Markers of Synovial Inflammation in Cohorts at Risk of Knee Osteoarthritis

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Abstract

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Background and objectives. Knee osteoarthritis (KOA) is a leading cause of disability in the developed world. Additionally, it is possibly linked with premature mortality. Low-grade inflammation is associated with a high risk of non-traumatic KOA incidence but also with metabolic syndrome. Knee injury is a major risk factor for KOA and is associated with an inflammatory response. Heterogeneity of both the symptoms and progression makes early identification of individuals at risk difficult. The aim of this thesis was to examine the diagnostic value of selected biomarkers to identify microscopic synovitis, examine their predictive value for KOA development and any association with excess premature mortality.

Methods. Four serum biomarkers, COMP, resistin, C3M and CRPM, all linked with both knee pain and synovial or systemic inflammation, were selected and an additional sonographic marker was tested. Data from two cohorts has been utilised for the purpose of this thesis: the Oxford Knee Injury Cohort, a prospective study of NHS patients with recent ACL and/or meniscal injuries, and the Chingford women's study, a community-based prospective cohort with over 23 years of follow-up.

Results. Cross-sectionally, sonographic markers correspond well with microscopically defined post-traumatic synovitis. Two biomarkers, COMP and resistin, were associated with development of incident radiographic KOA (RKOA), while two others, C3M and CRPM, predicted development of painful RKOA independently to age and BMI. High C3M levels and presence of painful RKOA were associated with premature mortality. Knee pain alone, especially in presence of RKOA was an independent predictor of mortality, but RKOA without pain was not.

Conclusion. These results support the use of the studied biomarkers in complex predictive models for development of KOA and associated premature mortality. Taking the competing risk of death into account is an important consideration in this field

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Abbreviations

ACL	anterior cruciate ligament
AP	anteroposterior
ASFP	anterior suprapatellar fat pad
BMI	body mass index
C3M	matrix metalloproteinase degraded collagen type III
CC	correlation coefficient
CI	confidence interval
COMP	cartilage oligomeric matrix protein
CRP	C-reactive protein
CRPM	matrix metalloproteinase degraded C-reactive protein
CV	coefficient of variability
ELISA	enzyme linked immunosorbent assay
EULAR	the European league against rheumatism
FPM	fractional polynomial models
GEE	general estimating equation
HRT	hormone replacement therapy
hs-CRP	high-sensitivity C-reactive protein
ICC	intra-class correlation
IQR	inter-quartile range
KOA	knee osteoarthritis
KOOS	knee injury and osteoarthritis outcome score
K/L	Kellgren and Lawrence
LLMR	lower limit of measurement range

MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
NSAIDs	nonsteroidal anti-inflammatory drugs
Non-ASA	non-aspirin
NRS	numerical rating scale
OA	osteoarthritis
OMERACT	the outcome measures in rheumatology group
OP	osteoporosis
OR	odds ratio
PTKOA	post-traumatic knee osteoarthritis
PROMS	patient reported outcome measures
RA	rheumatoid arthritis
REC	research ethical committee
RKOA	radiographic knee osteoarthritis
SD	standard deviation
SE	synovial effusion
SH	synovial hypethrophy
SR	suprapatellar recess
TKR	total knee replacement
US	ultrasound
ULMR	upper limit of measurement range
U/L	units per litre
QT	quadriceps tendon
VAS	visual analogue scale

Chapter 1 Introduction and literature review

1. Introduction

Knee osteoarthritis (KOA) is thought to be one of the leading chronic joint diseases in terms of health burden. Incidence of osteoarthritis (OA) is rising due to an ageing population and the obesity epidemic [1, 2]. Pathological changes at all stages of KOA include localised loss of cartilage, remodelling of the adjacent bone and inflammation. Treatment strategies mainly focus on improving knee function by alleviating pain through either pain relieving or anti-inflammatory agents, in conjunction with physical activity interventions, or – in the case of non-responding joint failure – knee arthroplasty [3]. There are currently no preventive treatment strategies in the high-risk groups. The economics of KOA is considerable, with its physical activity limitations associated with worse economic outcomes [4, 5] and a substantial increase in incidence in deprived communities [6].

As a result, the majority of studies have focused on better management of painful RKOA. While understanding treatment outcomes in the affected population is very important, there is a limited amount of research looking at effective prevention of incidence of painful KOA. This is primarily due to the relatively slow progression of KOA, leading to not only significant costs in terms of following up patients, but also a poor understanding of the key factors in OA pathogenesis. It is suggested that acute, chronic or repetitive injuries disturbing

joint homeostasis is a trigger for the repair processes that often result in remodelling, with a structurally altered but symptom-free knee joint.

The pathogenesis of painful RKOA is still poorly understood, but there is growing evidence that systemic metabolic dysregulation most likely predisposes patients to painful KOA by upregulating local inflammatory processes, which accelerates cartilage loss and development of osteophytes. Ability to more accurately profile groups at risk of KOA will potentially make primary and secondary preventive intervention trials feasible. Systemic and synovial inflammation have both been indicated as independently predictive factors for RKOA [7, 8]. Association with systemic inflammation could also potentially explain the increased risk of cardiovascular problems and premature mortality in individuals with KOA [9, 10]. Biomarkers associated with inflammation and knee pain could potentially increase the ability to predict both the incidence of early KOA and also its long-term consequences.

The aims of this chapter are to summarise current literature looking at:

1. the disease burden and known risk factors
2. inflammation, both systemic and localised, in KOA pathogenesis
3. potential inflammatory biomarkers in populations at risk.

1.1 Size and nature of disease burden

The lifetime risk of diagnosed painful KOA is around 45%, occurring relatively early, with a median age of 55 years at diagnosis [11]. KOA rates increase continuously with age and peak at around the seventh decade [12]. It is estimated that 10% of the UK population over 55 years has disabling symptoms due to KOA [13]. The lifetime risk of receiving a total knee replacement is between 8.1% for men and 10.8% for women [14] and substantially less than the risk of developing symptomatic KOA [15]. With an aging population and epidemic of obesity, the size of the future scale of problem is likely to be underestimated even after recent sharp increase in total knee replacements [16].

1.1.1 Known risk factors

KOA has a multifactorial aetiology, but there are several well-recognised strong risk factors for the incidence of KOA. These include age, sex, body mass index (BMI), quadriceps strength, previous injuries, bone density, smoking and malalignment [17].

1.1.1.1 Age and gender

Age and gender are strong independent factors for both general RKOA and painful RKOA [12, 18]. KOA is more common in females, with the risk increasing almost linearly after age 55 and peaking in the seventh decade [12, 19, 20].

1.1.1.2 Body mass index

Higher BMI is associated with increased risk of developing KOA [12, 21-23]. It has been estimated that up to 50% of that association could be explained by the effect

of the fat tissue inflammation signalling in the elderly population [24]. A recent study by Reyes et al. has suggested over threefold increased risk of KOA retrospectively with obesity [21]. Interestingly, it has been shown that weight loss reduces the risk of developing symptomatic KOA by up to 50 % [25], but also KOA symptoms in a dose-response manner [18]. All other risk factors add only modestly to the prediction of KOA incidence using age, gender and BMI in people above 55[26].

1.1.1.3 Knee Injuries

It is clear that joint damage predisposes patients to joint pain and OA, but also that the severity of this injury bears little relation to the severity of pain and risk of KOA after the injury. Anterior cruciate ligaments (ACL) and meniscal injuries are well-recognised risk factors for the development of post-traumatic KOA (PTKOA). Approximately 12% of the overall prevalence of KOA is attributable to PTKOA [27] that typically develops in middle-aged adults, and can affect quality of life in the early forties [28]. ACL tear and meniscal injuries are strongly associated with the development of post-traumatic KOA. A recent systematic review showed that up to 50% of subjects develop RKOA more than 10 years after ACL reconstruction [29]. A significantly higher prevalence of RKOA was reported in this review for those who had a combined ACL and meniscal injuries [30].

1.1.1.3.1 Anterior cruciate ligaments

PTKOA is common after ACL injuries, irrespective of treatment. Cartilage quality is significantly affected, with loss of up to quarter of proteoglycans within three weeks, with this not replaced even two years after ACL reconstruction [31].

Recently published results after 15 and 20 years of follow-up in a cohort with ACL reconstruction reported that 42% had evidence of RKOA [32]. Additionally, ACL tears are 20% more common in adults with painful KOA [33].

1.1.1.3.2 Meniscus

Oiestad and colleagues demonstrated in their systematic review that meniscus injury and partial meniscectomy results in increased risk of radiographic KOA [29]. This is consistent with a previous report by Professor Stefan Lohmander's group [34], which compared a cohort with partial meniscectomy to those with a conservative treatment and found a significantly increased risk of RKOA and symptomatic RKOA associated with meniscectomy. Meta-analysis observational studies by Muthuri and colleagues, looking at the association between history of knee injuries and KOA, suggested a very high association between not only meniscus damage, meniscectomies and ligamental knee injuries, but also femur fractures and risk of KOA, with a fivefold increased risk.

1.1.1.4 Biomechanical factors and genetics

Biomechanical factors include abnormal joint loading, knee misalignment, muscle weakness and occupation[35]. Malalignment has been reported as a significant factor in some studies, although in one large cohort, Farmington study, this was not a case [36-38].Traditionally, knee trauma and high BMI were also solely associated with abnormal biomechanics. However, restoring knee stability after trauma has not been proven to protect against KOA[32, 39], and half the association between elevated BMI and knee OA able to be explained by fat tissue inflammation [24].

There are multiple genes that have been associated with the development of OA[40-44]. The most convincing epidemiological data comes from the fact that hand OA has been repeatedly shown to be an independent predictor for the future development of hip and knee OA [12, 45]. Smoking has been shown to have some protective effect on incidence of OA independently to BMI[46-48].

1.2 Discordance between radiographic and painful knee osteoarthritis

Discordance between knee symptoms and radiographic changes has long been a well-known phenomenon. Around half of the subjects from the general population who meet radiographic criteria are asymptomatic or have no signs of clinical disease [49]. Cobb et al. (1957) published a paper comparing pain with RKOA and reported that only 30 percent of the population studied in Pittsburgh with X-ray changes consistent with RKOA reported knee pain on the same side in the previous five years. In 1966, Lawrence at al observed that only 39 percent of men and 46 percent of women with a Kellgren and Lawrence score of 3 or 4, reported knee pain. Meanwhile, this applied only to below 10 percent of those with grades 1 and 2 [50]. Lawrence also concluded that radiographic changes are more likely to be painful in obese individuals. Knee pain itself is a poor predictor of radiographic changes; interestingly the relation between RKOA and likelihood of knee pain holds a similar predictive value in the general population [51]. Even radiographic features often associated with key aspects of OA, including joint space narrowing (JSN), are not very sensitive to cartilage changes defined by

arthroscopic examination [52]. A systematic review of existing literature by Bedson and Croft has showed a significant variation in the degree to which knee pain relates to RKOA and the reverse. This can partly be explained through selection of the examined populations, variation of knee pain and radiographic changes in different age groups and between genders, but is also complicated by the use of different knee pain questions, including recall options [53].

Nevertheless, a seminal 2009 BMJ paper has indicated that those radiographic changes are associated with frequent and more severe knee pain in a dose response manner, with JSN being the strongest predictor of knee pain in general [54]. The association with frequent knee pain was particularly strong for advanced radiographic changes, namely a Kellgren and Lawrence score of 3 and 4, relatively weak for grade 2 and statistically non-significant for grade 1. Furthermore, even mild radiographic changes have a predictive value for RKOA progression [26, 55]. Additional X-ray projections and modification of the questions by adding a recall option over time could increase this association [56].

In patients with KOA and equal radiographic grades in both knees, sonographic defined effusion in the suprapatellar pouch correlates better with knee pain then radiographic changes [57]. Furthermore, studies looking at the MRI features of KOA have suggested synovitis as a key feature associated with knee pain [58-61]. These indicate that severity of knee pain in RKOA is related to synovial inflammation. Painful RKOA is not a single disease entity, but a common end-stage feature of joint dysfunction linked to a number of pathological processes, where synovium pays a significant role in symptom generation.

The question of whether OA should be defined radiographically or via clinical assessment is a controversial one [53, 62, 63]. Therapeutic options such as surgery or knee injection for knee pain tend to be considered in the presence of symptoms and radiographic abnormalities.

In summary, this review suggests that synovitis might provide an explanation of discordance between radiographic changes and pain.

1.3 Synovial membrane inflammation and pathogenesis of knee osteoarthritis

In 1949, Dr Robert Soeur reported characteristics of microscopic appearance of normal synovial membrane of the knee in humans and animals [64]. He described in his observations that the 'synovial membrane consists generally of a single internal layer of cells, resting on an external adipose layer', and that underlying tissue has very few 'free cells', that consist mainly of occasional plasmocytes or lymphocytes.

More recently published work suggested that normal synovial membrane consist of 1-2 layers[65]. Unlike human cartilage or meniscus, normal synovium has a good nerve supply, that includes the autonomic nervous system and those nerves tend to follow routes around vascular networks[66-69]. Knee synovium provides lubrication of cartilage, but is also responsible for maintaining synovial fluid volume and providing nutrition of chondrocytes[65]. Relationship between the oxygen pressure in the synovial fluid and synovial function is not very clear, but

observational studies suggested that following joint trauma, in osteoarthritis, but especially in active rheumatoid arthritis oxygen levels in synovial fluid are significantly lowered, and association with synovial inflammation have been suggested as a main cause [70, 71]. Arthroscopically defined medial parameniscal synovitis correlated with more severe cartilage changes and progression of chondropathy 12 months later [72].

More interestingly MRI defined synovial inflammation has been shown to be associated with risk of developing RKOA independently to other MRI changes in cohort studies of individuals at risk of KAO [8, 73].

Sonographic and MRI defined synovitis has been strongly associated with knee pain and pain sensitisation process [57, 59, 74-84]. A systematic review of literature by Hosny et al. looking at presence of synovitis in end stage knee and hip OA, concluded that the body of evidence supports a notion of end-stage painful OA correlating with presence of inflamed synovium [85].

In most of the studies examining microscopic changes in the synovial tissue in KOA, material was obtained either post-mortem or at the time of surgery for end stage joint failure. Late stage KOA synovial tissue is often fairly unspecific and difficult to differentiate between KOA and inflammatory conditions [86]. A number of studies compared microscopic synovial changes in different arthritic conditions and post-traumatic synovitis. In blinded studies, it was confirmed that it is very difficult distinguish the microscopic features of rheumatoid synovium from synovium obtained in patients with OA based on the histological

samples alone [87, 88]. Revell et al. described tissue from the knees of patients with either osteoarthritis or mechanical knee problems and concluded that synovium in the KOA is associated with inflammatory infiltrate[89]. No specific pattern of infiltration characteristic for OA was found in this study. Similar difficulties with non-specific inflammatory response were reported by others [90, 91].

These studies support the notion that qualitatively there is a little difference between microscopic synovial changes in inflammatory arthritis and OA. Nevertheless, in rare conditions synovial histology might be diagnostic in cases of undifferentiated arthritis[92] and new immuno-staining [93] helps to identify dominant inflammatory pathways, yet it remains to be shown that microscopic analysis of the synovial tissue early in disease has an independent value in identifying patients' long term diagnosis and outcomes in both suspected RA and OA.

Examination of post-traumatic knee synovium has a long history. Rene Patry reported microscopically defined chronic synovial inflammatory process around injured meniscal tissue in 1926[94]. Burman and Sutro (1933) described synovial changes around degenerative meniscus and suggested that 'nutrition' that both knee cartilage and meniscus receive from synovial fluid might be a contributing factor[95]. In 1997 Comín et al. described a case series of patients with chronic post-traumatic knee pain due to localised synovitis and treated successfully with arthroscopic synovectomy [96]. Benito et al. described changes in the synovium of individuals with knee pain and no RKOA and compared them with end stage KOA synovium[97]. He concluded that the first group's synovium was

characterised by an increased mononuclear cell infiltration when compared with the second.

In summary, the presence of microscopic defined synovitis with non-specific inflammatory infiltrate is well established in painful knees without RKOA, post-traumatic knees and KOA. In individuals at risk of developing KOA, synovitis predicts future cartilage loss and incidence of RKOA.

1.4 Role of Systemic Inflammation

There is growing evidence that the pro-inflammatory environment created by excessive visceral adipose tissue plays a role in the development of OA and may help to explain the association between obesity and increased risk of OA especially in non-weight bearing joints [98, 99]. Furthermore, even after adjustment for age, sex and BMI, hand OA has been shown to be an independent predictor for the future development of KOA [45]. Serum concentrations of IL-6 and TNF-α have been associated with increased prevalence of KOA and cartilage loss[99, 100]. IL-6 is associated with an increased risk for incidence of RKOA in a BMI-adjusted model[7]. Findings from several, but not all, studies have shown significant association between polymorphisms in IL-6 and IL-6 promoter genes, and susceptibility to hand, knee or hip OA[101-103]. The evidence on association between hsCRP and risk of developing KOA is still inconsistent[104-107]. High levels of hsCRP are more likely to be associated with knee pain rather than RKOA[98, 99].

High systemic levels of IL-6 and hsCRP have been found to predict cardiovascular problems in healthy individuals and also in patients with coronary artery disease or diabetes [108-112]. Elevated serum markers of systemic inflammation have been linked with development of metabolic syndrome, especially in women[113], and higher levels of inflammation have been associated with a high risk of poor outcomes[114]. This could explain the association between OA and an increased risk of CVD[115, 116]. Individuals with components of metabolic syndrome are more likely to suffer from KOA, and accumulation of these components is related to incidence of KOA [117, 118]. Adipose tissue cytokines are associated with low-level systemic inflammation [119-121]. There are four main adipokines discussed in the literature when considering KOA and development of the metabolic syndrome: leptin, adiponectin, Resistin and Visfatin. Fowler-Brown and colleagues recently found that almost half of the association between elevated BMI and knee OA could be explained by the leptin levels in an elderly population [24]. Associations between leptin and other markers and KOA are presented in table 1.1.

Table 1.1. The relationship between specific adipokines, RKOA and knee pain based on the current literature.

Adipokine	Radiographic OA (cross sectional, incidence or progression)	Joint Pain
Leptin	↑in♀[122-124]; ↑*[125] ↔[126];↔in♂[123];	↑in♀ & ↔ in♂[127]; ↑*[128, 129]
Adiponectin	↓[130, 131];↔[123, 132]; ↑[133]	↑in♀ & ↔ in♂[127]; ↓*[129];
Resistin	↑[124, 134]	↔in♀ & ↑in ♂[127]
Visfatin	↑[135]	↔*[129]

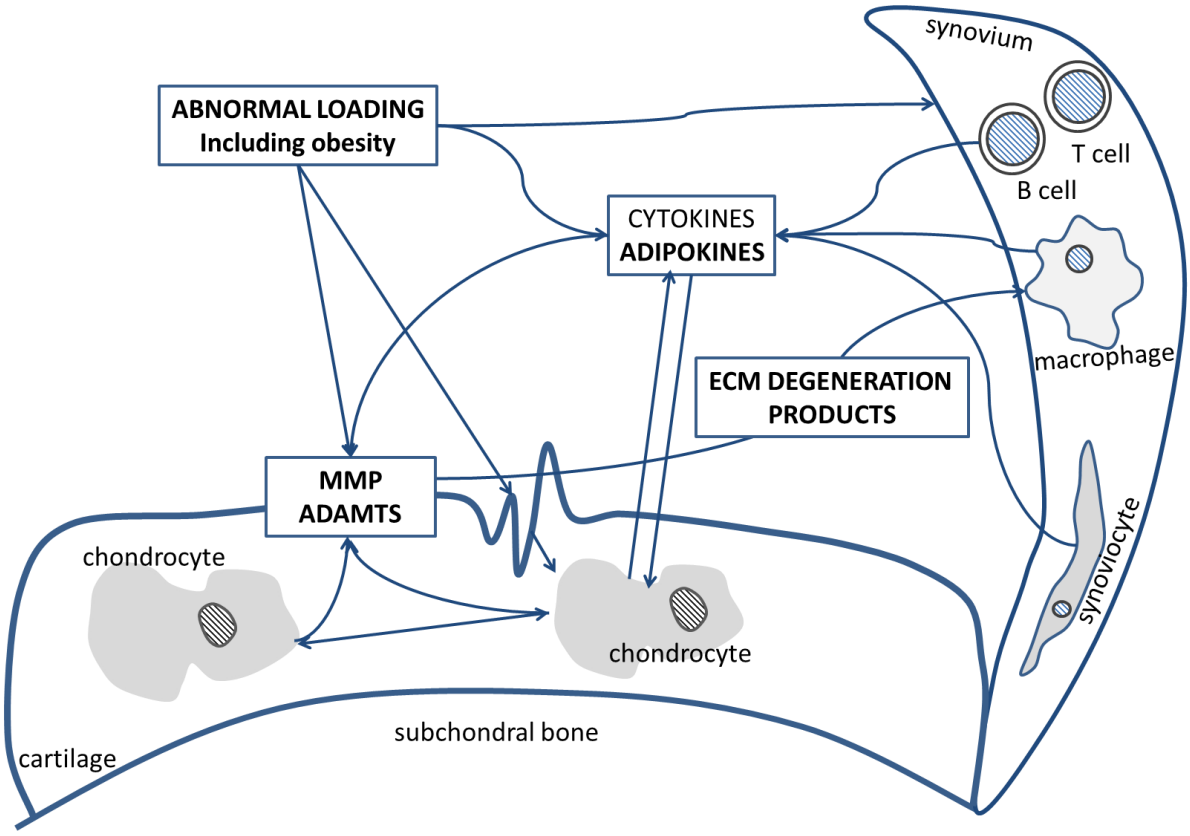
'↑' = positive association; '↓' = negative association; '↔' = no association;

*in synovial fluid concentration

'♀' = female population, '♂' = male population

In summary, elevated systemic markers of inflammation are linked with adipose tissue cytokines, components of metabolic syndrome and an increased prevalence of RKOA and arthritic pain. KOA development is closely associated with systemic metabolic dysregulation and increased cardiovascular disease risk; they share a common pathological process: an excessive adipose tissue associated inflammation.

Figure 1.1. Interplay between systemic, biomechanical and joint-specific inflammatory pathways in early OA. Based on the figure by Otero, Loeser and Goldring 136 and modified by Kluzek et al. 9.



1.5 Excess mortality associated with knee osteoarthritis

The altered secretion patterns of pro-inflammatory adipokines are present in obesity, the general elderly population and post-menopausal women, who present as high-risk groups for both OA and metabolic syndrome. Individuals with symptomatic knees have an increased prevalence of metabolic dysregulation. The most biologically plausible mechanism for this causal association is that knee pain results in sedentary behaviour, obesity and poor cardiovascular fitness. This is supported by the fact that only 7.7% women with

KOA meet the recommended Physical Activity guidelines [137]. Moreover, women with KOA have higher levels of markers of atherosclerosis [138] and some reports have indicated that increased walking-related disabilities, use of walking aids and poor baseline function are associated with excess all-cause mortality in individuals with hip and knee OA symptoms[139]. The association between KOA and excess mortality has been reported in a few observational studies, with the strongest evidence stemming from a study by Nüesch et al., where patients with symptomatic radiographic KOA and/or hip OA were found to be at increased mortality risk compared to the general population [140]. A more recent study demonstrated a borderline significant association between excess mortality and symptomatic (but not radiographic) KOA alone [141]. A systematic review on this subject found moderate evidence of increased mortality among participants with OA compared with the general population [142].

The main limitations of the previous studies are relatively short follow-up and a tendency for participants with symptomatic radiographic OA to be included only. This creates a potential selection bias and comparison with controls from the population mortality registers or participants with no ROA, with no study using a comparison group without pain and ROA.

In addition, musculoskeletal pain in unadjusted population based models has been previously associated with an increased risk of early mortality [143, 144].

It is currently unclear whether symptomatic or radiographic only KOA is uniquely associated with excess premature mortality. Low-grade inflammation,

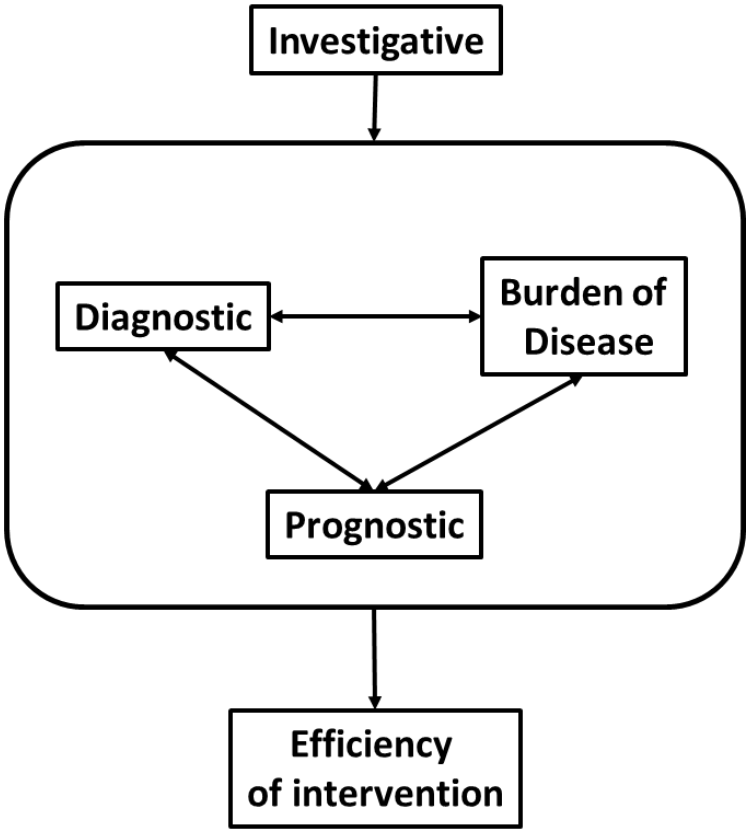
which has been previously associated with higher KOA incidence and the metabolic dysregulation, might be a contributing factor on the causal path.

1.6 Biomarkers associated with knee pain, and localised and systemic inflammation

One of the overall aims of this thesis is to identify predictive biomarkers that could be used for profiling individuals at risk of KOA for the purpose of future interventions. This requires proposed biomarkers to detect early changes with long term consequences. Biomarkers of inflammation associated with knee pain are a promising tool for detection of early-stage OA and individuals at risk. At the moment, knee radiography remains the method of choice for grading OA severity, but shows little or no changes in early disease. Discordance between RKOA and pain is another limiting factor but it is unclear whether RKOA without knee pain has any clinical significance. Ability to detect KOA in its early stages could facilitate our understanding of the pathogenesis and allow us to differentiate phenotypes within a heterogeneous KOA population.

In 2006, Bauer et al. proposed six categories of osteoarthritis biomarker classification to represent their development. These were captured in the acronym 'BIPEDS': Burden of disease, Investigative, Prognostic, Efficacy of intervention, and Diagnostic and Safety [145](figure 1.2).

Figure 1.2. Development process of OA biomarkers based on the figure by Bauer et al. [145]



The literature review presented in this chapter has so far suggested that the biomarkers associated with knee pain, knee synovitis and systemic inflammation might play a significant role in identifying individuals at risk.

In this thesis, serum biomarkers were investigated for their diagnostic ability to identify knee synovitis, the incidence of KOA and the burden of disease by assessing their possible association with the premature mortality linked to KOA. For that reason, only established biomarkers with the existing available serum assays were considered.

1.6.1 Inflammatory biomarkers – literature search

This study targeted serum based biomarkers associated with knee pain and localised and/or systemic inflammation. The aim was to select only four of the most promising biomarkers, to avoid statistical problems with multiple testing. A comprehensive search of electronic databases was carried out on Pubmed, the Cochrane Central Register of Controlled Trials, CINAHL and reference lists of relevant articles. The PubMed literature database was searched using MeSH index terminology. The following broad search terms were included: biomarkers, serum, knee pain, OA and/or post-traumatic in combination with inflammation, obesity, adipokine and adiponectin. This was complimented by hand searching OA journals for all bibliographic references from the key papers in this field. The search was limited to human-focused results written in the English language. Limits regarding publication date were applied, with only results from between January 1985 and January 2014 considered. A total number of 104 papers was found, with 22 biomarkers meeting basic criteria.

Two additional exclusion criteria were applied:

1. Non in vivo human studies were excluded.
2. Studies with experimental ELISA assay without published reproducibility of results in human subjects were rejected.

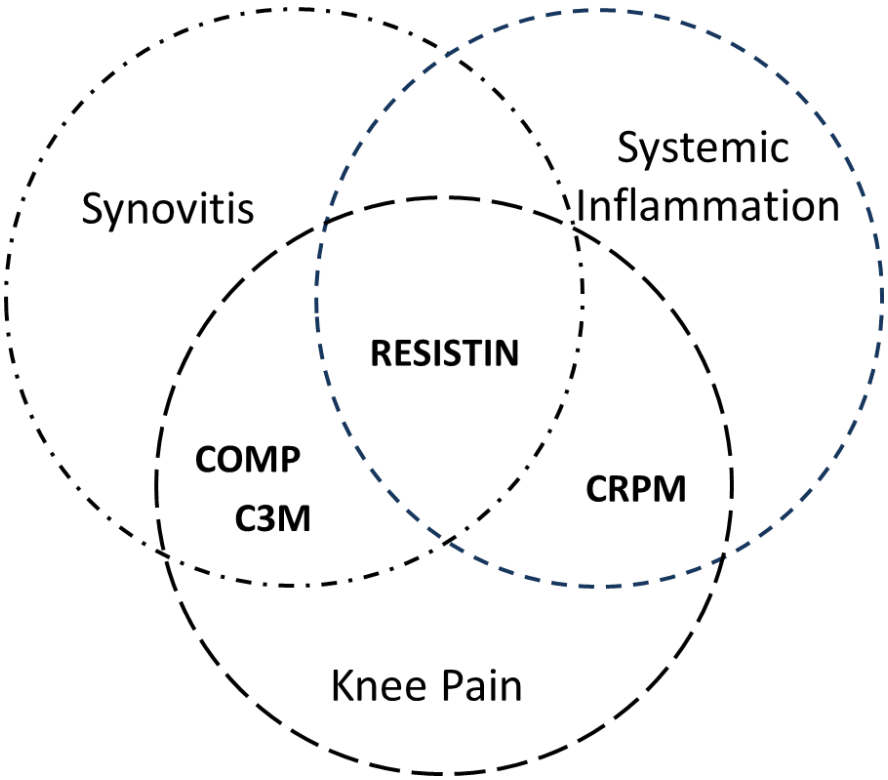
This resulted in a limitation to 11 biomarkers. Out of them, four were selected as the most promising in terms of association with metabolic dysregulation, or with either effusion or synovitis.

The serum-based biomarkers selected for this thesis were:

1. Cartilage oligomeric matrix protein (COMP): a well-established KOA biomarker associated with knee pain and clinical knee inflammation.
2. Resistin: an adipokine linked with knee pain, synovitis and systemic inflammation.
3. Matrix metalloproteinase (MMP)-generated collagen type III (C3M): a novel biomarker associated with knee pain and presence of microscopic synovitis.
4. Matrix metalloproteinase (MMP)-dependent degradation of CRP (CRPM): a novel biomarker associated with knee pain and systemic low-grade inflammation.

An additional sonographic biomarker assessing effusion and synovial hypertrophy in the suprapatellar recess (SR) has been developed, based on the modification of currently published protocols and the Outcome Measures in Rheumatology group (OMERACT) definition of synovial hypertrophy[146].

Figure 1.3. Schematic association of selected biomarkers associated with knee pain, localised and systemic inflammation based on existing literature.



1.7 Selected serum biomarkers

1.7.1 Cartilage oligomeric matrix protein

COMP is one of the few well-established predictive serum biomarkers in KOA research. It has been associated with cartilage degeneration, but also knee pain [147] and clinically detected knee synovitis [148, 149].

COMP is a pentametric glycoprotein that was first identified in hyaline cartilage and fibrocartilage [150, 151]. Despite its name, it is derived from several tissue types,

including cartilage, synovium and menisci, and composed of five equal subunits, each 110 kDa. Chondrocytes, but also synovial cells activated by proinflammatory cytokines, are responsible for COMP synthesis [152]. The role of COMP is not fully understood, but it is hypothesised that it might be involved in regulating collagen production and storage of vitamin D in the tissue. The presence of a pentametric COMP complex increases the collagen fibril formation, while the presence of the monomeric COMP decreases it [153]. Mutations of the COMP gene are linked with multiple epiphyseal dysplasia, a disorder of cartilage and bone development [154]. On the basis of COMP structure and biochemical properties, it has been suggested that COMP might be involved in tissue storage of vitamin D [155, 156]. It is also the case that synovial COMP concentrations have been correlated with serum COMP in patients with KOA [157]. The precise factors associated with variation of serum COMP levels remain incompletely understood. The results from The Johnston County Osteoarthritis Project demonstrate that ethnicity, sex and RKOA are associated with a variation in serum COMP levels [158]. Age, weight and mechanical loading all affect the level of COMP in healthy individuals [159-161]. Serum COMP levels increase significantly from 1 h after rising from bed and are affected by exercise [162, 163].

Elevated levels have been observed after knee injuries, as well as in active synovitis and KOA [148, 149, 164]. Several studies have shown a cross-sectional association of COMP with RKOA [162, 165]. The association with painful RKOA is more complicated, with higher serum concentrations linked to self-reported pain [147]. Similarly, meta-analysis of cohort and case-controlled studies have concluded

that COMP levels were significantly higher in patients with knee osteoarthritic symptoms [166]. Significantly higher levels in painful RKOA than in age and sex-matched healthy controls were reported[165]. However, there have been a few other studies showing no correlation between COMP severity of KOA symptoms [161, 167]. Furthermore, in a population with knee pain, no association was found between COMP levels and structural radiographic or MRI changes [168]. In longitudinal studies, low COMP levels in individuals with KOA had a significantly lower rate of medial cartilage volume loss [169] and higher COMP levels have been associated with increased risk of subsequent cartilage loss [170] and found to be at risk of rapidly progressing debilitating joint disease [161]. High baseline COMP levels have predicted the development of RKOA over 10 years in middle-aged women [171]. The longitudinal associations of baseline COMP levels with incidence of RKOA in more than 10 years and incidence of painful KOA are unknown.

1.7.2 Resistin

The adipokine Resistin is a small cysteine-rich 12.5 kDa polypeptide, that was initially mainly investigated as an insulin resistance inducing factor in mice [172]. In humans resistin is mainly produced by macrophages, both within and outside the adipose tissue [173, 174]. The receptor for Resistin remains unknown, but stimulation of the tissue with Resistin has strong pro-inflammatory properties and has been shown to increase concentrations of other proinflammatory cytokines such as TNF- a,IL-1b and IL-6 [175].

Serum Resistin is elevated in obese individuals [176] but this correlation is obscured in patients with advanced KOA [177]. In humans, Resistin correlates better with

subclinical inflammation than with insulin resistance [119]. The current concept is that Resistin can increase inflammatory responses to mechanical insults and result in fast progression to joint failure. In KOA, Resistin can be detected locally in the synovium and correlates with microscopically defined synovitis [134]. Both serum and synovial fluid concentrations are elevated in post-traumatic knees and associated with the release of cytokines and cartilage degeneration [178]. In an early symptomatic KOA cohort, Resistin levels have been shown to be positively associated with synovial biomarkers (sPIIINP) and high-sensitivity CRP (hs-CRP) but, more interestingly, with the incidence of RKOA [124, 179]. Those findings have been independent of BMI and of the concentration of hs-CRP as a potential confounder.

1.7.3 Selected matrix metalloproteinase – degraded products

MMP-degraded products are examples of neo-epitopes as post-translational modifications of proteins formed by processes associated with matrix metalloproteinases cleavage and are very specific to activity of the tissues containing both substrate and enzymes. They can be described as tissue-specific proteins and accumulate in the inflamed tissue, before being gradually released into the circulation [180]. A description of the specific neo-epitopes and assays is presented in chapter 2, section 2.2.3.

1.7.4 Matrix metalloproteinase-degraded collagen III fragments

The synovial membrane lining layer is composed mainly of type III collagen [65]. The C3M assay detects an MMP-9-derived fragment of type III collagen released from synovium, and is a good marker of connective tissue turnover [181]. It is also

high in fibrotic diseases [182]. A recent study has suggested that macrophages-like synoviocytes are necessary for activation of synovial MMP-3 and the subsequent release of C3M [183]. This suggests that C3M is a marker of synovial inflammation rather than increased turn-over of matrix. An association with synovial inflammation is consistent with the findings of a study on rheumatoid arthritis (RA) [184] and OA [185, 186] patients, where this marker was substantially elevated. In case of RA patients, a significant reduction was noticed after the use of an anti-synovial treatment. C3M is also associated with joint damage in ankylosing spondylitis and, in combination with other markers, has been shown to predict structural changes [187]. In a study looking at association between central sensitisation and KOA synovitis, C3M was positively associated with MRI based synovitis, but negatively associated with central sensitisation [188]. This marker was reported to have higher values in older individuals, but no gender differences were reported [188].

1.7.5 Matrix metalloproteinase degraded C-Reactive protein fragments

MMP-3 dependent degradation of C-Reactive Protein assay (CRPM) has shown to be specific for a neo-epitope associated with MMP-3 activity, as it does not recognise other protease cleavages of CRP, intact CRP or other proteins. Serum CRPM levels are elevated in patients with symptomatic KOA, independent of radiographic grade [186]. Moreover, Arendt-Nielsen and colleagues showed that the levels of CRPM correlated very well with the degree of central pain sensitisation but not with K&L grade in a group of patients with painful KOA [106]. In patients with active joint synovitis, CRPM has been shown to be decreased as a

result of an anti-IL-6 intervention [184]. Unsurprisingly, similar to full length CRP, differences in CRPM levels have been shown to have slight gender and age variation [106, 189].

In summary, this review has identified and described four promising selected available assays of the serum based biomarkers associated with knee pain and localised and/or systemic inflammation.

1.7.6 Selected sonographic markers

Knee ultrasound (US) is a promising technique able to provide direct information on soft tissue and can reliably detect synovial hypertrophy and effusion in painful RKOA [190, 191]. SR is particularly interesting in the context of knee effusion and synovitis. The suprapatellar pouch has a variable size and is located between the quadriceps tendon/muscle and the prefemoral fat pad. The only reliable data examining the volume of this structure comes from a study of SR xeroarthrograms performed on a population with chronic knee synovitis [192]. This showed a significant variation from around 1.2 cm^2 to 21 cm^2, with the volume directly related to the amount of synovial fluid in the knee. Sonographic changes in the SR have been found to be positively associated with knee pain in patients with equal radiographic grades of OA [57]. In addition, suprapatellar inflammation has been found in patients with painful RKOA, but not in a group with asymptomatic RKOA or healthy controls [57, 193]. Other studies have shown that the presence of SR effusion-synovitis is predictive for the development of RKOA[8].

Sonography of the SR is a potentially attractive alternative method to assess effusion-synovitis in populations at risk of KOA. However, sonography is an operator-dependent technique and reliability of existing protocols is not very good [194].

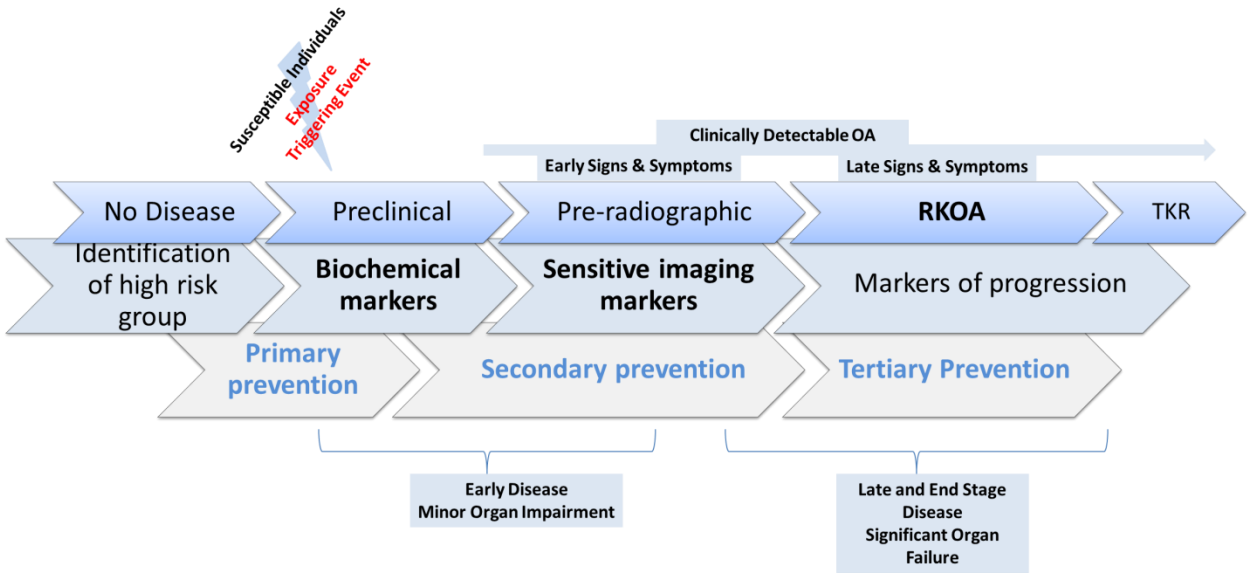
A standardised US protocol of the suprapatellar pouch was developed for this study, based on existing examples, and the reliability of the new protocol was tested. Both the detailed review of the literature and the development and testing of the new protocol are presented in chapter 3.

1.7.7 Summary of the introduction

Pain in KOA has been linked with systemic and localised inflammation. Synovitis might provide an explanation for discordance between radiographic changes and knee pain. Low-grade inflammation and MRI-defined synovitis have been associated with a higher risk of non-traumatic KOA incidence. Metabolic dysregulation and KOA share a common pathway of upregulated adipose tissue inflammation, and both have been previously associated with excess mortality. Heterogeneity of both the symptoms and progression makes early identification of individuals at risk of KOA difficult. Taken together, these findings suggest that biomarkers, associated with knee pain and either systemic or local inflammation, have a potential role for predicting development of KOA and explaining possible premature mortality. Following this literature review, four serum based biomarkers have been selected for this thesis and an additional sonographic marker has been proposed.

The selection of biochemical and sensitive imaging biomarkers has the potential to provide an excellent tool for selecting individuals for preventive treatment trials (figure 1.4).

Figure 1.4. Osteoarthritis continuum, type of biomarkers and potential associated interventions.



Aim of this Thesis

The overall aim of this thesis is to examine if selected biomarkers correlate with knee synovitis, predict incident KOA and identify those at risk of premature death in cohorts at risk of KOA.

Objectives of the Thesis

- Modify and validate the sonographic assessment of suprapatellar knee effusion and synovial hypertrophy in an acute knee injury cohort.
- Determine diagnostic ability of each of chosen biomarkers to detect microscopically defined knee synovitis in an acute knee injury cohort.
- Determine the predictive ability of selected biomarkers for incident KOA in a cohort of middle-aged women.
- Determine the prognostic ability of selected biomarkers for potential excess mortality in a cohort of middle-aged women.

Chapter 2 The chosen cohorts at risk of knee osteoarthritis

Longitudinal studies of individuals without radiographic knee osteoarthritis (RKOA) might provide invaluable information not only regarding the risk of knee osteoarthritis (KOA) incidence in the future and the long term complications associated with the painful knee, but also help to identify new risk factors and test biomarkers associated with knee osteoarthritis (KOA) pathogenesis.

It is not unreasonable to assume that it may become possible to re-grow and/or regenerate the damaged cartilage in advanced KOA as a standard procedure at some point in the future. However, the scale of this problem means effective preventive strategies are more likely to be cost-effective, feasible and positively change the health of the aging population.

Ideally, the onset of KOA should be detected at the very earliest stage, but it is still unclear whether the process leading to progression needs to be stopped in order to prevent the further structural and behavioural changes associated with knee pain or the mechanisms linked to the successful joint remodelling being stimulated and enhanced.

For the purpose of this thesis, data from two cohort studies have been utilised:

1. The Oxford Knee Injury Cohort (OxKIC)
2. The Chingford 1000 Women Cohort Study

The aim of this chapter is to describe the cohorts, recruitment processes, in addition to the predictive variables, confounders and specific outcomes in each study presented in this thesis. It will also discuss the suitability and reliability of the specific variables and outcomes and potential problems and biases associated with using them.

This chapter consists of three parts: The first part summarises how the OxKIC study was established and discusses the study protocol, recruitment and data entry procedures. It also offers a description of the important variables used in the results chapter. The second approaches the Chingford Cohort study in a similar manner, starting with a general description of the study, its protocol and recruitment, and the variables and outcomes used in the results thesis. The third and final section describes the collaboration with Nordic Bioscience Lab and the technical aspects of the biomarker measurements for both cohorts.

2.1 The Oxford knee injury cohort

The Oxford knee injury cohort (OxKIC) was established to examine cross-sectional associations between both the selected serum ('wet') and sonographic ('dry') biomarkers with microscopic synovitis and symptoms . This study has arisen from collaboration between the Arthritis Research UK Centre for Sport, Exercise and Osteoarthritis, represented by Dr Stefan Kluzek, Dr Julia Newton and Professor Nigel Arden, with Professor Andrew Price, Dr Fiona Watt, Professor David Beard and Ms Loretta Davies. Professor Andrew Price and Dr

Fiona Watt are part of Arthritis Research UK Centre for osteoarthritis pathogenesis.

2.1.1 Introduction

Anterior cruciate ligament (ACL) injuries and/or meniscal injuries are common and frequently followed by pain, swelling and episodes of instability. Furthermore, such injuries can lead to poor reported quality of life and decreased activity levels [195] [32]. They are also associated with an increased risk of KOA, irrespective of treatment [30]. Cohorts with ACL and/or meniscal injuries offer a unique model of KOA, as the onset of the disease is well defined. It is clear that joint damage predisposes patients to joint pain and OA, but also that the type rather the severity of this injury is associated with severity of pain and risk of KOA after the injury[196-198].

The menisci – intra-articular fibrocartilage wedges – have a well-recognised function in load sharing and secondary stabilisation [199-202], joint lubrication and nutrition of articular cartilage [203-205]. Meniscal tearing is the most common knee injury, with the incidence of meniscectomy reported as 61 out of 100,000 in the population per year [206]. Since 1970, partial or total meniscectomy has been associated with the early development of RKOA in up to 50% of the patients within 5-15 years post procedure [207-210].

Similarly to meniscal tears, the management of ACL injuries includes both surgical and conservative treatments and often depends on the presence of persistent mechanical symptoms after the injury. It is also associated with a high risk of KOA [29] and it is unclear whether stabilising the knee surgically produces

any further long-term benefit in comparison with conservative interventions [211-214]. This issue remains a problem in guiding the choice of treatment.

The large variation in the management of this patient group has been demonstrated in the UK [215, 216]. A recent randomised controlled trial by Frobell et al [211, 217] in Scandinavia has proven the effective use of rehabilitation in reducing the frequency of surgery while achieving satisfactory outcome in young active adults with acute ACL injury (diagnosed within 1 month).

There is growing evidence that inflammation plays a significant role in the pathogenesis of OA [97, 218, 219], but its role in incidence of post-traumatic KOA is unknown. To address this gap, but also assess the different NHS treatment pathways (ACL reconstruction surgery versus rehabilitation based only), the OxKIC study protocol was designed and proposed in late 2013. The main future outcome of this will be a change of the patients' reported symptoms and the reported difficulties experienced following knee injury. To quantify these, the Knee Injury and Osteoarthritis Outcome Score (KOOS) questionnaire was used as a main outcome. The aim is to follow up all participants for two years.

2.1.2 Ethics

The OxKIC study ethics application was submitted in May 2014 through the Integrated Research Application System (IRAS), with a project ID of 150239. The IRAS is a single online system for applying for permissions and approvals for health research in the UK. Following the Research Ethical Committee (REC) meeting (WALES REC 7) held on 20 August 2014, a favourable ethical opinion by the REC was granted on 15 October 2014 (REC reference number: 14/WA/1108).

A Site-Specific Information (SSI) Form was then submitted to the Research and Development (R&D) office for review as part of the R&D application. After application approval by the R&D office, a final collaboration agreement between The University of Oxford and the Oxford University Hospital Trust was signed on 14 January 2015. The final permission letter issued by the R&D department was signed by R&D Lead Ms Heather House on 28 January 2015. The first study participant was recruited on 5 February 2015. Dr Stefan Kluzek and Ms. Loretta Davies both led this process, starting from the first draft of the study protocol and then progressing through IRAS applications, REC meetings and corrections to the application, before finishing on a collaboration agreement and application for amendments.

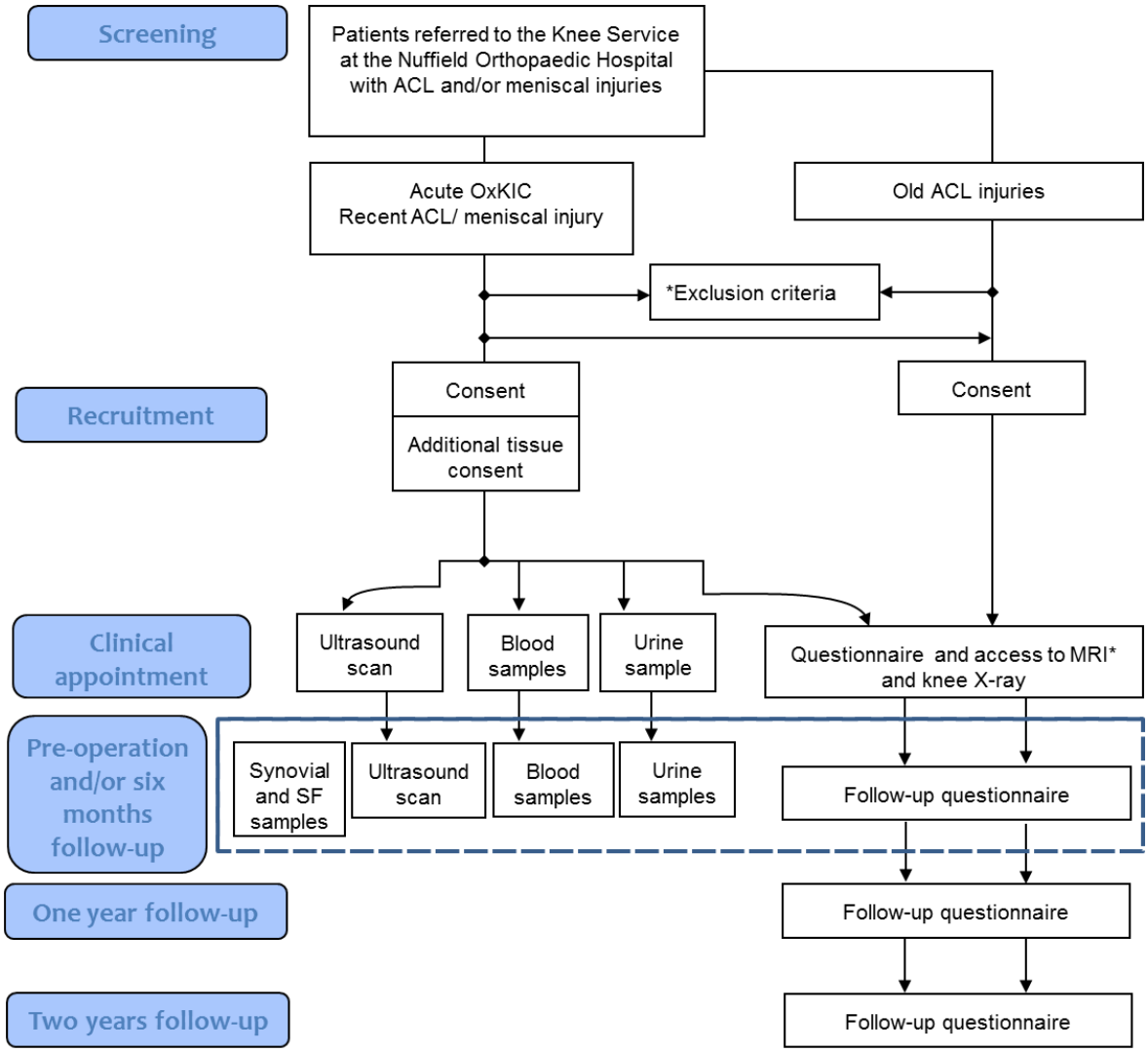
Amendments regarding additional questionnaires, allowing for the collection of more than one single synovial biopsy per patient and qualitative interviews with healthcare professionals were submitted in March 2015 and positively reviewed by the REC (Wales REC 7) on 1 July 2015.

Each study participant provided written informed consent before participating in this study. These were placed in the OxKIC study trial master file, containing all essential documents for the study, including the participants' consent forms and paper versions of questionnaires. This master file continues to be securely stored in a locked file cabinet in secure university facilities, with restricted access to key personnel and supervised by the principal investigator(s) of the study.

2.1.3 Study protocol

The OxKIC study constitutes a single centre prospective cohort of participants referred to the Knee Service at the Nuffield Orthopaedic Hospital with anterior cruciate ligament (ACL) and/or meniscal injuries. It measures the patients' reported outcome measures of the different NHS treatment pathways (ACL reconstruction surgery versus rehabilitation-based only) as the main predictor variables, but also examines the other potential baseline risk factors, including inflammatory biomarkers and sonographic markers of suprapatellar recess (SR) in predicting long term changes. To quantify these, the Knee Injury and Osteoarthritis Outcome Score (KOOS) questionnaire has been used. This validated patient-reported outcome measure consists of subscales that cover pain, symptoms, difficulty in sports/recreational activities and quality of life. Most participants have already reached the six-month follow-up stage and all of them will be followed up for two years. The standard of care treatment for all participants entering the cohort study will follow the usual practice and pathways at the University Hospitals NHS Trust Oxford.

Figure 2.1. OxKIC study recruitment and protocol plan



* Three 3T MRI scanners in the NHS Oxford University Trust were used to perform clinically indicated MRI scans of the affected knee. All of them had used the same protocol described in the appendix. For the purpose of this thesis, results of MRI scans reported either by a consultant radiologist or reviewing clinician were utilised to check eligibility for recruitment only.

The Oxford Knee Injury assessment booklet used as part of the routine management of this patient group formed the baseline assessment questionnaire. This consists of:

a. Basic epidemiological data, focusing on known risk factors for development of KOA.

b. The Knee Injury and Osteoarthritis Outcome Score (KOOS) questionnaire.

c. Tegner Activity Scale – scale of physical activity*.

d. The Numerical rating scale (NRS-11) for Pain.

e. EQ5D5L health questionnaire – health related quality of life questionnaire*.

f. Questions about productivity loss*.

g. MSK questionnaire*.

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* Results of the marked items were not used in this thesis. Therefore, their properties and results will not be discussed in this chapter.

A group of consecutive participants with recent knee injuries, defined as presenting in under 12 weeks after the injury and with no baseline radiographic KOA, have each been asked for additional permission to collect body fluid samples, tissue samples and undergo further scans of their knees with ultrasound (US). This group is referred to as the Acute OxKIC.

The additional tests have now been performed for the majority of patients during the routine outpatient visit and six months later during a follow-up appointment or directly before the scheduled routine operation.

These additional optional tests and procedures include:

a. Ultrasound of the knee SR to assess thickening of the synovium and knee effusion.

b. Blood tests for biomarkers of joint inflammation and joint degeneration (further information on collection, processing and storage parameters are described in section 2.1.1.2 and the appendix.

c. Urine sample for markers of cartilage injury and joint degeneration*.

d. Mouth swabs for specific genes associated with a higher risk of OA*.

e. Optional minimally invasive US-guided knee lining biopsy at six to 12 months after injury for non-surgically treated patients.

f. Intraoperative knee synovial biopsy for patients treated surgically for knee injury at any point.

g. Knee joint (synovial) fluid sampling during elective operation.

*Results of marked items were not used in this thesis. Therefore, their properties and results will not be discussed in this chapter.

For the purpose of this thesis, cross-sectional data came from baseline assessments, as well as surgical and US-guided biopsy follow-ups.

Though urine, blood and joint fluid samples were collected, only serum levels of new biomarkers associated with joint specific inflammation were assessed for this thesis.

Depending on the chosen pathway, all participants have been consented at baseline, with surgically managed patients giving permission for an intraoperative biopsy during their routine operation and patients managed non-operatively consenting to a US-guided biopsy of the knee joint lining. In cases of US-guided biopsies, this has either been coordinated with the patient's routine follow-up appointment or led to an invitation to attend a separate research appointment at the NIHR clinical trial unit, housed within the Nuffield Orthopaedic Centre (figure 2.1).

Some patients were listed for an elective knee operation before recruitment commenced in the clinic. These patients were identified and formally assessed for eligibility by a clinical team. If eligible, they received the Participant Information Leaflet with a routine scheduled admission letter for the operation. This included a detailed explanation about the study design, rationale and implications of taking part. During their admission, they have had an opportunity, while on the ward, to discuss further details of this study and given the option to participate. In keeping with the procedure for other participants in the cohort, a clinical research team member has been present before the operation to provide further

verbal explanation for those considering participating and, if applicable, obtain consent. The same information and tests have been collected as for the rest of the cohort with acute injuries.

There has been a maximum of two time points follow-ups for the collection of tissue samples and additional scans for each participant. Whenever possible, these have been coordinated with patients' routine visits.

2.1.4 Subjects

There were 102 consecutive eligible patients attending knee services at the Nuffield Orthopaedic Hospital in Oxford who agreed to take part in the study and were subsequently recruited between the beginning of February 2015 and the end of January 2016. Screening, recruitment and basic demographics of the OxKIC participants are shown below.

2.1.4.1 Inclusion and exclusion criteria

OxKIC study inclusion criteria included:

1. Knee injury involving anterior cruciate ligament and/or meniscal injury.

2. Participant were willing and able to give informed consent for participation in the trial.

3. Male or Female, aged 18 years or above.

For the Acute OxKIC additional criteria were added:

1. Men or women between 18 and 55 without primary knee OA, history of inflammatory arthritis or connective tissue disease.

2. Seen/reviewed within 12 weeks after the injury.

3. In the Investigator's opinion, is able and willing to comply with all study requirements.

OxKIC study Exclusion Criteria

The participant may not enter the trial if ANY of the following apply:

1. Participants who are unable to provide informed consent.

For the Acute OxKIC study:

1. Participants with established knee osteoarthritis* in either knee before injury.

2. Participants with known inflammatory or crystal arthropathy affecting any joint.

3. Participants who had a knee surgery, or knee injection within the last three months before last injury.

4. Participants with other knee injury, either side, within 3 months before the ACL rupture, ACL tear and/or meniscal injury that led to the patient's referral to the clinic.

5. Participants with severe infection 2-3 weeks before the clinic.

6. Participants with other severe joint injury or joint arthritis affecting the patient's daily activities on most days in the last month

7. Participants with decreased or altered mobility before injury due to paralysis, cerebral palsy or congenital lower limb deformities

8. Participants with reduced ability of the blood to clot due to long term anticoagulant treatment (vitamin K antagonists, Heparin and derivative substances or direct factor Xa inhibitors) or bleeding disorders that significantly increase risk of bleeding

9. Female participant who is pregnant, lactating or planning pregnancy during the course of the study

10. Participant with a significant renal or hepatic impairment.

11. Participant with life expectancy of less than 6 months.

12. Any other significant disease or disorder which, in the opinion of the Investigator, may either put the participants at risk because of participation in the study, or may influence the result of the study, or the participant's ability to participate in the trial.

* Knee osteoarthritis defined as using the Kellgren-Lawrence (K/L) radiographic score, where definite radiographic changes are described as K/L score 2 or more, or severe medial/lateral knee osteoarthritis on baseline MRI scan reported by clinical musculoskeletal radiologist.

2.1.4.2 Recruitment for the OxKIC study

There were 234 potential participants identified during the initial screening process, with 120 meeting eligibility criteria. The majority of the individuals who

did not meet eligibility criteria did not have an ACL or meniscal injuries. Out of 119 participants who met the initial eligibility criteria, there were 16 who were identified as being missed during the recruitment process. The most common reasons for this were that the patient in question had a change of appointment time or was being seen by a clinician not involved in the study. Three eligible potential participants refused to take part in the study (figure 2.4).

2.1.5 Data collection and data entry

The Oxford Knee Injury assessment booklet contained the English version LK1.0 of the Knee injury and Osteoarthritis Outcome Score (KOOS)[18, 220, 221].

All questionnaires, including follow up questionnaires have been entered on the electronic data entry system based on the RedCAP platform designed by Dr Antonella Delmestri in collaboration with Dr Stefan Kluzek. The RedCAP platform is a web-based survey and database tool that can be used with the internet. It is an open-source application developed for academic use by the REDCap Consortium, hosted at Vanderbilt University, locally approved by the University of Oxford and implemented by Dr Antonella Delmestri, database manager at the Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences (NDORMS). Dr Delmestri has helped to build the study data collection interface for this study, whilst Dr Stefan Kluzek, Clinical Research Fellow, Ms Lorretta Davies, Clinical Trial Coordinator and Mr James van Santen, the Centre Administrator for Arthritis Research UK Centre for Sport, Exercise and Osteoarthritis, carried out data entry. To minimise data entry mistakes, 90

minutes of data entry training was provided on 8 September 2015. This included a review of online and paper forms and different versions of the questionnaire.

2.1.5.1 Data entry checks

Mr James van Santen and Dr Stefan Kluzek were involved in 82% of data entry. Two main checks were performed: a) an assessment of data completeness b) a random review of the selected questions from the main outcome measure.

The RedCap system automatically shows whether all questions have been entered and prompted during data entry and reviews entries without any answers before proceeding to the next section.

A review of the Redcap system on 12 February 2016 confirmed that all baseline questionnaires have now been completed. Ten random baseline electronic questionnaires were checked and no unfilled fields were found. Additionally, RedCap generated a dataset based on those entered questionnaires and this was later analysed through Stata statistical software. This indicated that all answers from the original questionnaires had been entered on the electronic system, including missing values. Table 2.2 shows the descriptive characteristics of the OxKIC cohort.

To assess the reproducibility of the data entry, 10 random study questionnaires entered by Dr Stefan Kluzek and an additional random 10 questionnaires entered by Mr James van Santen were examined. Answers to a randomly selected one of 17 KOOS questions regarding the "Function and Daily living" (questions A1-A17) from each questionnaire were selected, based on random string of numbers

generated by an online random number generator [222] [223]. Those answers were then recorded by Dr Stefan Kluzek and compared with the results from the same questions already entered on the RedCap system. Weighted kappa statistics were utilised to establish the agreement. These indicated that intra-rater and inter-rater reproducibility were almost perfect. Only one answer out of 20 questionnaires was different in the re-recorded questionnaire.

Inter-rater agreement		
Agreement	**Kappa**	Std Error
100%	1.0	0.22
Intra-rater agreement		
Agreement	**Kappa**	Std Error
96.67%	0.92	0.23

Overall, this suggests high quality of data entry without any major differences between data entry personnel with regard to a small randomly selected group of questions from the main outcome measures questionnaire.

2.1.6 Population Demographics

The OxKIC cohort consisted of an acute OxKIC group including participants with recent ACL/meniscal injuries defined as presenting below 12 weeks on the day of potential recruitment and a non-acute group including chronic ACL injuries that could comprise any ACL injury regardless of time it occurred. It also included those who had an acute injury but were not keen on having an additional test as per the protocol.

Almost 90% of the OxKIC cohort has been based on the Acute Knee injury cohort. Compared to the rest of the study participants this group were slightly younger,

were more likely to be students and play high-level sport. The acute knee injury cohort participants were recruited between 11 and 70 days after the injury with median value of 26 days. In the further chapters of this thesis, only the baseline characteristics of individuals included in the specific analysis will be included.

Table 2.2. Descriptive characteristics across knee status categories

Characteristic	Old +Acute OxKIC (n=102)	Acute OxKIC (n=90)
Age, years, mean (SD)	33 (11.6)	32.4 (10.3)
Gender, n (%)		
Male	66 (65)	57 (63)
Female	35 (35)	33 (37)
Body Mass Index, n (%)		
Non-obese (<25 kg/m²)	48(47)	48 (53)
Overweight or obese (≥25 kg/m2)	5(5)	5 (5.5)
Missing data	49 (48)	37(41)
Affected knee, n (%)		
Right	57	49
Left	45	41
Knee Injury		

ACL only, n (%)	40(39)	35(39)
ACL and meniscus, n (%)	44(43)	37(41)
Meniscus/menisci tear, n (%)	18(18)	18(20)
Occupation, n (%) if retired or unemployed, previous occupation		
Student	36 (35.1)	36 (40)
Non-Manual	54 (53.0)	44 (49)
Manual	11 (10.9)	10 (11)
Physical activity before the injury: based on the Tegner score*, n (%)		
Tegner score >6 (high level competitive sports)	59 (58)	58 (65)
Tegner score >4 (competitive sport, recreational sport and heavy labour)	93 (91)	85(94)
Missing	4 (4)	0
Non-ASA NSAIDs use in the week before baseline assessment, n (%):		
No	17 (17)	17 (19)
Yes	41 (40)	41 (45.5)
Missing	44 (43)	32 (43)

* Tegner score is a validated and reproducible measure of activity levels[224]

2.1.7 Covariates and outcomes

For the purpose of this thesis, cross-sectional data from the Acute OxKIC study has been analysed, both from baseline and biopsy (including pre-operation) follow-up appointments, to examine the correlation between microscopically defined synovial inflammation scores with inflammatory biomarkers, and association between 'wet' and 'dry' biomarkers and symptoms.

The covariates and outcomes of interest for this thesis are:

1. Knee Injury and OA Outcome Score: two pain questions.
2. Numeric rating scale (NRS).
3. Results of the US protocol regarding the SR of the injured and contralateral side.
4. Results of serum COMP Enzyme Linked Immunosorbent Assay (ELISA).
5. Results of serum Resistin metabolic assay.
6. Results of serum C3M ELISA.
7. Results of serum CRPM ELISA.
8. Microscopic evidence of suprapatellar synovial inflammation.

2.1.8 Knee Injury and osteoarthritis outcome score

Knee injury and Osteoarthritis Outcome Score (KOOS) is well-validated patient-reported outcome measure that has been developed to be used after knee injuries that can result in post-traumatic OA [217, 225-228]. It consists of 42 questions in five

separately scored subscales: Pain, Symptoms, Function in daily living, Function in Sport and Recreation, and knee-related Quality of Life [220].

For the purpose of this thesis, only two out of nine pain questions were utilised from the KOOS pain subscale. This was based on previous research suggesting that frequency of the knee pain and knee pain on sitting might be associated with knee synovitis[229, 230]. The first regarded frequency of experienced knee pain: "How often do you experience knee pain?" and there were five potential answers: "Never", "Monthly", "Weekly", "Daily" and "Always". The second question regarded knee pain on sitting or lying. This was chosen on the basis of the reported association of that type of knee pain with knee synovitis [231, 232]. Participants were asked: "What amount of knee pain have you experienced in the last week during sitting or lying?", with the Likert scale response options: "None", "Mild", "Moderate", "Severe" and "Extreme".

In 2012, additional recommendations regarding scoring of the KOOS were published [233]. The most relevant aspect of those recommendations in terms of the questions utilised in this thesis relate to the answer-marks placed outside the answer-boxes or situations when two answer-boxes were marked. In the first situation, based on those recommendations, the closest box was chosen and, in the second scenario, the one indicating the more severe problem was used as an answer.

2.1.9 The Numerical rating scale

All OxKIC participants were asked to comment on their knee pain at the time of filling out the questionnaire, using a numeric rating scale (NRS). The numeric rating scale used measures pain intensity in one-dimension. This scale from 0 to 10 (NRS-11) has two extreme categories labelled under the numeric values: ''No pain at all'' and ''Worst imaginable pain". It is very similar to visual analogue scores (VAS), which asks participants to place an X at a 10 cm line that represents their general pain intensity for that injured knee. A score of 0 represents no pain, while 100 represents the worst possible pain intensity. Both of these scales are fairly commonly used in studies looking at knee pain [58, 128, 234-238]. A systematic review comparing VAS and NRS by Hjermstad et al. showed a good correlation between NRS and VAS. However, the same review showed NRS was preferred by the participants and viewed as therefore more likely to increase compliance [235].

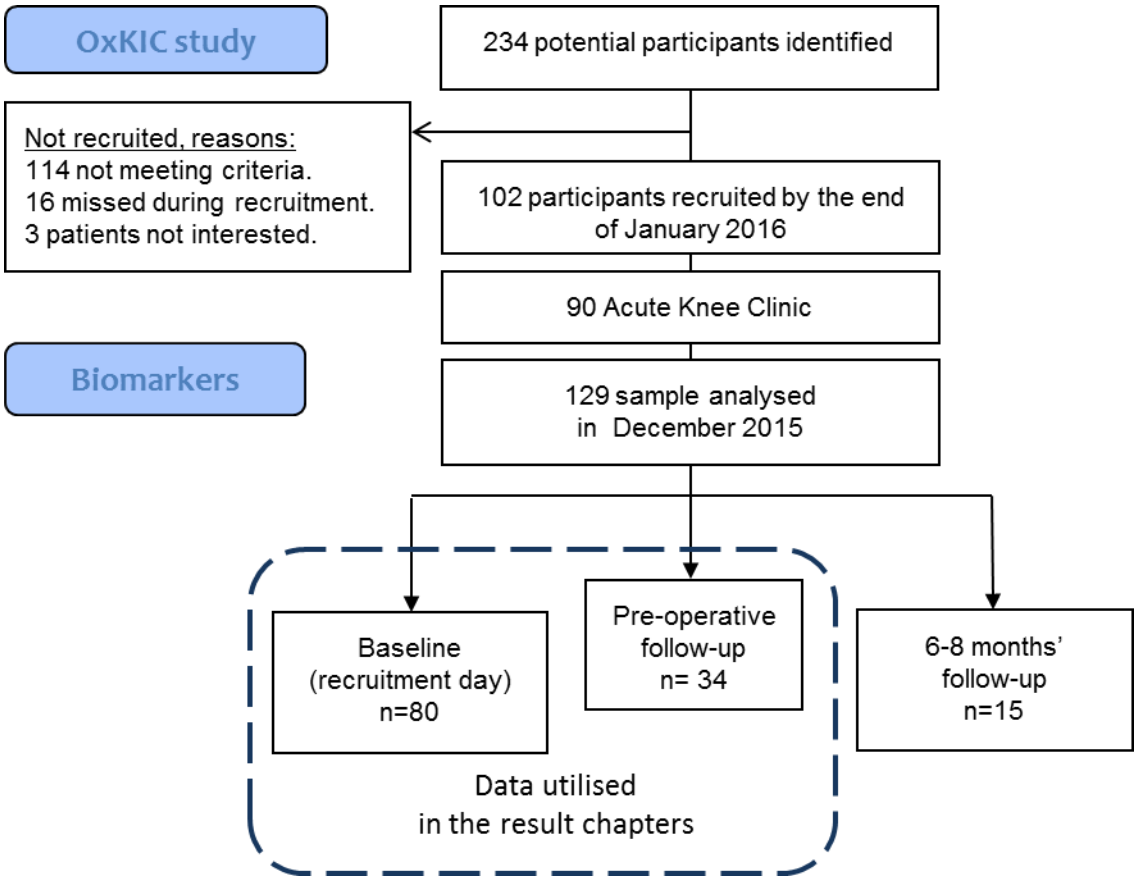
2.1.10 Biomarkers

In this section, serum collection, processing and measurements of selected biomarkers are outlined. Descriptive statistics were used to describe the basic features of the biomarker data in a study. Detailed tissue processing and storage of the blood samples is explained in the appendix section .

Blood collection was performed by either Dr Stefan Kluzek or Ms. Pam Lovegrove, the CLRN Research Nurse helping with recruitment. Ms. Erin Paterson a senior research technician, Ms. Kathrine Groves, Tissue Coordinator

(both working within the Centre for Osteoarthritis Pathogenesis) or Dr Stefan Kluzek were responsible for tissue processing.

Figure 2.4. OxKIC study recruitment and available serum biomarkers measurements per follow-up.



In December 2015, serum concentrations of COMP, Resistin, C3M and CRPM in 129 currently OxKIC study samples were measured by the Nordic Bioscience group represented by Dr Christian Thudium and Dr Anne-Christine Bay-Jensen (figure 2.4). The reproducibility and variation of the ELISA biomarker results are reported in section 2.3 for both the OxKIC and Chingford studies together.

Additionally, sonographic measurements of synovial effusion and synovial hypertrophy in the SR were measured in the affected and contralateral knee during each appointment. Reproducibility of that protocol is discussed in chapter 3.

Of 129 serum samples, there were 80 collected at baseline, during the recruitment visit. Those samples were non-fasted and collected between 1 and 4 pm in the outpatient department. Most of the patients had decreased mobility and were still using mobility aids in the form of knee braces and/or crutches at this stage.

From the total number of serum samples, 34 were collected during pre-operative follow-up. Of these, 30 participants had serum samples collected including at baseline and before the operation. Four participants had only operative samples taken under general anaesthesia, due to needle phobia. All pre-operative samples were fasting morning samples, taken between 7 and 11 am just before scheduled operations. Although 26 out of 34 participants at that point were mobilising independently, all could be classed as not very mobile for at least 7-8 hrs before the sample collection, due to sleep and hospitalisation from 7am.

Overview and descriptive statistics of biomarker data

Gender, age, BMI, type of injury, use of anti-inflammatory medications and time of collections are important factors to consider when examining biomarker data. Although only cross-sectional data from two-collection time-points (the baseline appointment and pre-operation follow-up) is analysed in his thesis, this provide

us with opportunity to examine longitudinal change of biomarker values in the subgroup who had samples take on both occasions.

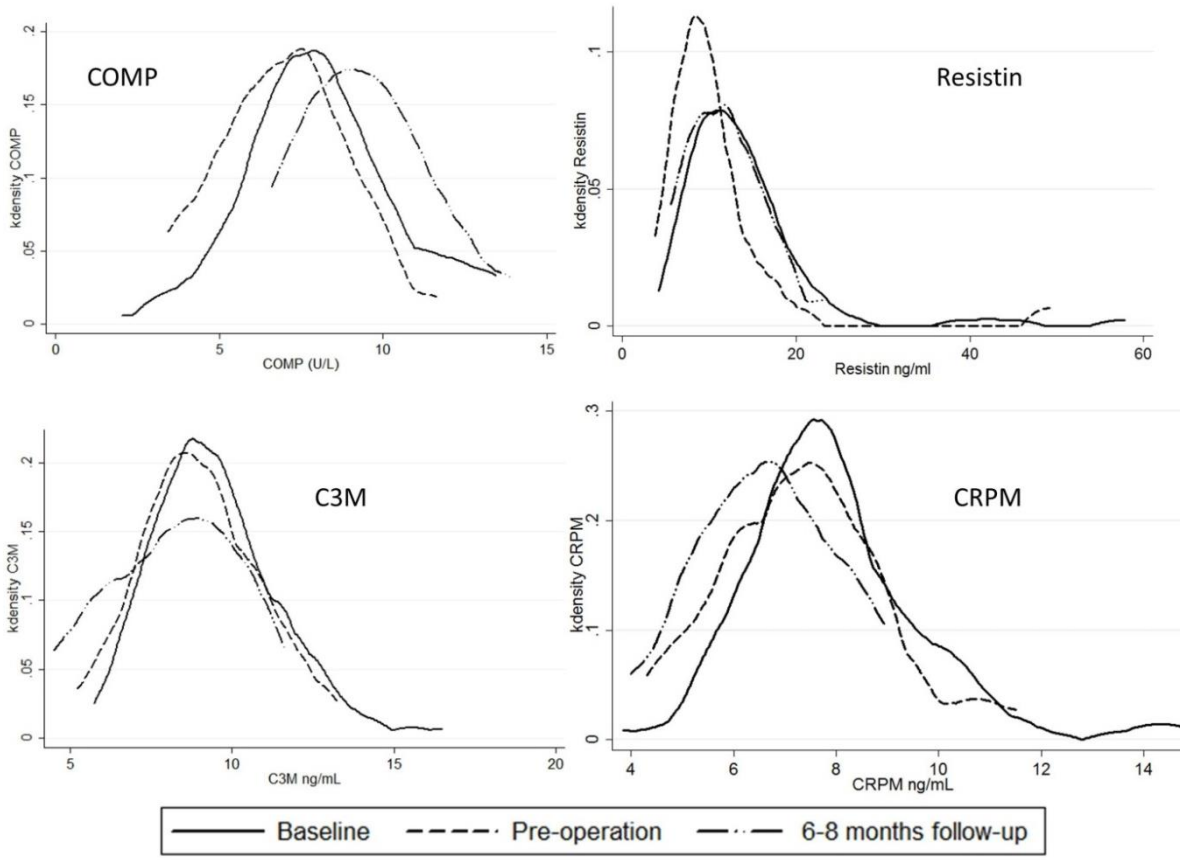
From the total samples, there were 15 collected during follow-up at six to eight months after recruitment. Results of those samples were not utilised in this thesis and are therefore not discussed in detail in this chapter and presented only for comparison.

Simple statistical methods were utilised, with the paired t-test to compare parametric data and the Wilcoxon signed-rank test compared non-parametric. The association between changes of the biomarker values with time (the delta between collections expressed in days) were assessed using Pearson's for associations that were linear or Spearman for those that were monotonic.

There were some variations in terms of the mean values of all biomarker concentrations between baseline, pre-operative and/or follow-up samples, especially for COMP and Resistin, but those could be explained by different groups and sample sizes (figure 2.5).

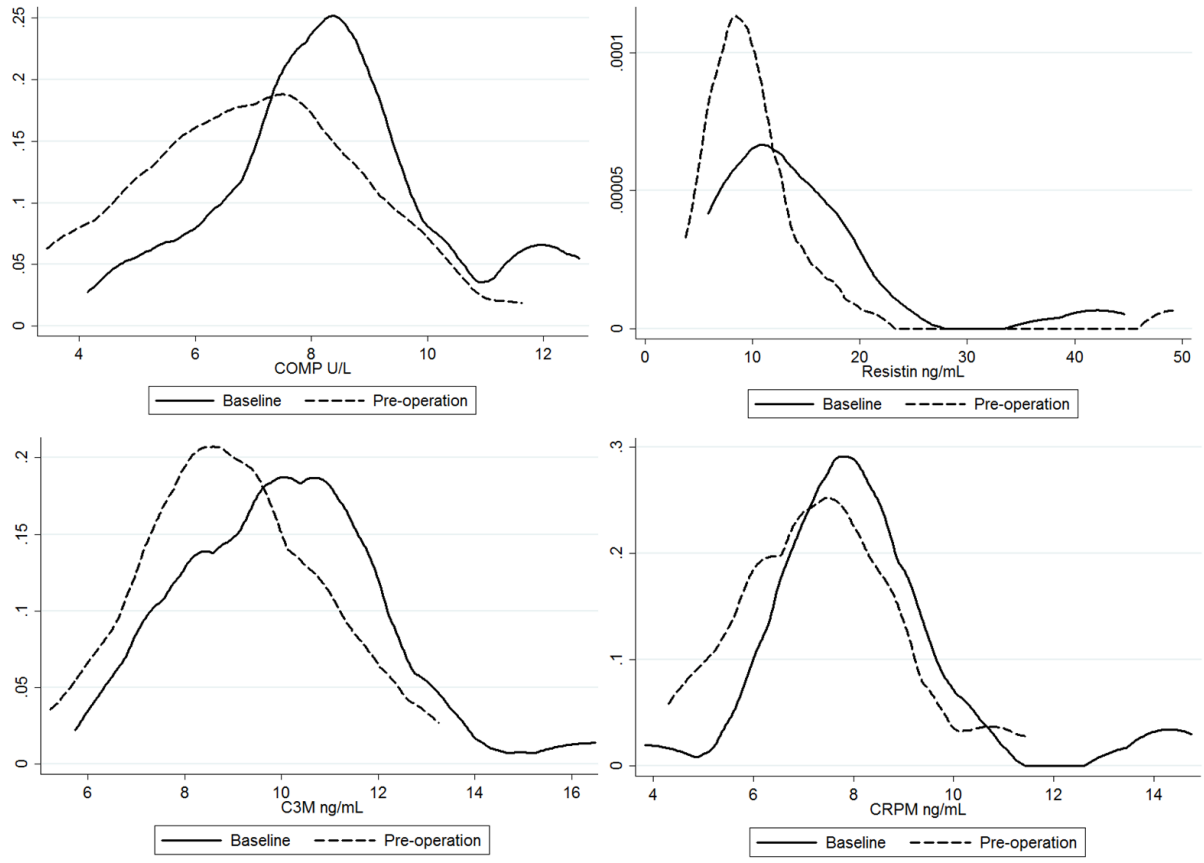
To assess differences in the same group, levels of the biomarkers in participants who had baseline and pre-operative samples collected were compared (figure 2.6).

Figure 2.5. Comparison of distributions of samples collected at baseline (n=80), before the operation (n=34) and during the six to eight-month follow-up visit (n=15).



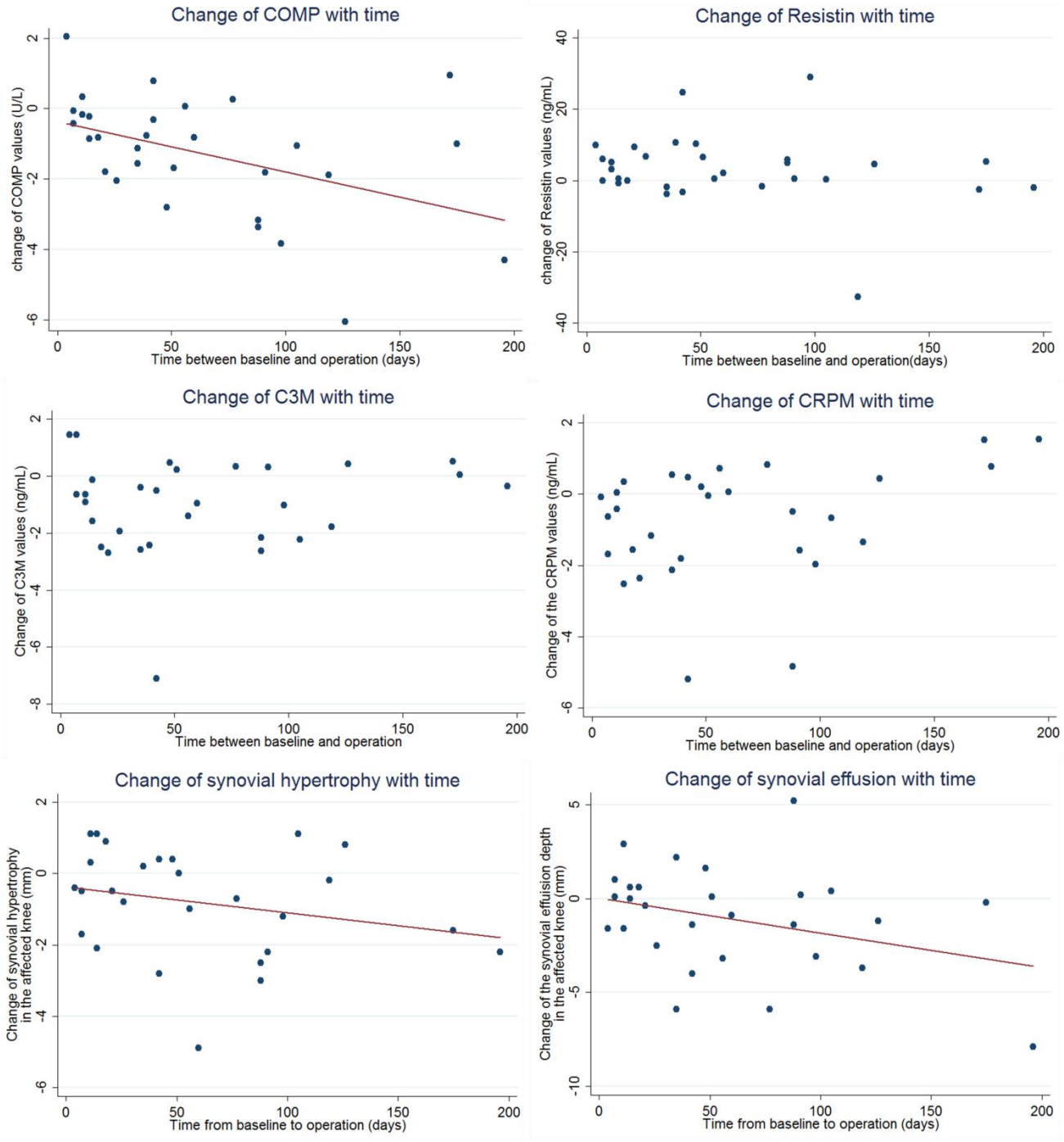
In the paired samples, similar differences were seen (as presented in figure 2.5). In general, there was a trend for the samples in the pre-operative group to have lower values than baseline samples. Interestingly, there were no significant differences between effusion at baseline and before the operation, with a p value of 0.7, using a non-parametric Wilcoxon rank-sum test.

Figure 2.6. Comparison of distributions of samples collected at baseline and before the operation in the same participants (n=30). The Y-axis in each graph is a kernal density estimate of the biomarker data.



The time between sample collections has been reported as an important covariant in changes of biomarker concentration [239]. The association between changes of the biomarker values with time (the delta between collections expressed in days) were assessed using either Pearson's or Spearman correlations.

Figure 2.7. Association between change of the biomarkers from baseline to operation and time in the same participants (n=30).



Mean baseline COMP values were significantly lower before the operation when compared with baseline samples. There was a significant negative linear correlation with the time (p-value 0.013) and this is presented in figure 2.7. Resistin showed significantly decreased pre-operative values (p-value 0.033) but no correlation with time (Figure 2.7). Meanwhile, baseline C3M and CRPM concentrations were significantly higher than before the operation (p-value 0.002 for C3M and 0.014 for CRPM). However, the change was not associated with time in this small sample, with p-values of 0.56 and 0.09 retrospectively (figure 2.7). Synovial hypertrophy (SH) was significantly lower before the operation (p-value of 0.009) but, despite this trend, the association of the change with time was not statistically significant (p-values 0.21, figure 2.7). Mean values of synovial effusion were lower before the operation (p-value 0.05) and there was a trend of linear association between the change of the effusion and time that was borderline significant (p value 0.079).

Age showed a positive association with COMP levels (p=0.052) but not other biomarkers. There were no significant differences between levels of biomarkers in different genders apart from higher levels of CRPM in women (p 0.057). Individuals classified as overweight (BMI≥25 kg/m^2) had higher levels of COMP and Resistin. Use of NSAIDs was recorded during the baseline visit only, with analysis showing a borderline significant association between their use during the week before recruitment and lower Resistin levels (p-value 0.07). There was no significant association between the types of injuries and pre-operative levels of the any of the biomarkers. Both baseline and pre-operation visits were

characterised by decreased mobility before the sample collections, and preoperative samples were fasted (figure 2.4).

In summary, all examined 'wet' and 'dry' biomarkers decreased from baseline to the operation. Both COMP and synovial effusion decreased with time in a linear manner. This was consistent with the previously published observation that high levels of COMP have been reported in individuals with effusion, trauma and increased loading activities [147]. There was no association between change of COMP values and either change of synovial effusion or hypertrophy. Decreased levels of Resistin before the operation could be explained by decreased effusion but big confounding effect is fasting that can affect levels of Resistin. C3M and CRPM are lower before the operation but in this small sample no association of change of those biomarkers with change of time.

It is important to highlight the potential limitations associated with the relatively small group of participants followed-up so far and the fact this might be a chance finding. There is also a potential for follow-up bias towards individuals who have not improved as much deciding to have an operation. With such a small sample, only some small differences regarding sex, BMI and use of NSAID were seen.

2.1.11 Microscopic synovial inflammation

2.1.11.1 Sampling knee synovium

The synovial tissue samples were obtained from the suprapatellar recesses of 37 OxKIC study participants patients with recent ACL or/medial meniscal injuries, normal radiographs and no history of inflammatory arthritis. This group consisted of consecutive patients from the OxKIC study who were listed for clinically indicated routine operations in the first eight months after starting OxKIC recruitment or US-guided synovial biopsies during their six-month follow-up visits. Either one or three suprapatellar synovial samples were collected from each knee, with rigid forceps, by the surgeon at the beginning of the routine arthroscopic procedure. Before amendments, only one synovial sample from the knee was collected. The aspect of the SR with the largest depth of SH was identified before each procedure using the protocol described in chapter 3. The surgeon performing the arthroscopy was asked to either collect one sample from the aspect of the SR identified as containing the largest SH or from central, medial and lateral aspects of SR without prior knowledge about ultrasound finding after the amendments. Each sample was fixed and embedded separately, then marked so that a record of the collection location within the SR was available.

OxKIC participants who were not scheduled for a surgical procedure were asked for additional permission to collect samples of suprapatellar synovium during US-guided biopsy procedures at the six-month visit. As a result, one participant

has had synovium collected using this method. Subsequently, 65 synovial samples from the SRs were obtained during 36 routine arthroscopic procedures and four synovial samples were acquired during one US-guided suprapatellar biopsy session.

There were some differences between the techniques that could potentially have affected results. Intraoperative samples were collected from patients during the routine operation and the usual settings were applied, including the application of a tourniquet bandage over the upper thigh at a fixed inflation pressure of 300 mm Hg to decrease bleeding [240]. All patients had general anaesthesia. After an attempt to collect synovial fluid samples, but before the synovial biopsies, all participants had their knee joints inflated with a sterile saline solution to expand the joint. Hypoxia associated with the use of the tourniquet lasted for a minimum of 15 minutes, before samples were collected, but less than 60 minutes. Little is known about the effect ischaemia has on synovial tissue and synovial fluid in the first 20 to 60 minutes. This can potentially affect cytokine production, and is probably particularly important for levels of inflammatory cytokines in the synovial fluid. To lesser extent, it is probably also important for infiltration of synovium with inflammatory cells. Some studies have suggested that, after the operation, there are both systemic reactions to reperfusion and hypoxia, but also a localised inflammatory response[240].

The US-guided synovial biopsies are not affected by potential hypoxia. In these biopsies there are potential changes from the use of lidocaine and normal saline solution.

US-guided biopsy is an efficient, safe and reliable technique that allows a significant amount of synovium to be obtained from the supra-patellar pouch. A protocol produced by Dr Steven Kelly [241] that has been tested and reported as a very safe and acceptable method for obtaining biopsies has been adopted in this thesis [242]. Following a discussion with Dr Steven Kelly, Dr Stefan Kluzek attended two practical sessions in London, observing three procedures. Prior to the first supervised biopsy by Dr Karen Partington, Consultant Radiologist, Dr Stefan Kluzek organised two supervised training sessions with Dr James Teh, Consultant Radiologist, to practice this technique. Following obtaining formal consent before the procedure, US-guided biopsies were performed at the Oxford Clinical Trials Research Unit. The participant had the usual sonographic assessment using standard protocol before a US-guided biopsy and this identified the aspect of the SR with largest SH. Four US-guided samples were collected, with a minimum of one from each aspect of SR. using 14 and 16G TEMNO Biopsy needles. The standard operating procedure produced by Dr Steven Kelly was followed[243].

2.1.11.2 Preparation of histology slides

The five main stages in the general preparation of histology slides are: fixing, processing, embedding, sectioning and staining.

A chemical fixation with 10% formalin (Aldehyde) was performed to stabilise the proteins, nucleic acids and other parts of the tissue by making them insoluble.

Tissue processing was performed by senior research histology technicians based at the Centre for Osteoarthritis Pathogenesis at the Kennedy Institute of Rheumatology: Dr Ida Parisi, Dr Bryony Stott and Ms Marcia Curtinha.

During processing, tissue water was replaced with paraffin, a medium that solidifies and sets to become hard. As a result, biological tissue was supported in a hard solid matrix to enable sufficiently thin sections to be cut. Before cutting, tissue was manually put into blocks in a process known as 'embedding', when the tissue is covered with molten paraffin. Then blocks were then sectioned into five-micrometre- thick sections, using a microtome with a disposable blade and a water bath with the temperature set at 37°C. Those slices were then picked up on a glass microscopic slide and placed in a warm oven for about 15 minutes to help the section adhere to the slide.

The Hematoxylin and eosin staining was performed using the standard method by an automated staining machine [244]. The automated protocol as well as solutions and reagents used are presented in the appendix. Hematoxylin stains acidic components of tissues, such as the cellular nuclei, while eosin stains the basic cellular components, such as the cytoplasm and the connective tissues. The majority of typical problems resulting from this technique are associated with the hematoxylin staining and often linked to a variation in the batch number of the hematoxylin, a change of supplier or pH differences. For the purpose of decreasing variability of the staining, each new batch in this study was checked for efficacy against current or earlier batches.

2.1.11.3 Analysis of synovial tissue

Although rheumatoid arthritis and classical inflammatory conditions are often defined by clinical and histological synovitis, classical microscopic evaluation of synovium is not an established method of differencing classical inflammatory arthritis from other forms of arthritis or posttraumatic-synovitis. A number of studies compared synovial histological findings in different forms of arthritis. In blinded studies, authors confirmed that it is very difficult to distinguish the histological features of rheumatoid synovium from synovium obtained in patients with OA based on the histological samples alone[87, 88].

Revell et al. obtained tissue from the knees of patients with all spectrum diseases and confirmed that synovium in the KOA is associated with inflammatory infiltrate[89], but could not identify any specific pattern of infiltration characteristic for OA. Similar difficulties with non-specific inflammatory response has been reported by other authors[90, 91].

These studies showed the significant degree of variation of different features within each disease category. Overall, they support the notion that qualitatively there is a little difference between synovial changes in various types of arthritic diseases. Especially in early disease it is difficult to establish diagnosis based on microscopic features of synovium. In rare conditions, synovial histology might be diagnostic in cases of undifferentiated arthritis[92] and immuno-staining helps to identify dominant inflammatory pathways, yet it remains to be shown that

microscopic analysis of the synovial tissue early in disease has an independent value in identifying patients' long term diagnosis and outcomes in both suspected RA and OA.

2.2 The Chingford cohort

The Chingford 1000 Women Study is a prospective, population-based cohort study of middle-aged women. It has been shown to be representative of this group in England in terms of basic characteristics, but with a lower rate of current smokers [245-249]. It is a unique cohort of predominantly asymptomatic individuals, with over 70% of them having no signs of RKOA in either knee at baseline and, more importantly, long-term and detailed follow-up data on symptoms and radiographic changes.

The study was approved by the Outer North East London Research Ethic Committee, formerly Barking & Havering and Waltham Forest RECs, LREC (R&WF) 96. Each study participant provided written informed consent before participating. In 2013, South Central – Oxford A Research Ethics Committee gave a favourable opinion regarding the further application that created the Chingford Cohort resource. This also included the ongoing maintenance of data, sample collection and analysis (REC reference number 13/SC/0156).

2.2.1 Population demographics and recruitment

All women were registered in the same large general practice in Chingford, North-East London. The study was set up in June 1989 to determine prevalence rates of OA and osteoporosis (OP). Out of almost 11000 patients from the same practice, 1353 women were contacted and asked to participate in the study. Just over 1000 (1003, 78% response rate) attended the baseline visit and have since been examined annually [245, 246].

This is a predominantly Caucasian cohort. At baseline, participants had an average age of 54.2 years, with an age-range of 44 to 67. Almost 12% had an evidence of RKOA at baseline. The mean BMI was 25.6 and 17% had a medical history of hypertension, while 1% had a history of diabetes[246, 248, 249]. Detailed demographics for the selected groups and comparison with the rest of the cohort are presented before each new analysis in this thesis.

The Chingford Cohort was a uniquely recruited group of middle-aged women, rather than a symptomatic or elderly population. Each visit year included a combination of health questionnaires, including detailed musculoskeletal questionnaires, physical examination, X-rays, and blood and urine collection, depending on the follow-up year. Structured interviews were conducted over the phone annually between the appointment visits. Questionnaires and interviews included queries regarding risk factors for OA, including age, smoking, family history of arthritis, information on current and previously used medications, location and frequency of the knee symptoms, injury history and physical activity. Physical examination included weight, height, blood pressure, but also

examination of the knee joints including assessment for bony swelling, knee effusion, tenderness and knee pain on flexion.

As this thesis aimed to investigate the role of selected serum biomarkers associated with knee pain and inflammation and risk of developing KOA, a subgroup of the Chingford Cohort with no baseline RKOA, defined as a Kelgren and Lawrence score of zero in both knees, was selected for the main analysis. There were some significant differences between this sub-group and women with either general RKOA or painful RKOA and these are described in detail in chapter 7.

2.2.2 Covariates and outcomes

The previously collected data over the duration of follow-up and the new data collected specifically for this research was analysed for the purpose of this thesis, namely new biomarker data from the visit's serum samples.

2.2.2.1 Biomarkers

Sera were obtained from venous fasting blood during the second and third year follow-ups and stored at -80°C. 94% of serum samples used in this research were collected during the second year visit. COMP, Resistin, C3M and CRPM serum concentrations for majority of the study participants were measured by the Nordic Bioscience group represented by Dr Christian Thudium and Dr Anne-Christine Bay-Jensen. COMP was measured in 2009 and Resistin, C3M and CRPM at the end of 2015. The reproducibility and variation of the ELISA

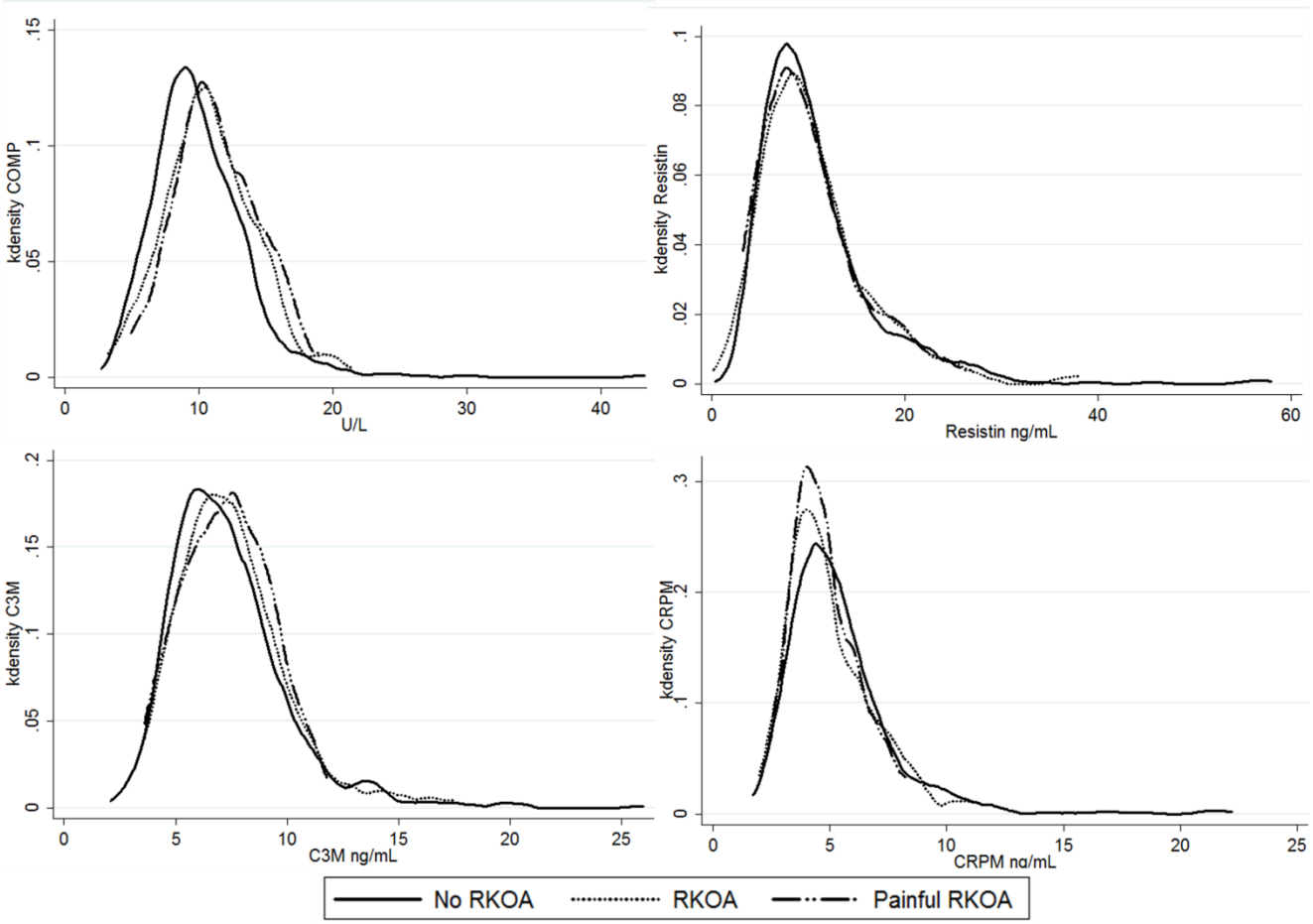
biomarker results are reported in section 2.3 for both the OxKIC and Chingford studies together.

Table 2.3. Biomarkers measured in the Chingford study, with 'Total' indicating all participants with measurements of a specific biomarker, by subgroups based on knee status.

Biomarker	**Total**	**K&L = 0 Both knees**	**K&L >0**	**K&L≥2**	**K&L≥2 and pain**
			Uni or bilateral RKOA		
COMP	745	594	151	110	48
C3M	849	700	149	109	47
CRPM	857	708	148	108	47
Resistin	841	697	144	104	45

Results of biomarkers in participants with no RKOA in both knees were used for the analyses. Small differences in terms of the number of participants who had their C3M, CRPM and Resistin measured (range 697-708) arose from the fact that CRPM was measured first, while Resistin was the last biomarker. Six samples were not sufficient, while a further three had too much variation in terms of repeated measures and were therefore not included in the analysis.

Figure 2.8. Kernel density plots for serum concentrations of selected biomarkers in individuals without and with RKOA, and painful RKOA.



To analyse if the differences between groups were significant, a non-parametric Wilcoxon rank-sum test was deployed to compare those different sub-groups for each of the biomarkers. Results showed no significant differences for most of those subgroups apart from COMP values being higher in RKOA and painful RKOA when compared to the no RKOA group (p-values of 0.006 and 0.002 respectively).

In the group with no RKOA, obesity was associated with slightly higher values of COMP, Resistin and CRPM. Higher age was also associated with a mild increase of COMP and C3M, but not other biomarkers.

Table 2.4. Association between selected biomarkers, obesity (BMI > 30 kg/m^2) and age at year 3. '↑' indicates higher levels associated with either obesity or higher age. '↔' indicates no difference between groups.

	Obesity	p-value	**Age**	p-value
COMP	↑	0.02	↑	< 0.005
Resistin	↑	0.04	↔	0.46
C3M	↔	0.41	↑	< 0.005
CRPM	↑	<0.005	↔	0.23

In non RKOA subgroup COMP, Resistin and CRPM concentrations were higher in group with BMI >30, and that was statistically significant when medians were compared a non-parametric Wilcoxon rank-sum test. In the same group only COMP and C3M levels were significantly higher in older individuals.

2.2.2.2 Knee Pain

Knee pain was evaluated via questionnaire at baseline and years 3, 5, 10 and 15.

The baseline questionnaire used the following question: "Have you had knee pain within the last month?" During clinic visits starting at years 3, 5, 10 and 20, participants were asked if they had felt, in the last month, any knee pain, the number of painful days of knee pain (if applicable) and which knee (left or right) was painful.

2.2.2.3 X-ray protocols

Bilateral anteroposterior weight-bearing radiographs were taken at baseline and years 2, 3, 5, 10, 15 and 20. A standard protocol of obtaining knee x-rays was established at baseline and utilised during all subsequent visits by experienced radiographers. The same equipment was used during each follow-up time-point. From baseline to year 15 of follow-up, a specific protocol using cassette was utilised. This is described in detail in a paper written by Dr Debbie Hart in 1999 [48].

Digitalised x-rays were taken at year 20 with a very similar protocol, and a calibration object was used for comparison of new X-rays and digitalised X-ray films [250].

2.2.2.4 Grading knee x-rays

Radiographs were graded by blinded researchers unaware of clinical information, using the Kellgren and Lawrence global score (table 2.5) [251].

Table 2.5. Descriptions of Kellgren and Lawrence grades

Grade	
0	Normal
1	Possible osteophyte, no joint space narrowing (JSN)
2	Definitive osteophyte, possible JNS

3	Multiple osteophytes, definite JNS, sclerosis and possible deformity of bone ends
4	Large osteophytes, marked JNS, severe sclerosis and definite deformity of bone ends

Baseline and year 5 radiographs were scored by Prof. Tim Spector and Dr Deborah Hart (DH). Year 10 and 15 knee x-rays were read by DH alone. Year 20 radiographs were read by Dr Kirsten Leyland, who was taught by DH. These demonstrated good reproducibility [250, 252].

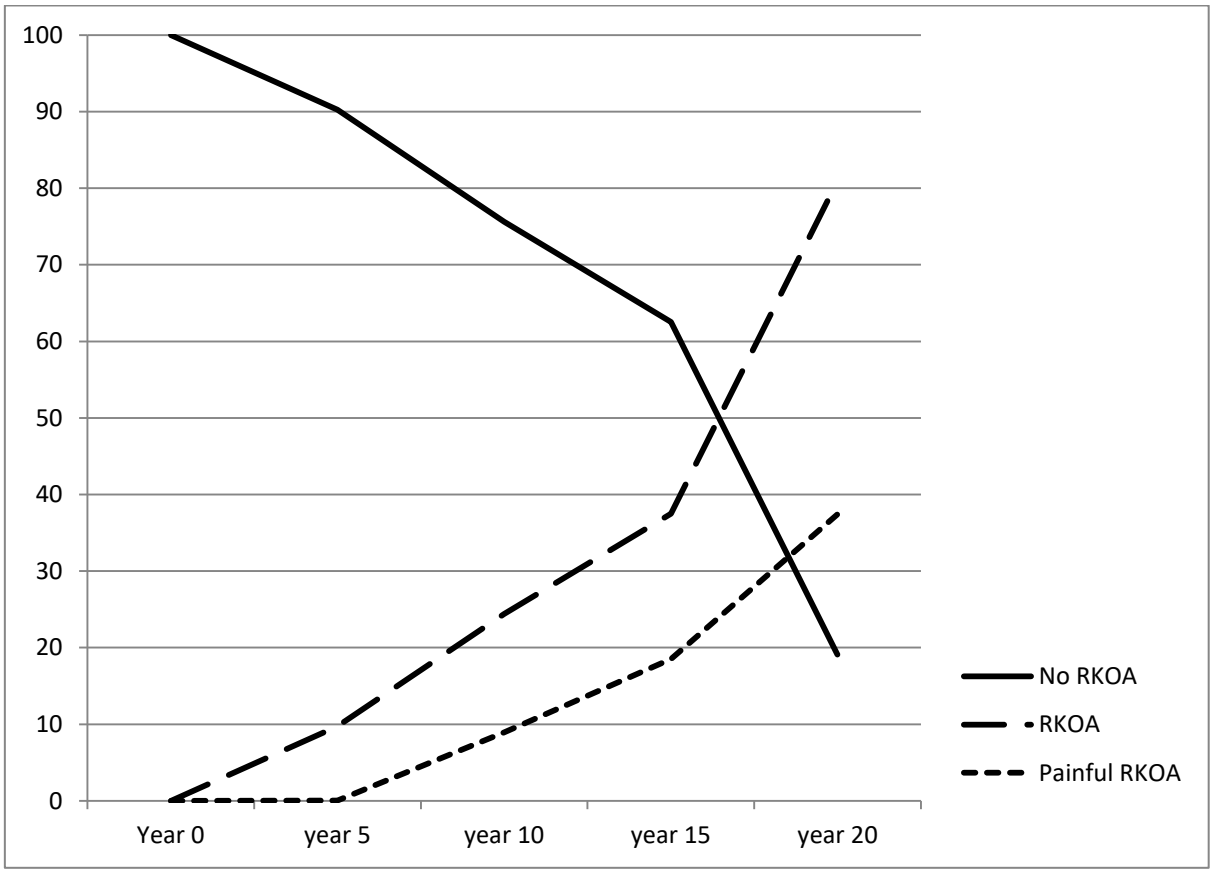
2.2.2.5 Incident radiographic knee osteoarthritis

Incident RKOA was calculated at the subject level and separately for every five-year follow-up time-point. It was defined as having a K/L grade of 0 in both knees at baseline and a grade 2 or above in any knee at the any of the one of the five-year time-points during the 20-year follow-up.

2.2.2.6 Incident painful radiographic knee osteoarthritis

Incident RKOA was calculated at the subject level and was defined as any side specific K/L grade of 2 and above at any of the five-year time-points follow-up and presence of any knee pain in the last month on the same side.

Figure 2.9. The diagrams illustrate the relationship between RKOA and painful RKOA during each –of the five-year time-points during the 20-year follow-up in a subgroup with no RKOA at baseline (n=750).



	Baseline	year 5	year 10	year 15	year 20
No RKOA	750	583	474	297	72
RKOA	0	63	153	178	305
Painful RKOA	0	14	56	88	141
Participants attending the follow-up	750	646	627	475	377
Percentage of initial group attending follow-up	100	86.1	83.6	63.3	50.3

This cohort has been followed-up now for over 20 years now. Over 50% of original group with no baseline RKOA attended follow-up 20 years after recruitment. Taking into account almost 20% mortality in that group by year 20, just over 70% of original population who survived till that time-point attended the follow-up after two decades. This is a great achievement for any study, and it presents a remarkable opportunity to test biomarkers on development of KOA. At any of the five-year time-points during the 20-year follow-up, less than half of individuals who had developed RKOA reported any knee pain in the affected knee in the last month. This is consistent with a previous cross-sectional study looking at a large population and discordance between RKOA and knee pain [51].

2.2.2.7 Excess of Premature Mortality

The all-cause and cause-specific mortality from CVD and cancer were based on available data up to August 2014. The Health and Social Care Information Centre provided detailed mortality information on the Chingford cohort, based on information collected by the Office for National Statistics from civil registration records. The cause of death was based on information from death certificates. For cause-specific mortality, we reviewed all CVD and cancer- related deaths data from death certificates. In seven cases of cardiovascular mortality, the cause of death was changed to cancer-related mortality, if the direct cause of death was likely a complication of cancer or its treatment.

The cumulative number of deaths over the 23 years (median 21.7 years (IQR: 21.2–22.3)) of follow-up was 223 women (22.2%) out of the total sample of 1003. CVD mortality accounted for 29% (n=64), with cancer and other diseases at 45% (n=100) and 26% (n=59) respectively in terms of all-cause mortality.

2.3 Serum biomarkers

745 participants had their COMP levels measured in 2009 by the Nordic Bioscience Laboratory research group as a part of international collaboration aiming to identify correlation between OA and specific biomarkers, 594 of participants who had their biomarkers tested had no baseline RKOA. At the end of 2013, a new collaboration between the Rheumatology Research Department of Nordic bioscience, represented by Dr Morten Karsdal, Dr Anne-Cristian Bay-Jensen and Dr Christian Thudium, and the epidemiological group at the Nuffield Department of Orthopaedics Rheumatology and Musculoskeletal Sciences, represented by Dr, Julia Newton, Dr Stefan Kluzek and Professor Nigel Arden, was established to investigate predictive ability of selected biomarkers. An additional 103 samples in the pool of baseline Chingford samples were identified and sent to the Nordic Bioscience Lab for all other biomarkers (Resistin, C3M, CRPM) to be measured with previously available serum samples.

Apart from the Chingford COMP measurements mentioned above, all other measurements for both this and the OxKIC study were performed at the end of 2015. All results came from the same (Nordic) laboratory and all of them were performed using the same equipment and following the procedures separately

for each biomarker. All those assays were validated before testing the samples. This process included the sample dilution, determination of the measurement range and day-to-day variation. For those reasons, and also to make comparisons between relatively old serum samples from Chingford and 'new' samples from the OxKIC study, reproducible results from both cohorts are presented together for each biomarker.

2.3.1 Collaboration with the Nordic Bioscience

Collaboration agreements between the Nuffield Department of Orthopaedics, the Rheumatology and Musculoskeletal Sciences department based in Oxford and Nordic Bioscience, Biomarkers & Research based at Herlev, Denmark were signed at the beginning of 2014. Furthermore, the Material Transfer Agreements (MTAs) for transferring biologic samples from baseline (Year 2 and Year 3) pool of the Chingford Women study samples, as well as the baseline and follow-up samples from the OxKIC study, were developed and accepted by both sides. Samples were send to the Nordic Bioscience at the end of 2015 and analyses of serum concentrations of COMP, Resistin, C3M and CRPM measured between December 2015 and February 2016.

For all results, in order to assess the reproducibility of the selected biomarkers assays, both the intra- and inter-assay coefficient of variability (CV) were measured:

$$\mathrm{CV} = \frac{Standard\ Deviation}{Mean}$$

2.3.2 Measurements of serum COMP

Serum COMP was measured using AnaMar Medical ELISA kit (Uppsala, Sweden). This is a one-step sandwich ELISA (Human COMP® ELISA) to detect COMP. The intra- and inter-assay variations were below 4% and 8%, respectively. The samples were measured in 2009 for Chingford and in 2015 for OxKIC study. The quantification range was determined internally by the lab as 2 (lower limit of quantification) to 35 (upper limit of quantification) U/L. All samples were measured in duplicate and within the measuring range when diluted between 1 in 4 and 1 in 10. Samples were rerun if intra assay coefficients of variability (CVs) were above 15% or if their values were outside the quantification range. Each plate included two kit controls, as well as three internally identified quality control samples. From these, the inter-assay CVs were monitored and controlled for being 5-10%. No measurements were discarded by a researcher performing the procedures above.

2.3.3 Measurements of Resistin

The serum Resistin was measured using Human Resistin MSD metabolic assay (Meso Scale Diagnostics, USA). The MSD plate contains capture antibodies and use of solution containing the detection antibodies conjugated with electrochemiluminescent label. The intra- and inter-assay variations were below 4% and 8%, respectively. The quantification range was determined internally by the lab as 275 (lower limit of quantification) to 58000 (upper limit of quantification) pg/mL. Twelve plates were used and all samples were measured in the duplicates and within the measuring range when diluted 10 times.

Samples were rerun if intra assay coefficients of variability (CVs) were above 15% or if their values were outside the quantification range. Each plate included two kit controls, as well as three internally identified quality control samples. From these, the inter-assay CVs were monitored and controlled for being 5-10%. No measurements were removed by the lab.

2.3.4 Measurements of C3M and CRPM

Serum concentrations of C3M and CRPM, for both the Chingford and OxKIC studies, were measured by the Nordic Bioscience Lab, where those assays were developed. This took place at the end of 2015. The neo-epitope of the C3M assay is KNGETGPQGP at amino acid position 610-619 and the assay has been biologically validated [186]. The neo-epitope of the CRPM assay is KAFVFPKESD at amino acid position 25-34 and has been shown to be specific for this fragment, as it does not recognise other protease cleavages of CRP[187].

For C3M, streptavidin pre-coated 96-well plates (Roche Diagnostics, Mannheim, Germany) were coated with 0.4 ng/mL of KNGETGPQGP-biotin and left for 30 min at 20°C. After washing, the calibrators, controls and serum samples (diluted 1:1 in incubation buffer) were added. This was followed by peroxidase-conjugated antibody NB51-G12. The mixture of sample–antibody was incubated at 20°C for 60 min. The sample–antibody mix was washed off the well plate and peroxidase reaction was visualized by 3,3′,5,5′-tetramethylbenzidine (TMB, Kem-En-tec, Taestrup, Denmark) at 20°C and stopped with sulfuric acid after 15 min and measured. Calibration curves were plotted using a 4-parametric mathematical fit model.

CRPM measurement followed the same procedure as C3M; however, applying a different peroxidase-conjugated antibody (NB94-1A7) and coater (KAFVFPKESDK-biotin).

Both the intra- and- inter assay coefficients of variability (CV) were measured. For Intra-assay CV% sample results in double determination was accepted below 15 %. If the sample results were within lower limit of measurement range (LLMR) and upper limit of measurement range (ULMR) but CV% above 15 %, the sample was re-analysed. Each plate included two kit controls, as well as three internally identified quality control samples.

For C3M assay analytic measurement range was between 1 and 22 ng/ml (pre-dilution), 38 ELISA plates were used for the Chingford study samples (analysed in 6 different runs), and 6 ELISA plates were used for the OxKIC study samples (1 run). Inter- and intra-assay CVs were below 15%, between 9.8% to 13.6% for the Chingford, and 1.2% to 14.6% for the OxKIC.

For CRPM assay analytic measurement range was between 1.7 and 23 ng/ml (pre-dilution), 33 ELISA plates were used for the Chingford study samples (analysed in 5 different runs), and 6 ELISA plates were used for the OxKIC study samples (1 run). Inter- and intra-assays CVs were below 15%, 9% for inter and 10% for intra-assays CVs for the Chingford study, and 5% for inter and 8% for intra assays CVs for the OxKIC study.

Normal values of C3M and CRPM were reported before and are similar to those seen in Chingford and OxKIC studies [187, 189].

Chapter 3 The Ultrasound of Suprapatellar Recess

3.1 Background

Synovial changes, including persistent synovial hypertrophy (SH) and synovial effusion (SE), are commonly found in post-traumatic knees [253]. Traditionally, conventional radiography was the diagnostic method of choice for evaluation of the painful knee. However, structural changes associated with knee pain, namely synovial changes and bone oedema, but also poorly innervated intraarticular structures like articular cartilage or menisci, cannot be directly evaluated using this method. Recent studies also suggest that SH, especially when seen in the suprapatellar pouch or Hoffa's fat pad, might be an independent predictor for the development of radiographic KOA in a middle-aged population at risk of developing KOA, as indicated by being overweight, having current knee symptoms or a history of knee injury or surgery[8, 59, 254]. Knee ultrasound (US) is a promising technique able to provide direct information on soft tissue, and can reliably detect SH and SE in painful KOA [190, 191]. Sonographic changes in the suprapatellar recess have been found to be positively associated with knee pain in patients with equal radiographic grades of OA [57]. In addition, suprapatellar inflammation has been found in patients with painful radiographic KOA (RKOA), but not in a group with asymptomatic RKOA or healthy controls [193]. Other studies have shown that the presence of suprapatellar pouch effusion is

associated with the high risk of pain [230]. In the context of acute knee trauma, sonographic assessment has been utilised to detect lipochromatosis, tendon injuries and to some degree meniscal and osteochondral lesions [255, 256].

The aims of this chapter are:

1. To evaluate the reproducibility of a modified standardised sonographic assessment of the suprapatellar recess in participants without radiographic KOA but either unilateral injured knees or post-surgical knees (post-surgery on ligaments, cartilage and/or tendons)
2. To compare sonographic findings between symptomatic (injured or post-surgical knees) with contralateral knee joints.

3.1 Ultrasound Protocol of the Suprapatellar Recess

3.1.1 Limitations of Existing Methods

Previous studies have shown that US can identify patterns of synovial inflammation in the suprapatellar recess [257, 258]. Sonography of the suprapatellar recess is a potentially attractive alternative method to assess effusion-synovitis in populations at risk of KOA and also help with the identification of pre-clinical and pre-radiographic KOA. However, there are some major challenges associated with this method: sonography is an operator-dependent technique and,

additionally, the physical properties of sound limit US's ability to assess the deeper structures of the joint.

There is still no widely agreed standardised definition of sonographic findings or acquisition protocols for KOA or post-traumatic knee sonographic screening. Following a review of published methodology, reports by the European League Against Rheumatism (EULAR) and the Outcome Measures in Rheumatology group (OMERACT), a new practical protocol based on selected published definitions and methods was produced and tested for this thesis.

OMERACT define synovial hypertrophy (SH) as "an abnormal hypoechoic (relative to subdermal fat, but sometimes may be isoechoic or hyperechoic) intraarticular tissue that is non-displaceable and poorly compressible and which may exhibit Doppler signal" [146, 259]. Although this definition explicitly refers to rheumatoid arthritis, it has also been applied to OA, as the differences in synovial inflammation between the two diseases are largely quantitative [260].

The position of the knee and muscle tone during sonographic knee examination has been debated in recent years [261, 262]. The position of the knee and probe affects evaluation outcomes (26). EULAR's Standing Committee for International Clinical Studies Including Therapeutics Trials report on the use of ultrasonography in painful KOA recommended to use the probe over the suprapatellar recess for image acquisition with the knee in semi-flexed position in 45 degrees [190].

The detection of SH is potentially more sensitive and specific for detecting synovitis than the detection of SE. Theoretically, intra-articular pressure associated with muscle activation could affect the volume of synovium. However, based on SH definition, a mild increase of the synovial fluid pressure through quadriceps contraction should not decrease SH thickness substantially. Our initial findings agreed with published methods suggesting that SE depth in the suprapatellar recess seems to be significantly increased during quadriceps muscle contraction in the neutral position when compared with a measurement of SE in the same knee with 30 degrees' flexion and relaxed quadriceps. The movement of the synovial fluid into the suprapatellar pouch is associated with better differentiation of synovial tissue from surrounding fat tissue in the suprapatellar recess.

The reported inter-rater reliability of sonographic SH measurements utilising semi-quantitative scores for SH in the knees have been suboptimal[146]. Semi-quantitative scores have been often based on quantitative measure of hypertrophy/effusion depth in millimetres by utilising different cut-off points [57, 232, 258, 263, 264]. The quantitative measurements in mm have been used more often recently as well [231, 265-267].

Table 3.1. The metric methods for classifying and quantifying synovial hypertrophy with greyscale US used in previous research.

Publication	Synovial grade (if applicable)				
	0	1	2	3	4
Rubaltelli et al. (1994)	absent	< 5mm	5-7 mm	≥ 8mm	N/A
Walther et al. (2001)	N/A	No SE/SH	Mild SE/SH	Moderate SE/SH	Marked SE/SH
Szkudlarek et al. (2003)	No changes	Mild SH	Moderate SH	Large SH	N/A
D'Agostino et al. (2005)	< 4mm	≥ 4mm	N/A	N/A	N/A
Wu et al. (2012)	< 4mm	≥ 4mm	N/A	N/A	N/A
Hall et al. (2014)	< 4mm	≥ 4mm	N/A	N/A	N/A
Bevers et al. (2014; 2015	<2mm	≥2mm	N/A	N/A	N/A
Abbasi et al. (2016)	< 4mm	≥ 4mm	N/A	N/A	N/A
Abou-Raya et al. (2014)	Measured in millimetres				
Chan et al. (2014)	Measured in millimetres				
Riecke et al. (2014)	Measured in millimetres				
Keen et al. (2015)	Measured in millimetres in the longitudinal and transverse planes and then calculating the mean of the two				

Measurement in millimetres has an advantage of producing a continuous variable. To improve reproducibility, analysis of the obtained sonographic pictures should be standardised and preferably based on fixed non-compressible anatomical structures that can be used to orientate the measurement axis. This has been successfully implemented in the measurement of meniscal extrusion, where a perpendicular straight line from the outermost edge of the medial meniscus to a line connecting the femoral and tibial cortices has been used [268] and resulted in high reproducibility. However, arbitrary cut-off points are not necessarily universal for KOA and post-traumatic knees. To address this issue, analysis of the distribution of SE and SH in the injured and non-affected knees is needed.

The advantage of testing this protocol on a population where the majority of participants have unilateral knee changes has allowed us to put into practise (or utilise) the current understanding of human visual recognition patterns. Humans are much quicker to identify objects that appear bigger than expected based on their previous experience [269, 270]. For that reason, this protocol starts by scanning the unaffected knee first, as our hypothesis was this reinforces a perception of normal sonographic anatomy of the suprapatellar pouch and allow faster identification of abnormal structures in the affected knee. This could potentially create a context bias as results could be classified as abnormal or normal based on the scanned side[271]. This was minimised by using quantitative measurements.

3.1.2 Hypothesis

A standardised US protocol of the suprapatellar pouch is a reproducible method for identifying post-traumatic synovial hypertrophy and effusion.

3.1.3 Protocol

Two main aims of this protocol are to identify the following, using greyscale US Scanning technique (GSUS):

1. The largest area of synovial hypertrophy within the suprapatellar recess (SR).

2. The maximal suprapatellar knee effusion depth within the SR.

Aspects discussed in the background of this section have informed the development of this protocol.

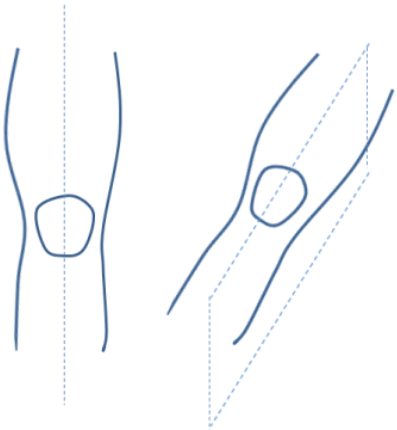
1. Participants are examined in the supine position.

2. Both knees are examined starting with the one not injured or painful (if available), or less painful if both knees are affected. This is to reinforce the perception of normal sonographic anatomy for the participant before examining the affected knee.

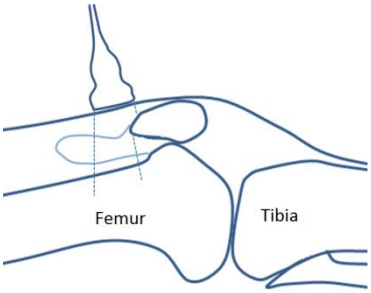
3. Advised position of the knee: neutral position (0°), with quadriceps contraction, or as close to neutral position as possible, with quadriceps contraction. Contraction and extension should be guided by the patient's symptoms and the patient should stay within the comfort zone.

4. EULAR/OMERACT definitions only of SH and SE have been utilised for the purpose of this study.

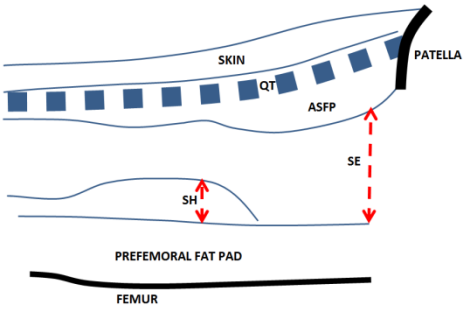
5. The examiner identifies the sagittal plane by scanning across the mid-patella while the participant is resting on a bench with active extension of the knee. The mid-patella line was based on the direction of a palpable string from the quadriceps tendon at attachment to the superior border of the patella, occurring during a change of muscle tone from non-contracted to contracted.



6. Sagittal images are obtained in the midline while keeping the distal edge of the probe over the patella display the quadriceps tendon.

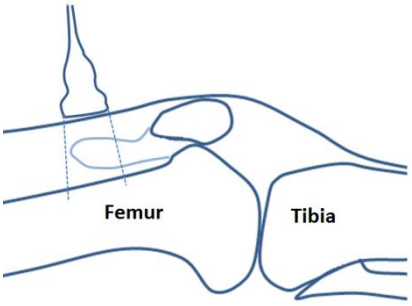


7. All the important structures of the SR are identified: patella, quadriceps tendon (QT), anterior suprapatellar fat pad (ASFP), SE, SH, pre-femoral fat pad and femur.

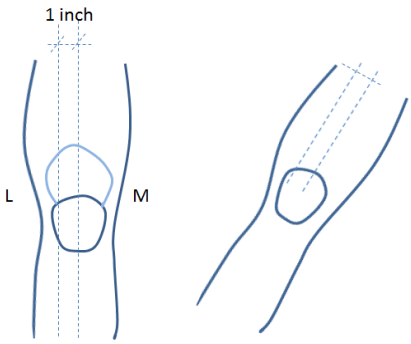


8. Examiners aim to identify and take pictures of the two sections in that position, with the largest synovial hypertrophy and/or the maximal suprapatellar knee effusion depth in that position. It is important that reflection of the ultrasound beam back from the femur is visible on each picture.

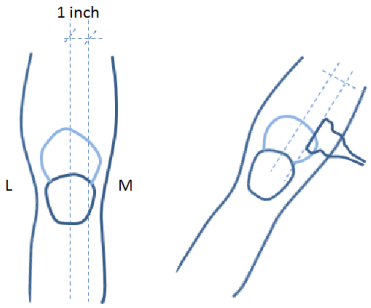
9. If the proximal aspect of the SR is not visualised, the examiner moves the probe longitudinally towards the hip joint, identifying any new aspects of the SR and takes pictures as described in point 8 until the proximal aspect of the suprapatellar recess aspect is reached. If the proximal aspect of the SR is visualised, the examiner should follow the protocol from point 10.



10. If the lateral border of the SR is not visualised, the examiner moves the probe at the longitudinal line above the patella around one inch laterally to a parallel longitudinal mid-patellar position and repeats steps 8 and 9 until the medial and proximal aspects of the SR have been assessed. If the lateral border of the SR is visualised, the examiner follow the protocol from point 11.

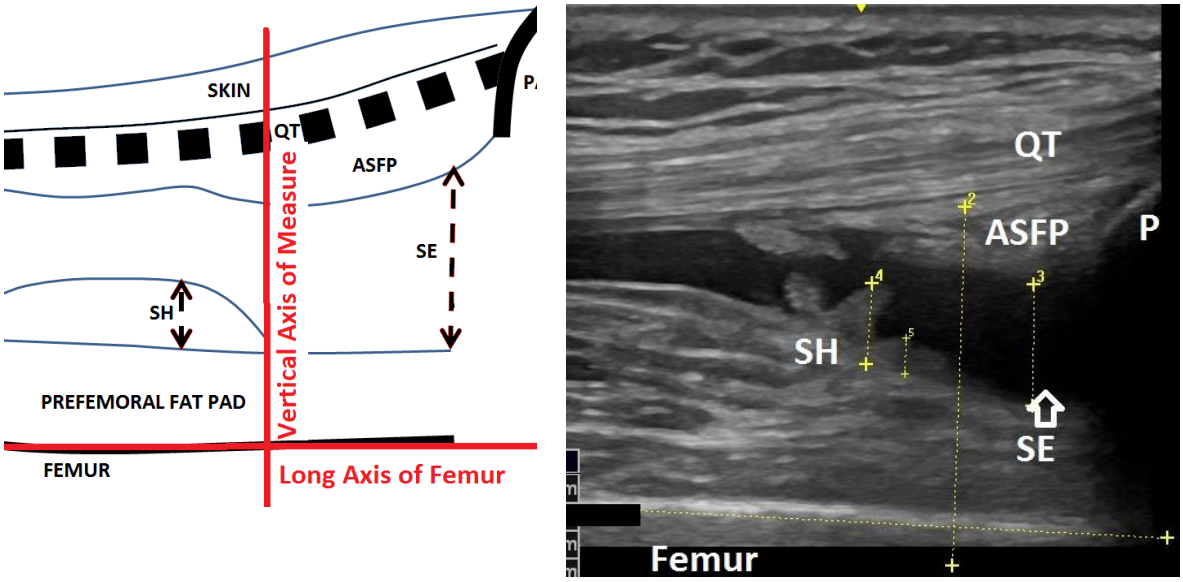


11. If the medial border of the suprapatellar recess is not visualised during steps 8 and 9, the examiner moves the probe at the longitudinal line above the patella around one inch medially to a parallel longitudinal mid-patellar line position and repeats steps 8 and 9 until the medial and proximal aspects of the SR have been assessed. If the medial border of the SR is visualised, the examiner follow the protocol from point 12.



Analysis of the sonographic pictures

12. All saved pictures are analysed straight after the examination.
13. A long line is drawn over the reflexion of the femur (long axis). A perpendicular straight line to the line based on the femur reflexion (long axis) is then used for defining the vertical axis for measurement of both synovium and effusion. All SH and SH measurement lines are parallel to this. The depth of the SE and SH is measured by the maximal anterior-posterior diameter of the suprapatellar recess.



14. All results are recorded on the results form (preferably online), with an example used in this study attached to the appendix.
15. The examiner is asked to confirm that all important anatomical structures have been identified on a long-axis plane of the suprapatellar region: quadriceps tendon, along with reflexion of the femur and the pre-femoral fat.

16. The largest thickness of synovial hypertrophy within the suprapatellar recess is recorded in millimetres and categorised, on the basis of the results, into one of the following four grades:
    - Grade 0 synovial tissue (< 4mm).
    - Grade 1 Hypertrophic synovial tissue (4mm to < 8 mm).
    - Grade 2 Hypertrophic synovial tissue (8mm to < 11 mm).
    - Grade 3 Hypertrophic synovial tissue (≥11mm).

Unless it is very easy to measure, any identified SH below 1mm is recorded as "1".

17. The maximal suprapatellar knee effusion depth within the suprapatellar recess is recorded in millimetres and categorised, on the basis of the results, into one of the four grades:
    - Grade 0 No effusion (< 4mm).
    - Grade 1 Mild effusion (4mm to < 8 mm).
    - Grade 2 Moderate effusion (8mm to < 11 mm).
    - Grade 3 Marked effusion (≥11mm).

3.1.4 Participants

To address the first aim of this chapter, 57 patients undertaking a structured knee rehabilitation programme were recruited from a single centre over a six-month period, for the separate study of molecular mechanisms and pathways of chronic inflammatory and degenerative diseases.

All participants gave written informed consent, and the study received positive review by the Riverside Research Ethics Committee (R REC number 07/H0706/81).

The exclusion criteria for this part of the study included: joint replacement, known inflammatory arthritis or known radiographic KOA, defined as a Kellgren and Lawrence grade of two or more in the affected knee.

The inclusion criteria were men or women over 18 years of age with unilaterally injured knees or post-surgical knee joint, and with no history of inflammatory joint disease and with radiographs with a K/L grade below two and/or an MRI report without evidence of osteophytes.

Table 3.2. Characteristics of individuals included in the study.

Total number of participants: 57 individuals					
Age groups	< 25	25-34	35-44	45-54	55-64
	25%	36%	16%	16%	7%
Gender	number		percentage		
Male	34		60%		
Female	20		35%		
Missing data	3		5%		
The duration of joint symptoms					
Below 1 month	6		11%		
1-6 months	10		18%		

7-12 months	11	19%
1-3 years	9	16%
4-6 years	3	5%
> 10 years	4	7%
Missing data	14	24%
Smoking status		
Non-smokers	34	60%
Ex-smokers	9	16%
Smokers	11	19%
Missing	3	5%

3.1.5 Equipment and Training

All sonographic examinations were performed utilising the commercially available GE Healthcare LOGIQ e US machine with the GE 12L Linear Transducer US Probe (3.7 – 11.3 MHz) with a 37.4 mm field of view. This is a probe with a flat head that produces sound waves in a straight line. It has a 42 x 10mm footprint and the width of the transducer grip of the transducer head is around 25 mm. The protocol was initially tested on four healthy volunteers and then later on three volunteers with recent knee injuries. All the results were then compared and discussed during meetings with Dr Eugene McNally, FRCPI, FRCR Consultant Radiologist specialising in musculoskeletal radiology, and Dr Thamindu Wedatilake (TW), a Sport and Exercise Medicine Physician and tutor

at the postgraduate musculoskeletal ultrasound courses at Bournemouth University. Each reader independently performed and scored at least 10 Suprapatellar Recesses while keeping a detailed record of scores, pictures and more importantly specific findings that were found to be problematic. All the results were then compared and discussed during a training session. Three one-hour training sessions were conducted by Dr Thamindu Wedatilake.

3.1.6 Blinding

The patients were instructed not to communicate any clinical information about their knee injuries or operations to the examiners. Examiners were not allowed to discuss any aspects of the results before recording them on the system.

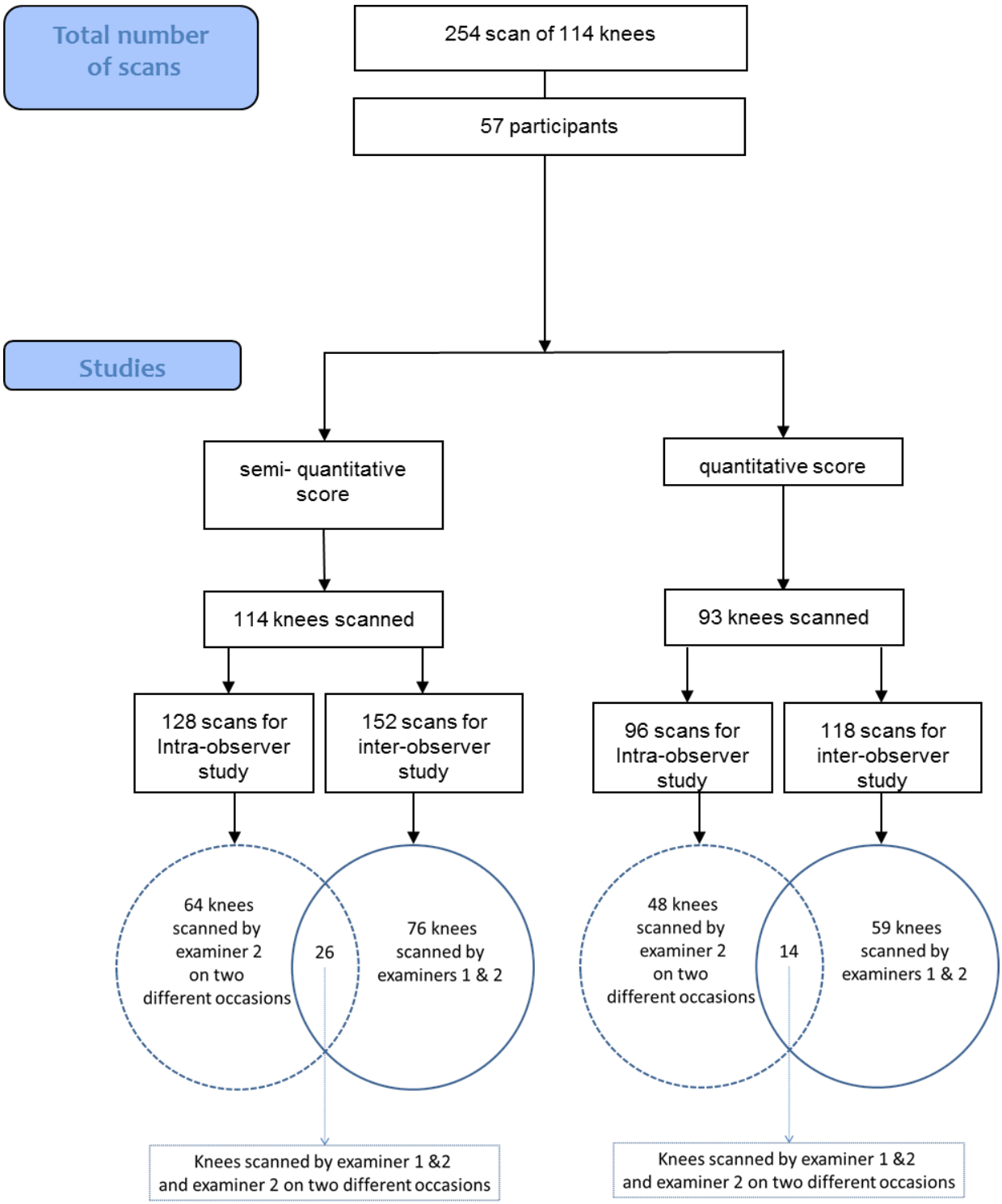
For the intra-rater reproducibility study, the groups with a minimum of two patients (four knees) were seen by the second examiner on two different occasions, 72hrs apart, to minimise the risk of bias associated with familiarity with the subject. Seventy two hours was decided as it was the most likely suitable time for the examiner not to recall findings from the previous session and also minimised the chance of significant change of the SH and SE within the short timeframe.

3.1.7 Reproducibility

Once the protocol was agreed and training was complete, two researchers, examiner 1 (TW) examiner 2 (SK) performed and scored 254 scans of 114 knees in 57 participants.

Scans of 93 knees had both results recorded as semi-quantitative scores and the measured value. Detailed information describing how the knee scans' results were utilised for both semi- quantitative and quantitative score reproducibility studies is presented in Figure 4.1.

Figure 4.1. The knee scans' results utilisation for both semi- quantitative and quantitative reproducibility studies.



3.1.8 Methods for reproducibility studies

Participants had US scans of their knees on one occasion performed by each of the examiners and again within a maximum 72 hour time interval by either one or two examiners. To minimise the risk of bias, the examiner who performed the US examination was unaware of the clinical results before performing the examination. The examiner recorded results of the US examination in writing or through the online RedCAP platform.

Each participant had a) US assessments of both knees performed on the same day by two examiners trained in performing musculoskeletal US and/or b) two US assessments performed by the same physician three days apart. Data was collected on the first 16 participants through paper forms that were recorded, collected and sealed in separate envelopes. The same form was then reproduced on the REDCap platform and all results directly collected by each examiner through filling in the online form after each examination. RedCAP platform and data entry are discussed in the 1st section of chapter 2. To avoid any bias associated with data entry by the examiners, Mr Richard Smith, a Research Fellow at the NDORMS, who was not involved in the project design or data collection, entered data from the first 16 participants into the RedCAP platform. Out of these, five forms were randomly selected, printed out from the RedCAP database and then compared with the original forms. No errors associated with data entry were found.

3.1.9 Statistical Methods

The reproducibility of continuous variables was tested using interclass correlations (ICCs) and 95% confidence intervals using the type of ICC based on the decision tree described by Muller and colleagues[272]. The variables were maximum depth of the suprapatellar effusion (SE) and maximum suprapatellar hypertrophy (SH).

For this reliability study the required number of scans was 42.

This was based on the formula published by Bonett et al [273] and the fact that in the pilot study 75% of affected knee scans can identify SH and SE.

Formula for sample size calculation is:

$$n = 8z_{\alpha/2}^2 \left\{(1-\tilde{p}_I)^2(1+(k-1)\tilde{p}_I)^2\right\} / \left\{k(k-1)w^2\right\} + 1$$

Where:

α = significance level = 0.05

K = number of raters = 2

β = Power = 80% = 0.2

$\tilde{p}_I$ = ICC = 0.85

W = width of confidence interval = 0.17

Therefore sample size is:

$= 8(1.96^2)[(1 - 0.85)^2(1 + (2 - 1)0.85)^2] / [2(2 - 1)0.1.7^2] + 1 = 41.6$

For 42 participants estimated ICC = 0.85 95%CI (0.76-0.93)

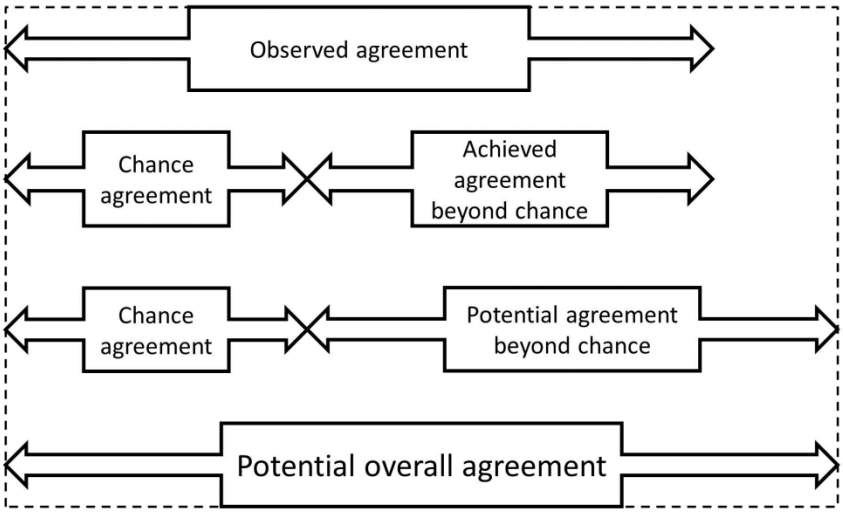
For 50 participants estimated ICC = 0.85 95%CI (0.77-0.93)

The difference between within-subjects scores cannot be measured by comparing average values from each reader. The first recommended step is to examine data by plotting results of continuous values against each other. In a 1986 issue of The Lancet, Altman and Bland advocated the use of a graphical method to plot the difference results of two measurements against the mean for each subject. In normally distributed data we would expect most of the differences to lie between – 2 and + 2 standard deviation of the differences. Those are often referred to as the "limits of agreement".

Kappa was used to measure agreement between semi- quantitative SH and SE grades. Kappa indicates the proportion of agreement beyond that expected by simple chance of agreement.

$$\text{k}= \frac{\text{observed agreement}-\text{chance agreement}}{1-chance\ agreement}$$

Figure 4.2. Schematic representation of observed agreement to potential overall and chance agreements. Adapted from Rigby[274].



Weighted kappa reflects better the degree of disagreement for ordinal data, by using methods that attach greater emphasis to large differences between ratings[275].

A standard interpretation of kappas has been based on the description by Landis and Koch (1977).

Standard of strength of agreement for the kappa		
Kappa range	Landis and Koch[276]	Landis scale adapted by Douglas Altman[277]
0	Poor	Poor
0.01-0.2	Slight	
0.21-0.4	Fair	Fair
0.41-0.6	Moderate	Moderate
0.61-0.8	Substantial	Good
0.81-1	Almost perfect	Very Good

Factors that can influence the magnitude of kappa include [278]:

1. The low or high prevalence of the attribute, which can directly affect chance agreement.
    - To address this separate sensitivity within this study, analyses were performed to assess reproducibility of higher (less common) grades.

2. The degree to which the raters agree on the proportion of cases, often described as the 'bias index'.

3. The non-independence of ratings. This requires the subject of the investigation to be independent (i.e. each knee can contribute only one pair of ratings) and for each observer to generate a rating without knowledge of the other observers' ratings.
    - To address this, examiners in this study were not aware of either the clinical details, each was blinded to the assessment of the other.

3.1.10 Results of the reliability study

3.1.10.1 Inter-observer reliability for the synovial effusion an hypertrophy

Seventy-six knees were examined for the inter-rater reliability study of SE and SH. The results for all 76 scans were available for SE reproducibility. However, only 75 scans were utilised for SH reproducibility, due to missing data in one SH-E form.

Table 3.3. Summary and cross tabulation of results for SE and SH semi-quantitative scores.

	Second examiner (SK)				
First Examiner (TW)	**SE scores**				Total
SE score	**0**	**1**	**2**	**3**	
0	38	0	1	0	39
1	4	15	0	0	19
2	0	0	7	0	7
3	1	0	2	8	11
Total	43	15	10	8	76

	Second examiner (SK)				
First Examiner (TW)	**SH scores**				Total
SH score	**0**	**1**	**2**	**3**	
0	50	2	0	0	52
1	0	17	0	0	17
2	0	2	0	0	2
3	0	0	1	3	4
Total	50	21	1	3	75

Agreement for both SH and SE were very good with weighted ƙ value of 0.90 and 0.95.

Table 3.4. Results of inter-reliability of SE and SF scores semi-quantitative scores.

	Weighted kappa	**Standard error**
SE	0.87	0.09
SH	0.85	0.09

These results were heavily influenced by the SE and SH normal (zero) grade that represented 56- 67% of the results.

When weighted ƙ were calculated for results with scores 1-3 only, the agreement was good with weighted ƙ values from 0.76 for SE and 0.78 for SH. As expected the standard error increased at the same time to 0.11 and 0.18.

3.1.10.2 Intra-observer reliability for the synovial effusion an hypertrophy

Sixty-four knees were examined for the intra-rater reproducibility study of semi-quantitative scoring of SE and SH. Results for where there was examination of a single knee were not included in the analyses due to missing data.

Table 3.5. Summary and cross tabulation of results for SE and SH semi-quantitative scores.

	Second Examination (SK)				
First examination (SK)	**SE scores**				Total
SE Score	**0**	**1**	**2**	**3**	
0	40	1	0	0	41
1	3	11	1	0	15
2	0	0	5	1	6
3	0	0	0	1	1
Total	43	12	6	2	63

	Second Examination (SK)				
First Examination (SK)	**SH scores**				Total
SH Score	**0**	**1**	**2**	**3**	
0	47	0	0	0	47
1	1	10	3	0	14
2	0	0	1	0	1
3	0	0	0	1	1
Total	48	10	4	1	63

Table 3.6. Results of intra-reter reliability of SE and SF scores semi-quantitative scores.

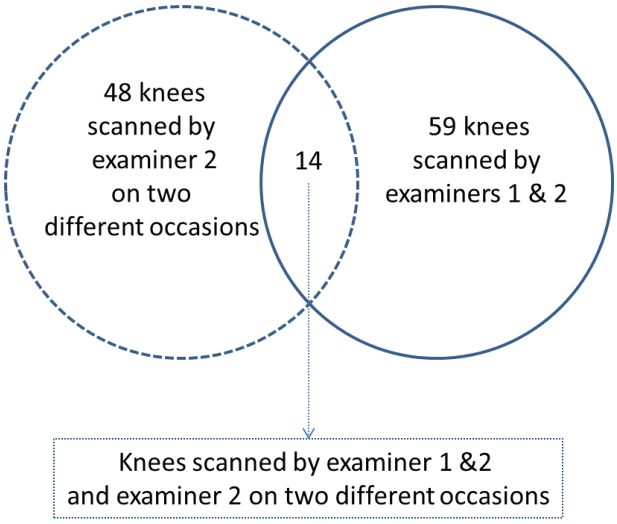
	Weighted kappa	**Standard error**
SE	0.77	0.1
SH	0.87	0.1

When weighted ƙ were calculated for results with scores 1-3 only, the agreement was good, with weighted ƙ values from 0.78 for SE and 0.61 for SH. As expected, the standard error increased at the same time to 0.21 and 0.19.

3.1.10.3 Evaluation of quantitative scoring system

Out of 254 scans performed for this study, 228 knee scans had both semi-quantitative and numeric values recorded.

Figure 4.3. Overlap between knee scans performed for intra-rater and inter-rater reliability studies of quantitative score.



3.1.10.4 Inter-observer for the synovial effusion an hypertrophy

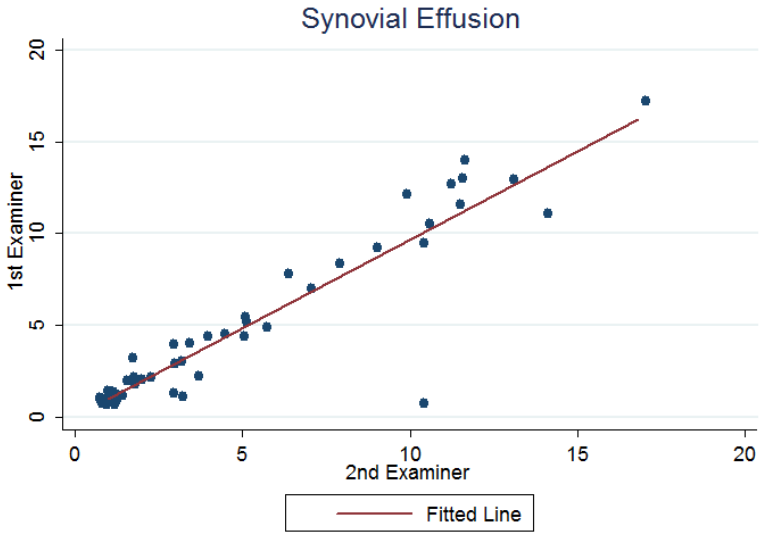
Published results of existing methods in the literature described ICCs for SE in the suprapatellar recess in KOA as 0.87and 0.94 [266, 267].

In this study, inter-rater reliability for both SE and SH were very good.

	ICC	95% CI
SE	0.91	0.87 – 0.84
SH	0.95	0.93 - 0.97

For visualization of the agreement, scatter and Bland-Altman plots were used.

Figure 4.4. Scatter plots of results for the synovial effusion and hypertrophy by two examiners.



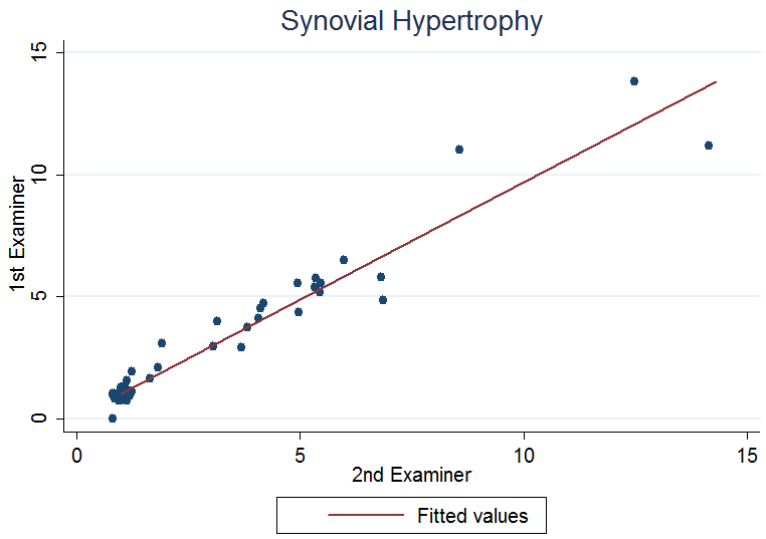
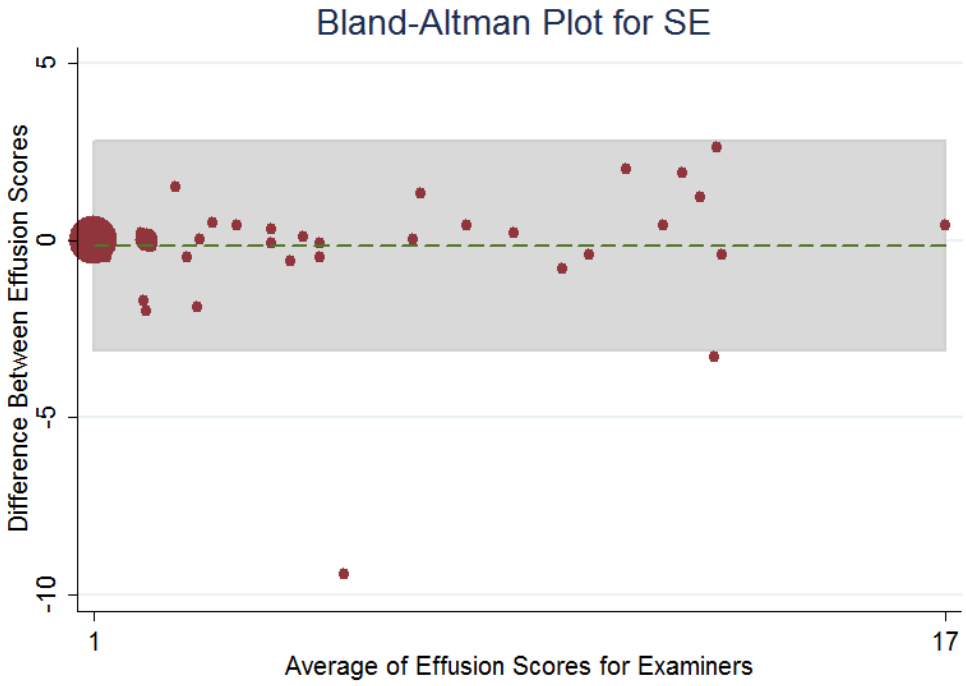
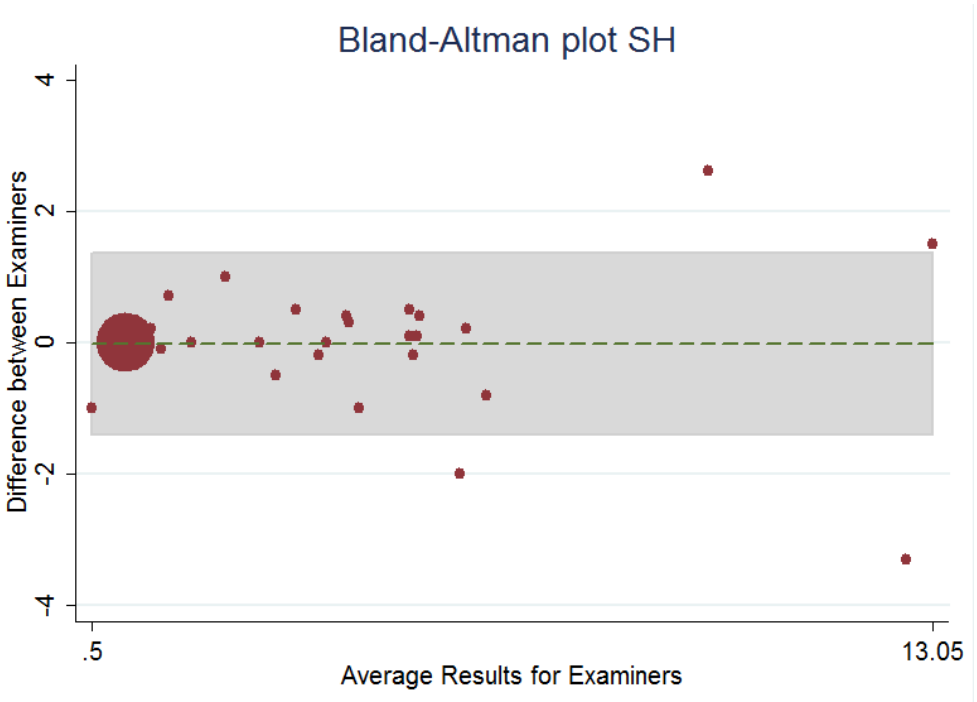


Figure 4.5. Bland-Altman plots of results for the synovial effusion and hypertrophy **by two examiners.**





The outliers for the results of SH and SE were investigated. Review of three out of four cases suggested that the differences were associated with the imaging acquisition from the lateral aspect of the SR. It seems that the lateral aspect of the SR was not well visualised and the largest SH and maximal SH depth present in this region were not measured by one of the examiners.

In summary, the results of the reproducibility study for quantitative methods showed very good intra-rater reliability. The few significant differences were most likely associated with acquisition rather than measurement problems, where large changes in the lateral compartment were missed by one of the examiners.

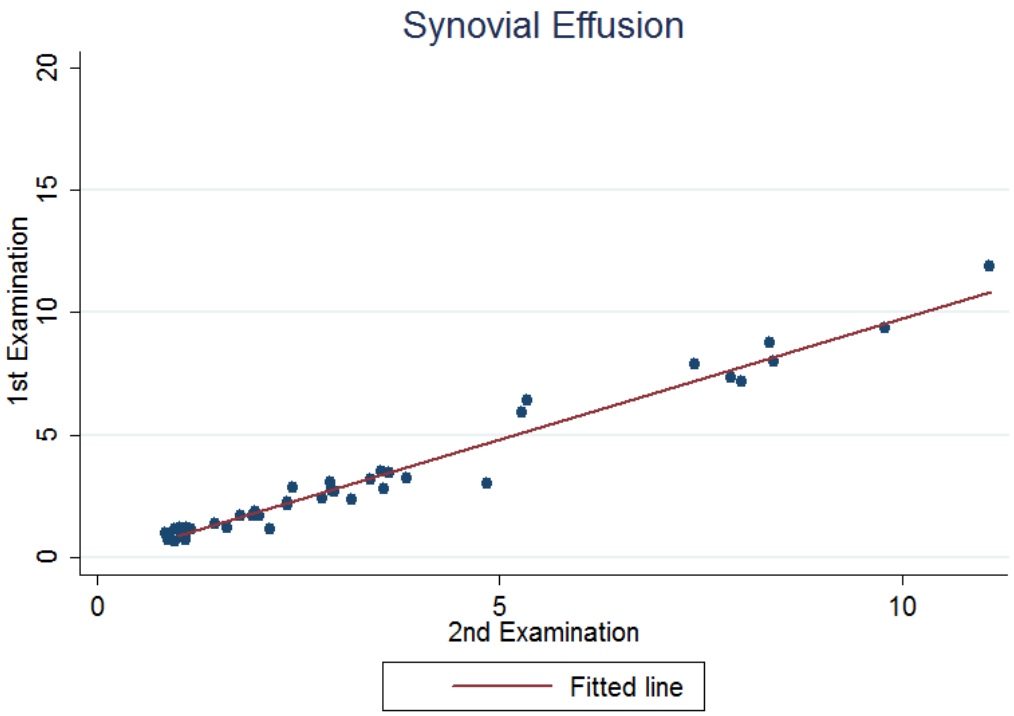
3.1.10.5 Intra-observer reliability for the synovial effusion an hypertrophy

Scans for intra-rater reliability, test re-test reliability study, were performed at a maximum of 72 hours apart.

In this study, the inter-rater reliability for both SE and SH was very good.

	ICC	95% CI
SE	0.98	0.98 – 0.99
SH	0.99	0.99 - 0.99

Figure 4.6. Scatter plots of results for the synovial effusion and hypertrophy by the same examiner.



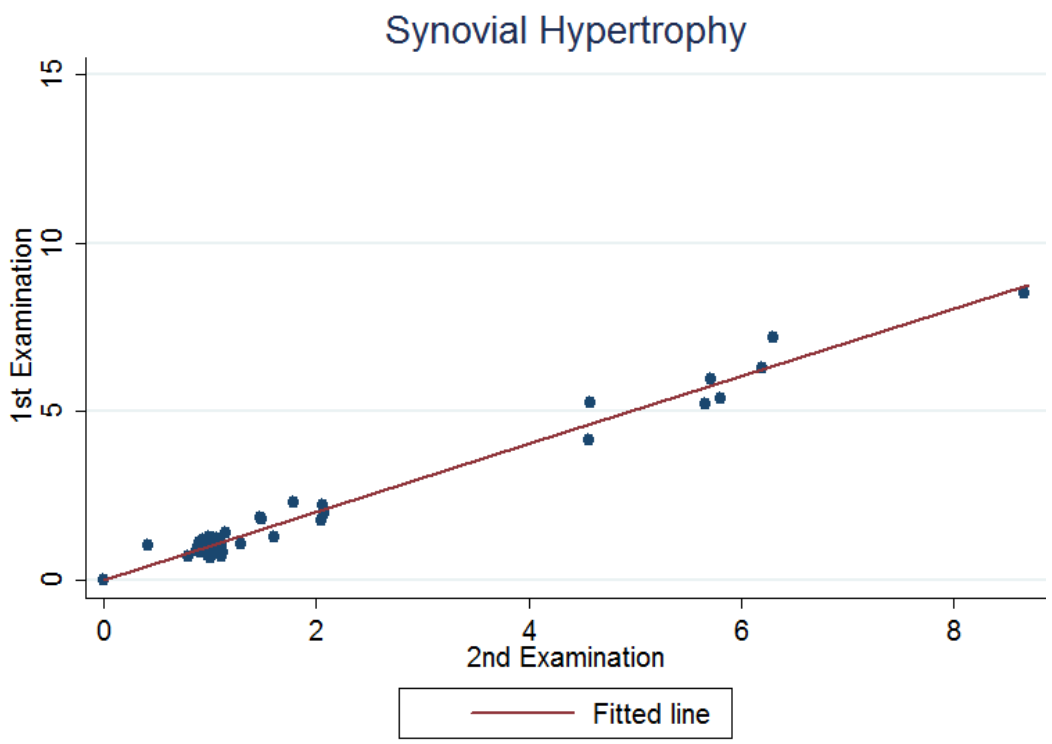
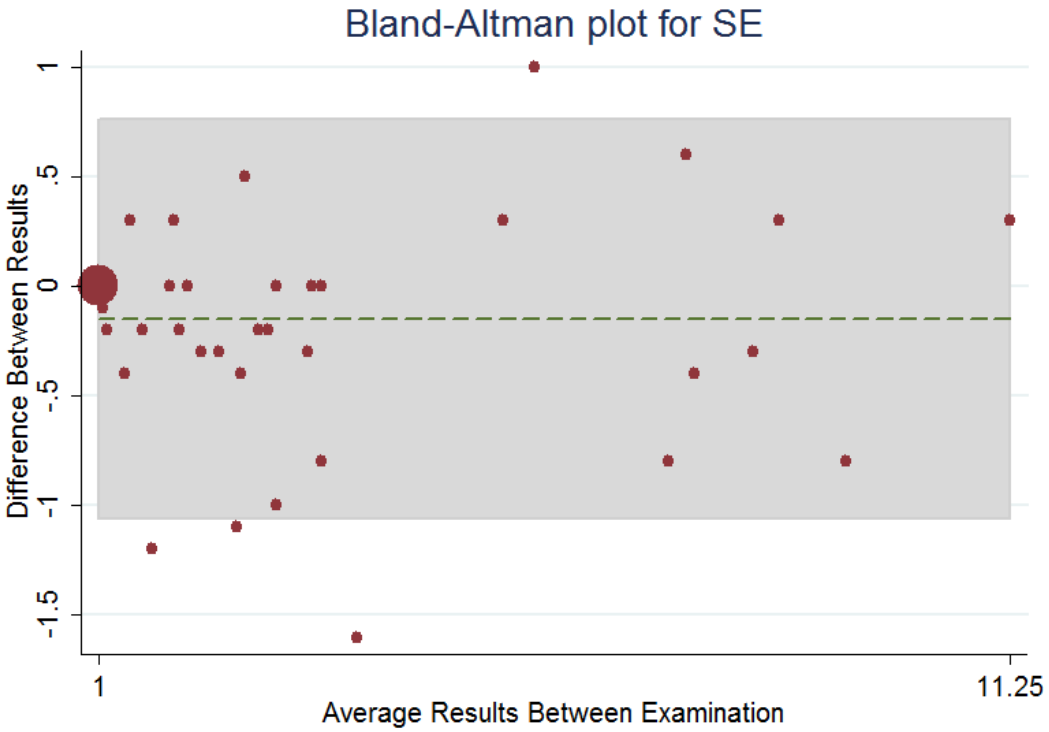
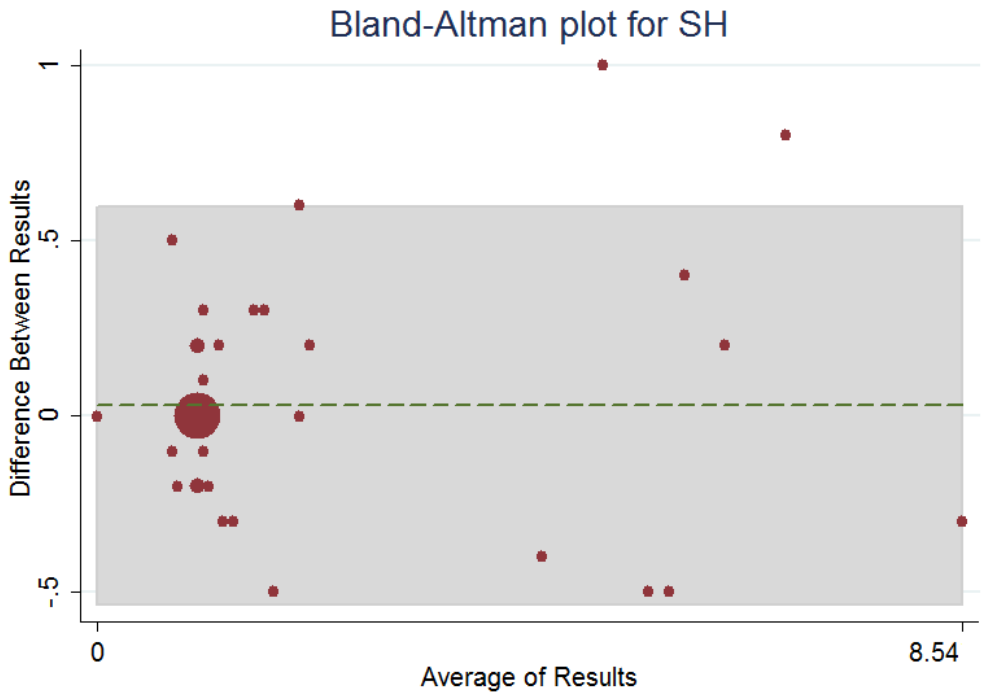


Figure 4.7. Bland-Altman plots of results for the synovial effusion and hypertrophy by the same examiner.





In summary, results of the reproducibility study for quantitative methods showed very good intra-rater reliability. Patients were assessed by the same examiner 72 hrs apart. Variation of the SE results in the intra-rater reproducibility study was higher than for SH. This could be explained by the increased likelihood for SE to change within three days when compared to SH, variability of SE reading with different voluntary quadriceps contraction, but also examiner and technique dependent bias. If this protocol would be used to monitor a change associated with treatment, rehabilitation or with time, controlling for intraarticular pressure would be required. This would require understanding of relationship between the intraarticular pressure, muscle contraction and how versatile is the synovial space in the knee overtime.

Despite the effort to ensure independence of examinations, there was a chance the results of intra-rater reproducibility could have been affected by potential

increased familiarity with the participants and their examined knees during the second exam. To address this, groups with a minimum of two patients (four knees) were seen on two different occasions by the second examiner.

3.1.11 Strengths, assumptions and potential limitations

The aim of this sonographic protocol is to identify the largest SH and the maximal suprapatellar SH depth within the SR. This protocol has been found to have both a 'very good' and 'good' intra-rater and inter-rater reproducibility for both semi-quantitative score and quantitative scores. The cut off values for SH for the semi-quantitative score were based on previous studies on KOA. Measurement differences could be associated with either the differences in terms of acquiring the images or measuring them. Review of the outliers in the semi-quantitative scores in this study have suggested that it is the acquisition rather than the analysis of obtained images that is responsible for these differences.

Looking back at the factors possibly associated with differences in acquisition, we found that those occurred on busy days, when three or four participants were scanned one after the other during the morning session. This might have created a pressure to obtain images quickly and lead to examiners missing the largest fragments in the SR. The optimal time of of acquiring the sonographic results from each participant to maximize the reproducibility of this method is unknown. The assumption was that this this would take around 10-15 minutes, based on the test scans. During the OxKIC recruitment, the duration of scanning

was measured during eight consecutive scans by an examiner. Results showed that scanning of both knees in the same participant took an experienced examiner between 15 and 22 minutes (with a mean figure of 17.5 minutes). The examiner was coordinating, and was therefore aware, that his scanning time was being measured.

This ultrasound technique was used to measure the largest area of synovial hypertrophy and the maximal suprapatellar knee effusion depth within the SR. The assumption was that SE depth and SH size would be closely associated with microscopic synovitis, which is considered a gold standard measurement for synovitis. Previously published MRI-based studies looking at the knee synovium and its predictive ability have been based on measuring the size of synovial tissue; their validity was constructed not only by comparing a volume score with microscopic synovial scoring, but also with the pain score in subjects with established KOA.

Most previous ultrasound studies looking at SE and SH in the SR used a cut of 4mm to describe abnormal SH in the SR. Again, those were based on subjects with established KOA, and there is no data for post-traumatic knees. Maximum size of normal SE or SH in the sagittal plane of the SR has not yet been investigated. There may be no correlation with either microscopic synovial inflammation or pain levels in subjects without KOA.

When compared to previously reported protocols, this method focuses solely on suprapatellar recess, has much more strict criteria of how images should be

obtained, and measured using the reflection of the ultrasound beams back from the femur, which is used as a reference point in each image. This increased substantially the reproducibility of his protocol. External validation of the findings assessing practicality and the reproducibility of this protocol is needed. Further correlation between sonographic findings and the macroscopic and microscopic features of synovial membrane inflammation in populations at risk of developing KOA will allow us to establish the diagnostic role of this protocol in the future.

3.2 Synovial hypertrophy and synovial effusion in the normal knee

There are no published studies looking at the validation of MRI or ultrasound based synovial scoring systems within the SR of post-traumatic non-arthritic knees or 'early' non-radiographic KOA.

In a study on painful KOA, D'Agostino and colleagues reported significantly increased synovial thickness in participants with effusion (≥4mm). For the whole group (n=600), the mean value of synovial thickness measured was 2.1 (SD 2.5) mm. The median (range) of SH in painful knees with effusion was over 2.9 (0-29) and, for comparison, 0.6 (0-10.2) for in the group with no effusion [190]. Chan et al. reported that SH in painful KOA was 4.36 (SD 2.21) mm [230]. The common (normal) value for SH in non-arthritic subjects with unilateral knee symptoms is not known. Mildly increased SH in other joints of asymptomatic individuals is quite common [279]. The prevalence of the knee SH in asymptomatic individuals is unknown, as is the cut off point for normal synovial hypertrophy levels in asymptomatic non-arthritic knees. Regarding painful knees, there have been

some reports suggesting knee pain, increased age and female gender were found to be predictive factors for high SH in a population based study [229].

In this section, we will look at SH in non-arthritic knees and compare values obtained from individuals with unilateral knee pain or injuries and/or after post-surgical procedures. Those individuals represent a group at high risk of developing KOA due to injury, abnormal mechanics but also potentially abnormal inflammatory response to mechanical insult.

The final analysis will test if SE or SH have independent association with affected knees independently to age and gender.

3.2.1 Study design and participants

To compare sonographic findings between symptomatic (injured or post-surgical knees) with contralateral non-affected knee joints, data from two studies was combined.

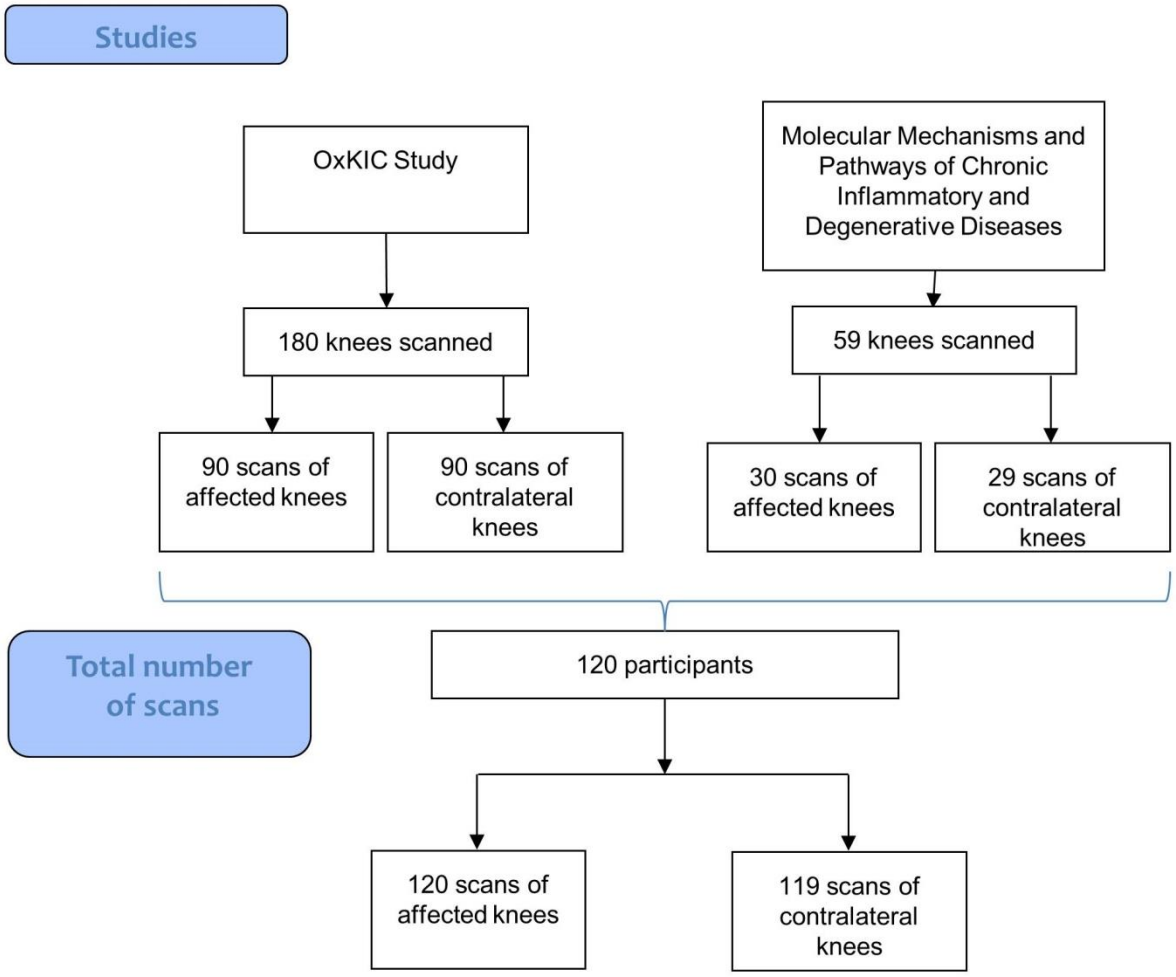
Non-affected knees from the study of Molecular Mechanisms and Pathways of Chronic Inflammatory and Degenerative Diseases, along with the OxKIC study, were considered to be non-arthritic knees with kelgren and Lawrence score below 2 at baseline. Similarly, post-traumatic or post-surgical knees were considered to be affected/controlled knees with abnormal SH and SE.

The median values for affected and normal knees have been compared using available data from the reliability study and baseline data from the OxKIC study. The OxKIC study population is described in detail in Chapter 4.

Sonographic data on the largest amount of SH and the maximal SE depth within the SR in the affected and non-affected knees have been combined.

The results of 239 knee scans from 120 participants have been presented and analysed for this thesis. Only participants with a full dataset have been included: 59 scans from 30 participants of the reproducibility study and 180 baseline scans from 90 OxKIC study participants.

Figure 4.8. This figure shows how knee scan results from both studies were utilised for the analysis of potential cut-off values for normal SE and SH values in the SR.



3.2.2 Statistical methods

Descriptive statistics of the sonographic variables in the affected and non-affected knees, in addition the Wilcoxon matched-pairs signed-ranks tests (Wilcoxon

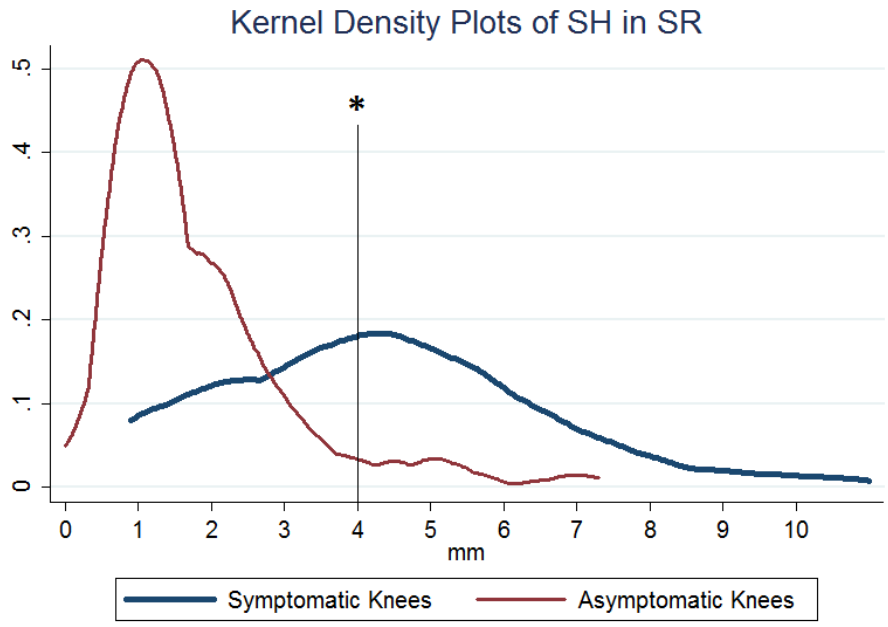
1945) were used to compare medians of SE and SH in affected and non-affected knees were applied.

To test association between the largest SH or maximal SE depth in the SR, and the affected or asymptomatic knees, logistic regression models adjusted for age and gender were applied.

3.2.3 Results

The SH score in the affected knees was normally distributed with a mean value of 4.25mm and SD of 2.1. SH and SE score datasets for asymptomatic knee, as well as SE for affected (symptomatic) knees, were positively skewed. There were no statistically significant associations between normal SH or SE values and age or gender in the examined groups.

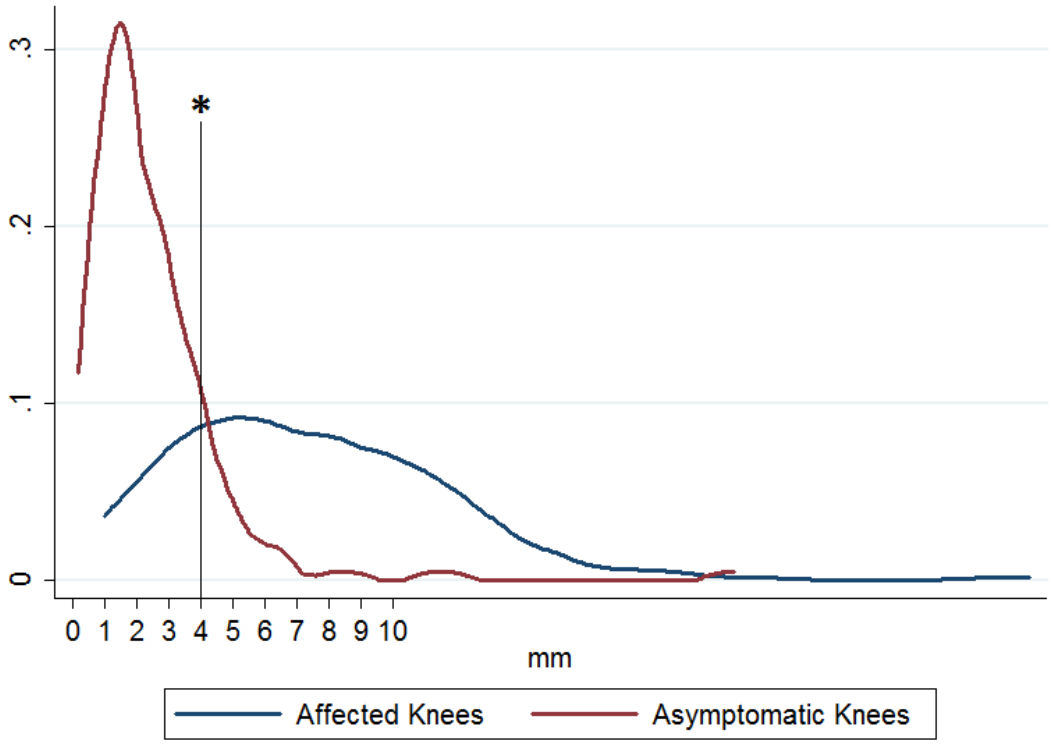
Figure 4.9. Kernel densities for synovial hypertrophy in the affected and asymptomatic knees. The asterisk marks current cut off point used for differentiating abnormal SH (synovitis) in the SR**.**



Ninety percent of the SH data from the non-affected knees was below or equal to 3mm, and 75% below 2.5mm (figure 4.9).

SE in affected and asymptomatic knees showed a similar distributions to SH and even better discriminative ability and smaller overlap of the values, results are presented in Figure 4.10.

Figure 4.10. Kernel densities for synovial effusion in the affected and asymptomatic knees. The asterisk and line below mark the current cut-off used for differentiating abnormal SE from normal values in the SR.



The 4mm cut-off point seems to discriminate well SE values from the non-affected and affected knees. However, further research comparing sensitivity and

specificity of those thresholds against a gold standard of inflammatory changes in the SR would be required to validate these findings.

As expected, the results of the Wilcoxon matched-pairs signed-ranks tests indicated that medians for both SE and SH in affected (symptomatic) and asymptomatic knees were statistically very different with the p value below <0.001.

The results of association between the largest SH or maximal SE depth in the SR and the affected or asymptomatic knees were statistically significant and independent to age and gender for both SE and SH. For SE odds ratio [OR] was 1.81 with 95% confidence interval [CI]: 1.55-2.13), and the association was stronger for SH with OR 2.40 (95% CI: 1.9-3.02) in an adjusted models.

3.2.4 Strengths, assumptions and potential limitations

This is the first study looking at the largest SH or maximal SH depth in the SR, measured as a continuous variable in non-affected, uninjured and non-painful knees and then comparing them with injured or post-operative non-arthritic knees.

This study indicated that injured or postoperative knees showed an abundance of large sonographic SH and SE lesions in the SR. There was a strong and independent association between the largest SH or maximal SH depth found in the injured or post-operative, non-arthritic knees.

The distribution of the sonographic SH data in the affected and asymptomatic knees suggests that the cut off point for abnormal SH semi- quantitative score in

the post-traumatic knees could be potentially lowered down to 3 or even 2.5mm. Assessing the sensitivity and specificity of new cut off points could lead to better differentiation of abnormal SH in non-arthritic knees.

Potential limitations are worth mentioning. The SH and SE score in the injured knees from the OxKIC study could be very different from the SH and/or SE results of individuals undertaking knee rehabilitation programme following knee injuries or after a knee operation. The OxKIC study participants at baseline were using mobility aids and were less mobile overall, thereby making them more likely to depend heavily on their normal, non-affected knees. This could have been more associated with the SR changes connected to the increased loading and more SH than it was within the population already going through intensive supervised knee rehabilitation programmes after knee operations or injuries. In addition to this, the OxKIC participants were recruited following recent knee injuries and, in many cases, this was associated with hemarthrosis and large effusions.

To assess the above, the analysis of the relationship between the largest SH or maximal SE depth in the SR of the normal and affected knees from both studies were performed, with results from each study population then compared to one another. The Wilcoxon matched-pairs signed-ranks test was then used to compare the median figures representing SE and SH in affected and non-affected knees from both studies. These tests indicated no statistically significant differences between the studied groups. Indeed, the normal knees had very similar values. Small differences were seen in the affected knees, with a mean SH

value of 3.8mm (SD 2.7 in the validation group, and 4.4 (SD 1.9) for the OxKIC Cohort, but those were not statistically significant.

The radiographic status of the non-affected knees was not assessed and it is possible that knees with radiographic KOA were included in the control/asymptomatic subgroup.

It is also likely that the largest SH or maximal SE depth in the SR of asymptomatic knees was increased, due to gait changes following an injury in the contralateral knee.

Our absolute minimal value for measuring SH was 0.9 mm and values below that were recorded as "1". This has affected the distribution of SH around the value of 1mm, especially in the non-affected knees.

3.3 Summary

The results in this chapter demonstrate that the reproducibility results of a modified standardised sonographic assessment of the SR in participants without radiographic KOA but either unilateral injured knees or post-surgical knees were either 'good' or 'very-good' when either semi-quantitative and quantitative scores were used. This technique is able to identify significant SE and SH differences in the affected knees when compared to contralateral non-affected knee joints. The cut-off value for normal SH in the SR could be potentially lowered down to 3mm.

In summary, this study has indicated that this standardised sonographic assessment of the SR is a reproducible technique and that the results of this ultrasound assessment of non-affected knees significantly differ from injured or post-operative knees in terms of magnitude of US inflammatory findings. Three and 4mm thresholds, for normal SH and SE in the SR retrospectively, seem to be very sensibly based on the distribution of those values in the injured and non-affected knees. Further studies, evaluating the potential SE and SH cut-off points that are able to indicate the well discriminated values associated with microscopic synovitis and normal synovium, are required to validate these current findings.

4. Microscopic synovial assessment

Microscopic assessment is considered the gold standard for analysis of synovial changes in classical inflammatory and 'degenerative' joint diseases. It is also used to rule out other pathologies associated with synovial hypertrophy such as amyloidosis, haemochromatosis or multicentric reticulohistiocytosis, to validate surrogate measures of synovitis and as an outcome measure in clinical trials [83, 280, 281].

Synovitis has been linked with pain and disease progression in KOA [72, 282]. When reporting on chronic synovial changes with radiographic KOA, Stoppiello et al. found that the grade of microscopic synovitis, rather than cartilage loss, was associated with symptoms [283].

Given the link between synovitis and pain, disease progression and disability in patients with KOA, synovial inflammation has now been implicated in the pathogenesis of OA[60, 284, 285]. In subjects without radiographic KOA, synovitis, especially in the suprapatellar pouch, seems to be a precursor of radiographic changes independently to other intra-articular lesions [8, 18]. It is unclear whether synovitis in the post-traumatic knee correlates well with knee pain and/or disability.

Microscopic assessment of KOA synovium has been based on samples taken from different joint locations, including peri-meniscal synovial tissue. Synovitis of the peri-meniscal samples is often associated with a chronic degeneration of

the meniscus. There are no published studies looking at suprapatellar synovial changes in post-traumatic knees.

Reproducibility is one of the main principles underpinning any scientific method and an important step in evaluating its quality. Many histological methods have been shown to have poor especially inter-rater reproducibility[286]. Two commonly used methods of assessing reproducibility are: 1) inter-rater agreement to evaluate reproducibility between graders, and 2) intra-rater agreement to assess the reproducibility of a result from single observer.

The propositions are that, firstly, synovial inflammation in the suprapatellar pouch, as defined by microscopic synovial scoring, is associated with knee pain in post-traumatic knees and, secondly, that similar microscopic changes are present in the suprapatellar synovium in end-stage KOA and post-traumatic knees.

Therefore, the aims of this chapter are to evaluate the following:

1. Reproducibility of the synovial scoring method.
2. Differences between synovial scoring of post-traumatic synovitis and synovial changes in end-stage KOA.
3. Association between synovial scoring and knee pain.

4.1 Reproducibility of synovial scoring method of suprapatellar synovium

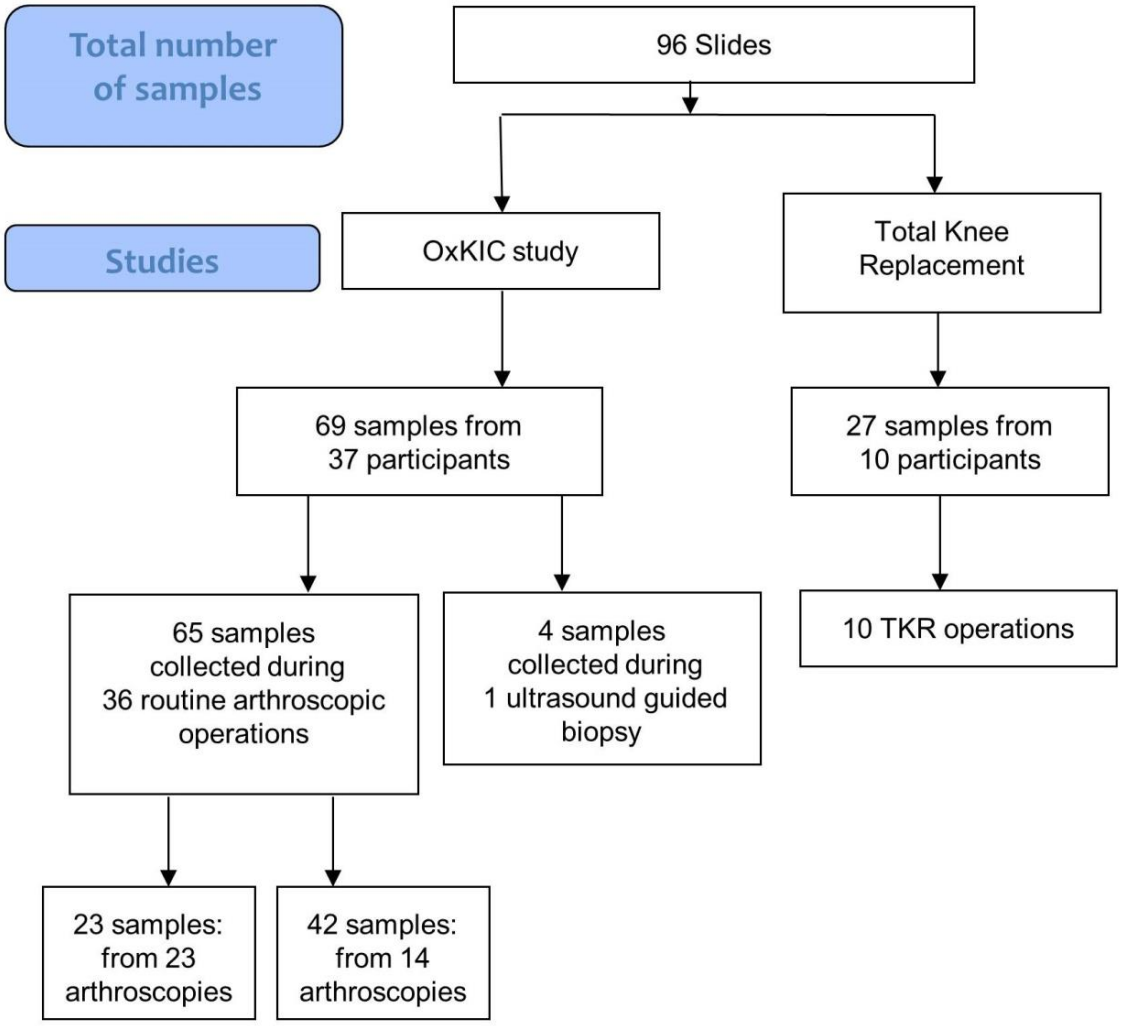
4.1.1 Material and methods

Synovial samples from the suprapatellar recess of 47 individuals were analysed. The synovial tissue samples were obtained from the suprapatellar pouches of:

- 37 patients with recent ACL or/medial meniscal injuries, normal radiographs and no history of inflammatory arthritis (post traumatic knees)
- 10 patients with KOA, undergoing total knee joint arthroplasty (end-stage KOA).

The first group consisted of consecutive patients from the OxKIC study who were listed for clinically indicated routine operations in the first eight months after starting OxKIC recruitment or ultrasound guided synovial biopsies during their six-month follow-up visits. The second group was recruited from a randomly selected ten patients, who were undergoing a routine TKR for end-stage KOA at the Nuffield orthopaedic Centre in Oxford and agreed to donate their synovial samples for research between March and June 2014 (HTA Licence 12217, Oxfordshire Research Ethics Committee C, 09/H0606/11)

Figure 5.1. This figure shows how the synovial samples were collected for microscopic evaluation in this thesis.



4.1.2 Oxford knee injury cohort (OxKIC)

The OxKIC procedures, including synovial biopsies, collection, storage and analysis protocols have received a favourable Research Ethics Committee opinion (REC: Wales REC 7; REC Reference: 14/WA/1108).

Either one or three suprapatellar synovial samples were collected from each knee, with rigid forceps, by the surgeon at the beginning of the routine

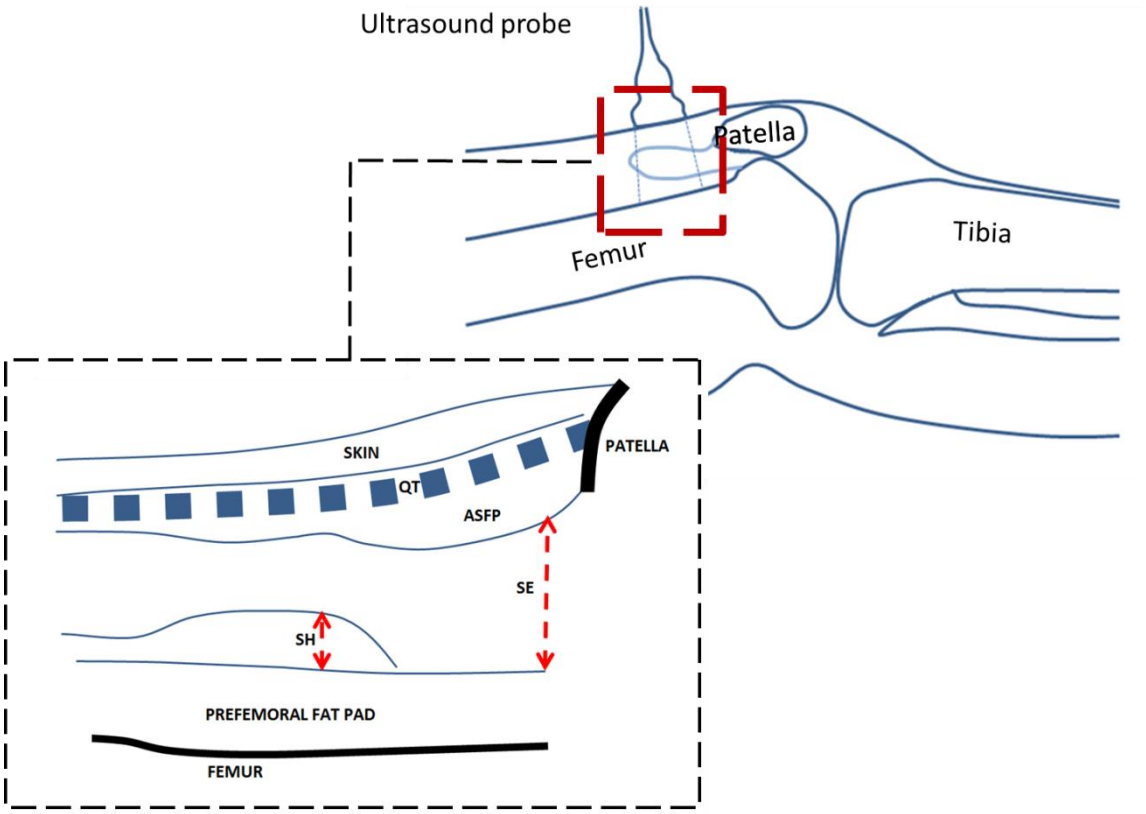
arthroscopic procedure. Each sample was fixed and embedded separately, then marked so that a record of the site of collection was available.

OxKIC participants who were not scheduled for a surgical procedure were asked for additional permission to collect samples of suprapatellar synovium during ultrasound-guided biopsy procedures at the six-month visit.

Sixty-five synovial samples from suprapatellar recesses were obtained during 36 routine arthroscopic procedures, for recent meniscal and/or ACL injuries. Four synovial samples were acquired during one ultrasound guided suprapatellar biopsy session and then later analysed.

Twenty-two out of 69 synovial samples from the OxKIC study were single intraoperative biopsies collected from the aspect of the suprapatellar pouch (ASP) identified to have the largest amount of synovial hypertrophy on the sonographic assessment performed just before the operation.

Figure 5.2. This shows a tibiofemoral joint in the sagittal plane with the ultrasound probe above the suprapatellar recess, which is marked with a light blue bold line.



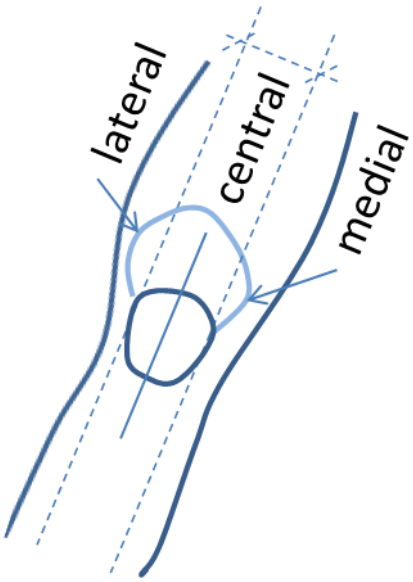
*The highlighted area illustrates the structures that can be identified on ultrasound: skin, shadows of the patella and femur, Quadriceps tendon (QT), Anterior superior femoral pad (ASFP), synovial effusion (SE), synovial hypertrophy (SH) and pre-femoral fat pad.

The sonographic protocol used to identify the region of the suprapatellar pouch with the largest amount of synovial hypertrophy, including the reliability study, is presented in chapter 4 of this thesis.

Surgeons performing the procedure were asked to collect synovial samples from either central, medial or lateral aspect of the suprapatellar pouch (ASP),

depending on sonographic findings. Those ASP regions were identified according to the position of the supero-medial and supero-lateral margins of the patella and image lines running vertically up the surface of the anterior thigh, parallel to the long patellar axis, passing through the superior-lateral or medial margin of the patella.

Figure 5.3. This shows a right knee joint from the anterior aspect. The light blue bold line represents the suprapatellar pouch position above the patella. The dashed lines represent divisions between the central medial and lateral aspects of the suprapatellar pouch (ASP). Those lines are based on the position of superior lateral or superior medial margins of the patella and parallel to the long patellar axis.



In 15 out of 37 synovial biopsies in the OXKIC study, three samples were taken from each suprapatellar pouch during each procedure. These were taken from different aspects of suprapatellar pouch(ASP): medial, lateral and central. The results of the ultrasound scan performed before the procedures were not disclosed to the surgeons performing the operation.

4.1.3 End stage knee osteoarthritis

End-stage KOA synovial samples were collected according to Oxford Musculoskeletal BioBank ethics (HTA Licence 12217, Oxfordshire Research Ethics Committee C, 09/H0606/11).

4.1.4 Methods

All samples were taken from consented consecutive patients undergoing routine total knee joint arthroplasties for end-stage KOA, on the days when Ms Eleanor Mawbey-Adamson, the tissue coordinator from the NDORMS biobank was available. She identified eligible patients, who were then informed and gave consent before the procedure. The surgeon performing the operation was also informed about tissue collection before the operation.

Following synovial tissue extraction, the surgeon handed over the tissue to Ms Mawbey-Adamson, who then fixed each sample in the neutral 10% formalin solution within 10-15 minutes. Ten large synovial samples from the SR were obtained and then processed by Dr Bryony Stott, Senior Research Histology Technician, in The Kennedy Institute of Rheumatology. This entailed coordinating the blocks preparation and the cutting and staining of the samples.

Due to size of the samples, each one was divided into two or three pieces before paraffin embedding. As a result, 27 paraffin blocks were sectioned for the final analysis.

4.1.4.1 Histology

All samples were fixed in 10% formalin at room temperature and embedded in Paraffin. Fixation preserves the micro-architecture of the tissue and supports high quality and reproducible staining. The Paraffin-embedded tissue samples were cut in 5 µm-thick sections on a microtome with a disposable blade and a water bath with temperature set at 37°C. All the blocks and slides were kept together. The Hematoxylin and eosin staining was performed using the standard method by an automated staining machine [244]. Preparation of histology slides and staining process are described in chapter 2 (section 2.1.1.2).

For the reproducibility study, 96 slides with two to three sections of histologic specimens from the same block were stained with hematoxylin and eosin.

Sections were assessed using a Zeiss Axio Imager-M1microscope (Carl Zeiss, Welwyn Garden City, UK).

All stained sections were examined independently by two observers (LW and SK), later referred as the first reader (LW) and the second reader (SK).

Each slide contained two to three sections from the same paraffin block. Each section on the slide was referred as a 'field' and assessed individually. The

highest score from any 'field' on the slide was considered the main result for the whole slide.

4.1.5 Blinding

All reference numbers to clinical details were printed at the top of each slide before staining of the slides. Before scoring of the synovial samples, the printed labels were covered with a non-transparent tape by the second reader. The slides' order was randomly changed by the first reader after the printed labels were covered. This was followed by marking of the covered slides with consecutive numbers 1 to 96 by the second reader, based on the new position in the slides' filing system. Those numbers were used as slide identification numbers for all reproducibility checks. Only after all reproducibility results were available, were masking tapes with new identifiers removed from each slide, with reference numbers to the clinical data read and input into the database. This allowed correlation of the scoring system with the TKR or OxKIC study numbers.

4.1.6 Characteristics of the participants

Ninety-six slides with synovial specimens were analysed and scored for this study. This involved assessment of 27 slides with synovial samples from 10 patients who underwent TKR and 69 slides with synovial samples from 37 OxKIC study participants.

The TKR group was on average 40 years older than the OxKIC study group. Gender distribution was similar, with 60 percent being male participants.

Eighty-four percent of participants from the OxKIC study group had ACL injuries, while 55 percent of them had associated meniscal injuries as well. Mean BMI for this group was 26.2 and 86 percent had high physical activity levels before the knee injury.

Table 4.1. Characteristics of individuals from total knee replacement (TKR) and knee injury (OxKIC) groups who had a synovial samples analysed.

	OxKIC (n=37)	**TKR** (n=10)
Age, mean (SD)	30 (8.7)	70.1 (10.9)
Gender		
Male, n (%)	66 (65)	6 (60)
Female, n (%)	35 (35)	4 (40)
BMI, mean (SD)	26.2 (5.3)	No data
Affected knee		
Right, n (%)	21(57)	5(50)
Left, n (%)	16(43)	5(50)
Injury		
ACL, n (%)	14 (37.8)	No data
ACL and meniscus, n (%)	17 (45.9)	No data
Meniscus/menisci tear	6 (16.2)	No data

Other		
Activity levels before the injury		
Tegner score >6 (competitive sports), n (%)	24(65)	No data
Tegner score >4 (competitive sport, recreational sport and heavy labour), n (%)	32(86)	No data
numeric rating scale (NRS) knee pain score at presentation, mean (SD)	3.5 (2.7)	No data

4.1.7 Histology scoring method

Many scoring systems for grading synovial inflammation have been developed over the years (table 4.2). These share two common domains:

1. Assessment of synovial lining cell hyperplasia.
2. Assessment of inflammatory infiltrate.

Table 4.2. Domains of existing synovial scoring methods.

Modified Goldenberg and Cohen score	FitzGerald score; method used by Loeuille and colleagues to validate MRI enhanced results[82, 83, 287]	Krenn et al.[288] scoring system adapted recently Lange-Brokaar and colleagues[289].	Haywood and Walsh scoring system [283, 290-292]
1. synovial lining cell hyperplasia (0-2); 2.villous hyperplasia (0-3), 3.cellular infiltration by (perivascular) mononuclear and poly-morphonuclear leukocytes (0-5).	1. the thickness of synovial lining layer, 2.subsynovial infiltration by lymphocytes and plasma cells, 3. Surface fibrin deposition, 4. Blood vessel vasodilation and blood vessel proliferation, 5. fibrosis, 6.perivascular edema.	1. lining cell layer 2. synovial stroma 3. inflammatory infiltrate.	1. synovial lining 2. inflammatory infiltrate 3. presence of the lymphoid aggregates.

A semi-quantitative Haywood and Walsh method of assessing synovium has been adopted for this study. This method has been used in the past to assess synovium in OA [290-292] and has been shown to have a good reliability [283].

All sections were initially assessed in low power fields (objective lens 2.5 and 10 folds). The pattern of the synovial lining proliferation and infiltration was defined in high power fields (objective lens 20-40 fold). Synovitis was graded from normal to severe. The additional score was defined if no synovial lining was identified in the section of tissue.

Table 4.3. Grades of microscopic synovitis based on the Haywood and Walsh scoring system.

GRADE	Criteria
Normal	synovial lining <4 cells thick, sparse cellular distribution, with few or no inflammatory cells.
Mild inflammation	synovial lining 4 or 5 cells thick, increased cellularity with some inflammatory cells.
Moderate inflammation	synovial lining 6 or 7 cells thick, dense cellularity with inflammatory cells, but not lymphoid aggregates.
Severe inflammation	synovial lining >7 cells thick, dense cellularity and inflammatory cell infiltration, may contain perivascular lymphoid aggregates.
No synovial lining	No synovial lining was identified in the field.

Samples displayed a range of synovial inflammation, from normal to severe, with lining hyperplasia, infiltration and lymphoid aggregates.

4.1.8 Training

The first reader, Dr Laura Wyatt from the University of Nottingham provided expertise in this method of synovial scoring. Dr Wyatt was trained in synovial grading methods by Dr David Walsh, a leading expert in synovial changes in knee osteoarthritis, and her reproducibility has been tested and published previously [283]. Dr Laura Wyatt kindly accepted an invitation to provide formal training of the second reader (SK) in this method and to test inter-rater reproducibility.

The second reader (SK), who was familiar with the microscopic features of synovitis, reviewed examples of each microscopic score from available published examples of those scores [290-292]. Ten slides were randomly selected from the pool of 86 slides for the reproducibility study and scored by the second reader, based on available examples without prior consultations with the first reader.

After the first reader scored the same 10 slides, results were compared and differences discussed. The percentage of agreement was used to test differences between the first and the second reader's results. The disagreements between scores were mainly between the normal and mild grade of synovitis.

4.1.9 Reproducibility results

For reproducibility analyses, the weighted kappa statistic with standard errors in addition to percentage agreement was used for synovial scores. Stata software version 12.1 was used for all statistical analyses and graphics generation.

Table 4.4. Distribution of the different synovitis scoring results in each sample of slides (n=96), for reproducibility study.

	Frequency	Percent
Normal	20	20.83
Mild	14	14.58
Moderate	42	43.75
Severe	13	13.54
No synovial lining	7	7.292
Total	96	

4.1.9.1 Intra-observer reproducibility

Intra-observer reliability of the first reader for histological scoring was checked beforehand and described as good, using the kappa statistics [283].

For the baseline scores, the first reader assessed 86 slides, while the second reader assessed 96 slides. Intra-rater observer reliabilities of the readers were assessed by rescoring a randomly selected 35 percent of baseline slides on two separate occasions, two days apart. On each occasion, a random string of numbers was

generated by an online random number generator [222] [223]. Intra-observer reliability for the second reader in the medium-term was measured for the second reader by assessing a randomly selected 30 percent of baseline slides six weeks after baseline assessment.

Samples with no synovial lining were all identified correctly by both readers on each occasion. Therefore, very strong intra-rater and inter-rater agreements were found for the slides with those samples. To check if the reproducibility study was affected by those samples, analyses were performed both with and without those readings for both readers.

In terms of rescoring of slides by the first reader, 85% were given the same grade, while this only applied to 79%of the rescored slides looked at by the second reader.

Table 4.5 demonstrates that the scoring method generally had very good reproducibility in the short- and medium-term, with kappa results ranging between 0.69 to 0.80. More experienced reader 1 (LW), had a higher intra-rater reproducibility compared with reader 2.

Medium-term readings showed a similar 'good to very good' level of intra-rater reproducibility, with weighted kappas ranging from 0.69 to 0.71.

Table 4.5. Intra-rater reproducibility of synovial scoring.

	Short-term Reproducibility			**Medium-term Reproducibility**		
	Agreement	**Kappa**	Std Error	Agreement	**Kappa**	Std Error
Reader 1	93%	0.80	0.11	Not assessed		
Reader 2	94.6%	0.77	0.11	93%	0.71	0.13

	After removing '**No synovial lining'** scores					
	Short-term Reproducibility			**Medium-term Reproducibility**		
	Agreement	**Kappa**	Std Error	Agreement	**Kappa**	Std Error
Reader 1	92.5%	0.75	0.12	Not assessed		
Reader 2	92.3%	0.69	0.13	91%	0.65	0.13

4.1.9.2 Inter-observer rater reproducibility

Inter-rater reproducibility between the first and the second reader was also very good (Table 4.6). All slides without the synovial lining (n=7) were scored the same by both readers. Eighty percent of the rest of the samples also had the same result. When only samples with synovial lining were scored, the reproducibility was still 'very good' (Table 4.6).

Table 4.6 Inter-observer reproducibility of synovial scoring.

Inter-rater reproducibility		
Agreement	**Kappa**	Std Error
94.50%	0.81	0.064

After removing '**No synovial lining'** scores		
Agreement	**Kappa**	Std Error
92%	0.76	0.072

4.1.10 Summary of the results

Good reproducibility is a key feature of research tool quality. Performed analyses showed a very good intra-rater and inter-rater reliability for the chosen microscopic synovial scoring method. The feature with the lowest kappa was the second reader's medium-term reproducibility, with a value of >0.65, which is still considered to be high.

Tissue without synovial lining is relatively easy to classify and all of these slides (n=7) were scored the same way by both readers each time. To avoid reproducibility results being affected by the results from this subgroup, weighted kappas were also analysed for the samples with synovial lining only. As expected this led to a mild decrease of kappa values.

4.2 Comparison of microscopic synovial grading between different aspects of the suprapatellar recess in the OxKIC study participants.

4.2.3 Introduction

Along with macroscopic synovial changes in the suprapatellar pouch in post-traumatic non-arthritic knees, ultrasound-detected grayscale synovial hypertrophy showed a patchy distribution of synovial hypertrophy, examples of arthroscopic images of SR from OxKIC study participants are presented in the appendix. It is unclear whether synovial biopsies from different aspects of the suprapatellar pouch (ASP) have similar microscopic synovial scores. This section of the thesis will analyse microscopic differences between biopsies taken from different ASP in post-traumatic non-arthritic knees.

4.2.4 Methods

Microscopic synovial scoring results from 45 slides of 15 participants from the OxKIC study were compared. Each participant had biopsies taken from the medial, lateral, and central ASP (Table 4.7). The surgeons performing the biopsies were unaware of the sonographic findings. Samples were usually taken in the following order: central, lateral and medial ASP.

The weighted kappa statistic with standard errors in addition to the percentage agreement was used to evaluate differences between semi-quantitative scores from a different ASP.

4.2.5 Results

A total 53% of individuals had the same microscopic synovial scores in all three aspects of suprapatellar pouch. Another 40% had a mild synovitis score in one or two ASP and normal synovium in the rest. In one case, synovium was scored as moderate in two ASP and indicated as a mild inflammation in other.

Table 4.7 Comparison between site of the biopsy and synovial scoring

Synovial score	Site of biopsy (ASP)			
	central	**Lateral**	**medial**	Total
Normal	5	4	6	15
Mild	3	3	2	8
Moderate	6	6	5	17
No synovial lining	1	2	2	5
Total	15	15	15	45

Table 4.8. Comparison between the site of the biopsy and synovial scoring in each participant. The last column shows the ASP identified as having the most marked synovial hypertrophy on ultrasound prior to biopsy. The bold boxes mark the highest synovial score in any of the ASPs of each knee.

	Microscopic Biopsy Score			ASP with the most marked Sonographic SH
Patient's id (different to the study number)	Central ASP	Lateral ASP	Medial ASP	
1	No lining	No lining	No lining	Lateral
2	Moderate	Moderate	Moderate	Lateral
3	Normal	Normal	Normal	Lateral
4	Moderate	Moderate	No lining	Lateral
5	Mild	Normal	Normal	Central
6	Moderate	Moderate	Moderate	Lateral
7	Normal	Mild	Normal	Central
8	Moderate	No lining	Mild	Central
9	Mild	Normal	Normal	Central
10	Normal	Mild	Mild	Lateral
11	Normal	Normal	Normal	Central
12	Moderate	Moderate	Moderate	Lateral
13	Moderate	Moderate	Moderate	Central
14	Mild	Moderate	Moderate	Lateral
15	Normal	Mild	Normal	Lateral
Total	n=15	n=15	n=15	n=15

In almost all cases (six out of seven) of biopsies from knees were there were differences between microscopic scores from different ASPs the highest microscopic synovial scores were present in the aspects of SR containing the most marked SH defined through the ultrasound scan by Dr Stefan Kluzek.

Microscopic synovial score agreements between compartments of the same knee were substantial, with kappa values between 0.60 and 0.75 (Table 4.9).

Table 4.9 Weighted kappa values between microscopic synovial samples from different aspects of suprapatellar recess.

Aspects	Agreement	**Kappa**	Std Error
Central vs Lateral	84.44%	0.60	0.1883
Central vs Medial	88.10%	0.68	0.2068
Lateral vs Medial	90.48%	0.75	0.195

Mild scores in one or more ASPs often coexisted with normal scores in the other ASP (33%). When all microscopic scores with mild inflammation were grouped together with normal synovium, 13 out of 15 patients had the same synovial scoring in all ASP.

4.2.6 Summary

The majority of the synovial samples showed the same synovial scoring in all three different ASP in the same knee of individuals with recent ACL and/or meniscal injuries and no background of KOA. Reliability of a single biopsy to

reflect microscopic synovial changes in other aspects of suprapatellar pouch is good or very good based on kappa statistics (kappa values from 0.60 to 0.75). Biopsies from ASP identified by sonographic assessment as containing the largest synovial hypertrophy are very likely to represent the highest microscopic synovial score in the knee, where differences exist. US-defined ASP with the most marked SH corresponded well with the highest histological synovial scores within the suprapatellar pouch. Based on those results, a single biopsy sample guided by US findings performed just before the operation would be justified.

Microscopically defined moderate synovitis seemed to be distributed around the suprapatellar pouch. This is based on the samples taken from different aspects of the suprapatellar pouch. It is likely that surgeons performing the procedure were aiming for the most inflamed patch in each ASP. Alternatively, equal argument might be presented where, due to constraints associated with the use of rigid forceps, it was more likely that the most convenient fragment in the lateral and medial aspects of the suprapatellar pouch were biopsied.

Mild and normal synovial scores often coexisted in the same knee. In up to 40% of examined knees, normal synovium in one or more ASP was accompanied by a mildly inflamed synovium in the other ASP. In all but one case, sonographic assessment was able to identify the ASP with highest inflammation when differences were found between different ASPs.

4.3 Comparison with end-stage KOA histological findings.

4.3.3 Introduction

There has previously been little knowledge regarding synovial inflammation of the SR in post-traumatic knees and how this compares to synovial changes in advanced KOA. In this section, we will compare synovial changes in the suprapatellar recess in non-arthritic post-traumatic knees with end-stage KOA.

4.3.4 Methods

Descriptive statistical analysis of the semi-quantitative synovial scoring was performed for each group to summarise and describe differences.

4.3.5 Results

The qualitative assessment of synovitis features was the same in both groups with significant hyperplasia of the lining cells and generalised inflammatory infiltrate. Unlike post-traumatic synovium, the majority of end-stage synovial samples showed a presence of lymphoid aggregates. Even with the similar appearance of lining or infiltration in the rest of the field, these were the biggest microscopic differences driving a higher score in those samples. As a result, advanced KOA cases displayed higher synovial scores in suprapatellar pouch synovium.

Table 5. Microscopic synovitis scores of the suprapatellar recesses synovial samples from the OxKIC and TKR participants.

Grade of synovitis	**OxKIC**	**TKR**
Severe	1	7
Moderate	21	2
Mild	9	0
Normal	5	1
Total	**n=36**	**n=10**
Mode for each study	Moderate	Severe

In summary, all features of synovial inflammation seen in end-stage KOA were found in non-arthritic knee injury samples from the suprapatellar pouch. Lymphoid aggregates in the perivascular tissue were rare in the post-traumatic knee synovitis group and common in end-stage KOA.

4.4 Correlation between knee pain and the severity of microscopic synovial inflammation following knee trauma

Little is known about the association between suprapatellar synovitis and knee pain in individuals with recent ACL and/or meniscal injuries. There are multiple

questions regarding knee pain, its frequency and link with specific activities. The KOOS questionnaire itself contains nine different questions regarding knee pain. The correlation between suprapatellar synovial hypertrophy on US assessment in this study suggested that VAS knee pain, frequency of the knee pain and knee pain on sitting might be associated with knee synovitis [229, 230]. Numeric rating knee pain scale (NRS) used in the OxKIC study correlates well VAS [293]. To avoid multiple testing, those three questions were selected for comparison with microscopic synovial scoring.

4.4.3 Study Design

This is a cross-sectional analyses of selected knee pain questions and microscopic synovial Inflammation in the OxKIC study.

4.4.4 Methods

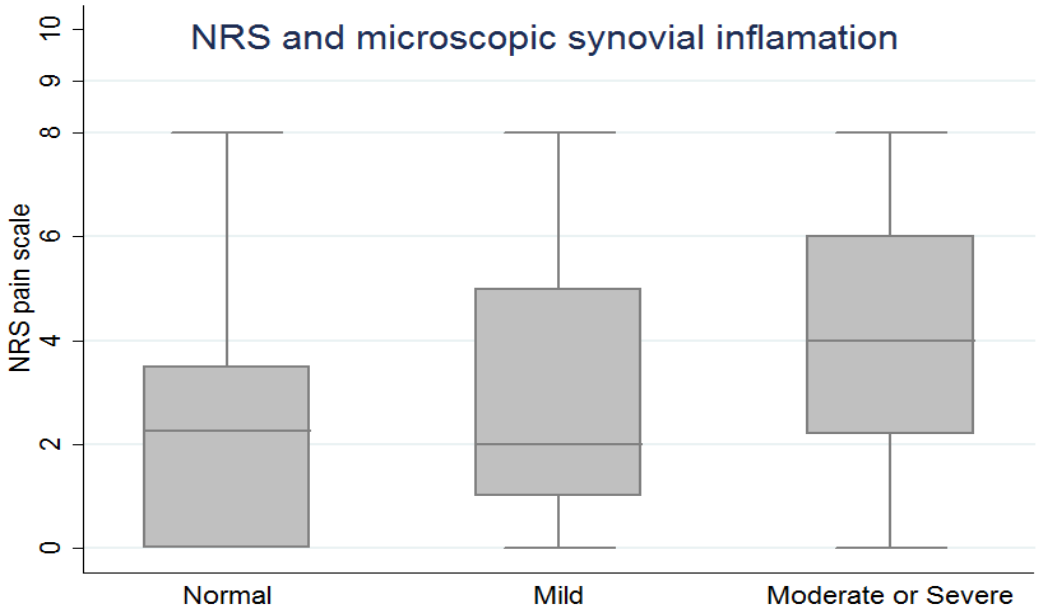
Data available from OXKIC study included assessments of microscopic synovitis, NRS average knee pain scores and an American-English version of the KOOS questionnaire including a pain domain which had questions on frequency of knee pain in the last week and pain on sitting or lying.

To examine the relationship between patients' NRS pain and synovial inflammation, logistic regression models were used. For association between KOOS pain and synovial inflammation, a chi square (X2) statistic was used to investigate whether distributions of categorical variables differ from one another. Only synovial samples with synovial lining were included in the analyses. Severe

inflammation was present in only one case and this was not included in this particular analysis.

4.4.5 Correlation with the NRS Pain Scale

Figure 5.4. This Box and Whisker diagram shows distribution of the NRS pain scale in each synovial inflammation category. Segments inside the rectangle show the medians of the NRS results for each category.



There was a significant overlap between pain levels in normal and mildly inflamed synovium. As presented in the previous section of this chapter, normal and mildly inflamed synovium is often found in different ASPs of the same knee. For these reasons, and to increase statistical power, results with normal and mildly inflamed synovium were merged together before analysis. Severe

synovitis (n=1) was merged with moderate synovitis (n=21) and defined as a positive outcome in the logistic regression.

Logistic regression was used to see if the NRS score is associated with the degree of synovial inflammation. The NRS score was an independent ordinal variable in this analysis and synovitis, defined as moderate or severe microscopic synovial score, was a dependent variable. As expected, results showed a positive association, with Odd Ratio of 1.4 and 95% Confidence interval from 1.04 to 1.89.

4.4.6 Correlation with KOOS pain scale

Frequency of knee pain and whether it occurred during sitting or lying has been previously associated with knee synovial inflammation.

The OxKIC participants were asked to mark a frequency of the knee pain from "Never" to "Always" using the Likert scale.

When the two categories were compared, the association between the frequency of knee pain and microscopic synovial inflammation was not statistically significant (p value = 0.243).

Table 5.1. Distribution of the different pain frequency scores, across microscopically defined synovial inflammatory groups.

Question: **"How often do you experience knee pain?"**				
	Microscopic Synovial Scoring			
Pain	**Normal**	**Mild**	**Moderate**	Total
Never	1	0	0	1
Monthly	0	2	2	4
Weekly	0	3	4	7
Daily	3	3	12	18
Always	1	1	3	5
Total	5	9	21	35

Participants were also asked prior to synovial biopsy about the amount of knee pain during sitting or lying activities. Similarly, no association was found between knee pain on sitting or lying, and microscopic synovial scoring (p-value = 0.259). Interestingly, people without or with mild synovial inflammation very rarely experienced moderate or severe pain (7%) during sitting or lying activities, while participants with moderate inflammation reported it frequently (48%).

Table 5.2. Distribution of pain on sitting or lying scores, across microscopically defined synovial inflammatory groups.

Question: "**What amount of knee pain have you experienced in the last week during sitting or lying**?"				
	Microscopic Synovial Scoring			
Pain	**Normal**	**Mild**	**Moderate**	Total
None	2	4	7	13
Mild	3	4	4	11
Moderate	0	1	7	8
Severe	0	0	3	3
Total	5	9	21	35

When values of 'none and mild' for the pain, and 'normal and mild' for the synovitis score were combined, the association between knee pain during sitting and lying activities and synovial scoring was statistically significant (p= 0.012) (Table 5.3 and Table 5.4).

Table 5.3. Distribution of the pain on sitting or lying scores, across microscopically defined synovial inflammatory groups, were merged together to form mild/normal or moderate.

Question: **"What amount of knee pain have you experienced in the last week during sitting or lying?"**			
	Microscopic Synovial Scoring		
	Normal/Mild	**Moderate**	
Pain			Total
None/Mild	13	11	24
Moderate/ Severe	1	10	11
Total	14	21	35

4.5 Summary of results

These results showed that the reproducibility of the chosen microscopic synovial scoring system was good to excellent. Moreover, reliability of a single biopsy to reflect microscopic synovial changes in other aspects of the suprapatellar pouch was also good to very good.

A range of normal to severe synovial inflammatory changes in the suprapatellar pouch were found in both post-traumatic non-arthritic knees and end-stage KOA. Post-traumatic synovium displayed less severe inflammatory features. In 60% of examined knees, there was severe or moderate microscopic synovitis in the SR. In up to 40% of post-traumatic knees without radiographic KOA, there

was a mild synovial inflammation in one aspect of the suprapatellar pouch accompanied by normal synovium in the other.

The relevance of structural synovial changes to knee pain was examined only in the post-traumatic knee group. A statistically significant association between moderate inflammation and the NRS pain score was found. No association with the frequency of pain was seen. Similar to previous studies in established KOA, moderate or severe self-reported knee pain on sitting and lying in the last week reported in individuals with post-traumatic but non-arthritic knees was associated with a moderate or severe synovitis in the suprapatellar pouch [230].

This semi-quantitative Haywood and Walsh method of assessing synovium is a highly reproducible technique that is useful for defining clinically important synovitis in patients post injury and with end-stage knee OA.

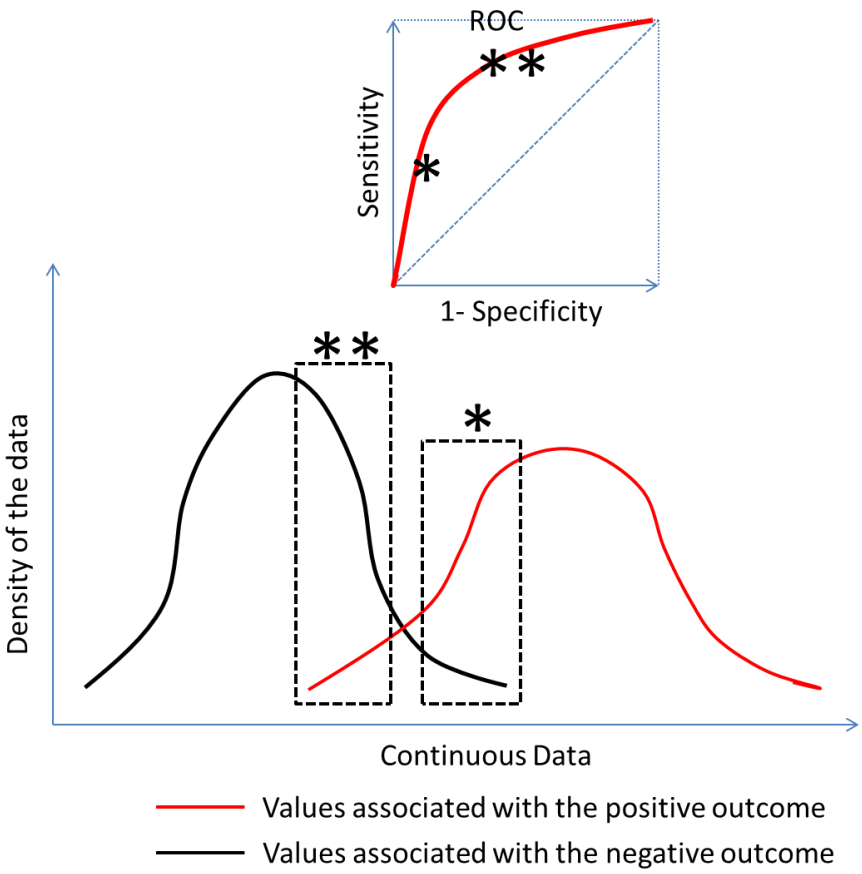
5 Diagnostic ability of biomarkers for knee synovitis

Recent MRI based studies have suggested that knee synovitis precedes development of radiographic knee osteoarthritis (RKOA) and is an independent risk factor in this process, when compared with other MRI changes [8, 73]. Arthroscopically defined knee synovitis is associated with more development of more severe cartilage changes 12 months later[72]. In post-traumatic knees, chronic synovitis is associated with pain [96]. It is often assumed that, after trauma, inflammation in the knee joint gradually settles down after the joint stability and function are restored. A recently published study by Ahlen et al. has shown that this is not the case and inflammatory cytokines coupled with markers of cartilage degeneration are significantly elevated in the knee joints even eight years after successful ACL reconstruction [294].

All biomarkers selected for this thesis are associated with knee pain and inflammation and some of them with synovitis in knee osteoarthritis (KOA). The correlation with microscopically defined knee synovitis in post-traumatic knees is unknown. For any diagnostic test, understanding its diagnostic accuracy is crucial. The sensitivity and specificity, as well as the receiver operating characteristic (ROC curve), are commonly used to describe this. The sensitivity of the biomarker is often described in terms of the proportion of positive tests correctly identifying the affected individuals. Meanwhile, specificity relates to the proportion of unaffected patients correctly identified by the test. The ROC curve

plots the true positive (sensitivity) values against false positives (1 minus specificity). A perfect test, in which values from the groups linked with positive or negative outcome do not overlap at all, shows a sensitivity decrease from 1 to 0, with no change to the perfect specificity[295]. Here, the resulting area under the ROC Curve (AUC) would be 1.

Figure 5.1. Figure showing association between distributions of values linked with either a positive or negative outcome, or the ROC curve when those values do overlap. Asterisks mark parts of the ROC curve that overlap with values linked with positive and negative outcome.



Aims of this chapter are to evaluate the following:

1. Diagnostic accuracy of modified selected 'wet' and 'dry' biomarkers to detect microscopically defined synovitis in the OxKIC study participants with unilateral injured knees and without radiographic KOA.
2. Potential optimal values for biomarkers associated with microscopic synovial inflammation that are sensitive and specific to knee synovitis.
3. The cross-sectional correlation between a biomarker that is both sensitive and specific to microscopic synovitis and the rest of the biomarkers at the baseline visit of the OxKIC study.

5.1 Diagnostic value of biomarkers

5.1.3 Methods

The continuous data based on either serum biomarker levels or results of sonographic assessment was compared with the dichotomised gold standard assessment for synovitis: microscopic assessment of the synovium. Mild, moderate and severe synovitis were each categorised as one and described as positive outcomes, while normal synovium was described as a negative outcome. The intra- and inter-reader reliabilities of the sonographic and microscopic assessments were classed as 'good' to 'very good' and described in chapters 4 and 5. Meanwhile, reliability of the serum biomarker assessment, including CV, were classed as 'good' to 'very good' and were described in chapter 3.

The mean values of the selected serum based biomarkers and/or thresholds for synovitis in the population with established KOA have been described before and are summarised in table 5.4 below. The published cut off-values for synovial hypertrophy (SH) and synovial effusion (SE) of the suprapatellar recess (SR) in KOA are presented in chapter 4.

Table 5.4. Reported mean values of selected serum based biomarkers in KOA groups.

Biomarker	Painful KOA	Publication
CRPM	11.2 (10.8-11.6) ng/mL	Siebuhr et al. 2013[296]
C3M	19.2 (18.5-19.9) ng/mL	Siebuhr et al. 2013
COMP	11.1 (10.4-12.8) U/L	Chua et al. 2008 [297]
Resistin	4.25 (4.12) ng/ml	Zheng et al. 2015[298]

Data from the OXKIC study, containing microscopic synovitis coupled with the results of selected serum and sonographic biomarkers collected on the same day before the operation, was utilised to calculate sensitivity and specificity, along with the area under the curve, the standard error and normal confidence intervals. Receiver operating characteristic (ROC) plots were used to illustrate the performance of each biomarker in identifying microscopic synovitis.

To aid in understanding the ability of markers to distinguish between participants with synovitis and without, logistic regression was carried out, with synovitis status (0 or 1) as the dependent variable, and sonographic or serum based biomarkers as the predictor variables.

Sonographic measures of SH and SE in the injured knees, with or without microscopically defined synovitis, were compared using an unpaired t-test for parametric data and a Mann-Whitney test for non-parametric data.

5.1.4 Sample size calculations

Despite recommendations, it is uncommon for diagnostic studies to report a priori calculation of sample size [299]. To calculate the required sample for the ROC curve, a minimal precision of the anticipated area under the curve must be given, along with an estimated ratio of positive and negative outcomes. In this study, the potential sample size was calculated according to the anticipated area under the ROC curve of above 0.75. The power size calculations were performed on the basis of the formula suggested by Hanley et al. [300]. Minimum sample sizes for anticipated variance and the area under ROC curve are presented in table 5.5. For the α-level (type 1 error), a value of 0.05 was selected and for β-level (type 2 error) the selected value was 0.20 (with 80% power). Based on the initial review of 10 synovial samples from the OxKIC study, the ratio of sample sizes in negative/positive groups was around 0.7, with four samples not displaying any significant signs of inflammation. The expectation, based on review of the

outcomes of patients referred to the acute knee clinic a year before, was that 30 to 40 patients would have an operation within the first eight months after enrolment for the OxKIC study. Based on the sample size calculations, biomarkers with an AUC above 0.75, that offer 'fair' and 'excellent' accuracy, would be evaluated with sufficient statistical rigour [301, 302].

Table 5.5. Sample size formula for estimating sensitivity, specificity and area under the ROC curve.

AUC	0.85	0.82	**0.80**	**0.78**	**0.76**	**0.75**	0.70	0.65	0.60
Sample size	19	23	**26**	**31**	**36**	**40**	60	116	266
Positive cases	11	13	15	18	21	23	37	68	156
Negative cases	8	10	11	13	15	17	26	48	110

A commonly classification for a diagnostic test based on AUC is summarized in table 5.6.

Table 5.6. The common interpretation of the diagnostic value of a test based on the AUC.

The AUC range	Accuracy of the diagnostic test
0.90 -1	Excellent
0.80- 0.89	Good
0.70 – 0.79	Fair
0.60 - 0.69	Poor
0.50 -0.59	Fail

5.1.5 Accuracy of the selected biomarkers in detecting suprapatellar knee synovitis.

5.1.6 Sonographic markers

Thirty-six OxKIC participants had data available with both sonographic biomarkers and synovial scoring from the same day and the same SR. Characteristics of those participants are described in chapter 5 of this thesis. All of them had SH and SE measured in the affected and contralateral (unaffected) knees before the operation. There were significant differences between affected and contralateral knees in terms of SE and SH levels. There were no significant differences between mean values of SH and SE measured before the operation in

36 participants and mean values of SH and SE in OxKIC participants at baseline (n=80). A table with characteristics of both groups is presented in the appendix. This indicates a small difference in terms of age and type of injury, with meniscal injuries more common in the group containing patients who had undergone surgery.

Figure 5.2. Kernel density estimate plots of synovial hypertrophy distribution in the affected and contralateral knees at baseline and on the day of operation.

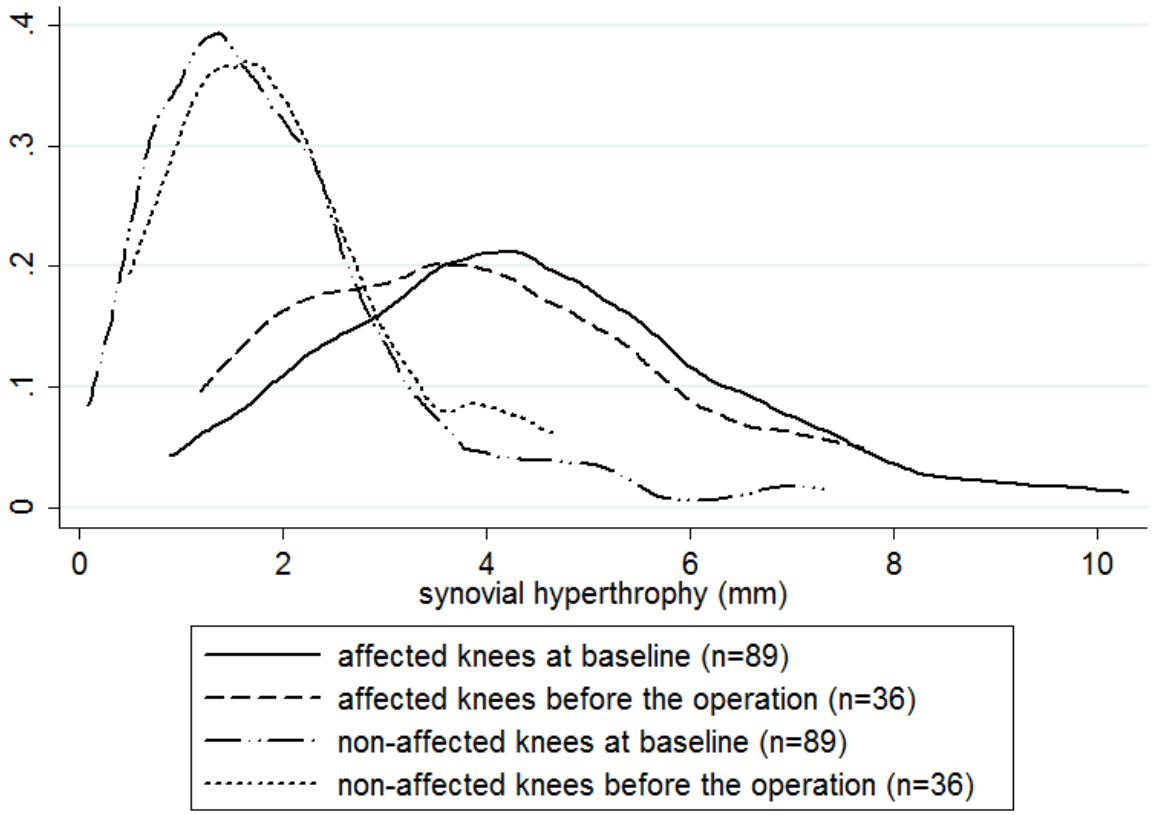
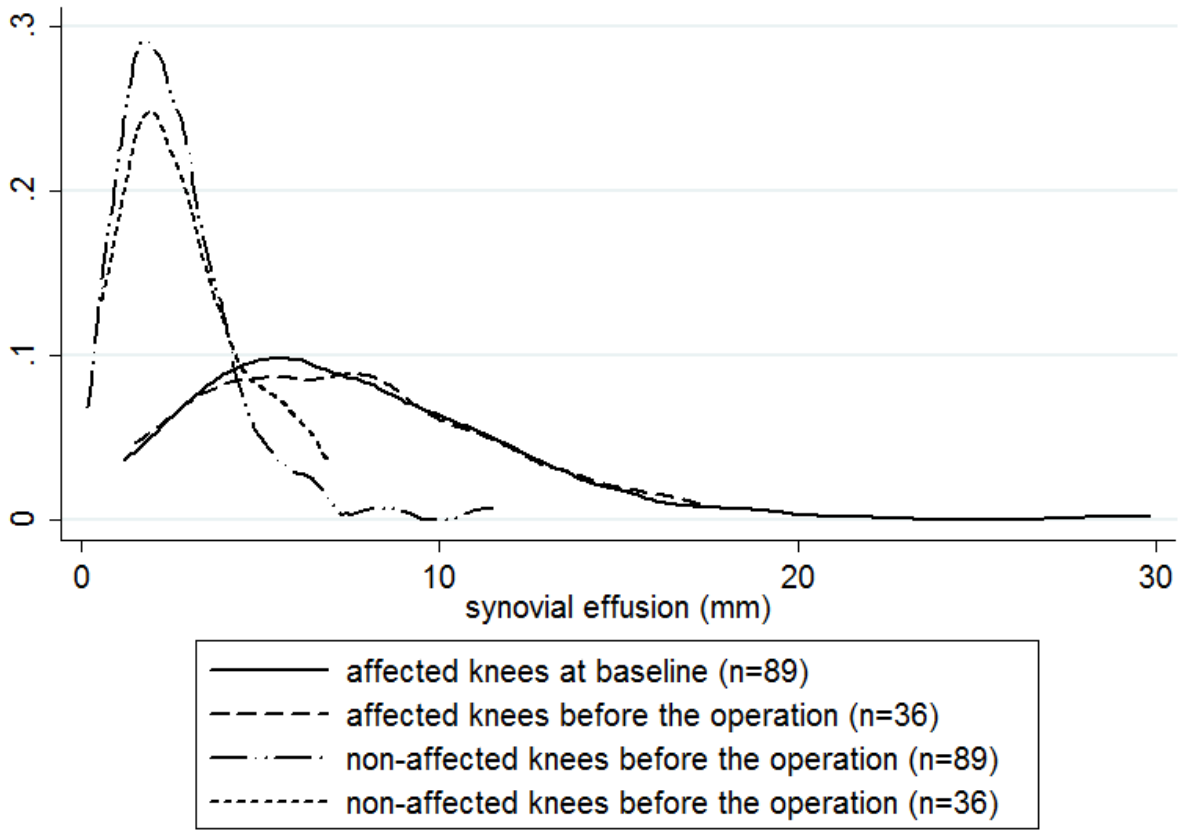


Figure 5.3. Kernel density estimate plots of synovial effusion distribution in the affected and contralateral knees at baseline and on the day of the operation.

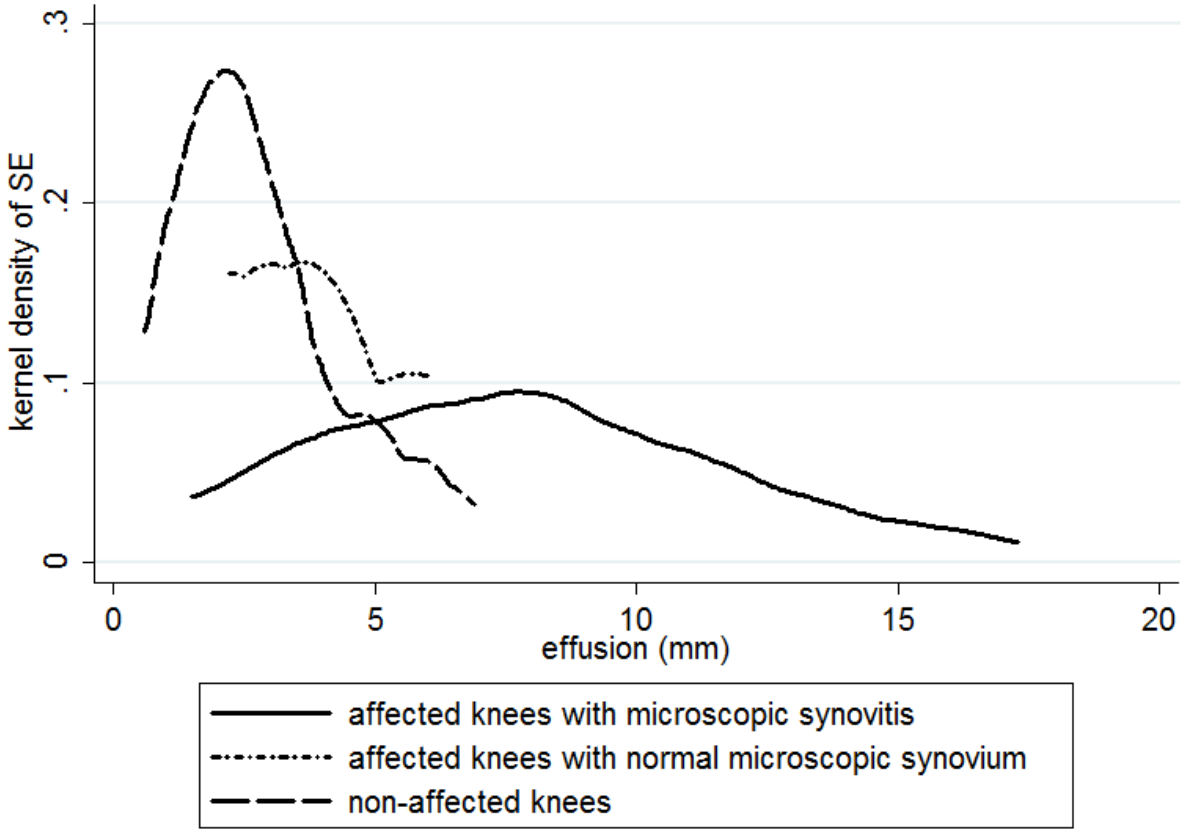


The univariate logistic regression model in the pre-operative sample showed a positive and statistically significant association between SE as a continuous variable and microscopic synovitis, with an Odd Ratio of 1.5 (95% CI of 1.01-2.32).

Mean values of SE in an injured knee with microscopic synovitis and without were statistically different (p-value 0.003). A similar distribution of SE readings in both the non-affected and injured knees without synovitis was found, as seen in figure 5.4. This suggest that SE, which is a function of produced and reabsorbed synovial fluid within the knee joint, is similar in injured knees

without microscopic synovitis and contralateral uninjured knees but different from injured knees with microscopic synovitis.

Figure 5.4. Kernel density estimate plots of synovial effusion distribution in the affected knees with or without microscopic synovitis and contralateral knees on the day of operation.



The AUC for SE in the affected knee in detecting microscopic synovitis was 'good' 0.82, with a standard error of 0.08 and a 95% confidence interval (95% CI) of 0.66 to 0.97.

This was despite a relatively small number of biopsies with normal synovium. The cut-off point equal or higher than 5 mm represented a very good potential threshold, with sensitivity of 80% and specificity of 68%.

Figure 5.5. The ROC curve of synovial effusion in the affected knee on the day of operation and microscopically defined synovitis.

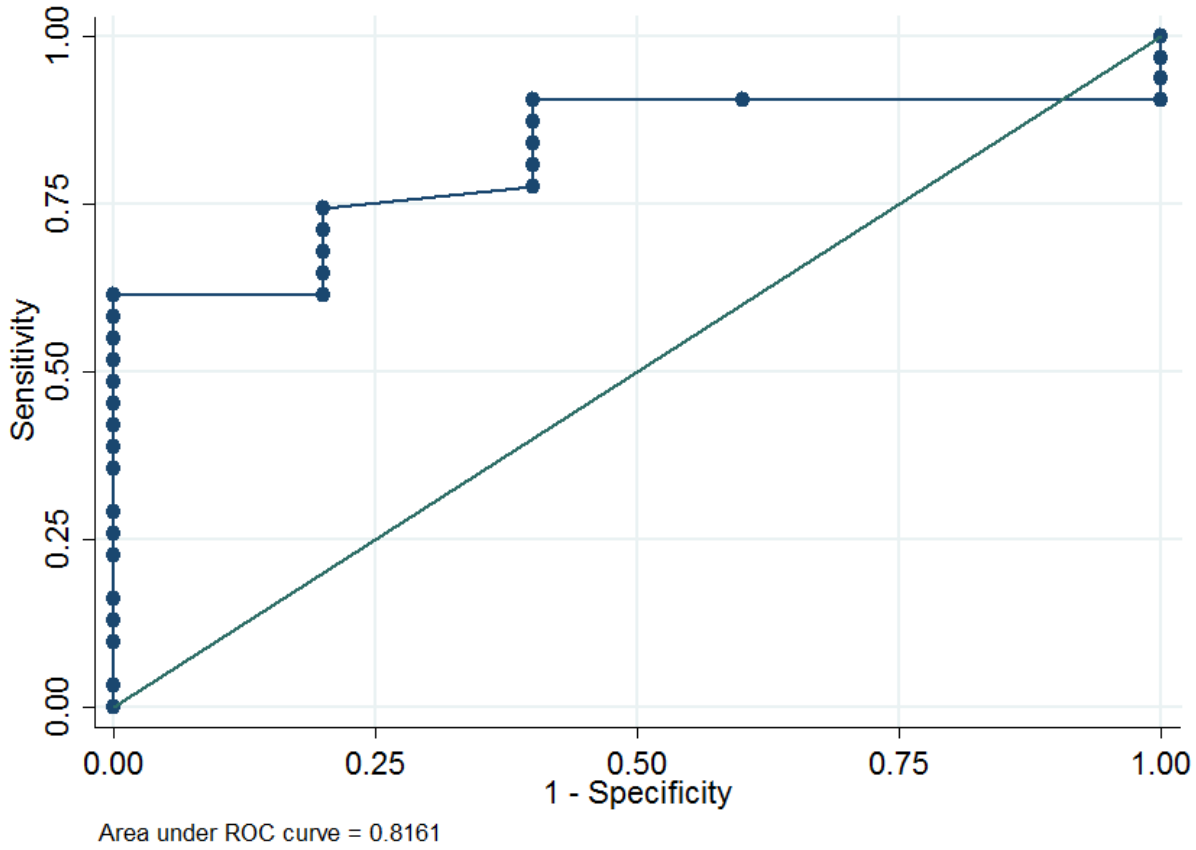
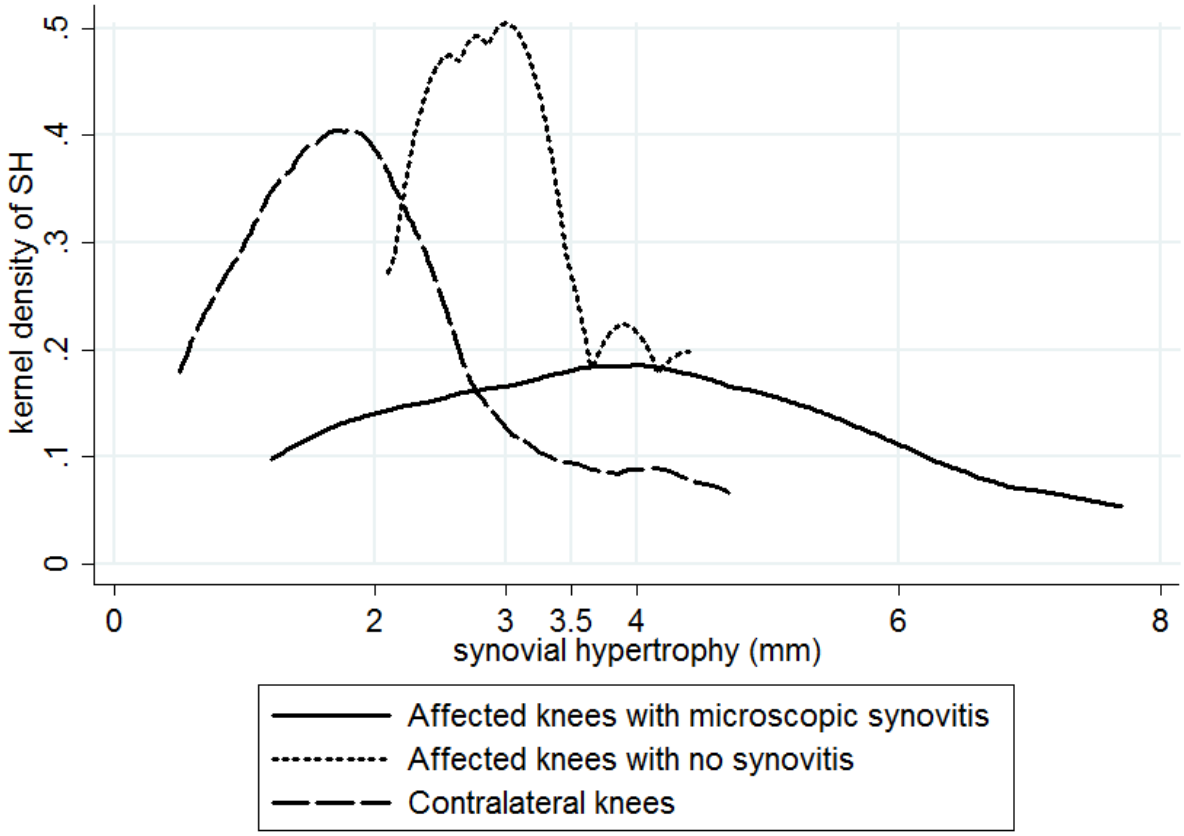
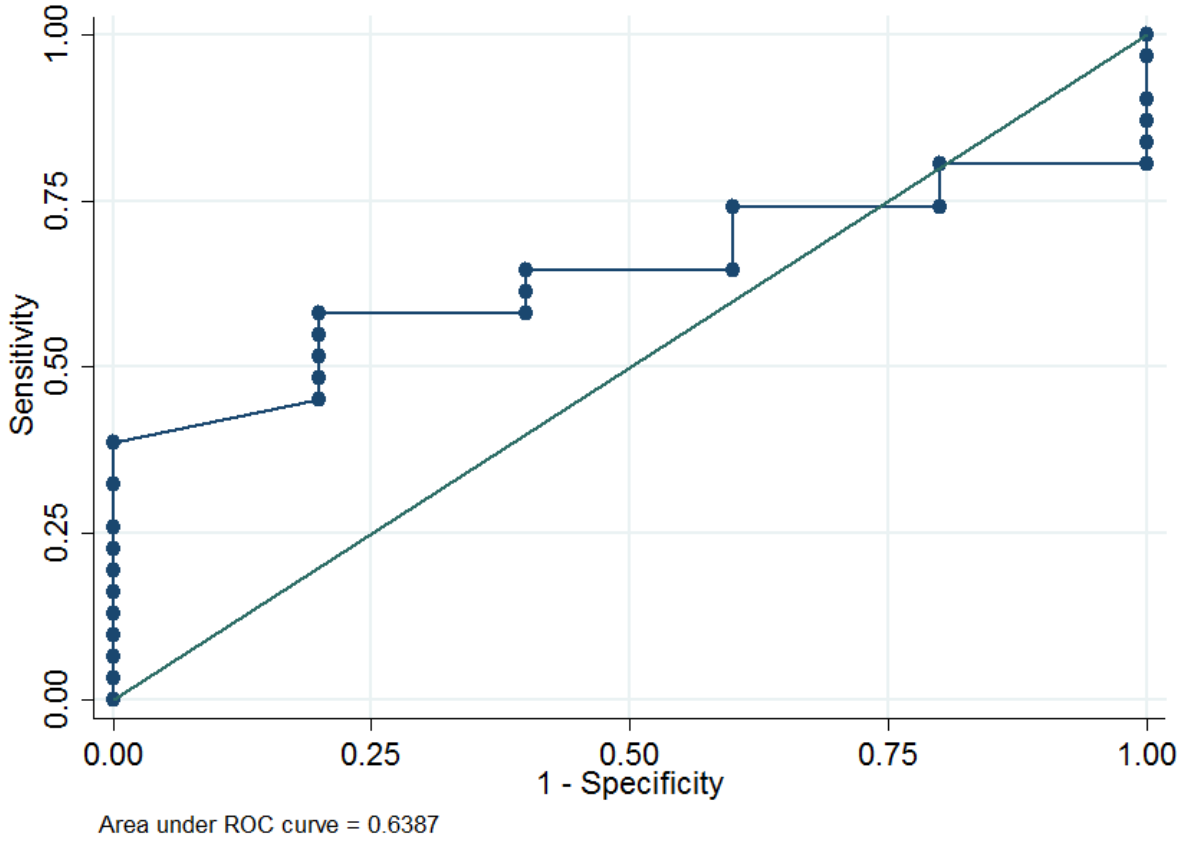


Figure 5.6. Distribution of SH in the affected and contralateral knees at baseline and on the day of operation.



Diagnostic accuracy of SH was analysed in a similar manner, with the area under the curve for SH in the affected knee showing at 0.64, with a standard error of 0.1 and 95%CI between 0.45 and 0.83.

Figure 5.7. The ROC curves of synovial hypertrophy in the affected knees on the day of operation and microscopically defined synovitis.



In this small sample, the mean values of SH in injured knees, both with and without microscopic synovitis, were different. However, this was only borderline significant, with p-values of 0.09. Despite a significant overlap in terms of the range of values between SH in the injured knees without synovitis and SH distribution in the contralateral knees, mean values were significantly different (p-value of 0.04), as seen in figure 5.6. This suggested that SH in injured knees without microscopic synovitis was not similar to the values of SH in the

contralateral uninjured knees, but probably different from SH in the injured knees where microscopic synovitis was present (as seen in figure 5.6).

5.1.7 Selected serum biomarkers

Thirty-four OxKIC participants had data available with both a serum-based biomarkers and microscopic synovial scoring from the same day and the same suprapatellar pouch. Five participants had normal synovium. For α-level (type 1 error), a value of 0.05 was selected and for β-level (type 2 error), 0.20 (80% power). This sample size would provide adequate power to analyse any markers with a ROC of 0.81 or higher.

Analysis performed on serum concentrations of COMP, CRPM, C3M and Resistin showed either 'poor' or no diagnostic value regarding those markers in terms of detecting knee synovitis.

Table 5.7. The AUC serum based biomarkers and microscopically defined synovitis.

Biomarker	AUC	Standard error	95 % Confidence interval
Resistin	0.64	0.09	0.46 - 0.82
COMP	0.55	0.13	0.28 - 0.82
C3M	0.47	0.14	0.19 - 0.74
CRPM	0.41	0.12	0.17 - 0.65

A sensitivity and specificity table for Resistin is presented in the appendix.

5.1.8 Summary

Synovial effusion showed 'good' diagnostic accuracy, while synovial hypertrophy and Resistin were 'poor' in terms of detecting microscopic synovitis in a population with post-traumatic knee joints without RKOA. The power from this sample size would only detect with significant certainty diagnostic values for tests with the AUC above 0.75. The most promising cut-off point for SE to identify knees with microscopic synovitis was equal to and above 5mm.

5.2 The relation between suprapatellar effusion and selected biomarker levels

The diagnostic accuracy of SE in the SR for detecting microscopic knee synovitis is good and the cut-off value of 5mm has potentially good sensitivity and specificity.

The OxKIC study dataset contained cross sectional data of all selected serum and sonographic biomarkers at baseline (n=80) on the day of operation (n=34). Distribution of SE in the affected and contralateral knees at baseline and on the day of operation was very similar, as demonstrated in the figure 5.1.

Correlation between levels of the serum biomarkers in the OxKIC cohort with the sonographic biomarker will indirectly allow an insight into the association between those serum-based biomarkers and SR synovitis in the larger sample.

5.2.3 Methods

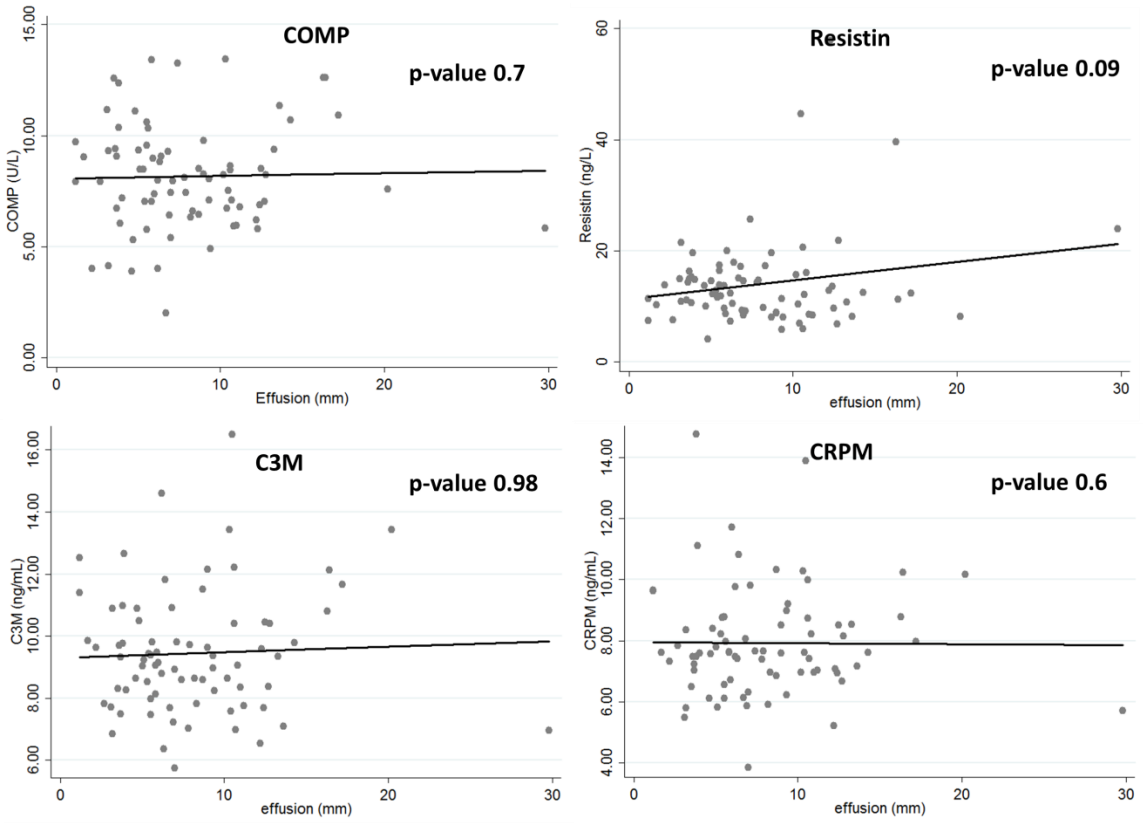
In the first instance, two sample t-tests for parametric and Mann –Whitney two-sample statistic test for non-parametric were performed to establish if any differences in baseline and operation follow-up for sonographic and selected serum based biomarkers exist. Logistic regression models were utilised to assess the strength of the association between serum based biomarkers and dichotomised SE, based on the ≥ 5mm cut-off point for both baseline and pre-operative samples.

5.2.4 Results

The median number of days between the baseline and operation was 42. There were 80 participants with available baseline serum and sonographic samples and 36 of them had the same markers repeated before the operation. The effect of time on the biomarker values in paired samples is presented in chapter 2.

Comparing baseline mean values for C3M, CRPM and SE (n=80) with mean values of the same biomarkers before the operation (n=36) showed statistically significantly lower COMP (p-value of 0.005) and Resistin (p-value of 0.001) levels in the pre-operative sample group. Analysis of baseline biomarkers (n=80) showed that COMP values were lower in females when compared to males (p-values 0.009), with this also being the case for SE (p-value of 0.048).

Figure 5.8. Scatterplots of serum based biomarkers from baseline vs synovial effusion as an independent variable. P-values for linear Pearson or Spearman coefficient, dependent on linear or monotonic relationship are presented in the top right corner of each graph.



Univariate logistic regression models for baseline data were utilised to evaluate the association between selected serum based biomarkers and dichotomised SE. Positively skewed Resistin data was transformed using logarithmic transformations. As the dependent variable (SE) showed some significant gender differences, sensitivity analyses were performed for each gender.

For baseline biomarkers (n=80), no statistically significant associations between levels of the biomarkers and SE ≥ 5mm in the SR for the whole baseline group or

in the gender sub-analysis were found. Results for each baseline serum biomarker are presented in table 5.8.

Table 5.8. Association between baseline levels of biomarkers and dichotomised synovial effusion, with a cut-off point equal to or above 5mm for synovial effusion in the SR.

Biomarker	OR (95% CI)		Females, OR	(95%CI)	Males, OR	(95%CI)
Resistin	1.34	(0.4-4.5)	1.12	(0.2-6.2)	2.1	(0.4-12)
COMP	0.97	(0.8-1.2)	1.05	(0.7-1.7)	0.9	(0.7-1.2)
C3M	0.93	(0.7-1.2)	0.95	(0.6-1.4)	0.9	(0.6-1.3)
CRPM	0.95	(0.7-1.3)	1.2	(0.7-1.8)	0.8	(0.5-1.2)

Pre-operative samples showed only a positive association between COMP levels and high levels of SE in the SR, as presented in table 5.9. This association was not statistically significant when the model was adjusted for gender. There were only a few women in the pre-operative group and therefore the subgroup analyses for gender were not performed, as these results would not be statistically reliable.

Table 5.9. Association between pre-operative levels of biomarkers and dichotomised synovial effusion, with a cut-off point equal to or above 5mm for synovial effusion in the suprapatellar recess.

Biomarker	OR (95% CI)	
Resistin	0.6	(0.1-3.0)
COMP	**1.8**	(1.1-3.2)
C3M	1.1	(0.7-1.7)
CRPM	0.82	(0.5-1.3)

5.2.5 Strengths, assumptions and potential limitations

This is the first piece of research looking at the cross-sectional association between serum COMP, Resistin, C3M and CRPM levels and coexisting SE in post-traumatic knees without RKOA collected at two different time-points: at baseline and before the operation. It showed the positive association between SE and COMP levels, but only in an unadjusted model and in the samples collected before the operation.

There are limitations within this study. Serum samples were collected at different times of day. The baseline samples were non-fasted and collected between 1 and 4 pm in the out-patient clinic. Most of the patients were still using mobility aids in the form of knee braces and/or crutches at this stage. This would be associated with not only an alteration of the gait and potentially different serum levels of biomarkers, due to abnormal loading in the contralateral knee, but also decreased activity. Pre-operative samples were fasted and collected between 7 and 10 am.

Most of the participants were mobilising independently without an aid at this stage and had reassumed most of their daily activities, but due to hospitalisation, their activity on the day was limited.

COMP and SE showed some statistically significant gender associated differences, with higher values in males. This is partly supported by published literature reporting a small gender difference and potential relationship between oestrogens and synovial inflammation [303-306]. Unfortunately, we were not able to assess the influence of hormonal changes, as the information on the phase of the menstrual cycle, use of birth control pills or HRT were not collected.

5.3 Summary

A synovial effusion was shown to be a good diagnostic marker of post-traumatic synovitis in the suprapatellar pouch, with 5mm being a threshold associated with good sensitivity and specificity. Synovial hypertrophy and Resistin were also potentially 'fair' diagnostic markers of post-traumatic knee synovitis, but the study was not powered enough to reject or confirm this. The ROC curve analyses were powered to investigate accurately the diagnostic ability of tests, with the AUC levels over 0.75. Larger number of biopsies coupled with sonographic and serum based biomarker data is needed to confirm or discredit the diagnostic value of biomarkers with AUC levels below 0.75.

In the second section of this chapter, the high SE values, above or equal to 5mm were categorised and utilised as proxy markers of synovitis for all serum based

biomarkers from the baseline OxKIC study assessment. None of the selected markers were independently associated with the high values of SE in the SR. Gender differences associated with SE and COMP levels support a hypothesis proposing a possible mediating role of oestrogens in synovial response to joint injury.

6 Predictive ability of biomarkers for future incidence of knee osteoarthritis

6.1 Background

The main risk factors for the incidence of knee osteoarthritis (KOA) are age, gender, body mass index (BMI) and injury. These are based on results from large epidemiological studies [26, 307, 308]. There is a currently existing panel of other risk factors, including genetic and biochemical markers. However, together, these add only modestly to the prediction of KOA incidence [26].

The relationship between radiographic KOA (RKOA) and knee pain is still poorly understood. A significant proportion of the population will develop RKOA, but less than half of these affected individuals will report any concurrent knee pain [51, 309].

However, there is a correlation between knee pain and the development of KOA [310]. The serum-based biomarkers selected for this thesis are linked with not only knee pain, but also disturbed cartilage and/or synovium homeostasis. The next step is to evaluate the predictive ability of these biomarkers for the development of KOA.

The predictive ability of any biomarker could do more than simply allow for the stratification of the population at risk for future interventions; it could also

enable the identification of potential pathological pathways as future targets for interventions.

One of the overall aims of this thesis is to identify predictive biomarkers that could be used for profiling individuals at risk of KOA for future interventions. Dichotomising continuous variables has often enhanced utility and ease of use of biomarkers in daily clinical practice and interventional research. There are different methods of setting up optimal thresholds. To assess the accuracy of predictive models using categorised independent variables, results will be compared with outputs of the models, using continuous biomarker data.

The aim of the analyses described in this chapter is to evaluate the association between baseline levels of the selected biomarkers in a population of middle-aged women with no RKOA in either knee at baseline who later develop radiographic or painful RKOA.

The meet those objectives, specific analyses were performed to:

1. evaluate the predictive ability of selected serum-based biomarkers for the development of RKOA and painful RKOA over 20 years in a longitudinal cohort of middle-aged women
2. compare the diagnostic ability of different potential cut-off points for continuous independent biomarker variables.

6.2 Methods

6.2.3 Population

The Chingford cohort study, which has been shown to be representative of middle-aged women in England, has been used for this set of analyses. A description of the cohort, the population selected and outcomes are included in chapter 2.

6.1.1.1 Inclusion and exclusion criteria

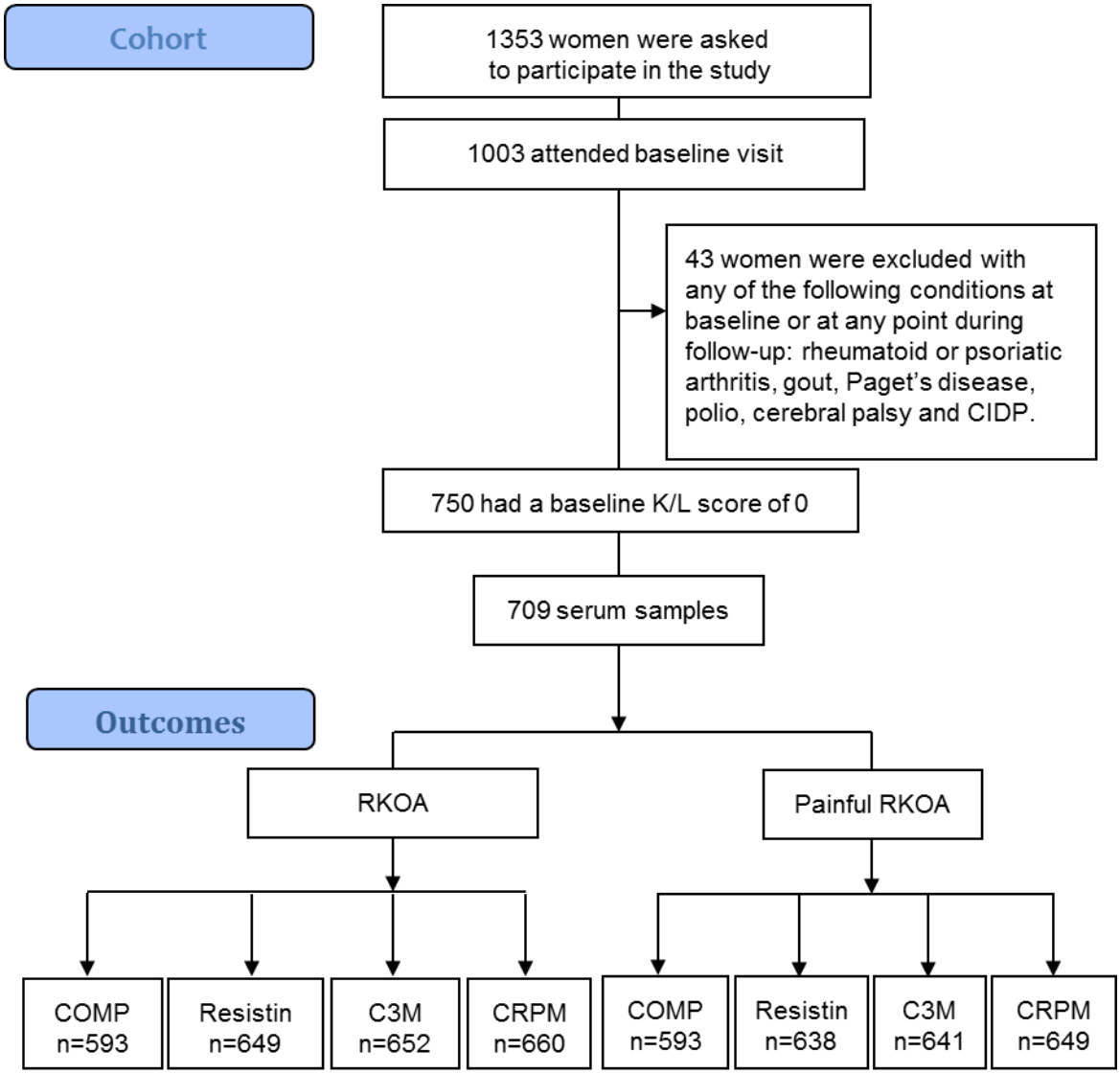
Only participants with no RKOA in both knees at baseline (Kellgren and Lawrence score of 0) were included in the analyses. In addition, women with any of the conditions associated with increased risk of joint degeneration at baseline or at any point during follow-up were excluded. These included rheumatoid arthritis, psoriatic arthritis, gout, Paget's disease, polio, cerebral palsy and chronic inflammatory demyelinating neuropathy. All participants were required to have data regarding pain and RKOA at baseline and during at least one five-year interval over a 20-year follow-up.

6.2.4 Independent variables and outcomes

Out of 1003 participants at year one, 750 had a Kellgren and Lawrence score of 0 in both knees. Out of those participants, 709 had serum samples collected during the same follow-up. Almost 600 had COMP and around 700 participants had a serum concentration of C3M, CRPM and Resistin measured from the same serum samples from year 2 of the study follow-up. All participants with COMP had other biomarkers measured from the same serum samples. Serum COMP

concentration was measured first. After the results of this measurement were available, an additional 103 samples in the pool of baseline Chingford samples were identified and shipped to the Nordic Bioscience Lab for all other biomarkers (Resistin, C3M, CRPM) to be measured with previously available serum samples.

Figure 6.3. Number of participants included in each analysis.



A group of Chingford participants with radiographic KOA had biomarkers concentrations measured. Breakdown of study participants who had their biomarkers measured is presented below (table 6).

Table 6. Number of participants from the Chingford Cohort Study with biomarker measurements based on radiographic knee status and painful RKOA.

Biomarker	**All**	**K&L = 0 Both knees**	**K&L >0**	**K&L≥2**	**K&L≥2 and pain**
			Uni or bilateral RKOA		
COMP	745	594	151	110	48
C3M	849	700	149	109	47
CRPM	857	708	148	108	47
Resistin	841	697	144	104	45

Coefficients of variation (CVs) were used to assess inter-assay reliability and are presented in chapter 2 (section 2.3), together with information about the lab, equipment and assays used.

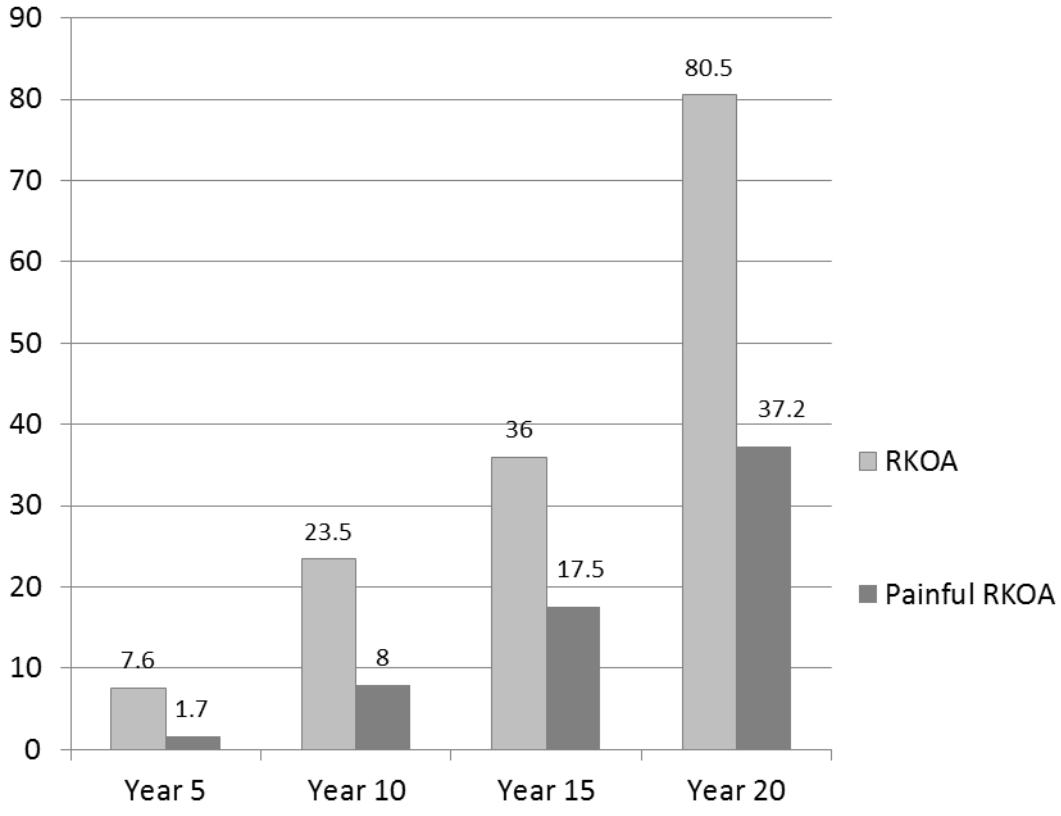
All selected serum biomarkers in individuals with no KOA at baseline had positive skewed distributions with a long tail and continuous data was log-transformed in the presented analyses. Kernel density estimate plots of the baseline distributions for each biomarker before and after log transformation are presented in the appendix.

The outcomes of interest were the incidence of:

1. RKOA, determined using a K/L score of 2 or more at any point during follow-up with radiographic data

2. painful RKOA, defined as knee pain reported in the past month in the knee joint with RKOA.

Figure 6.2. Percentage of radiographic and painful knees during each follow-up visit of the cohort, with no RKOA at baseline. Numbers above the bars represent the percentage of the subgroups during each follow up.



Methods defining pain and scoring radiographs, as well as the development and reproducibility of the biomarker data are presented in detail in the methods section of this thesis (chapter 2, section 2.2).

Between 79 and 88% of all eligible participants in the Chingford cohort study were included in the analyses. There were no significant differences between a) the baseline characteristics of those who were included in the analyses or b) those not included due to a lack of biomarker, pain and/or radiographic data. A comparison of characteristics, with no baseline RKOA, for participants not included in the analyses (due to a lack of biomarker, pain and/or radiographic data) is presented in the appendix section.

6.2.5 Methods of testing conformity for a linearity gradient

Logistic regression analysis uses a binary dependent variable and does not make as many key assumptions as linear regression or general linear models based on ordinary least squares algorithms. This is because logistic regression is based on a form of binomial distribution, known as the Bernoulli distribution, which can only have two possible outcomes: 0 and 1. Logistic regression can handle different relationships between the dependent and independent variables, as it applies a non-linear log transformation to the predicted odds ratio (OR) [311]. However, logistic regression is based on the assumption that the continuous variable conforms to a linear gradient, which is often tested by analysing how an independent variable is linearly related to the log of the OR (a way of expressing probabilities). A recent review of papers published in two major epidemiology journals has suggested that less than 20% of published articles reported linearity gradient conformity for the continuous variable [312]. The definition of the OR is explained below.

Table 6.1. Relationship between probabilities in exposed and non-exposed groups, followed by the formulas defining the odd ratio.

	Affected	**Unaffected**
Exposed	Affected and exposed (probability 1)	Unaffected and exposed (probability 2)
Not Exposed	Affected and not exposed	Unaffected and not exposed

$$Odd\ ratio = \frac{(Affected\ \&\ exposed)/(Affected\ \&\ not\ exposed)}{(Unaffected\ \&\ exposed)/(Affected\ \&\ not\ exposed)}$$

$$Odd\ ratio = \frac{probability\ 1/(1 - probability\ 1)}{probability\ 2/(1 - probability\ 2)}$$

There are several methods of assessing whether an interval variable fits the linear assumption of logistic regression. The conformation of a linear gradient is frequently assessed by categorising a variable into equal dichotomous units on the variable's scale and then taking these several dichotomous variables to compare estimated coefficients against the midpoint of each one. The graph then tracks the relationship between the independent and outcome variable, and any significant differences between both lines suggests a non-linear relationship [313].

The fractional polynomial (FP) modelling is another common and popular method to reveal non-linear associations [314, 315]. In the case of skewed data, the linearity assumption might be doubtful, so mathematical transformations, such as those that are logarithmic, are commonly used in prognostic modelling to make a linearity assumption plausible. An alternative and common method is to categorise the continuous variables, which results in a simplification of the analysis but is also associated with decreased power and risk of 'Type 1 error' [316-319].

To assess the conformity of these biomarkers to the linear gradient, FP models were used and the probability against the independent variable methods was assessed, confirming conformity for all biomarkers. Graphical results of those analyses are presented in the appendix (section 10.3).

The other important assumption of logistic regression is that the model includes all the relevant variables. This makes it vital that the model is fitted correctly and does not include any variables that should not be present. In the analyses below, all participants of the same gender and age, as well as BMI, were added to the models as potential confounders.

6.2.6 Methods to define cut-off points

It is sometimes useful to divide continuous variables into categories, as this can offer enhanced utility and ease of use of biomarkers in interventional research. It is also sometimes necessary, especially when the independent variable fails to fulfil the linearity gradient conformity for the logistic regression model. An

adverse consequence of this type of method is decreasing granularity of the data, hence decreasing power, which could result in the creation of biologically unrealistic models[316, 320]. This also results in a situation where the results of different studies cannot be compared directly. However, clinically, this might be the easiest approach for utilising the biomarker discovery in the stratification of potential participants for future clinical trials. One of the major challenges in categorising a continuous variable is determining the cut-off points. There is no one generally accepted method for doing this, and several problems associated with data categorisation have been highlighted before. In this section, we will determine thresholds for continuous biomarker by identifying:

a) quartiles based on biomarker data distribution

b) the median value of the biomarker concentrations in the RKOA group

c) the best fitting cut-off point in terms of the predictive effect of continuous biomarker variables, by utilising fractional polynomial models (FPM) of association between baseline biomarkers' data and incidence of RKOA and painful RKOA at year 10.

6.1.1.2 Quartiles

Quartiles are four equal groups into which a given sample of the population can be divided. The quartiles for each serum based biomarker are presented in the table below (table 6.2).

Table 6.2. Quartiles of COMP, Resistin, C3M and CRPM in the Chingford cohort, with no baseline KOA.

COMP quartiles (U/L)	Min	Max	Resistin quartiles (ng/mL)	Min	Max
1	2.676	7.653	1	0.275	6.64
2	7.665	9.428	2	6.65	9.19
3	9.435	11.845	3	9.20	12.86
4	11.887	43.283	4	12.87	57.91

C3M quartiles (ng/mL)	Min	Max	CRPM quartiles (ng/mL)	Min	Max
1	2.086	5.516	1	1.736	3.972
2	5.528	6.824	2	3.992	4.948
3	6.84	8.444	3	4.952	6.212
4	8.464	26	4	6.22	22.192

6.1.1.3 Biomarkers in individuals with RKOA

The choice of cut-off point in a study such as this one is often based on informed judgment made in association with important clinical outcomes. In this study, the aim was to identify individuals at risk of KOA, and information on the median

concentrations of selected biomarkers in the group with RKOA within the same cohort was utilised in order to inform the cut-off points for each biomarker with a specific outcome. The median values in the group with painful RKOA were similar to those with RKOA in general. Therefore, the median values in RKOA group informed cut-off points for categorising continuous biomarker data in a group with no RKOA.

As there were no differences in median CRPM results between the groups with RKOA and those without, no cut-off point was established for CRPM based on this criterion.

Table 6.3. Median values of selected serum based biomarkers in groups without KOA, with general RKOA and painful RKOA.

Biomarker Median	**No RKOA**		**RKOA**		**Painful RKOA**	
	in the Chingford Cohort Study					
COMP(U/L)	9.4	n=594	10.7	n=110	11.1	n=48
Resistin (ng/ml)	9.2	n=697	9.7	n=104	9.0	n=45
C3M (ng/mL)	6.8	n=700	7.2	n=109	7.2	n=47
CRPM (ng/mL)	4.5	n=708	4.54	n=108	4.4	n=47

All selected cut-off points based on the RKOA median values were within the third and fourth quartiles of selected biomarkers with no RKOA. When comparing distributions of serum biomarkers in the groups with RKOA and no RKOA, only COMP had statistically different results (p value 0.035) in the RKOA

category only. Despite different median values of Resistin and C3M in the groups with RKOA and no RKOA, overall differences between RKOA and no RKOA were not statistically significant. This is consistent with recently published data [298]. Whisker box plots for each subgroup of biomarkers based on radiographic and knee pain status are presented in the appendix.

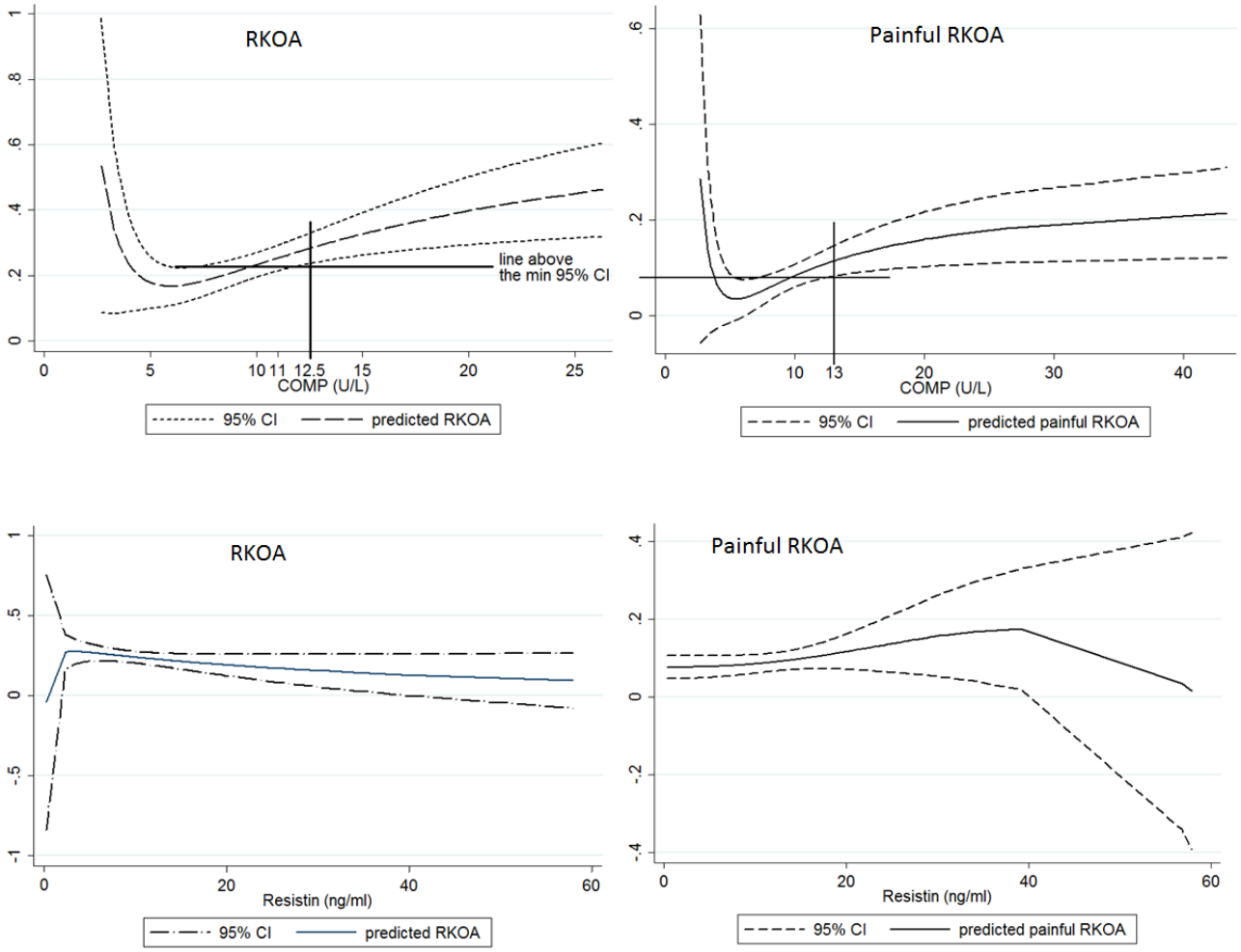
The expected potential disadvantages of choosing cut-off points based on the levels of selected biomarkers in the group with RKOA was that it would divide the sample into unequal groups and also might not be representative for the whole population or the levels of biomarker associated with an incidence of RKOA in the early stages.

6.1.1.4 Fractional polynomial models

The final cut-off point was based on fractional polynomial models (FPM) looking at the association between baseline biomarkers and RKOA or painful RKOA at year 10 (Figure 6.4 and Table 6.4).

This method was considered likely to maximise the fit to the sample data and expected to overestimate the predictive importance by increasing 'Type I' error rates. For that reason, results based on this analysis should be used with caution and would require validation in an external cohort.

Figure 6.4. Relationship between predictive probability of incidence of RKOA at year 10 and serum concentration of biomarkers. Solid black lines suggest a new threshold based on the point where there is a clear difference between the upper margin of a 95% confidence interval (95%CI) of the lowest predictive value and the lowest margin of a 95%CI for the most predictive cut-off point.



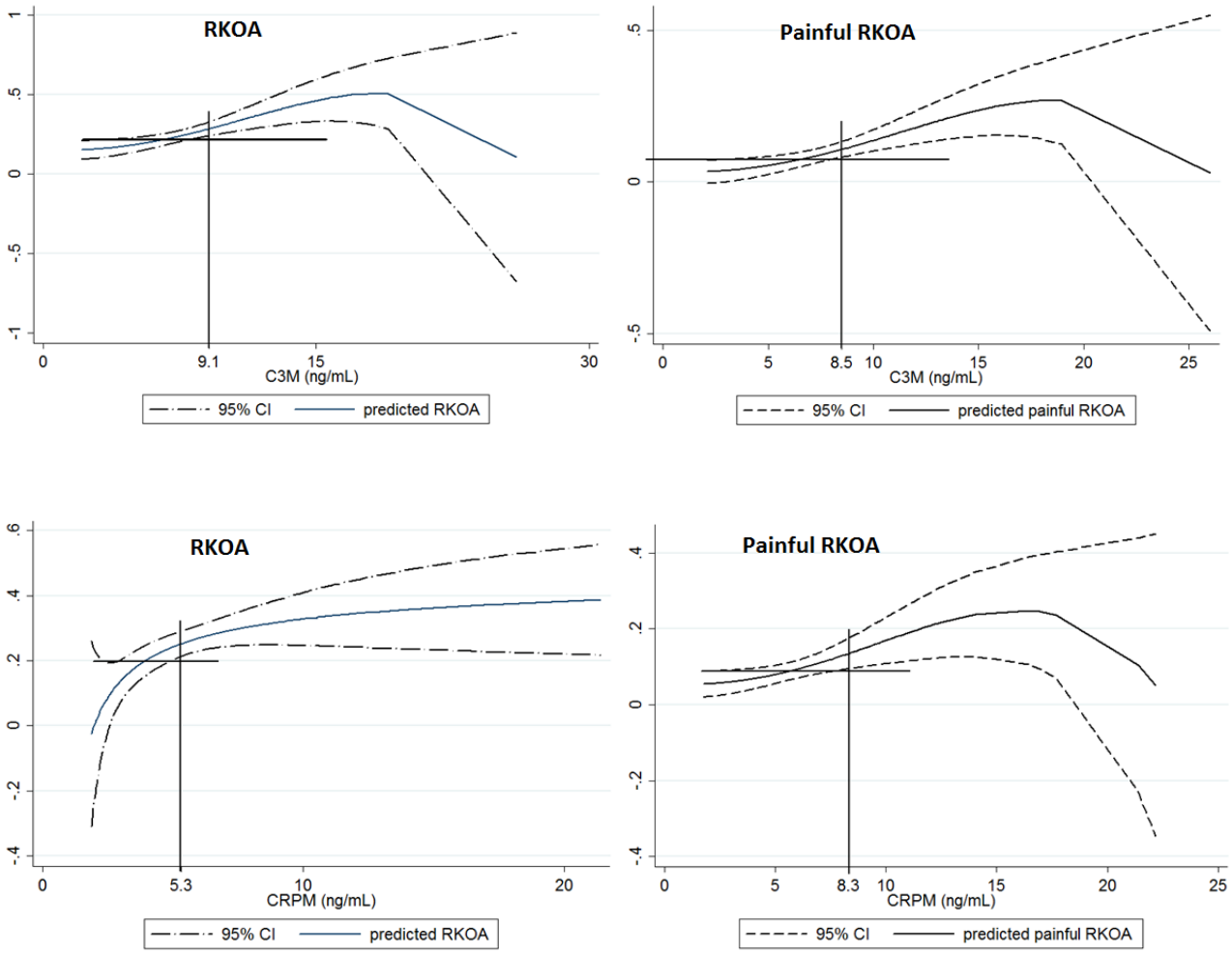


Table 6.4. Thresholds based on fractional polynomial models.

Biomarker	FPM based cut-off points	
	RKOA	Painful RKOA
COMP(U/L)	12.5	13
Resistin (ng/ml)	No cut-off identified	No cut-off identified
C3M (ng/mL)	9.1	8.5
CRPM (ng/mL)	5.3	8.3

6.1.1.5 Categorising biomarkers - conclusions

Quartiles and FPM based thresholds use the same dataset. The thresholds based on median value of the biomarkers in the RKOA group are also based on the Chingford cohort, but these use different data that is not included in the main predictive analysis. It is reassuring that those FPM and RKOA-based thresholds do have similar values (Table 6.5). However, even small differences in thresholds can affect the outcomes of models. Ultimately, comparing the results of predictive models using categorised independent variables with the results themselves utilising continuous variables, allows the best methods of categorising the data to be determined. The models utilising continuous variables are considered to be the most powerful form of analysis and therefore the 'gold standard'.

Table 6.5. Thresholds for data-dependent categorisation of continues biomarker variables are presented for each biomarker. Thresholds are based on quartiles, median values in individuals with established RKOA and FP models.

Biomarker	**Quartiles**		**RKOA - median values**	**FPM**	
	4th	**1st**		**for RKOA**	**for painful RKOA**
COMP(U/L)	>11.89	<7.65	10.7	12.5	13
Resistin (ng/ml)	>12.9	<6.6	9.7	No cut-off	No cut-off

C3M (ng/mL)	>8.5	<5.5	7.2	9.1	8.5
CRPM (ng/mL)	>6.22	<3.97	No cut-off	5.3	8.3

6.2.7 Statistical methods used in this chapter

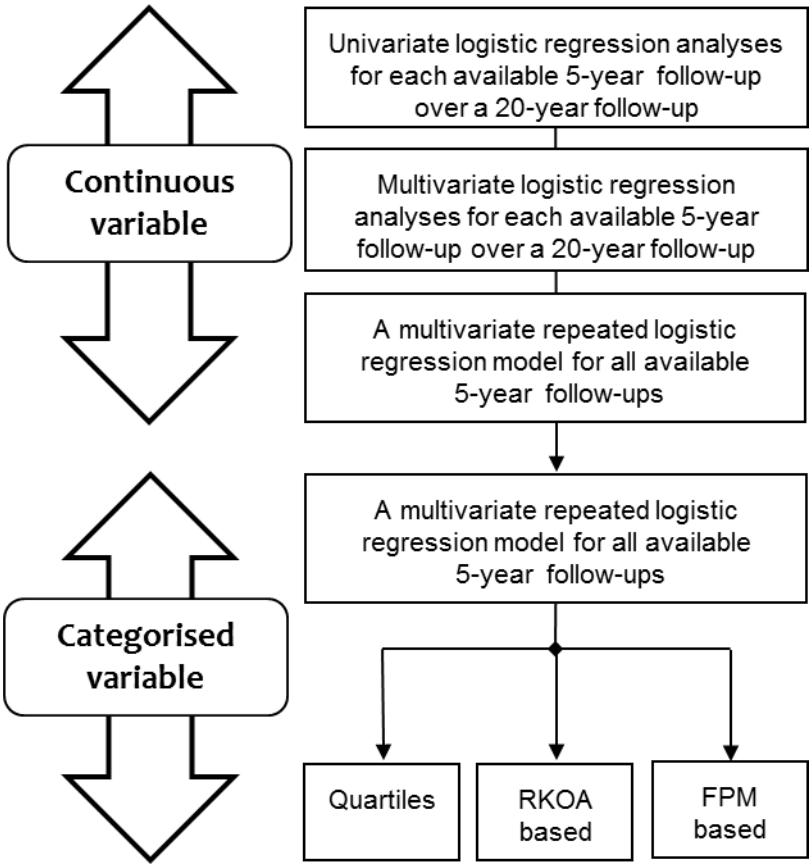
Repeated measures logistic regression models were used where the outcomes were recorded at 5, 10, 15 and 20-year follow-ups to identify variables potentially able to predict the incidence of RKOA generally or painful RKOA. The primary independent variable in those models was the presence of baseline serum concentrations of selected biomarkers. One of the assumptions of the repeated measures logistic regression model is that there I no interaction with time, i.e. association with outcome is constant over follow-up time points.

The repeated measures logistic regression models estimated the impact of predictors on the average outcome over the four follow-up time-points. It is reasonable to assume that biomarkers might only have a long, medium or short-term predictive ability. Therefore, assessment of associations at different follow-up time-points was needed to compare predictive trends. Only consecutive follow-ups with outcomes showing similar associations were included in the repeated measures logistic regression models. For doubtful cases, where wide confidence intervals raised the possibility of unusual trends being incidental findings, a regression model was used to assess interaction with time.

The results of the uni and multi-variate models were presented and compared. Risks were reported using ORs and adjusted predictions for each biomarker category in the quartile or dichotomised models during 5-year intervals.

Multivariate repeated-measures logistic regression models were used where the outcomes were recorded at 5, 10, 15 and 20-year follow-ups or for the selected follow-up time-points with a similar association with the outcomes. All identified selected cut-off points were utilised to categorise continuous variables. Both continuous and categorised independent variables based models were used to examine the association between biomarkers and RKOA or painful RKOA.

Figure 6.5. Analyses performed for each biomarker, separately for RKOA and painful RKOA.



Two univariate analyses were conducted to determine the relationship between BMI, age at baseline and either RKOA and painful RKOA. The association

showed that BMI and age have conformed to the linearity gradient. Results of those analyses are presented in the appendix.

6.3 Incidence of radiographic knee osteoarthritis

The overall aim of this part of the study was to examine whether each of the selected biomarkers can independently predict RKOA, but also how statistical models using three different methods of categorising biomarker data perform against the models utilising a continuous biomarker variable.

6.3.3 Introduction

While association between some of the selected biomarkers and 10 years' risk of incidence of RKOA has been investigated before [171], the relationship with the risk of developing RKOA over 20 years in 5-year intervals has not been studied in a cohort of middle-aged women.

In the COMP analyses below, all the results are presented in tables and graphically, using figures. In the analyses of other biomarkers, only figures with significant or interesting results are presented.

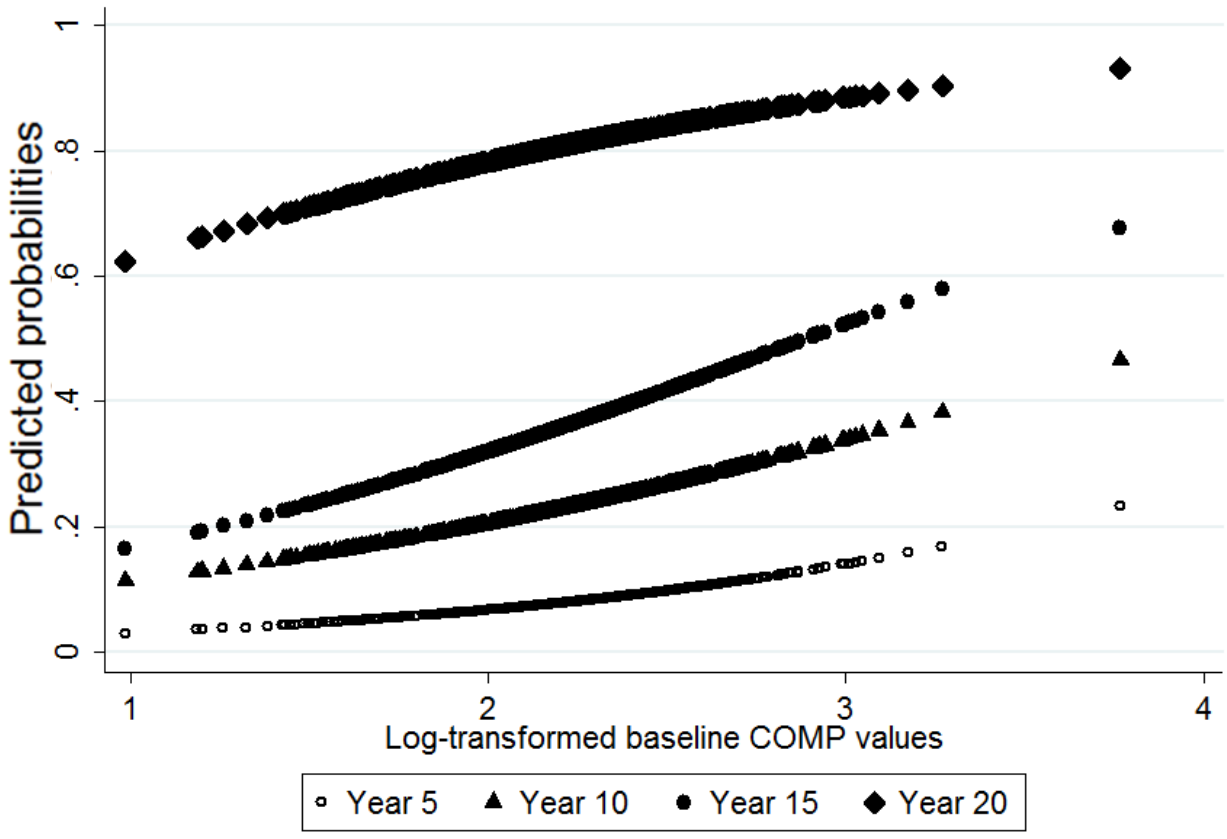
6.3.4 Serum COMP

6.3.4.1 Continuous variable models

The univariate analysis of the incidence for the COMP predictive model showed a strong statistically significant predictive value for the incidence of RKOA during follow-up appointments at 10 and 15 years. The association between the

independent biomarker and the outcome have similar magnitude and distribution during each follow-up appointments in both univariate and multivariate models (Figure 6.6 and Figure 6.7).

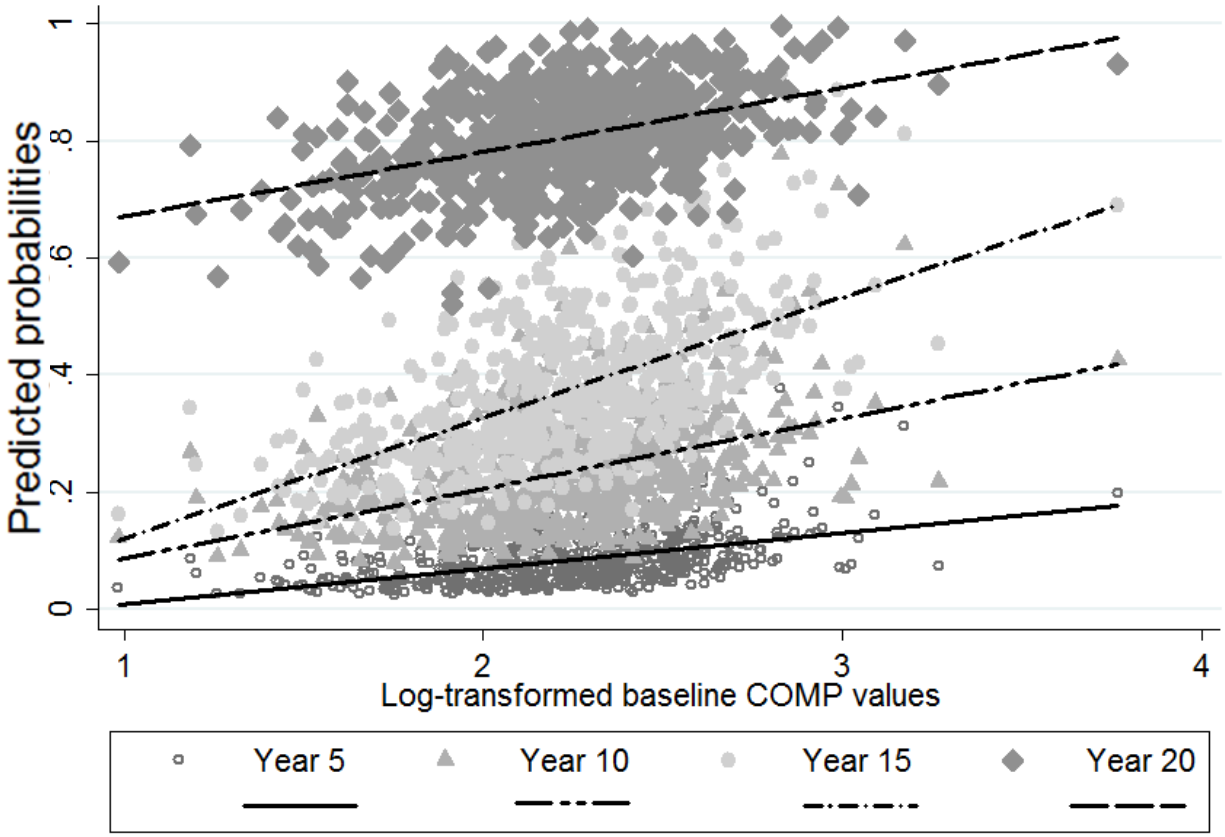
Figure 6.6. Univariate models looking at the associations between COMP values and probability of developing RKOA during each of the four follow-up points, with the results of univariate logistic regression for each time-point of follow-up below.



	Year 5	Year10	Year 15	Year 20
COMP OR(95%CI)	2.3 (0.93 -5.44)	2 (1.13 -3.52)	2.33 (1.3 -4.2)	2.13 (0.97 -4.7)

Univariate analysis showed a similar association during each follow-up time, with a statistically significant association for years 10 and 15.

Figure 6.7. Multivariate models looking at associations between baseline COMP values and the probability of developing RKOA during each of the four follow-up points in the models adjusted for baseline age and BMI, with results of multivariate logistic regression for each time-point of follow-up below.

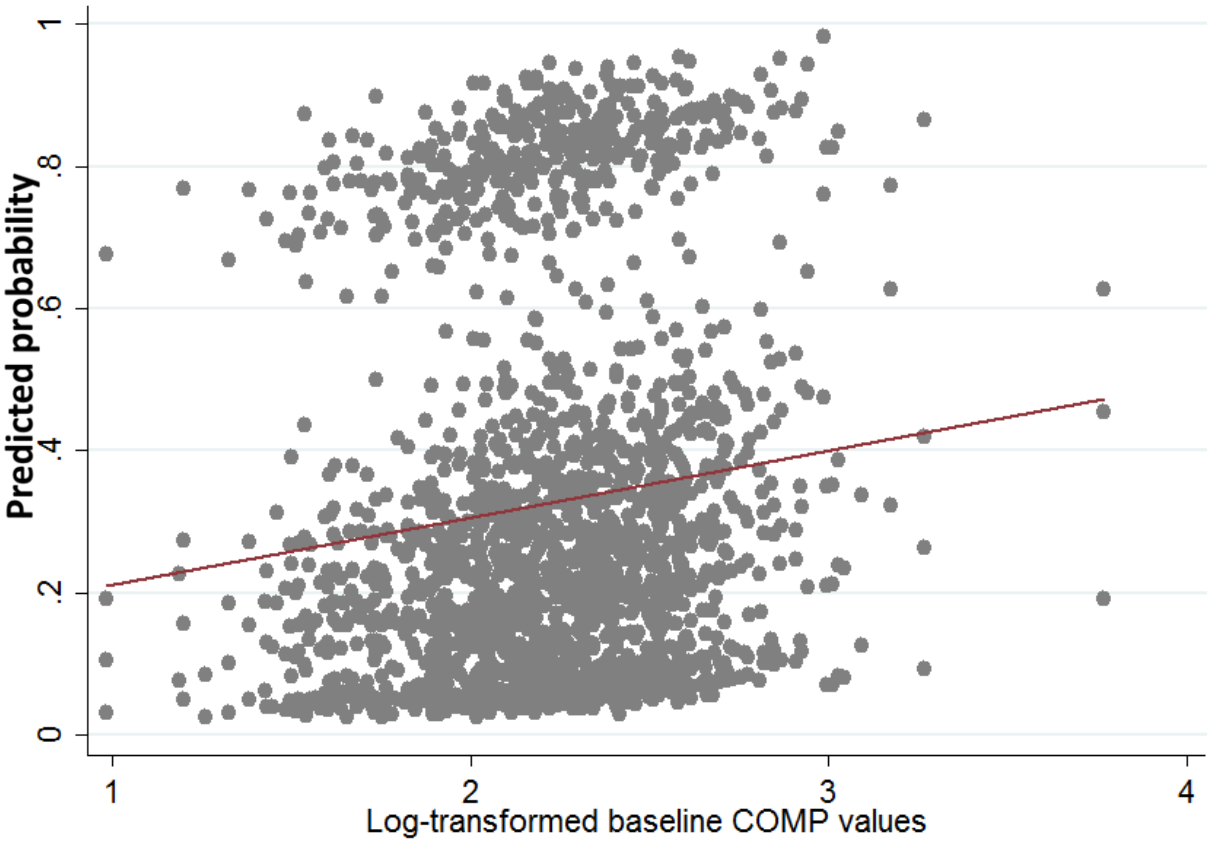


	Year 5	Year10	Year 15	Year 20
COMP OR (95%CI)	1.5 (0.6-3.77)	1.38 (0.76-2.48)	1.8 (0.99-3.32)	1.18 (0.73-3.66)

As highlighted in the table below figure 6.7, when logistic regression was performed for each follow-up in multivariable analyses, the results were borderline significant. A repeated logistic regression model for all time-points (5, 10,15 and 20 years), adjusted for age and BMI, showed a positive association between baseline serum COMP values and the risk of developing painful RKOA, with a cumulative OR of 1.55 and 95%CI 1.03- 2.32(p value 0.03).

A repeated logistic regression model had a greater power when compared to logistic models based on individual follow-up. Even after adjusting for BMI and age, it was still statistically significant (Figure 6.8).

Figure 6.8. Predictive probability of developing RKOA over 20 years based on baseline COMP serum concentrations and using multivariate repeated measures logistic regression model.



6.3.4.2 Categorised variable models

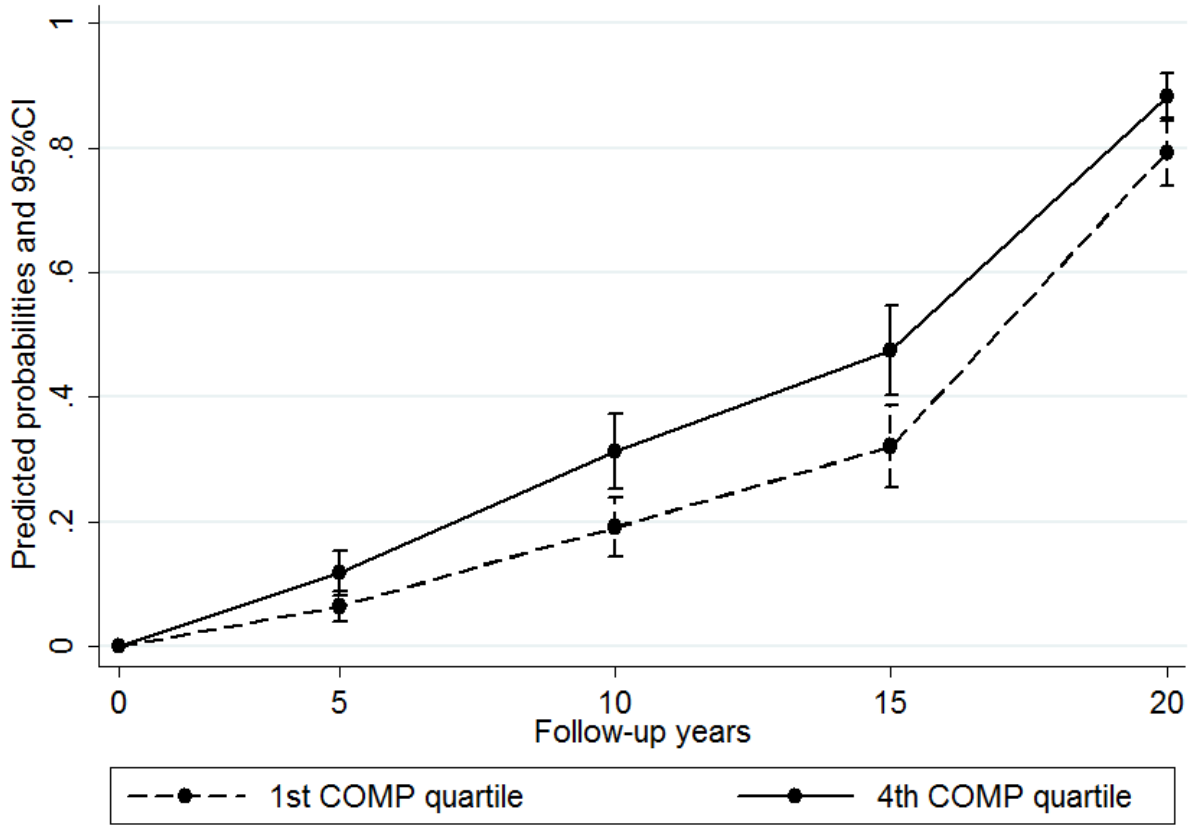
All models based on categorised independent variables were adjusted for baseline age and BMI. Results for each analysis are presented in the table below (table 6.6). The OR was consistently around 1.8.

Table 6.6. Summary of the predictive ability of three models with a categorical COMP variable defined by three different methods: quartiles of the COMP distribution, median value in the affected group (RKOA) and a fractional polynomial model (FPM).

	Quartiles (Q2-4) Q1: reference	**RKOA based**	**FPM**
COMP OR(95%CI)	Q2: 1.2 (0.8 -1.8) Q3: 1 (0.7 - 1.5) Q4: 2 (1.3 - 2.9)	1.7(1.3-2.2)	1.8 (1.3 - 2.5)

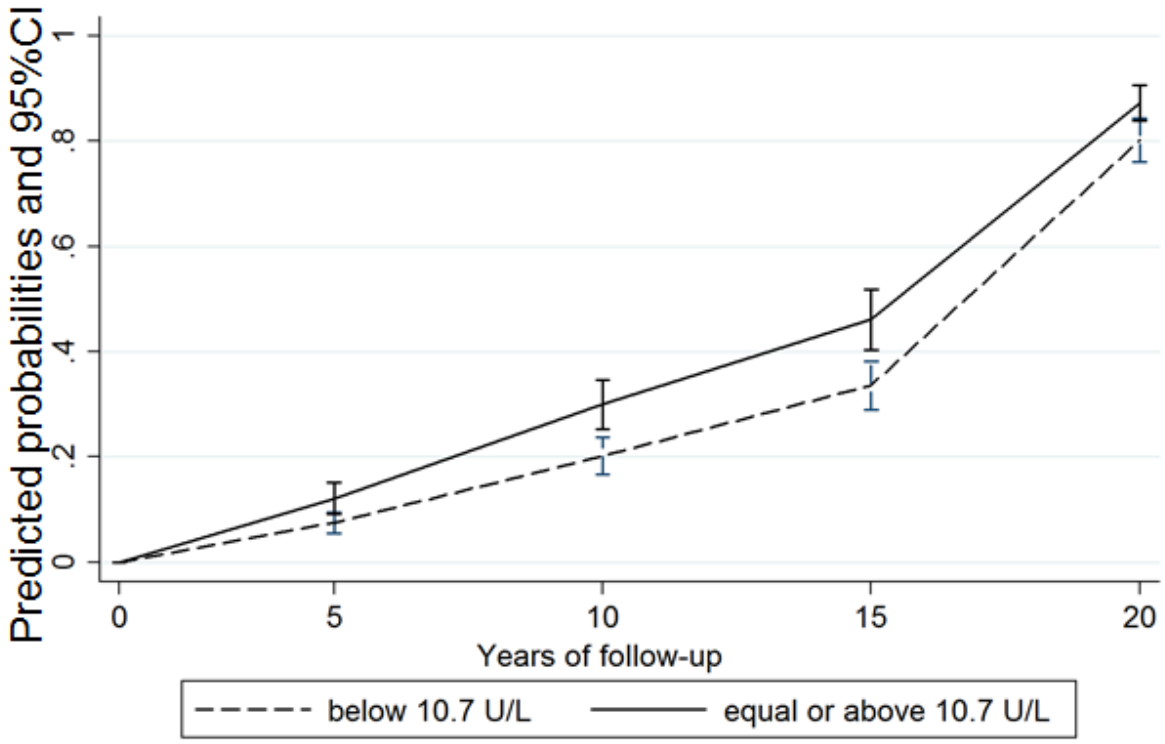
The model based on quartiles had the highest OR, but only when the 4th quartile was compared with the first. Results of the analysis for each time-point of follow-up are presented below (Figure 6.9).

Figure 6.9. Probability of developing RKOA during each follow-up visit for highest and lowest COMP quartile. Results adjusted for baseline age and BMI.



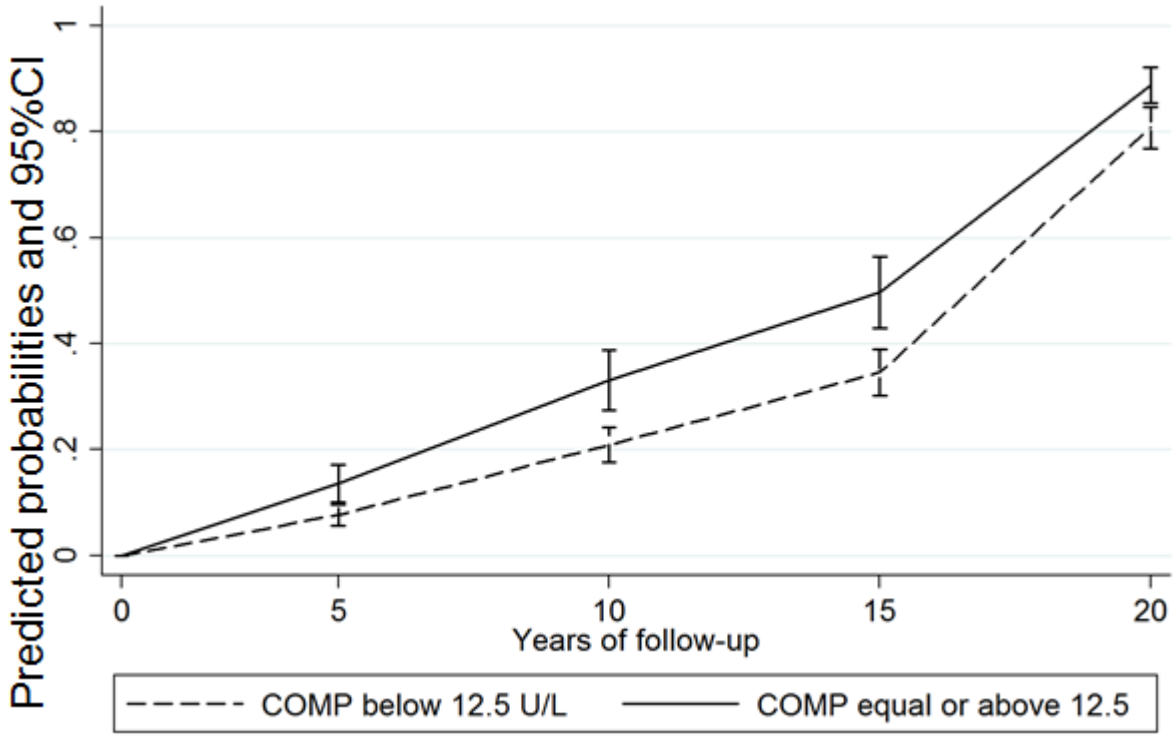
Categorising the COMP variable on the basis of the median value of COMP from the affected group with RKOA gave similar results, but unlike the model based on the highest and lowest quartile, this utilised the whole spectrum of biomarker data (Figure 6.10 and Figure 6.10).

Figure 6.10. Probability of developing RKOA during each follow-up visit for dichotomised COMP variable based on median value in the KOA group. Results adjusted for baseline age and BMI.



Here, we were able to identify a threshold associated with a higher risk of future incidence of RKOA: as predicted, the cut-off based on the fractional polynomial model performed better than the model using the cut-off point based on the median COMP value in the established RKOA group (Figure 6.11).

Figure 6.11. Probability of developing RKOA during each follow-up visit, for dichotomised COMP variable based on FPM. All results adjusted for baseline age and BMI.



6.3.4.3 Results

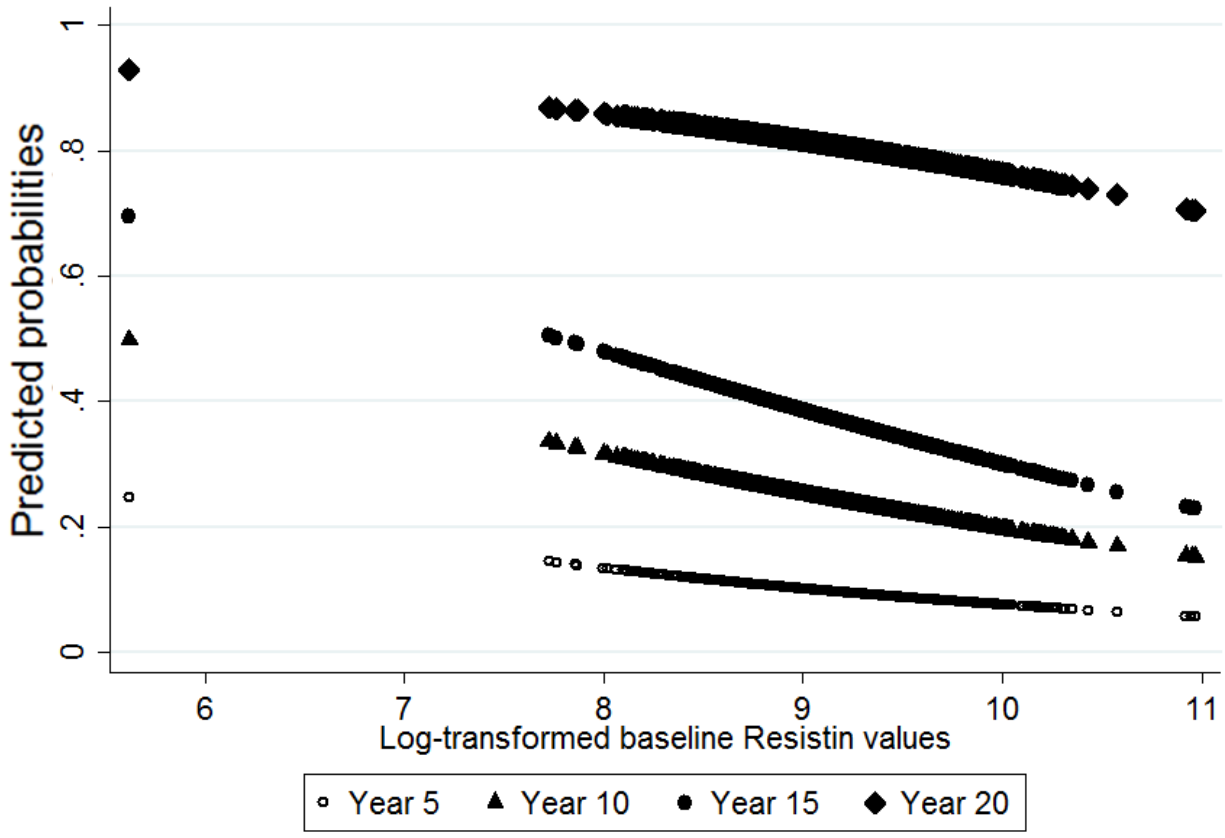
The high serum COMP concentration predicts the incidence of RKOA over 20 years, even when adjusted for baseline age and BMI, in a cohort of middle-aged women with no baseline RKOA. Continuous variables suggested an OR of 1.55, while categorised models showed similar results of OR from 1.7 and 2, with the model based on clinical association with RKOA being the closest to the results, using the continuous variable analysis.

6.3.5 Serum Resistin

6.3.5.1 Continuous variable models

There was a negative association between the higher serum Resistin concentration and incidence of RKOA, and this was similar for each of the follow-ups in unadjusted models (Figure 6.12), all showing a similar magnitude to that association.

Figure 6.12. Univariate models looking at the associations between baseline level of Resistin and subsequent risk of developing RKOA at each of the follow-up time-points.



	Year 5	Year10	Year 15	Year 20
Resistin OR(95%CI)	0.73 (0.4- 1.2)	0.73 (0.51-1.0)	0.7 (0.5 -1.0)	0.7(0.4- 1.2)

Multivariable analyses showed similar results for each of the follow-ups, with this being especially significant at the 10 and 15 year marks (Figure 6.13).

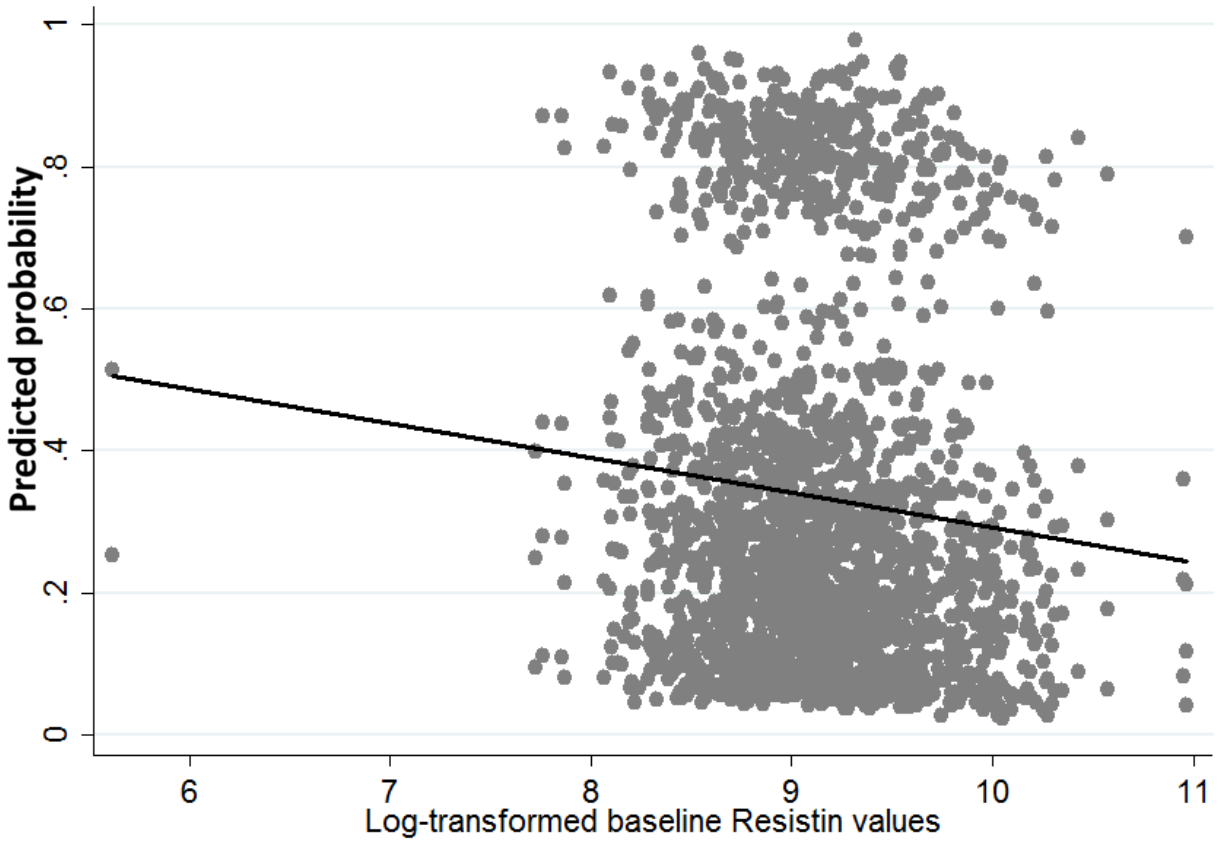
Figure 6.13. Multivariate models on the associations between baseline Resistin values and probability of developing RKOA during each of the four follow-ups in the models, adjusted for baseline age and BMI, with the results of multivariate logistic regression for each time-point of follow-up below.



Resistin	Year 5	Year10	Year 15	Year 20
OR (95%CI)	0.71 (0.4- 1.2)	0.7 (0.47-1.0)	0.68 (0.5 -1.0)	0.68(0.4- 1.2)

The final model in the analysis of Resistin as a continuous variable included all the follow-up points and was adjusted for age and BMI.

Figure 6.14. Predictive probability of developing RKOA over 20 years based on baseline Resistin levels and using multivariate repeated measures logistic regression model.



Results of the multivariate repeated- measures logistic regression model showed a statistically significant association, with overall OR of 0.73 and 95%CI 0.56 – 0.94 (p value 0.014). This is a very interesting, as Resistin is a relatively new

biomarker and has not been previously shown to have a role in the development of RKOA.

6.3.5.2 Categorised variable models

Cut-offs based on quartiles and on the median value for the RKOA group were used. Again, analyses of the quartiles confirmed the protective value of higher baseline Resistin concentration for incidence of RKOA over 20 years independently to age and BMI.

A dichotomised model based on the median value of Resistin in RKOA showed a similar trend but this was not significant.

	Quartiles (Q2-4) Q1: reference	**RKOA-based**
Resistin OR(95%CI)	Q2: 0.62 (0.4-0.9) Q3: 0.91 (0.6-1.3) Q4: 0.52 (0.4-0.7)	0.83 (0.64 -1.1)

6.3.5.3 Results

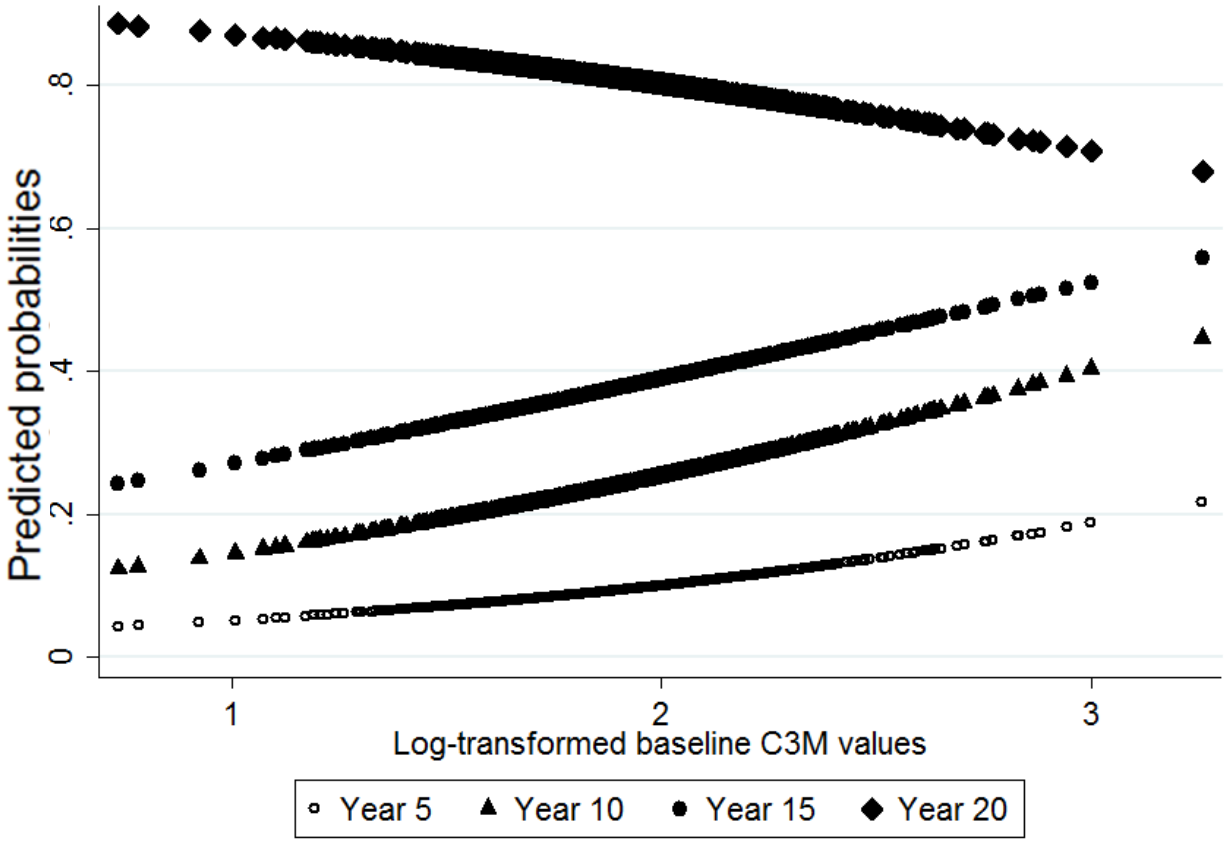
Higher baseline serum Resistin concentrations are protective against future development of RKOA. This has been confirmed using models adjusted for age and gender and using categorised data (quartiles) and a continuous variable (repeated logistic regression model).

6.3.6 Serum C3M

6.3.6.1 Continuous variable models

Due to the positive skewness of the data, the log-transformed values were utilised in the predictive models. The univariate analysis showed statistically significant positive associations for years 5, 10 and 15 of follow-up, but negative associations with the incidence of RKOA at year 20, which was not statistically significant (Figure 6.15).

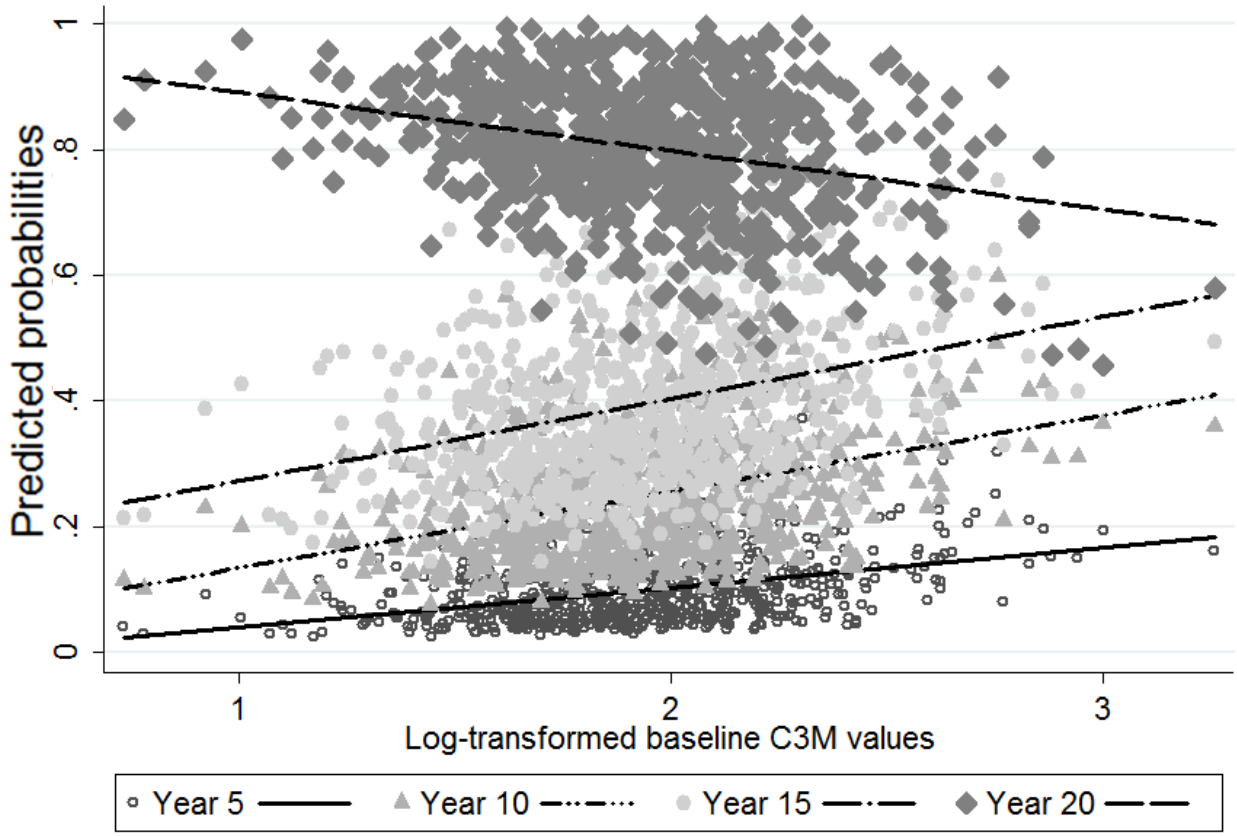
Figure 6.15. Univariate models looking at the associations between baseline level of C3M and subsequent risk of developing RKOA for each follow-up point.



	Year 5	Year10	Year 15	Year 20
C3M OR(95%CI)	2.1 (0.93- 4.6)	2 (1.1-3.5)	1.7 (0.96 -3.1)	0.6(0.2- 1.4)

To investigate this further, multivariate analyses were undertaken. These showed similar trends, with year 20 of follow-up exhibiting a negative trend and higher values of C3M (Figure 6.16). This suggested that C3M may be predictive in the short and medium-term, but not after 20 years of follow-up.

Figure 6.16. Association between baseline level of C3M and subsequent risk of developing RKOA for each of the follow-up points after adjusting models for baseline BMI and age.



	Year 5	Year10	Year 15	Year 20
C3M OR(95%CI)	1.6 (0.7- 3.6)	1.6 (0.9-2.8)	1.5 (0.8 -2.7)	0.5(0.2- 1.2)

The results of multivariable logistic regression for follow-up at years 5 to 15 were similar, with OR between 1.6 and 1.5. Adjusting for age and BMI moved this trend from being statistically significant to not significant. Results for year 20 again indicated a different association (Figure 6.11 and 6.12). The regression analysis confirmed this significant interaction with time for the year 20 follow-ups, and for that reason the final multivariate repeated measures logistic regression analysis included only years 5,10 and 15 of follow-up. This statistical model, adjusted for age and BMI, showed a positive but borderline significant association between baseline serum C3M values and the risk of developing RKOA over 15 years, with a cumulative OR of 1.35 and 95%CI 0.83- 2.20 (p value 0.22).

6.3.6.2 Categorised variable models

All models based on categorised independent variables were adjusted for baseline age and BMI and showed similar results, with an average OR of 1.25 (Table 6.8). Models based on quartiles had the highest OR, but only when the highest quartile was compared with the lowest.

Table 6.8. Summary of predictive ability of three models with categorical C3M variables, defined and based on three different methods: quartiles of the C3M distribution, the median value in the affected group (KOA) and the fractional polynomial model (FPM).

	Quartiles (Q2-4) Q1: reference	**KOA based**	**FPM**
C3M OR(95%CI)	Q2: 0.8 (0.56-1.2) Q3: 0.92 (0.6-1.3) Q4: 1.3 (0.93-2)	1.1 (0.9-1.5)	1.35 (0.98-1.84)

6.3.6.3 Results

The analyses performed suggested that C3M is a weak predictive biomarker for RKOA in middle-age women. Adjusting C3M models for the baseline age and BMI was associated with similar trends but those were not statistically significant. The repeated logistic regression model based on continuous biomarker variable showed a positive association between C3M and the probability of incidence of RKOA over 15 years, with an OR of 1.35 and again this was not statistically significant. Interestingly, logistic regression for year 20 showed a very different trend to other follow-up points. Considering that previous analyses of COMP and Resistin showed consistent associations at each 5-year follow-up and C3M models are based on the same outcome and dataset, year 20 results are unlikely to be explained by losses to follow-up. It is likely that C3M has only a medium term predictive ability, or there is another potential

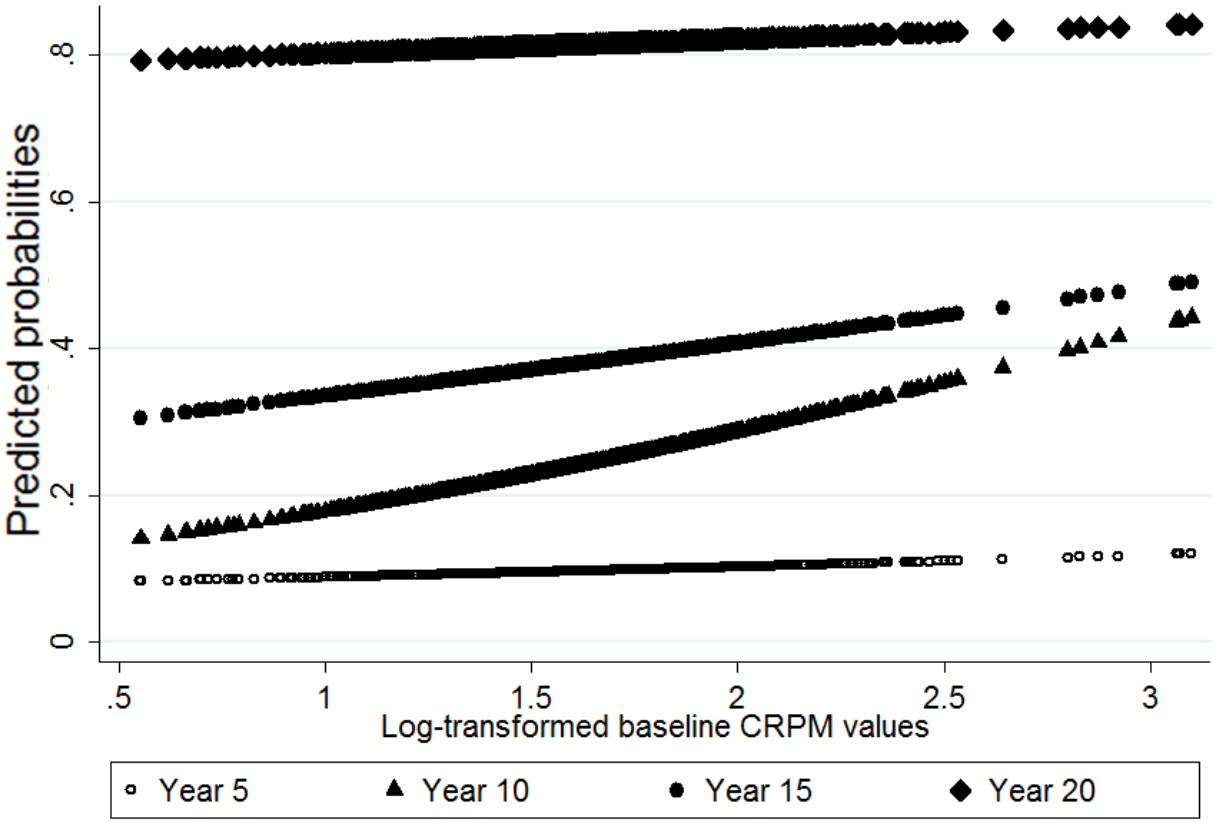
residual confounder linked with high levels of C3M. Both age and gender are weakly associated with C3M levels, but they do not explain the different association with year 20 follow-up.

6.3.7 Serum CRPM

6.3.7.1 Continuous variable models

The univariate analysis showed a positive association for predicting the incidence of RKOA after five, 10, 15 and 20 years. The association was much stronger for years 10 and 15 of the follow-up, with similar results (figure 6.17).

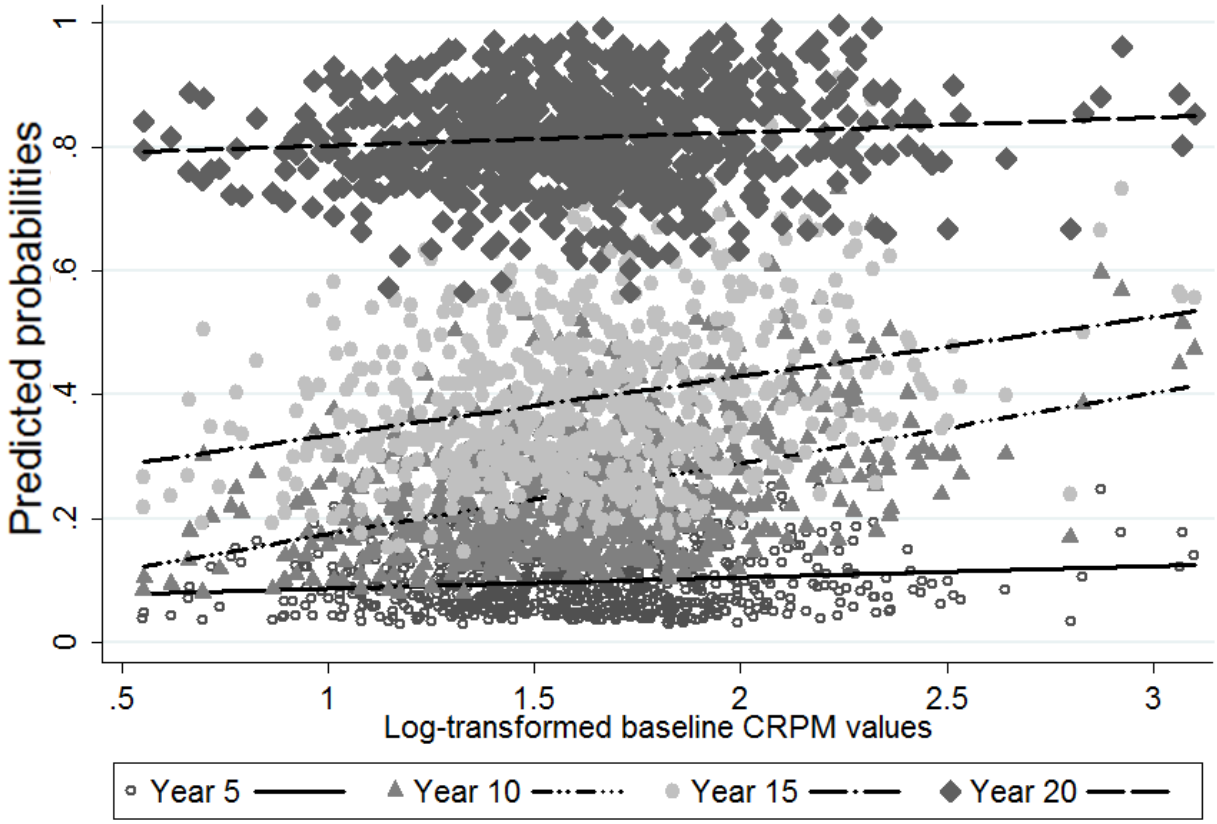
Figure 6.17. Association between probability of developing RKOA during four follow-up points and baseline concentrations of CRPM. Results of univariate logistic regression analyses for each year of follow-up are presented below the table.



	Year 5	Year10	Year 15	Year 20
CRPM OR(95%CI)	1.9 (0.46-7.5)	2.3 (1.1- 4.8)	1.9 (0.99-3.5)	1.2 (0.65-2.1)

Differences were yet more pronounced in the multivariable model, with year 5 and 20 exhibiting much weaker association and statistical power (Figure 6.18).

Figure 6.18. Association between the probability of developing RKOA during four follow-up points and baseline levels of CRPM, adjusted for baseline age and BMI.



	Year 5	Year10	Year 15	Year 20
CRPM OR(95%CI)	1.3 (0.32-5.6)	1.8 (0.8-3.9)	1.6 (0.8-3.0)	1.1 (0.6 -2.0)

Based on the fact that only year 10 and 15 of follow-up showed similar associations with incidence of RKOA, data from these follow-up was included in the multivariate repeated logistic regression model. This showed a positive trend

that was not statistically significant, with a cumulative OR of 1.4 and a 95% CI 0.88 - 2.15 (p value 0.16).

6.3.7.2 Categorised variable model

Baseline CRPM results were categorised on the basis of distribution, using quartiles, and dichotomised on the basis of the cut-off point derived from the FPM. As there was no significant difference in terms of median values of serum CRPM between individuals with RKOA and individuals without it, no threshold based on this criterion was established or tested.

Table 6.9. Summary of the predictive ability of two models with a categorical CRPM variable based on two different methods: quartiles of the CRPM distribution and the fractional polynomial model (FPM).

	Quartiles (Q2-4) Q1: reference	**FPM**
CRPM OR(95%CI)	Q1: 1.1 (0.7-1.8) Q2: 1.5 (0.9-2.4) Q3: 1.4 (0.8-2.2)	1.4 (0.9-1.9)

Interestingly, FPM provided the highest predictive value from all models based on the categorisation of the independent variable. However, the result was very similar to the outcome of CRPM as a continuous variable (Table 6.9).

6.3.8 Summary of results

The analysis of the four selected serum-biomarkers suggested the good independent predictive ability of high serum COMP levels with regard to the short and long-term development of RKOA, independently of baseline age and BMI, in a middle-aged population of women with no baseline RKOA, followed up for 20 years. There was an opposite association, with higher values linked to a lower risk of incidence of RKOA, with regard to Resistin, this being statistically significant in multivariable models.

C3M and CRPM also initially suggested good predictive and statistically significant associations with the incidence of RKOA. However, this was only seen in some unadjusted models. Moreover, both C3M and CRPM showed only medium-term predictive value (15 years and below) in terms of incidence of RKOA. This cannot be explained by the loss of follow-up bias, as the same data set showed similar trends in each follow-up time-point for other biomarkers. The assumption underlying this thesis is that measuring biomarkers associated with inflammatory response and knee pain at baseline would enable us to identify the different trajectories of KOA risk and associated mortality. It is plausible that, for some biomarkers, a single measure is only able to predict mid-term outcomes or there is another potential residual confounder.

Results of the multivariable models based on the categorised data were very similar to the same models with independent continuous biomarker data (Table 6.10). More importantly, none of the results based on categorised biomarker data

were outside the 95% confidence interval for the results based on statistical model using continuous data.

Tables 6.10. Comparison of results from multivariable models, using continuous and categorised independent variable. Highlighted boxes contain data with the result closest to the model based on the continuous variable.

	Continuous variable, OR (95% CI)	**Quartiles, OR**	**KOA, OR**	**FMA, OR**
COMP	1.5 (1.03-2.23)	2	1.7	1.8
Resistin	0.73(0.56–0.94)	0.5	0.83	N/A
C3M	1.35(0.83- 2.20)	1.3	1.1	1.35
CRPM	1.4 (0.86 - 2.13)	1.45	N/A	1.42

Considering that categorised models are far easier to utilise in clinical practice and interventional research, this provides evidence that, for these biomarkers, this is a reasonable method of utilising categorised biomarkers.

6.4 Incidence of painful radiographic knee osteoarthritis

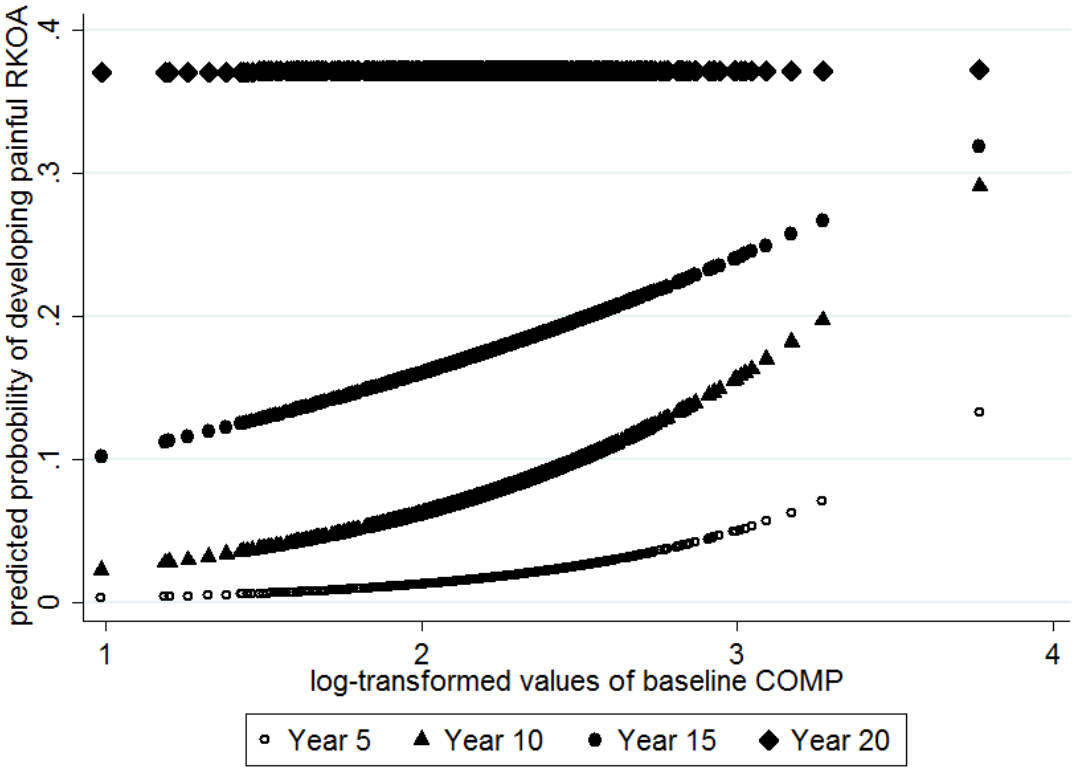
The overall aim of this part of the study was not only to examine whether each of the selected biomarkers would independently predict painful RKOA, but also consider how statistical models based on three different methods of categorising biomarker data would perform against the models utilising continuous biomarker variables.

6.4.3 Serum COMP

6.4.3.1 Continuous variable models

Univariate analyses of baseline COMP concentration showed a similar trend, with the risk of painful RKOA for follow-up time-points 5 to15 years of follow-up. However, year 20 follow-up gave a different result, showing no association between the independent variable and incidence of painful RKOA (Figure 6.20).

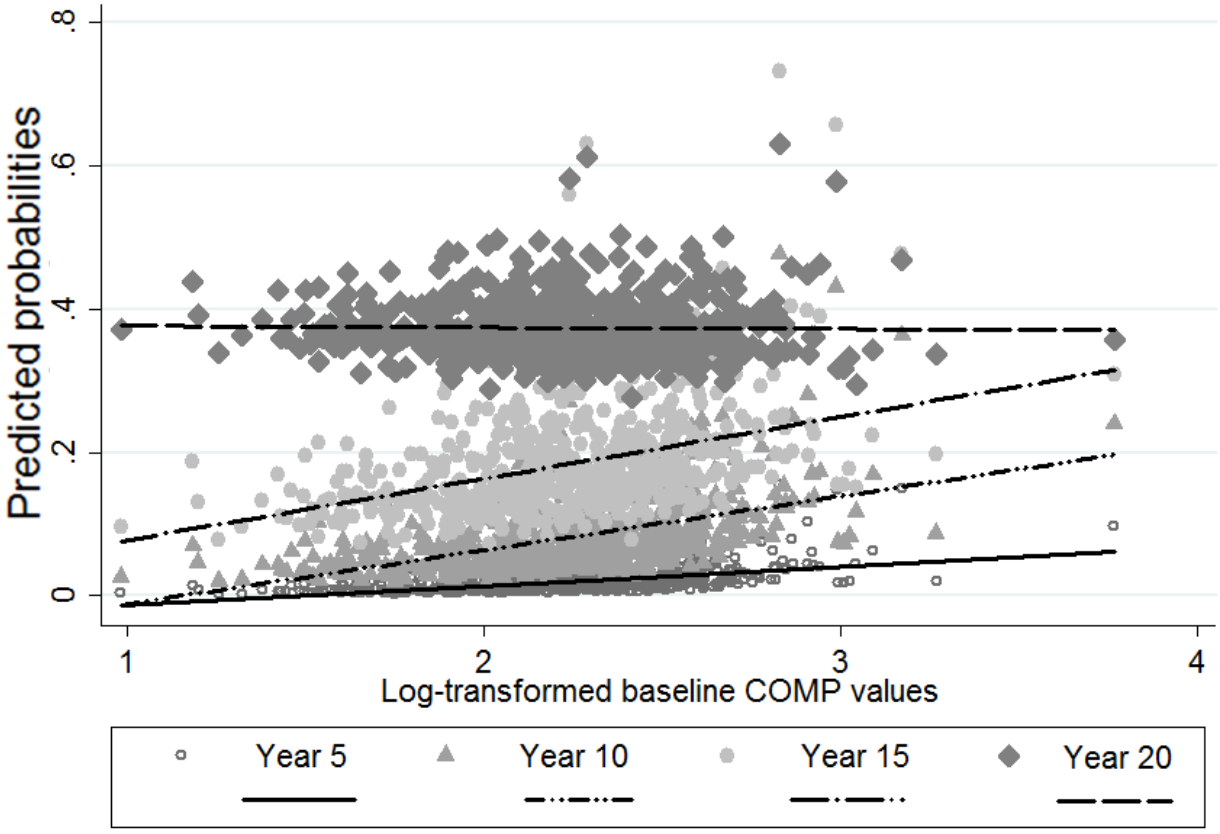
Figure 6.20. Association between baseline levels of COMP and probability of developing painful RKOA at four follow-up time-points.



	Year 5	Year10	Year 15	Year 20
COMP OR(95%CI)	4 (0.7-23)	2.8 (1.2-6.7)	1.7 (0.8-3.4)	1 (0.5-1.9)

After adjusting for baseline BMI and age, follow-ups at years 10 and 15 showed simmilar associations, unlike years 5 and 20 (Figure 6.21).

Figure 6.21. Association between baseline levels of COMP and probability of developing painful RKOA over four follow-up points, adjusted for baseline age and BMI.



	Year 5	Year10	Year 15	Year 20
COMP OR(95%CI)	2.3 (0.4-14.9)	1.8 (0.7-4.5)	1.3 (0.6-2.8)	0.9 (0.5-1.7)

For that reason, only data from years 10 and 15 was included in the repeated logistic regression model adjusted for age and BMI. This showed a positive, though not statistically significant, trend in relation to baseline serum COMP values and the risk of developing painful RKOA, with a cumulative OR of 1.43 and 95%CI 0.73-2.8 (p value 0.3).

6.4.3.2 Categorised variable models

Results of the repeated logistic regression model, adjusted for baseline age and BMI and using data categorised according to the distribution, mean value in the RKOA group and FPA were similar. None of these were statistically significant, apart from dichotomised model based on median values in RKOA group (Table 6.11).

Table 6.11. Summary of the predictive ability of three models with the categorical COMP variable, based on two different methods: quartiles of the COMP, median value of COMP in RKOA and the fractional polynomial model (FPM).

	Quartiles (Q2-4) Q1: reference	**KOA based**	**FPM**
COMP OR(95%CI)	Q2: 0.8 (0.4-1.6) Q3: 1.0 (0.5-1.9) Q4: 1.7 (0.9-3.2)	**1.7 (1.1-2.7)**	1.1(0.6-2)

6.4.3.3 Results

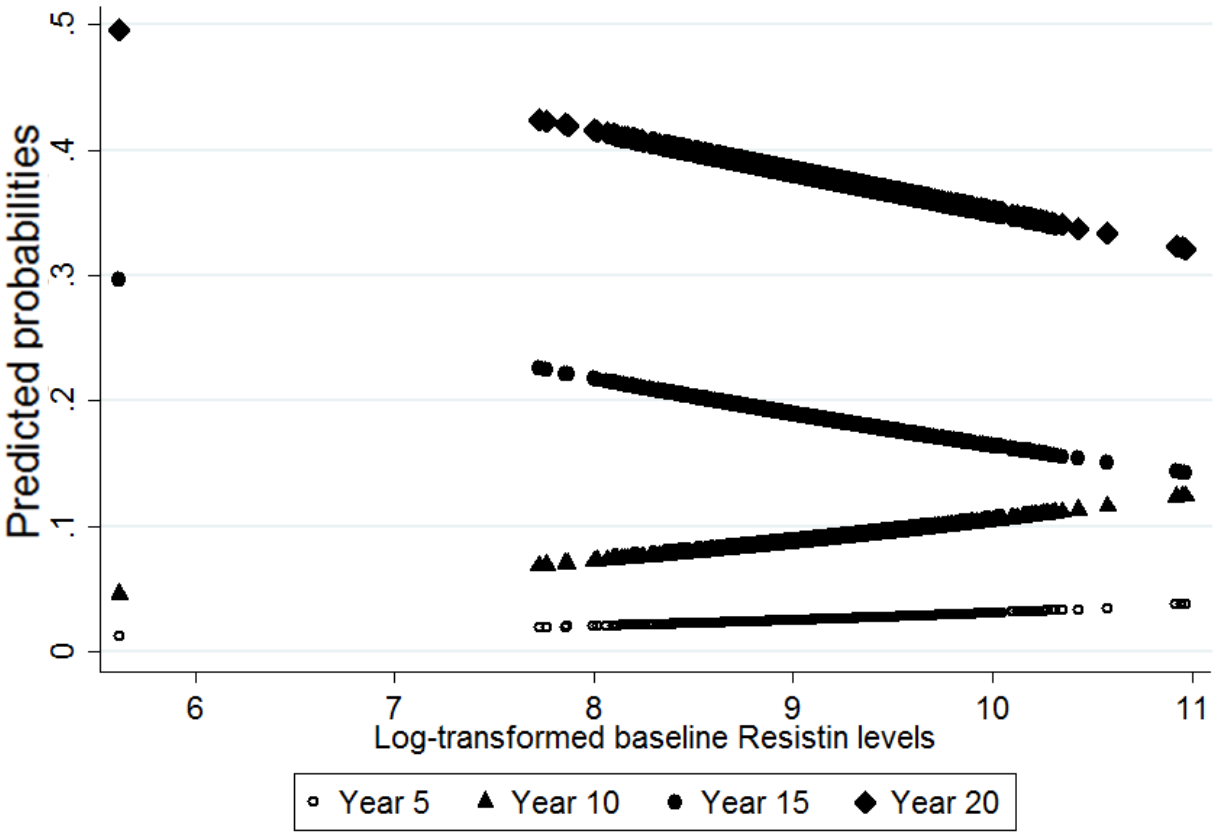
Baseline serum COMP levels in a population without RKOA seemed to have a mild positive predictive ability for 10-15 years' incidence of RKOA. This was only significant in a univariate model but, interestingly, also in a multivariable model based on the median value of COMP in RKOA group. This suggests that COMP is a better predictor of radiographic changes rather than painful RKOA.

6.4.4 Serum Resistin

6.4.4.1 Continuous and categorised variable models

The analyses examining trends between baseline levels of Resistin and the probability of developing painful RKOA, suggested that during the 5 and 10 year of follow-up, the association was mildly positive, but during the 15 and 20 years of follow-up it has a negative (Figure 6.22). The univariate logistic regression models confirmed that and showed that these associations were not statistically significant.

Figure 6.22. The univariate logistic regression models looking at associations between baseline levels of Resistin and the probability of developing painful RKOA.



	Year 5	Year10	Year 15	Year 20
Resistin OR (95%CI)	1.2 (0.4-3.4)	1.2 (0.7-2.1)	0.8 (0.5-1.3)	0.9 (0.6-1.37)

To assess if the trends above were associated with significantly different results in the combined multivariable models, regression analysis was performed for interaction with time. This showed no significant interaction with time between follow-up time points. Subsequently, a combined model with all four time-points

was tested. Results of this repeated logistic regression model, adjusted for baseline age and BMI showed OR 0.97 and 95%CI 0.7-1.3. Results of multivariable statistical models with categorised biomarker variable were similar (Table 6.12).

Table 6.12. Summary of the predictive ability of two models using a categorised Resistin level based on quartiles and the median value of Resistin in RKOA.

	Quartiles (Q2-4) Q1: reference	**RKOA-based**
Resistin OR(95%CI)	Q2: 0.8 (0.5-1.2) Q3: 0.8 (0.5-1.3) Q4: 0.9 (0.6-1.4)	0.9 (0.6-1.2)

6.4.4.2 Results

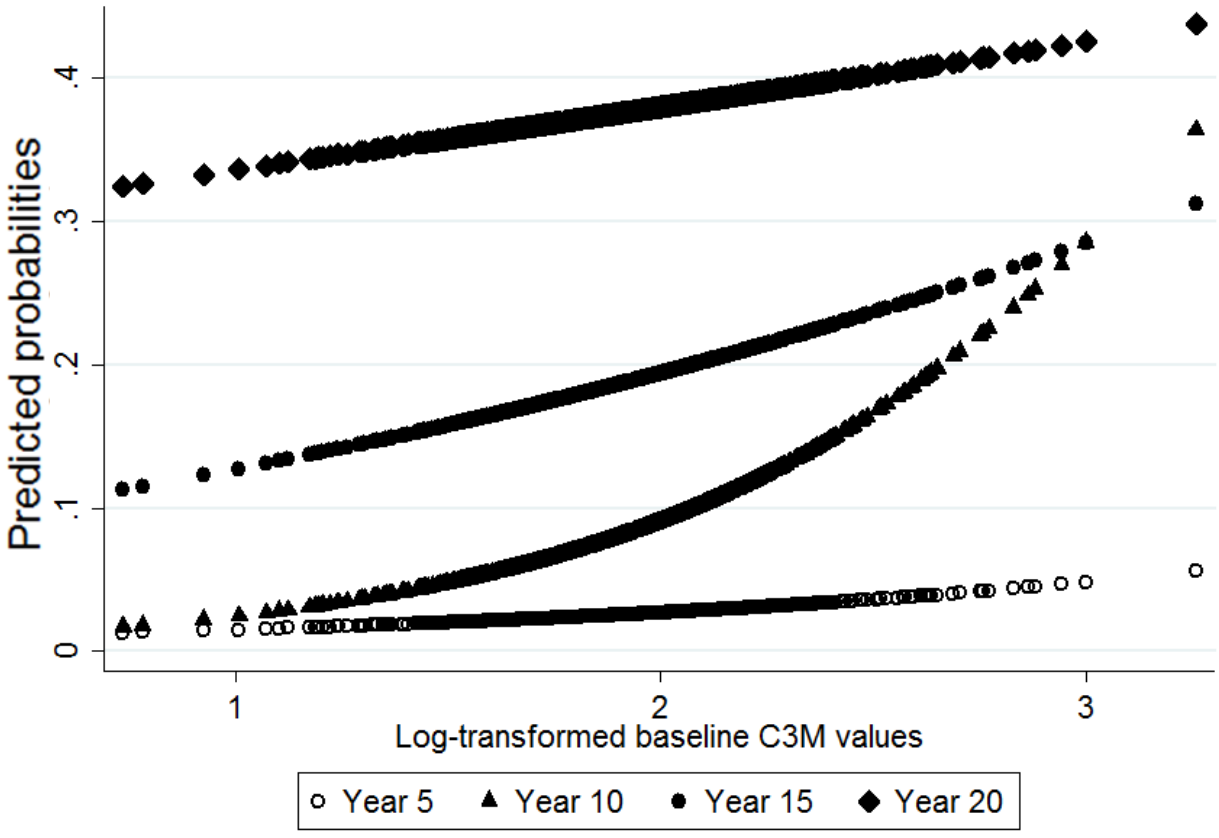
Baseline serum Resistin levels did not predict the incidence of painful RKOA over 20 years in the study's population of middle-aged women with no baseline RKOA, even in unadjusted models. Serum concentration of Resistin might have a protective role for incidence of RKOA, but it is not associated with an incidence of painful RKOA.

6.4.5 Serum C3M

6.4.5.1 Continuous variable models

The univariate analyses suggested that C3M has a predictive ability for incidence of RKOA over 10 years (Figure 6.23). The association between baseline levels of this biomarker and the risk of development of RKOA 10 years later was found to be positive in an unadjusted model with an OR of 4.0 (95%CI 1.7-9.4). This association was still very significant after adjusting for baseline age and BMI (OR 3.2, 95%CI 1.34-7.6).

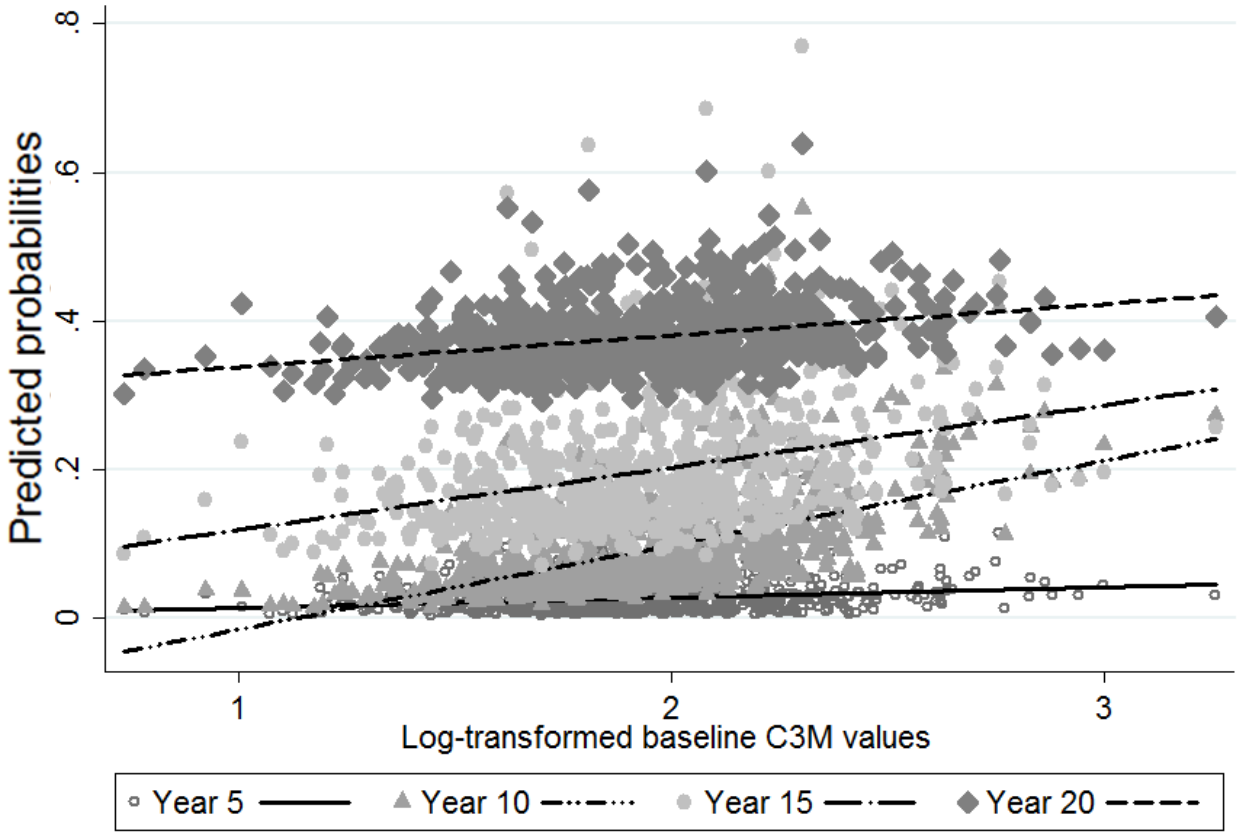
Figure 6.23. Univariate models looking at the associations between baseline levels of C3M and the probability of developing painful RKOA at four follow-up time-points, in an unadjusted model.



	Year 5	Year10	Year 15	Year 20
C3M OR (95%CI)	1.8 (0.4-8.9)	4 (1.7-9.4)	1.6 (0.8-3.36)	1.2 (0.6-2.4)

Multivariable models confirmed that the association between baseline levels of C3M were strong for outcomes over 5 years. The association with year 5 outcomes was different and, for that reason, was not included in the repeated logistic regression model (Figure 6.24)

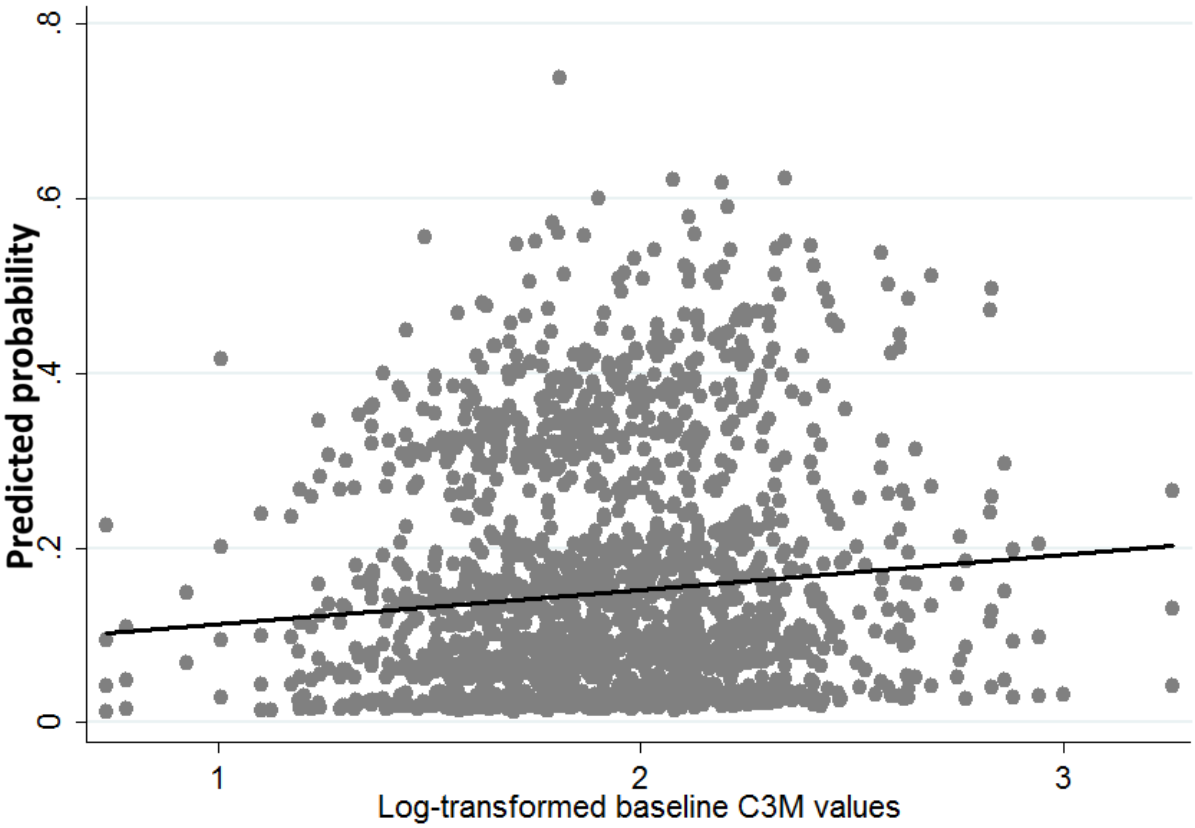
Figure 6.24. Multivariate models looking at the associations between baseline levels of C3M and the probability of developing painful RKOA over four follow-up points, adjusted for baseline age and BMI.



	Year 5	Year10	Year 15	Year 20
C3M OR(95%CI)	1.3 (0.3-6.2)	3.2 (1.3-7.6)	1.5 (0.7-3.14)	1.2 (0.6-2.3)

Results of the repeated logistic regression model adjusted for baseline age and BMI for continuous variables showed a borderline significant OR of 1.62 with 95%CI between 0.95 and 2.7 (p value 0.07) for combined follow-up outcomes at 10, 15 and 20 years.

Figure 6.25. Association between baseline levels of C3M and probability of developing RKOA over 20 years. Model adjusted for baseline BMI and age.



6.4.5.2 Categorised variable models

Baseline C3M results were categorised according to distribution, by using quartiles and dichotomised on the basis of the cut-off points. In one case, this used the mean value in the RKOA group and was derived from the FPM in the other. Similar results to the combined model based on the continuous independent variable were obtained but, interestingly, those were more statistically significant (Table 6.13).

Table 6.13. Summary of predictive ability of three models adjusted for age and BMI, with the C3M variable categorised on the basis of three different thresholds: quartiles, the median value of C3M in KOA group and the fractional polynomial model (FPM).

	Quartiles (Q2-4) Q1: reference	**KOA based**	**FPM**
C3M OR(95%CI)	Q2: 0.8(0.4-1.4) Q3: 1.0 (0.6-1.9) Q4: 1.7(0.9-2.9)	**1.6 (1.1-2.5)**	**1.7 (1.1-2.8)**

6.4.5.3 Results

Serum concentration of C3M was found to be predictive for the subsequent development of painful RKOA during the 10-year follow-up. This was still highly significant, even after adjusting for baseline age and BMI. The repeated multivariable logistic regression models, where cut-off points were based on the

median value in the RKOA group or the FPM model, taking into account follow-up results above the 5-year mark, showed a similar positive independent and statistically significant associations. The same model using independent continuous C3M variable showed similar association but borderline significant (p value 0.07).

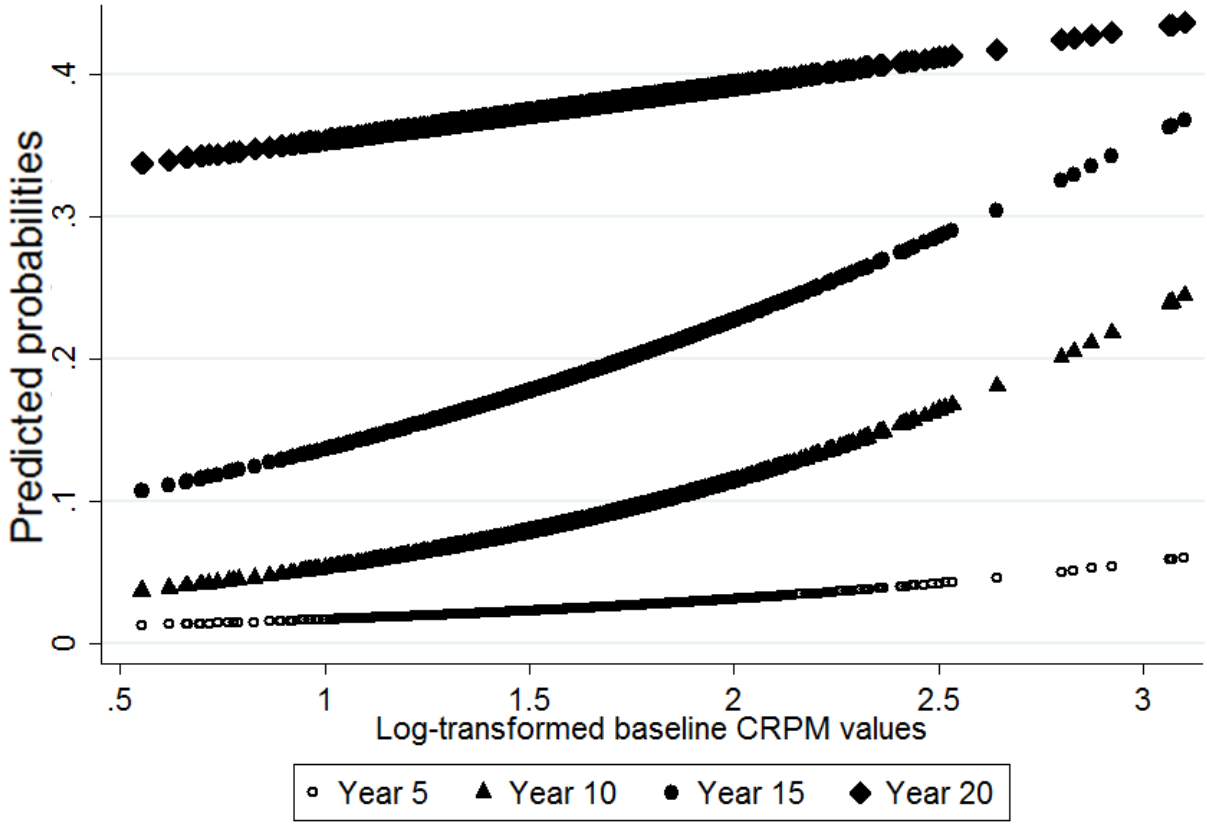
In summary, C3M is a good predictive marker for the incidence of painful RKOA in both the medium and long-term, but it has the strongest predictive value for 10-year risk.

6.4.6 Serum CRPM

6.4.6.1 Continuous variable models

The univariate analyses showed a positive association for predicting the incidence of RKOA after 5, 10, 15 and 20 years. However, the association with years 5 and 20 of follow-up (Figure 6.26) was not as strong as with 10 and 15.

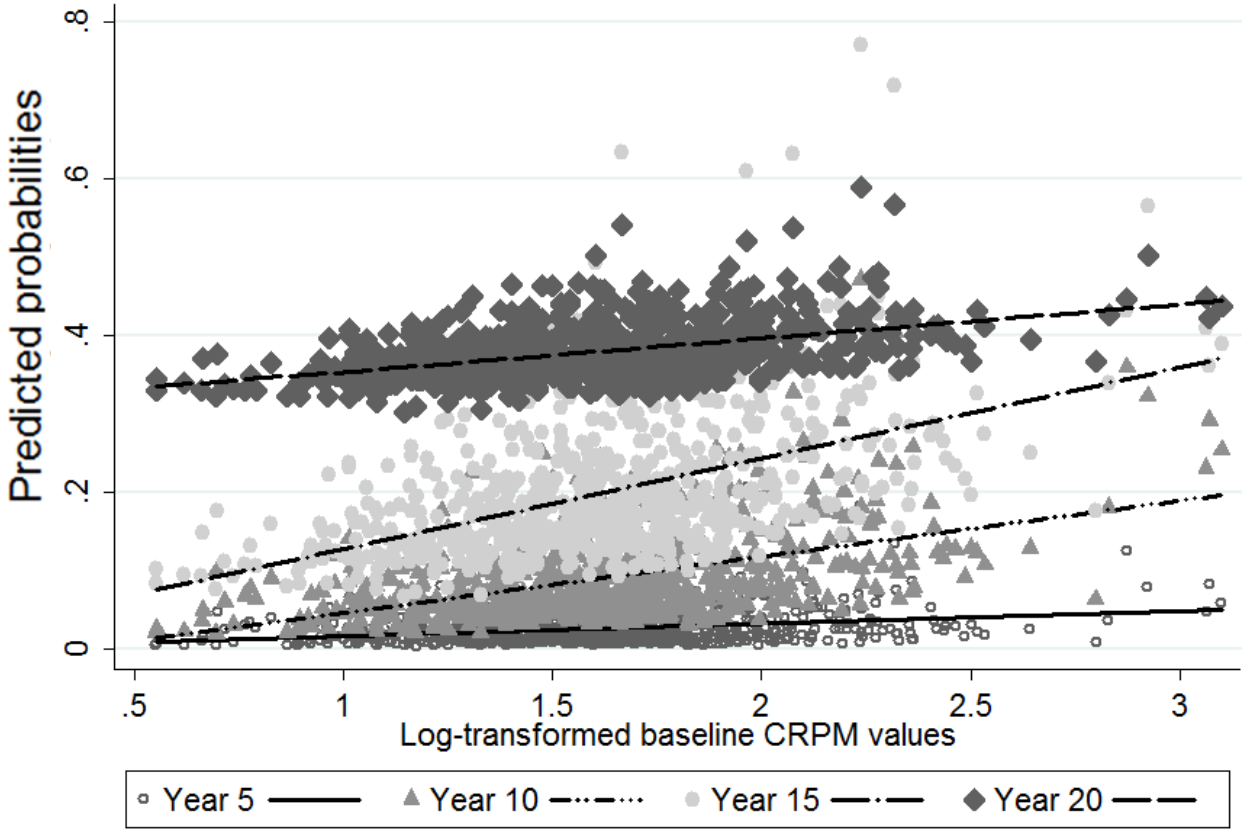
Figure 6.26. Univariate models looking at the associations between the probability of developing painful RKOA during four follow-up points and baseline levels of CRPM.



	Year 5	Year10	Year 15	Year 20
CRPM OR(95%CI)	1.8 (0.5-1.32)	2.3 (1.1-4.8)	1.89 (0.99-3.47)	1.2 (0.6-2.15)

Adjusting for age and BMI further emphasises those differences (Figure 6.27).

Figure 6.27. Multivariate models looking at the associations between baseline levels of CRPM and the probability of developing painful RKOA over four follow-up points, adjusted for baseline age and BMI.



	Year 5	Year10	Year 15	Year 20
CRPM OR(95%CI)	1.3 (0.3-5.6)	1.8 (0.9-3.9)	1.6 (0.8-3.0)	1.1 (0.6-2)

For that reason, the repeated logistic regression model combining results from follow-up appointments at years 10 and 15. The statistical model adjusted for baseline age and BMI, showed a positive, though borderline significant, association with an OR of 1.73 with 95%CI between 0.98-3.1.

6.4.6.2 Categorised variable models

Baseline CRPM results were categorised on the basis of distribution, by using quartiles, and then dichotomised according to the FPM defined cut-off point. Those analyses utilised showed very similar borderline statistically significant positive association with the incidence of painful RKOA (table 6.14).

Table 6.14. Summary of the predictive ability of two models adjusted for baseline age and BMI, with the C3M variable categorised according to two different thresholds: quartiles and the FPM.

	Quartiles (Q2-4) Q1: reference	**FPM**
CRPM OR(95%CI)	Q2: 1.0 (0.5-1.9) Q3: 1.3 (0.7-2.6) Q4: 1.4 (0.8-2.7)	1.7 (0.9-3.2)

6.4.7 Summary of results

This part of the thesis assessed the effect of baseline levels of selected biomarkers on the incidence of painful RKOA in a population of middle-aged women with no RKOA at baseline.

COMP and Resistin did not show significant predictive abilities while high C3M and CRPM serum concentrations showed positive associations in relation to the future risk of developing painful RKOA. Those positive associations were borderline statistically significant after adjusting for age and BMI. Interestingly, there were significant differences in terms of associations for between biomarkers as specific follow-up time-point. C3M showed a strong independent association with the incidence of RKOA at year 10 and, to a lesser extent, with regard to follow-ups at years 15 and 20, while CRPM only exhibited a positive association with follow-ups at years 10 and 15.

To address the secondary aim of this chapter, additional analyses looking at the results of statistical models based on categorised biomarker variables were performed. These again showed similar results to the statistical models based on the continuous variables (table 6.15), with results based on median values in the KOA group performing very well.

Tables 6.15. Comparison of results from multivariable models using the continuous and categorised independent variable. Highlighted boxes contain results with the closest ORs to the models based on the continuous variable.

	Continuous variable, OR (95% CI)	**Quartiles, OR**	**KOA, OR**	**FMA, OR**
COMP	1.43 (0.73-2.8)	1.71	1.73	1.1
Resistin	0.97 (0.7-1.3)	0.91	0.94	N/A
C3M	1.62 (0.95-2.7)	1.7	1.6	1.7
CRPM	1.73 (0.98-3.1)	1.4	N/A	1.7

6.5 Summary of results and discussion

The primary aim of this study was to examine the predictive value of selected biomarkers in terms of incidence of RKOA and painful RKOA. The secondary aim was to compare the results of the models based on categorised biomarker data with those with a continuous biomarker variable.

The major findings of this chapter were that serum concentration of COMP and Resistin are good predictive biomarkers of RKOA, with C3M and CRPM, are positive associations with painful RKOA (table 6.16). Those were independent of baseline age and BMI, in a middle-aged population of women.

Table 6.16. Predictive value of selected biomarkers in terms of incidence of RKOA and painful RKOA.

	RKOA (years of follow-up included)		**Painful RKOA (years of follow-up included)**	
	OR	95%CI	OR	95%CI
COMP	**1.55 (5-20)**	1.03- 2.32	1.43 (10-15)	0.7- 2.8
Resistin	**0.73 (5-20)**	0.56 – 0.94	0.97 (5-20)	0.7-1.3
C3M	1.35 (5-15)	0.83-2.2	**1.6* (10-20)**	0.95-2.5
CRPM	1.4 (10-15)	0.9-2.1	**1.7 (10-15)**	0.98-3.1

*Here, a strong independent predictive ability for year 10 of follow-up was found, with OR 3.2 (95%CI 1.3-7.6)

COMP and Resistin associations with RKOA occurred over 20 years, with similar trends seen for each of the follow-ups. C3M and CRPM associations with incidence of painful RKOA were similar, with regard to being significant at years 10 and 15, and additionally at year 20 of follow-up for C3M.

To address the second aim of this chapter, the independent continuous biomarker was categorised on the basis of either the distribution (using quartiles), median values of the biomarker levels in KOA groups as thresholds or based on cut-off points from fractional polynomial models. All three methods showed similar results and were not vastly different from the results of models utilising continuous variables (table 6.9 and 6.15). More importantly, none of the results based on the categorised biomarker data was outside the 95%CI of the corresponding models based on continuous data. The quartiles-based model performed more poorly out of three different tested methods in terms of producing similar results to the models based on the continuous biomarker. In most cases (five out of six available), dichotomisation of the continuous variable based on the median values in RKOA groups showed results closest to the statistical models based on a continuous biomarker variable.

Associations of selected biomarkers with incidence of RKOA and painful RKOA groups differed significantly in this study, and this might improve

understanding of the discordance between RKOA and knee pain. COMP and Resistin serum concentrations were associated with an increased risk of developing RKOA over 20 years. This did not translate into an increased risk of painful RKOA. Meanwhile, C3M and CRPM, the biomarkers associated with synovial, cartilage and systemic inflammation, predicted the risk of painful RKOA after 10 and 15 years but did not predict RKOA alone.

RKOA is a permanent change, which could explain why COMP and Resistin exhibited the same trends in the risk at each 5-year follow-up point over 20 years, despite having an opposite effect on the increase of RKOA. Pain in RKOA tends to fluctuate, but is associated with more advanced RKOA changes [321]. This could potentially explain why the risk of incidence of painful RKOA occurs only after 10 years.

Another important consideration is loss during follow-up [322]: biomarkers associated with systemic inflammation, higher BMI [323] and painful RKOA might profile individuals with an increased risk of early mortality and therefore this group might be underrepresented in the long term follow-up. Individuals with painful KOA have been reported to have substantially increased premature mortality, with some reports suggesting that this is not a case for non-painful RKOA [109, 140, 142, 324]. This could affect rate of follow-up at year 20 for those who developed painful KOA early and potentially could explain why there is a substantially decreased risk of painful RKOA for CRPM and to some extend C3M the last follow-up.

6.6 Strengths and potential limitations

This cohort study is representative of the UK population of middle-aged women. With five-year interval follow-up knee radiographs obtained over 20 years and self-reported knee pain data collected contemporaneously, this is by far the longest observational study looking at the predictive value of those selected serum biomarkers for the development of KOA.

This study had some limitations, including losses to follow-up and results being limited to women only. There is potential for bias due to subjects' mortality or withdrawal and the general selection of a healthier cohort to attend the follow-up visits. To minimise this healthier selection bias, the statistical model maximising the utility of follow-up data was used. The discrete-time survival analysis models utilised results from all individuals, who met baseline inclusion criteria and attended any five-year interval follow-up over 20 years.

The serial knee radiographs were not obtained in the semi-flexed AP view, as these were not available 25 years ago when the study started. Therefore, for consistency, the same technique was used during follow-up. Levels of biomarkers can reflect morphological and pathological changes in any cartilaginous joint, and tested models were not adjusted for this.

The potential association between, not only selected biomarkers, but also KOA and excess mortality needs to be examined. This could potentially introduce bias towards a null hypothesis for a good predictive marker in the long-term follow-up cohorts due to loss of follow-up in the group at risk.

7 Premature mortality and inflammation

7.1 Introduction

Identification of the predictive role of the biomarkers examined for the development of painful radiographic knee osteoarthritis is complex, as the same biomarkers may also be linked to premature mortality[325]. This would make estimations of long-term predictive ability more difficult, but could potentially explain some differences seen in the trends of the long-term follow-up presented in the previous chapter.

There is growing evidence that low-grade systemic inflammation, often linked with adipose tissue, is associated with not only development of cardiovascular diseases (CVD) [326-328], but also joint pain [99, 329, 330] and incidence of radiographic osteoarthritis (RKOA) [24].

The analysis of the predictive value of this thesis' selected biomarkers for development of knee osteoarthritis (KOA) highlighted a significant long-term loss of follow-up (Chapter 6, section 6.3) [322]. During the last follow-up visit, this came to just under 50%, with 20% mortality. This could potentially explain different associations with long term (20 year) follow-ups, but would not explain why other biomarkers analysed using the same dataset, namely Resistin and COMP, did not follow the same pattern. One plausible explanation is that the high baseline levels of inflammation, measured by the selected biomarkers, may

have resulted in increased frailty and/or premature mortality of affected individuals. The effect of a non-random loss to follow-up might result in underestimation of the risk by failing to account for the competing risk of death [331].

The other important consideration for this analysis is premature mortality in the individuals who developed KOA during the study duration. Individuals with KOA have previously been reported to have substantial excess premature mortality but this relationship has been less consistent for non-painful RKOA [109, 140, 142, 324]. In this study, CRPM and C3M were positively associated with an increased risk of painful RKOA at year 10 of the follow-up. This could potentially introduce bias towards a null hypothesis for a good predictive marker of painful RKOA in the long-term follow-up cohorts due to loss of follow-up in the group at risk.

The strongest evidence of a relationship between OA and mortality comes from an observational study by Nüesch et al., where the mortality rates of a large selected population-based sample of men and women with symptomatic radiographic knee and/or hip OA were compared with age and sex standardised mortality ratios after a median of 14 years' follow-up [140]. A significant excess in all-cause, CVD-related and dementia-related mortality was reported. Meanwhile, Liu et al. found that participants with symptomatic KOA but not RKOA alone had a higher risk of early mortality [141]. However, Cerhan et al. found that RKOA was associated with decreased survival of middle-aged women who worked in the radium dial-painting industry, when compared with participants without

radiographic changes consistent with OA [332]. A more recent study, based on a large Swedish healthcare register, showed no evidence of increased premature mortality for those who had received a healthcare professional diagnosis of KOA in comparison to the rest of the population [333]. The authors of this report, similarly to Hochberg and Lee before, have highlighted that lack of association between KOA and mortality might be due to a potential misclassification of KOA in the studies based on healthcare databases, which are likely to be biased towards the null hypothesis [142, 334].

It is often assumed that discordance between RKOA alone and painful RKOA can be at least partly explained in relation to limited physical activity; knee pain in the population with RKOA might be limited by restricting physical activity (PA). If excess premature mortality is associated with low cardiovascular fitness, then it is likely that both painful and non-painful RKOA groups have an increased association with excess mortality when compared to individuals without RKOA and no knee pain. However, if only knee pain is associated with mortality while non-painful RKOA is not, then there is no substantial limitation of PA in the RKOA only group. The alternative explanation is that knee pain, with or without RKOA, is an early sign of metabolic dysregulation, which is associated with a state of higher systemic inflammation and, as a consequence, premature mortality.

The hypotheses here are as follows: firstly, selected inflammatory biomarkers are associated with excess mortality in a group without RKOA; secondly, painful RKOA, but not RKOA alone, is associated with excess premature mortality;

Therefore, the aims of this chapter are to evaluate the relationship between:

1. Serum concentration of the selected biomarkers and the risk of the risk of premature mortality.
2. The risk of mortality in a longitudinal community-based cohort with 23 years of follow-up in a subgroups based on knee pain and radiographic KOA.

7.2 Methods

The unadjusted log-rank tests were utilised to compare the survival distributions of the selected subgroups and the Kaplan-Meier graphs were used to present survival curves.

Cox proportional hazards regression model was used to assess the risk of mortality between the exposed and non-exposed groups. The basic Cox model associated time-dependent hazard ratio (HR) is based on the relation of hazard rates of those exposed (i) and not exposed (0). Here, the hazard rate is represented by λ(t) and the rest of equation is the basic Cox PH model.

$$HR: \quad \lambda_i(t)/\lambda_0(t) = e^{\beta X_i(t)}$$

The Cox proportional regression model assumes that the effects of the predictor variables are constant over time. Furthermore, there should be a constant linear relationship between the endpoint and predictor variables. The linearity of the relationship was confirmed and is presented in the appendix. Predictor variables had a skewed distribution and required logarithmic transformation to reduce the effect of extreme values.

The analysis based on this Cox model can develop flaws when the basic model assumptions are not met. Two main assumptions are:

1. The hazard rate of the exposed individual at time t, λ_i (t) is proportional to the hazard rate for any individual at time t, λ_0 (t). That is, the level of effect of the exposure and other covariates on the hazard are constant over the study period.
2. Exposure and other covariates contribute linearly to the natural log of the HR.

An evaluation of proportional hazards assumption was examined by Schoenfeld residuals in each analysis presented in this chapter [335]. There was no evidence that the hazards were not proportional over the follow-up period studied. The linearity to the natural log of the HR was assessed by fractional polynomial models and covariates were categorised if a non-linear relationship was found (appendix table). Potential confounders were compared between the subgroups using the Mann–Whitney U test for a nonparametric unpaired continues data and the chi square tests for categorical data.

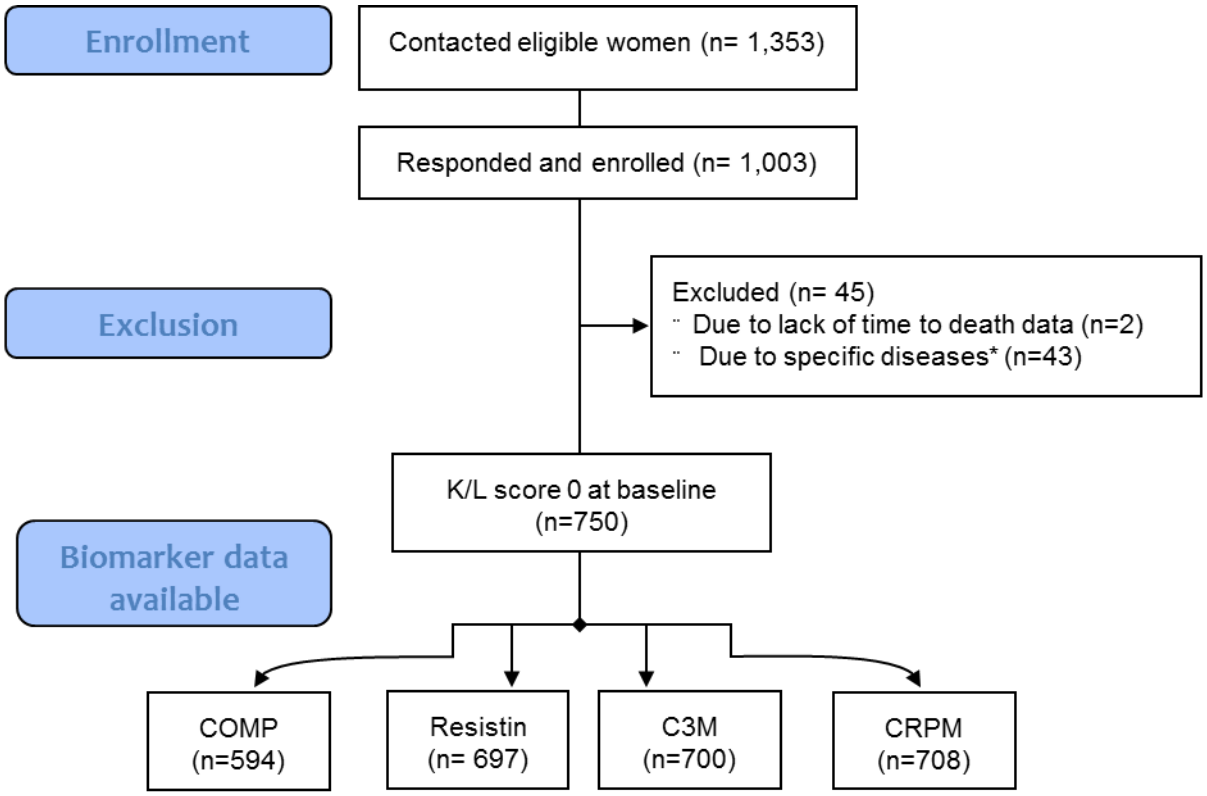
7.2 Serum biomarkers associated with inflammation and risk of premature mortality

The aim of this section was to examine the association between baseline serum concentrations of biomarkers and the risk of premature mortality in a longitudinal community-based cohort, with over 20 years of follow-up. The hypothesis was that high levels of those biomarkers are associated with increased premature mortality.

7.2.3 Subjects

The same longitudinal dataset from the Chingford Cohort Study that was utilised for predicting RKOA and painful RKOA, with additional mortality data (described in chapter 2) provided by the Health and Social Care Information Centre, was utilised to examine the potential relationship between baseline serum concentrations of selected biomarkers and the risk of overall mortality. All participants who had COMP measured was a group that had all four biomarker measured (figure 7.1).

Figure 7.1. Flow diagram of participants included and excluded from each analysis.



* Rheumatoid arthritis, psoriatic arthritis, gout, Paget's disease, polio, cerebral palsy and chronic inflammatory demyelinating neuropathy

A comparison of women's characteristics with no baseline RKOA who were not included in the analyses is presented in the appendix section. This shows no significant differences when compared to those included.

Table 7.1. A comparison of women's characteristics with no baseline RKOA included in each biomarker analysis.

Characteristic	COMP (n=594)	Resistin (n=697)	C3M (n=700)	CRPM (n=708)	p-value*
Mortality during the follow, n	93	139	139	139	
Age, years, mean (SD)	55.2 (5.7)	55.4 (5.8)	55.3 (5.8)	55.3 (5.8)	0.2
Occupation, n (%)					0.9
Manual	102 (17.3)	121 (17.5)	123 (17.7)	126 (17.95)	
Non-Manual	486 (82.6)	570 (83.5)	571 (82.3)	576 (82)	
HRT, n (%)					
No	358 (64.1)	419 (65.67)	420 (65.52)	425 (65.7)	
Yes	200(35.8)	219(34.4)	221(34.5)	222(34.3)	
Smoking, n (%)*					0.85
Never/ex-smoker	470(79.1)	543 (77.9)	543(77.6)	550(77.7)	
Smoker	124(20.9)	154(22.1)	157 (22.4)	158 (22.3)	
Physical activity at age 30, n (%)					0.8
No	119 (20.3)	131 (19.1)	132(19.2)	133 (19.1)	
Yes	466 (79.7)	554 (80.9)	556 (80.8)	564(80.9)	
Body mass index, n (%)					0.78

Non-obese (<30 kg/m²)	362 (88.5)	409(87.5)	408(87.7)	413(87.7)	
Obese (≥30 kg/m²)	47(11.5)	58(12.5)	57(12.3)	58(12.3)	
Previous CVD disease, n (%)					0.87
No	537 (96)	611 (95.5)	614 (95.5)	620 (95.5)	
Yes	22(4)	29 (4.5)	29 (4.5)	29 (4.5)	
BP medication, n (%)*					0.76
No	512(87)	583(86.9)	587 (87)	593 (87)	
Yes	76 (12.9)	87(13)	87(13)	89(13)	
Non-ASA NSAIDs medication, n (%)					0.73
No	564 (96)	624(95.6)	645(95.7)	653(95.7)	
Yes	24(4)	29 (4.4)	29 (4.3)	29(4.3)	
Systolic BP, mmHg, mean (SD)*	125.4 (18.9)	125.3(19.3)	125.2(19.2)	125.3(19.2)	
Total cholesterol, mg/dL, mean (SD)*	6.6 (1.4)	6.6 (1.4)	6.8 (1.4)	6.6(1.4)	0.7
HDL-cholesterol, mg/dL, mean (SD)*	1.7 (0.4)	1.7 (0.4)	1.7 (0.4)	1.7 (0.4)	0.6
Glucose, mmol/L, mean (SD)	4.9 (0.6)	5.0 (0.5)	5.0(0.5)	5.0(0.5)	0.23

*One-way ANOVA analysis (Tukey's multiple comparison test) was used for continues and Chi-squared for categorised variables.

There were no significant differences between the baseline characteristics of those who were included in the analyses and those who were not included due to a lack of biomarker, or radiographic data. There were some statistically significant differences between biomarker subgroups based on the thresholds associated with a higher risk of developing painful RKOA. The higher serum concentrations of COMP and C3M were associated with increased age, cholesterol and higher blood pressure. Higher levels of Resistin and CRPM were associated with increased age, but also BMI and glucose. The comparison of the baseline characteristics of those subgroups are presented in the appendix section.

7.2.4 Potential confounders

Information on potential confounders utilised in the analyses was collected at year 3 of follow-up. *Socio-demographic covariates* included age and occupation ('Manual' versus 'Non-manual'). *Health-related behaviours* were physical activity at age 30 and smoking. Physical activity (PA) at age 30 was assessed by the following question: 'Were you a physically active person at age 30?'. Smoking habits were classified through the categories of 'never smoked/ex-smoker' or 'currently smoking'. *Current and past CVD* was ascertained by asking the women if they had any CVD. We included all risk factors for heart disease that are part of the Framingham Heart Study Risk Assessment [336]. The *biological factors* incorporated were body mass index (BMI), systolic blood pressure (BP), total and high-density lipoprotein (HDL) cholesterol and glucose. Body mass index (BMI)

was calculated at baseline and had a 'J' shape association with mortality, leading it to be divided into two groups: 'non-obese' (BMI<30 kg/m^2) and 'obese' (BMI ≥30 kg/m^2). This was consistent with previously published studies [337, 338]. Total and HDL cholesterol, and glucose were measured via fasting serum samples taken [249]. For the analysis, systolic BP, total and HDL cholesterol, and glucose were treated as continuous variables. BP medication, anti-inflammatory drugs (NSAIDs) and hormone replacement therapy (HRT) use were coded as dichotomous variables.

7.2.5 Outcomes of interest and independent variables

The outcomes of interest were all-cause mortality between year 3 of follow-up to August 2014.

7.2.6 Statistical analysis

Kaplan-Meier survival curves for all-cause mortality were estimated using categorised biomarkers data based on the threshold grounded in the median value in the KOA group or fractional polynomial model as described in chapter 6. Statistical models based on that categorised independent biomarker data showed a very similar predictive value for painful RKOA as models based on the continuous biomarkers.

The association between RKOA and pain subgroups and all-cause mortality was assessed using continuous Cox proportional hazards regression models to estimate HR and 95% confidence intervals (95%CI). Those models used a log-

transformed continuous biomarker variable to reduce the effect of extreme values.

Four models were used to assess this association:

Model 1) unadjusted

Model 2) age-adjusted

Model 3) added risk factors from the Framingham Risk Score (smoking, total cholesterol, HDL-cholesterol, systolic BP and BP medication).

Model 4) added the remaining potential confounders (occupation, HRT use, past physical activity, BMI, current/previous CVD disease, Non-ASA NSAIDs and glucose levels).

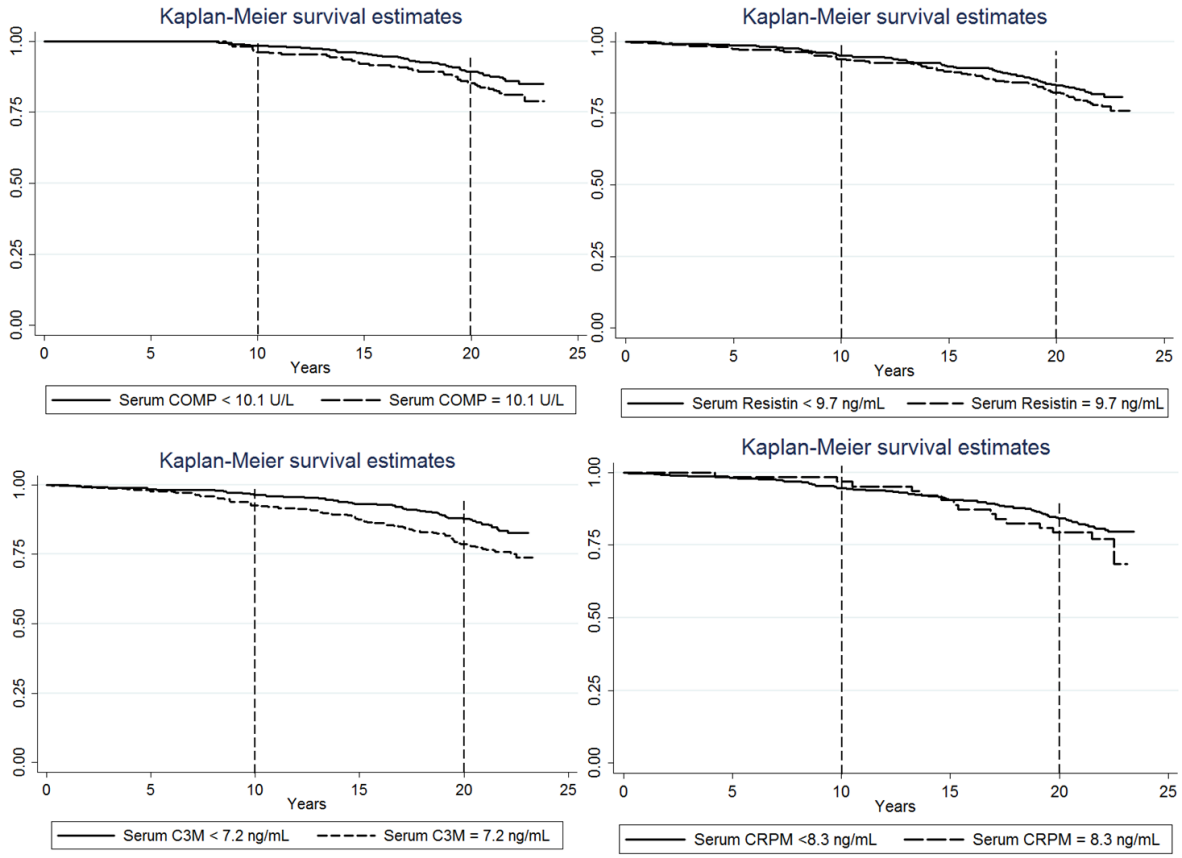
Multiple imputation using chained equations was used to investigate the impact of missing potential confounders, while 100 imputed datasets were generated using all potential confounders (including all-cause mortality and log-transformed survival time) and estimated parameters were combined using Rubin's rules. The proportional hazards assumption was examined by Schoenfeld residuals.

7.2.7 Results

Higher level of baseline biomarkers showed a trend associated with increased risk of earlier mortality when compared with lower levels of biomarkers (figure 7.2). Log-rank tests suggested small differences between groups based on the thresholds linked with increased risk of developing painful RKOA (presented in

chapter 6). The only statistically significant differences were observed for baseline serum C3M, with the group with lower concentration accounting for 20.5% less deaths than expected, with a log-rank test p-value ≤ 0.005. This is confirms that the group with high level of C3M at baseline is under-represented in the long-term follow-up due to higher risk of early mortality. The differences between groups were expected to be minimal at year 10 of follow-up, but more substantial by year 20 (figure 7.2).

Figure 7.2. Kaplan-Meier survival plots of all-cause mortality for each selected biomarker stratified by thresholds associated with increased risk of painful RKOA.



Results from Cox proportional hazard models estimating the mortality risk according to each selected log-transformed continuous biomarker are shown in table 7.2. These results confirmed association seen in the log-rank tests, but using continuous variables, and allowed for adjusting for potential confounders. In the unadjusted model, one unit change for C3M (log-transformed) was associated with an 80% increased risk of all-cause mortality [HR 1.80 95%CI 1.09-2.98; p-value=0.022]. After adjusting for age, the statistical trend was the same but the results were not statistically significant. Further adjustment for additional risk factors made this association non-significant with no trend suggesting increased risk. The unadjusted COMP model showed a similar positive trend, but was not statistically significant. No other significant associations between the levels of the biomarkers and risk of mortality were found in the fully adjusted models. Results for each of the analyses are presented in table 7.2 below.

Table 7.2. Results of Cox-regression models for each biomarker. Hazard Ratio (95%CI) of all-cause by unit increase of each biomarker.

Outcome	No. of deaths (%)	Model 1		P-value	Model 2		P-value	Model 3		P-value	Model 4		P-value
All-causes mortality		**HR**	**95%CI**		**HR**	**95%CI**		**HR**	**95%CI**		**HR**	**95%CI**	
COMP	94 (16)	1.52	0.84-2.80	0.15	1.14	0.63-2.05	0.66	0.91	0.47-1.76	0.78	0.80	0.43-1.62	0.55
Resistin	139 (20)	1.17	0.84-1.62	0.35	1.18	0.84-1.65	0.34	1.05	0.71-1.54	0.81	1.02	0.68-1.54	0.93
C3M	139 (20)	**1.80**	**1.09-2.98**	**0.02**	1.46	0.88-2.43	0.14	0.97	0.53-1.76	0.91	0.97	0.53- 1.80	0.94
CRPM	139 (20)	0.89	0.57-1.39	0.62	0.91	0.58-1.41	0.68	0.84	0.50-1.43	0.53	0.83	0.48-1.43	0.51

Model 1: unadjusted

Model 2: adjusted for age

Model 3: Model 2 + Smoking, total cholesterol, HDL-cholesterol, systolic BP and BP medication (Framingham Risk Score Factors)

Model 4: Model 2 + occupation, BMI, HRT use, past physical activity, current/previous CVD disease, Non-ASA NSAIDs and glucose levels.

7.2.8 Summary of the results

Higher levels of C3M are associated with an increased risk of long-term premature mortality, and this should be taken into account when analysing the predictive value of this biomarker over 10 years. C3M does not independently predict mortality, adjusting for age, smoking, cholesterol and systolic BP explained in the regression model this association. COMP showed a similar trend in the unadjusted model. However, those results were not statistically significant and this trend was not present in the model adjusted for age and other confounders.

To some extent, these results might explain differences seen in associations between C3M and the incidence of painful RKOA after 10 and 20 years. Higher levels of this biomarker are associated with increased overall mortality and this is likely to contribute towards the higher rates of loss of follow-up in this group and therefore introduce bias towards a null hypothesis for a good predictive marker of painful RKOA in the long-term.

The results of these analyses also suggest that this was not the case for CRPM and decreased association with painful RKOA in the long-term term could not be explained by higher loss of follow-up due to mortality in the group at risk of KOA.

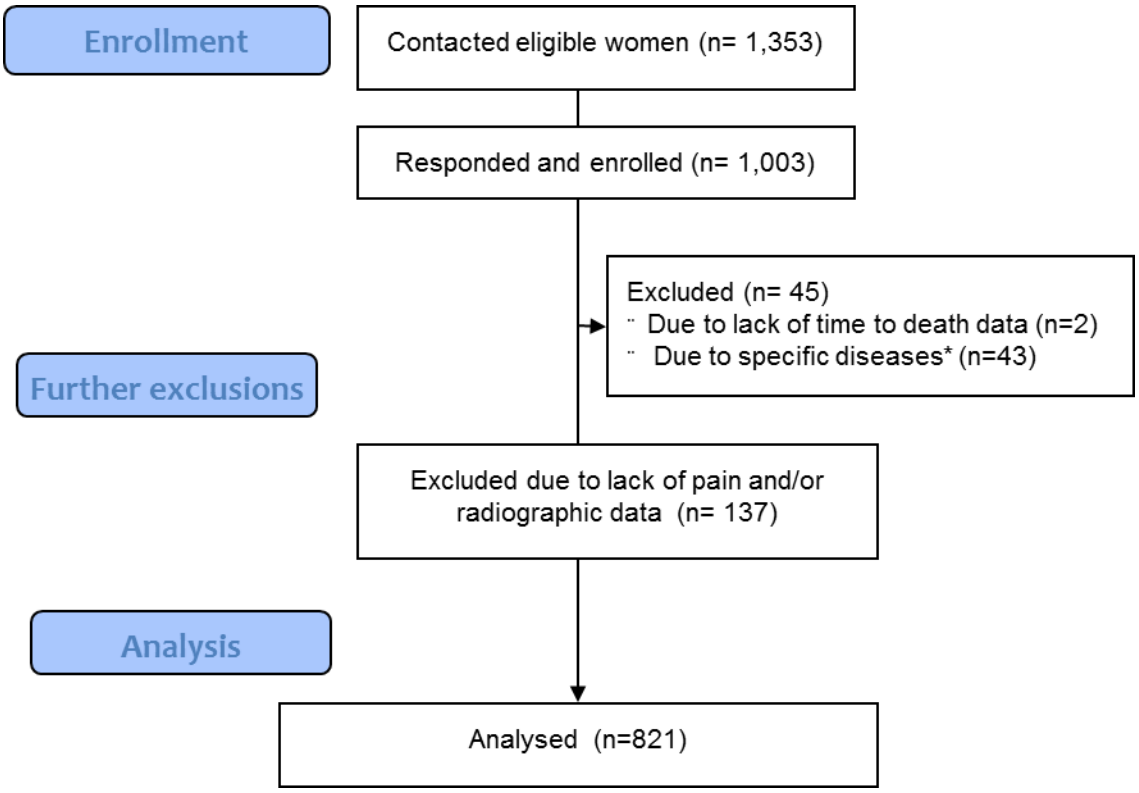
7.3 RKOA and knee pain as a predictor of early mortality

The potential confounder between selected biomarkers and increased mortality is painful RKOA. The aim of this section was to examine the relationship between knee pain, RKOA and the risk of mortality in a longitudinal community-based cohort, with over 20 years of follow-up. Participants were divided into four subgroups based on the presence or absence of knee pain and RKOA.

7.3.3 Subjects

The longitudinal data from the Chingford Cohort Study was utilised to examine the potential relationship between pain and/or RKOA and risk of mortality. There were 821 women included for analyses, with available data on both pain and RKOA. Women with the diseases associated with increased mortality (n=43) at baseline or at any point of follow-up were excluded from analyses. These diseases were rheumatoid arthritis, psoriatic arthritis, gout, Paget's disease, polio, cerebral palsy and CIDN. Furthermore, two participants were excluded due to lack of time-to-death data. Another 137 women were excluded due to lack of pain and/or radiographic data at baseline are shown in figure 7.3, while descriptive characteristics across knee categories are presented in table 7.4. Characteristics of the participants without baseline radiographic or pain data were similar to those included for analyses and these are presented in the appendix.

Figure 7.3. Flow diagram of participants included and excluded from each analysis.



* Rheumatoid arthritis, psoriatic arthritis, gout, Paget's disease, polio, cerebral palsy and chronic inflammatory demyelinating neuropathy.

7.3.4 Outcomes of interest and variables

The outcomes of interest were all-cause and cause-specific mortality, categorised as CVD and cancer and based on available data up to August 2014. This data was provided by the Health and Social Care Information Centre. Detailed information about mortality outcomes are presented in chapter 3.

The weight-bearing anteroposterior knee radiographs at baseline (year 1) were graded using the Kellgren-Lawrence (K/L) OA grading system. The protocols for

radiographic grading and reproducibility of this method are presented in chapter 3.

Knee pain was assessed at year 3 as a part of a self-administrated questionnaire. Women were asked if they had experienced any knee pain in the past month and the number of days this had occurred. Knee pain was classified as positive if the answer was 'yes' and/or more than 0 days of knee pain was reported.

Joint-specific KOA and pain was classified into four subgroups based on the presence and absence of pain and RKOA:

1. Neither RKOA nor knee pain (Pain−/RKOA−: reference category[339]).

2. RKOA only (Pain−/RKOA+).

3. Pain only (Pain+/RKOA−).

4. Painful RKOA (Pain+/RKOA+: joint(s) with RKOA and pain on the same side).

Table 8.1. Four subgroups based on the presence or absence of the pain and RKOA, described later as knee categories.

		Knee Pain	
		+	**-**
RKOA	**+**	Pain **+** **RKOA+*** n=57	Pain **-** **RKOA +** n=64
	-	Pain **+** **RKOA -** n=176	Pain **-** **RKOA -** n=524

*only if pain and RKOA were present in the same side.

Potential confounders used in those analyses were categorised the same way as in the biomarkers study (Chapter 7, section 7.3.2, page).

Table 8.2. Descriptive characteristics across knee status categories

Characteristic	Pain−/RKOA- (n=524)	Pain−/RKOA+ (n=64)	Pain+/RKOA- (n=176)	Pain+/RKOA+ (n=57)	p-value*
Age, years, mean (SD)	55.2 (5.8)	58.7 (6.3)	56.4 (5.8)	59.6 (4.9)	**0.000**
Occupation, n (%)					**0.042**
Manual	79 (15.1)	11 (17.2)	40 (22.7)	8 (8.8)	
Non-Manual	440 (84.0)	52 (81.3)	135 (76.7)	52 (91.2)	
HRT, n (%)					**0.045**
No	343 (65.5)	44 (68.8)	100 (56.8)	39 (68.4)	
Yes	164 (31.3)	16 (25.0)	74 (42.1)	17 (29.8)	
Smoking, n (%)*					0.706
Never/ex-smoker	401 (76.5)	53 (82.8)	137 (77.8)	45 (79.0)	
Smoker	123 (23.5)	11 (17.2)	39 (22.2)	12 (21.1)	
Physical activity at age 30, n (%)					0.328
No	101 (19.3)	13 (20.3)	25 (14.2)	8 (14.0)	
Yes	413 (78.8)	50 (78.1)	150 (85.2)	49 (86.0)	
Body Mass Index, n (%)					**0.002**
Non-obese (<30 kg/m^2)	316 (60.3)	39 (60.9)	118 (67.1)	34 (59.7)	
Obese (≥30 kg/m^2)	41 (7.8)	14 (21.9)	25 (14.2)	13 (22.8)	

Previous CVD disease, n (%)					0.436
No	489 (93.3)	58 (90.6)	163 (92.6)	53 (93.0)	
Yes	18 (3.4)	2 (3.1)	11 (6.3)	3 (5.3)	
BP medication, n (%)*					**0.008**
No	454 (86.6)	51 (79.7)	149 (84.7)	40 (70.2)	
Yes	70 (13.4)	13 (20.3)	27 (15.3)	17 (29.8)	
Non-ASA NSAIDs medication, n (%)					**0.010**
No	507 (96.8)	63 (98.4)	163 (92.6)	51 (89.5)	
Yes	17 (3.2)	1 (1.6)	13 (7.4)	6 (10.5)	
Systolic BP, mmHg, mean (SD)*	124.8 (20.5)	129.7 (17.6)	127.1 (17.7)	132.8 (19.7)	**0.029**
Total cholesterol, mg/dL, mean (SD)*	6.6 (1.4)	6.7 (1.3)	6.8 (1.4)	7.1 (1.2)	0.072
HDL-cholesterol, mg/dL, mean (SD)*	1.7 (0.4)	1.6 (0.4)	1.7 (0.4)	1.7 (0.5)	0.263
Glucose, mmol/L, mean (SD)	4.9 (0.6)	5.0 (0.8)	4.9 (0.7)	5.1 (0.6)	0.130

*One-way ANOVA analysis (Tukey's multiple comparison test) was used for continues and Chi-squared for categorised variables

7.3.5 Statistical analysis

Kaplan-Meier survival curves for all-cause mortality in joint-specific OA and pain subgroups were estimated. The association between RKOA and pain subgroups and all-cause and cause-specific mortality was assessed using Cox proportional hazards regression models to estimate HR and 95% confidence intervals (95%CI). In the analyses of cause specific mortality, deaths attributed to other causes were treated as censored at the time of death. Proportional hazards assumption was examined by Schoenfeld residuals.

Three models were used to assess this association:

Model 1) was age-adjusted.

Model 2) added risk factors from the Framingham Risk Score (smoking, total cholesterol, HDL-cholesterol, systolic BP and BP medication).

Model 3) added the remaining potential confounders (occupation, HRT use, past physical activity, BMI, current/previous CVD disease, Non-ASA NSAIDs and glucose levels).

One hundred imputed datasets were generated using all potential confounders (including all-cause mortality and log-transformed survival time) and estimated parameters were combined using Rubin's rules.

Three sensitivity analyses were conducted:

1. Women who underwent joint-specific surgery at any point during follow-up were excluded.

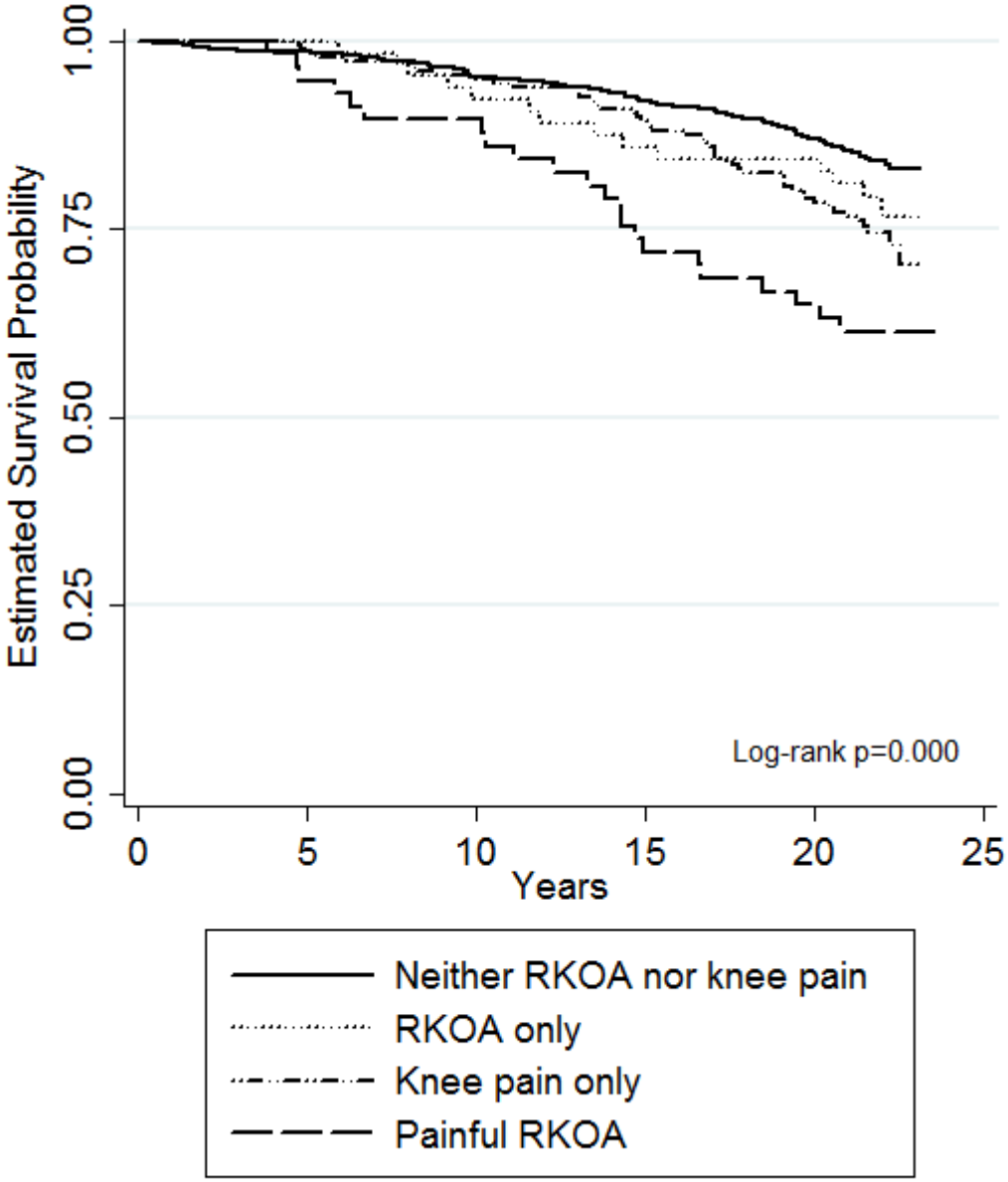
2. All deaths occurring during the first 12 months of follow-up were excluded.

3. Available crude measures of PA were assessed through the following question: 'How many times per week do you engage in activity that makes you sweat?'. This was added as a covariate to the fully adjusted Cox regression (model 3).

7.3.6 Results

Kaplan-Meier survival curves for all-cause mortality according to knee subgroups are presented in figure 7.4. Women with painful knee RKOA had a greater risk of all-cause mortality compared to women with neither knee pain nor ROA (log-rank test p-value ≤ 0.001). These results suggested significant differences in mortality rates between those who have painful RKOA and other groups.

Figure 7.4. Kaplan-Meier survival plots of all-cause mortality in a subgroup stratified by RKOA and knee pain status.



Results from Cox proportional hazard models estimating the mortality risk according to RKOA and pain categories are shown in table 8.2. In the age-adjusted model, women with knee pain and no RKOA had a 49% increased risk of dying from all-cause mortality, compared to those with neither knee pain nor

ROA [HR 1.49 95%CI 1.04-2.14; p-value=0.029]. The mortality risk was stronger among women with painful RKOA, reaching a 97% increase in mortality risk in an age-adjusted model [HR 1.97 95%CI, 1.23-3.17; p-value=0.005] (Model 1; Table 8.3). After adjustment for the factors from Framingham Risk Score Factors (Table 2; Model 8.3), the HRs increased slightly for painful knee subgroups (ROA+, ROA-) (1.55 [95%CI, 1.07-2.22] and 2.06 [95%CI, 1.27-3.33], respectively) (Model 2; Table 8.3). The relationship remained unchanged even after further adjusting for all other covariates (Model 3; Table 8.3). In contrast, RKOA alone was not associated with mortality in any of the three models.

The magnitude of association between knee pain subgroups (Pain+/ROA- and Pain+/ROA+) and mortality was the strongest for CVD-specific mortality in all models. Compared to women with neither knee pain nor ROA, the HR associated with knee pain only (Pain+/ROA-) was 3.25 [95%CI, 1.64-6.43], while the HR associated with painful knee ROA (Pain+/ROA+) was 4.19 [95%CI, 1.87-9.40], after adjustment for Framingham Risk Score factors (Model 2; Table 8.3). Further inclusion of all other covariates produced an almost negligible reduction in the HR for CVD-specific mortality (Model 3; Table 8.3). No statistically significant associations were found for cancer-specific mortality.

No substantial differences in the results were observed when women died during the first 12 months of follow-up (n=2), or when women who underwent knee replacement (for knee analysis) during the follow-up (n=29) were excluded (data not shown).

Table 8.3. Results of Cox-regression models for each knee category. Number of deaths (N) and Hazard Ratio (95%CI) of all-cause and disease specific mortality by knee OA categories.

Outcome Knee OA status	No. of deaths (%)	Model 1		P-value	Model 2		P-value	Model 3		P-value
		HR	95%CI		HR	95%CI		HR	95%CI	
All-causes										
Pain−/RKOA-	84 (16.0)	1.00			1.00			1.00		
Pain−/RKOA+	14 (21.9)	0.95	0.54-1.69	0.865	1.01	0.57-1.80	0.968	1.05	0.58-1.88	0.876
Pain+/RKOA-	46 (26.1)	1.49	1.04-2.14	0.029	1.55	1.07-2.22	0.019	1.44	0.99-2.08	0.055
Pain+/RKOA+	22 (38.6)	1.97	1.23-3.17	0.005	2.06	1.27-3.33	0.003	1.97	1.20-3.22	0.007
CVD disease										
Pain−/RKOA-	17 (3.2)	1.00			1.00			1.00		
Pain−/RKOA+	4 (6.3)	1.14	0.38-3.43	0.811	1.47	0.48-4.49	0.498	1.45	0.47-4.48	0.521
Pain+/RKOA-	18 (10.2)	2.78	1.43-5.41	0.003	3.25	1.64-6.43	0.001	2.93	1.47-5.85	0.002
Pain+/RKOA+	10 (17.5)	3.98	1.81-8.76	0.001	4.19	1.87-9.40	0.001	3.57	1.53-8.34	0.003
Cancer disease										
Pain−/RKOA-	44 (8.4)	1.00			1.00			1.00		

Outcome	No. of deaths (%)	Model 1		P-value	Model 2		P-value	Model 3		P-value
Pain−/RKOA+	7 (10.9)	1.08	0.48-2.41	0.860	1.11	0.49-2.51	0.801	1.10	0.48-2.51	0.818
Pain+/RKOA-	19(10.8)	1.23	0.72-2.12	0.444	1.23	0.72-2.12	0.451	1.12	0.64-1.94	0.693
Pain+/RKOA+	6 (10.5)	1.17	0.49-2.78	0.719	1.33	0.56-3.16	0.523	1.18	0.49-2.88	0.712

Model 1: adjusted for age

Model 2: Model 1 + Smoking, total cholesterol, HDL-cholesterol, systolic BP and BP medication (Framingham Risk Score Factors)

Model 3: Model 2 + occupation, BMI, HRT use, past physical activity, current/previous CVD disease, Non-ASA NSAIDs and glucose levels

7.3.8 Summary of the knee osteoarthritis as a predictor of early mortality

Painful RKOA is associated with significant increase of mortality risk when compare to individuals without KOA, or RKOA without pain.

Further analysis that included all known risk factors for mortality suggested that knee pain with or without RKOA – but not RKOA alone – is associated with significantly increased risk of all-cause and CVD-specific mortality in middle-aged women. This suggests that knee pain, more so than the structural changes of OA, is the main driver of excess mortality in patients with OA.

Knee pain seems to be a potent factor in cardiovascular mortality. Limited PA has been postulated to be a major factor contributing to this. RKOA has been strongly correlated with knee pain. It is commonly assumed that patients with RKOA will limit their activity to minimise the level of knee symptoms provided they are able to perform basic activities in their daily lives. If the RKOA-only group (Pain-/RKOA+) limited their activities based on their knee symptoms, and the assumption is that poor PA in KOA contributes to increased mortality, we would have expected to see similar differences in mortality ratios in both this group and those with knee pain with or without RKOA. However, this was not a case, suggesting either the group with RKOA only did not limit their activity substantially and RKOA represented in this group a successful remodelling process , or that decreased PA in KOA does not explain excess mortality.

Increased mortality in the painful RKOA (Pain+/RKOA+) group when compared to the control group could potentially be explained by the higher mean age and BMI, at least in unadjusted models. However, in the multivariable models adjusted for age and BMI and other risk factors, this association is still significant. Furthermore, the painless RKOA group (Pain−/RKOA+) was larger than the painful RKOA group, though with similar baseline characteristics. However, it showed no positive association with excess mortality in the fully adjusted model.

Additional analyses focused on hand OA were performed and, similarly to the analyses presented above, significant mortality differences in hand OA were found in an unadjusted model. However, adjusting for all the covariates resulted in finding no additional mortality differences, that could not be explained by basic characteristics [10]. This suggests those models were adjusted for all-important covariates.

7.4 Strengths and potential limitations

Some potential limitations in this study are worth mentioning. Firstly, the findings are not generalizable to the whole population but limited to middle-aged and, predominantly, Caucasian women. In our multivariable analysis of the groups, we used baseline values of covariates, but those values may have changed over time and have time-dependent effects on OA and mortality outcomes that we would not be able to comment on in this analysis. Secondly, this analysis is likely to underestimate the absolute risk of the exposure groups, due to the fact that participants from the control group (ROA-/Pain-) in this

study remained in the original group even if they developed pain and/or ROA over time. In this study, all exposure categories have this immortal time period [340].

Another important limitation of this study to be considered is the fact that radiographs of the knees were taken three years before knee pain assessments. To mitigate against this, outcomes were measured from year 3 onwards. Potential misclassifications will have led to a bias towards a null hypothesis, as control groups may have included participants with RKOA. Over five years, incidence of RKOA was around 7.5% in this cohort, and over three years it was likely to be much smaller. PA and function was another area for potential residual confounding. We performed a sensitivity analysis using an available crude measure of PA. When adjusted for, this did not attenuate the findings. CVD-specific mortality was a relatively rare outcome in the group without RKOA at baseline, consisting of around 30% of all cause deaths during this period of follow-up.

7.5 Summary of the chapter

Painful RKOA is also associated with increased risk of premature death. After adjusting for known risk factors linked with premature death, knee pain with or without RKOA, but not RKOA alone, was also associated with a significantly increased risk of all-cause and CVD-specific mortality in middle-aged women.

High baseline serum concentrations of C3M are associated with a high risk of developing painful RKOA, but also with an increased risk of early mortality. This could provide an explanation for the change of predictive trend for C3M at year 20 of follow-up. High levels of COMP and Resistin were also associated with a mildly increased risk of mortality, but those trends were not statistically significant.

Taking into account for the competing risk of death should be an important considerations when studies are looking at predictive value of biomarkers are analysed or planned.

8 Discussion

8.1 Main findings

The overall aim of this thesis was firstly to examine if selected 'wet' and 'dry' biomarkers correlate with microscopic knee synovitis and predict incident knee osteoarthritis (KOA). Secondly, to investigate the associations between these biomarkers and risk of premature death and identify whether painful radiographic KOA is linked with early mortality.

Results from the Oxford Knee Injury study (OxKIC) confirmed the diagnostic value of synovial effusion, as a marker, in identifying the presence of microscopic synovitis in the post-traumatic knee. This marker is based on the reproducible sonographic protocol of the suprapatellar recess. This protocol also confirmed that microscopic synovitis in that area is associated with knee pain cross-sectionally.

High levels of selected serum biomarkers were able to identify individuals at risk of developing radiographic KOA (RKOA) and painful RKOA, independently of baseline age and BMI. Cartilage oligomeric matrix protein (COMP) was associated with an increased risk of developing RKOA, while resistin was protective. Both COMP and resistin showed no significant association with an increased risk of developing painful RKOA. High serum concentrations of biomarkers associated with localised inflammation, MMP-degraded collagen III

(C3M) and MMP-degraded C-Reactive Protein (CRPM), were associated with an increased risk of developing painful RKOA. This suggests that MMP-degraded products, associated with an intra-articular inflammation, are more specific for radiographic changes associated with pain.

This thesis emphasises the usefulness of new sonographic protocol to detect microscopic synovitis in the post-traumatic knees, both in terms of identifying the abnormal structure associated with symptoms but also potentially monitoring response to any knee specific treatment.

Painful RKOA in the middle-aged population of women was an independent risk factor for excess premature mortality. In the unadjusted model high serum concentration of C3M was also linked with an increased risk of early mortality. It is likely that painful KOA is either a confounder or that there is an interaction with C3M levels and excess premature mortality.

Inflammation is an important factor associated with higher risk of developing KOA and potentially increased risk of premature mortality. Osteoarthritis is a heterogeneous disease, and selected biomarkers might help to identify high-risk individuals and will potentially make primary and secondary preventive intervention trials feasible.

This thesis adds to our understanding of the discordance between knee pain and RKOA. Although, the risk of individuals with non-painful RKOA progressing to painful RKOA is still unknown, it is unlikely to be higher than for the rest of the age-matched population. Non-painful RKOA in a large cohort of middle-aged

women, was not linked with an increased risk of mortality after adjusting for baseline covariates. Assuming that decreased physical activity due to knee pain is a factor linking KOA and mortality, this suggests that individuals with non-painful RKOA are unlikely to limit their activity substantially more than their peers. This indicates that a large proportion of individuals develop structural changes that are clinically silent and are not associated with significant negative outcomes. Preventive KOA strategies should take this into account as a potential positive outcome.

The development of preventive strategies in high-risk groups, including those recovering after knee trauma, has been limited by an inability to identify subsets of individuals at the highest risk. It is likely that some inflammatory response is associated with successful joint remodelling, but either excessive or inadequate response might result in chronic pain and joint failure. The selected biomarkers associated with knee pain and either local and/or systemic inflammation might be able to address this gap in future research.

8.2 Future work

This thesis demonstrates the usefulness of biomarkers associated with knee pain and either systemic and/or local inflammation to predict KOA independently of age and body mass index (BMI). The results offer several new research directions. The understanding of association between C3M, CRPM with post-traumatic synovitis required a larger sample of matched synovium with serum and sonographic biomarkers to compare them. It is also plausible that

immunohistochemistry staining focused on macrophages or the Dickkopf WNT signalling inhibitor 1 pathway, will be able to define association between specific patterns of inflammation and serum biomarkers.

Validation of the sonographic protocol and findings in a second cohort is needed and there are expressions of interest by another acute knee injury research group. Finally, one and two-year follow-up outcomes will allow us to examine the associations between baseline samples and long-term consequences. To increase power, but also to validate the OxKIC findings externally, collaboration with investigators of other knee injury cohorts have already started. The overall aim is to synchronise collected variables across currently running knee injury cohorts in order to answer important questions about role of inflammation in development of post-traumatic KOA. The change of the biomarkers with time, their associations with patient-reported symptoms in the OxKIC longitudinal study and the effect of covariates will be examined when results of all follow-up samples are available.

The modified ultrasound (US) protocol has been designed to allow quick and reliable assessment of suprapatellar effusion and synovitis in clinical settings. While a significant association between synovial effusion and microscopically defined synovitis has been confirmed in the post-traumatic knee cohort, associations between those measures in the population with established KOA remains unknown. Further reliability tests, assessment of association with microscopic synovial inflammation and a study of symptoms in the group of participants with KOA is needed. This could then be translated into clinical

practice in primary and secondary care, where sonographic assessments are already often used for diagnostic purposes. However, there is a lack of standardised method to allow us to compare results and monitor treatment outcomes using more objective measures than monitoring the presence or absence of knee effusion clinically.

Further research area is the additive value of using multiple biomarkers to enable better phenotyping of individuals at risk of KOA.

Finally, serum samples collected for both cohorts can be utilised for identification of new biomarkers. Our recent analysis of metabolomics data generated from the Chingford cohort[341] has suggested a strong cross-sectional associations between specific molecules linked with the carbohydrate metabolism and KOA. Further investigation of those results might provide invaluable information on good new biomarker targets for predicting incidence of KOA.

Identifying individuals at risk with those biomarkers will potentially make primary and secondary preventive intervention trials feasible. Considering that MMP-cleaved products have been able to predict painful KOA, use of MMP inhibitors, such as doxycycline, could potentially offer a logical next step in terms of future intervention.

Appendices

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Appendix 1: Publications associated with this thesis

Journal Articles

1. Kluzek, S., Newton, J. L., and Arden, N. K. (2015) Is osteoarthritis a metabolic disorder?, Br Med Bull 115, 111-121. doi: 10.1093/bmb/ldv028.

2. Kluzek, S., Arden, N., and Newton, J. (2015) Adipokines as potential prognostic biomarkers in patients with acute knee injury, Biomarkers, 1-7. 2015;20(8):519-25. doi: 10.3109/1354750X.2014.948914.

3. Williamson, W., Kluzek, S., Roberts, N., Richards, J., Arden, N., Leeson, P., Newton, J., and Foster, C. (2015) Behavioural physical activity interventions in participants with lower-limb osteoarthritis: a systematic review with meta-analysis, BMJ Open 5, e007642. doi: 10.1136/bmjopen-2015-007642.

4. Kluzek, S., Sanchez-Santos, M. T., Leyland, K. M., Judge, A., Spector, T. D., Hart, D., Cooper, C., Newton, J., and Arden, N. K. (2015) Painful knee but not hand osteoarthritis is an independent predictor of mortality over 23 years follow-up of a population-based cohort of middle-aged women, Ann Rheum Dis. 2016 Oct;75(10):1749-56. doi: 10.1136/annrheumdis-2015-208056.

5. Kluzek, S., Sanchez-Santos, M. T., Leyland, K. M., Judge, A., Newton, J., and Arden, N. K. (2016) Response to: 'Painful knee but not hand osteoarthritis is an independent predictor of mortality over 23 years follow-up of a population-based cohort of middle-aged women' by Gao et al, Ann Rheum Dis. 2016 Apr;75(4):e23. doi: 10.1136/annrheumdis-2015-209029

6. Kluzek, S., Bay-Jensen, A. C., Judge, A., Karsdal, M. A., Shorthose, M., Spector, T., Hart, D., Newton, J. L., and Arden, N. K. (2016) Serum cartilage oligomeric matrix protein and development of radiographic and painful knee osteoarthritis. A community-based cohort of middle-aged women, Biomarkers, 1-8. doi: 10.3109/1354750X.2015.1105498.

Conference Papers

1. Kluzek, S., Bay-Jensen, A. C., Spector, T., Hart, D., Leyland, K., Sanchez-Santos, M., Arden, N., and Newton, J. (2014) Higher serum levels of cartilage oligomeric

matrix protein (COMP) are associated with self-reported knee pain, Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society 22, S74.

2. Bacardit, J., Mobasheri, A., Kluzek, S., Edwards, M., Spector, T., Hart, D., Cooper, C., and Arden, N. (2016) Metabolic profiles associated with radiographic knee osteoarthritis in a population-based cohort of middle-aged women, Osteoarthritis and Cartilage 24, S70-S70.

3. Leyland, K. M., Sanchez-Santos, M. T., Kluzek, S., Prieto-Alhambra, D., Spector, T. D., Hart, D. J., Newton, J., and Arden, N. K. (2014) Subjects with self-reported baseline injury have an increased risk of symptomatic radiographic knee osteoarthritis over twenty-years, Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society 22, S385.

4. Kluzek, S., Wedatilake, T., McNally, E., Price, A., Judge, A., Watt, F., Arden, N., and Newton, J. L. (2016) Suprapatellar synovial hypertrophy and reliability of the novel ultrasound assessment of suprapatellar recess in patients with recent knee injuries or early osteoarthritis, Osteoarthritis and Cartilage 24, S299-S300.

Appendix 2: Laboratory and imaging protocols

9.1 Serum samples

For both the OxKIC and Chingford studies serum samples were obtained by venepuncture into a red top serum tubes with no additive. The cells were removed by centrifugation at 1,200×*g* for 15 min at 10°C. Serum aliquots were stored at −80°C until analyzed.

9.2 H&E staining

There are number of H&E protocols available, most of them follow the same regime with some significant variation in terms of timing. All samples from OxKIC study followed the same protocol below:

1. Start with de-paraffined sections.
2. Two changes of xylene, for 5 minutes each.
3. Two changes of absolute alcohol, for 1minute long each.
4. Wash in running tap water for 1 min.
5. Haematoxylin Harris, for 6.30 minutes.
6. Wash in running tap water for 2 mins.
7. Differentiate in 1% acid alcohol for 40 seconds (for 1 L of solution: 990ml 70% EtOH + 10 ml Concentrate HCl).
8. Wash in running tap water for 2 mins.

9. Bluing in 0.2% ammonia water or saturated lithium carbonate solution for 1 minute.
10. Wash in running tap water for 1 mins.
11. Counterstain in eosin-phloxine solution for 1 minute 45 seconds.
12. Wash in running tap water for 3 minutes.
13. Three changes of absolute alcohol, for 1 minutes each.
14. Two changes of xylene, for 1 minutes each.
15. Mounting the coverslips with *Di-N*-Butylphthalate in Xylene (DPX) mounting media.

9.2 Solution and reagents

Solution and reagents used in tissue processing and staining

	Details	**Manufacturer**
Formalin	10%, neutral buffered	Sigma-Aldrich Chemie GmbH
Alcohols solutions	Reagent Alcohol	Sigma-Aldrich Chemie GmbH
Xylene	Xylenes	Sigma-Aldrich Chemie GmbH
Paraffin	Paraffin for embedding	CellPAth
Hematoxylin	Hematoxylin Harris Solution	Surgipath, Leica

Eosin	Eosin Y stain 1% (Aqueous)	CellPAth

9.3. The OxKIC study: collection, tissue processing and storage

The recruitment visit: non-fasted and collected between 1 and 4 pm in the outpatient department of the Nuffield Orthopaedic Centre.

The pre-operative collection: fasting morning samples, taken between 7 and 11 am just before scheduled operations.

The 6-8months follow-up visit: non-fasted and collected between 1 and 5 pm in the outpatient department of the Nuffield Orthopaedic Centre.

	Blood	**Urine**	**Synovial Fluid**	**Synovial tissue**
Collection	During each appointment, 20-40 * ml of venous blood collected from the anterior cubital vein with BD Vaccutainer™ and adjusted 30cm Tub and BD Vacutainer™ Plastic Blood Collection Tubes with	During each appointment, a 15-50 mL mid-stream urine sample was collected	During the routine operation appointment, 0.5-6.5mL of synovial fluid were collected.	During the routine operation appointment, one or three samples were corrected – depending on consent

	Hemogard™ Closure.			
Processing	Centrifuge for 15 minutes at 1600g (RCF) for 15 mins at 4°C in a refrigerated centrifuge with sealed buckets. The serum and plasma were pipetted the into a clean plastic 0.5 mL cap vials and the labels were attached.	Centrifuge for 20 minutes at 3000g (RCF) for 20 minutes at 20°C to remove cells. The serum and plasma were pipetted the into a clean plastic 0.5 mL cap vials and the labels were attached.	Centrifuge for 15 minutes at 1600g (RCF) for 15 mins at 4°C in a refrigerated centrifuge with sealed buckets The urine was pipetted the into a clean plastic 0.5 mL cap vials and the labels were attached.	Each sample was fixed, processed as described (in section 2.1.7.6) and embedded separately.
Storage	The blood was kept in an 80°C freezer at the Kennedy Institute of Rheumatology (KIR), Oxford.	The urine was kept in a -80°C freezer at the KIR.	The synovial fluid was kept at -80°C freezer at the KIR.	The synovial tissue was kept at room temperature in a lockable cupboard based at the histology department at the KIR.
*Before the amendments described in section 2.1.2, 20 ml of blood was taken				

during each visit.
**RCF (relative centrifugal force) is calculated using rotational speed (RPM) and the diameter of the rotor (mm). This allows the settings to be translated accurately between centrifuges with different rotor sizes and speed limitations. See attached for the equation from our centrifuge manufacturer (Hettich). RCF can be calculated using the formula below. $$RCF = \left(\frac{RPM}{1000}\right) * r * 1.118$$

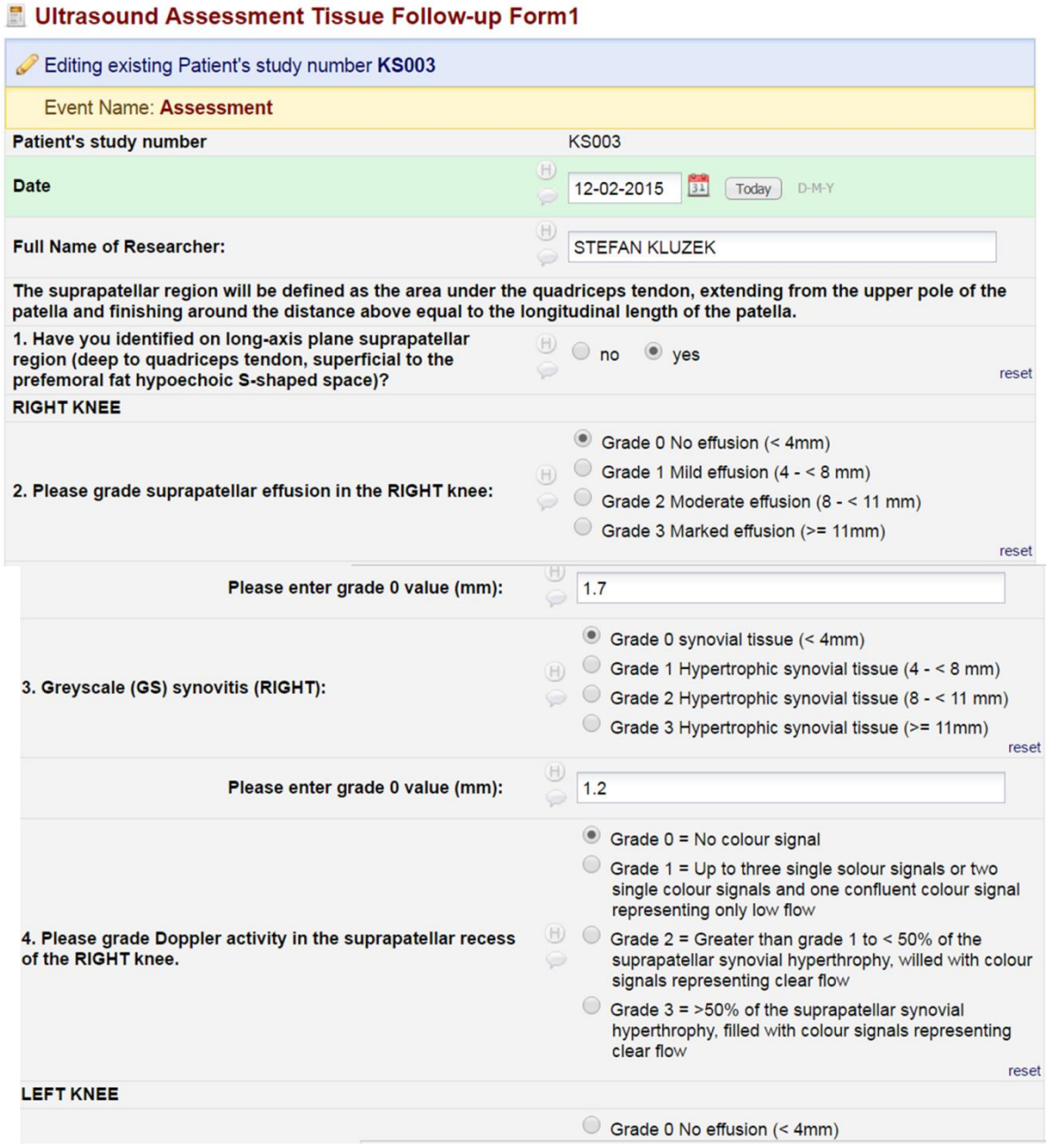
9.4. MRI protocols used in the OxKIC study

The protocol for knees across the NHS Oxford University Hospitals was performed on 3T MRI scanners unless the patient was claustrophobic or too large. Sequences used:

1. Sagittal and Coronal axial proton density (PD) weighted sequence.

2. Sagittal gradient-echo imaging.

9.5. The Ultrasound of Suprapatellar Recess: an example the online RedCAP form for recording the assessment results.

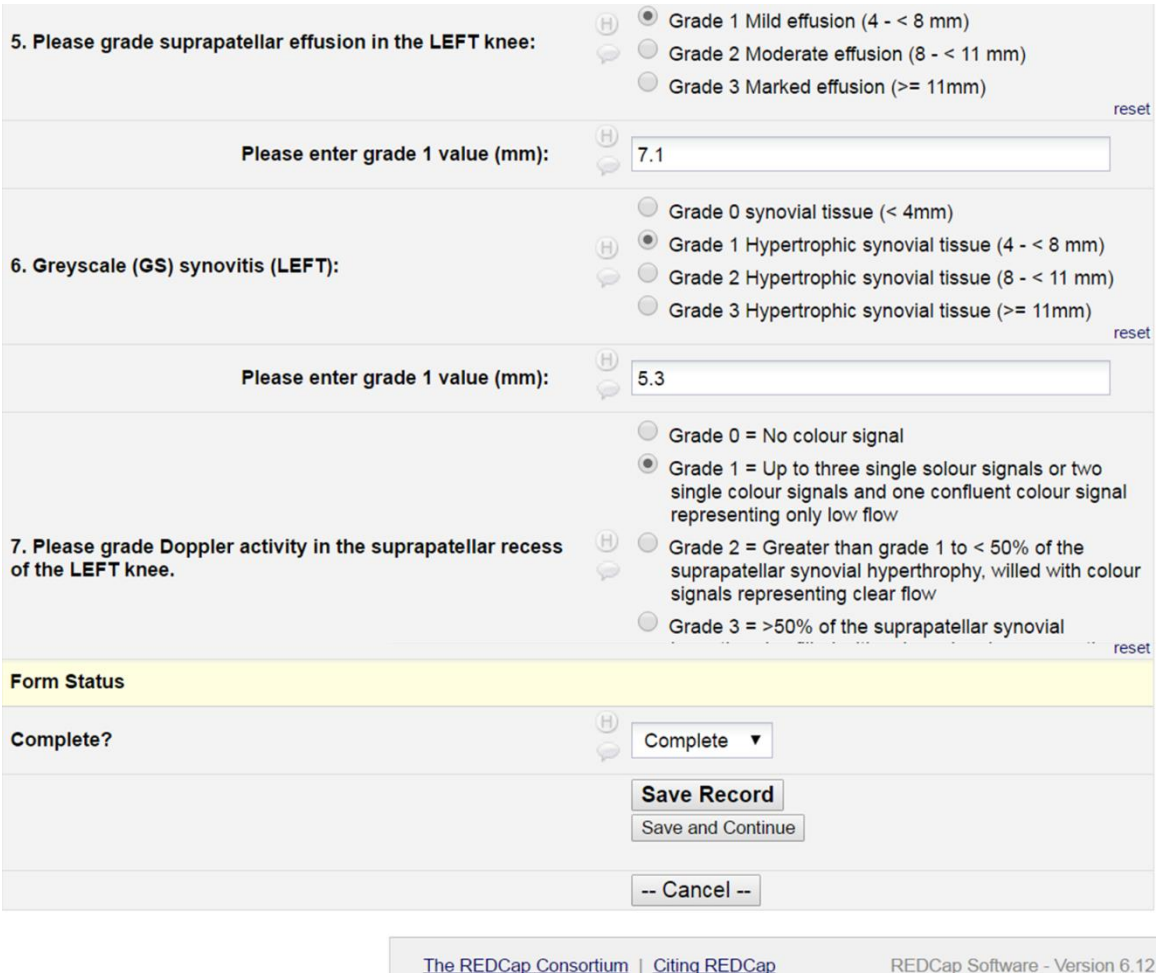


Ultrasound Assessment Tissue Follow-up Form1

Editing existing Patient's study number **KS003**

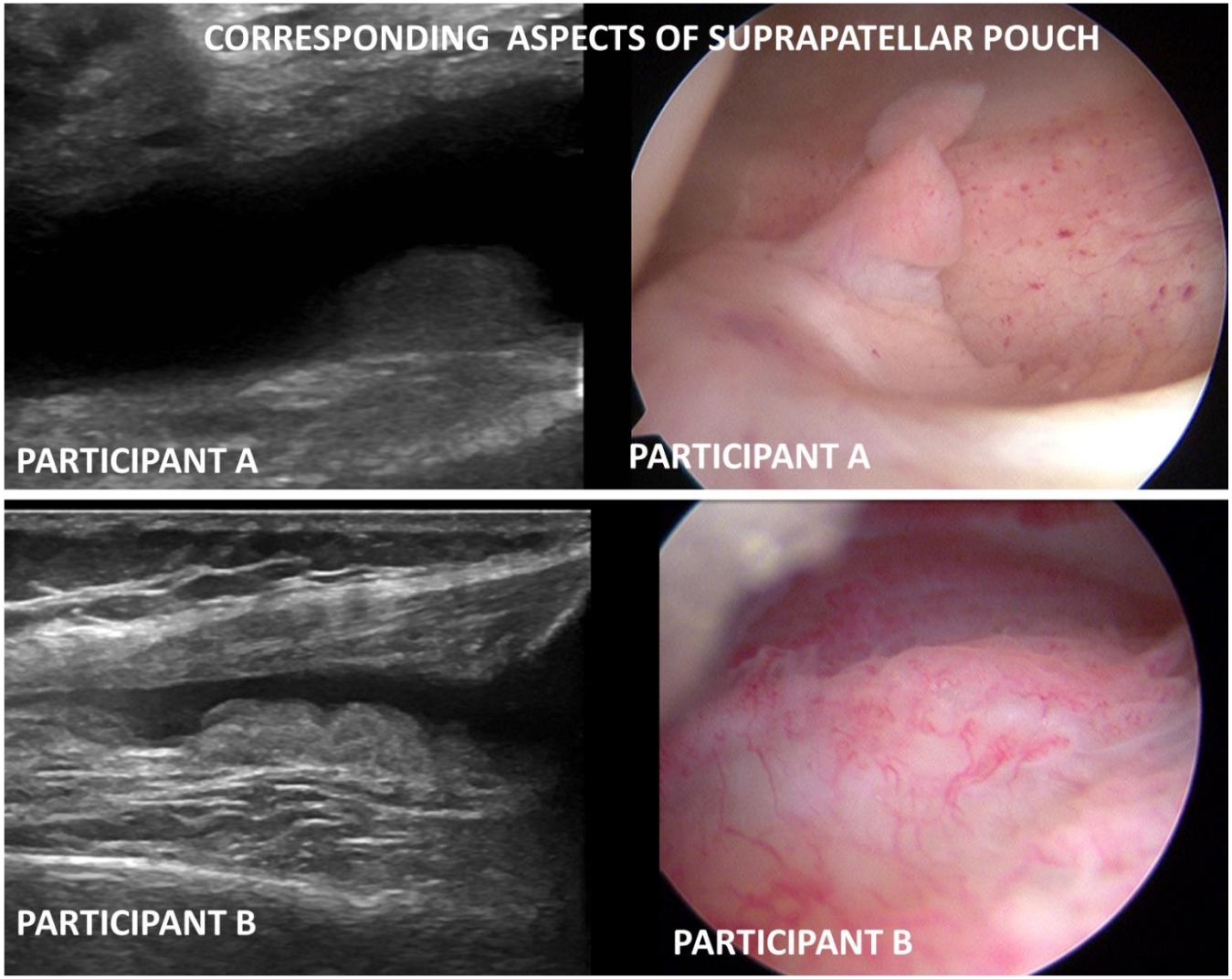
Event Name: **Assessment**

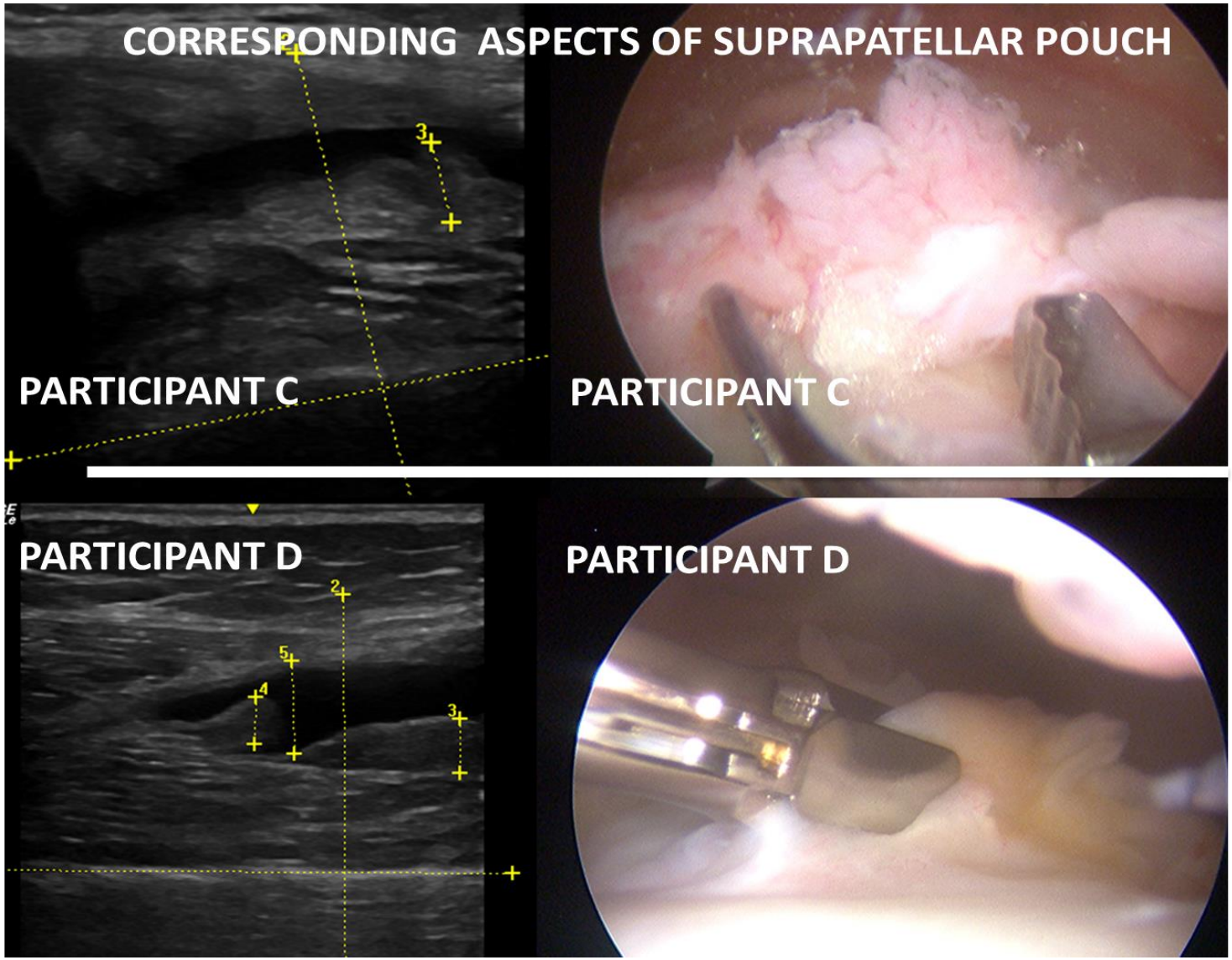
Patient's study number	KS003
Date	12-02-2015 Today D-M-Y
Full Name of Researcher:	STEFAN KLUZEK
The suprapatellar region will be defined as the area under the quadriceps tendon, extending from the upper pole of the patella and finishing around the distance above equal to the longitudinal length of the patella.	
1. Have you identified on long-axis plane suprapatellar region (deep to quadriceps tendon, superficial to the prefemoral fat hypoechoic S-shaped space)?	no / yes (reset)
RIGHT KNEE	
2. Please grade suprapatellar effusion in the RIGHT knee:	Grade 0 No effusion (< 4mm) Grade 1 Mild effusion (4 - < 8 mm) Grade 2 Moderate effusion (8 - < 11 mm) Grade 3 Marked effusion (>= 11mm) (reset)
Please enter grade 0 value (mm):	1.7
3. Greyscale (GS) synovitis (RIGHT):	Grade 0 synovial tissue (< 4mm) Grade 1 Hypertrophic synovial tissue (4 - < 8 mm) Grade 2 Hypertrophic synovial tissue (8 - < 11 mm) Grade 3 Hypertrophic synovial tissue (>= 11mm) (reset)
Please enter grade 0 value (mm):	1.2
4. Please grade Doppler activity in the suprapatellar recess of the RIGHT knee.	Grade 0 = No colour signal Grade 1 = Up to three single solour signals or two single colour signals and one confluent colour signal representing only low flow Grade 2 = Greater than grade 1 to < 50% of the suprapatellar synovial hyperthrophy, willed with colour signals representing clear flow Grade 3 = >50% of the suprapatellar synovial hyperthrophy, filled with colour signals representing clear flow (reset)
LEFT KNEE	
	Grade 0 No effusion (< 4mm)



9.6. The Ultrasound of Suprapatellar Recess: a comparison with arthroscopic images.

Four examples of arthroscopic images of suprapatellar synovial hypertrophy with ultrasound based pictures of the corresponding regions.


CORRESPONDING ASPECTS OF SUPRAPATELLAR POUCH
PARTICIPANT A
PARTICIPANT A
PARTICIPANT B
PARTICIPANT B


CORRESPONDING ASPECTS OF SUPRAPATELLAR POUCH
PARTICIPANT C
PARTICIPANT C
PARTICIPANT D
PARTICIPANT D

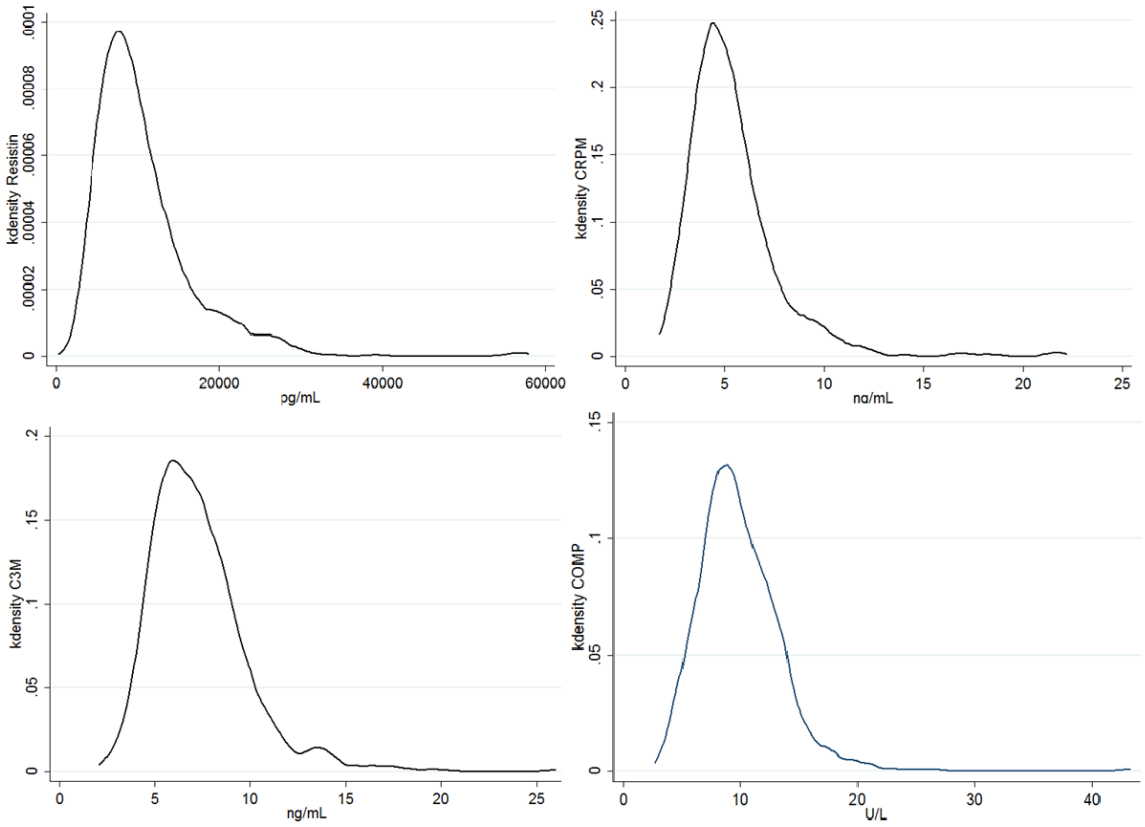
Appendix 3: Tables comparing subsets of selected cohorts

10.1 The OxKIC study, chapter 4. Characteristics of individuals from knee injury (OxKIC) groups who had a synovial samples analysed and all acute OxKIC study.

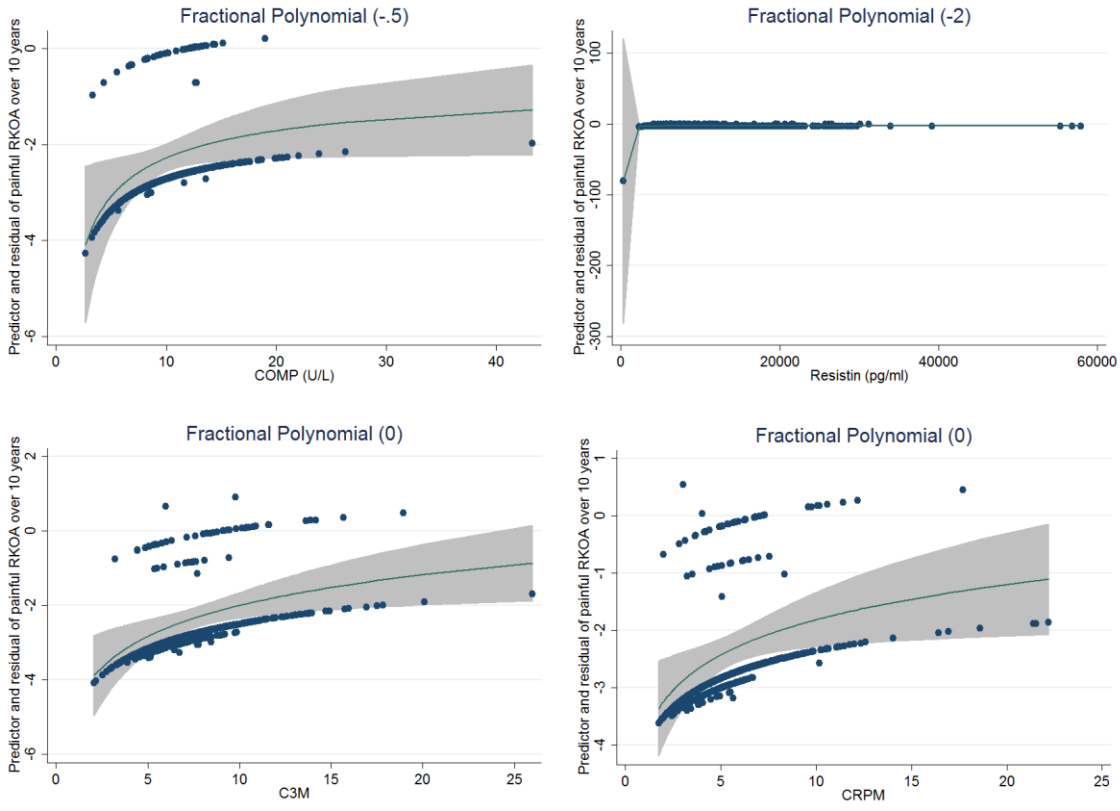
	Subset of Acute OxKIC who had biopsies (n=37)	**Acute OxKIC (n=90)**
Age, mean (SD)	30 (8.7)	32.4 (10.3)
Gender		
Male, n (%)	66 (65)	57 (63)
Female, n (%)	35 (35)	33 (37)
BMI, mean (SD)	26.2 (5.3)	26.8 (4.5)
Affected knee		
Right, n (%)	21(57)	49 (54)
Left, n (%)	16(43)	41 (46)
Injury		
ACL, n (%)	14 (37.8)	35(39)
ACL and meniscus, n (%)	17 (45.9)	37(41)

Meniscus/menisci tear	6 (16.2)	18(20)
Other		
Activity levels before the injury		
Tegner score >6 (competitive sports), n (%)	24(65)	58(65)
Tegner score >4 (competitive sport, recreational sport and heavy labour), n (%)	32(86)	85 (94)

10.2 The Chingford study, chapter 6. The baseline distributions for each biomarker in the Chingford study with baseline K/L grade of zero in both knees, before and after log transformation.

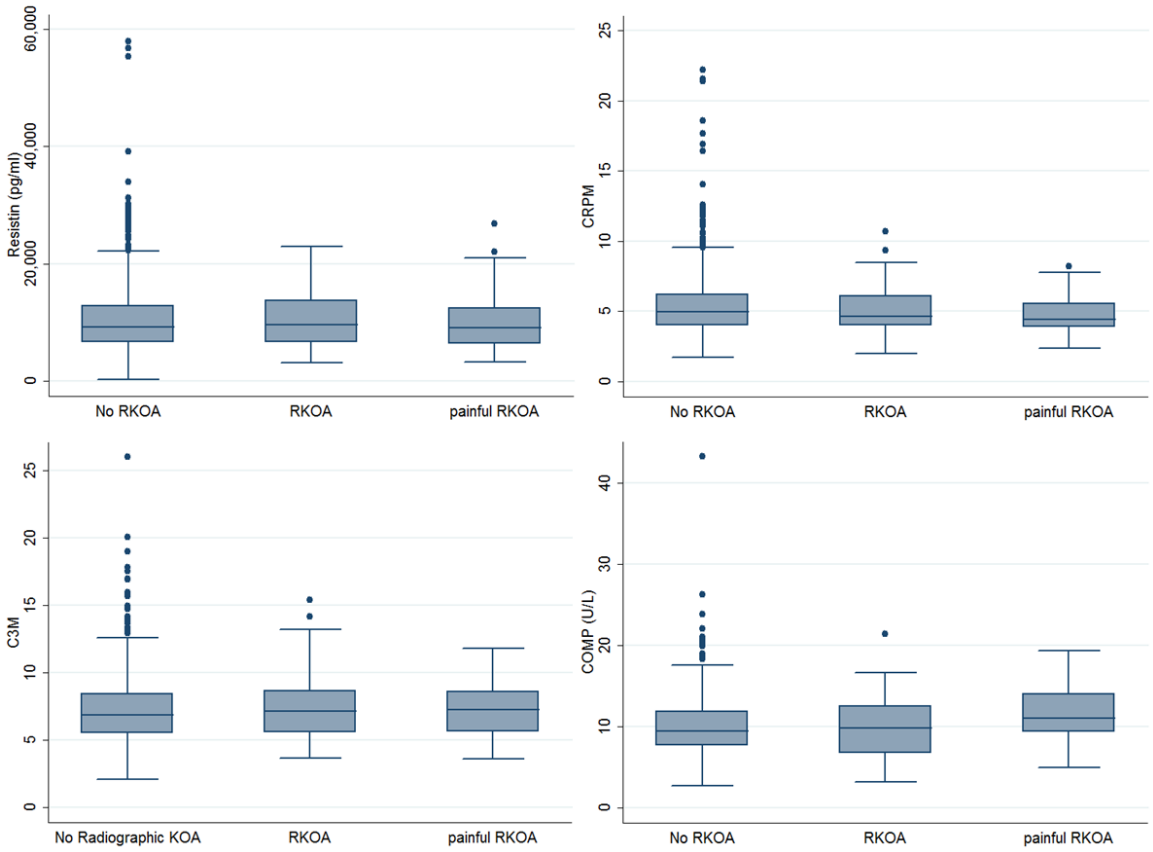


10.3 The Chingford study, chapter 6. The association between baseline levels of the biomarkers in the Chingford study and incidence of RKOA. FPM used to assess conformity with the gradient.

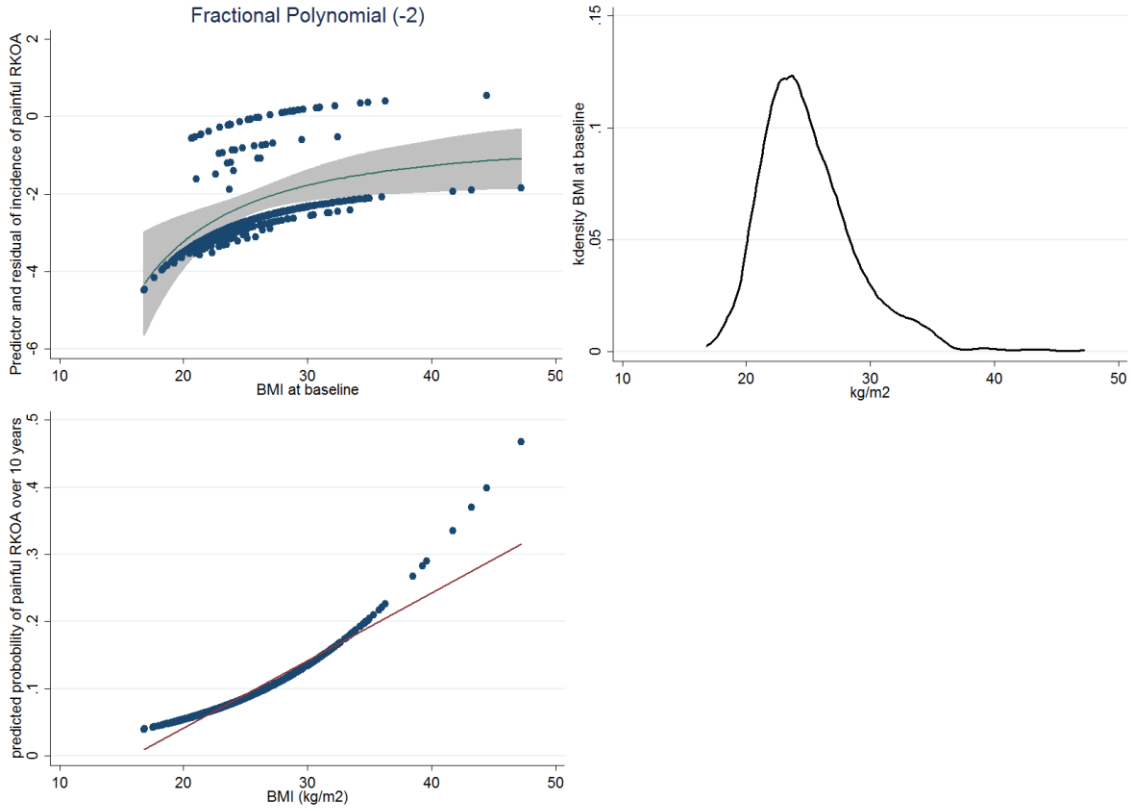


10.4 The Chingford study, chapter 6.

Whisker box plots for each subgroup of biomarkers based on radiographic and knee pain status are presented in the appendix



10.5 The Chingford study, chapter 6.Association between BMI and the outcome of interest (RKOA) at year 10 of the follow-up.



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