscle strength precedes or is associated with ROA of the knee[53].

The findings regarding the association between BMI and knee cartilage loss have been far from consistent, with previous studies suggesting no association with loss of tibial cartilage

volume[54], significant association with loss of patellar cartilage volume[55], or a significant association with loss of tibial cartilage volume only in those having greater cartilage volume at baseline[56]. These are in stark contrast to the consistent and significant associations between BMI, bone marrow lesions and cartilage defects[37, 57-59].

1.1.6 Biomechanics and knee OA

The medial compartment is by far the most affected compartment in knee OA[60], possibly because it normally bears 2.5 times as much load as the lateral compartment[61, 62]. Biomechanically the external knee adduction moment (EKAM) is the primary determinant of load distribution on the medial compartment of the knee[63], and it is a predictor of knee OA severity[64] and progression[65]. In addition, higher body weight has been associated with higher knee joint load and EKAM[66], but this finding has not been consistent in all studies[67]. Biomechanical studies have demonstrated a strong association with weight as the principal cause of more extreme medial compartment loading during obese gait. Thus, rather than thigh[68] or abdominal fat distribution, it seems to be the obese state that increases medial compartment loading[69].

However, joint overload fails to fully explain the association between obesity and OA, as hand OA is twice as likely to occur in obese patients[70]. Obesity-related cytokines may enhance the activity of MMPs causing cartilage degradation[71]. Expression of COX-2, IL-1β and PGE_2 are increased when cartilage explants are exposed to mechanical stresses[72] and IL-8/Kc expression is stimulated by mechanical stretching in fibroblast-like synoviocytes[73]. Overall it is difficult, given the current evidence, to fully establish whether obesity is a causative factor of OA or a result of degenerative disease and sedentary lifestyle or, more likely, a combination of both. However many studies correct for BMI when analysing the

efficacy of their results[74] and this information is crucial when evaluating any biomarker given the significance of the role of BMI in OA.

1.1.7 Treatment of knee OA

Broadly speaking, treatment of knee OA can be divided into 3 categories; conservative and lifestyle, pharmacological and surgical. Conservative treatment in the form of exercise can provide moderate short term improvements in pain and function at least equivalent to treatment with non-steroidal anti-inflammatory drugs[75] and is recommended by the OA research society international (OARSI) as an intervention suitable for all patients with OA[76]. In terms of weight management there is good evidence to suggest that weight loss can reverse early cartilage change[77] and can reduce pain and levels of cartilage oligomeric matrix protein (COMP), a commonly-investigated marker of cartilage degradation[78]. OARSI recommend both oral and topical NSAIDs in specific subtypes of OA to reduce pain and improve function[76]. Although some studies advocate the efficacy of glucosamine[79] and intra-articular hyaluronic acid injections[80], these are not currently recommended by OARSI. For end-stage OA the mainstay of treatment remains arthroplasty, and knee arthroplasty, although a major surgical procedure, has a satisfaction rate of 82–89%[81].

1.2 Overview of cartilage structure and degradation

1.2.1 Articular cartilage

Articular cartilage, otherwise known as hyaline cartilage, consists of an avascular extracellular matrix (ECM) principally composed of water and protein. It contains one cell type, the chondrocyte, that is responsible for both synthesis and degradation of the ECM. Type II collagen and proteoglycans provide the molecular basis for the load-bearing and friction-reducing function of articular cartilage. Chondrocytes obtain nutrients and oxygen

via diffusion from synovial fluid and subchondral bone, and waste products of bone degradation and cellular metabolism are cleared via the same process[82].

Articular cartilage is characterised by its limited ability to self-repair and chondrocytes are not thought to significantly change in number in normal adult cartilage[83, 84] However mitosis may occur in diseased or traumatised cartilage presumably as a repair response and a decline in chondrocyte number is reported with ageing[85-87].

Chondrocyte activity alters in response to mechanical stimulus with noticeable changes in the secretion of ECM constituents, inflammatory cytokines and matrix-degrading enzymes[88]. Indeed the duration, force and frequency of mechanical loading can positively and negatively influence the synthesis of ECM with higher static forces leading to a reduction in ECM production[89, 90].

1.2.2 Aggrecan

There are 3 broad groups of proteoglycan and the ones found in the ECM of articular cartilage are the chondroitin sulphate group of which aggrecan is the major proteoglycan[91].

Proteoglycans create large osmotic pressures and draw water into cartilage. Negatively-charged anionic groups on the glycosaminoglycan (GAG) chains draw in positive ions producing an osmotic imbalance as the proteoglycan molecule is too large to freely move and re-distribute itself. This water-swollen matrix is biomechanically compressible, but due to the presence and interaction of collagen, the network is resistant to deformation[92].

Aggrecan consists of a core protein 250-kDa in size which is attached covalently to chondroitin sulphate and keratan sulphate GAG chains. Aggrecan core protein has three

major globular domains (G1, G2, and G3) (Figure 1). Between the G1 and G2 domains there is an inter-globular domain (IGD), which is the major site of cleavage by MMPs and aggrecanases [93].

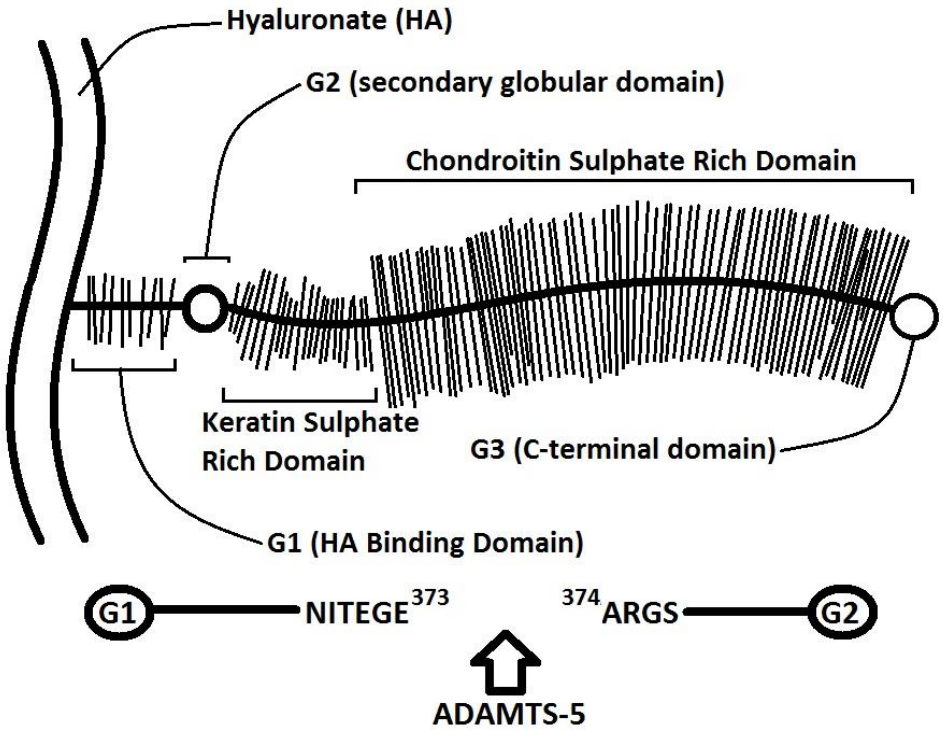

Figure 1 Aggrecan interacting with hyaluronan and the ADAMTS-5 cleavage site

1.2.3 Aggrecan proteolysis and ADAMTS-5

The turnover of aggrecan has been estimated to be 3–120 days *in vivo*[94] and 3–20 days *in vitro*[95]. Both chondrocytes and synovial cells are sources of the MMPs and aggrecanases involved in the healthy turnover of cartilage[96, 97]. The discovery of the family of proteins known as a disintegrin and metalloprotease with thrombospondin-like motifs (ADAMTS) which are involved in the inflammatory process[98], led to further research which revealed that some of these enzymes, namely ADAMTS4 and ADAMTS5, had critical roles as aggrecanases[99]. Several aggrecanase cleavage sites have been identified in the aggrecan

core protein and in the context of ADAMTS-5 this is at Glu373–Ala374, producing fragments containing two neoepitopes; PRNITEGE373 and 374ARGSVI[100, 101].

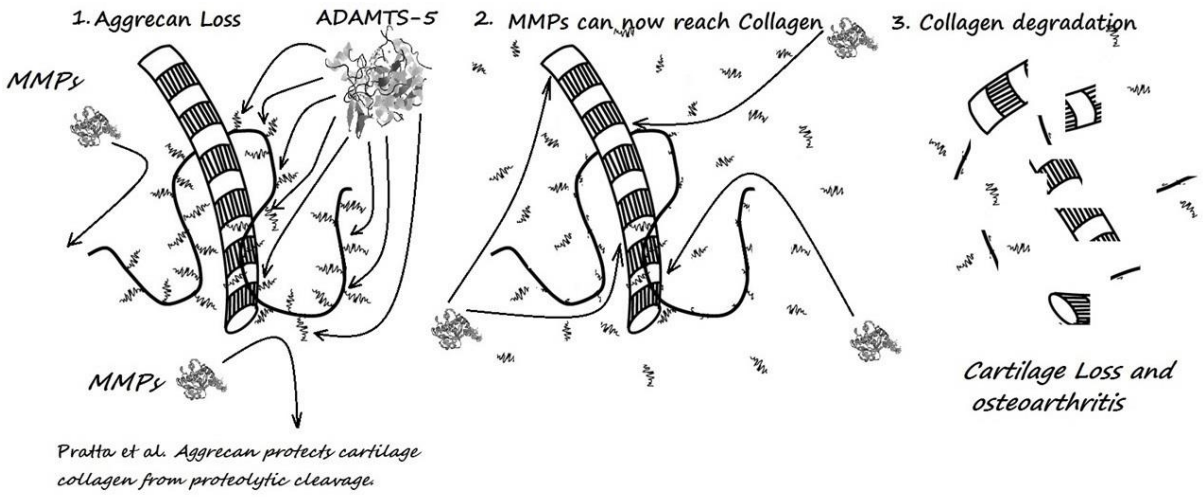

Figure 2 Degradation of collagen and aggrecan in OA

Aggrecan may prevent breakdown of type II collagen by blocking access to the collagen matrix (Figure 2)[102] and in mice the ADAMTS-5 enzyme has been shown to be the major aggrecanase involved in cartilage breakdown[103]. Consequently there has been an emphasis on trialling preclinical inhibitors that target ADAMTS-5 activity, and on establishing a suitable human model for such trials[104].

1.2.4 Aggrecan biomarkers

It has been determined previously that the ARGS neoepitope is increased in human knee OA and that this is a more specific marker than non-cleavage derived aggrecan measurements[105, 106]. However the ARGS neoepitope was not associated with radiographic progression of knee OA in patients with previous meniscectomy on plain radiography[107]. Aggrecan loss has been determined to be a reversible effect of cartilage damage in OA making its potential role as a biomarker even more valuable[108]. Although serum and urinary ARGS neoepitope levels have been reported to be increased in patients

undergoing total knee replacement compared to healthy controls[109], another study demonstrated than non-progression of OA was likely to be associated with higher levels of ARGS neoepitope[110].

1.2.5 Models of cartilage degradation

Cartilage explant experiments allow the *ex vivo* evaluation of the physiological behaviour of chondrocytes in response to potential disease therapeutics either from an organic source[111, 112], nutritional supplement[113] or a specific artificial therapeutic[114]. Therapeutic agents that alter the degradation of cartilage by both MMP-selective[115] and non-selective inhibition[116, 117] have been investigated with measurable decreases in enzymatic end products of MMPs.

Chondrocyte behaviour changes within the same joint depending upon the condition of the surrounding cartilage[118]. This suggests that within a joint there is a localised process accounting for at least some of the biological effects of OA, and studies have shown that macroscopically normal cartilage from an OA joint does not have the same protein signature as cartilage from a non-OA joint[119]. Therefore *ex vivo* and *in vivo* response to therapeutics may vary dependent upon the microanatomical source of explanted cartilage.

Chondrocytes can also be collagenase digested from cartilage explants and cultured in monolayer for *in vitro* studies. Advantages of monolayer culture include removal of effects caused by differences in the protein content of different areas of the joint and their greater utility for population expansion[120]. However monolayer chondrocytes can de-differentiate impacting on their response to stimuli and their representation of an *in vivo* cell[121, 122].

1.3 Measurement of OA

1.3.1 Imaging and outcome measures in OA

The broad definition of OA and lack of any biological marker reaching clinical efficacy in its diagnostic or prognostic attributes[123] has meant imaging is the most utilised outcome measure for OA clinical decision making and research. Clinical outcome scores or questionnaires have been devised based upon areas of importance to patients and are known as patient recorded outcome measures (PROMS). At present there are 4 scores of high prevalence out of 47 instruments (KSS – Knee Society Score, OKS – Oxford Knee Score, KOOS – Knee Injury and Osteoarthritis Outcome Score and WOMAC – Western Ontario and McMaster University Arthritis Index) which have been administered to patients as PROMS and a recent review was unable to determine which has the best validity[124].

1.3.1.1 Plain radiography

Sub-classification of rheumatological diseases in terms of anything other than joint pain and functional deficit took a large leap forward after Roentgen's discovery of X-rays. OA-associated radiological changes were formally classified by Kellgren and Lawrence in 1957[125] and this has become the cornerstone of how we objectively view OA. Plain radiography remains very much a relevant and useful tool for both diagnosis and treatment pathway decisions for advanced OA.

However, much of the evidence is inconsistent and studies show both reliable[126] and unreliable relationships[127, 128] between radiographic measures and cartilage status at arthroscopy. Joint space narrowing (JSN) does give a good indication of surgical prognosis and risk of undergoing knee replacement[129]. Originally JSN was thought to only represent cartilage thinning but it is now known that meniscal extrusion can contribute to this change[130-132].

Clinically, the occurrence of nocturnal knee pain has been reported as 3.6%, 6.9%, 19.4%, 32.7%, and 75.0% in Kellgren Lawrence grades 0, 1, 2, 3, and 4 respectively[133]. However in regard to clinical outcome scores both JSN and tibio-femoral cartilage loss were found to be poor predictors of KOOS and WOMAC scores, although JSN and tibio-femoral cartilage loss measures were highly correlated[134]. A literature search does not reveal any studies relating OKS and imaging.

1.3.1.2 MR imaging

Plain radiography has remained the gold standard of OA clinical imaging whilst other modalities such as MRI have received little application outside of research except to exclude co-existing pathology. The reason for this is a combination of practicality, availability and actual necessity, yet markers from MRI are likely to be more sensitive than radiology in detecting joint changes in OA[135]. Recently MRI has received significant research interest in the evaluation of OA as it may be more sensitive at diagnosing early disease[136]. MRI has been shown to predict joint replacement surgery[137] and be superior to JSN in predicting knee replacement[138]. Furthermore, intervention at an early stage can affect quantifiable measures of cartilage pathology on MRI, such as volume and signal intensity. One recent study has shown a linear relationship between weight loss and reduction in medial cartilage volume loss[139]. The reversibility of cartilage pathology associated with OA has been demonstrated using MRI[77, 140] and this would not be possible with standard radiographic measures.

1.3.1.2 Bone scintigraphy

Other imaging methods have been useful in quantifying the burden of disease. Addison *et al.* identified a link between bone scintigraphy and COMP biomarker, and demonstrated that the biomarker is representative of the burden of disease[141].

1.3.1.3 Imaging summary

Imaging is often referred to as a structural biomarker and provides one of the most important outcome measures in clinical OA. Furthermore, imaging measures are essential when assessing the utility of serological markers of disease while allowing some degree of inter-patient comparison. Table 2 shows the relative strengths and weaknesses of plain radiographs and MRI. There are few comparative studies and a recent summary of a debate on the strengths and merits of these forms of imaging concluded that studies, if possible, should include both modalities such that their relative performance can be assessed[142]. It must be remembered that in comparison to radiography the evidence base for MRI is smaller and it is not as practical a tool to implement in a clinical or research environment, however it does provide significantly more information.

Table 2 Comparison of imaging modalities

	Plain Radiograph	MRI
Primary outcome	Joint space width loss	Cartilage volume loss
Positioning	Crucial for reproducibility	Relatively unimportant
Clinical outcome correlation	Poor	Poor when using cartilage volume Good when using synovitis, effusion
Versatility	Joint space, osteophytes, sclerosis	Meniscal, ligaments, bone marrow lesions, alignment, signal intensity measurements, synovitis
Cost & availability	Cheap, Widely available	Expensive, availability more restricted
Evidence base	Extensive	More restricted, especially longer term studies

1.3.2 Patient-recorded outcome measures (PROMs)

Research in many areas of medicine and surgery has shown that the patient can provide reliable and valid judgements of their health status and of the benefits of treatment[143]. As a result, evaluation of the outcome of knee surgery has led to the development of multiple PROMs including the OKS[144] and the WOMAC[145]. Furthermore scores have been devised to assess types of pain such as neuropathic pain (painDETECT)[146], and pain specific to OA (ICOAP)[147]. These outcome scores have been used to evaluate imaging[148] and biological markers[149] as well as the efficacy of OA surgery and non-surgical treatments. Evaluation of clinical measures as well as structural markers could provide a more comprehensive interpretation of serological markers, and is crucial to improving the decision-making process of OA. Multiple studies however have failed to show a relationship between imaging and clinical measures[134, 150-154] or function[155] and one study has demonstrated a slight association between decreased cartilage and less pain[148]. However other studies have correlated PROMs to other MRI features including meniscal injury[156], bone marrow lesions[157] and effusion[158].

1.4 Biomarkers

1.4.1 Overview

Healthy cartilage undergoes constant turnover of ECM components and this equilibrium between degradation and synthesis is disrupted in OA tissue[159]. Early detection and objective evaluation have been critical barriers to development of a prevention strategy in OA. Fragments of structural ECM proteins can be detected in synovial fluid, blood and urine and are the basis of many serological biomarkers of OA. However these ECM-derived biomarkers may also be associated with bony changes and cartilage synthesis. The National Institutes of health (NIH) define a biomarker as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes or pharmacological responses to treatment"[160]but they can also be thought of as risk factors for disease. Currently about 20 biomarkers of blood and urine are being trialled in OA for at least one of the following criteria: burden of disease, investigative, prognostic, efficacy of intervention and diagnostic (BIPED criteria)[161]. However, although many studies show significant differences in the detectable levels of these biomarkers between OA patients and controls there is still considerable overlap in their absolute values. Van Spil *et al.* demonstrated in a meta-analysis that around 50% of the trialled biomarkers demonstrated efficacy according to BIPED criteria. However none of these biomarkers were sufficiently discriminating to aid diagnosis or prognosis of OA or performed consistently enough to act as an outcome for clinical trials[162]. Given the lack of singularly discriminatory biomarkers it is likely that measuring a combination of biomarkers may improve disease prediction and clinical utility[123].

1.4.2 Creatinine and urinary biomarkers

Levels of urinary biomarkers vary significantly between individuals due to: age[168], gender[110], burden of disease within the body[141], ethnicity[169], medication[170], diurnal patterns[163, 164, 167, 171], and activity[163, 165, 166]. Correction for these variations are made by dividing biomarker values by each sample's creatinine level[164, 171-176]. This method is effective if the mechanism of renal excretion of biomarker is similar to that of creatinine[177] which is not currently established for most OA biomarkers. Creatinine level varies diurnally within healthy individuals (without renal impairment) by as much as 6.6-fold and between individuals by as much as 9.2-fold[178]. Serum hyaluronic acid (HA) has been found to vary diurnally by 95%[163] and therefore there can be a large variation within a single individual especially when combining these two factors. Currently there are no published studies on the effects of correction of urinary or any OA biomarker by establishing patient lean body mass for which there is an established relationship to creatinine excretion[179]. Variation in 24 hour creatinine excretion in urine of 10.1–14.4% exists from day to day with a range of greater than 50% of the mean even after adjusting for lean body mass[180]. The creatinine excretion rate is higher for men than for women[181], decreases with age[181], and increases with exercise[182, 183], muscle mass[184], and intake of meat[185-187]. Notably many of these factors also influence OA. Furthermore the excretion rate varies due to diurnal causes decreasing significantly at night[188], seasonal factors[189] and menstruation in women[190, 191].

Heerspink *et al.* found that first morning albumin-to-creatinine ratio (ACR) was modestly stronger at associating with clinical outcome (renal impairment) than several other methods including the gold standard (24 hour urine collection) in diabetics[192]. Further value of the marker was added by using creatinine for correction which, at lower concentrations in urine,

is associated with adverse cardiovascular events and mortality in diabetic patients as low muscle mass (and hence lower creatinine excretion) is associated with more advanced disease, i.e. is also a biomarker for diabetic renovascular disease[193, 194]. In some papers correction of urinary biomarkers with creatinine[195] has raised the possibility of it being a confounding factor as creatinine can be linked to the disease process for which the biomarker is being evaluated[196, 197]. It may well be the case that creatinine which is related strongly to muscle mass may be acting as a secondary marker for OA especially as many papers have cited a connection between these two factors. It is therefore worth considering these factors when analysing and interpreting the results of urinary biomarkers.

1.4.3 Exercise and OA biomarkers

Transient but significant decreases in the level of OA biomarkers or related cytokines (COMP, IL-10) for several hours after exercise with gradual resolution has been observed[165, 166]; this suggests that there is reasonable clearance of these proteins from the blood in healthy individuals. Gordon *et al.* did not demonstrate significant correlation with activity for c-telopeptide of type II collagen (CTX-II)[163] and cited high renal clearance of this biomarker as a possible reason. On the other hand creatinine excretion is also affected by the level of physical activity, with increases of 50%[182] and 60–80% during strenuous activity[198] although subsequent work has indicated that higher intensity exercise resulted in lower creatinine excretion than lower intensity exercise[199]. Furthermore creatinine excretion is higher in more active rather than sedentary individuals[200]. In patients experiencing a greater degree of pain the levels of activity could be affected, thereby influencing both creatinine excretion and OA biomarker production.

1.5 Summary

OA is a leading cause of disability in developed countries[201] and has been established as an independent risk factor for mortality[202]. A report from a EULAR ad hoc expert committee defining OA research priorities identified four priority research areas, including imaging and biomarkers, stating “Robust biomarkers are required for both improving clinical trial outcomes and stratifying interventions”[16].

When characterising any novel biomarker there should be comparison and correlation against existing measurements of disease. In OA these standards are clinical outcome measures and imaging as no biological marker has yet been validated to such an extent that it can be considered a benchmark of OA.

1.6 Overview of project

1.6.1 Overall aim

A disorder can be diagnosed when the best and earliest effective tests are positive. In OA, the degeneration of cartilage is likely to be initiated years before the onset of symptoms by which time the process of reversing the pathobiology is impossible. Systematic biomarker research in OA is crucial in generating a system of diagnosis and objective evaluation especially in a disease process which remains poorly defined and understood.

This project seeks to investigate the utility of the ARGS neoepitope as a novel biomarker in patients with early OA or post-injury OA and in end-stage OA patients undergoing arthroplasty. The ARGS neoepitope will be assessed for capacity as a biomarker of disease burden and for value in determining potential interventional outcome.

Evaluation of ARGS neoepitope released from cartilage explants treated with a humanised anti-ADAMTS-5 monoclonal antibody (mAb) will ascertain the potential of this mAb as an OA therapeutic (Figure 3) [203, 204].

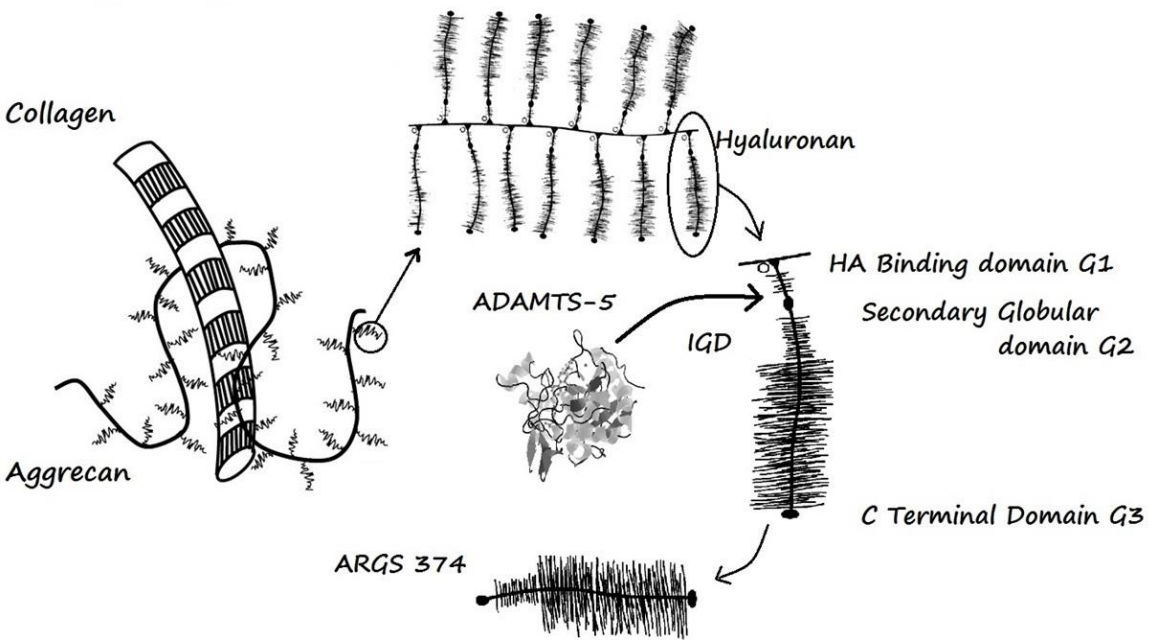

Figure 3 Measuring activity of ADAMTS-5

1.6.2 Project objectives & hypotheses

Primary

1. Evaluate ARGS neoepitope levels in blood and urine of patients with early and late OA pre- and post-surgery and compare them to synovial fluid levels.

2. Compare ARGS neoepitope levels to imaging biomarkers obtained from analysis of pre-operative MRI scans.

3. Evaluate the ARGS neoepitope release of *ex vivo* cartilage explants treated with an ADAMTS-5-specific mAb.

Secondary

1. Characterise the temporal and diurnal variation of serological ARGS neoepitope levels.

2. Evaluate performance of the ARGS neoepitope against PROMs in end-stage OA.

3. Evaluate the performance of imaging biomarkers against PROMs in end-stage OA.

Broad hypothesis:

Serological levels of ARGS neoepitope are related to extent of imaging-derived OA severity in human knee disease.

Null hypothesis: There is no relationship between level of OA defined by loss of cartilage volume in human knee disease and serological levels of ARGS neoepitope.

Specific Hypotheses

i) An elevation of serological levels of ARGS neoepitope is associated with increased cartilage loss on quantitative measurement from corresponding MRI.

Null hypothesis: There is no association between serological levels of ARGS neoepitope and increased cartilage loss on quantitative measurement from corresponding MRI.

ii) Explants treated with ADAMTS-5-specific mAb will suppress levels of ARGS neoepitope release compared to isomer control.

Null hypothesis: Explants treated with ADAMTS-5-specific mAb will not suppress ARGS neoepitope release compared to isomer control.

Chapter 2 Methodology

2.1 Study Design

2.1.1 Study overview

This is a prospective observational cross sectional study investigating the efficacy of a novel biomarker, the ARGS neoepitope, in the evaluation of OA in knee surgical patients. The overall aim is to establish whether serological levels of ARGS neoepitope reflect MRI outcome measures, in particular cartilage volume. We will also investigate the ability of an ADAMTS5-specific mAb to reduce the production of ARGS neoepitope from *ex vivo* human cartilage.

The study was split between 2 sites: patient enrolment, sample collection, follow up and early processing was performed at the Oxford site, then analysis of samples for ARGS neoepitope took place at the GSK Stevenage site. Data was collated in an encrypted database accessible to those within the research group, all patient details and identifiers were anonymised prior to samples entering research and this was overseen by the Oxford Musculoskeletal Biobank (OMB). Project samples were collected under the OMB ethics(Oxford REC09/H0606/11).

2.2 Study Design

The study involved a total of 50 subjects, including an 'End Stage OA' cohort of 30 subjects at the time of knee replacement surgery and an 'Early OA or injury' cohort of 20 subjects with minimal OA who were not anticipated to require joint replacement in the coming year. Surgical and thus diagnostic decision making was based on symptomology, clinical examination and imaging.

Sample size is not based on statistical sample size calculation, but on feasibility. All statistical analyses were fundamentally exploratory, although hypotheses were tested, and provide information for powering future clinical studies. Published data indicate that a sample size of 20 is sufficient in an exploratory study [2], since the precision gain is less pronounced after the sample size has reached 20. Hence, it was anticipated that at least 16 evaluable OA subjects were required to assess ARGS neoepitope utility as a biomarker. Additionally, 20 early OA patients were recruited and sampled to evaluate the quantifiability and characteristics of the biomarker in another group at an earlier stage of disease. A timetable is shown in Figure 4.

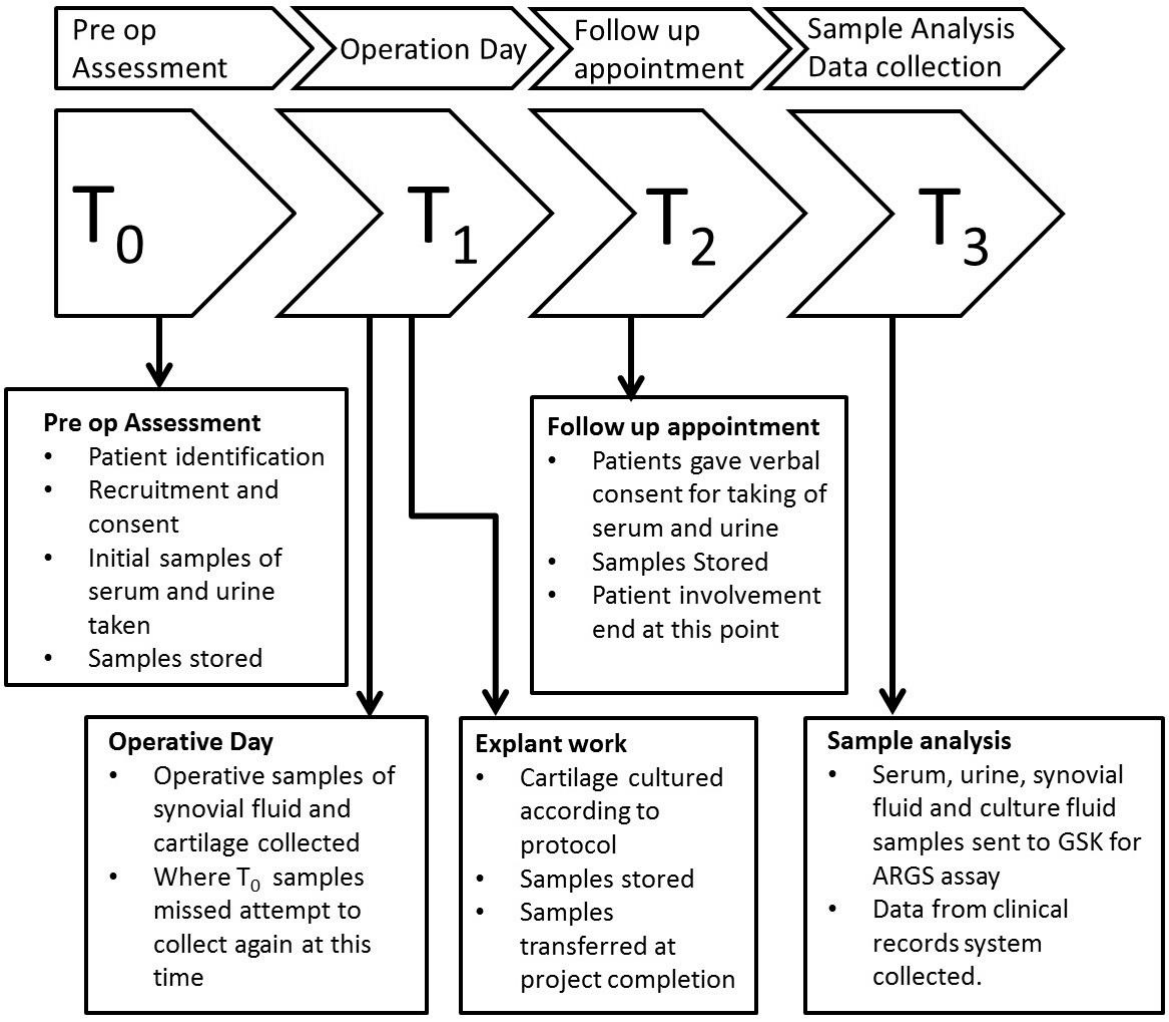

Figure 4 Sample collection timeline

Patients were consented on the basis that they met inclusion/exclusion criteria (see section 2.1.4) which was determined from the electronic patient record system. Serum and urine were collected and processed at preassessment(T_0) and stored (Figure 5). Operative samples were collected on the day of surgery (T_1) and a further serum and urine sample was collected at patient follow-up (T_2). Bone cuts were collected from the 'End Stage OA' cohort at surgery for *ex vivo* experiments. When all samples were collected, frozen samples were shipped to GSK for analysis on dry ice (T_3).

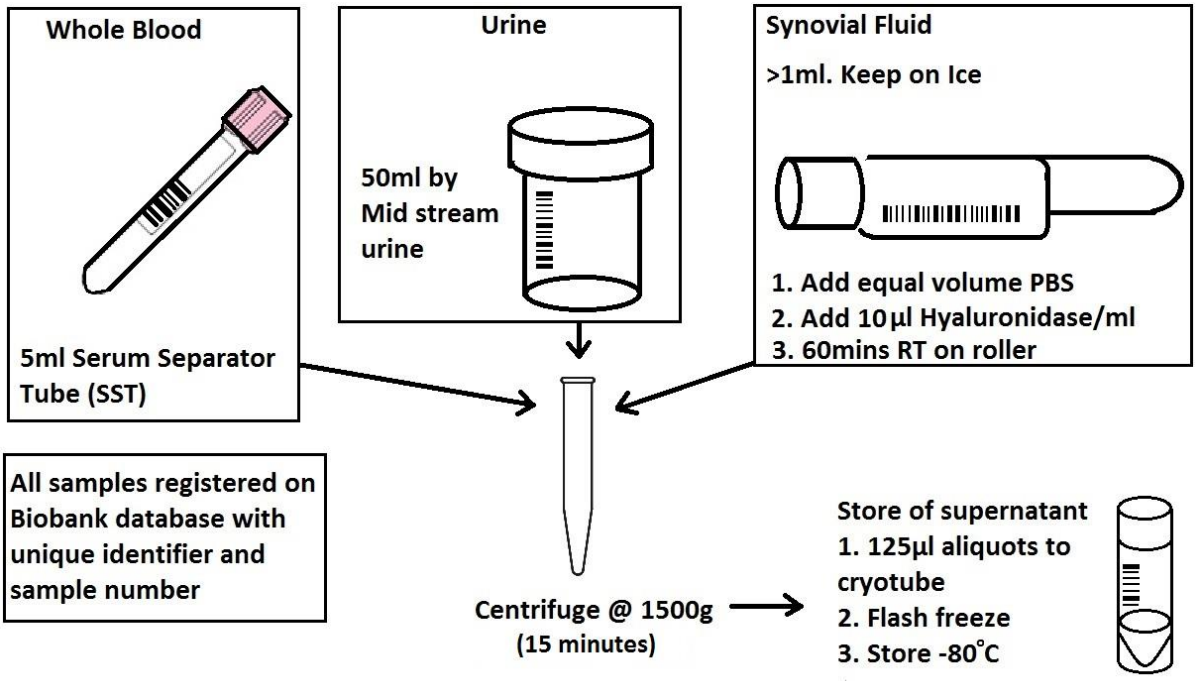

Figure 5 Sample processing for serological samples

In addition, MRI data were also collected. Predictors and potential confounders including age and BMI were collected in all cases. Previous MRI imaging studies revealed a link between cartilage volume and gender and height, and corrective models can be applied to adjust for this[205]. It was our intention to arrange sample collection for patients around the time of their medical appointments so as to enhance recruitment and hopefully

enhance collection of full sample sets. In a non-interventional study where there is no possible benefit to their enrolment in the study this is the most reasonable way to collect samples.

2.3 Outcomes

Primary outcomes: assessment of synovial fluid, serum and urinary ARGS neoepitope levels.

Secondary outcomes:

i) MRI-derived markers of OA including, but not limited to, cartilage volume and intensity.

ii) ARGS neoepitope release from cartilage explants treated with a novel monoclonal antibody against ADAMTS-5 (GSK2394002).

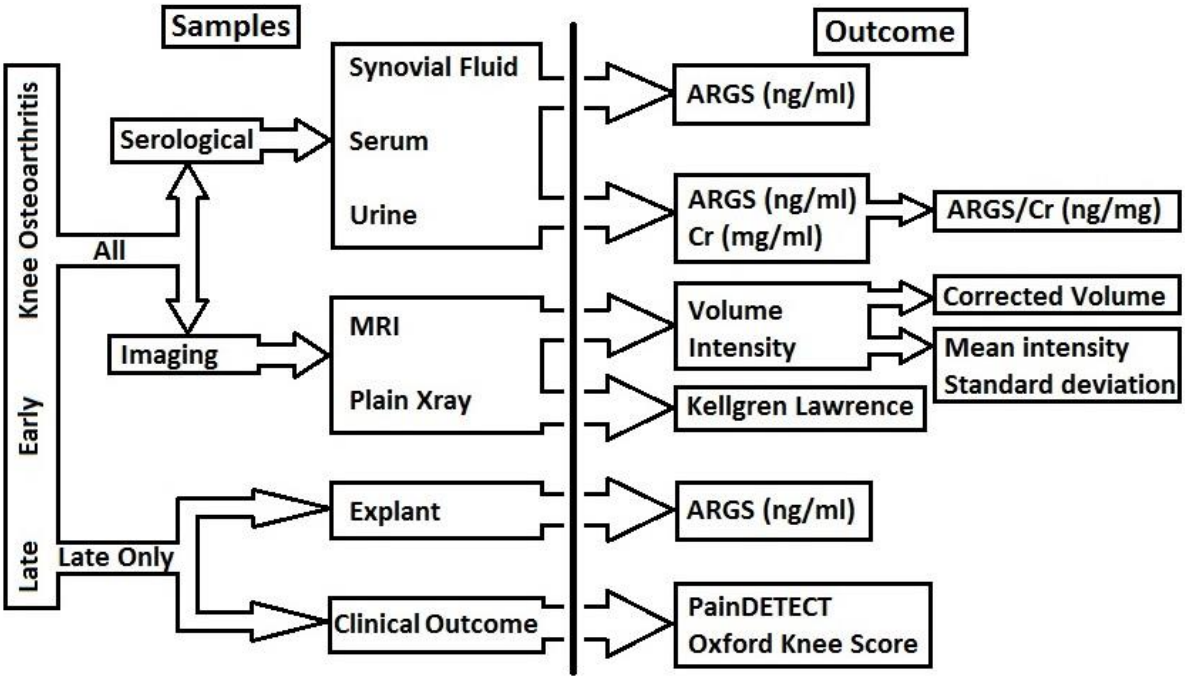

Figure 6 Diagrammatic representation of samples and outcomes

Additional specific measures include patient reported outcome scores principally the Oxford knee scores (OKS) and PainDETECT scores. Samples and outcomes are illustrated above (Figure 6).

2.3.1 Overview of data collected

A sufficient number of subjects were enrolled so that samples were obtained from at least 30 end-stage OA patients and 20 early OA/injury patients.

Patients recruited:	196
Patients included in study analysis:	95
Ex vivo explant study recruited:	31
Ex vivo explant study samples analysed:	18
Imaging and MRI analysis:	89

2.3.2 Study size

2.3.3 Inclusion criteria

A subject was eligible for inclusion in this study only if all of the following criteria applied:

1) Male or female subjects aged 18 or over at the time of signing consent.
    i) End-stage OA cohort: Diagnosis of OA and scheduled for partial or total knee replacement.
    ii) Early OA/injury cohort: Diagnosis of early OA or recent knee injury requiring surgical intervention. Generally regarded to be in 'good health', aside from the presence of OA, as determined by medical history and judgment of the investigator. Subjects with non-life threatening medical conditions (e.g. hypertension, asthma, diabetes mellitus) that are well controlled through medical or lifestyle management were eligible.

2) Capable of giving written informed consent, including compliance with the requirements and restrictions listed in the consent form.
3) Must have had an MRI scan of the affected knee in the year prior to signing the consent form.

2.3.4 Exclusion Criteria

A subject was not be eligible for inclusion in this study if any of the following criteria applied:

1) Use within previous three months of immunomodulators, corticosteroids, intra-articular hyaluronate, calcitonin, teriparitide.
2) Current history of rheumatoid arthritis, autoimmune disease, Lyme arthritis, psoriatic arthritis or any other non-osteoarthritis arthropathy.
3) Previous meniscectomy in affected joint.

2.4 Ex vivo assessment of ADAMTS5-specific mAb activity

2.4.1 Cartilage preparation

Cartilage was collected from theatre under the supervision of the consultant surgeon having verified the patient details and confirmed consent for tissue to be retrieved for research under project ethical constraints. Cartilage was processed in the tissue culture laboratory and handled with sterile instruments. Slices of cartilage of uniform thickness were removed from bone cuts (Figure 11) and cut with a leather punch into 3 mm explants (Figure 7) before distribution into a 96-well plate containing DMEM supplemented with 10% foetal calf serum (FCS), penicillin (100 $IUmL^{-1}$) and streptomycin (100 $mgmL^{-1}$) (Lonza Group Ltd, Basel, Switzerland). Samples were equilibrated for 48 hours at 37^{o}C and 5% CO_2.

After 48 hours incubation medium was replaced with fresh medium containing test compounds (Figure 8). Aspirated medium was stored at -80^{o}C for ARGS neoepitope analysis. Two hundred microlitres of medium, with or without test reagents, was added to the relevant wells and plugs were incubated for 72–96 hours before sampling of 100 µL of medium for neoepitope reagent analysis. A fresh 100 µL aliquot of reagent-containing medium was then added to the corresponding wells before a further incubation for 72–96 hours, the end of which constituted the next sampling time-point. The reagents used were: GRITS27601, which was an isomer control, GSK2394002, a specific ADAMTS-5 inhibitor and GSK571949, a small molecule enzyme inhibitor (GSK, Brentford, Middlesex, UK), concentrations used are shown in Figure 8.

After time-point 8 (24–28 days), plugs were stimulated with interleukin 1 (IL-1) and oncostatin M (OSM) for 48 hours to confirm response to cytokines and viability of cells. Plugs were disposed of in line with standard operating procedures and collected medium was stored at -80^{o}C prior to transfer for ARGS neoepitope analysis.

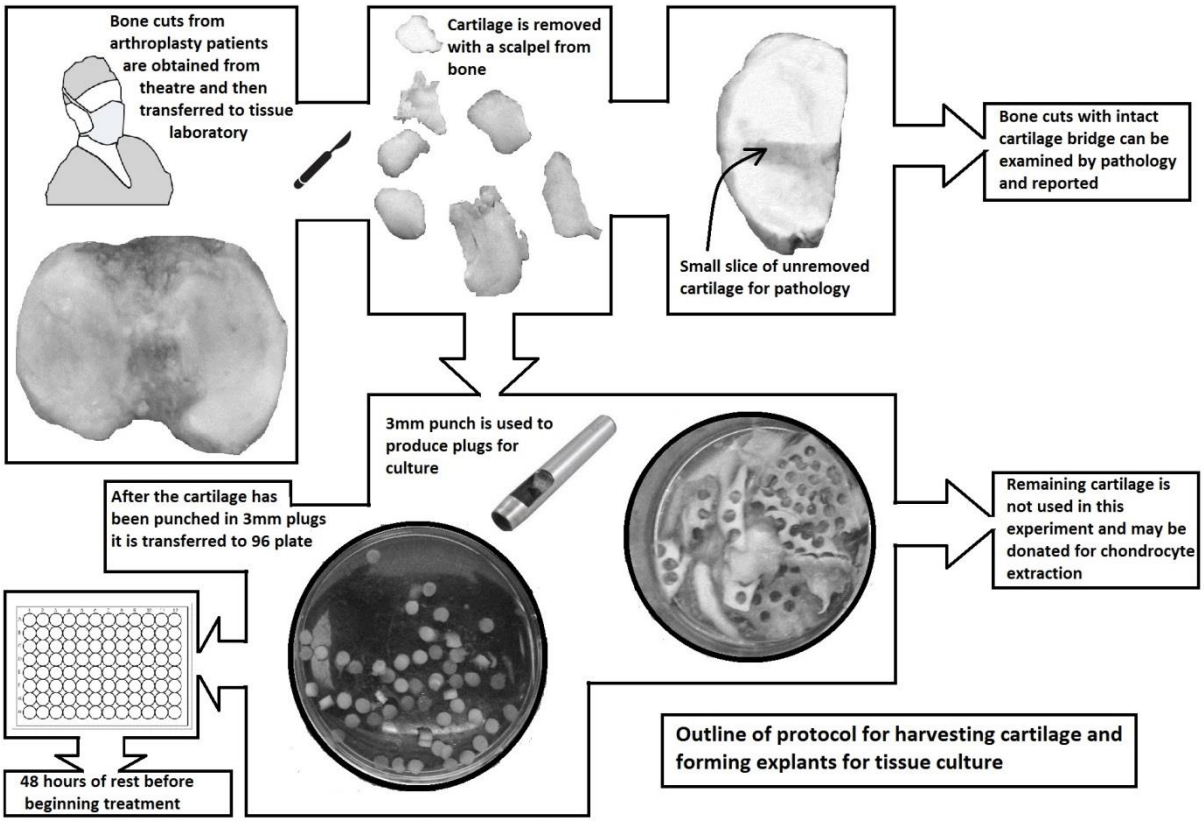

Figure 7 Process for generating explants

An overview of this protocol is shown in Figure 6 and full details are contained within appendix 1 (section 10.3.1).

<table>
<tr><td></td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td></tr>
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<tr><td>B</td><td>Sterile H_20</td><td colspan="9">200 µg/mL GRITS27601
(Humanised Isotype Control)</td><td rowspan="7" colspan="2"></td><td>Sterile H_20</td></tr>
<tr><td>C</td><td>Sterile H_20</td><td colspan="9">200 µg/mL GSK2394002
(anti-ADAMTS-5 monoclonal antibody)</td><td>Sterile H_20</td></tr>
<tr><td>D</td><td>Sterile H_20</td><td colspan="9">50 µg/mL GSK2394002
(anti-ADAMTS-5 monoclonal antibody)</td><td>Sterile H_20</td></tr>
<tr><td>E</td><td>Sterile H_20</td><td colspan="9">12.5 µg/mL GSK2394002
(anti-ADAMTS-5 monoclonal antibody)</td><td>Sterile H_20</td></tr>
<tr><td>F</td><td>Sterile H_20</td><td colspan="9">2 µM GSK571949
(small molecule inhibitor of ADAMTS-5)</td><td>Sterile H_20</td></tr>
<tr><td>G</td><td>Sterile H_20</td><td colspan="9">Control Medium</td><td>Sterile H_20</td></tr>
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</table>

Figure 8 Tissue culture plate layout for explant work.

2.5 Data sources and measurements

2.5.1 Serological samples

Samples were processed immediately after acquisition, blood was centrifuged and serum extracted; synovial fluid had hyaluronidase(1 U/mL) added along with an equal volume of Dulbecco's phosphate-buffered solution and all biological samples awaiting analysis were stored at -80^{o}C prior to transfer (Figure 5 and appendix 1). Samples were analysed by electrochemiluminescence immunoassay using meso-scale discovery (MSD) at GSK using a monoclonal antibody recognising the N-terminal neoepitope "374ARGS" (ARGS neoepitope) developed and tested by GSK [206]. In addition urine samples underwent analysis using a

plate-based colorimetric assay for urinary creatinine concentration (Cayman chemical co., Ann Arbor, MI, USA) according to the manufacturer's protocol. *Ex vivo* cartilage explant cultures generated eight time-point samples of medium that were stored at -80^{o}C until transfer to GSK for ARGS neoepitope analysis.

2.5.2 MRI analysis

2.5.2.1 MRI acquisition

MRI scans were obtained with permission from Oxford University Hospitals NHS Trust (OUH) under special dispensation from the Cauldicott Guardian. A secure link was used to download and anonymise patient scans at source so the DICOM files could then be analysed using Dynamica© software made by Image Analysis™ (Image Analysis, Lonodn, UK). MRI scans were clinical scans and therefore three different sequences of scan were used in this study (PD FS, PDW SPAIR, WATS).

2.5.2.2 Regions of interest (ROI)

MRI scans were analysed using Dynamica© software. Cartilage was manually delineated on each MRI slice creating a region of interest (ROI) (Figure 9).

Figure 9 Image analysis MRI ROIs

Each slice may contain several ROIs including the femoral, medial and lateral tibia and patellar cartilage. Any effusions were also segmented out to give an estimate of fluid within the joint. When each segment containing either cartilage or effusion had been analysed the software processed these ROIs collectively.

2.5.2.3 MRI compartments

Sagittal scans were processed by manually segmenting the different compartments of the knee. For the purposes of clarity the femoral volume was not separated into compartments but analysed as a whole; the rest of the knee cartilage was subdivided into medial and lateral tibia and patella. The effusion volume was also measured in all knees; however bone marrow lesions were not assessed.

2.5.2.4 Imaging variables

Following combination of ROIs, the data contained within each compartment were analysed (i.e. volume, minimum and maximum intensity, mean intensity and standard deviation of

intensity). Measurements of combined intensity and volume referred to as NORMI were obtained. Although figures for each slice within each compartment were calculated, overall we felt this data would be more difficult to correlate and used the overall readings for each compartment in the analysis.

2.5.2.5 Kellgren Lawrence scoring

Via the same system used to obtain MRI scans, plain radiographs of the knee being operated on were obtained. Although this was not included in the inclusion criteria as that may have limited potential participants for the project, almost all knee patients had plain films. Overall 84 patients had a corresponding plain radiograph leaving 11 without (Late n = 4, early n = 7).

2.5.2.6 User analysis

MRI and Kellgren-Lawrence analysis were performed by a single user who was blinded to the participant identification and received training for this purpose.

2.5.3 Patient information and clinical scores

Patient clinical outcome scores (OKS and painDETECT) were collected prospectively both before and after treatment, and additional data were collected retrospectively if relevant to the study, including medication, surgical findings, involvement of other joints with OA and other previous joint replacement surgery. Data on adverse events were collected prospectively although no adverse events were documented.

2.5.4 Study bias

To be eligible for the study the patients must have had a clinical MRI scan within one year of recruitment so the imaging could be quantified and compared against ARGS neoepitope levels. It would have been costly and also potentially drastically reduced recruitment to ask for MRI scans for research purposes and therefore it was decided to recruit only those with

pre-existing MRI imaging. This however may have led to bias as patients may have undergone scans for diagnostic or management uncertainty indicating a less advanced level of OA and potentially skewing the end-stage cohort.

2.6 Statistics

2.6.1 Statistical methods

Statistical analysis was performed using SPSS package version 20 (IBM Corp., Armonk, NY, USA) to identify variables which may be associated with the ARGS neoepitope levels in serum, synovial fluid and urine. The variables included X-ray, clinical outcome scores and measures derived from quantitative MRI analysis as well as patient clinical details and demographic data.

Univariate analyses were then performed on patient demographic data and biomarker information before looking at the potential associations with imaging data. Data were assessed for distribution and analysed accordingly. Significant differences between means were determined by independent and paired t-tests as appropriate using Levene's test to determine presence of normal variance. Pearson's test was applied to data sets to determine correlations. Multiple linear regression was used to analyse data sets where n values were greater than 30 to maintain accuracy.

2.6.2 Statistical representation

When representing the data we have used tables of figures with either R^2, mean values or median values if the data were discrete. For boxplots we used a standard output of the median with the box representing the first and third quartiles and the error bars representing the 95% confidence intervals. With scatter plots we have used Pearson's

correlation coefficient and a line of best fit to represent the data. Where there are significant differences or correlations, we have included in the figures a *P* value which is based on the results of a paired or unpaired t-test. A P-value of <0.05 was considered statistically significant.

2.7 Summary of risk management

Since this study did not involve administration of pharmacologic agents (investigational or non-investigational), no specific risks to subjects were anticipated. Blood samples were obtained from subjects by venipuncture, a procedure that is associated with minimal risk to subjects (primarily bleeding or bruising, irritation, redness at the venipuncture site). A synovial fluid sample was obtained from subjects in the end stage OA cohort at the time of knee replacement surgery and similarly patients undergoing non-arthroplasty procedures would have synovial fluid drawn after prepping the patient for surgery. Samples would be taken at the discretion of the surgeon. No additional risks to subjects due to sample collection were anticipated.

Chapter 3 Overview of results

3.1 Results introduction

The results of this study have been put into 6 chapters, each chapter contains a results and discussion section:

- **Chapter 3 Overview of results** provides information on the study population, overall results in the categories of ARGS neoepitope, MR imaging and a comparison of the overall figures from these categories. It will also include data on lowest limit of quantification (LLOQ) values.
- **Chapter 4 Behaviour of ARGS neoepitope** provides results which chronicle the behaviour of this biomarker in different samples and patient groups.
- **Chapter 5 Imaging measures** provides results and interpretation for the analysis of imaging data and in particular the different outcome measures of MRI.
- **Chapter 6 Imaging measures and ARGS neoepitope** provides results from comparative analysis exploring the relationship between these 2 markers.
- **Chapter7 Clinical outcome scores** provides results from analysis to explore the relationship between clinical scores, and imaging and ARGS neoepitope.
- **Chapter 8 Explant work** provides results of the explant work and relates them where possible to other collected data from this study.

3.2 Study population

In total 196 patients were recruited for the study, however patients were later excluded from the study if we were unable to obtain paired pre- and post-operative serum and urine samples along with a synovial fluid sample obtained during surgery (Figure 10).

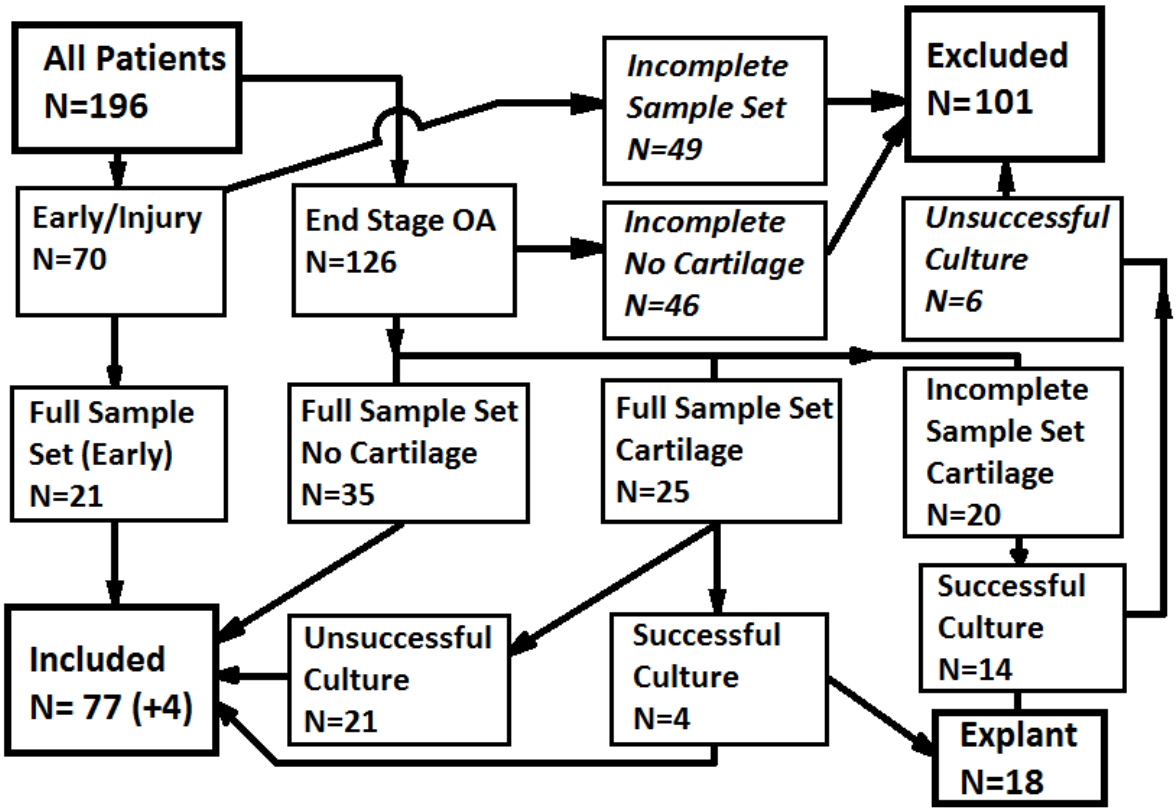

Figure 10 Breakdown of recruited participants

There were several areas of difficulty in collecting complete sample sets. Firstly inclusion criteria limited the number of patients who were suitable for the trial. Secondly collecting synovial fluid in theatre was often troublesome in closed procedures (arthroscopy and ACL repair) and this had implications for interpretation of results from this group. Thirdly explant studies were limited due the poor availability of cartilage from UKR participants. The study also had to exclude patients who were lost to follow up leading to incomplete sample sets.

3.2.1 High exclusion rate in the non-arthroplasty group

Study design is crucial to running a study that is hypothesis-driven and conforms to ethical standards. In arthroscopy patients, if simple aspiration was unyielding the procedure continued as routine and if there was any fluid from the scope insertion we took a sample, however in around two thirds of knees in this group we did not obtain a sample. Routine

care was never altered for the provision of a sample in this study. One further question remains: by excluding those without synovial fluid samples and in particular knees without effusion are we creating a bias towards more significantly-affected knees and hence not really achieving what we set out to achieve, a relatively mildly diseased cohort as a comparator?

3.2.2 UKR often provides insufficient cartilage to perform explant experiments

In Oxford, a greater emphasis is placed on the Oxford unicompartmental knee replacement, this procedure is performed by all knee surgeons in this hospital but in other hospitals it is seen as a specialist operation, and is arguably a more difficult procedure with poorer results found in non-expert units[207] but excellent results from high volume surgeons[208]. The unicompartmental knee replacement is designed for predominantly medial-sided disease of the knee although can be used laterally, however in this study the pertinent point is that the size of the surgical bone cuts from which cartilage is obtained is significantly smaller (see Figure 11).

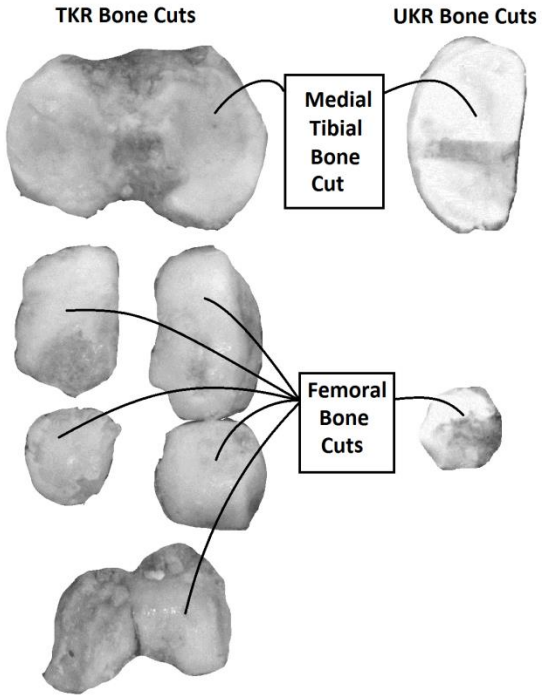

Figure 11 TKR and UKR bone cuts

One further point to make regarding this matter concerns the topographical position of the source of cartilage used for explants. From a perspective of zonal and topographical differences in chondrocyte behaviour and expression[209, 210] it is preferable to use cartilage derived from similar areas, however the medial compartments of TKR patients were in such poor condition it was often preferable to use other less-damaged areas in order to obtain viable cartilage and this could influence the results.

3.3 Demographics of study participants

Characteristics of the patients who were included in the study are shown below (Table 3) classified by demographics.

Table 3 Breakdown of the study participants' demographics

		Late		Early	
	ALL	TKR	UKR	SCOPE	ACL
N	95	30	43	16	6
Age	65.6 (13.6)	69.8 (7.3)	66.3 (11.1)	53.1 (15.4)	41 (12.9)
Height	170.3 (9.5)	170.3 (9.2)	170.1 (9.8)	169.6 (8.9)	173.3 (11.7)
Weight	88.3 (16.3)	89.7 (14.1)	86.4 (14.9)	94.8 (21.9)	78.2 (15.2)
BMI	30.5 (5.5)	31.0 (4.7)	29.8 (4.6)	33.1(8.1)	25.8 (3.0)
Male	50	17	22	7	4
Female	45	13	21	9	2

It is clear from these data that the main difference is that patients considered to have early OA have a lower mean age and this needs to be taken into account when interpreting the results.

3.4 Overview of ARGS and MR imaging values

If we look at the overall results for different patient types subcategorised by operation and showing the values for pre-operative serum and urine ARGS neoepitope, and the intraoperative synovial fluid ARGS neoepitope, we can demonstrate that synovial fluid in all groups contains the highest ARGS neoepitope concentration and that urinary ARGS, although corrected for creatinine, contains the lowest concentration. Table 4 shows that synovial fluid ARGS neoepitope levels are highest in the TKR groups and lowest in the arthroscopy group (SCOPE). The arthroplasty group display the highest levels of serum and urine ARGS neoepitope. Overall the ACL group showed the lowest values in each serological sample type.

Table 4 ARGS results by operation

ARGS Neoepitope	All	N	TKR*	N	MUKR*	N	ACL*	N	SCOPE*	N
Synovial Fluid (ng/mL)	18.66 (11.25)	64	20.30 (13.36)	16	17.19 (7.28)	26	15.48 (11.31)	5	19.56 (14.01)	12
Serum (ng/mL)	9.40 (7.64)	68	10.41 (4.64)	20	9.88 (10.36)	30	5.19 (4.21)	4	8.19 (3.76)	9
Urine (ng/mg)	3.62 (1.98)	48	3.63 (1.50)	14	3.87 (2.14)	18	2.82 (0.47)	3	3.33 (2.65)	10

***TKR – total knee replacement; MUKR – medial unicompartmental knee replacement; ACL – anterior cruciate ligament reconstruction; SCOPE - arthroscopy**

When we look at the MRI findings (Table 5) we find that cartilage loss is universally highest in the medial compartment regardless of the operative procedure being performed. Whilst the TKR group shows the highest cartilage loss, the next highest overall group is the arthroscopy group. As this group was intended to represent an earlier stage of OA, it shows that some caution must be applied when making any assumption regarding the patient's cartilage status based on their surgical diagnosis or classification. The anterior cruciate reconstruction patient group contains the smallest volume of cartilage loss and effusion volumes, and therefore represents the mildest category of degenerative knee disease in this study.

Table 5 MR imaging by procedure means (standard deviation)

MRI	All	N	TKR	N	MUKR	N	ACL	N	SCOPE	N
Total Loss (%)	-24 (16.8)	89	-34 (12.1)	27	-19 (19.3)	36	-10 (13.7)	6	-23 (12.4)	15
Femoral Loss (%)	-13 (19.1)	89	-21 (12.5)	27	-9 (22.6)	36	-1 (20.8)	6	-12 (16.4)	15
Medial Loss (%)	-47 (21.9)	89	-58 (23.6)	27	-45 (21.5)	36	-30 (24.4)	6	-41 (22.7)	15
Lateral Loss (%)	-39 (23.4)	89	-45 (21.6)	27	-32 (22.2)	36	-27 (13.1)	6	-36 (19.7)	15
Patella Loss (%)	-33 (22.1)	89	-44 (21.6)	27	-26 (23.4)	36	-18 (10.2)	6	-35 (15.1)	15
Covariance Intensity	37.4 (7.46)	89	39.7 (5.84)	27	38.1 (8.6)	36	34.5 (5.0)	6	33.9 (7.4)	15
Effusion (mL)	24.2 (20.2)	89	26.9 (19.9)	27	23.5 (18.5)	36	20.7 (22.2)	6	24.4 (25.6)	15

Synovial fluid has previously been demonstrated to contain higher levels of ARGS neoepitope than serum and urine samples; however this study also found higher levels in acute injury than in OA[105]. The ARGS neoepitope has been observed similarly to be higher in OA than in controls[109]. However as we see in the imaging data, an arthroscopic surgical procedure does not seem to represent a milder category of disease. From our data it appears that the ACL group is most representative of lower ARGS neoepitope levels and radiological disease signature.

3.4.1 The surgical classification of the patient does not seem to infer that the disease process is milder in the non-arthroplasty cohort excluding ACL.

Non-arthroplasty patients were recruited to represent mild disease and provide a group to contrast the findings of the arthroplasty cohort. However results from interpretation of imaging outcome measures suggest this group contains a heterogeneous group of patients including patients with significant cartilage volume loss. Therefore as a group they are not representative of a mild cohort of patients and the analysis should focus on other outcome variables for the purpose of identifying an early OA comparator cohort. One of the explanations for this phenomenon would be that by being unable to consistently aspirate synovial fluid samples from this group at surgery we have selected a more diseased population from our cohort leading to a bias. When we look at the data, TKR patients have the greatest cartilage loss and the highest serum and synovial fluid ARGS neoepitope levels, whereas ACL has the lowest cartilage loss and the lowest serum, synovial fluid and urine values of the group. However there is a significant age discrepancy between the two groups which also has ramifications with regard to the group comparison.

3.5 Overview of ARGS against patient demographic, imaging and clinical scores

Table 6 shows an overview of correlations between different sample types and outcome measures. We compared ARGS neoepitope levels against patient measures; age and BMI which are both considered to be independent risk factors of OA, and to other sample ARGS neoepitope levels, imaging measures and clinical outcome measures. In regard to clinical outcome scores we used pre-operative Oxford knee scores and pain scores from painDETECT™ (average of three initial scores on questionnaire).

Table 6 also contains results for all patients and also the largest surgical group which were the medial unicompartmental knee replacement (MUKR) group. The rationale for exploring the data in this group in particular was their relatively homogenous disease pattern. Values are for Pearson's correlation with *P*-values in brackets and significant results are in bold font.

Table 6 Overview of ARGS and outcome correlations

		All (N = 96)			MUKR (N = 38)		
		Synovial Fluid	**Serum**	**Urine**	**Synovial Fluid**	**Serum**	**Urine**
Age	All	**0.110 (0.008)**	**0.067 (0.034)**	0.061 (0.090)	0.034 (0.368)	0.071 (0.155)	0.020 (0.578)
	Male	0.057 (0.167)	0.061 (0.118)	0.036 (0.315)	0.012 (0.732)	0.094 (0.248)	0.005 (0.846)
	Female	**0.219 (0.010)**	**0.145 (0.050)**	0.190 (0.070)	0.156 (0.163)	0.175 (0.137)	0.114 (0.414)
BMI	All	0.005 (0.580)	0.011 (0.405)	0.013 (0.435)	0.012 (0.599)	0.020 (0.456)	0.043 (0.407)
	Male	0.015 (0.477)	0.29 (0.284)	0.044 (0.265)	0.003 (0.877)	0.041 (0.451)	0.077 (0.436)
	Female	0.001 (0.890)	0.023 (0.449)	0.007 (0.750)	0.044 (0.471)	0.051 (0.436)	0.000 (0.971)
Serum ARGS	All	0.002 (0.741)	x	**0.172 (0.006)**	0.011 (0.621)	x	**0.250 (0.034)**
	Male	0.006 (0.660)	x	0.059 (0.211)	0.001 (0.910)	x	0.068 (0.467)
	Female	0.034 (0.390)	x	0.212 (0.084)	0.043 (0.496)	x	0.464 (0.063)
Urine ARGS/Cr	All	0.005 (0.575)	**0.172 (0.006)**	x	**0.369 (0.023)**	**0.250 (0.034)**	x
	Male	0.034 (0.386)	0.059 (0.211)	x	**0.770 (0.022)**	0.068 (0.467)	x
	Female	0.007 (0.741)	0.212 (0.084)	x	0.037 (0.650)	0.464 (0.063)	x
Total % Cartilage Loss	All	0.000 (0.951)	0.004 (0.623)	0.003 (0.707)	0.099 (0.127)	0.002 (0.808)	0.021 (0.590)
	Male	0.000 (0.916)	0.000 (0.897)	0.103 (0.089)	0.212 (0.131)	0.014 (0.680)	**0.538 (0.024)**
	Female	0.024 (0.430)	0.112 (0.095)	0.044 (0.417)	0.000 (0.954)	0.213 (0.112)	0.003 (0.904)
Medial Comp % Cartilage Loss	All	0.033 (0.154)	0.006 (0.548)	0.002 (0.795)	**0.244 (0.012)**	0.022 (0.912)	0.037 (0.475)
	Male	0.011 (0.249)	0.024 (0.339)	0.131 (0.051)	**0.346 (0.044)**	0.027 (0.555)	**0.544 (0.023)**
	Female	0.110 (0.085)	0.033 (0.374)	0.011 (0.682)	0.245 (0.086)	0.019 (0.655)	0.011 (0.822)
Co variance intensity	All	0.004 (0.610)	0.017 (0.304)	0.003 (0.699)	0.014 (0.571)	0.014 (0.543)	0.021 (0.592)
	Male	0.001 (0.872)	0.005 (0.670)	0.064 (0.185)	0.001 (0.915)	0.065 (0.360)	0.088 (0.438)
	Female	0.011 (0.591)	0.111 (0.096)	0.044 (0.421)	0.117 (0.253)	0.183 (0.145)	0.147 (0.396)
Oxford knee score	All	0.061 (0.102)	0.000 (0.860)	0.033 (0.302)	0.005 (0.743)	**0.167 (0.025)**	0.098 (0.207)
	Male	0.091 (0.140)	0.015 (0.518)	0.005 (0.766)	0.018 (0.681)	0.078 (0.296)	0.010 (0.785)
	Female	0.050 (0.344)	0.129 (0.110)	0.206 (0.120)	0.026 (0.583)	0.147 (0.177)	0.070(0.526)
Average PAINDETECT (Noc)*	All	0.**094 (0.031)**	0.065 (0.069)	0.022 (0.372)	0.156 (0.094)	**0.177 (0.046)**	**0.412 (0.010)**
	Male	**0.146 (0.044)**	0.006 (0.669)	0.093 (0.147)	0.350 (0.072)	0.100 (0.293)	**0.465 (0.043)**
	Female	0.004 (0.776)	0.142 (0.092)	0.016 (0.648)	0.000 (0.969)	0.227 (0.164)	0.106 (0.530)

R^2 Values (Pearson Correlation); *P* values (paired T-test); significant values in bold.

Age shows some correlation with synovial fluid and serum ARGS neoepitope values, showing that synovial fluid ARGS neoepitope tends to decrease with advancing age ($R^2 = 0.11$, $P = 0.08$) while serum ARGS neoepitope increases with age ($R^2 = 0.067$, $P = 0.034$). We will look at these relationships in more detail later in Chapter 4 (section 4.2). BMI did not show any consistent relationship with ARGS neoepitope levels. Cartilage volume demonstrated some correlations but only in the MUKR group and only for synovial fluid and urine ARGS neoepitope. Intensity inhomogeneity as expressed by co-variance of intensity did not correlate with ARGS neoepitope in any group, however there were some relationships with clinical outcome scores.

3.5.1 Imaging and ARGS values show poor overall correlation

The correlation statistics for overall figures of imaging and biochemical representation of disease burden were not significant, however with a wide cohort of patients in terms of age, BMI, co-morbidity and probable disease burden there are multiple influencing and uncorrected factors which may be contributing to this finding. As shown in the table there are particular areas of promise in terms of focused statistical exploration. In particular the medial compartment, which is the most commonly and severely affected compartment[211], could become a focus of interpretation, especially in participants with predominantly this form of disease.

3.5.2 Selecting a homogenous group of participants (MUKR) reveals some links between imaging and biochemical markers

We will be discussing specific correlations further in Chapter 6, but it would seem clear that in general, the data only showed trends rather than clear relationships. Overall there are no relationships between serological, imaging and clinical markers of disease. OA is so multifactorial that invariably in order to produce significant results cohorts are going to have

to be highly selective or have high study numbers. In selecting the more homogenous MUKR cohort we can see more trends and correlations appearing which may help identify the relationship between ARGS neoepitope and imaging measures by reducing the impact of other patient-related factors.

3.6 Lowest limit of quantification (LLOQ) and missing data

Approximately 25% of urinary samples, 13% of serum samples and 18% of synovial fluid samples did not contain sufficient quantities of the ARGS neoepitope to determine an accurate value and were under the LLOQ which was set at a value of 0.9765625 ng/mL. Consequently, we looked at the relationship between creatinine and uncorrected urinary ARGS neoepitope (which in Figure 12 is rounded to the nearest whole number) and found that creatinine levels were associated with uncorrected levels of urinary ARGS, and that in particular levels below the LLOQ were very low suggesting a dilutional component.

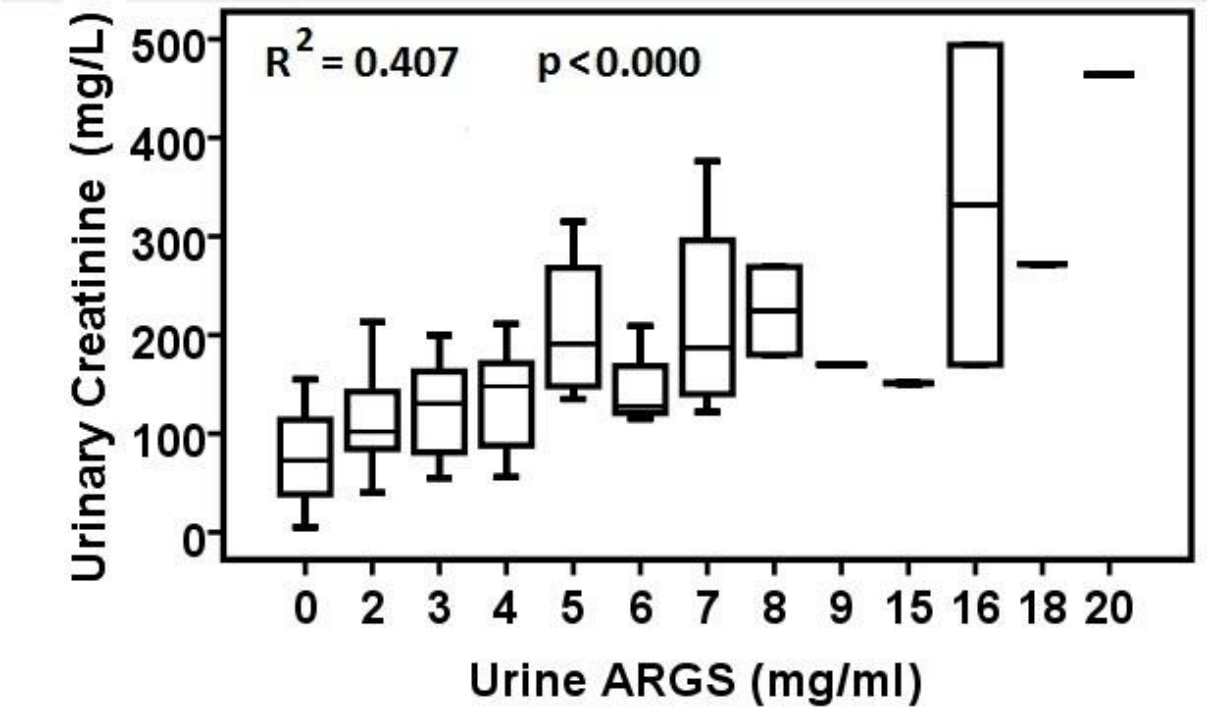

Figure 12 Urinary ARGS compared to creatinine

3.6.1 ARGS neoepitope levels below the LLOQ in urine are related to low creatinine values

One of the limitations of this study is the inability of the assay to detect very small quantities of biomarker, this has previously been described where around one third of samples were not measurable[212]. This has caused a degree of interpretation issues, in that the reason for the low levels found in urine is the high urinary flow rate leading to dilute samples. They have been excluded from the results as statistical analysis of the values determined that arbitrarily assigning an LLOQ value was not representative of the true value and likely to skew results. The best time for collection of urinary biomarkers has not yet been established; however potentially, collecting an early morning sample would produce more concentrated urine due to the effects of ADH and therefore allow more reliable detection of relatively small amounts of biomarker. It could also reduce potential effects of physical activity on both urinary creatinine levels[198, 199] and OA-related biomarkers[165].

3.7 Summary of results overview

The study has had some setbacks in how samples are obtained and these to a large extent were unavoidable, it is technically difficult to retrieve synovial fluid from knees even under anaesthetic if there is little or no effusion. However as effusion is related to underlying pathology, this should be considered during interpretation of results. Many of the other overall findings will be discussed in further depth over the next four chapters but it is surprising in some ways to find that our arthroscopy cohort, which was recruited to act as a model of a relatively mild level of disease, has shown large amounts of cartilage loss with correspondingly high ARGS neoepitope results indicating that surgical classification alone would not be suitable for analysing whether ARGS neoepitope is higher in more advanced disease.

Chapter 4 Behaviour of ARGS neoepitope

4.1 ARGS neoepitope in different sample types

One of the primary objectives of the study was to analyse the relationship between levels of ARGS neoepitope in synovial fluid, serum and urine. Synovial fluid is specific to a joint, whereas serum and urine are regarded as peripheral fluids, which may be influenced by arthritis of other joints and other physiological processes. In general synovial fluid contained far higher levels of ARGS than peripheral serological samples (Figure 13).

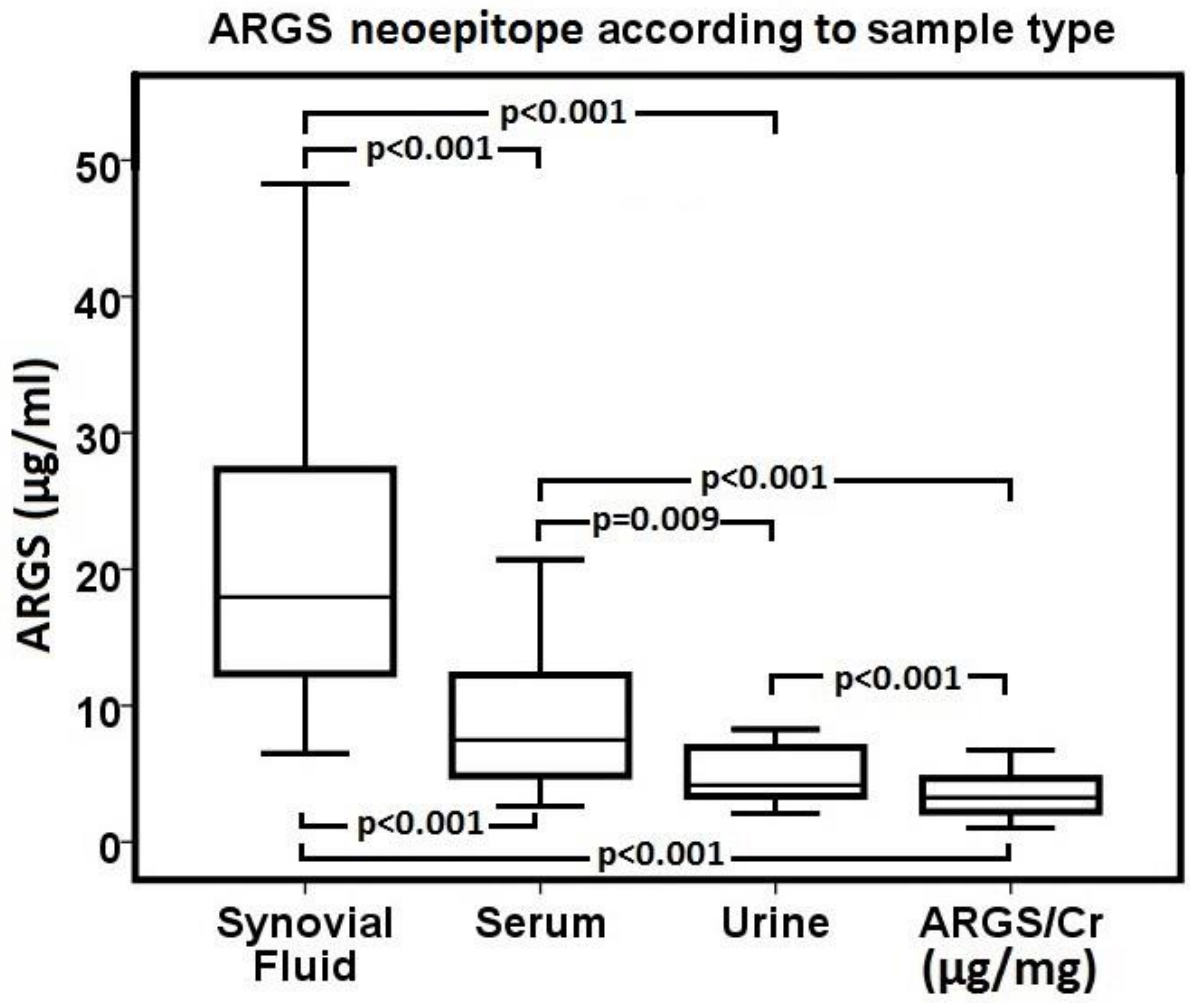

Figure 13 ARGS by sample type

Urine samples were adjusted for creatinine to adjust for urinary flow rate, and therefore a direct comparison to other sample types should take this into account. Physiologically, urinary creatinine may be influenced by several factors related to significant joint disease and therefore it is possible that the picture of disease it represents will differ from that of serum. In testing we attempted to use a Thompson correction[213] as an alternative to adjusting for urinary flow rate; however the calculation was prone to generating occasional large outlying figures and therefore these results were not included[177].

4.1.1 Synovial fluid in general contains the highest concentration of ARGS neoepitope

When analysing the behaviour of an endogenous biomarker there are many things to consider. We would assume that the sampled joint would have the highest concentration of the biomarker in question in relation to the other fluids sampled and this is largely what we have observed here. Although synovial fluid has been previously observed to contain far higher levels of ARGS than serum and urine[212], our results did not reveal the same ratios and we found synovial fluid levels to be only 1.98 times overall higher than serum levels, as opposed to 20 times. There are several factors that may explain this, the first of which is that the study in question used samples from different patient groups. One group consisted of acute and chronic injury patients for whom the age group was not stated[214], a second sample group included healthy patients (mean age 32), acute injury patients (mean age 37) and OA patients (mean age 48)[215], and a third study from which samples were used contained patients with acute injury, OA and inflammatory arthritis which represented different underlying pathologies to our cohort[106]. According to our findings, higher synovial fluid to peripheral sample ARGS neoepitope ratios are seen in younger more acute patients and explains why the difference between synovial fluid and serum ARGS

neoepitope is less marked in our older and predominantly end-stage OA cohort. The study also did not include any participants with inflammatory joint disease which has been shown to produce the highest quantities of ARGS neoepitope[106].

When we analysed the relationship between different sample types (Figure 14) we found a very poor correlation with synovial fluid and a weak but significant correlation between serum and urine (R^2= 0.172, *P* = 0.006).

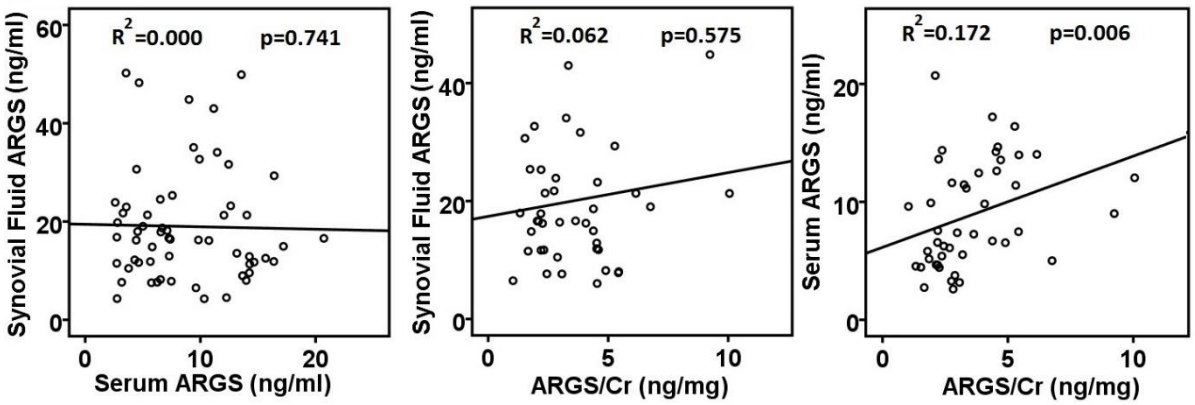

Figure 14 Correlation between sample types

There are many potential reasons for this discrepancy, including unmeasurable factors such as the contribution of other joints in the body to serum and urine ARGS neoepitope levels.

4.1.2 There is a poor relationship of synovial fluid to peripheral fluid and a modest relationship between serum and urine ARGS neoepitope levels

Although we could correlate the serum and urine levels, we were not able to correlate the synovial fluid to serum as described previously by Larsson *et al*.[212]. This may be partly due to the fact that serum and urine samples were mainly taken at preoperative assessment rather that at the same time as surgery, which was normally 4 weeks prior. However the biggest factor is possibly explained by thinking of synovial fluid as a marker of individual joint disease, and peripheral markers as a marker of total body burden of disease. When

assessing the correlation between the levels of the marker in the blood and urine we did find a weak but significant correlation, which was consistent with previous work on ARGS neoepitope[109]. Peripheral serological biomarkers have a relationship based on production at source which is principally determined by burden of disease but also physical activity in the case of OA biomarkers[163, 165]. Furthermore, correction for urinary creatinine remains a crude model for adjusting for urinary flow as it relates to age[181], gender[181], exercise[199] and pre-existing muscle mass[179]. This may require more robust urinary flow correction techniques, most of which require at least one 24 hour urine collection[177].

However there was slightly more consistency between the two peripheral sample types.

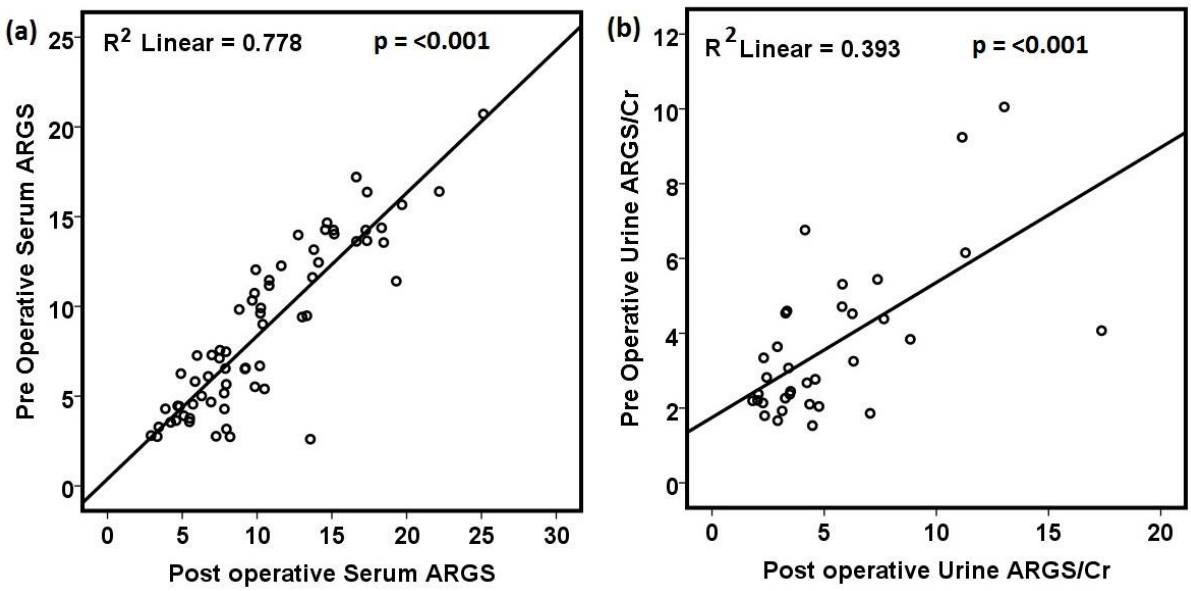

Figure 15 Relationship between pre-operative and post-operative ARGS neoepitope levels

We can see that there is a fairly consistent relationship between the levels of ARGS neoepitope in pre-operative and post-operative samples, especially in serum (Figure 15(a)), but also in urine (Figure 15(b)). In general there was a propensity for ARGS neoepitope levels to increase after surgery in late-stage OA.

4.1.3 Pre-operative ARGS neoepitope levels correlate with post-operative levels

Germaschewski *et al*. described good consistency in serial measurements of serum ARGS neoepitope in patients over the course of a single day and over multiple visits; these results also agree with our findings. Furthermore our findings suggest intra-patient serum samples seem to be more consistent than urine samples[109].

4.2 ARGS neoepitope levels in different age groups

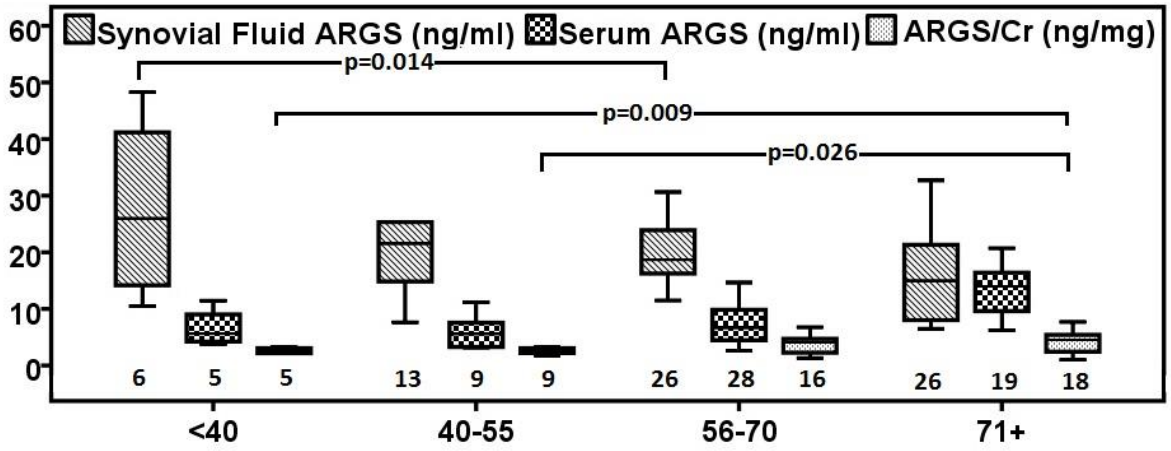

Figure 16 ARGS levels in different age-groups for all patients

Synovial fluid ARGS neoepitope shows a general decline with advancing age, concomitant with a general increase in serum ARGS neoepitope levels (Figure 16). In essence a single joint is producing less ARGS neoepitope in the elderly but resulting in higher overall serum levels, which suggests there is another source of ARGS neoepitope other than the specific joint from which synovial fluid was aspirated. From the patient records we analysed the number of joints replaced and looked for signs of other areas of OA including large joints and spine; however the results did not reveal any significant relationships.

4.2.1 Synovial fluid ARGS neoepitope levels tend to decrease with age while peripheral sample neoepitope levels tend to increase with age

When analysing the results we can see that ARGS neoepitope levels decrease with age in peripheral fluids. The sources of this marker may be other tissues[216] or processes like angiogenesis or fracture healing[217] as well as other joints or intervertebral discs[218], factors which are more prominent in aged patients. Indeed studies have correlated serum cartilage oligomeric protein (COMP) to disease activity on bone scans which allow estimation of the total disease process of the entire patient[141]. Invariably this disease burden increases with age and hence the serum and urinary biomarker levels rise disproportionately to the synovial fluid levels. There is also the issue that acute injury causes large increases in biomarker levels, presumably as a result of increased chondrocyte activity[173, 219], and the proposed relative senescence of chondrocytes in more aged individuals[220] suggests relatively lower cartilage activity in these individuals and hence lower synovial fluid ARGS neoepitope levels. Therefore the marker may be showing specific age-related characteristics.

4.3 ARGS neoepitope in different operation types

When analysing pre-operative and post-operative samples (Figure 17) we found a tendency for late OA participants to undergo a rise in the ARGS neoepitope levels post-operatively

with little change in the early cohort from pre- to post-op. Possible explanations for this may be linked to other physiological processes within the body.

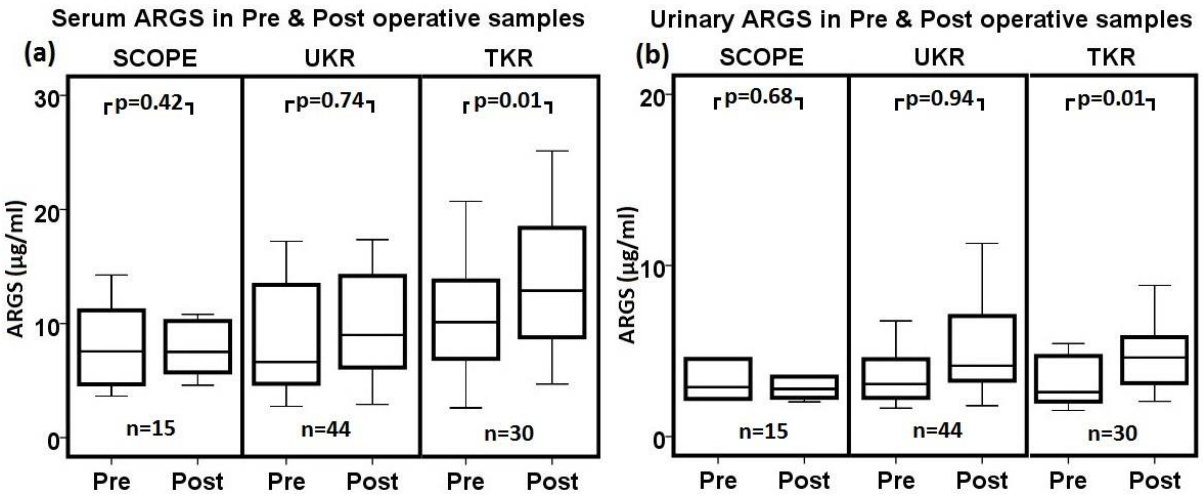

Figure 17 Pre- and post-operative ARGS neoepitope levels

The post-operative serum and urine samples did not show a reduction in ARGS neoepitope levels after removing OA-affected cartilage by arthroplasty in TKR and UKR patients, and in TKR patients there appears to be a significant increase instead (Table 7). Samples were acquired postoperatively at 6 weeks for the majority of study patients.

Table 7 Pre-operative and post-operative ARGS neoepitope levels

Scope	N	Mean pre-operative	Mean post-operative	*P* value (t-test)
Serum	15	8.194107	7.60979	0.424791
Urine	15	3.334527	3.835948	0.678226
UKR	**N**	**Mean Pre**	**Mean Post**	***P* value (t-test)**
Serum	44	9.801921	10.71365	0.741843
Urine	44	3.86145	5.464312	0.939416
TKR	**N**	**Mean Pre**	**Mean Post**	***P* value (t-test)**
Serum	30	10.40596	12.84817	0.006035
Urine	30	3.63454	4.629868	0.013788

In a cohort of those patients with end stage OA, BMI did show a negative correlation with advancing age (R^2 = 0.086; *P* = 0.011) and there was a more pronounced negative correlation between age and BMI in the TKR cohort (R^2 = 0.403; *P* = 0.000) but there was no association with ARGS neoepitope levels.

4.3.1 Serum and urine ARGS neoepitope levels increase significantly after TKR surgery

When analysing serum ARGS neoepitope levels alone we observed an increase from pre-operative values to post-operative values, and this change was more pronounced in TKR patients (Figure 18).

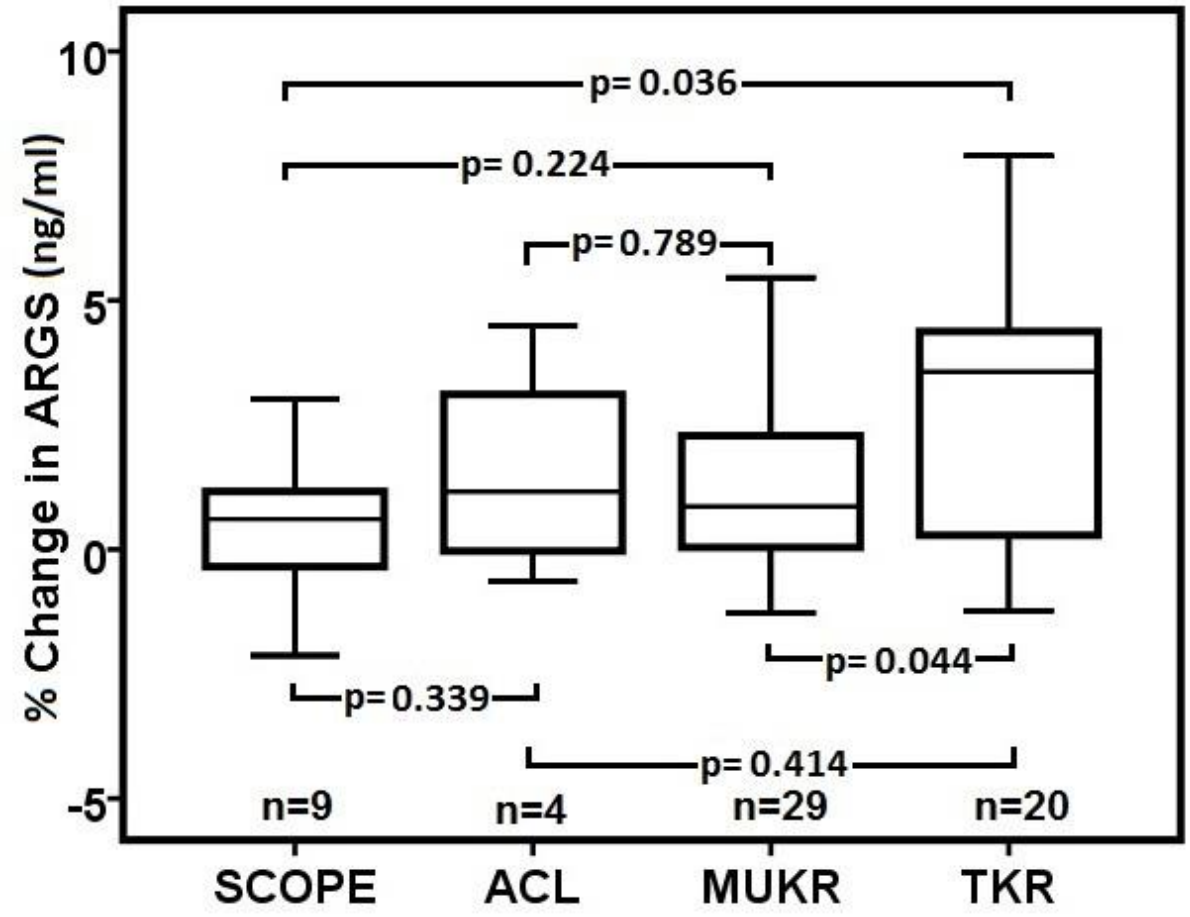

Figure 18 Change in ARGS Pre and Post Surgery

This shows the more invasive TKR procedure generates a higher ARGS value despite the removal of the greatest amount of diseased cartilage. Certainly due to the effect of LLOQ the numbers are smaller in the non arthroplasty groups ,and therefore a statistical comparison is not as accurate.

As TKR necessitates greater bone and soft tissue healing than the other procedures, it is likely that the increase in systemic levels of ARGS neoepitope may be due to increased activity of ADAMTS-5 at the site of healing whether soft tissue or bone. ADAMTS-5 is involved in the soft tissue healing[216] and also there is evidence it is involved in the

fracture healing process and when its function is impaired then callous formation is reduced[221].

In terms of study design the implication of this finding is that where an active tissue healing process is occurring the peripheral serological markers are likely to be significantly affected. This means that collecting early post-operative samples is unlikely to result in increased understanding of the behaviour of this biomarker. In a single case in the trial, serum ARGS marker levels were over twice the next highest value and actually decreased by 11.5 ng/mL over the same time-period as the other patients, the notes indicated concomitant healing of a proximal humerus fracture.

4.4 Gender differences in ARGS neoepitope levels

We also analysed gender differences between patients, but found no significant difference for either synovial fluid or serum (Figure 19).

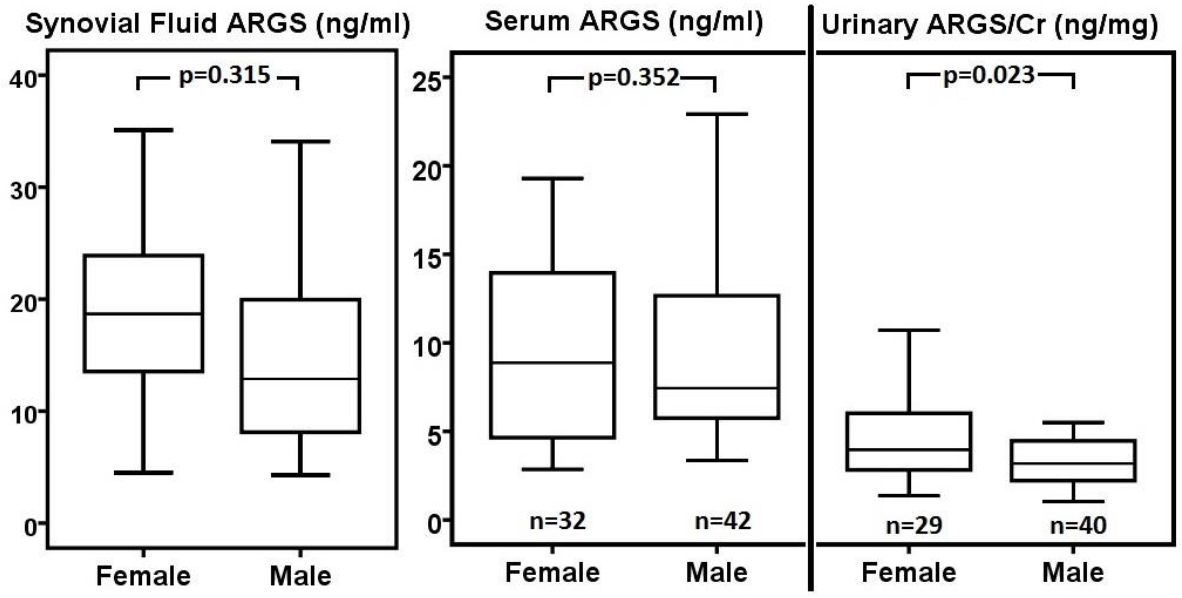

Figure 19 Gender differences in sample types

There was a significant difference when comparing urinary samples (*P* = 0.023), with urinary ARGS neoepitope levels being higher in female than male patients. However when urinary creatinine and urinary ARGS were analysed separately, (Figure 20) we found that the difference was in the creatinine levels rather than the ARGS neoepitope levels.

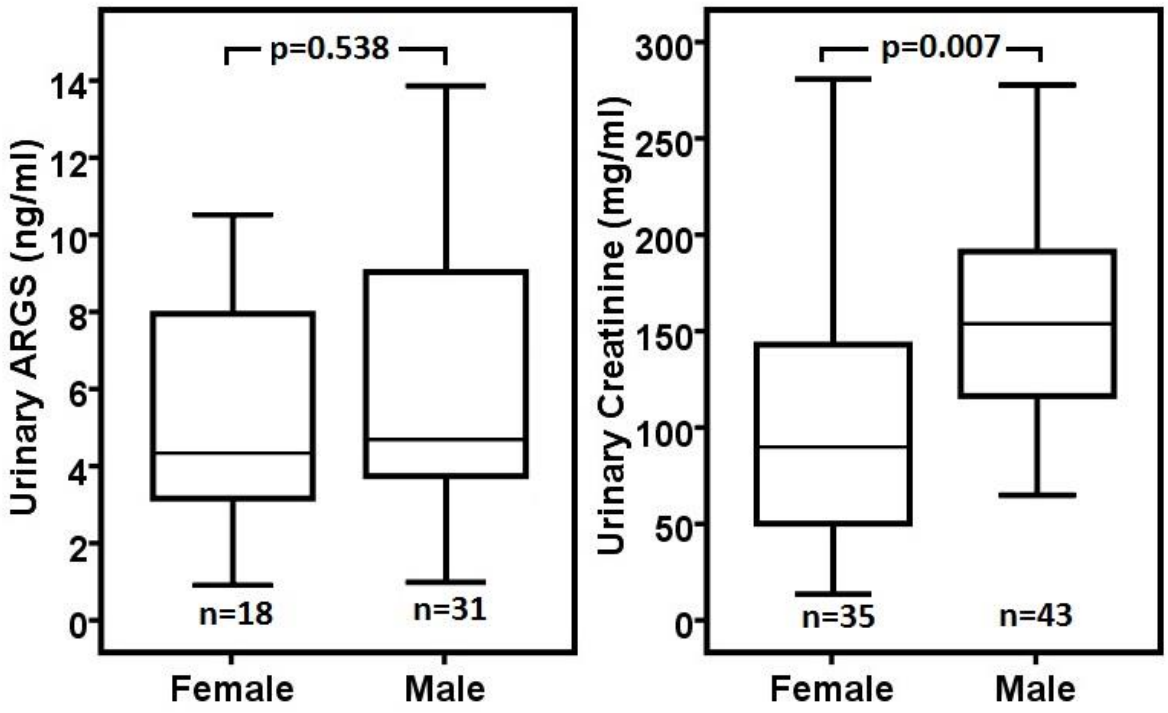

Figure 20 Urinary ARGS and creatinine by gender

Comparison of age group and gender of ARGS neoepitope levels in different sample types showed an increase in peripheral sample (serum and urine) ARGS neoepitope levels with increasing age and a decrease in synovial fluid ARGS (Figure 21).

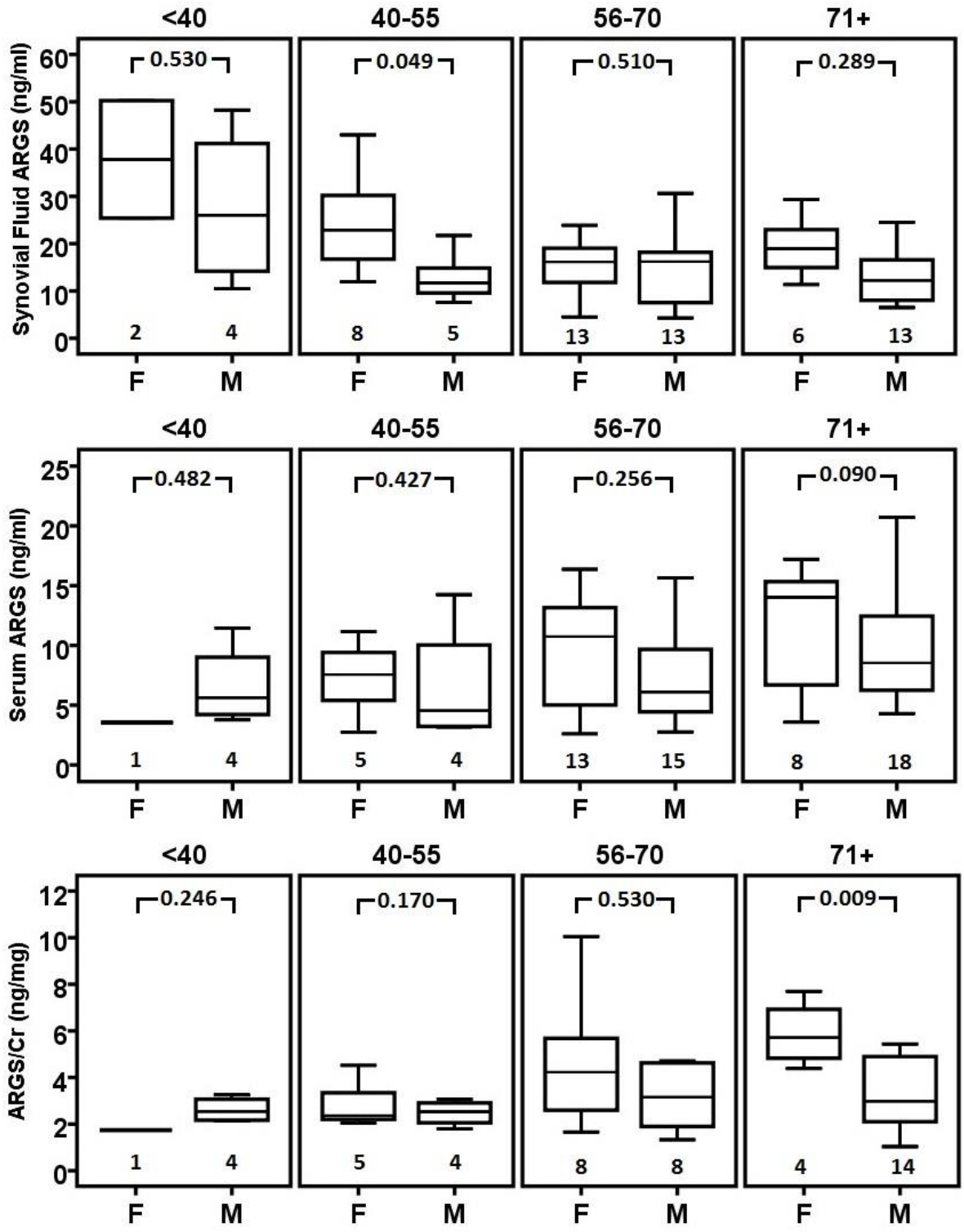

Figure 21 ARGS neoepitope levels by age group and gender

There were no significant differences in ARGS neoepitope levels when discriminating by gender with the exception of urinary ARGS in the oldest group of patients (P = 0.009) which will be discussed later.

4.4.1 Gender difference in urine but not serum ARGS neoepitope levels

With regard to gender association we found only a slight difference in the urinary ARGS levels between samples. Previous studies on ARGS neoepitope have indicated higher synovial fluid levels in men[107]; however a more recent study indicated no difference in ARGS neoepitope between genders[109]. The correction for urinary creatinine is essentially an inaccurate process and to analyse urinary samples more precisely we need to perform studies on individual biomarkers to look at the relationship of biomarkers to creatinine excretion[177]. It is possible to measure creatinine without encountering an effective LLOQ because of its relative abundance in urine but the same is not true for the ARGS neoepitope, and arbitrarily assigning an LLOQ value to very dilute samples creates a bias and those values were then excluded. This produces a discrepancy in the n values between the 2 groups (Figure 21). As more men reached the LLOQ, two explanations are possible, either women have a higher urinary flow rate or men produce more ARGS in their urine samples. As no difference was found in the serum values there is little evidence to support the latter. There are many factors we cannot correct for when looking at these results, most notably the differences in muscle mass, as it is known that creatinine excretion is highly correlated to muscle mass[179] and lean body mass differs significantly according to gender. Furthermore, this lean body mass in women further diminishes after the menopause[222], which may be the cause of the increase in some biomarkers after the menopause[223, 224]. Overall these factors need to be taken into account when interpreting the results of any

studies on urinary biomarker in OA, as low muscle mass may be a risk factor for disease severity[224].

4.5 Summary of ARGS neoepitope behaviour

The highest concentrations of ARGS neoepitope are found in synovial fluid, with diminishing concentrations as it passes systemically via the bloodstream to the urine. However this process is not seemingly predictable as synovial fluid and peripheral fluid ARGS neoepitope do not correlate very well. There does appear to be an age-related association and also in urine, a gender association, although this is almost certainly related to differences in creatinine levels. What is interesting, is the rise in serum ARGS neoepitope after surgery, particularly in TKR surgery, which as the proposed source of the biomarker has been effectively removed, implies there is another process releasing ARGS neoepitope into the bloodstream.

Chapter 5 Imaging measures

Measurements obtained from clinical imaging of patients are outlined below (Figure 22). Imaging is principally focused around MRI and within this type of imaging the central focus is corrected cartilage volume.

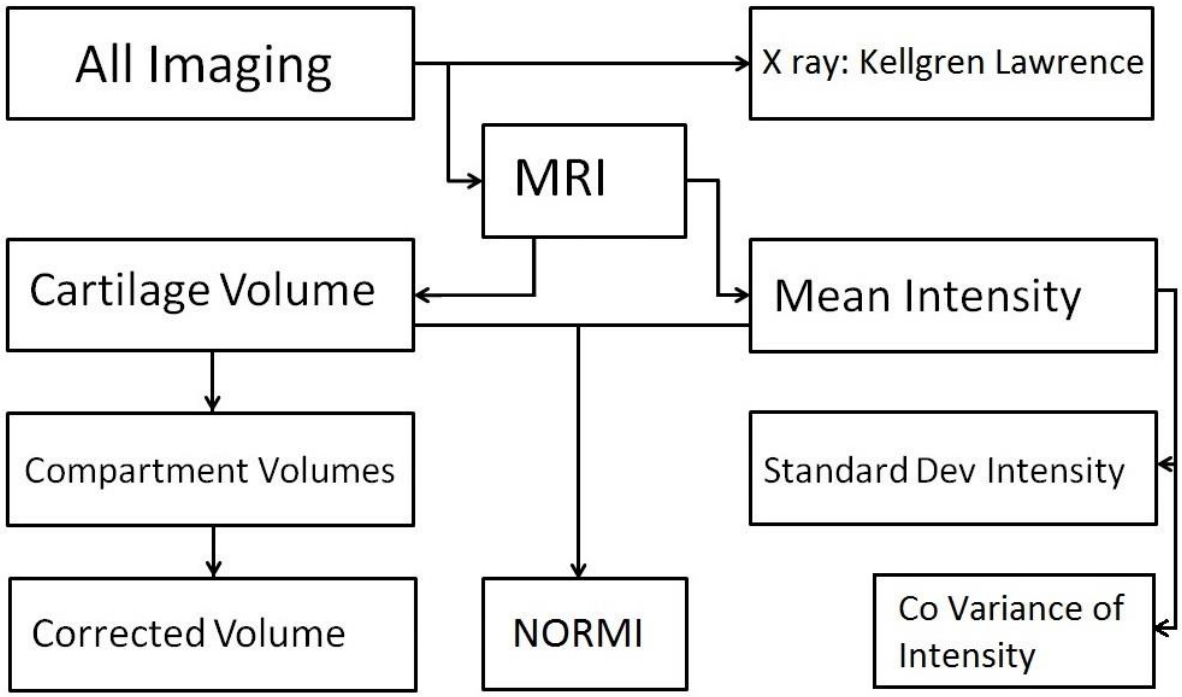

Figure 22 Breakdown of imaging outcomes

Cartilage volume is linked to height and gender in patients with no history of joint disease[205] and from this work it is possible to estimate the predicted volume of cartilage in any given compartment of the knee. This can then be compared against the actual volume on MRI to give an estimate of the percentage cartilage loss. Secondary outcome measures include intensity-derived measures and Kellgren Lawrence scores from plain X-ray images.

5.1 Analysis of scan types

Figure 23 shows volume (i), intensity (ii) and co-variance measures for different scan types(PD FS, PDW SPAIR and WATS), and then a comparison of volume (iv) and co-variance (v) measures for different compartments. Overall from looking at the results it is very apparent that after analysing the scans, although volumes do not vary between scan protocols, intensity and any derivative thereof is affected. So results for volume can be compared but results involving intensity should be analysed within scan types only.

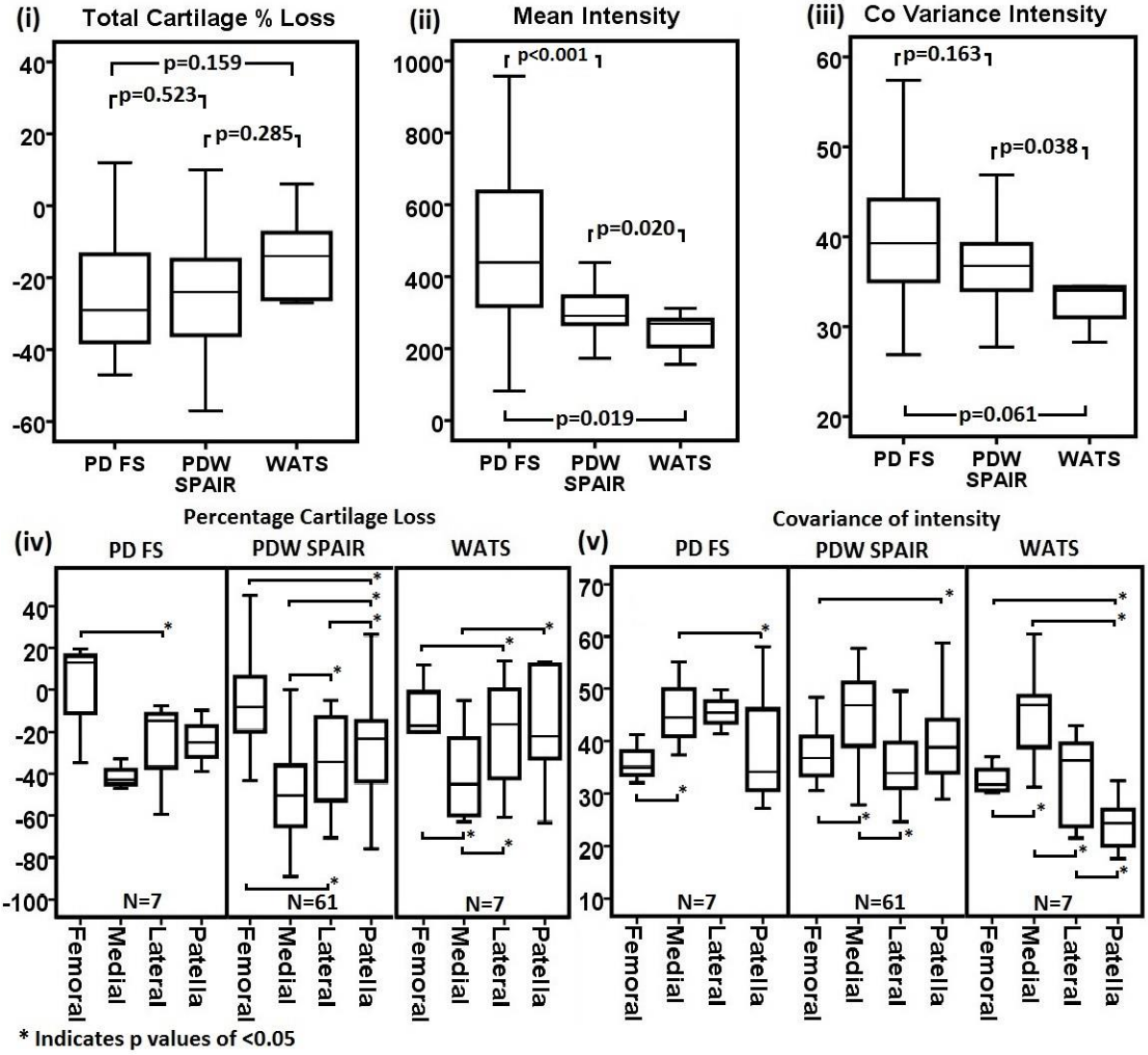

Figure 23 Volume and Intensity Measures for different scan types

Mean intensity scores were found to differ between different scan types and therefore standard deviation of intensity was normalised to the mean to provide a covariance value; this was done to allow comparison of cartilage homogeneity even if scan sequences were different. In Figure 23 (iv) and (v) we can see a breakdown of the compartmental MRI analysis, this shows that medial compartment disease is manifested as low cartilage volume with high inhomogeneity of the cartilage itself which is seen consistently across different scan sequences. We can also see there is great variation in the relative compartmental volume losses and intensity inhomogeneity between compartments.

5.1.1 Volume but not intensity are comparable on different MRI sequences

When we compared different scan types we were able to demonstrate consistency in calculation of volume but significant differences in the intensity values, which is why we preferred to use the covariance value to allow comparison between the different groups. However a significant difference was demonstrated between PD FS and WATS scans in terms of covariance. One of the reasons for this may be due to the scan bias as WATS scans were exclusively used in association with MUKR procedures, whereas PD FS was used in several categories but as the number was small it was relatively easy to exclude this small number from some of the statistical analyses.

5.2 Cartilage Volume Measurements

As previously discussed, cartilage volume was measured in terms of compartment, after which values were compared to a relative to a predicted value based on height and gender assuming, no OA was present, and a percentage loss was calculated from this.

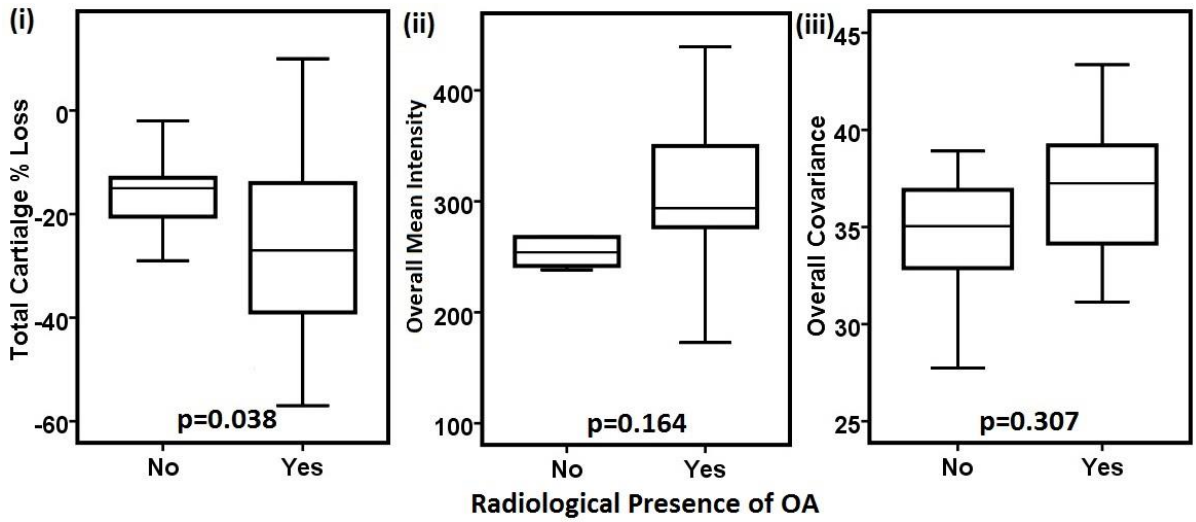

Figure 24 Comparison imaging measures in radiological OA

Figure 24 shows a comparison of the radiological presence of OA against overall % cartilage loss (i), overall intensity of cartilage for patients with PDW SPAIR scans (ii) and covariance of intensity (iii). Patients are classified as having radiological OA if MRI showed the presence of full thickness cartilage loss in one or more compartments or non-radiological OA by absence of full thickness disease. These results show a significant decrease in cartilage volume but non-significant increases in intensity and covariance of intensity. Unfortunately there is a confounder in the age of the two groups with a significant average age difference (21.5 years, $P = 0.000$). Using a contralateral knee scan is a potential alternative to using predicted values however as OA is a condition which takes many years to become symptomatic there

is a high chance of finding cartilage loss in the other joint adding another variable to the analysis.

5.2.1 Significant difference in volume of cartilage between OA and non-OA patients

This finding in itself is expected in that surgical intervention is strongly associated with volume of cartilage loss[225]. Results comparing mild and non-OA against volume of cartilage have been less consistent[226] and generally in a more recent report the advantages of MRI over plain radiographs in terms of sensitivity to change were noted but it was still recommended that both were useful in research[142]. Our results demonstrate that a KL score as low as two will show a significant difference in total volume of cartilage compared to normal cartilage. However we should bear in mind that evaluation of KL scoring is not an exact science and there can be disagreement between users with regard to grade[227].

5.3 MRI volume and intensity patterns in different operations

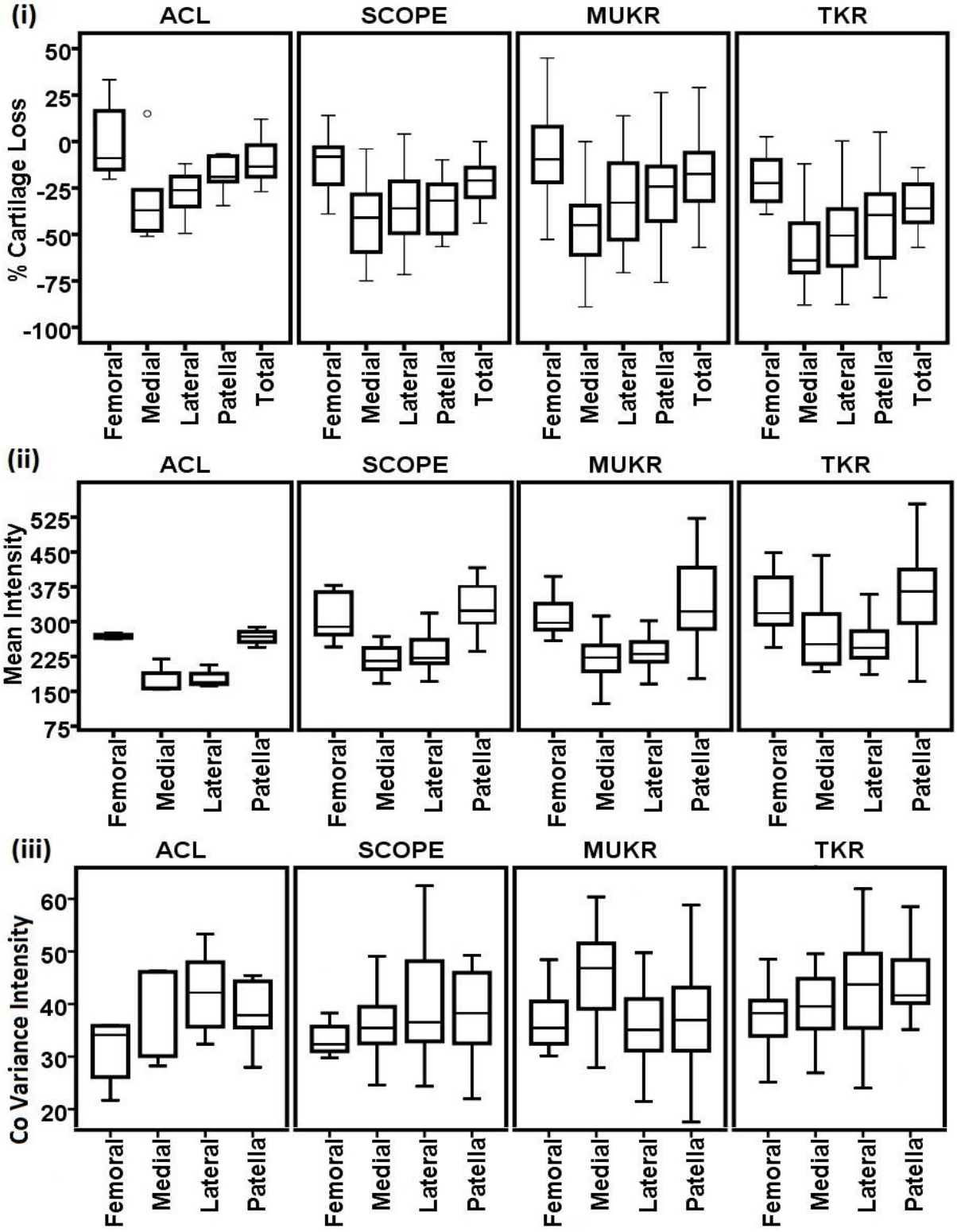

Figure 25 Relationship between operation types and MRI outcome by compartment

Figure 25 (i) shows percentage cartilage loss which was based on gender- and height-predicted knee cartilage volume in healthy knees [205]. Figure 25 (ii) Shows the mean intensity for patients in the same MRI sequence (PDW SPAIR only), and (iii) shows the covariance of intensity results. When looking at these figures it is also important to realise that the patients undergoing arthroscopy (SCOPE group) and ACL also had some degree of knee disease including, in some cases, quite significant OA, where arthroplasty was recommended post procedure. There were significant differences in all cartilage volume measurements between ACL and TKR groups, while in the SCOPE and TKR groups, the differences in medial (P = 0.016), lateral (P = 0.042) and total cartilage loss (P = 0.008) were significant. When comparing the ACL, SCOPE and MUKR groups, no significant differences were observed with regard to cartilage volume loss. However, when comparing the MUKR and TKR groups, there was significantly higher volume loss in all compartments in TKR patients compared to MUKR patients. Intensity and co variance of intensity measures seemed to also show distinct patterns in the variation of measurement, in particular intensity measures were decreased in the medial and lateral compartments compared to the patella and femoral compartments.

5.3.1 Medial compartment loss is a feature of knee joint disease in all surgical patients

Our study shows that the vast majority of patients have medial compartment disease and in terms of interpreting results there is more agreement regarding medial compartment disease in terms of radiological and clinical outcome measures. It has been shown that in around 85% of OA cases, the medial compartment is involved in disease progression[211] and in our study the medial compartment was involved in approximately 92% of cases.

It has been established that the medial compartment is thinner in healthy patients than the lateral compartment[228]. In addition it has been previously suggested that tibial cartilage may be adequate as a disease measure as it relates to the corresponding femoral compartment, but is easier to quantify[229]. For these reasons it is worth considering that in most patients the medial compartment may be a more useful inter-patient comparator for joint disease than other compartments.

5.3.2 TKR shows significantly higher volume of cartilage loss compared to other groups while MUKR is not significantly different in volume loss to other groups

It is interesting that MUKR is associated with a significantly less-pronounced cartilage loss than TKR in this cohort. Figure 26 shows that there is no significant difference between non-arthroplasty participants and MUKR patients in terms of cartilage loss. It has been found

that central medial compartment cartilage loss is a strong radiological predictor of joint replacement[230].

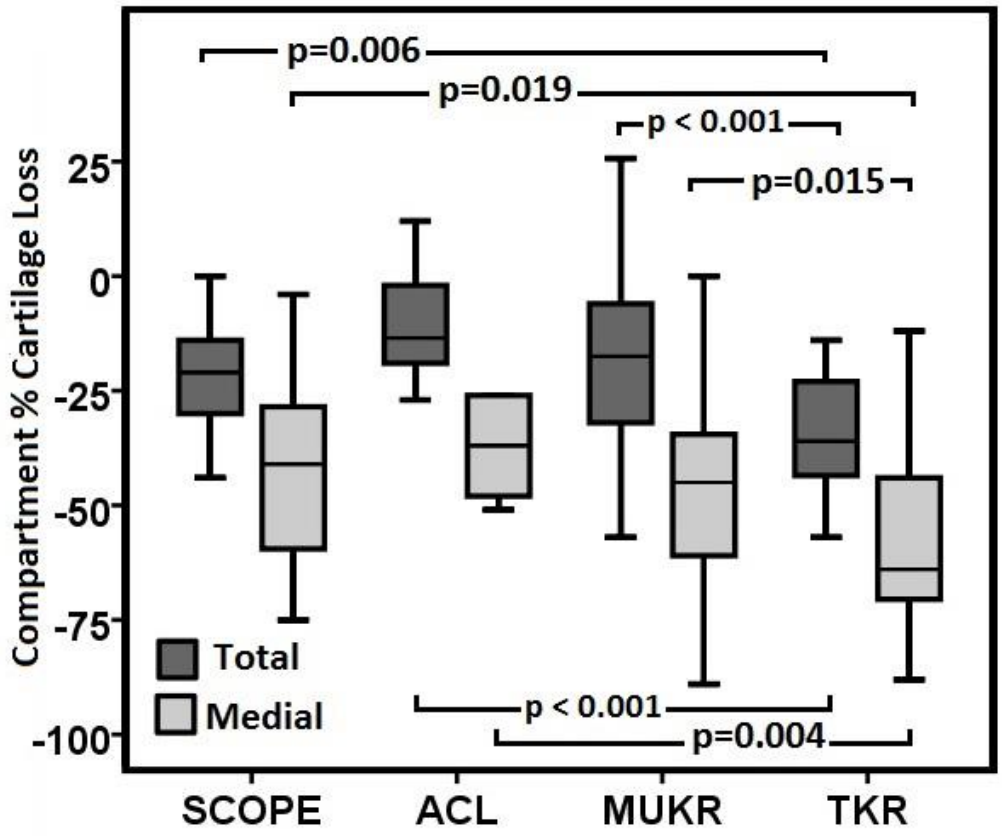

Figure 26 Percentage cartilage loss by surgical procedure

MUKR patients therefore represent a milder stage of OA in terms of MRI-derived measures of cartilage volume loss and this reflects the level of arthroplasty especially in Oxford where there is a local bias towards a certain type of implant[211]. As clinical outcome scores show, there is no difference in clinical change between the MUKR and TKR groups, this suggests that both groups benefit equally (Figure 27) showing no difference in improvement in clinical scores evaluated by painDETECT(Noc) (0-10), painDETECT ^{TM} (0-35) and Oxford Knee score (0-48). However it has been shown that there are some benefits to MUKR over TKR in particular the reduction in soft tissue damage[231].

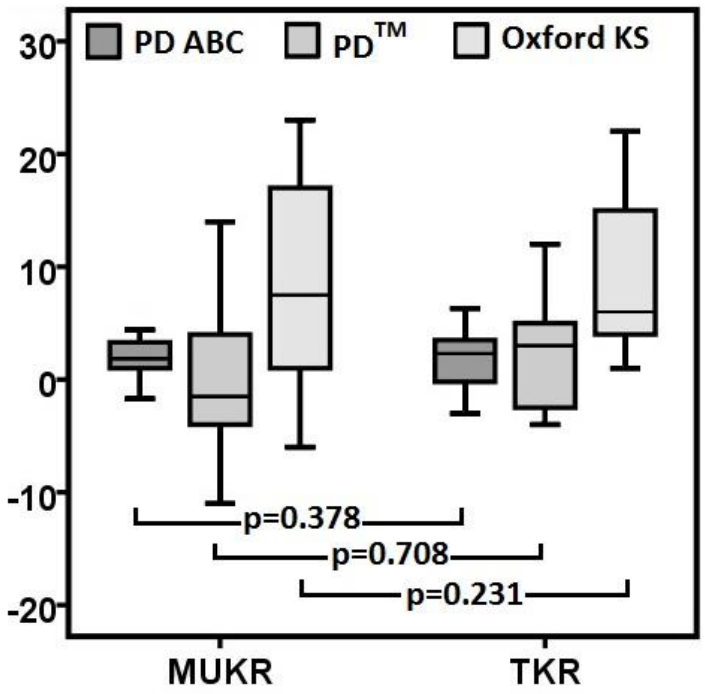

Figure 27 Clinical outcome score changes pre-and post-operatively for MUKR and TKR groups

5.4 Kellgren-Lawrence score comparison to MRI markers

Figure 28 (i) shows the comparison between KL scores for all patients for total compartment volume loss and medial compartment volume loss. We found a weak correlation between KL score and medial tibial cartilage loss (R^2= 0.106; *P* = 0.004) although we have to bear in mind that KL scoring is based on measurements other than joint space narrowing (JSN).

Figure 28 (ii) shows the comparison of KL with overall co variance of cartilage intensity and medial tibial cartilage covariance of intensity.

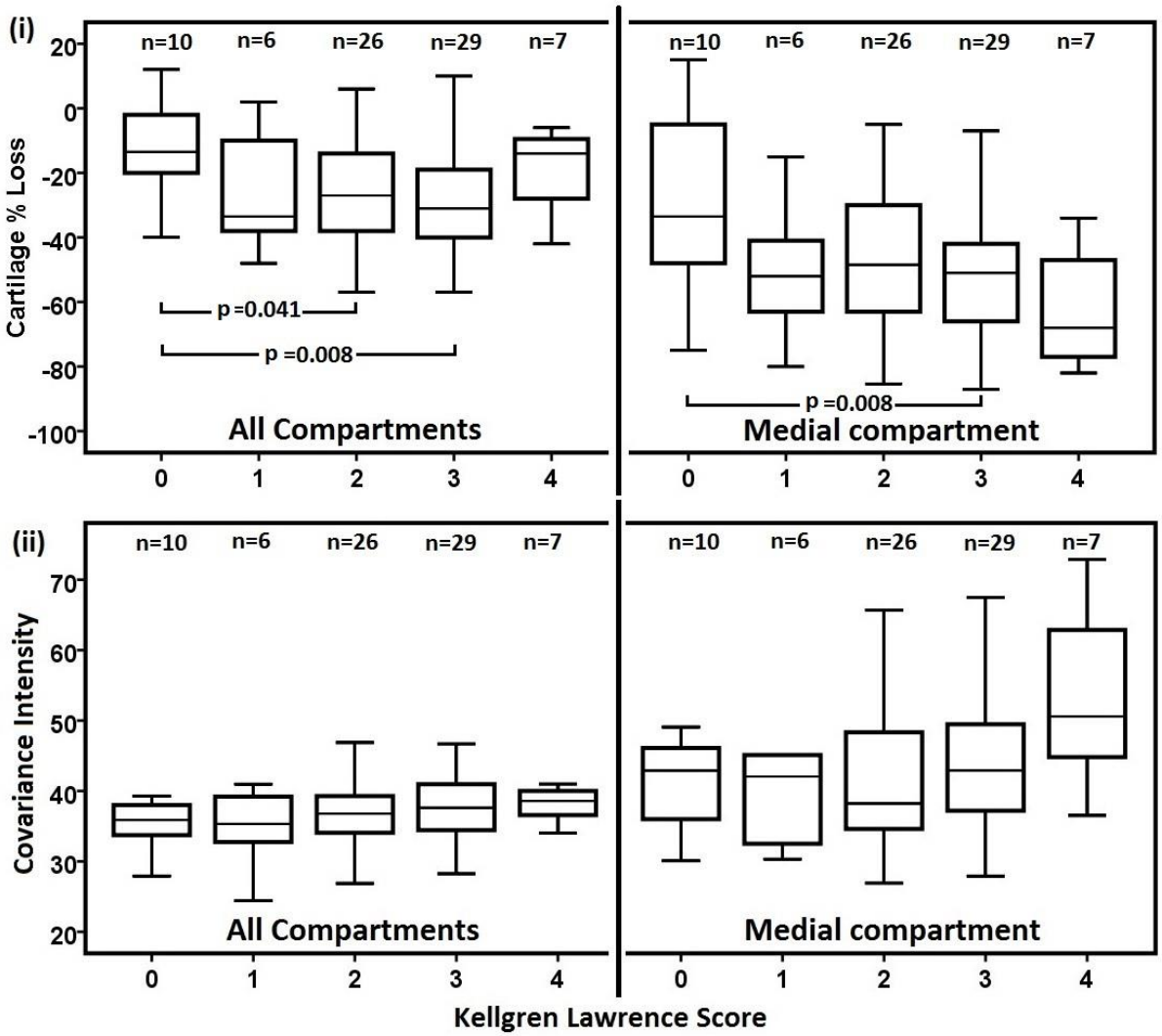

Figure 28 KL score comparison to MRI.

The relationship of co-variance to the KL score shows that there is a trend towards increasing cartilage inhomogeneity with advancing radiographic evidence of disease (R^2= 0.045;*P* = 0.062).

5.4.1 Kellgren Lawrence scores demonstrate a superficial relationship with volume and inhomogeneity measurements

When it comes to comparing both volume of cartilage and covariance of intensity to Kellgren Lawrence score, we observed a relationship with medial compartment values rather than overall measurements, and although the analysis suggests increased inhomogeneity in more advanced disease this was not significant. Previous studies have shown inhomogeneity to be useful in distinguishing between healthy controls and those with OA but not at distinguishing disease severity[232]. Once radiographic changes are detected, significant disease is already present; when grade 1 joint space narrowing is detected, 11–13% of cartilage has already been lost[233]. However recently one study has shown a linear relationship between weight loss and reduced medial cartilage volume loss[139] and the reversibility of cartilage pathology on MRI associated with OA has been demonstrated in other work after weight loss[77, 140] which is only possible if the disease is detected at an early stage before cartilage loss. Our data suggests that by the time there is a detectable KL score of more than 0 then there is significant cartilage loss.

5.5 Summary of imaging measures

This chapter has highlighted potential strengths in terms of imaging and the good correlation between volume measurements, although this was not the case for intensity-related measures. The combination of volume and intensity measures show a consistent drop in the values of the medial compartment in all patients. Whilst imaging can tell part of the story, cartilage is not uniformly lost within a joint[234] and this can be related to other structural factors e.g. ACL integrity[235]. These cartilage damage patterns may have similar overall volume and intensity measurements but have different disease “phenotypes” which may correlate to more active future cartilage loss or turnover.

Chapter 6 Imaging measures and ARGS neoepitope levels

6.1 Analysis of ARGS neoepitope by surgical diagnosis

If we categorise OA as either mild or severe and then compare the levels of detectable ARGS neoepitope across sample types, we see that synovial fluid ARGS neoepitope does not distinguish these two groups. Figure 29 shows the comparison of ARGS neoepitope levels between "severe OA", defined in this context as full thickness cartilage loss on either MRI or intra operatively, and partial thickness or no detectable abnormality, classified as "mild".

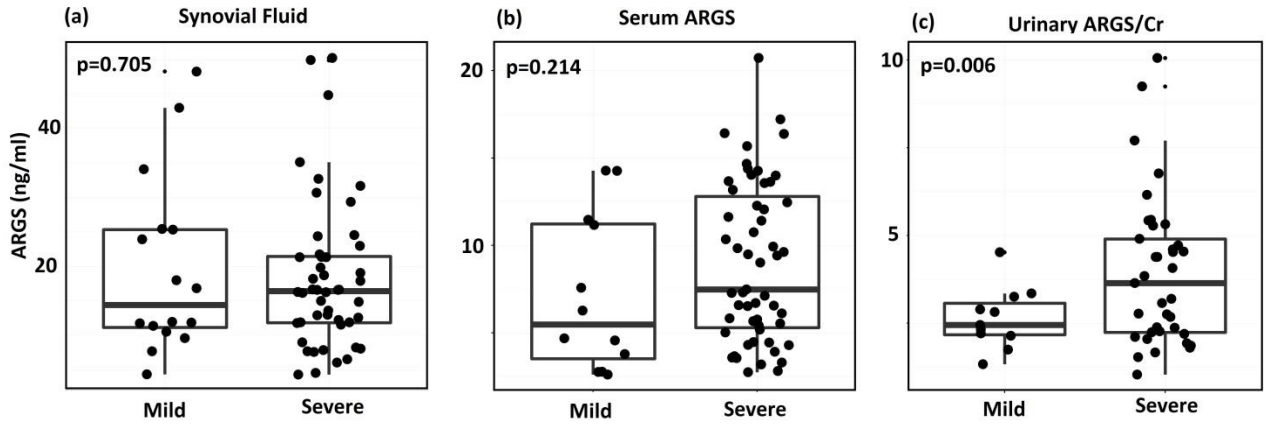

Figure 29 ARGS neoepitope in relation to mild and severe OA.

The major confounder here is that the mild cohort contains patients with subacute injury who tended to have relatively high levels of ARGS production. Secondary to this, the arthroscopy patient group contained many patients with relatively high levels of cartilage loss and this is why a comparison is made on the basis of MRI diagnosis of OA rather than surgical findings. Serum and urine show values a greater ability to differentiate between grades of disease severity but only urine levels were statistically significant.

6.1.1 Urinary ARGS neoepitope levels discriminate between mild and severe disease

In assessing the value of the ARGS neoepitope for disease diagnosis, we found that there was no significant difference in levels of ARGS neoepitope in synovial fluid and serum

between patients with mild and severe disease. However a significant difference was determined in the urinary ARGS neoepitope measurement which appears to be in contradiction to previous findings suggesting serum levels are more discriminatory[236]. However this study does not contain a control group of patients. In theory, since urine and serum are both peripheral fluids, a product of the whole body's synthesis of any biomarker should behave similarly in these two fluids in the analysis and indeed there was a correlation between the two sample types (section 4.1.1). However the subtle difference between them is that creatinine itself may have properties related to OA. Creatinine is related to lean body mass[179, 184] and exercise[182, 183] both of which may be reduced in OA.

6.2 Analysis of the effect of age on ARGS neoepitope levels and volume of cartilage

In Figure 30 (a) we can see the relationship between synovial fluid ARGS neoepitope level divided by serum ARGS neoepitope level compared to age. The ratio of synovial fluid ARGS neoepitope compared to serum ARGS neoepitope decreases with age. This suggests that the

specific joint from which the synovial fluid originates is contributing less to the total amount of ARGS neoepitope in serum as an individual ages.

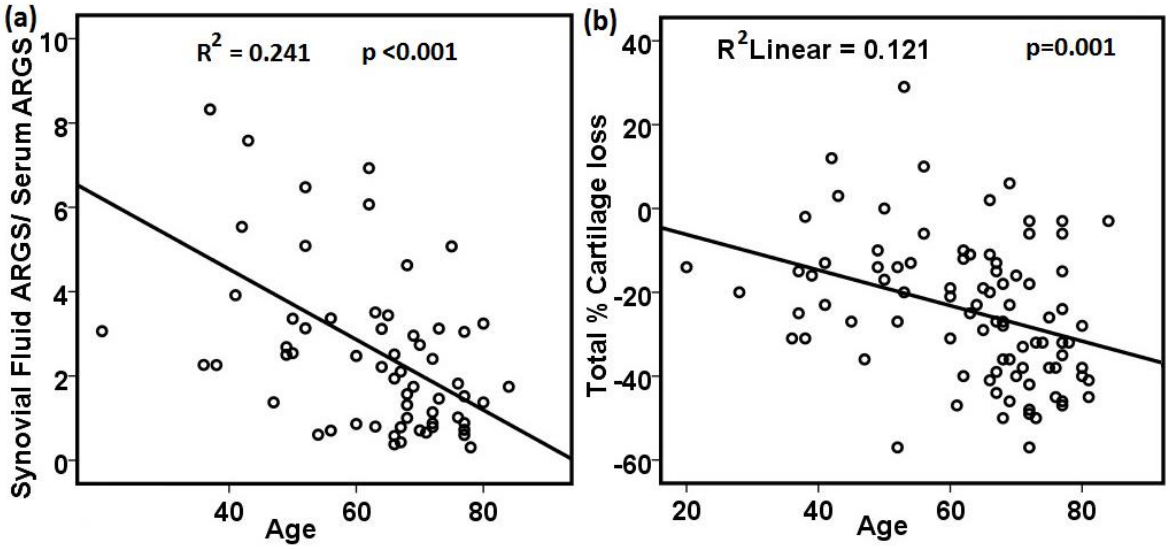

Figure 30 Relationship between age, ARGS and cartilage volume

In Figure 30 (b) we can see the relationship of cartilage volume to age, which shows that overall, cartilage loss increases with advancing age. In only examining the radiological disease pattern in a single joint, it is difficult to make assumptions about how much ARGS neoepitope is present, and this is demonstrated here.

6.2.1 Age is related to an increase in the ratio of peripheral to synovial fluid ARGS neoepitope and an increase in cartilage volume loss

Our results show that the relative level of synovial fluid ARGS neoepitope to peripheral fluid (serum and urine) ARGS neoepitope decreases with age. As discussed previously in section 4.2, there are other processes which may increase serum and urine ARGS neoepitope[216-218]. Indeed studies have correlated the serum levels of biomarkers to bone scan activity which estimate the total disease process of the entire patient[141]. Invariably this disease burden increases with age and hence the resulting disproportionate rise in serum and urinary ARGS neoepitope levels compared to the synovial fluid levels. There is also the issue

that acute injury causes large increases in cartilage turnover activity, presumably as a result of increased chondrocyte turnover[173, 219], and the proposed relative senescence of chondrocytes in more aged individuals[220] meaning that cartilage turnover is reduced in older individuals.

6.3 Relationship between serological markers and cartilage volume

Figure 31 shows ARGS levels compared to cartilage volume; showing total percentage cartilage loss (i), (iv) and (vii), medial compartment percentage cartilage loss (ii), (v) and (viii) and medial compartment percentage cartilage loss in MUKR patients (iii), (vi) and (ix) and the relationship to synovial fluid ARGS (top row), serum ARGS (second row) and urinary ARGS (bottom row). Analysis of the synovial fluid, serum and urine ARGS neoepitope levels against total volume loss revealed no relationship between these factors.

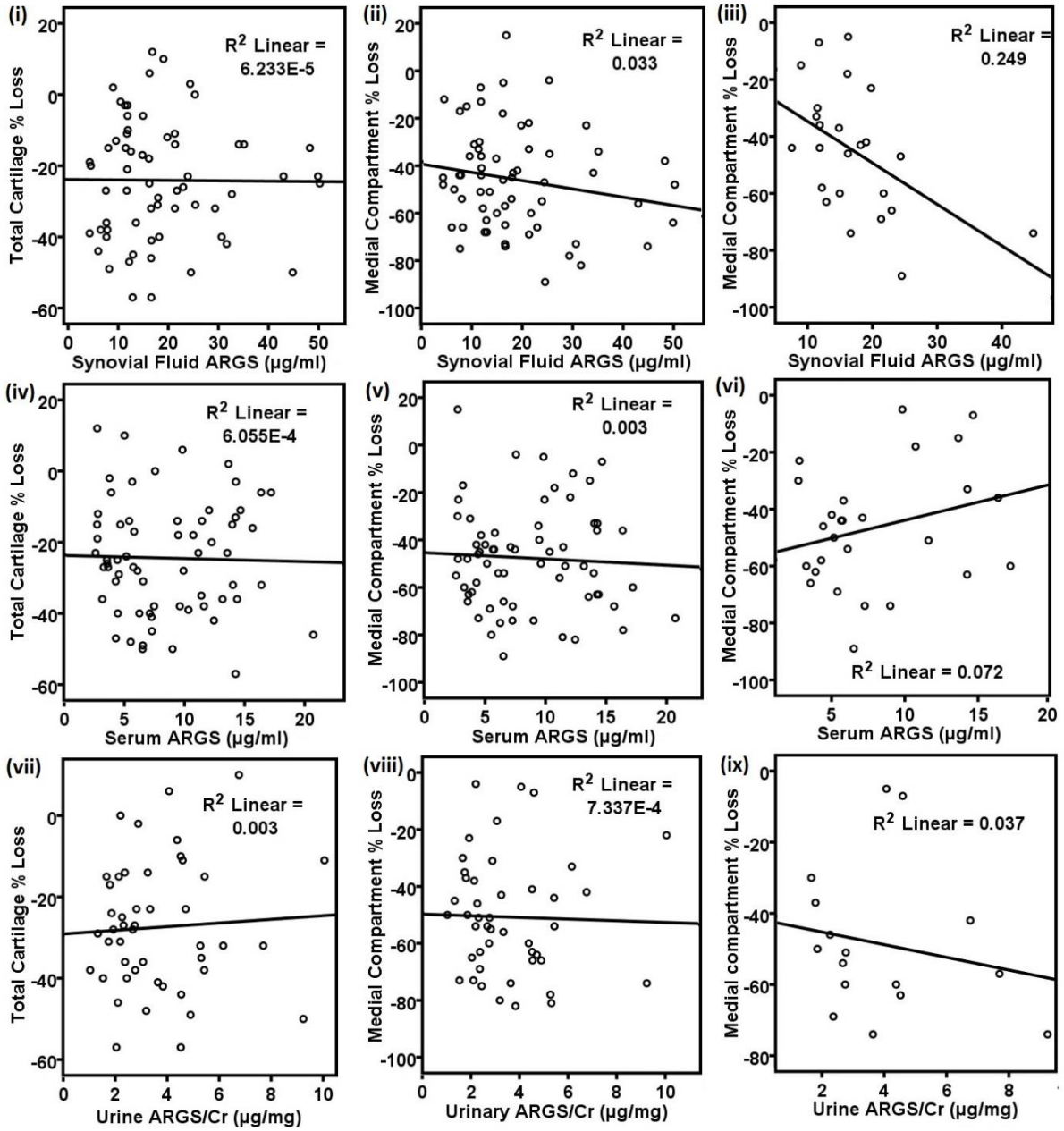

Figure 31 Relationship between ARGS neoepitope and volume of cartilage loss

Consequently we analysed subgroups of more homogenous patient types, in view of the fact that other variables could be affecting the data. The largest patient cohort was the patients undergoing medial unicompartmental knee replacement(MUKR) in which there were 38 patients. Analysis of the MUKR group revealed a statistically-significant relationship between medial compartment percentage cartilage loss and synovial fluid ARGS neoepitope level (R^2= 0.249; *P* = 0.012). When synovial fluid ARGS neoepitope level was compared

against levels in other compartments, we found no correlations in femoral (R^2= 0.066; *P* = 0.216), lateral (R^2= 0.049; *P* = 0.287), patella (R^2= 0.049; *P* = 0.292) or total (R^2= 0.099; *P* = 0.127) groups. There was no relationship between serum and urine ARGS neoepitope level and medial compartment loss in MUKR patients.

6.3.1 Synovial fluid ARGS neoepitope level shows a direct correlation with volume in the medial compartment of MUKR patients

When addressing the results in the specific subset of patients undergoing MUKR the goal was to create a more homogenous patient group and hopefully reduce the number of confounders. The MUKR group did exhibit some interesting trends in terms of cartilage volume results. The relationship between rising serum cartilage markers (COMP, CTX-II) and cartilage volume loss has been identified previously[237, 238]; however there are few studies comparing cartilage volume and synovial fluid biomarkers. Urinary markers, whilst displaying a greater efficacy as biomarkers overall[162], do not have the same associations in females as in a similar cohort of men[239], and this may be related to the creatinine production itself which changes during the course of the menopause[224]. As such it is possible that urinary biomarkers are less reliable in the female cohort.

With imaging markers in general we did not find an encouraging relationship between ARGS neoepitope level and intensity or volume measurements overall. MRI findings have previously been compared to those of static plain radiography with some degree of correlation in terms of OA disease measures[240] but most studies look at the longer-term effects[238] with previously-reported static values showing poor correlation to progression of disease[241]. Long-term studies involving multiple imaging time points are more robust at determining future disease progression; however in a clinical setting, single time-point analysis is more convenient for patients and more cost effective. Therefore if a biomarker

were available which could establish at a single time-point the severity and likely progression of disease it would be more useful from a medical interventional standpoint and ideally this should be in the early course of disease when more treatment options are available.

6.4 Summary of imaging measures and ARGS neoepitope levels

There is evidence of a relationship between the volume of cartilage and ARGS neoepitope levels, but the relationship is not robust and is related to other factors, in particular patient age. The patterns of the two measured variables have to be taken into account when analysing these results and it is possible we would see more correlation in more homogenous study participants.

Chapter 7 Clinical outcome scores

7.1 Introduction to clinical outcomes

Clinical outcome scores refer to the subjective functional and pain scores given by patients usually on a questionnaire basis. They are often based on what the patient, rather than the clinician, determines as important when evaluating the success of certain procedures, and these are known as patient-recorded outcome measures (PROMs). Table 8 shows the clinical outcome scores used in the study.

Table 8 Clinical outcome scores used in study

Clinical score	Questions	Best Score (least pain /functional impairment)	Worst Score (most pain /functional impairment)
Oxford Knee Score (OKS)	12	48	0
PainDETECT* (Nociceptive)	3	0	30
PainDETECT* (Neuropathic)	7	0	35 (+3)
***In study painDETECT (nociceptive) as "painDETECT(Noc)" and painDETECT (neuropathic) as painDETECT**			

Patient clinical outcome scores were collected at pre-operative assessment and by follow-up questionnaire for painDETECT scores sent out by the Oxford Musculoskeletal Biobank, and for Oxford Knee Score at clinic appointments pre- and post-operatively by surgeons, although only in patients undergoing arthroplasty. PainDETECT score is a pain rather than functional score not designed specifically for knees and is specifically designed to look for neuropathic pain but provides an initial nociceptive score on the questionnaire[146]. Oxford Knee Score is designed specifically to look at the functional as well as pain outcomes in patients undergoing arthroplasty and is not suitable for non-arthroplasty assessment[144].

7.2 Cartilage volume and clinical outcome scores

Whilst there was an improvement in the Oxford Knee Score between pre- and post-surgery scores (overall *P* = 0.000, TKR *P* = 0.003, UKR *P* = 0.0002), the follow up score was obtained at 4–8 weeks, and there is evidence to suggest that this is too early to obtain optimal results. With the painDETECT scores the results were less clear (overall *P* = 0.606, TKR *P* = 0.562, UKR *P* = 0.851).

There was no significant difference in Oxford Knee Score (*P* = 0.969) (Figure 32) or medial tibial cartilage loss (*P* = 0.930) when comparing upper and lower quartiles of cartilage loss for all patients (with upper representing least cartilage loss). In terms of painDETECT(Noc) score, total cartilage loss did not show any significant difference (*P* = 0.179); however medial tibial cartilage loss did show a difference between the highest and lowest quartiles (*P* = 0.034).

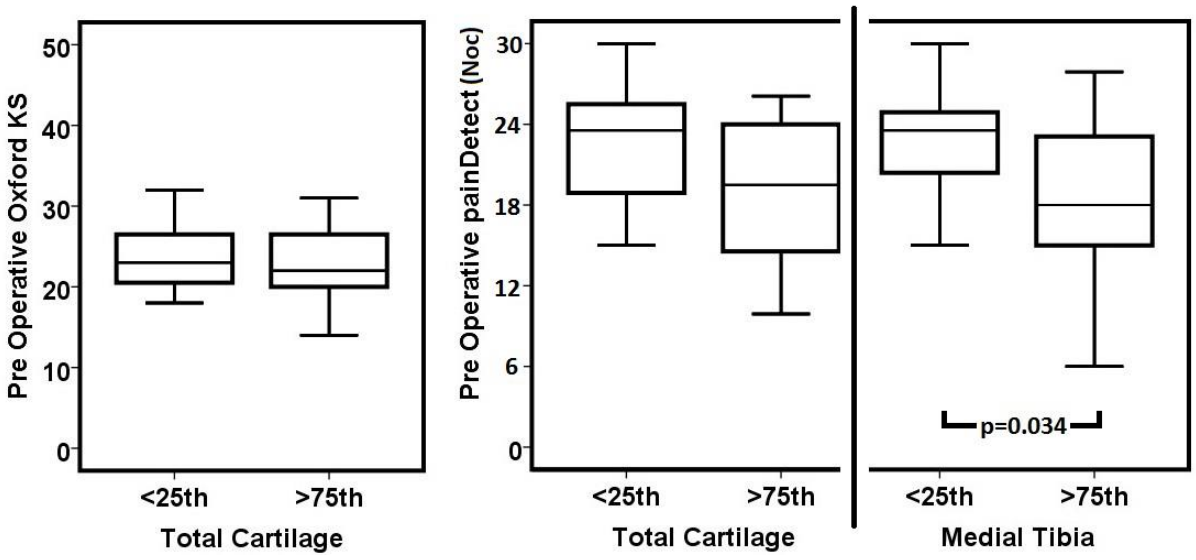

Figure 32 Cartilage volume and clinical outcome scores

Overall there was no correlation between total cartilage loss and OKS (R^2= 0.000, *P* = 0.897), painDETECT (R^2= 0.000, *P* = 0.957) or painDETECT(Noc) (R^2= 0.029, *P* = 0.162) or between medial cartilage loss and OKS (R^2= 0.000, *P* = 0.913), painDETECT (R^2= 0.009, *P* = 0.418) or painDETECT(Noc) (R^2= 0.031, *P* = 0.162). It was therefore decided to compare the upper and lower quartiles.

7.2.1 Medial compartment volume was related superficially to clinical outcome

In terms of clinical outcome, an association was demonstrated with the loss of medial compartment cartilage and overall pain scores from painDETECT(Noc) but not the neuropathic painDETECT or OKS. Multiple studies have failed to show a relationship between imaging and clinical outcome scores[134, 150-154] or function[155] and have even shown a slight association between decreasing cartilage and less pain[148]. However studies have correlated many other MRI features including meniscal injury[156], bone marrow lesions[157] and effusion[158] to clinical outcome scores.

7.3 ARGS levels and clinical outcomes

When we compared highest and lowest quartiles (Figure 33) we found no difference in the Oxford Knee Score ($P = 0.192$) or the painDETECT score ($P = 0.230$); however we did find a significant difference in the painDETECT(Noc) scores ($P = 0.026$).

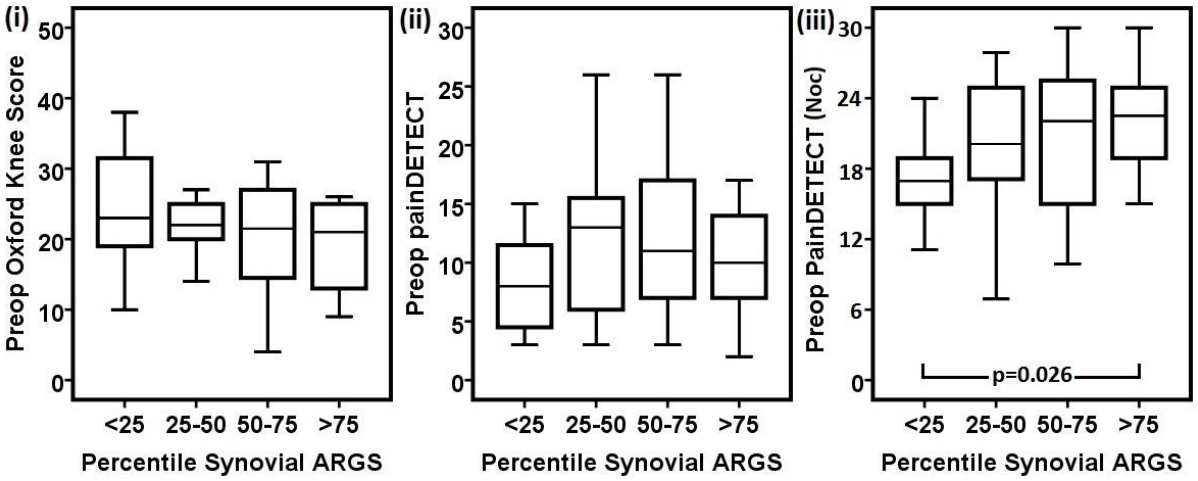

Figure 33 ARGS levels and Clinical outcomes

7.3.1 Increased ARGS neoepitope levels in synovial fluid is associated with higher pain scores

Our results showed that there is a relationship between synovial fluid ARGS neoepitope levels and painDETECT pain scores. In the literature there is ambiguity over the relationship between cartilage volume and clinical scores[148], although ARGS neoepitope level in synovial fluid has previously been shown to correlate with aspects of WOMAC[109]. Patterns of increasing values of other biomarkers (COMP and CTX-II) have also been associated with deteriorating scores in previous work but not tied to static values[242]. Static values of urinary GlcGal-PYD and serum osteocalcin were associated with WOMAC scores in another study echoing our findings, showing that biomarkers may be related to patient pain and function[243]. There appears to be no comparison of painDETECT and Oxford Knee Scores against biochemical OA markers in the evidence base. The explanation

for this relationship may be that the underlying disease pathology is related to inflammation and repair; the resultant increased cartilage turnover associated with these processes may also be linked to pain. One of the limitations of these results is that pain and function scores for joint replacement are more likely to have reached full improvement by a year rather than the 4-8 weeks in this study.

7.4 Pre-operative and Post-operative Clinical Outcomes

Whilst there was no significant relationship between clinical outcome scores and volume or intensity measurements there was a correlation between effusion volume and improvement in OKS (Figure 34(c), (d)).

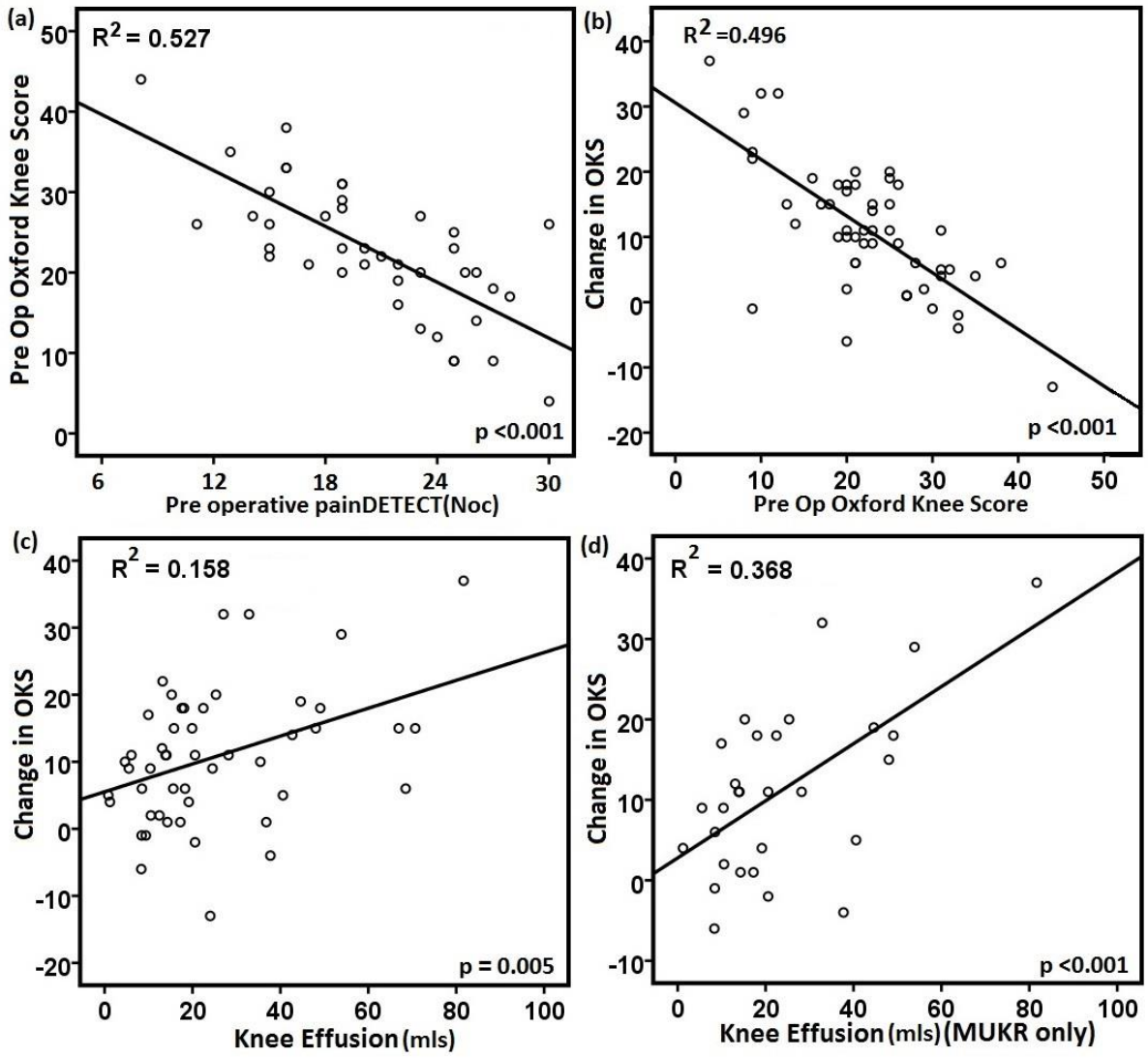

Figure 34 Comparison of clinical outcome scores

We also found a good correlation between painDETECT(Noc) and pre-operative OKS (R^2= 0.527, *P* < 0.001), as well as between the painDETECT score and pre-operative OKS (R^2= 0.162,*P* < 0.001) (not shown). Overall the best predictor of clinical improvement was the pre-operative OKS (R^2= 0.496, *P* < 0.001). Effusion was linked to clinical improvement as determined by OKS (R^2= 0.158, *P* = 0.005) and this was more noticeable in MUKR patients (R^2= 0.368, *P* < 0.001).

7.5 Summary of clinical outcome scores

Overall the clinical outcome scores appear to be more tied to radiological measures than to ARGS neoepitope level. There was an association between medial cartilage loss and synovial fluid ARGS neoepitope levels and pain scores. These data sets show that there is good agreement between outcome scores in general, and that improvement is best predicted by pre-operative clinical outcome score.

Chapter 8 Explant analysis

8.1 Introduction to explant analysis

Culture of *ex vivo* cartilage was performed to determine the response of ARGS neoepitope to a monoclonal antibody inhibitor of ADAMTS-5 (GSK2394002). Table 9 shows a breakdown of the cartilage donors for this experiment. Cartilage loss was calculated from segmental analysis of preoperative MRI.

Table 9 Explant samples: patient demographics

Gender	Age (*P*)	BMI (*P*)	% Cartilage Loss (*P*)
Male (7)	66.6 (0.775)	28.8 (0.311)	42.3 (0.135)
Female (11)	68.2 (0.775)	31.1 (0.311)	22.7 (0.135)

In the calculation of response we used the isomer (Iso) and inhibitor (949) levels to determine the change in ARGS production as follows (where T_{200} is the test reagent);

Δ ARGS = $T_{200} - T_{949} / T_{Iso} - T_{949}$

8.2 Results

In the figures below the response to GSK2394002 is plotted for two time-points (T4 and T8 indicating 12 and 24 days) to demonstrate the relative responses. Although we sampled the medium every three days initially, we found little change in the earlier timepoints (T1,T2, T3) and hence the analysis was mainly around the timepoints thereafter in particular T4 and T8). Figure 18 shows the % response compared directly to the control and demonstrates a dose-related response. At T4 there was a significant reduction in ARGS production following treatment with GSK2394002 at 200 µg/mL in comparison to both 50 µg/mL (*P* = 0.05) and

12.5 µg/mL (*P* = 0.001). At T8 there was a significant reduction in ARGS production in the 200 µg/mL GSK2394002 group compared to the 12.5 µg/mL group (*P* = 0.003) but not to the explants in the 50 µg/mL group (*P* = 0.115).

8.2.1 Overall response

Figure 35 shows the percentage reduction of ARGS secretion following treatment with different concentrations of GSK2394002 compared to an isotype control (T4; 200 µg/mL (-43.6% SD:23.2 *P* < 0.000), 50 µg/mL (-33.5% SD:24.8 *P* < 0.000), 12.5 µg/mL (-18.9% SD:33.8 *P* < 0.108). T8 200 µg/mL (-31.4% SD:26.8 *P* < 0.000), 50 µg/mL (-20.4% SD:30.2 *P*<0.000), 12.5 µg/mL (-6.1% SD:33.6 *P* = 0.173)). In reviewing the results overall we decided to look at time points 4 and 8 representing the middle and end of the experiment in terms of absolute decrease in ng/mL. Both the 200 µg/mL and 50 µg/mL concentrations of GSK2394002 produced significant reductions in ARGS neoepitope compared to the control at the same time points.

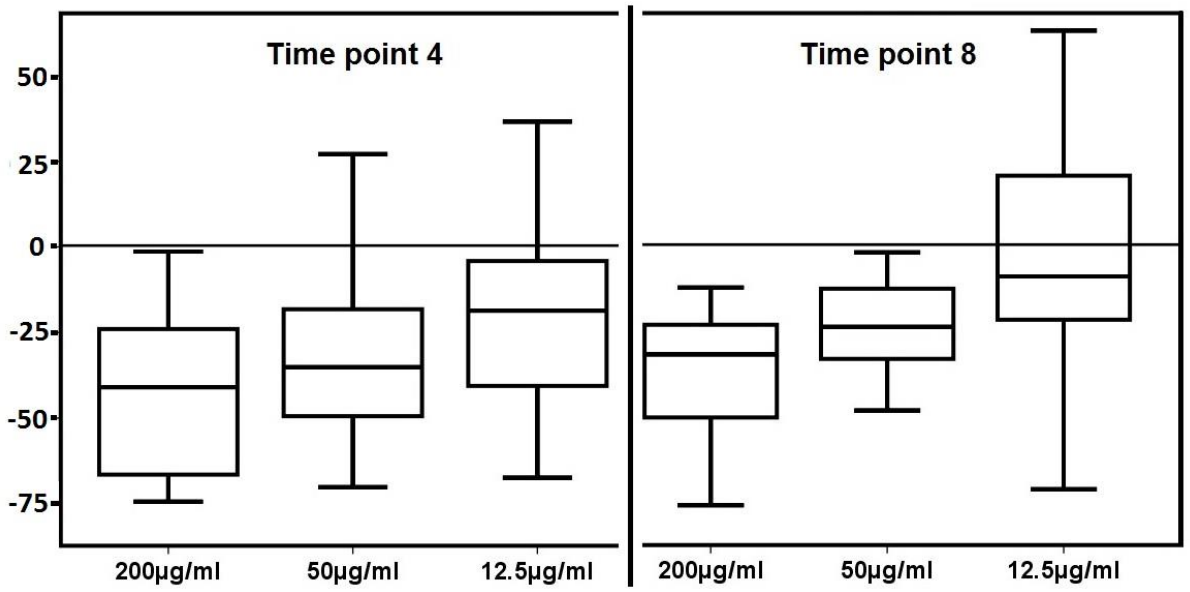

Figure 35 Percentage reduction of explant ARGS neoepitope level with different concentrations of GSK2394002

We also analysed the response of ARGS neoepitope relative to the baseline level of untreated samples to analyse the potential differences in the size and chondrocyte populations of the plugs.

The formula used was as below:

Δ ARGS (relative to baseline) = $((T200_{T0} / T200_{T4}) - (T949_{T0}/ T949_{T4})) / ((TIso_{T0} / TIso_{T4})-(T949_{T0})/ T949_{T4}))$

These results also demonstrated a consistent pattern which correlated with a dose-dependent response. A difference between the relative change from baseline in ARGS neoepitope production from T_0 to T_4 was compared against the change seen in the isotype control and still yielded significant results (T4: 200 µg/mL, *P* = 0.005, 50 µg/mL, *P* = 0.041, 12.5 µg/mL, *P* = 0.089; T8: 200 µg/mL, *P* = 0.052, 50 µg/mL *P* = 0.091, 12.5 µg/mL *P* = 0.253) although the relative differences appeared less significant when compared to baseline explant ARGS neoepitope production.

8.2.2 Explants show a dose-dependent response to GSK2394002 in the reduction of ARGS neoepitope

Cultured cartilage explants showed a dose-related response to GSK2394002. This finding has also been documented in other studies looking at proteoglycan production and release into medium after treatment with glucosamine in an effort to inhibit chondrocyte-derived proteolytics, and these also seem to have a ceiling of inhibition[244]. Even with 1000-fold increases in reagents such as chondroitin, the relative response did not correlate with the concentration of aggrecan degradation product[245]. There was a gradual increase in ARGS neoepitope production in all experiments, even those with the small molecule inhibitor GSK571949, causing an overall accumulation of ARGS neoepitope with time, and similar

findings were observed by Deng *et al.* over a similar time-frame[246]. It has been reliably observed that there is a relationship between mechanical loading and chondrocyte proteoglycan physiology[247], and absence of physical stress may reduce proteoglycan synthesis[248] which may explain the gradual increase of protein release over time in a non-mechanically-stimulated *ex vivo* environment.

8.2.3 Response over time

Figure 36 (a) shows again the dose-related percentage reduction of ARGS neoepitope in the explants over time compared to isotype control. All explants were hyper-stimulated at the end of the project by the addition of interleukin 1 (IL-1) and oncostatin M (OSM) and demonstrated significant ARGS neoepitope release. Time-points T1 though T3 were largely untested as the cartilage response from the initial experiments showed very little change in ARGS production until time-point 4 (day 12).

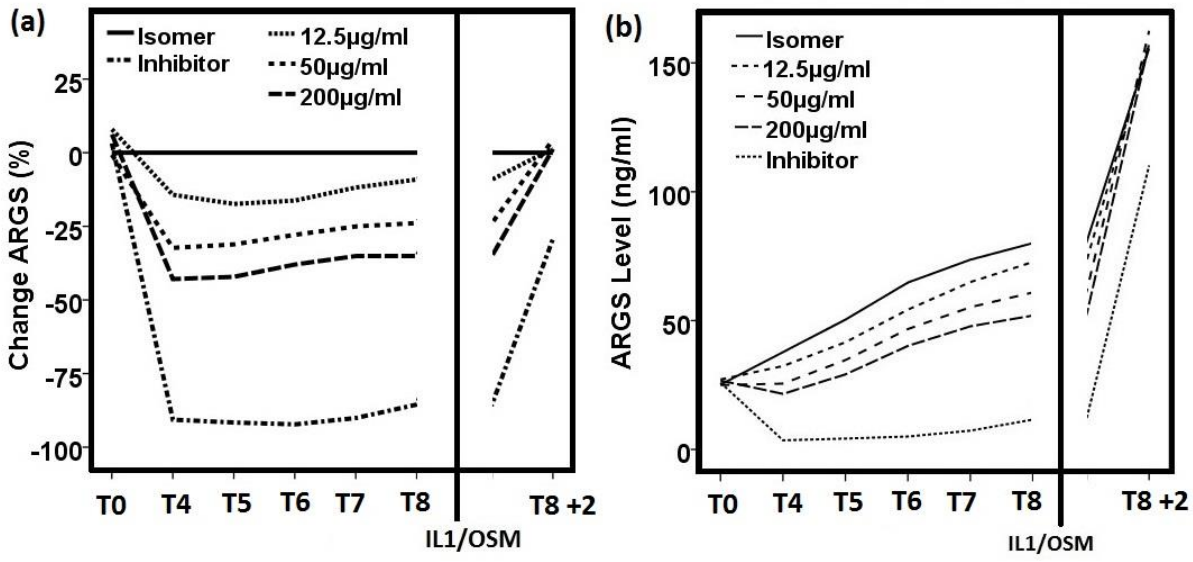

Figure 36 ARGS neoepitope production at all time-points.

Figure 36 (b) shows that absolute ARGS neoepitope production in culture gradually increased over time. It is also apparent that the small molecule inhibitor GSK571949 is more effective than the antibody inhibitor GSK2394002.

Figure 37 shows the reduction of ARGS neoepitope production caused by different concentrations, plotted with a line of best fit for time points T4 and T8.

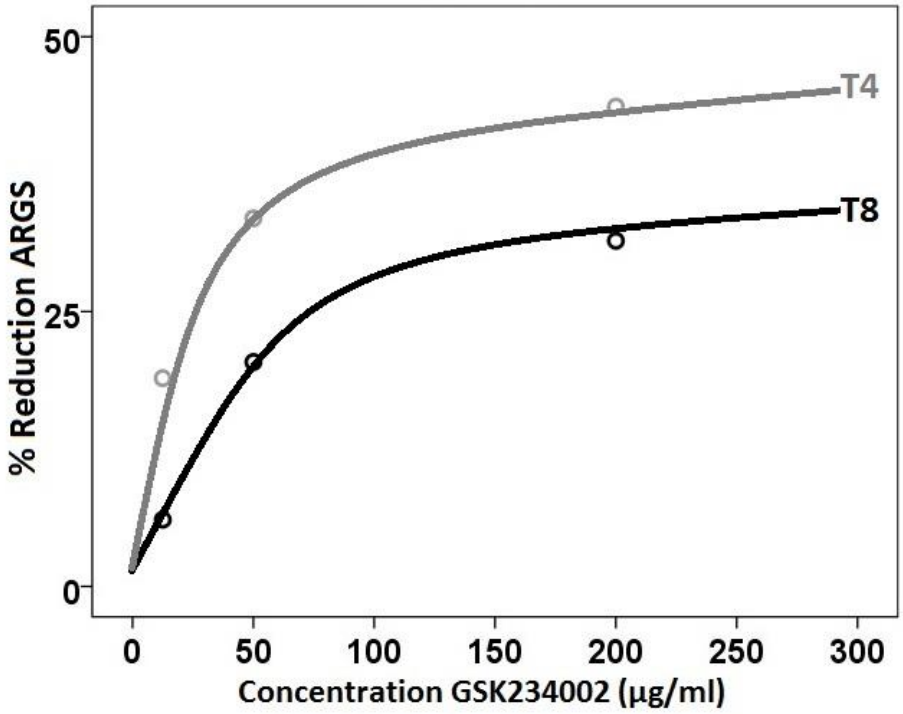

Figure 37 Mean inhibition of the ARGS neoepitope with different concentrations of reagent GSK2394002

Despite a 4-fold increase in GSK2394002 the reduction in ARGS neoepitope production was not proportional. At T4 the 4-fold increase in concentration of GSK2394002 from 12.5 µg/mL to 50 µg/mL resulted in a 1.8-fold reduction of ARGS neoepitope and over the increase from 50 µg/mL to 200 µg/mL concentration the effect was 1.3-fold. At T8 the effect between 12.5 µg/mL and 50 µg/mL was 3.4-fold and between 50 µg/mL and 200 µg/mL the effect was 1.5-fold.

8.2.4 Compared to a small molecule inhibitor there appears to be a less effective upper limit of GSK2394002 on ARGS neoepitope production.

The cartilage explant experiment did demonstrate a reduction in ARGS neoepitope production in a dose-dependent manner; however the 4-fold incremental increases in the reagent did not translate into 4-fold decreases in ARGS production *in vitro*. Instead a maximum reduction of 50% was observed. Therefore it is likely another enzymatic process is at play; previous studies suggest this is most likely to be ADAMTS-4[249], although this would contradict the assertion that ADAMTS-5 is 100 times more active than ADAMTS-4[250], or that MMPs may be responsible for the remaining degradation, although previous work has suggested their role to be substantially secondary to aggrecanases[251]. It may also be possible that the wide spectrum inhibitor can penetrate better into the cartilage explant than the ADAMTS-5 blocker. Cartilage matrix has been shown to be impermeable to large molecules such as full-size immunoglobulins[252] and as such this potential therapeutic may require radiolabelling to determine penetrance[253].

8.2.5 Response in different operation types

Figure 38 (a) shows that there is a difference in absolute production of ARGS neoepitope in explants derived from TKR and MUKR patients, but whilst this is significant in the 50 µg/mL treatment group, when looking at relative reductions there is no suggestion that there is a significant difference between the two groups (b).

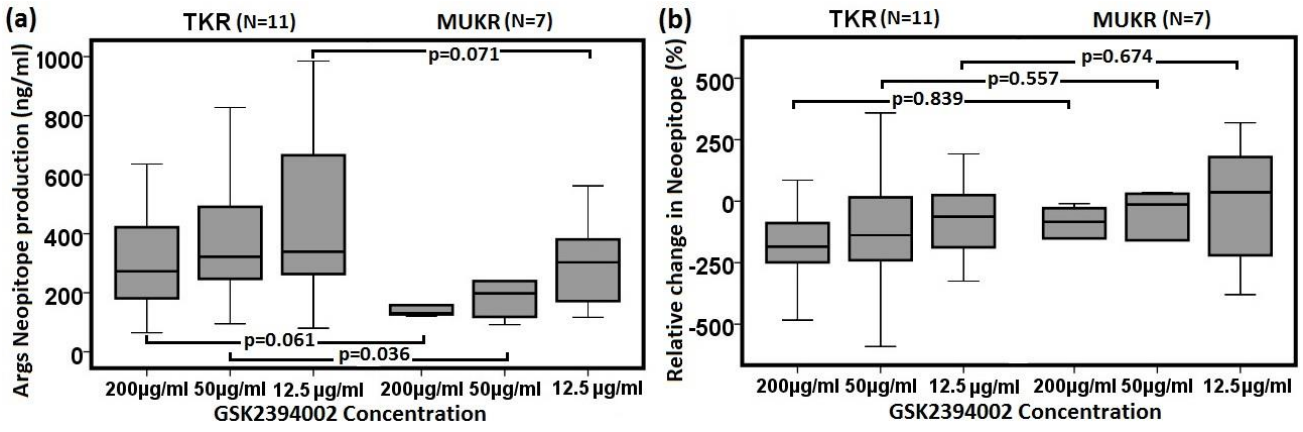

Figure 38 Absolute and relative ARGS neoepitope reduction in TKR and MUKR

8.2.6 Response correlation to patient characteristics

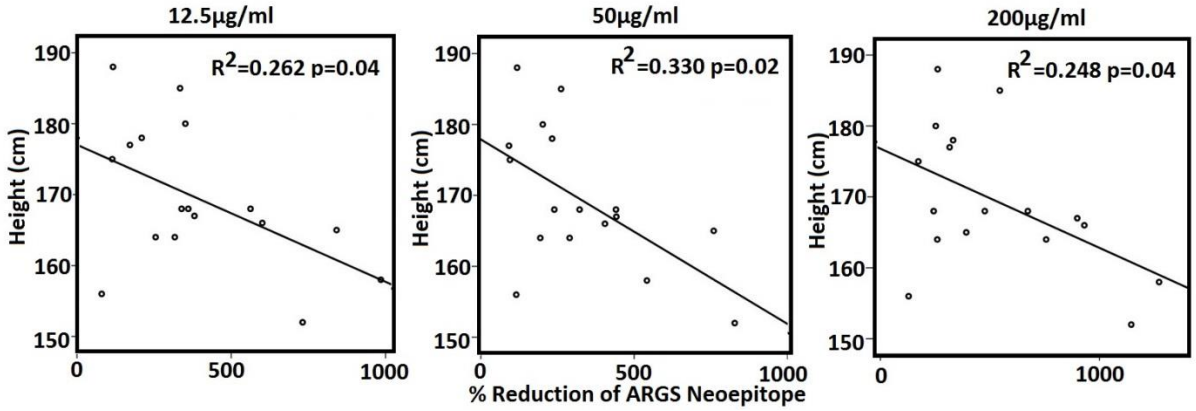

Figure 39 Change in ARGS neoepitope from T0 to T8 compared to patient height

We also compared the response of ARGS neoepitope to the height of the patient (Figure 39). Interestingly, we found that the two factors seemed to consistently correlate and seemed completely unrelated to patient gender or operation type. Although this was not part of the original study design and analysis plan, there is merit to assessing the population characteristics associated with the greatest effect in the case of a potential therapeutic. Interestingly, this pattern was not present in the medium control or isomer control but seems to indicate a greater percentage increase in ARGS neoepitope production in shorter individuals i.e. a reduced responsiveness to the GSK2394002 antibody. However it is also

possible that the shorter participants had less cartilage for processing into explants and therefore the plugs were more likely to have some more advanced OA changes and therefore produce more ARGS overall; further investigation is required to shed light on this result.

8.3 Summary of explant analysis

Overall the human explants displayed a measurable response compared to control in *ex vivo* conditions, however they produced an incomplete response when compared to the small molecule inhibitor GSK571949. The penetration of the cartilage matrix by the monoclonal antibody inhibitor GSK2394002 is as yet unknown and potentially it may be worth investigating this by using monolayer culture or radiolabelling.

Chapter 9 Conclusions and limitations

9.1 Conclusions

9.1.1 Broad hypothesis:

Serological levels of ARGS neoepitope are not related to level of OA severity in human knee disease, therefore the hypothesis is rejected.

Null hypothesis: There is no relationship between level of OA defined by loss of cartilage volume in human knee disease and serological levels of ARGS neoepitope.

If the principle outcome measure of human knee disease in this study is volume of cartilage then the null hypothesis cannot be rejected as overall there is insufficient evidence that total volume of cartilage is linked to synovial fluid, serum or urine markers in a cross-sectional cohort of patients undergoing knee surgery.

9.1.2 Specific hypotheses

i) Hypothesis: An elevation of serological levels of ARGS neoepitope is associated with increased cartilage loss on quantitative measurement from corresponding MR imaging.

Hypothesis rejected.

Null hypothesis: There is no association between serological levels of ARGS neoepitope and increased cartilage loss on quantitative measurement from corresponding MR imaging.

Although the correlations between volume of cartilage or indeed any other outcome measure and ARGS neoepitope level are poor when assessing the late OA cohort collectively, analysis of specific subsections of the cohort and adjustment for known confounders revealed a correlation between loss of medial cartilage volume in MUKR

patients and synovial fluid and urine ARGS neoepitope levels. Further investigation of the mechanisms underlying ARGS neoepitope production and its correlation with imaging measurements of cartilage disease are necessary. Many other studies have focused on the other aspects of the BIPED criteria, usually in trying to investigate the value of these markers to predict future disease, and this study cannot address this due to the short duration. Broadly speaking the ARGS neoepitope level or MRI-derived markers did not determine with any accuracy the surgical intervention in this study; this infers poor reliability as a diagnostic marker at present. Further study on ARGS neoepitope as a biomarker or indeed any novel biomarker would benefit from the findings of this trial in designing the project, and recommendations will be outlined in section 9.3.

ii) Hypothesis: Explants treated with a specific ADAMTS-5 mAb will show suppressed levels of ARGS neoepitope compared to isomer controls. **Hypothesis accepted**

Null hypothesis: Explants treated with a specific ADAMTS-5 mAb will not suppress levels of ARGS neoepitope compared to isomer control.

The ADAMTS-5 specific mAb (GSK2394002) caused a dose-dependent reduction in the production of ARGS neoepitope from cartilage explants. Other factors must be considered before further study can be directed towards a local or indeed systemic therapeutic agent, which would include drug penetrance, drug delivery and most importantly overall safety of the therapeutic.

9.2 Study Limitations

Due to the short duration for this initial exploratory study we could not obtain information on prognostic factors that are necessary for selecting candidates for intervention and primary prevention. The study is almost cross-sectional and therefore can give some indication on the relationship of markers to diagnosis and disease burden. Furthermore this study can help to direct future investigations, for instance long-term studies into biomarker behaviour over time.

A missed opportunity in this regard would have been to assess ARGS neoepitope levels alongside other available and more widely-researched biomarkers. Performance of other more studied markers may have provided a yardstick for interpretation as to how effective this marker is in the short term. Whilst it is not really feasible to initiate a long-term study on the validity of a relatively unstudied marker this could aid with interpretation as to whether it holds value in comparison to a more studied marker, in particular serum COMP and urinary c-telopeptide of type II collagen (uCTX-II). Additionally a combinatorial pattern of expression of a number of biomarkers may add more power to allow discrimination between disease subtypes and potential intervention.

A further limitation is the bias introduced by recruiting patients with MRI scans particularly in those undergoing arthroplasty for whom there may have been diagnostic uncertainty and unclear or earlier stage multi-compartmental disease.

In line with other studies, many factors remain difficult to control and these are in principal the relationship between patient activity factors, the measurement of total burden of OA and genetic components.

9.3 Further study recommendations and future work

9.3.1 Time point harmonisation

It is difficult to compare biochemical markers without having quantitative imaging data available from a relatively similar time point. Scans used in this study were clinical and there was a significant time-period from the scan date to the operation date (mean scan-date-to-operation-date interval was 212 days; median 185 days). Whilst this allowed for higher recruitment and reduction in costs there is an element of error produced by both the delay itself and the variability in delay length.

9.3.2 Post-operative serum and urine samples

In this study post-operative samples did not provide additional information and caused the loss of a significant number of sample sets. The average follow-up length was 62 days (median 50 days) and no significant differences or trends in ARGS neoepitope level were detected other than a general slight increase in the TKR cohort. This finding in itself is interesting because the removal of a potential source of ARGS neoepitope resulted in a greater production of this marker. However it does suggests that early follow-up may not provide new information and it is therefore necessary to assess ARGS neoepitope at later time points (e.g. 1 year) to assess whether levels are altered.

9.3.3 Acknowledging the different characteristics of different sample types

The ARGS neoepitope levels in the serological samples assessed (serum, urine, synovial fluid) did not show any strong correlation. Certainly a significant factor is the site of ARGS neoepitope production whether local, in the case of synovial fluid, or general, in the case of serum and urine. Further analysis, e.g. 24-hour urine collections, is required to assess the correlation with serum samples. It should also be considered whether creatinine has a link to OA due to gender, which the results of this study suggest could be a factor (section 4.1.4),

as well as body composition and muscle activity. Indeed body composition in relation to biomarkers has been poorly studied and future studies should make some effort to assess this via bio-impedance or by DEXA scanning. These markers tell different stories and whilst synovial fluid gives good results for research purposes it is the least feasible as a long-term marker or potential screening tool. We should investigate further the normalisation techniques used for urinary assessment of biomarkers and the role of creatinine by carrying out 24-hour collection studies. Urine is cheap and safe to collect and we could be missing vital information which could enhance the use of potential biomarkers both in prospective and previous studies[224].

9.4 Overall Conclusion

This project demonstrates some correlations between ARGS neoepitope levels and the burden of OA in a heterogeneous knee surgical cohort. This is the first study to assess a potential OA biomarker in relation to a range of imaging and clinical outcome measures. The findings of this study may help direct further investigation into the utility of the ARGS neoepitope and other OA biomarkers.

As a secondary outcome, *ex vivo* cartilage explants showed a reduction in ARGS neoepitope production when treated with the ADAMTS-5 targeting antibody GSK2394002 and this may be useful in future therapeutic development. Alongside animal models suggesting that abolition of the activity of ADAMTS-5 in joints leads to delayed or absent cartilage degeneration, our work suggests it may be a viable localised therapy. In order to avoid potential off-target effects of cartilage protease inhibitors, an intra-articular approach may be the most logical initial approach[254]. This could potentially avoid significant systemic

side-effects such as musculoskeletal syndrome[255]. According to the findings of this project, it would also be worth investigating whether any particular demographic may have a better response (section 8.2.4).

To date no biomarker for OA has shown proven clinical utility and this echoes our findings from this work. The difficulties in studying OA, in particular lack of standardised outcomes, remain significant factors in generating a sensitive and specific biomarker. This work further highlights the requirement and difficulty in harmonising patient groups to provide accurate comparative data.

ARGS neoepitope is potentially a useful marker of cartilage turnover via its representation of ADAMTS-5-driven cartilage degradation. However this and other work suggests it may represent other as-yet not properly studied physiological processes, in particular bone and soft tissue healing, which need to be considered in the design and analysis of future studies.

In closing, the ARGS neoepitope has shown some association with imaging and clinical outcome scores; however these are insufficient to predict diagnosis or treatment without further study and validation. GSK2394002, the mAb targeting ADAMTS-5, inhibited degradation of aggrecan *ex vivo* and therefore has some potential to be developed further as a therapeutic.

Chapter 10 Appendices

10.1 Planned publication abstracts

10.1.1 Paper 1: Association between the aggrecan biomarker ARGS neoepitope and quantitative MRI imaging in patients with knee osteoarthritis.

Abstract

Aggrecan is one of the first cartilage proteins to be degraded during degenerative joint disease, principally as a result of the activity of the protease ADAMTS-5. The ARGS biomarker has been developed with a proposed role of quantifying disease activity and evaluating the clinical effect of agents used to reduce the disease process.

In this study ARGS neoepitope was measured in serum, synovial fluid and urine in a cohort of patients who were undergoing knee surgery for either injury or osteoarthritis in either early or end-stage patients. These results were then compared to radiological and clinical outcome measures to identify whether the measurement of this degradation product of ADAMTS-5 has any effective role as a biomarker of osteoarthritis.

Results of this study indicate that ARGS neoepitope levels are tied to disease activity of joints whether this is acute injury or end stage arthritis. Urinary ARGS shows a significant difference between mild and severe OA (P = 0.006), while medial compartment volume of cartilage loss is correlated to synovial fluid ARGS (R^2= 0.249, P = 0.012). Although multiple correlations were found in the course of this study, the validity of this biomarker in assessing diagnosis, disease burden and efficacy of intervention is not proven.

OA has proven a very difficult disease to assess due to multiple factors influencing the behaviour of biological markers including the burden of disease and lack of categorical OA phenotypes. The ARGS biomarker does show potential in specific circumstances to act as a potential disease marker in terms of disease burden and diagnosis; however this needs to be evaluated in the context of the patient.

10.1.2 Paper 2 Reduction in aggrecan proteolysis using ADAMTS-5 blocking therapeutic in *ex vivo* human cartilage model.

Abstract

Aggrecan degradation has been shown to be an early process of cartilage loss in degenerative joint disease and is a target for pharmaceutical intervention in osteoarthritis. This process is mainly thought to be driven by the activity of ADAMTS-5 and subsequently provided a focus for the aforementioned drug therapy.

The aim of the study was to investigate the activity of a specific blocker of ADAMTS-5 in an ex vivo human cartilage model. The study compared drug therapy of different concentrations against inhibitor and isomer control. The main outcome of the study was the enzymatic degradation product of aggrecan (ARGS neoepitope).

The ADAMTS-5 blocker produced a dose-dependent reduction in ARGS neoepitope when compared to the isomer control. The mean reduction at 12 days was 43.6% while at 24 days it was 31.4%. Despite stepwise fourfold increases in the reagent concentration this did not translate into a proportional response in reduction of ARGS neoepitope.

This inhibitor of ADAMTS-5 shows efficacy in reducing ARGS neoepitope release, suggesting possible efficacy as an osteoarthritis disease-modifying agent. However aggrecanolysis inhibition was far from complete, suggesting activity of other enzymes most likely ADAMTS-4. Other findings in terms of the donor factors in relationship to explant response are also discussed.

10.1.3 Paper 3 Comparison of quantitative imaging measures from clinical MRI to serological and clinical outcomes.

Abstract

Osteoarthritis is a leading cause of disability in developed countries and is independently associated with increased mortality. Measures of disease burden consist predominantly of imaging and clinical outcome scores.

We aimed to show the viability of analysis of different MRI sequences across different knee surgical patients in the evaluation of knee disease. We intend to examine the patterns of disease present in different knee diseases and compare them to clinical outcome measures and plain radiography findings.

In total the MRI images of 89 patients undergoing knee surgery were analysed. The results revealed a decrease in cartilage volume and increase in cartilage intensity inhomogeneity with advancing age. Medial tibial cartilage volume was associated with Kellgren-Lawrence score but intensity measures were not. Overall medial compartment disease showed the best association with other measures although these were modest. Change in Oxford knee score was significantly associated with effusion size overall (R^2 = 0.167, P = 0.005) and in MUKR patients (R^2 = 0.368, P = 0.000).

Whilst it is possible to utilise different scan types in the evaluation of quantitative MR-derived outcomes, it is the quantitative outcomes themselves which demonstrate poor correlation to other disease measures.

10.2 Acronyms & Abbreviations

ACL	Anterior Cruciate Ligament
ADAMTS	A Disintegrin And Metalloprotease With Thrombospondin-Like Motifs
AGE	Advanced Glycation End Products
ARGS	ARGS374 Neoepitope (cleavage neoepitope of ADAMTS-5)
BMI	Body Mass Index
CDC	Centre for Disease Control
COX 2	Cyclo-Oxygenase 2
CO_2	Carbon Dioxide
COMP	Cartilage Oligomeric Matrix Protein
Cr	Creatinine
CTX-II	C Telopeptide of Collagen type II
DEXA	Dual-energy X-ray absorptiometry
DICOM	Digital Imaging and Communication in Medicine
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EKAM	External Knee Adduction Moment
ELISA	Enzyme-Linked Immunosorbent Assay
ESR	Erythrocyte Sedimentation Rate
EU	European Union
EULAR	European League Against Rheumatism
FCS	Foetal Calf Serum
GAG	Glycosaminoglycan
GDP	Gross Domestic Product
GlcGal-PYD	Urinary Glucosyl-Galactosyl-Pyridinoline

GSK	Glaxo Smithkline (Pharmaceutical company)
HA	Hyaluronic Acid
IGF	Insulin–like Growth Factor (cytokine)
IGD	Inter Globular Domain
IL	Interleukin (Cytokine)
IU	International Unit
JSN	Joint Space Narrowing
Kc	Keratinocyte chemoattractant (murine equivalent of IL8)
KDa	Kilo Dalton
KL	Kellgren Lawrence score
KOOS	Knee Osteoarthritis Outcome Score
KSS	Knee Society Score
LLOQ	Lowest Limit of Quantification
LUKR	Lateral Unicompartmental Knee Replacement
mAb	Monoclonal Antibody
MMP	Matrix Metalloproteinase
MRI	Magnetic Resonance Imaging
µL	Microlitre
MUKR	Medial Unicompartmental Knee Replacement (Oxford)
ng	Nanogram
NICE	National Institute for Health and Care Excellence
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
OKS	Oxford Knee Score (0-48) (0 = Worst Outcome, 48 = Best)
OSM	Oncostatin M
OUH	Oxford University Hospitals
PainDETECT	Paindetect (neuropathic) score (0-36) (0 = Least pain)

PainDETECT(Noc)	Paindetect (nociceptive) Score (0-10) (0 = Least pain)
PD FS	Proton Density Fat Saturation
PDW SPAIR	Proton Density Weighted Spectral Adiabatic inversion Recovery
PGE_2	Prostaglandin E_2
PFKR	Patellofemoral Knee Replacement
PROM	Patient-Recorded Outcome Measure
RF	Rheumatic Factor
ROA	Radiographic Osteoarthritis
ROI	Region of Interest
ROS	Reactive Oxygen Species
SCOPE	Arthroscopy (Abbreviation)
SF	Synovial Fluid
SF OA	Synovial Fluid Signs of OA
TKR	Total Knee Replacement
TNF α	Tumour Necrosis Factor α (cytokine)
UK	United Kingdom (of Great Britain and Northern Ireland)
UKR	Unicompartmental Knee Replacement
WATS	Water Only Scan (MRI sequence)
WOMAC	Western Ontario and McMaster University Arthritis Index

10.3 Appendices

10.3.1 Appendix 1 Serological sample processing

Serum Collection

1. Approximately 6 mL blood/patient will be collected at each time point.
2. Collect blood into 6 mL serum separator (SST) tubes (yellow top) and invert 5–6 times. Leave to coagulate at ambient temperature for 60 minutes.

3. After clotting for 60 minutes, centrifuge in a swing bucket centrifuge at ≤ 1300 x *g* for 10 minutes at 2-8°C.
4. Harvest serum using a fine-tipped pipette and aliquot 125 µL into labeled polypropylene 2 mL screw-cap cryotube. Aim to collect 10 aliquots per patient per visit.
5. Freeze samples at -80°C until shipment on dry ice to GSK.

Urine Collection

1. Minimum of 10 mL/patient at both time-points.
2. Collect mid-stream samples into a labelled 30 mL sterile urine collection pot.
3. Invert sample to mix and transfer to a 15 mL centrifuge tube and spin at 1500 x *g* for 5 minutes at room temperature within 2 hours of collection.
4. Aliquot 125 µL into an appropriately-labelled 2 mL screw-cap cryotube. Aim to collect 10 aliquots per patient per visit.
5. Freeze samples at -80°C until shipment on dry ice to GSK.
6. Samples also kept at Oxford for colorimetric assay, protocol found at https://www.caymanchem.com/pdfs/500701.pdf

Synovial Fluid Collection

1. Collect synovial fluid samples (minimum of 1 mL, no maximum volume) into 25mL polypropylene tubes and keep on ice for no longer than 30 minutes, if processing is not immediate

   .
2. Dilute the sample with an equal volume of Dulbecco's PBS.

3. Prepare a 100 U/mL stock solution of hyaluronidase and store at 2–8°C out of direct sunlight for no more than 8 weeks. Add 10 µL of stock to each mL of synovial fluid to give a final hyaluronidase concentration of 1 U/mL.
4. Place tubes containing synovial fluid and hyaluronidase on a roller at room temperature for 60 minutes (± 5 minutes) and centrifuge at 1500g for 10 minutes at room temperature.
5. Remove any small flocculate matter (if present) with a pipette tip.
6. Remove supernatant and transfer to a clean 15 mL centrifuge tube.
7. Spin at 2500 rpm for 10 minutes at room temperature.
8. Harvest supernatant and aliquot 125 µL into an appropriately-labelled 2 mL screw-cap cryotube.
9. Freeze samples at -80°C until shipment on dry ice to GSK.

10.3.2 Appendix 2 Cartilage culture

Cartilage preparation

Note: All procedures should be performed in appropriate biohazard containment cabinet using universal precautions.

1. Carefully unpack cartilage sample container being cautious of leaks around the container lid.
2. Prepare workspace inside biohazard cabinet with absorbent diapers, plentiful paper towels, appropriate sterilization spray, ~400 mL of 80% ethanol in beaker (for instrument sterilization), several sterile scalpels, sterile forceps, sterile surgical scissors, sterile dissection plates (square petri dishes), and sufficient volume of culture medium (DMEM + 10% foetal calf serum + 1× antibiotics + 1× L-glutamine).
3. Sterilize all instruments in the 80% ethanol solution before use.

4. Open cartilage container and remove individual sample pieces for preparation. Keep additional sample pieces moist in medium until use.
5. Using a scalpel, shave thin slices of cartilage from the sample removing any fatty or connective tissues. Approximate cartilage thickness should be < 1 mm and as uniform as possible.
6. Place cartilage slices in a new sterile petri plate in a sufficient volume of fresh culture medium to keep the cartilage submerged.
7. Repeat steps 3 thru 5 for each sample piece until all cartilage has been collected.
8. Return un-used sample pieces to the sample container and decontaminate with 100% bleach for 2 × 5 minutes aspirating between steps. Aspirate all liquid from the sample container following final decontamination. Wrap sample container and all shipping materials which were in contact with sample inside an absorbent diaper and dispose in appropriate biohazard waste container.
9. At this point, cartilage can be placed in a sterile dish with medium until preparation of plugs.

Preparation of cartilage plugs

Note: All procedures should be performed in appropriate biohazard containment cabinet using universal precautions.

1. Prepare workspace inside biohazard cabinet with absorbent diapers, plentiful paper towels, appropriate sterilization spray, ~400 mL of 80% ethanol solution in beaker (for instrument sterilization), sterile scalpel, sterile forceps, sterile surgical scissors, hand-held leather punch, sterile dissection plates (square petri dishes), and sufficient volume of culture medium (DMEM + 10% foetal calf serum + 1× antibiotics + 1× L-glutamine).

2. Sterilize all instruments in the 80% ethanol solution before use.
3. Carefully transfer cartilage sample slices into a sterile square petri dish containing culture medium.
4. Using forceps and a hand-held leather punch, cut cartilage plugs from the sample slices taking care not to overlap the punches or punch too close to the edges of the cartilage where it is thinner and less uniform. Plugs should be ~3 mm in diameter.
5. Place cartilage plugs in a sterile square petri plate containing culture medium until loading into the assay plate.

Loading Assay Plate

1. Label appropriate number of sterile, polystyrene, 96-well, flat-bottomed plates with sample ID number and date.
2. Pipet 200 µL of culture medium into each well of each plate.
3. Using forceps carefully place one cartilage plug into each well of the plate.
4. Place plates in an incubator at 37^{o}C, 5% CO_2, 95% relative humidity.

Assay Setup and Sampling

1. Allow cartilage explant plates to equilibrate for 48 hours in the incubator before starting the assay.
2. Remove the total volume from each well and transfer to a separate 96-well plate for analysis as a Baseline sample. Cover and seal the sample plate edges with parafilm. Store sample plates at -20^{o}C until all time points have been collected for analysis.
3. Dilute the appropriate volume of each control or experimental antibody and after removing and storing Baseline sample, pipet 200 µL of appropriate diluted antibody into each explant well according to the plate template.

4. Additionally, pipet 100 µL of the diluted antibody into a separate 96-well plate as a Day-0 time-point. Cover and seal the sample plate edges with parafilm. Store sample plates at -20°C until all time-points have been collected for analysis.
5. At desired time points, sample 100 µL from each well of the explant assay plate (every 3–5 days) and store at -20°C until all time-points have been collected for analysis.
6. Replace sampled volume with fresh assay medium in each well.

Culture Template

With the plates prepared for the experimental assay, the assays are prepared and added according to the plate as follows:

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td>Sterile H_20</td><td>Sterile H_20</td><td>Sterile H_20</td><td>Sterile H_20</td><td>Sterile H_20</td><td>Sterile H_20</td><td>Sterile H_20</td><td>Sterile H_20</td><td>Sterile H_20</td><td>Sterile H_20</td><td>Sterile H_20</td><td>Sterile H_20</td></tr>
<tr><td>B</td><td>Sterile H_20</td><td colspan="8">200 µg/mL Humanised Isotype Control (GRITS27601)</td><td rowspan="8">Baseline time-point</td><td>Sterile H_20</td></tr>
<tr><td>C</td><td>Sterile H_20</td><td colspan="8">200 µg/mL GSK2394002</td><td>Sterile H_20</td></tr>
<tr><td>D</td><td>Sterile H_20</td><td colspan="8">50 µg/mL GSK2394002</td><td>Sterile H_20</td></tr>
<tr><td>E</td><td>Sterile H_20</td><td colspan="8">12.5 µg/mL GSK2394002</td><td>Sterile H_20</td></tr>
<tr><td>F</td><td>Sterile H_20</td><td colspan="8">2 µM GSK571949</td><td>Sterile H_20</td></tr>
<tr><td>G</td><td>Sterile H_20</td><td colspan="8">Control Medium</td><td>Sterile H_20</td></tr>
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Samples are then taken according to the protocol every 3 days up to time-point 8 (T8). After the T8 time-point where all remaining medium is removed, the samples are treated with IL-1 and OSM (R&D Systems) to check explant viability and these are then sampled and the plates discarded. All samples are stored immediately at -80^{o}C up to the point when they are shipped to the GSK site for sample analysis.

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