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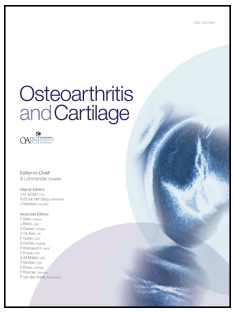

Molecular expression patterns in the synovium and their association with advanced symptomatic knee osteoarthritis

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Molecular expression patterns in the synovium and their association with advanced symptomatic knee osteoarthritis

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ABSTRACT (246/250 words)

OBJECTIVE: Osteoarthritis (OA) is a major source of knee pain. Mechanisms of OA knee pain are incompletely understood but include synovial pathology. We aimed to identify molecular expression patterns in the synovium associated with symptomatic knee OA.

DESIGN: Snap frozen synovia were from people undergoing total knee replacement (TKR) for advanced OA, or from post-mortem (PM) cases who had not sought help for knee pain. Associations with OA symptoms were determined using discovery and validation samples, each comprising TKR and PM cases matched for chondropathy (Symptomatic or Asymptomatic Chondropathy). Associations with OA were determined by comparing age matched TKR and PM control cases. Real-time quantitative PCR for 96 genes involved in inflammation and nerve sensitisation used TaqMan® Array Cards in discovery and validation samples, and protein expression for replicated genes was quantified using Luminex bead assay.

RESULTS: Eight genes were differentially expressed between asymptomatic and symptomatic chondropathy cases and replicated between discovery and validation samples (P<0.05 or > 3-fold change). Of these, matrix metalloprotease (MMP)-1 was also increased whereas interleukin-1 receptor 1 (IL1R1) and vascular endothelial growth factor (VEGF) were decreased at the protein level in the synovium of symptomatic compared to asymptomatic chondropathy cases. MMP1 protein expression was also increased in OA compared to PM controls.

CONCLUSION: Associations of symptomatic OA may suggest roles of MMP1 expression and IL1R1 and VEGF pathways in OA pain. Better understanding of which inflammation-associated molecules mediate OA pain should inform refinement of existing therapies and development of new treatments.

KEY WORDS: Osteoarthritis, Pain, Synovitis, Gene expression

INTRODUCTION

Knee osteoarthritis (OA) is a complex disease involving all joint tissues. Mechanisms of OA knee pain are incompletely understood, but can include synovial pathology [1-3] and subchondral bone [4]. Inflammatory mediators from the synovium activate or sensitise nociceptors through downstream signalling pathways. Nerve terminal sensitisation leads stimuli that would not usually elicit pain to be perceived as painful. Understanding molecular expression patterns that contribute to symptomatic OA is crucial to developing new analgesic treatment strategies, and to focus disease modification strategies on those which are most likely to improve symptoms.

Numerous inflammatory mediators such as cytokines, chemokines, growth factors, and matrix metalloproteinase (MMPs) released from synoviocytes during inflammation might contribute to OA pain. Key roles have been suggested for the pro-inflammatory cytokines interleukin (IL)-1β and tumour necrosis factor (TNF)-α in mediating pain through the release of other downstream inflammatory mediators such as matrix metalloproteases (MMPs) and cytokines [5]. The effects of IL-1β are mediated through binding to IL-1 receptor type 1 (IL1R1). Pain has been associated with increased TNF-α [6], chemokine ligand 2 (CCL2), chemokine ligand 4 (CCL4), IL-6 and interferon-γ [7] in synovial fluid. Vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis involved in neuropathic pain [8], is increased in OA synovium [9, 10] and associated with OA pain and progression [11]. MMP1 is an interstitial collagenase that is elevated in synovial fluid from people with OA [12]. The nuclear factor kappa-B (NF-κB) is part of a downstream signalling pathway which contributes to the up-regulation of various pro-inflammatory and angiogenic factors [13].

Recent work has identified differences in gene expression patterns of inflammatory cytokines between inflamed and non-inflamed areas of synovia from people with OA [14, 15]. We

hypothesised that specific molecular patterns in the synovium are associated with symptomatic OA, indicating possible molecular mechanisms of OA pain. Gene and protein expression patterns in the synovium were compared between groups of people with similar macroscopic appearances of the tibiofemoral articular surfaces who had either sought TKR for OA symptoms (symptomatic chondropathy) or had not sought help for OA knee pain before death (asymptomatic chondropathy), and between people with or without OA. The rationale for comparing people with or without OA was to define whether signatures identified as characteristic of symptomatic OA were also characteristics of OA. Pain in OA might be due to aspects of OA pathology which mediate pain, or due to concurrent pathology which, in the context of OA, is painful.

METHOD

This cross-sectional study was approved by Nottingham Research Ethics Committee 1 (05/Q2403/24) and Derby Research Ethics Committee 1 (11/H0405/2).

Patients

Total knee replacement groups

Snap frozen synovium samples were collected at total knee replacement (TKR) surgery for symptomatic OA ('OA' or 'symptomatic chondropathy' groups). All TKR cases satisfied the American College of Rheumatology classification criteria for knee OA at the time of surgery [16] but groups differed only in that the OA group comprised cases aged-matched to post mortem (PM) controls, whereas the symptomatic chondropathy group was matched to the asymptomatic chondropathy group for macroscopic scoring of cartilage surface changes [17]. All in the OA group had a Kellgren Lawrence radiographic score ≥2.

Post-mortem (PM) groups

Three sample groups were selected from post-mortem (PM) cases who did not have arthritis and had not reported knee pain during the last year of their life ('PM control', 'non-arthritic control' and 'asymptomatic chondropathy' groups).

The PM control group were selected as consecutive aged matched cases to the OA group and did not include cases with macroscopic chondropathy lesions of grade 4 (subchondral bone exposure) in the medial tibiofemoral compartment [17].

Inclusion criteria for the non-arthritic control group were no osteophytes in the dissected knee, no Heberden's nodes (because these may be associated with knee OA incidence and progression [18]) and no macroscopic chondropathy lesions grade ≥3 in the medial tibiofemoral compartment [3].

Molecular associations with OA symptoms

Associations of gene expression with symptoms were determined using discovery (n=12/group) and validation samples (n = 10/group), each comprising symptomatic and asymptomatic chondropathy groups. Discovery and validation samples were combined to compare protein expression between asymptomatic (n=22) and symptomatic (n=22) chondropathy groups (supplementary figure 1A).

Molecular associations with OA disease status

The following age-matched (within 7 years) control PM groups and OA groups were compared to determine associations with OA disease status:

1) Non-arthritic control vs. symptomatic chondropathy (n = 10/group) for gene expression analyses (supplementary figure 1B).

2) PM control (n=10) vs. OA (n=11) for protein expression analyses (supplementary figure 1C).

Body mass index (BMI; kg/m^2) was available for TKR but not PM cases.

Tissue processing and grading

Surgeons and technician (RH) were instructed to collect synovium from the medial joint line from PM and TKR cases. Fresh synovium was snap-frozen in liquid nitrogen, without fixation, with replicate samples formalin-fixed and wax-embedded for haematoxylin and eosin staining and grading for synovitis [9]. Synovial inflammation was graded (0 to 3) only in samples with synovial lining present. Grade 0 = no synovitis, synovial lining < 4 cells thick, with few or no inflammatory cells. Grade 1 = mild synovitis, synovial lining 4 or 5 cells thick, with increased cellularity and some inflammatory cells present. Grade 2 = synovial lining 6 or 7 cells thick, dense cellularity with inflammatory cells (but no lymphoid aggregates). Grade 3 = severe synovitis; synovial lining more than 7 cells thick, with inflammatory cell inflammation which may include perivascular lymphoid aggregates and dense cellularity.

The extent and severity of articular cartilage loss of medial and lateral tibial plateaux and femoral condyles were graded [17] as follows; grade 0 = normal: smooth, unbroken surface, homogeneous white to off-white colour, grade 1 = swelling and softening: a light brown homogenous colouration, grade 2 = superficial fibrillation lightly broken surface, white to off-white/light brown in colour, grade 3 = deep fibrillation: coarsely broken cartilage surface, dark brown, grey or red in colour, grade 4 = subchondral bone exposure: stippled white and dark brown/red in colour. The proportion of each articular surface area corresponding to each grade was used to calculate a chondropathy score (0-100). Scores for each of the 4

compartments were summated to give a tibiofemoral chondropathy score (0; normal – 400; complete cartilage loss).

PM delay was calculated as the time (h) between death and opening of the knee for tissue collection. Cadavers were stored at 4°C.

Gene expression

Total RNA was extracted from snap frozen synovia, homogenised in 1ml of TRI reagent (Sigma, Poole, UK) and purified according to manufacturer instructions. Total RNA (100ng) was reverse transcribed to complementary DNA using Affinity Script Reverse Transcriptase (Agilent Technologies, Stockport, UK) and random primers, according to the manufacturer's protocol. The reaction was incubated at 25°C for 10 minutes, then 50°C for 60 minutes and terminated by incubation at 70°C for 15 minutes. The cDNA was in a total reaction volume of 28µl.

Gene expression profiling was performed using custom-made 384 well microfluidic cards (TaqMan® Array Card, Applied Biosystems, Waltham, MA). Each card consisted of 4 reference genes (Beta actin [*ACTB*], Glyceraldehyde 3-phosphate dehydrogenase [*GAPDH*], Hydroxymethylbilane Synthase [*HMBS*] and Ubiquitin C [*UBC*]) and 92 target genes, which were identified as possibly mediating pain through sensitising peripheral nerve terminals (supplementary table 1).

For each tissue sample a reaction mix was made using 100µl of diluted cDNA (1:4) and 100µl of TaqMan Universal PCR Master Mix. Reaction mix (100µl) was loaded into two adjacent ports in the microfluidic card which allowing duplicate runs on a 7900HT Fast Real-Time PCR system (Applied Biosystems). RNA expression values are reported as arbitrary units normalised to reference gene expression.

Protein expression

The Luminex screening human assay (10-plex) (LXSAH-10, R&D systems) was used to measure expression of CCL2, CCL5, CCL8, Chemokine ligand 10 (CXCL10), IL1β, IL1R1, MMP1, MMP7, TNFα, VEGF. Analytes selected for Luminex analysis were those that were either significantly ($P<0.05$) or > 3-fold different (in the same direction) between asymptomatic and symptomatic chondropathy groups in both the discovery and validation samples. In addition, we included two analytes previously hypothesised to be important in OA (TNFα and IL1β) [19], and two pro-inflammatory chemokines that were increased in symptomatic chondropathy compared to non-arthritic controls (CXCL10 and CCL5). ANXA1 and NFKBIA protein expression were excluded due to non-availability of compatible reagents. Discovery and validation samples on cases with RNA data in the current study were together used to compare protein expression between asymptomatic and symptomatic chondropathy groups.

Total protein was extracted from snap frozen synovia homogenised in 600µl of Cell Lysis buffer (R&D systems, Abingdon, UK) with protease inhibitor (Sigma), and centrifuged for 5 minutes. Total protein concentration was measured in supernatants (Pierce BCA-200 Protein Assay Kit, Fisher Scientific, Loughborough, UK). For Luminex analysis the remaining supernatant was diluted 1:2 with Calibrator Diluent RD6-52. The plate, standards (3-fold dilution series), microparticle cocktail, biotin antibody cocktail and streptavidin-PE were prepared according to the manufacturer's instructions. In brief, the plate was rinsed with wash buffer and liquid removed using a vacuum manifold. Tissue samples were incubated (2h, room temperature) with the microparticle cocktail on a microplate shaker, followed by incubation with Biotin antibody (1h) and Steptavidin-PE (30min), with triplicate washes between each step. Plates were read using a Bio-Plex® Multiplex Immunoassay System (Bio-

Rad, Hemel Hempstead, UK). Each analyte was adjusted for total protein concentration in each case. Protein expression is expressed as ng protein of interest per g total protein (ng/g).

Statistical analysis

Fold changes in gene expression levels were calculated for each tissue sample using the comparative C_t method ($2^{-\Delta Ct}$) where ΔC_t refers to C_t value of each individual target gene value minus C_t value of the reference gene. ΔC_t values are given as mean (95% confidence interval [CI]) and using Mann-Whitney U test (asymptomatic vs symptomatic chondropathy and PM control vs OA). Kruskal Wallis One Way ANOVA with post-hoc pair wise comparisons compared differences between non-arthritic controls, asymptomatic and symptomatic chondropathy). Fold increase in gene expression was calculated by dividing the mean of the symptomatic chondropathy group by the mean of the asymptomatic chondropathy group, and fold decrease as the inverse of the fold increase. Tissue samples were excluded from analysis where RNA could not be transcribed to cDNA, or where reference gene C_t values were outliers (Grubb's test, Graphpad, San Diego). $P< 0.05$ was considered statistically significant, and the false discovery rate (FDR) set at 5%, was used to correct for multiple testing [20].

NormFinder (Microsoft Excel add-in) was used to determine the most stable individual reference gene C_t values, or the most stable geometric mean of different combinations of reference genes to normalise gene expression.

Binary logistic regression compared between groups reference gene stability and gene expression associations with covariates (age, gender, BMI and PM delay, each separately tested in discovery and validation samples combined). C_t values were dichotomised about the

median as the dependent variable, and analyses adjusted for experiment number, to account for inherent variability between experimental runs as discovery and validation studies were conducted on different days. Spearman's rank correlation was used to determine associations between protein expression and each parameter (age, gender, BMI, PM delay), and separately to identify associations between reference gene expression and PM delay.

Multivariable testing was used to adjust for multiple covariates (age, gender and experiment number) combing discovery and validation sample RNA gene expression data for key analytes. All gene and protein targets were selected to share associations with inflammation or sensitisation, and therefore adjustments were not made for other genes or proteins measured in the same cases within each experiment.

Pseudo R^2 values are reported to explain logistic regression model variance (Cox and Snell R-square and Nagelkerke R-square), and percentages are reported for the number of cases correctly classified as asymptomatic chondropathy vs. symptomatic chondropathy. Receiver operator curve (ROC) analysis was used to determine sensitivity, specificity and 95% confidence intervals for determining classification of asymptomatic or symptomatic chondropathy cases (StataSE v15). ROC analyses were conducted using one gene at a time and binary logistic regression was undertaken to produce a predictive variable combining three genes together.

RESULTS

Patient demographics and joint pathology

Study groups were similar for sex, but symptomatic chondropathy groups were younger than asymptomatic chondropathy groups in discovery gene expression and proteomics studies (Table 1). Synovitis scores were higher in symptomatic (median (IQR); 1 (0-3) and 1.5 (0.25-

241 3)) than in asymptomatic (0 (0-0.5) and 0 (0-0)) chondropathy cases (P=0.05 and 0.005,
242 respectively for discovery and validation gene expression samples).

243 PM controls (n=10) selected for comparison of protein expression with OA cases (n=11)
244 displayed low macroscopic chondropathy scores (median [IQR]; 82 [45-111]) and their
245 demographics did not significantly differ from OA cases (median (IQR) ages 66 (59-70) and
246 61 (54-74) years, P=0.86; 60% and 27% male, P=0.20). Histological synovitis was absent
247 (grade 0) in 9/10 PM control cases and mild (grade 1) in 1 case. Cases in the OA group all
248 displayed moderate or severe synovitis (grades 2 or 3).

249 ***[TABLE 1]***

Reference gene expression

C_t expression for each of the 4 reference genes was not significantly different between PM and TKR cases (asymptomatic and symptomatic chondropathy groups, respectively, $P \geq 0.42$) and their geometric mean was used for normalisation (supplementary Table 2). PM delay (h) was not associated with the C_t values of any of the four reference genes; *ACTB, GAPDH, HMBS* and *UBC* (P = 0.98, 0.74, 0.70, 0.68). Final study numbers/group are reported in table 1, (see table 1 legend for an explanation of exclusions).

Synovial gene and protein expression patterns associated with symptomatic OA

Synovial gene expression in symptomatic OA

In the discovery samples, following corrections for multiple testing (FDR = 5%, $P \leq 0.01$) 8 genes were significantly upregulated and 12 significantly down-regulated in symptomatic compared to asymptomatic chondropathy cases (supplementary Table 3). In the validation samples, 2 genes were significantly up-regulated and one significantly down-regulated (supplementary Table 4). Table 2 shows genes which were differentially expressed in the same direction in both discovery and validation samples.

CCL2, CCL8 and *ANXA1* were up-regulated in symptomatic chondropathy cases in both discovery and validation samples (Table 2) but did not reach statistical significance after FDR correction. In addition, *MMP1* expression was >3-fold higher in symptomatic chondropathy cases across both samples, reaching statistical significance in the discovery sample. *IL1R1* and *NFKBIA* gene expressions were down-regulated in symptomatic chondropathy cases in both discovery and validation samples, *IL1R1* remaining significant after FDR correction in both samples. *MMP7* and *VEGFA* expressions were >3-fold lower in

symptomatic chondropathy cases in both discovery and validation samples, *VEGFA* was significantly downregulated in the discovery sample (P= 0.001).

[Table 2]

Synovial protein expression in symptomatic OA

Five analytes were significantly differentially expressed at the protein level between groups (Figure 1, Table 3). Of these, CCL5 and MMP1 were greater, whereas VEGF, CXCL10 and IL1R1 were each lower in symptomatic than in asymptomatic chondropathy cases.

[Figure 1]

[Table 3]

Synovial gene and protein expression patterns associated with OA disease status

In order to explore whether differences in gene and protein expression between symptomatic and asymptomatic OA represented characteristics of OA disease, we compared OA samples obtained at TKR with post mortem samples from people without known arthritis.

Synovial gene expression

Gene expression is compared between groups in supplementary table 4. Several genes were upregulated in symptomatic chondropathy compared to non-arthritic control groups (fold increase, *P*); *ANXA1* (1.90, P<0.001), *ANXA6* (2.30, P=0.001), *CCL2* (2.25, P=0.042), *CCL5*

(3.30, P=0.001), *CMKLR1* (4.25, P=0.02), *CTGF* (3.06, P=0.001), *CXCL10* (6.28, P=0.001) and *FOS* (6.87, P<0.001). *F2RL3* (32.25, P<0.001), *IL1R1* (1.84, P=0.02) and *NFKBIA* (3.52, P=0.04) were decreased in symptomatic chondropathy compared to non-arthritic control groups.

Synovial protein expression in OA

CCL8 and MMP1 protein immunoreactivities were significantly increased in the synovium of the OA compared to PM control groups (Figures 2B&C), whereas CCL2, VEGF, CXCL10 IL1R1 and CCL5 did not reach statistical significance (Figures 2A, D-G).

[Figure 2]

Contribution of synovial molecular expression to classification of symptomatic and asymptomatic chondropathy

To evaluate the possible direct contributions of synovial molecular expression to the presence or absence of symptoms in OA we first explored possible effects of measured confounding factors, and then evaluated the relative contributions of gene expression for 3 identified key molecules (*IL1R1, MMP1* and *VEGFA*) to classification of symptomatic and asymptomatic chondropathy cases.

Possible effects of age, gender, post-mortem delay or body mass index on protein or gene expression, separately were explored; only *IL1R1* gene expression was associated with age and *CXCL10* with gender (supplementary table 5).

When gene expression data from discovery and validation samples, analysed within a single model, were adjusted for age, gender and experiment number, the following were

significantly increased in symptomatic chondropathy cases compared to asymptomatic cases; *CCL2* (2.01-fold, $P = 0.01$), *CCL8* (4.46-fold, $P = 0.007$), *IL1β* (1.93-fold, $P = 0.021$) and *MMP1* (11.6-fold, $P = 0.03$). *IL1R1* and *VEGFA* RNA were significantly decreased (2.67-fold, $P = 0.016$, and 4.79-fold, $P = 0.017$, respectively) in symptomatic chondropathy vs. asymptomatic chondropathy. *CCL5, CXCL10, MMP7* and *TNFα* RNA were not significantly different between groups $P = 026$, 0.11, 0.17 and 0.26, respectively.

The logistic regression model exploring association of symptomatic vs. asymptomatic chondropathy with expression of each identified gene was adjusted for age, gender and experiment number. For *IL1R1* the model explained between 58% (Cox and Snell R-square) to 78% (Nagelkerke R-square) of the variance and correctly classified 87% of cases. For *MMP1* the logistic regression model explained between 49% (Cox and Snell R-square) to 65% (Nagelkerke R-square) of the variance and correctly classified 80% of cases. For *VEGFA* the logistic regression model explained between 49% (Cox and Snell R-square) to 66% (Nagelkerke R-square) of the variance and correctly classified 87% of cases. A combined logistic regression model (which included *MMP1, IL1R1* and *VEGFA,* adjusted for age, gender and experiment number), explained between 75% (Cox and Snell R-square) to 100% (Nagelkerke R-square) variance, and correctly classified 100% of cases in symptomatic and asymptomatic chondropathy groups. Similarly, ROC analyses indicated that 90% of cases were correctly classified using combined expression of the 3 genes, with sensitivity and specificity of 85-95% (supplementary table 6).

DISCUSSION

We have identified synovial molecular expression patterns that are associated with symptomatic OA by comparing TKR cases (symptomatic chondropathy) with PM cases with similar macroscopic joint surface appearances who had not sought help for knee pain (asymptomatic chondropathy). Additionally, we have identified synovial molecular expression patterns that are associated with OA disease status, by comparing aged-matched PM and TKR cases.

Up-regulation of MMP1 in concert with the down-regulation of VEGFA and IL1R1 might reflect molecular pathways that mediate OA symptoms. MMP1 (collagenase-1) is a secreted metalloproteinase which catalyses cleavage of matrix collagens in OA. MMP1 gene and protein expression were increased in association with OA disease status in the synovia of OA compared to PM controls. MMP1 is induced in synovial fibroblasts in response to pro-inflammatory mediators such as IL1β and TNFα [21]. Synovium, as well as chondrocytes, might contribute to increased synovial fluid MMP1 levels observed in OA [22]. Association of MMP1 expression with symptomatic disease is unlikely to be entirely explained by cartilage structural damage because our cases and controls were matched for severity of macroscopic chondropathy. Urinary collagen degradation products, generated by the action of collagenases, have also been associated with OA pain[23]. Increased MMP1 might be a marker of cytokine-driven inflammation, which may in turn lead to a cascade of events that sensitise peripheral nerve terminals in the synovium, whilst exacerbating cartilage damage.

IL1β is produced by OA synovium, even in early disease[24]. IL1β was upregulated in symptomatic compared to asymptomatic chondropathy cases. The pro-inflammatory actions of IL1β are exerted through binding its membrane receptor, interleukin-1 receptor (IL1R1). Increased IL1R1 expression was previously found in OA synovial fibroblasts, compared to

normal controls [25]. IL1R1 expression can be downregulated during activation by IL1β [26]. Our data suggest downregulation of IR-1R1 in OA synovium compared to non-arthritic controls, and, in particular in symptomatic compared with asymptomatic chondropathy, consistent with increased IL1β/IL1R1 pathway activity. Decreased IL1R1 mRNA in symptomatic chondropathy was replicated across both discovery and validation samples, and at the protein level. IL1β/IL1R1 pathway activation might therefore have particular relevance for OA symptoms. Studies using OA animal models report favourable benefits of IL-1 receptor antagonist therapy [27, 28]; however clinical trials in humans reported no improvement in pain ([29, 30]).Antibodies specifically targeted at IL1R1 did not achieve clinical important symptomatic benefit compared to placebo [29]. Our data raise the possibility that IL1R1 downregulation prior to treatment might have contributed to these negative results, and earlier phases of OA synovitis might respond differently to IL1β/IL1R1 pathway inhibition. Furthermore, IL1β/IL1R1 pathway inhibition might only be effective for a subset of people with OA whose pain is mediated by synovitis.

Increased VEGF in synovium, cartilage, synovial fluid and plasma might contribute to synovitis and osteophyte formation in OA[31]. VEGF might also contribute to OA pain through facilitating inflammation and by actions on sensory nerves[32, 33]. Perhaps surprisingly, we found that VEGFA was decreased at the gene and protein level in patients with symptomatic compared to asymptomatic chondropathy. VEGF exists as multiple isoforms dependant on alternative splicing of mRNA [34]. VEGFAa isoforms contribute to angiogenesis and pain, whereas VEGFAb isoforms might be anti-angiogenic and analgesic. Further studies should explore whether reduced VEGF expression observed in the current study reflects an alteration in isoform balance that might contribute to OA pain.

We found associations of symptomatic chondropathy with a range of additional chemokines, cytokines and metalloproteinase, although associations were less consistent at gene and

protein expression levels than with MMP1, IL1R1 and VEGF. The small sample sizes in the current study might have led us to overlook biologically important associations, although our repository of joint samples from >3000 cases was required to select sample groups with adequate matching for severity of structural chondropathy and other factors. Further research should explore mechanisms by which CCL2, CCL8, CCL5, CXCL10, TNF-α and MMP7 might contribute to OA pain.

CCL2 and CCL8 gene expressions were higher in symptomatic OA vs non-arthritic controls (CCL8 protein was also higher in OA vs. PM controls), and in symptomatic knee OA compared to chondropathy-matched asymptomatic post mortem cases. CCL2 and CCL8 each serve as ligands for chemokine receptor 2 (CCR2) [35]. CCL2 from synovial fibroblasts [36] recruits and activates inflammatory cells to sites of inflammation [37] and CCL2 mRNA and protein are upregulated in osteoarthritic tibiofemoral joints [38]. Synovial fluid CCL2 has been associated with OA knee pain severity, in addition to physical disability [39]. During inflammation, elevated expression of CCL2 might act on sensory nerves to activate transient receptor potential cation channel subfamily V member 1 (TRPV1) to induce hyperalgesia [40]. CCL8 has previously been detected in fibroblasts and macrophages in the synovial lining of arthritic patients [35]. Mice that lacked the CCL2 receptor (CCR2) were protected against movement-provoked pain following surgical induction of OA [41]. Together these data indicate the CCL2, CCL8 and CCR2 pathway as possible targets for OA pain.

Our study is necessarily subject to a number of limitations. Both RNA and proteins are susceptible to degradation by post-mortem processes, and RNA by RNAses [42]. However, we did not identify associations between gene or protein expression levels and time from death to tissue processing for any of the replicated genes taken forward for Luminex analysis. Furthermore, there were no significant differences in the expression of the 4 reference genes between surgical and post-mortem groups. Target gene expression was also normalised to

reference genes to compensate for any heterogeneity of quality between tissue samples. Genes might be activated post-mortem, however this has only been shown in animal studies and not yet with human tissue [43]. OA is strongly associated with age, and it can be difficult to distinguish between OA pathological change and age-related changes or senescence. However, we found associations of gene and protein expression with disease status in age-matched cases, and associations with symptomatic chondropathy were not affected by adjustment for chronological age, except for IL1R1. Gene expression and protein levels alone need not necessarily indicate protein activity. We validated key molecular targets identified through gene expression studies using a complimentary proteomics approach, but future studies should explore functional activity. We investigated a large number of proteins and genes, and some statistically significant associations might have occurred by chance. In order to reduce this risk, we undertook analyses to adjust for multiple testing by applying a correction for FDR [20]. Furthermore our study design comprised of initial exploratory analysis (discovery RNA study), which was then validated using a separate set of asymptomatic and symptomatic chondropathy cases. Our main conclusions are based on results from across independent case samples used for discovery and validation gene expression studies and supported by protein expression data. Genes and proteins were selected for study due to their potential roles in inflammation and neuronal sensitisation, and identified targets might be markers for other associated inflammatory or sensitising factors. The high pseudo R^2 values obtained in this study suggest that, when severity of chondropathy is matched, a high proportion of model variance for allocation to symptomatic or asymptomatic chondropathy groups might be explained by synovial gene and protein expression. This suggests that gene and protein expression might be biologically important, but targets identified through these studies require further exploration either as biomarkers, or

as treatment targets for managing OA pain. However, it is important to note that the high pseudo R^2 values may be representing an overfitted model.

In conclusion, symptomatic OA was associated with an up-regulation in synovium of MMP1 and decrease of IL1R1 and VEGFA compared to asymptomatic chondropathy cases with similar macroscopic joint surface appearances who did not seek TKR. Synovial inflammation is a feature of symptomatic OA, and better understanding of the gene expression patterns could lead to refinement of existing therapies and development of new treatments to reduce pain. This work was a target generating exercise. Further work is necessary to determine whether molecular targets that we have identified are biologically or clinically important, or may eventually lead to treatment strategies aiming to alleviate OA symptoms.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr Wyatt (laura.wyatt@nottingham.ac.uk) had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Substantial contributions to study conception and design. Wyatt, Wilson, Hill, Spendlove, Bennett, Scammell and Walsh

Substantial contributions to acquisition of data: Wyatt and Nwosu.

Substantial contributions to analysis and interpretation of data. Wyatt, Nwosu, Spendlove, Bennett, Scammell and Walsh

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1: Protein expression in synovia from chondropathy cases classified as either asymptomatic or symptomatic. Groups were matched for macroscopic chondropathy scores. **A**: CCL2 (chemokine ligand 2), **B**: CCL5 (chemokine ligand 5). **C:** MMP1 (matrix metalloprotease 1), **D**: VEGF (vascular endothelial growth factor-A), **E**: CXCL10 (chemokine ligand 10), **F**: IL1R1 (interleukin 1 receptor 1). Median (IQR) are shown. IL1β, TNFα, MMP7 and CCL8 immunoreactivities were below the lower limit of detection.

Figure 2: Protein expression for selected genes compared between PM control and OA cases undergoing arthroplasty. **A:** CCL2 (chemokine ligand 2), **B:** CCL8 (chemokine ligand 8) **C**): MMP1 (matrix metalloprotease 1), **D:** VEGF-A (vascular endothelial growth factor-A), **E:** CXCL10 (chemokine ligand 10), **F:** IL1R1 (interleukin 1 receptor 1) and **G:** CCL5 (chemokine ligand 5). Data expressed as median (IQR). MMP7, IL1β and TNFα immunoreactivities were below the lower limit of detection.

638 **TABLES**

639 **Table 1 Clinical and pathological characteristics of the study groups**

	Discovery sample				Validation sample			Protein expression		
	Chondropathy			Non-arthritic controls	Chondropathy			Chondropathy		
	Asymptomatic	Symptomatic	P		Asymptomatic	Symptomatic	P	Asymptomatic	Symptomatic	P
n	11	**11**		7	8	**9**		20	**21**	
Age, years	79 (65-88)	61 (54-73)	0.005	64 (49-74)	67 (52-78)	64 (55-72)	0.756	74 (64-85)	64(35-82)	0.026
% male	36	46	0.748	43	25	35	0.774	35	43	0.611
BMI, kg/m^2	NA	33 (31-39)	NA	NA	NA	31 (28-36)	NA	NA	32 (29-37)	NA
Post-mortem delay (h)‡	58 (29-89)	NA	NA	55 (29-64)	66 (44-79)	NA	NA	64 (35-82)	NA	NA
Macroscopic chondropathy score (scale range 0-400)	214 (204-229)	223 (213-239)	0.300	55 (44-97)	197 (163-204)*	195(171-203)^	0.001	205 (195-223)	208(188-231)	0.698

640 Tissues were obtained at the time of total knee replacement for OA (symptomatic chondropathy) or were obtained post mortem (asymptomatic chondropathy
641 and non-arthritic controls). Results are reported for groups following exclusions for outlier reference genes, or inability to transcribe RNA to cDNA. In the
642 discovery RNA study, 1 asymptomatic chondropathy case was excluded due to inability to transcribe RNA to cDNA (low RNA concentration) and one
643 symptomatic chondropathy case due an outlier reference gene Ct value (final numbers, 11/group). In the validation study, the following were excluded from the
644 final analysis; 3 non-arthritic controls, (low RNA concentration), 2 asymptomatic chondropathy cases (one low RNA concentration, the other due to an outlier
645 reference gene Ct value) and 1 symptomatic chondropathy cases (low RNA concentration). Final numbers for the validation study were 7 non-arthritic controls,
646 8 asymptomatic chondropathy and 9 symptomatic chondropathy. Protein expression conducted on one extra asymptomatic chondropathy and symptomatic
647 chondropathy case that were excluded from the final RNA analysis (due to outlier reference genes). Asymptomatic and symptomatic chondropathy cases were
648 successfully matched for macroscopic chondropathy scores. ‡Post-mortem delay was calculated as the time (h) between death and tissue collection. Data

649 expressed for included cases as median (IQR) or %. Differences between asymptomatic and symptomatic chondropathy groups in the discovery sample and in
650 the proteomics analysis were comparing using Mann Whitney tests. Differences between non-arthritic controls, asymptomatic chondropathy, and symptomatic
651 chondropathy groups in the validation sample were compared using Kruskal Wallis One Way ANOVA. *P = 0.006 vs non-arthritic controls, ^P = 0.003 vs
652 non-arthritic controls. BMI; body mass index, NA; not available.

653 **Table 2: Genes which were differentially expressed in the synovium of symptomatic chondropathy**
654 **cases compared to asymptomatic chondropathy cases in discovery and validation samples.**

	Discovery sample		Validation sample	
	Fold change	**P**	**Fold change**	**P**
Up-regulated				
ACE	2.05	0.01	1.81	.059
ANXA1	1.41	**0.04**	1.30	**.021**
CASP1	2.90	<0.001	1.45	.139
CCL2	1.65	**0.013**	3.57	**.004**
CCL3	2.02	0.056	3.21	.167
CCL4	1.91	0.023	1.98	.236
CCL5	1.40	0.034	1.07	.606
CCL8	**3.87**	**0.016**	**6.28**	**.000082***
CMKLR1	1.99	0.008	2.06	.167
CNR2	2.85	0.088	1.39	.481
CTGF	2.22	0.003	1.61	.139
CTSK	1.19	0.562	1.37	.236
CXCL10	5.81	0.133	2.08	.277
EPHX2	1.68	0.034	1.07	.370
FOS	2.03	0.056	5.16	.001
IL10	2.31	0.023	2.62	.059
IL1B	1.29	0.519	3.85	.036
IL6	1.01	0.401	2.40	.370
JUN	1.23	0.171	1.49	.139
MMP1	**13.93**	<0.001*	**4.66**	.888
MMP3	4.15	0.116	1.27	.606
S100A8	1.43	0.243	1.49	.888
TG	1.08	0.606	1.51	.963
TREM1	1.23	0.652	1.33	.743
TRPV4	1.45	0.088	1.25	.888
Down-regulated				
CX3CL1	2.72	<0.001	1.62	0.167
CXCL2	2.71	0.013	2.40	0.036
CXCL5	3.36	0.056	2.50	0.481
F2RL3	7.45	0.101	9.65	0.004
IL1R1	2.07	**0.001***	3.32	**0.001***
IL8	2.86	0.056	1.95	0.423
KDR	2.33	0.01	1.28	0.093
LTB4R	2.29	0.007	1.87	0.093
MMP7	**4.91**	0.034	**11.62**	0.541
MMP9	1.56	0.699	5.58	0.277
NFKBIA	2.37	**0.0003***	3.79	**0.006**
NOS3	2.20	0.019	1.42	0.423
S100A9	2.12	0.606	1.34	0.277
SOCS1	2.70	0.002	1.11	0.815
SOCS3	2.23	0.056	1.49	0.277
STAT3	1.11	0.652	2.06	0.114

TNFRSF11B	1.27	0.562	1.45	0.321
VEGFA	**8.15**	**<0.001***	**4.08**	0.139

Up or down regulation references symptomatic compared to asymptomatic chondropathy cases. Genes shown are those which were increased or decreased in the same direction in both discovery and validation samples; see supplementary tables 3 & 4 for additional analytes. Bold indicates genes selected for analysis of protein expression based on concordant findings between discovery and validation samples ($p<0.05$ or >3-fold difference between symptomatic and asymptomatic chondropathy groups). *$P<0.01$ after FDR (5%) corrections in the discovery sample and <0.0001 in the validation sample. Gene expression is normalised to the geometric mean of all 4 reference genes.

663 **Table 3: Overall summary of key molecular targets associated with symptomatic OA.**

Target	RNA Discovery		RNA Validation		Protein	
	Fold change	P	Fold change	P	Fold change	P
MMP1: Matrix Metalloprotease 1*	13.93 increased in Symptomatic chondropathy	**<0.001**	4.66 increased in Symptomatic chondropathy	0.888	2.92 increased in Symptomatic chondropathy	**0.001**
IL1R1: Interleukin 1 receptor, type I*	2.07 decreased in Symptomatic chondropathy	**0.001**	3.32 decreased in Symptomatic chondropathy	**0.001**	1.68 decreased in Symptomatic chondropathy	**0.003**
VEGF: Vascular endothelial growth factor A*	8.15 decreased in Symptomatic chondropathy	**<0.001**	4.08 decreased in Symptomatic chondropathy	0.139	3.63 decreased in Symptomatic chondropathy	**<0.001**
CCL2: Chemokine Ligand 2	1.65 increased in Symptomatic chondropathy	**0.013**	3.57 increased in Symptomatic chondropathy	**0.004**	1.46 decreased in Symptomatic chondropathy	0.192
CCL8: Chemokine Ligand 8	3.87 increased in Symptomatic chondropathy	**0.016**	6.27 increased in Symptomatic chondropathy	**<0.001**	NA	NA
IL-1β: Interleukin 1-beta	1.29 increased in Symptomatic chondropathy	0.519	3.85 increased in Symptomatic chondropathy	**0.036**	NA	NA
TNF-α: Tumour necrosis factor-alpha	3.86 increased in Symptomatic chondropathy	**<0.001**	1.25 decreased in Symptomatic chondropathy	0.815	NA	NA
MMP7: Matrix Metalloprotease 7	4.908 decreased in Symptomatic chondropathy	**0.034**	11.62 decreased in TKR	0.541	NA	NA

CCL5: Chemokine ligand 5	1.40 increased in Symptomatic chondropathy	**0.034**	1.07 increased in TKR	0.606	1.86 increased in TKR	**0.015**
CXCL10: Chemokine (C-X-C motif) ligand 10)	5.81 increased in Symptomatic chondropathy	0.133	2.08 increased in TKR	0.277	1.97 decreased in TKR	**0.019**

* Genes that satisfy the following criteria 1)increased in the same direction in both the original and replication RNA study, 2) P<0.05 or fold change >3 and 3) significantly differentially expressed at the protein level.

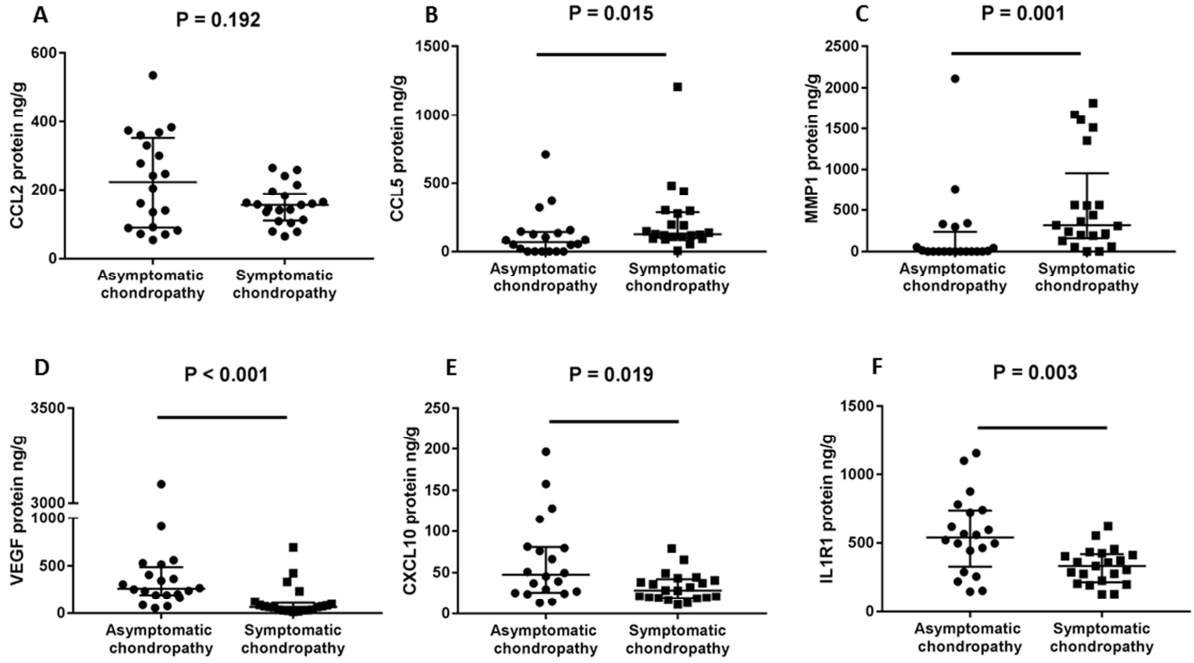
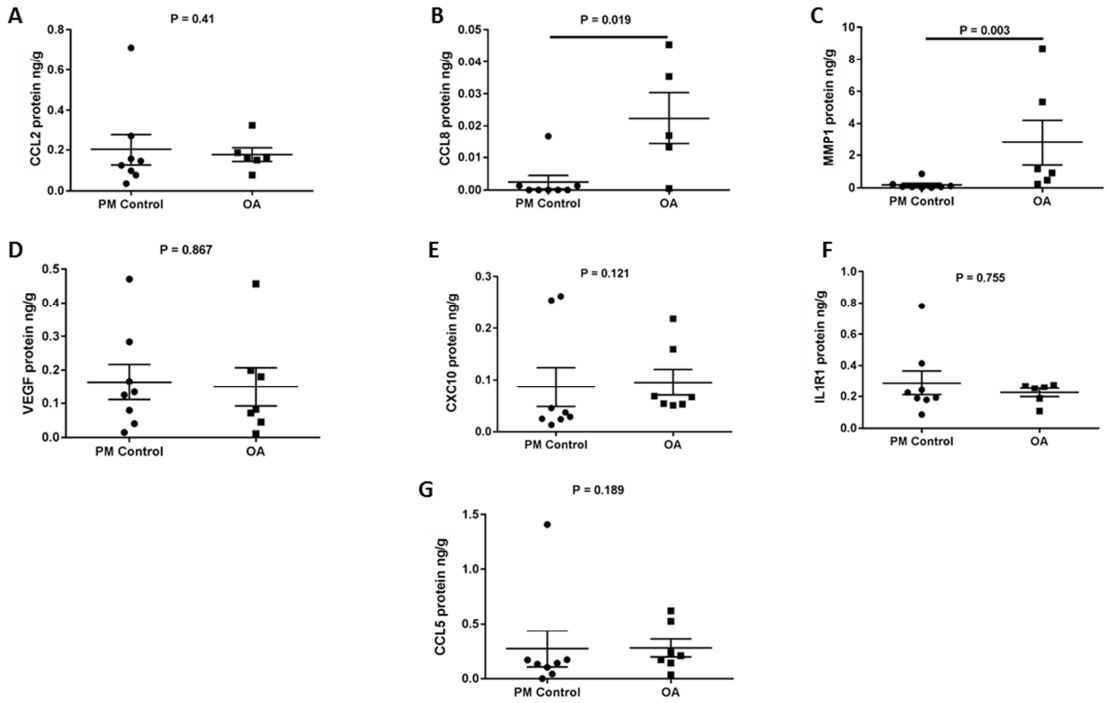


Figure 1: Protein expression in synovia from chondropathy cases classified as either asymptomatic or symptomatic. Groups were matched for macroscopic chondropathy scores. **A**: CCL2 (chemokine ligand 2), **B**: CCL5 (chemokine ligand 5). **C:** MMP1 (matrix metalloprotease 1), **D**: VEGF (vascular endothelial growth factor-A), **E**: CXCL10 (chemokine ligand 10), **F**: IL1R1 (interleukin 1 receptor 1). Median (IQR) are shown. IL1β (interleukin 1 beta), TNF-α (tumour necrosis factor alpha), MMP7 (matrix metalloprotease 7) and CCL8 (chemokine ligand 8) immunoreactivities were below the lower limit of detection.



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678 **Figure 2:** Protein expression for selected genes compared between PM control and OA cases
679 undergoing arthroplasty. **A:** CCL2 (chemokine ligand 2), **B:** CCL8 (chemokine ligand 8) **C**):
680 MMP1 (matrix metalloprotease 1), **D:** VEGF-A (vascular endothelial growth factor-A), **E:**
681 CXCL10 (chemokine ligand 10), **F:** IL1R1 (interleukin 1 receptor 1) and **G:** CCL5
682 (chemokine ligand 5). Data expressed as median (IQR). MMP7 (matrix metalloprotease 7),
683 IL1β (interleukin 1 beta) and TNF-α (tumour necrosis factor alpha) immunoreactivities were
684 below the lower limit of detection.

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