EXTENDED REPORT

Microarray analysis of bone marrow lesions in osteoarthritis demonstrates upregulation of genes implicated in osteochondral turnover, neurogenesis and inflammation

Anasuya Kuttapitiya,1 Lena Assi,1 Ken Laing,1 Caroline Hing,2 Philip Mitchell,2 Guy Whitley,3 Abiola Harrison,1 Franklyn A Howe,3 Vivian Ejindu,2 Christine Heron,2 Nidhi Sofat1

ABSTRACT

Objective Bone marrow lesions (BMLs) are well described in osteoarthritis (OA) using MRI and are associated with pain, but little is known about their pathological characteristics and gene expression. We evaluated BMLs using novel tissue analysis tools to gain a deeper understanding of their cellular and molecular expression.

Methods We recruited 98 participants, 72 with advanced OA requiring total knee replacement (TKR), 12 with mild OA and 14 non-OA controls. Participants were assessed for pain (using Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC)) and with a knee MRI (using MOAKS). Tissue was then harvested at TKR for BML analysis using histology and tissue microarray.

Results The mean (SD) WOMAC pain scores were significantly increased in advanced OA 59.4 (21.3) and mild OA 30.9 (20.3) compared with controls 0.5 (1.28) (p<0.0001). MOAKS showed all TKR tissue analysed had BMLs, and within these lesions, bone marrow volume was starkly reduced being replaced by dense fibrous connective tissue, new blood vessels, hyaline cartilage and fibrocartilage. Microarray comparing OA BML and normal bone found a significant difference in expression of 218 genes (p<0.05). The most upregulated genes included stathmin 2, thrombospondin 4, matrix metalloproteinase 13 and Wnt/Notch/catenin/chemokine signalling molecules that are known to constitute neuronal, osteogenic and chondrogenic pathways.

Conclusion Our study is the first to employ detailed histological analysis and microarray techniques to investigate knee OA BMLs. BMLs demonstrated areas of high metabolic activity expressing pain sensitisation, neuronal, extracellular matrix and proinflammatory signalling genes that may explain their strong association with pain.

INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis worldwide affecting more than 27 million adults in the USA alone and is a major cause of pain and functional disability. OA prevalence is set to rise globally with ageing populations accompanied by the rising epidemic of obesity. OA most commonly affects large weight-bearing joints, affecting the knees in up to 37% of adults over 60. Pain is a major symptom for people with OA, with 16.7% of US adults aged 45 years and above reporting pain as a predominant problem.

Pain in OA is thought to arise from several structures within the arthritic joint, including the synovium (from which prostaglandins, leukotrienes and inflammatory mediators are released), joint effusions, joint capsule involvement, tendon and muscle weakness that all contribute to pain and reduced function. Synovitis is often observed by MRI in OA and strongly correlates with pain. Cartilage degradation is one of the hallmarks of OA disease and exposes the structures from which pain is most likely arising as cartilage is an avascular, aneural structure composed largely of extracellular matrix (ECM) embedded sparsely with chondrocytes. Recent interest has grown in the importance of bone marrow lesions (BMLs) in relation to pain in OA. Epidemiological studies have shown a strong correlation between BMLs observed by MRI and OA-related knee pain in several large cohorts, with an OR of 3.2 for the association of BMLs with pain. The data outlined above demonstrate the multifactorial nature of OA and how pain mechanisms are supported by the biopsychosocial model of pain.

Recently, BMLs have been shown to be a very early biomarker of joint damage in OA with descriptions of their histology and histomorphometry. However, no previous transcriptomic studies of BMLs in OA are described. In the current study, we describe novel findings demonstrating BMLs have features of angiogenesis, fibrosis, new cartilage formation and increased bone turnover with disruption of the physiological osteochondral interface. Whole transcriptomic analysis of BML regions found upregulated expression of genes involved in neurogenesis, pain sensitisation, chemokine and cytokine signalling as well as cartilage remodelling pathways.

MATERIALS AND METHODS

All study procedures were carried out after ethical approval was granted (Health Research Authority approval number 12/LO/1970 and clinical trials.gov identifier NCT02603939). Participants attending the South London Elective Orthopaedic
Basic and translational research

Centre were recruited at assessment for total knee replacement (TKR), comprising the ‘advanced OA group’. For the ‘mild OA’ group, participants were recruited from rheumatology clinics at St George’s University Hospitals NHS Foundation Trust. For bone tissue controls, participants undergoing surgery following trauma, amputation or trochleoplasty were recruited (approval number 09/H0806/43) with no clinical or radiographic arthritis. Blood and urine samples were also obtained with full consent for biomarker studies.

Study criteria
Eligibility for participation included age of 35–90 years, presenting with pain and fulfilling ACR criteria for the diagnosis of knee OA. Participants continued to experience pain despite treatment for OA. All participants underwent baseline knee radiography to confirm knee OA with a Kellgren-Lawrence grade of greater than 2 in the affected tibio-femoral knee joint.

Clinical data collection
All scores were collected for participants with advanced OA and mild OA. For controls, Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) was not collected as participants underwent different surgeries. The primary pain score was the WOMAC with subscales for pain, stiffness and function. Participants were asked to score based on symptoms in the last 48 hours. Data were also collected for body mass index (BMI), Visual Analogue Scale pain rating 0–10 and the Hospital Anxiety and Depression Scale.

Molecular methods
Total RNA was isolated from approximately 200 mg of bone tissue. Amplified labelled cRNA samples (600 ng) were hybridised to Agilent whole human genome 60k microarray chips. Array signal intensities were analysed by the Agilent GeneSpring GX software. Significant differentially expressed entities between bone samples from healthy controls and OA participants were selected using a union of a Student’s moderated t-test corrected for multiple comparisons with the Bonferroni correction (p<0.05). Further methodical details are provided in the online supplementary methods.

STATISTICAL ANALYSIS
Data were anonymised for all analyses independently by the research team who were not involved in diagnosing or treating the study participants. To detect significant differences between groups at p<0.05, recruitment of at least 80 subjects was required, and we achieved n=98 participants. GraphPad Prism V.7 was used for all analyses, and significance was set at p<0.05 for all analyses. For microarray statistical analysis, refer to online supplementary methods.

RESULTS
Demographic data showed that our participants were representative of a knee OA population. Knee OA participants who underwent TKR had a high BMI and high pain scores measured by WOMAC (table 1). The mean (SD) WOMAC pain scores were significantly increased in advanced OA 59.4 (21.3) and mild

| Table 1 | Demographics showing characteristics of study population key. Data presented as means and SD. |
|---------|-----------------------------------------------------------------------------------------------|
| Advanced OA | Mild OA | Tissue control |
| Number* | 72 | 12 | 10 |
| Age range (Mean (SD)) | 51–88 | 49–79 | 21–88 |
| Gender Female N (%) | 55 (76.4) | 9 (75) | 9 (90) |
| Body mass index Mean (SD) | 32.5 (5.7) | 28.8 (3.9) | N/A |
| WOMAC pain Mean (SD) | 59.4 (21.3) | 30.9 (20.3) | N/A |
| WOMAC stiffness Mean (SD) | 62.8 (25.4) | 33.0 (29.7) | N/A |
| WOMAC function Mean (SD) | 59.8 (20.6) | 34.0 (24.3) | N/A |
| NRS pain Mean (SD) | 5.7 (2.3) | 2.6 (2.4) | N/A |
| HADS Mean (SD) | 12.6 (7.2) | 9.6 (6.7) | N/A |
| MOAKS* N (%) | | | |
| BML | MOAKS=0 | 9 (14.1) | 4 (57.1) | N/A |
| MOAKS=1 | 52 (81.3) | 3 (42.9) | 0 (0) |
| MOAKS=2 | 3 (4.6) | 0 (0) | |
| MOAKS=3 | 0 (0) | |
| Synovitis/effusion N (%) | MOAKS=0 | 2 (3.1) | 2 (28.6) | N/A |
| MOAKS=1 | 28 (43.8) | 2 (28.6) | |
| MOAKS=2 | 18 (28.1) | 1 (14.2) | |
| MOAKS=3 | 16 (25) | 2 (28.6) | |
| Cartilage damage N (%) | MOAKS=0 | 0 (0) | 4 (57.1) | N/A |
| MOAKS=1 | 16 (25) | 3 (42.9) | 0 (0) |
| MOAKS=2 | 41 (64.1) | 0 (0) | |
| MOAKS=3 | 7 (10.9) | 0 (0) | |
| Clinical Management | Underwent knee replacement surgery | Medical management | Underwent other surgery |

BML, bone marrow lesion; HADS, Hospital Anxiety and Depression Scale; MOAKS, MRI Knee Osteoarthritis Score; NRS, Numerical Rating Scale; OA, osteoarthritis; WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index.
OA 30.9 (20.3) compared with controls 0.5 (1.28) (p<0.0001), showing the advanced OA group had significantly more severe pain and functional impairment.

A mixture of OA participants was identified, and they were classified as severe or mild based on MRI. In the advanced OA group, 81.3% of participants had up to 33% of the bone volume (MOAKS score 1) forming a BML in at least one of the 21 measured regions, in addition to significant levels of synovitis and cartilage damage (table 1). MRI scans found BML areas to be invariably associated with regions of established cartilage damage, particularly in medial tibial regions, which were the focus of our tissue and microarray to maintain consistency of anatomical tissue lesions analysed. We found that 37.5% of grade 1 and 2 BML were in the medial tibial compartment, with 12.5% in the lateral tibial compartment. The remainder were distributed in the femur, trochlea and patella. For microarray, 50% samples were localised in the medial tibial compartment, 35.7% were found in the lateral tibial compartment and 14.2% crossed both tibial compartments.

Trends for WOMAC pain with individual MOAKS modalities showed higher WOMAC pain scores were associated with significantly greater BMLs in the advanced OA versus mild OA groups (see online supplementary figure S1). There was also a trend of increasing WOMAC pain with worsening MOAKS-scored synovitis, although these correlations did not reach statistical significance.

Histological analysis showed most normal bone marrow was adipocytic with adipocytes being the primary bone lining cells (figure 1). The bone volume fraction was starkly reduced in BML areas, with marrow replaced by new blood vessels, dense fibrous connective tissue, hyaline cartilage and fibrocartilage. Areas of aggressive resorption were found at the periphery of BML zones alongside regions of cartilaginous aggregates found at least 2 mm deep to the articular surface embedded within the bone compartment. Regions of vascular proliferation with fibrocartilage were interspersed with areas of de novo cartilage formation. Other BML regions exhibited a cellular infiltrate working through the osteoid network. Histological quantification found the BML group had increased vascular proliferation, cellular infiltration and trabecular thickening when compared with the non-BML (NBML) group (p<0.05).

Whole transcriptomic analysis identified 218 entities to be significantly differentially expressed between the OA BML and control bone samples (p<0.05) (figures 2 and 3). The most highly upregulated genes were stathmin 2 (STMN2), ATP-binding cassette protein, thrombospondin 4 (THBS4), matrix metalloproteinase 13 (MMP-13) and chromosome 21 open reading frame 1 (C21orf1) with functions in ECM organisation, cell adhesion and integrin-mediated signalling; and G protein coupled receptor (GPR158), which facilitates signal transduction and binds hormones/neurotransmitters and ATPase H+ transporting lysosomal (ATP6V0D2) gene expressed at axon termini and synaptic vesicles that is implicated in neuron projection. We also found upregulation of the DIRAS family, GTP-binding RAS-like 2 (DIRAS2) which is a Ras GTPase implicated in neurodegeneration. PC4 and SFRS1 interacting protein 1 (PSIP1) were also identified and are molecules involved in neuroepithelial stem cell differentiation, neurogenesis and apoptosis. Neuronal tyrosine phosphorylated phosphatase-3-kinase adaptor 2 (NYAP2) was also detected, which is a gene involved in neuronal development, interacting with WAVE1 proteins and is implicated in cytoskeletal modelling. We also found catenin (cadherin-associated protein) (CTNN2D2) upregulation, an adhesive junction associated protein implicated in bone, pain sensitisation, brain development and cancer formation.

Gene ontology analysis identified 166 of the 218 significantly differentially regulated entities to be associated with 59 canonical pathways. The angiogenic, Alzheimer disease-presenilin pathway, EGF/FGF/gonadotrophin signalling, inflammation mediated by chemokine and cytokine signalling with PDGF/Notch/vascular epidermal growth factor (VEGF) and Wnt signalling pathways were a few of which had the greatest number of entities related.

Quantitative polymerase chain reaction analysis confirmed STMN2, MMP-13 and THBS4 were significantly upregulated in BML regions compared with the control comparator group. THBS4 and STMN2 were the most highly upregulated genes between the BML and control bone groups (p<0.0001), reflecting comparable results to the microarray (figure 4). MMP-13 and STMN2 were upregulated within BML regions compared with NBML matched regions (p<0.0001). However THBS4 was found to be most upregulated in the NBML compared with both BML and control groups. Serum STMN2 levels were not significantly increased in mild/ advanced OA groups compared with controls. Protein quantification of STMN2 in BML tissue found control bone to have higher presence of STMN2 compared with BML bone (p<0.0001). Functional significance of MMP-13 protein activity, one of the highest array-expressed genes, found a significant increase in urine CTX-II levels, that is, cleavage products of type II collagen, in the advanced OA group compared with mild OA and control groups (p<0.001) (see online supplementary figure S2).

**DISCUSSION**

BMLs have been well described by MRI in knee OA, but very little is known about their transcriptomic expression. To our knowledge, our study is the first to use a multimodal approach with MRI to locate knee OA BMLs, followed by detailed histological analysis and whole transcriptomic techniques for a multi- variate interrogation of the changes seen within BMLs.

Bone marrow signal changes were first described on MRI by Wilson et al who used the term ‘bone marrow oedema’ to describe MRI findings in painful joints. Studies so far have focused on acquiring data from patients undergoing joint surgery of the knee and hip. Zanetti et al demonstrated histologically that BMLs contained normal fatty marrow with marrow necrosis, necrotic or remodelled trabeculae, oedema and bone marrow bleeding. The same group matched MRI changes to BML abnormalities in participants undergoing TKR and found regions of normal tissue alongside bone marrow fibrosis, oedema and bleeding. In a hip and knee OA study, Hunter et al reported increased bone volume fraction but decreased tissue mineral density within BML using light microscopy. Samples from the lesion area showed increased trabecular thickness, with granulation, oedema, necrosis, fibrinoid deposition and hyperplasia of blood vessels. Taljanovic reported one of the largest histological studies of hip OA BML, where regions of fibrosis and microfracture formation at different stages of

Kuttapilly A, et al. Ann Rheum Dis 2017;0:1–10. doi:10.1136/annrheumdis-2017-211396
healing were observed. Leydet-Quilici et al also described oedema, necrosis and fibrosis within BML biopsies. Using MRI, Roemer et al previously demonstrated that progression of disease and the development of BMLs correlated with an increased risk of cartilage loss within the same subregion and that regions without BMLs are associated with decreased risk of cartilage loss, changes that our work supported. Carrino et al reported 87% of subchondral cysts were associated with BML abnormalities, which our analysis confirmed on MRI and by histology. In comparison with other studies, our detailed MRI matching with histological techniques allowed improved visualisation of BMLs, with direct observation of areas appearing as BML-associated cystic structures on MRI and transcriptomic expression. We found higher WOMAC pain scores with greater MOAKS-measured cartilage damage, as suggested by previous studies.

Figure 1 (A) Coronal plane of MRI scan visualising BML and associated cyst. (B) Axial plane of MRI scan presenting BML and associated cyst. (C) Macroscopic view of tibial BML and cystic area. (D) Image of cross section cut through BML and cyst localised by MRI revealing a gelatinous aggregate. (E) H&E staining of cystic region presenting cellular infiltrate in marrow spaces. (F) H&E staining of subchondral cyst forming. (G) H&E staining of BML region with vascular proliferation and cellular infiltration. (H) H&E staining of BML visualising a chondrification centre near the tidemark. (I) H&E staining of adipocyte in bone compartment with a soft tissue infiltrate working through osteoid network. (J) H&E staining of BML showing areas of thickened trabecular adjacent to thinning trabeculae. (K) H&E staining of BML demonstrating areas of fibrotic cartilage formation within the subchondral bone compartment. (L) Quantification of histology analysing 50 BML FOVs and 40 non-BML (NBML) FOVs for blood vessels (BV), cartilage within bone compartment (Cart), cysts (Cys), myxoid/fibrous tissue (M/F), cellular infiltrate (Inf) and trabecular thickening (TT) (n=4). A percentage for the presence of each histological feature was determined for each group. Significance was tested between the groups using Friedman test (*p<0.05). (M) Magnification of each histological change within the bone compartment: BV within subchondral bone, Cart within bone compartment with a chondrification centre, Cys within subchondral bone, M/F adjacent to subchondral bone, Inf within the osteoid network and TT. BML, bone marrow lesion; FOV, field of view.
In our study, cystic BML areas were surrounded by regions of fibrosis, infiltration by inflammatory cells and vascular proliferation. Previous hypotheses that BMLs could be precystic but that not all BMLs become cystic is also supported by our histological findings, where we observed cystic structures within the areas defined as cysts using MRI, and also adjacent to areas of fibrocartilage, vascular proliferation, chondrogenesis and amorphous tissue deposition. We observed new cartilage forming deep within the subchondral bone compartment. The new cartilage tissue within the BML could be arising from mesenchymal stem cells (MSCs) in the marrow, which is seen by other groups. From our microarray, the highest upregulated gene was STMN2, a phosphoprotein involved in regulating microtubule function, responsiveness to nerve growth factor (NGF), neuronal growth and osteogenesis. Upregulation of STMN2 within BML could lead to new neuronal structures and expansion of the BML in OA, thereby causing pain. Stathmin 2 protein expression was higher in normal than BML bone, which could reflect increased stathmin 2 turnover in OA BMLs.

We also identified neuronal markers including thrombospondin 4 (THBS4), implicated in the inflammatory response to Central Nervous system (CNS) injury, presynaptic hypersensitivity and neuropathic pain states. In animal models of pain sensitisation, THBS4 levels are increased locally in dorsal root ganglion neurons and contribute to pain behaviour, which can be inhibited by the calcium channel modulator gabapentin.
Basic and translational research

Other upregulated genes involved in neuronal morphogenesis included $\text{ATP6V0D2}$, $\text{PSIP1}$, $\text{NYAP2}$, $\text{FERM}$ and $\text{PDZ}$ containing 4 ($\text{FRMPD4}$), implicated in CNS development and pain states. ECM genes were also represented in the array, including $\text{MMP-13}$ and collagens, $\text{COL16A1}$, fibronectins and growth factors, which are known to be bound within the ECM.
| Accession no | Symbol | Entity name | T↓ | Abs FC | Log FC | P Value* | P Value† |
|-------------|--------|-------------|-----|--------|--------|----------|----------|
| NM_007029  | STMN2  | Statmin 2   | Up  | 19.30  | 4.27   | 3.67 x 10^-6 | 1.6 x 10^-6 |
| NM_001163942 | ABCB5 | ATP-binding cassette, sub-family B (MDR/TAP), member 5 | Up  | 12.11  | 3.60   | 2.06 x 10^-6 | 8.86 x 10^-3 |
| NM_003248  | THBS4  | Thrombospondin 4 | Up  | 11.53  | 3.53   | 1.31 x 10^-6 | 7.35 x 10^-5 |
| NM_002427  | MMP13  | Matrix Metalloproteinase 13 (collagenase 3) | Up  | 11.18  | 3.48   | 2.78 x 10^-6 | 1.41 x 10^-5 |
| NR_037585  | C21orf37 | Chromosome 21 open reading frame 37 | Up  | 9.32   | 3.22   | 3.64 x 10^-6 | 1.65 x 10^-5 |
| NM_001167890 | EGFL6 | EGF-like-domain, multiple 6 | Up  | 9.07   | 3.18   | 2.69 x 10^-5 | 1.38 x 10^-5 |
| NM_001856  | COL16A1 | Collagen, type XVI, alpha 1 | Up  | 8.25   | 3.04   | 1.8 x 10^-5  | 9.08 x 10^-6 |
| NM_020752  | GPR158 | G protein-coupled receptor 158 | Up  | 8.21   | 3.04   | 1.13 x 10^-5 | 6.35 x 10^-6 |
| NM_012093  | AKS    | Adenylate kinase 5 | Up  | 8.01   | 3.00   | 5.77 x 10^-5 | 2.73 x 10^-5 |
| NM_174858  | AKS    | Adenylate kinase 5 | Up  | 8.01   | 3.00   | 3.33 x 10^-5 | 1.74 x 10^-5 |
| NM_152565  | ATP6V0D2 | ATPase, H+ transporting, lysosomal 38KDa, V0 subunit d2 | Up  | 7.89   | 2.98   | 4.11 x 10^-5 | 1.91 x 10^-5 |
| ALU1       |        | Alu 2 Element | Up  | 7.44   | 2.89   | 1.32 x 10^-4 | 5.82 x 10^-7 |
| NM_017594  | DIRAS2 | DIRAS family, GTP-binding RAS-like 2 | Up  | 7.14   | 2.84   | 2.8 x 10^-6 | 1.29 x 10^-6 |
| XR_245643  | LOC101929504 | Uncharacterized LOC101929504 | Up  | 7.02   | 2.81   | 3.79 x 10^-5 | 2.02 x 10^-5 |
| NM_021233  | DNASE2B | Deoxynucleoside II beta | Up  | 7.02   | 2.81   | 1.55 x 10^-5 | 7.86 x 10^-6 |
| NM_014980  | STXBPS1 | Syntaxin binding protein 5-like | Up  | 6.72   | 2.75   | 2.68 x 10^-6 | 1.24 x 10^-6 |
| NM_004789  | LHX2   | LIM homeobox 2 | Up  | 6.71   | 2.75   | 7.61 x 10^-6 | 4.23 x 10^-6 |
| NM_021144  | PSIP1  | PC4 and SFRS1 interacting protein | Up  | 6.57   | 2.72   | 3.62 x 10^-6 | 1.71 x 10^-6 |
| NM_020864  | NYAP2  | Neuronal tyrosine-phosphorylated phosphoinositide-3-kinase adaptor 2 | Up  | 6.48   | 2.70   | 2.53 x 10^-5 | 1.33 x 10^-5 |
| NM_001332  | CTNN2D | Catenin (cadherin-associated protein), delta 2 | Up  | 6.36   | 2.67   | 6.52 x 10^-6 | 3.19 x 10^-6 |
| NM_032532  | FNDC1  | Fibronectin type III domain containing 1 | Up  | 6.09   | 2.61   | 7 x 10^-5  | 3.91 x 10^-5 |
| NM_001426  | EN1    | Engrailed homebox 1 | Up  | 5.75   | 2.52   | 1.21 x 10^-6 | 5.56 x 10^-7 |
| NR_027054  | MIR31HG | MIR31 host gene (non-protein coding) | Up  | 5.64   | 2.50   | 1.21 x 10^-6 | 1.03 x 10^-4 |
| XLOC_006820 | FRMP4  | FERM and PDZ domain containing 4 | Up  | 5.48   | 2.45   | 9.05 x 10^-6 | 4.6 x 10^-6 |
| NM_014728  | TCONS_00014487 | Uncharacterized LOC101929450 | Up  | 5.34   | 2.42   | 3.09 x 10^-5 | 1.68 x 10^-5 |
| NM_022970  | FGFR2  | Fibroblast growth factor receptor 2 | Up  | 5.30   | 2.41   | 9.69 x 10^-6 | 4.97 x 10^-6 |
| NM_012152  | LPAR3  | Lysophosphatidic acid receptor 3 | Up  | 5.27   | 2.40   | 3.65 x 10^-5 | 2 x 10^-5 |
| NM_004370  | COL12A1 | Collagen, type XIII, alpha 1 | Up  | 5.27   | 2.40   | 1.32 x 10^-6 | 6.2 x 10^-7 |
| BC043571   | LOC613266 | Uncharacterized LOC613266 | Up  | 5.09   | 2.35   | 1.2 x 10^-7  | 5.25 x 10^-8 |
| NM_000170  | GLDC   | Glycine dehydrogenase (decarboxylating) | Up  | 5.00   | 2.32   | 6.11 x 10^-5 | 3.46 x 10^-4 |
| NM_031913  | ESYT3  | Extended synaptotagmin-like protein 3 | Up  | 5.00   | 2.32   | 3.61 x 10^-5 | 1.99 x 10^-5 |
| ALU1       | Alu 1 Element | Down | -5.02 | -2.33 | 3.17 x 10^-7 | 1.44 x 10^-7 |
| NM_025260  | C6orf25 | Chromosome 6 open reading frame 25 | Down | -5.82 | -2.54 | 5.35 x 10^-6 | 2.62 x 10^-6 |
| NM_080429  | AQP10  | Aquaporin 10 | Down | -6.92 | -2.79 | 6.26 x 10^-7 | 2.62 x 10^-6 |
| NM_005306  | FFAR2  | Free fatty acid receptor 2 | Down | -7.29 | -2.87 | 5.63 x 10^-10 | 3.06 x 10^-10 |
| AB305916   | TRBV28 | T Cell Receptor Beta Variable 28 | Down | -7.50 | -2.91 | 3.35 x 10^-10 | 1.55 x 10^-10 |
| NM_000517  | HBA2   | Hemoglobin, alpha 2 | Down | -7.64 | -2.93 | 7.61 x 10^-10 | 3.25 x 10^-10 |
| NM_000517  | HBA2   | Hemoglobin, alpha 2 | Down | -7.99 | -3.00 | 2.74 x 10^-10 | 1.1 x 10^-10 |
| NM_016509  | CLEC1B | C-type lectin domain family 1, member B | Down | -8.24 | -3.04 | 5.59 x 10^-10 | 2.33 x 10^-10 |
| NM_002620  | PF4V1  | Platelet factor 4 variant 1 | Down | -9.31 | -3.22 | 2.34 x 10^-10 | 1.04 x 10^-10 |

Continued
Our data demonstrate that BMLs are regions of high metabolic activity with increased cell turnover, bone remodelling, neuronal and inflammatory gene signatures. Gene ontological analysis revealed canonical pathways involved in chemokine, integrin and cytokine signalling. We found neurodevelopment, new nerve formation and pain mediation in BML tissue.

Table 2  Continued

| Accession no | Symbol | Entity name | ↑↓ | Abs FC | Log FC | P Value* | P Value† |
|--------------|--------|-------------|-----|--------|--------|----------|----------|
| NM_022468    | MMP25  | Matrix Metalloproteinase 25 | Down | −9.33  | −3.22  | 4.32 x 10^−1 | 2.28 x 10^−1 |
| NR_120522    | LOC102724484 | Uncharacterized LOC102724484 | Down | −10.04 | −3.33  | 1.01 x 10^−1 | 5.6 x 10^−5 |
| NM_001136503 | SMIM24 | Small integral membrane protein 24 | Down | −10.29 | −3.36  | 1.38 x 10^−1 | 6.73 x 10^−4 |
| NM_030773    | TUBB1  | Tubulin, beta 1 class VI | Down | −12.37 | −3.63  | 5.86 x 10^−2 | 2.34 x 10^−1 |
| HSJ1167H4    |        |             | Down | −13.17 | −3.72  | 3.71 x 10^−1 | 1.65 x 10^−1 |
| NR_001552    | TTY16  | Testis-specific transcript, Y-linked 16 (non-protein coding) | Down | −13.65 | −3.77  | 6.28 x 10^−1 | 3.34 x 10^−3 |
| NR_047499    | LINC00570 | Long intergenic non-protein coding RNA 570 | Down | −14.00 | −3.81  | 1.03 x 10^−4 | 8.67 x 10^−5 |
| NM_144673    | CMTM2  | C-type lectin domain family 1, member B | Down | −14.25 | −3.83  | 2.71 x 10^−1 | 1.36 x 10^−1 |
| NM_001557    | CXCR2  | Chemokine (C-X-C motif) receptor 2 | Down | −14.93 | −3.90  | 9.27 x 10^−1 | 4.34 x 10^−1 |
| NM_000519    | HBD    | Hemoglobin, delta | Down | −15.75 | −3.98  | 7.89 x 10^−1 | 2.74 x 10^−4 |
| NM_002100    | GYPB   | Glycoprotein B (MNS blood group) | Down | −16.15 | −4.01  | 1.03 x 10^−4 | 1.43 x 10^−4 |
| XM_005261527 | SEC14L3 | SEC14-like 3 (S. cerevisiae) | Down | −16.65 | −4.06  | 2.98 x 10^−1 | 1.5 x 10^−5 |
| AK128128     | FLJ46249 |        | Down | −16.90 | −4.08  | 6.19 x 10^−1 | 3.27 x 10^−5 |
| NM_016509    | CLEC1B | C-type lectin domain family 1, member B | Down | −17.06 | −4.09  | 1.34 x 10^−1 | 6.39 x 10^−6 |
| NM_016509    | CLEC1B | C-type lectin domain family 1, member B | Down | −17.67 | −4.14  | 4.83 x 10^−6 | 2.15 x 10^−6 |
| NM_002049    | GATA1  | GATA binding protein 1 (globin transcription factor 1) | Down | −19.55 | −4.29  | 7.87 x 10^−1 | 4.21 x 10^−5 |
| NM_005764    | PDZK1P1 | PDZK1 interacting protein 1 | Down | −20.36 | −4.35  | 7.59 x 10^−1 | 3.47 x 10^−4 |
| NM_006163    | NFE2   | Nuclear factor, erythroid 2 | Down | −22.54 | −4.49  | 3.22 x 10^−1 | 1.62 x 10^−5 |
| NM_002619    | PPIA   | Platelet factor 4 | Down | −31.42 | −4.97  | 1.26 x 10^−1 | 4.32 x 10^−8 |
| XLOC_013489  |        |             | Down | −31.94 | −5.00  | 1.26 x 10^−1 | 2.56 x 10^−5 |
| NM_000032    | ALAS2  | Aminolevulinate, delta-, synthase 2 | Down | −33.49 | −5.07  | 1.93 x 10^−1 | 9.3 x 10^−6 |
| NM_005980    | S100P  | S100 calcium binding protein P | Down | −33.56 | −5.07  | 1.11 x 10^−1 | 6.06 x 10^−5 |
| NM_005331    | HMB1   | Hemoglobin, theta 1 | Down | −34.07 | −5.09  | 3.58 x 10^−1 | 1.53 x 10^−5 |
| NM_002704    | PPBP   | Proplatelet basic protein (chemokine (C-X-C motif) ligand 7) | Down | −39.94 | −5.32  | 4.11 x 10^−8 | 1.3 x 10^−8 |
| NM_000517    | HBA2   | Hemoglobin, alpha 2 | Down | −41.07 | −5.36  | 2.47 x 10^−1 | 8.77 x 10^−8 |
| NM_001003938 | HBM    | Hemoglobin, mu | Down | −45.11 | −5.50  | 7.66 x 10^−1 | 4.05 x 10^−5 |
| NM_018437    | HEMG   | Hemoglobin | Down | −53.12 | −5.73  | 1.89 x 10^−1 | 7.66 x 10^−7 |
| NM_005621    | S100A12 | S100 calcium binding protein A12 | Down | −56.95 | −5.83  | 7.25 x 10^−1 | 3.81 x 10^−3 |
| NM_005621    | S100A12 | S100 calcium binding protein A12 | Down | −58.82 | −5.88  | 4.6 x 10^−1 | 2.34 x 10^−5 |
| NM_000559    | HBG1   | Hemoglobin, gamma A | Down | −88.82 | −6.47  | 1.94 x 10^−6 | 7.82 x 10^−2 |

Symbol, Entity Symbol. ↑↓, Regulation. Abs FC, Absolute Fold Change. Log FC, Log transformed Fold Change.* Adjusted Student T-test P value for microarray corrected for multiple testing by the Bonferroni FWER method.
†Adjusted Moderated T-test P value for microarray corrected for multiple testing by the Bonferroni FWER method.

osteoprogenitors. The same group reported that endothelial Notch activity promotes angiogenesis and osteogenesis in bone. We also demonstrated OMD in our BML tissue: Ninomiya et al showed that osteoclast activity induces OMD expression in bone, suggesting BMLs represent areas of active bone remodelling.

The expression of both osteogenic and angiogenic genes along with the tissue changes we identified may suggest that vascular proliferation and bone formation are likely to be coupled in BML formation. Since blood vessels are formed within neurovascular bundles, it is likely that increased neuronal pathway gene expression including STMN2, THBS4, PSIP1, NYAP2 and catenin, which were among some of the most highly expressed genes from our BML analysis, are implicated in neural pathway development, new nerve formation and pain mediation in BML tissue.
Our array also identified molecules within the Wnt signalling pathway, including catenin. Other studies have demonstrated a critical role for Wnt signalling in the production and persistence of neuropathic pain after nerve injury and bone cancer. Rodent models show that in nerve injury and bone cancer pain models, respectively, Wnt signalling is activated, which may contribute to pain by regulating pro-inflammatory cytokines interleukin-18 and tumour necrosis factor-alpha, as well as NR2B and subsequent Ca2+-dependent signals in the dorsal horn. We found a high representation of the inflammatory chemokines and cytokine signalling; other groups have also identified chemokines in OA pain, for example, CCR2 was recently reported to mediate pain in a murine model of OA. Our data suggest that chemokine pathway molecules could be pain sensitisers in BMLs. Walsh et al showed that OA neurovascular changes at the osteochondral junction, including vessels and both sensory and sympathetic nerves breaching the tidemark, could possibly be a source of joint pain. The genes we have identified in our BML transcriptome support the hypothesis of neurovascular gene upregulation in BML tissue.

One of our most highly expressed genes was MMP-13, an enzyme expressed in cartilage, involved in regulating ECM turnover and cartilage destruction in OA. Our data showed that type II collagen degradation products were increased in urine from our advanced OA population. The de novo cartilage formation observed within BMLs, coupled with the increased transcriptomic expression of MMP-13 observed using microarray and the detection of MMP-13 cleavage products, could suggest recapitulation of the embryonic bone development phenotype within OA BML regions.

Limitations of our study included the sample size for microarray, which although on a standard format of 24 samples, will benefit from larger studies. Future work for protein evaluation of the genes identified is needed, investigating which cells within BMLs are responsible for producing the genes identified and how BMLs develop with respect to the pathological changes identified in OA over time. Although we did not identify NGF, we found genes in neurotrophin pathways, including stathmin 2, which increases responsiveness to NGF, syntaxin, which regulates brain-derived neurotrophic factor and pituitary adenylate cyclase-activation polypeptide, implicated in neuronal development.

In conclusion, our work demonstrates that BMLs are regions of high metabolic activity, with expression of genes involved in neuronal development, pain, ECM turnover, cartilage/bone formation and angiogenesis. Our findings contribute to understanding of OA pathogenesis and could help lead to the development of new diagnostic tools and future therapies for this most common arthritic disease.

Acknowledgements We express our sincere gratitude to all patients who participated in this study.

Collaborators St George’s University Hospitals NHS Foundation Trust: Dr Virinderjit Sandhu, Dr Katie Moss, Dr Avind Kaul, Dr Patrick Kiely (Co-Investigators); St George’s, University of London: Ms Debbie Rolfe (Regulatory Manager), Dr Irina Chis...
REFERENCES
1 Lawrence RC, Felson DT, Helmick CG, et al. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II. Arthritis Rheum 2008;58:26–35.
2 Nicholls E, Thomas E, van der Windt DA, et al. Pain trajectory groups in persons with, or at high risk of, knee osteoarthritis: findings from the knee clinical Assessment Study and the Osteoarthritis Initiative. Osteoarthritis Cartilage 2014;22:2041–50.
3 Sofat N, Ejindu V, Kiely P. What makes OA painful? The evidence for peripheral and central pain processing Rheumatology. Rheumatology 2011;50:2157–65.
4 Roemer FW, Kassis Javadi M, Guermazi, A, et al. Anatomical distribution of synovitis in knee osteoarthritis and its association with joint effusion assessed on non-enhanced and contrast-enhanced MRI. Osteoarthritis Cartilage 2010;18:1269–74.
5 Roy S, Meachim G. Chondrocyte ultrastructure in adult human articular cartilage. Ann Rheum Dis 1968;27:544–58.
6 Felson DT, Chaissone CE, Hill CL, et al. The association of bone marrow lesions with pain in knee osteoarthritis. Ann Intern Med 2001;134:541–9.
7 Sowers MF, Hayes C, Jaymadar D, et al. Magnetic resonance-detected subchondral bone marrow and cartilage defect characteristics associated with pain and X-ray-defined knee osteoarthritis. Osteoarthritis Cartilage 2002;11:387–93.
8 Altman R, Aesch E, Bloch D, et al. The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the knee. Arthritis Rheum 1986;29:1039–49.
9 NIACE guidelines ‘Osteoarthritis: Care and Management’. https://www.nice.org.uk/guidance/cg177
10 Kelgiren JH, Lawrence JS. Radiological assessment of osteoarthritis. Ann Rheum Dis 1957;16:494–502.
11 Bellamy N, Hochberg M, Tabach F, et al. Development of multinaional definitions of minimal clinically important improvement and patient acceptable symptomatic state in osteoarthritis. Arthritis Care Res 2015;67:972–80.
12 Dworkin RH, Turk DC, Farrar JT, et al. Core outcome measures for chronic pain clinical trials: impact recommendations. Pain 2005;113:9–19.
13 Bjelland I, Dahl AA, Haug TT, et al. The validity of the Hospital anxiety and depression Scale. an updated literature review. J Psychosom Res 2002;52:69–77.
14 RNeasy mini Hand book isolation kit. Fourth Edition. Qiagen, 2012. https://www.qiagen.com/es/resources/resourcedetail?id=1ae7cbe-521a-4c7f-b8c-69f6a3e32484&lang=en
15 Microarray-Based Gene Expression Analysis. Version 6.9.1 https://www.agilent.com/cs/library/usermanuals/Public/G2505-90019. Scanncell_C_User.pdf, 2015.
16 Mi H, Prudel S, Munagajnan A, et al. PANTHER version 10: expanded protein families and functions, and analysis tools. Nucleic Acids Res 2016;44(D1):D366–D342.
17 Gannon P, Piperno M, Greytes E, et al. Cross sectional evaluation of biochemical markers of bone, cartilage, and synovial tissue metabolism in patients with knee osteoarthritis: relations with disease activity and joint damage. Ann Rheum Dis 2001;60:619–26.
18 Hunter DJ, Guerazzi A, Lo Gh, L, et al. Evolution of semi-quantitative whole joint assessment of knee OA: moaks (MRI Osteoarthritis Knee score). Osteoarthritis Cartilage 2011;19:990–1002.
19 Wilson AI, Murphy WA, Hardy DC, et al. Transient osteoporosis: transient bone marrow edema? Radiology 1988;167:757–60.
20 Zanetti M, Bruder E, Romero J, et al. Bone marrow edema pattern in osteoarthritic knees: correlation between MR imaging and histologic findings. Radiology 2000;215:835–40.
21 Talajianov MS, Graham AR, Benjamin JB, et al. Bone marrow edema pattern in advanced hip osteoarthritis: quantitative assessment with magnetic resonance imaging and correlation with clinical examination, radiographic findings, and histopathology. Skeletal Radiol 2008;37:423–31.
22 Leydet-Quillè H, Le Coroller T, Bouvier C, et al. Advanced hip osteoarthritis: magnetic resonance imaging and osteoarthritis correlations. Osteoarthritis Cartilage 2010;18:1429–35.
23 Roemer FW, Guermazi A, Javadik M, et al. Change in MRI-detected subchondral bone marrow lesions is associated with cartilage loss: the MOST study. A longitudinal multicentre study of osteoarthritis. Ann Rheum Dis 2009;68:1461–5.
24 Carrino JA, Blum I, Pareilledja JA, et al. MRI of bone marrow edema-like signal in the pathogenesis of subchondral cysts. Osteoarthritis Cartilage 2006;14:1081–5.
25 Zhang D, Johnson LL. Hsu HF, et al. Cartilaginous deposits in subchondral bone in regions of exposed bone in osteoarthritis of the human knee: histomorphometric study of PRG4 distribution in osteoarthritic cartilage. J Orthop Res 2007;25:873–83.
26 Campbell TM, Churchman SM, Gomez A, et al. Mesenchymal stem cell alterations in bone marrow lesions in patients with hip osteoarthritis. Arthritis Rheumatol 2016;68:1648–59.
27 Jin K, Mao XQ, Cuttell B, et al. Proteomic and immunochromatographic characterization of a role for stathmin in adult neurogenesis. Front J Neurosci 2004;18:287–99.
28 Liu H, Zhang R, Ko SY, Sy K, et al. Microtubule assembly affects bone mass by regulating both osteoblast and osteoclast functions: stathmin deficiency produces an osteopenic phenotype in mice. J Bone Miner Res 2011;26:2052–67.
29 Kim DS, Li KW, Boroujerdi A, et al. Thrombospondin-4 contributes to spinal sensitization and neuropathic pain states. J Neurosci 2012;32:8977–87.
30 Pan B, Guo Y, Wu HE, He W, et al. Thrombospondin-4 divergently regulates voltage-gated Ca2+ channel subtypes in sensory neurons after nerve injury. Pain 2016;157:2068–80.
31 Fouilles T, Wood JD. Pain genes. PLoS Genet 2008;4:e1000086.
32 Swaminathan A, Delage H, Chatterjee S, et al. Transcriptional coactivator and chromatin protein PC4 is involved in hippocampal neurogenesis and spatial memory extinction. J Biol Chem 2016;291:20303–14.
33 Sofat N. Analysing the role of endogenous matrix molecules in the development of osteoarthritis. Int J Exp Pathol 2009;90:463–79.
34 Hopwood B, Tsikyn A, Findlay DM, et al. Microarray gene expression profiling of osteoarthritic bone suggests altered bone remodelling, WNT and transforming growth factor-beta/bone morphogenic protein signalling, Arthritis Res Ther 2007;9:R100.
35 Chou CH, Wu CC, Song IW, et al. Genome-wide expression profiles of subchondral bone in osteoarthritis. Arthritis Res Ther 2013;15:R190.
36 Kusum AP, Ramsay SK, Adams RH. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. Nature 2014;507:323–8.
37 Ramsay SK, Kusum AP, Wang L, et al. Endothelial notch activity promotes angiogenesis and bone formation in vivo. Nature 2014;507:376–80.
38 Ninomiya K, Miyamoto T, Inai J, et al. Osteoarthritis improves osteogenous expression in osteoblasts. Biochem Biophys Res Commun 2007;362:460–60.
39 Zhang YK, Huang ZI, Liu S, et al. WNT signaling underlies the pathogenesis of neuropathic pain in rodents. J Clin Invest 2013;123:2268–86.
40 Miller RE, Tran PB, Das R, et al. CR2R chemokine receptor signaling mediates pain in experimental osteoarthritis. Proc Natl Acad Sci USA 2012;109:720–7.
41 Walsh DA, McWilliams DF, Turley ML, et al. Angiogenesis and nerve growth factor at the osteochondral junction in rheumatoid arthritis and osteoarthritis. Rheumatology 2010;49:1852–61.
42 Little CB, Barai A, Burkhardt D, et al. Matrix metalloproteinase-13-deficient mice are resistant to osteoarticular cartilage erosion but not chondrocyte hypertrophy or osteohyde development. Arthritis Rheum 2009;60:3723–33.
43 Kofuji T, Fujiwara T, Sanda M, et al. hPCN-1/syntain 1A and syntain 18 play distinct roles in neuronal survival. J Neurochem 2014;130:514–25.
44 Vauduy D, Gonzalez BJ, Basille M, et al. Neurotrophic activity of pituitary adenylate cyclase-activating polypeptide on rat cerebrospinal development. Proc Natl Acad Sci USA 1999;96:9415–20.