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EPIDEMIOLOGICAL SCIENCE

Linking chondrocyte and synovial transcriptional profile to clinical phenotype in osteoarthritis

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (<http://dx.doi.org/10.1136/annrheumdis-2020-219760>).

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Received 19 December 2020

Revised 30 March 2021

Accepted 11 April 2021

ABSTRACT

Objectives To determine how gene expression profiles in osteoarthritis joint tissues relate to patient phenotypes and whether molecular subtypes can be reproducibly captured by a molecular classification algorithm.

Methods We analysed RNA sequencing data from cartilage and synovium in 113 osteoarthritis patients, applying unsupervised clustering and Multi-Omics Factor Analysis to characterise transcriptional profiles. We tested the association of the molecularly defined patient subgroups with clinical characteristics from electronic health records.

Results We detected two patient subgroups in low-grade cartilage (showing no/minimal degeneration, cartilage normal/softening only), with differences associated with inflammation, extracellular matrix-related and cell adhesion pathways. The high-inflammation subgroup was associated with female sex (OR 4.12, $p=0.0024$) and prescription of proton pump inhibitors (OR 4.21, $p=0.0040$). We identified two independent patient subgroupings in osteoarthritis synovium: one related to inflammation and the other to extracellular matrix and cell adhesion processes. A seven-gene classifier including *MMP13*, *APOD*, *MMP2*, *MMP1*, *CYT11*, *IL6* and *C15orf48* recapitulated the main axis of molecular heterogeneity in low-grade knee osteoarthritis cartilage (correlation $\rho=-0.88$, $p<10^{-10}$) and was reproducible in an independent patient cohort ($\rho=-0.85$, $p<10^{-10}$).

Conclusions These data support the reproducible stratification of osteoarthritis patients by molecular subtype and the exploration of new avenues for tailored treatments.

INTRODUCTION

Our understanding of the molecular mechanisms that underlie the observed epidemiological and clinical heterogeneity in osteoarthritis (OA) is incomplete. The accessibility of primary disease tissues at the point of joint replacement surgery provides the opportunity to stratify patients based on tissue-specific molecular profiles. Such stratification may help provide mechanistic insights into the molecular processes underlying the disease and subsequently develop novel tailored treatments. By examining expression profiles in cartilage, two previous studies in small cohorts have identified two subgroups of patients: microarray data from 23 patients suggested subgroups with gene expression differences related to inflammatory response,

Key messages**What is already known about this subject?**

- Osteoarthritis (OA) is a disease with both clinical and molecular heterogeneity.
- However, it remains unclear whether molecular subgroupings of patients vary between joint tissue types, how they relate to clinical characteristics and whether they can be reproducibly captured by a molecular classification algorithm.

What does this study add?

- We carried out the first in-depth characterisation of molecular heterogeneity using patient synovium and cartilage in the largest cohort to date.
- We detected two patient subgroups based on low-grade (largely intact) OA cartilage, which were associated with sex and proton pump inhibitor prescription. Patient subgroups in synovium were associated with inflammation and, separately, extracellular matrix and cell adhesion, and were independent of the low-grade OA cartilage subgroups.
- A seven-gene classifier reproducibly recapitulated both the discrete assignment of knee low-grade OA cartilage subgroups and the main continuous spectrum of variation within the tissue.

How might this impact on clinical practice or future developments?

- These data demonstrate that molecular tissue profile in OA is associated with patient clinical characteristics, that this profile can be characterised using a limited panel of genes, and support the case for precision medicine approaches in OA.

leucocyte activation, regulation of cytokine production and chemokine activity¹; RNA sequencing data from 44 patients also suggested two subgroups, but with differences related to oxidative stress, innate-immune responses, Wnt signalling and chemokine signalling rather than classical inflammation.²

Several questions emerge from these studies: Do molecular profiles in different disease-relevant tissues define the same patient subgroups? Is the molecular subgrouping associated with any clinical characteristics? Is the molecular subgrouping



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To cite: Steinberg J, Southam L, Fontalis A, et al. *Ann Rheum Dis* Epub ahead of print: [please include Day Month Year]. doi:10.1136/annrheumdis-2020-219760

Table 1 Characteristics of patients in discovery and validation cohorts

Variable	Discovery total	Discovery cohort 1	Discovery cohort 2	Discovery cohort 3	Discovery cohort 4	Validation
No of patients (after QC)	113 (106)	12 (11)	20 (17)	11 (10)	70 (68)	60 (60)
Osteoarthritis joint	Knee or hip	Knee	Knee	Hip	Knee	Knee
Low-grade OA cartilage samples after QC	87	11	16	10	50	60
High-grade OA cartilage samples after QC	95	10	16	10	59	–
Synovium samples after QC	77	–	16	–	61	–
Females, n (%) after QC	63 (59)	2 (19)	12 (71)	8 (73)	41 (60)	27 (45)
Age, average years (range) after QC	69 (38–88)	69 (50–88)	70 (54–79)	61 (44–88)	70 (38–84)	72 (63–85)

OA, osteoarthritis; QC, quality control.

truly categorical or better reflected by a continuous spectrum of variation? Can a molecular classification algorithm reproducibly recapitulate both categorical subgrouping and the main continuous spectrum of variation? Here, we examine these questions through genome-wide transcriptional profiling of multiple primary patient tissues (low-grade OA cartilage, high-grade OA cartilage and synovium), integrating information from electronic health records, and substantially increasing the cohort size and hence power (doubling the size of the discovery sample and almost tripling the total number of patients with genome-wide data compared with past studies).

MATERIAL AND METHODS

Patients

We analysed tissue samples from 113 patients undergoing total joint replacement surgery (table 1). All patients gave informed written consent prior to participation (online supplemental methods). Matched low-grade and high-grade OA cartilage samples (ie, largely intact vs degraded tissue, respectively; see online supplemental methods) were collected from each patient and synovial lining samples from 90 knee OA patients (table 1). All cartilage samples were collected from weight-bearing areas of the joint, ensuring that any differences observed between low-grade and high-grade OA cartilage reflected disease severity rather than differential mechanical loading. Cartilage scoring, isolation of chondrocytes and synoviocytes, and RNA extraction are described in online supplemental methods.

For knee OA patients, we obtained clinical characteristics and prescribed drugs from the electronic patient records (online supplemental methods).

RNA sequencing

Multiplexed libraries were sequenced on the Illumina HiSeq 2000 or HiSeq 4000 (75bp paired-end reads). After quality control, we applied salmon 0.8.2,³ and tximport⁴ to obtain gene-level scaled transcripts per million estimates (online supplemental methods).

Molecular subgroups

We applied limma-voom⁵ to remove heteroscedasticity from gene expression data, permuted Surrogate Variable Analysis (pSVA)⁶ to remove technical variation and explicitly regressed out effects of known sample collection and sequencing batches. We then applied ConsensusClusterPlus⁷ to identify discrete molecularly defined subgroups ('clusters') of patients, with a sensitivity analysis to verify that clustering was similar when restricting to knee OA patients only (online supplemental text). We tested differential gene expression between clusters using limma,⁸ and applied SPIA⁹ and Goseq¹⁰ to identify associated biological processes (online supplemental methods).

Associations between molecular clusters and clinical characteristics

We tested for association between cluster assignment and clinical characteristics using a generalised linear model and applied a Bonferroni multiple-testing correction for the effective number of tests ($p < 0.0047$, online supplemental methods). As sensitivity analyses, we successively added the following covariates to the models: sex, age, OA joint, body mass index (online supplemental methods).

In-depth characterisation of molecular heterogeneity

To test for patient heterogeneity using a method that can detect both discrete clustering and a continuous spectrum of variation, we used Multi-Omics Factor Analysis (MOFA).¹¹ We investigated the correspondence between the discrete clusters and the continuous spectrum of variation identified by the MOFA and carried out extensive sensitivity analyses to verify robustness of the MOFA results (online supplemental methods).

Low-grade OA cartilage classifier

We used the 'Prediction analysis for microarrays for R' (PAMR)¹² package to construct a classifier which used a smaller subset of genes to recapitulate the main axis of molecular heterogeneity in knee low-grade OA cartilage (online supplemental methods). We validated the classifier using an independent publicly-available dataset from 60 knee OA patients² (table 1, online supplemental methods).

RESULTS

After quality checks, we analysed transcriptomic profiles of 259 tissue samples from 106 patients (table 1).

Do molecular profiles in different tissues define the same patient subgroups?

Using consensus clustering, we identified two robust patient clusters in synovium, each of which further formed two subclusters (figure 1A). We also identified two robust patient clusters within low-grade, but not high-grade OA, cartilage (figure 1B, online supplemental figure 1). Cartilage clustering was independent of the synovium clusters (Fisher's $p > 0.66$), and not associated with patient cohort nor with sequencing batches (χ^2 test, $p > 0.96$).

Signalling Pathway Impact Analysis⁹ showed that the differences between the two synovium patient clusters relate to inflammation, while differences between the sub-clusters relate to the extracellular matrix and to cell adhesion (figure 1C,D, online supplemental figure 2, table 1 and text). The differences between low-grade OA cartilage clusters were also strongly associated with inflammation, extracellular matrix-related and cell adhesion pathways (figure 1E, online supplemental table 1).

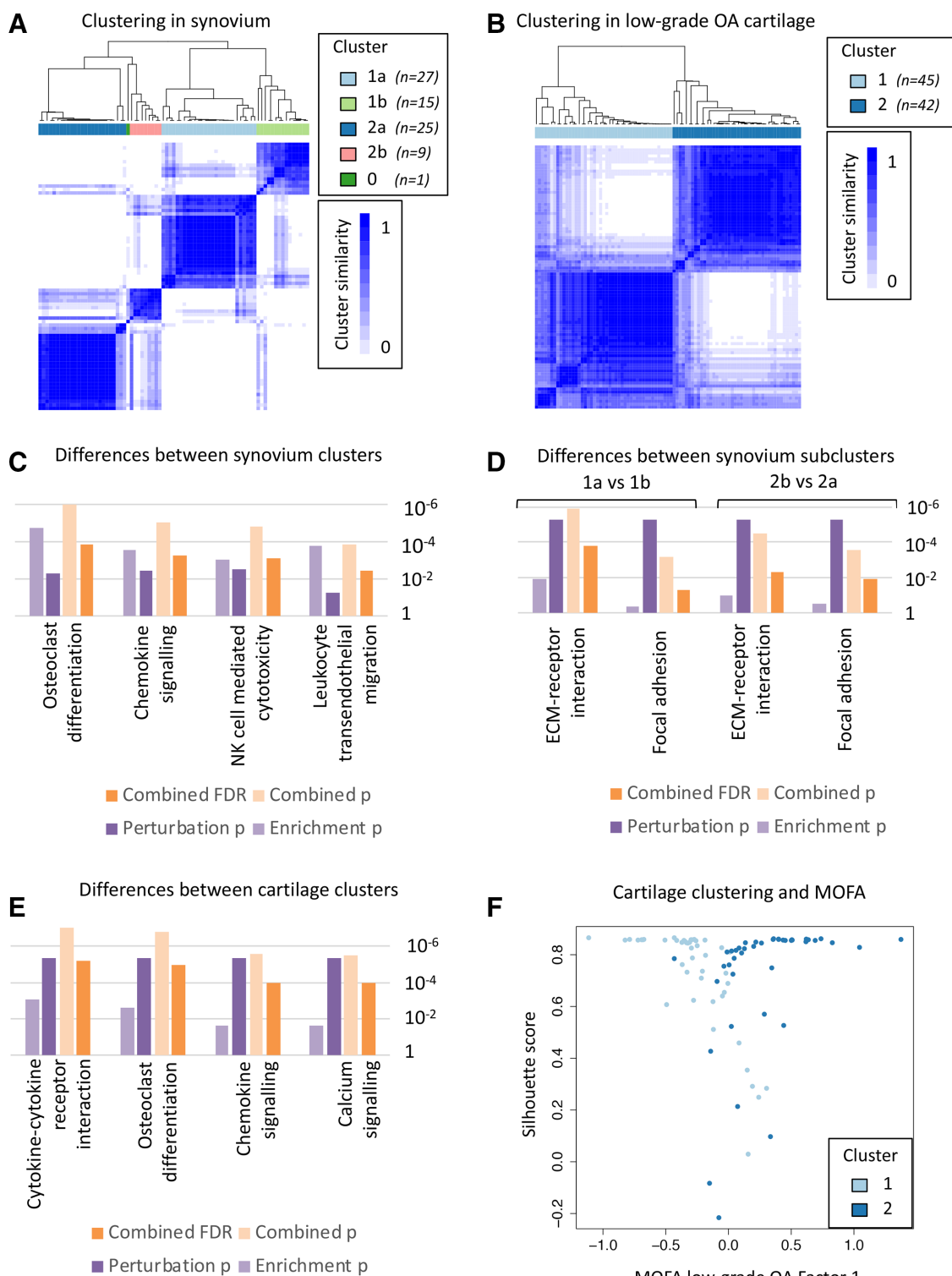


Figure 1 Distinct molecularly defined patient clusters identified in low-grade OA cartilage and synovium tissue. (A) Two clusters of patients based on consensus clustering of synovium RNA data. Each cluster formed two subclusters, with one outlier sample. (B) Two clusters of patients based on consensus clustering of low-grade OA cartilage RNA data. (C) Gene expression differences between synovium clusters show several significant (false discovery rate <5%) associations related to inflammation and osteoclast differentiation using Signalling Pathway Impact Analysis (SPIA). Here and below, P: p values based on enrichment of genes; perturbation of the pathway based on gene log-fold differences; or combining enrichment and perturbation. The associations shown are robust across several gene-level differential expression cut-offs (online supplemental table 1). (D) Gene expression differences between the synovium subclusters within each cluster show similar pathway associations, including to ECM–receptor interaction and focal adhesion pathways. (E) Gene expression differences between low-grade OA cartilage clusters show significant associations with inflammation and osteoclast differentiation pathways. (F) An analysis of low-grade OA cartilage samples using MOFA identifies a continuous spectrum of variation between samples, which corresponds to the identified clusters. Samples with intermediate MOFA factor 1 scores have lower Silhouette scores, showing more uncertainty in cluster assignment. For synovium, see online supplemental figure 3. ECM, extracellular matrix; FDR, false discovery rate; MOFA, Multi-Omics Factor Analysis; OA, osteoarthritis.

Is the molecular subgrouping associated with clinical characteristics?

We found that women were more likely to be members of the cartilage cluster characterised by higher inflammation (OR=4.12, $p=0.0024$; online supplemental table 2 and text). Patients in the high-inflammation cluster were also more likely to be prescribed proton pump inhibitors (PPIs; OR=4.21, $p=0.0040$; online supplemental text). We did not detect significant associations between synovium clustering and clinical characteristics (online supplemental table 2 and text).

Is the molecular clustering categorical or continuous?

MOFA¹¹ identified continuous axes of variation within synovium and low-grade OA cartilage tissue that correspond strongly with cluster assignment (figure 1F, online supplemental figures 3 and 4, online supplementary text). In low-grade OA cartilage, the first MOFA factor (ie, the main axis of variation) explained 28% of variation in gene expression levels; the gene expression weights for this first factor and the log-fold-differences between clusters had very high correlation (Pearson correlation $r=0.91$, $p<10^{-15}$; online supplemental figure 4). These findings were also recapitulated in synovium ($r=0.83$ – 0.96 for gene weights for the first two MOFA factors and the log-fold-differences between synovium clusters and subclusters; online supplemental figure 4). This suggests that the variation within these tissues is better represented as a continuous spectrum.

We verified robustness of the MOFA results using extensive sensitivity analyses (including restricting analysis to knee OA patients only or explicitly regressing out age and sex effects; online supplemental text).

Can a classifier reproducibly recapitulate both categorical clustering and the main axis of variation?

We used a soft-thresholding centroid-based method, PAMR,¹² to construct a tool that can recapitulate the clustering and main axis of heterogeneity in low-grade OA cartilage. As clinical and research applications would likely differ between OA joints, we restricted the

analysis to patients with knee OA. The resulting tool predicts probabilities of knee low-grade OA cartilage cluster assignment based on the expression levels of seven genes (figure 2A, online supplemental figure 5, Data availability): *MMP1*, *MMP2* and *MMP13*, which are involved in cartilage degradation¹³; *IL6*, a proinflammatory cytokine; *CYTL1*, a cytokine-like gene, loss of which has been found to augment cartilage destruction in surgical OA mouse models¹⁴; *APOD*, a component of high-density lipoprotein found to be strongly upregulated by retinoic acid,¹⁵ which is in turn regulated by *ALDH1A2*,¹⁶ an OA risk locus^{17 18} and *C15orf48*, of currently unknown function. Notably, the probabilities for cluster assignment generated by the classifier captured the main continuous spectrum of variation in this tissue (Spearman's correlation $\rho=-0.88$, $p<10^{-10}$; figure 2B). We validated the seven-gene classifier in an independent gene expression dataset of low-grade OA cartilage samples from 60 knee OA patients undergoing joint replacement surgery.² The posterior probabilities for cluster assignment had good correspondence to the main continuous spectrum of variation in the validation samples, supporting the predictive potential of the seven-gene classifier ($\rho=-0.85$, $p<10^{-10}$; figure 2C).

We also found that the seven-gene classifier had improved generalisability compared with a classifier developed in previous work²: the majority of genes in the previously developed classifier showed either discordant expression differences between the clusters in our larger dataset or high false discovery rates ($>30\%$; online supplemental table 3 and text).

DISCUSSION

Our findings indicate that molecular heterogeneity in OA cartilage and synovium is associated with similar biological processes (including inflammation), but molecularly defined patient clusters differ between tissues, potentially reflecting differences in tissue-specific dominant disease processes.

The clustering in low-grade OA cartilage agrees with two previous smaller studies.^{1 2}

We also identified an association between the cartilage high-inflammation cluster and female sex, which is consistent with the

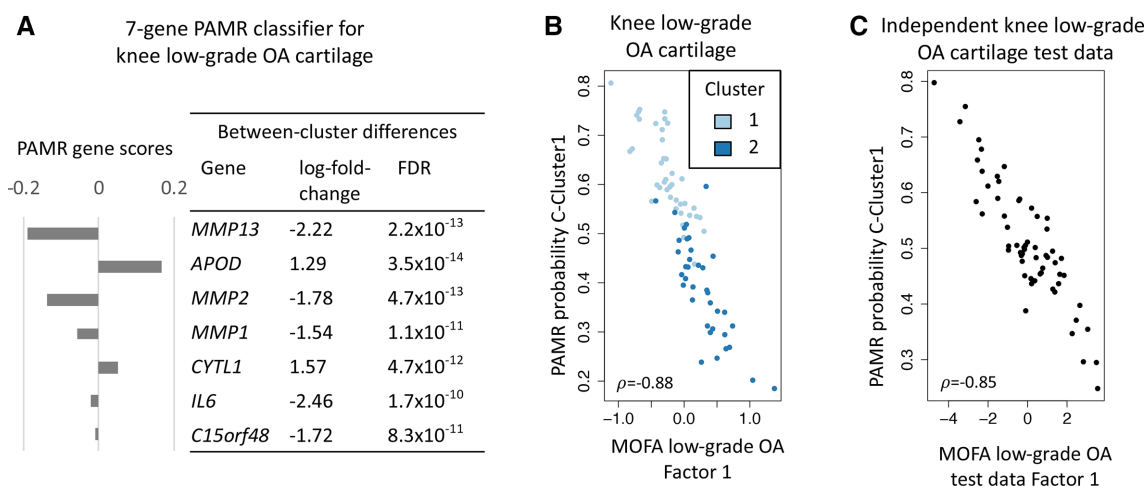


Figure 2 Clustering and main axis of variation within knee low-grade OA cartilage can be recapitulated using a seven-gene classifier. (A) PAMR scores for each gene in the seven-gene knee OA classifier (the difference between the standardised centroids of the two clusters) and the differential expression of the genes between the two low-grade OA cartilage clusters. See online supplemental figure 5 for classifier performance. (B) The PAMR posterior probabilities for cluster assignment are highly correlated with MOFA factor 1 scores for knee low-grade OA cartilage samples, capturing the main continuous spectrum of variation between samples. Inset: Spearman correlation, $p<10^{-10}$. (C) In an independent set of 60 low-grade OA cartilage samples from 60 knee OA patients, the posterior probabilities for cluster assignment from the seven-gene classifier are well correlated with the continuous spectrum of variation in these samples, as quantified by the first MOFA factor in an ab initio analysis. Inset: Spearman's correlation, $p<10^{-10}$. IL, interleukin; MOFA, Multi-Omics Factor Analysis; OA, osteoarthritis.

disproportionate increase in the incidence of OA in women after the menopause. This association might be explained by the lower concentration of oestrogen and androgens (which have established anti-inflammatory effects) in postmenopausal women.^{19 20} We speculate that our observed association between the high-inflammation cluster and PPI use could be explained by over the counter use of non-steroidal anti-inflammatory drugs for which PPIs are commonly coprescribed. We did not see discrete subgrouping in high-grade OA cartilage, perhaps indicating that there is less clear variation in molecular profiles in cartilage with advanced degeneration.

Our MOFA results further confirmed that the main axis of variation was related to inflammation in both synovium and cartilage. The seven-gene classifier generated using PAMR was able to place knee OA patients along the inflammatory endotype axis of variation and confirmed that such classification reflects continuous variation rather than categorical clustering, with validation in independent data. This finding has implications for the development of therapeutic strategies for OA, providing empirical evidence that responses might be expected to be heterogeneous along an axis of variation, rather than discrete. However, further study will be required to determine to what extent the inflammation axis is present in earlier disease stages, is stable across time or differs with disease activity, which cartilage scoring system is best suited to detect this axis, and whether the classifier can be applied to or modified for peripheral tissue (eg, saliva or blood). We anticipate that, looking ahead, this approach could underpin tailored therapy development and help improve patient care.

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Acknowledgements We thank the study participants who made this work possible by their generous donation of samples. The authors are grateful to Dr Iris Fischer for helpful edits.

Contributors Study design: EZ, JMW and JS. Collection of knee samples: MJC, RLI, DS, KMS and JMW. Collection of hip samples: RAB and AWM. Review of patient electronic health record data: AF and JMW. Patient stratification, Multi-Omics Factor Analysis, differential expression, pathway association and statistical analyses: JS. Writing – original draft: JS, LS, JMW and EZ. Writing – comments and review: all authors.

Funding This work was funded by the Wellcome Trust (206194). MJC was funded through a Centre for Integrated Research into Musculoskeletal Ageing grant (MRC 148985). RAB and the Human Research Tissue Bank are supported by the NIHR Cambridge Biomedical Research Centre. AM receives funding from Versus Arthritis; Tissue Engineering and Regenerative Therapies Centre (21156).

Competing interests None declared.

Patient and public involvement statement The biobank under which this project was conducted is overseen by a steering committee that includes two lay members who reviewed this project proposal prior to its initiation. The lay committee members had the opportunity to comment upon and make edits to the study design, as did the Sheffield Lay Advisory Panel for Bone Research (LAPBR). The conduct of the biobank and its outputs are also reviewed by the biobank lay committee members.

Patient consent for publication Not required.

Ethics approval The work was approved by the National Research Ethics Service (15/SC/0132).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement The RNA sequencing data reported in this paper have been deposited to the EGA (accession numbers EGAS00001002255, EGAD00001003355, EGAD00001003354, EGAD00001001331). Further data including results from consensus clustering, Multi-Omics Factor Analysis and PAMR analyses, as well as the scripts for PAMR classifier construction and application to test data, can be obtained online from <https://hmgubox.helmholtz-muenchen.de/d/f5be29c5123244359f73/>.

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Linking chondrocyte and synovial transcriptional profile to clinical phenotype in osteoarthritis

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Supplemental Information

Table of Contents

Supplemental text	3
Associations between clusters and biological pathways.....	3
Patient low-grade OA cartilage clusters identified in previous work	4
Continuous spectrum of variation within synovium and cartilage tissues corresponds to patient clusters.....	6
Associations between molecular clusters and clinical characteristics.....	8
Clustering and MOFA sensitivity analyses based on samples from knee OA patients only	10
MOFA sensitivity analyses of female and male patients separately, and analysis explicitly adjusting for sex and age	12
PAMR classifier construction based on low-grade cartilage samples from all patients.....	13
Supplemental methods.....	14
Patients.....	14
Sample clustering.....	19
Differential gene expression between tissue clusters.....	20
Associations between tissue clusters and clinical characteristics	20
Multi-Omics Factor Analysis (MOFA) and correspondence to sample clustering	22
Construction of classifier to recapitulate heterogeneity within knee low-grade OA cartilage	24
Validation of clustering classifier.....	26
Supplemental figures.....	27
Supplemental tables.....	40
Data available online	41

Supplemental text

Associations between clusters and biological pathways

To help interpret gene expression differences between clusters detected in synovium, we carried out pathway enrichment analyses using SPIA. This is a within-tissue analysis, thus different from comparisons of e.g. osteoarthritis tissue to health tissue. If a pathway is generally dysregulated in osteoarthritis disease tissue, the within-tissue analysis is expected to show no association. By contrast, we found multiple associations between synovium clusters that related to inflammation, including Chemokine signalling and Leukocyte transendothelial migration (figure 1c, online supplemental table 1). By contrast, differences between the sub-clusters related to the extracellular matrix and to cell adhesion (figure 1d, online supplemental table 1).

In an analogous comparison of differences between low-grade cartilage clusters, we also found associations with pathways related to inflammation, including Cytokine-cytokine receptor interaction and Chemokine signalling (figure 1e, online supplemental table 1). These results were supported by enrichment analyses based on Gene Ontology annotations (online supplemental table 1).

These results also agree with previous studies that have found low-grade cartilage clusters with differences associated with inflammation.[1, 2] As a limitation to this comparison, we note that the previously published studies used gene annotation data prior to 2014 and 2018, respectively, and these annotations would thus differ to those used in the current study.

Patient low-grade OA cartilage clusters identified in previous work

Previous studies in smaller patient sets have identified discrete subgroups by using gene expression arrays or RNA sequencing in low-grade OA cartilage.[1, 2] Here, we substantially increase sample size and hence power, and are able to test for overlap between clusters identified from different tissues, test for associations with clinical characteristics, and evaluate whether the clustering is truly categorical or better represented by a continuous spectrum of variation.

A previous study using array-based gene expression data across 23 patients identified two clusters in low-grade OA cartilage, with differences related to inflammation.[1] Of the 9 genes validated using qRT-PCR, 6 were also present in our data (including all 5 genes with significant differences in the original validation sample), and all showed significant differences in a concordant direction between the two cartilage clusters (online supplemental table 3). However, this previous study did not develop a classifier that could be applied to other data.

Another study used RNA sequencing data from low-grade OA cartilage of 44 knee osteoarthritis patients and applied a network-based approach to identify two patient groups, with differences related to inflammation, Wnt signalling, and calcium regulation.[2] This group assignment corresponded to the categorical cluster assignment from the 7-gene classifier constructed in this study for 70% of the samples (31 out of 44 samples with

available data). We further considered the certainty of the original group assignment as previously published[2]. Of the 18 samples with high silhouette scores (>0.7) signifying strong cluster assignment in the original analysis, 14 had a concordant dichotomous prediction by PAMR (78%).

To distinguish between the two patient groups, this previous study also constructed a classifier based on the expression of 10 genes in 44 patients; 7 of these genes also showed qPCR differences between two groups in a second cohort of 16 patients. Of these 7 genes, all were present in our data, but the majority showed a discordant direction of effect or higher false-discovery rates (33.5-99.8% FDR) between the low-grade OA cartilage clusters detected in our larger discovery cohort (83 patients; online supplemental table 3). By contrast, all 7 genes used by the 7-gene classifier constructed in the current work showed concordant differences between the two groups from the previous study (*MMP1*, *MMP2*, *MMP12* showed significant differences at 0.07% FDR; *APOD*, *CYTL1*, *C15orf48* at 11-15% FDR; *IL6* at 25.5% FDR), indicating improvement in transferability.

We also note that previous studies have focused their validation efforts on detecting differences in expression of individual genes using qPCR. By contrast, we validated the full 7-gene classifier in a completely independent gene expression dataset (patients samples collected in different hospitals, RNA sequenced and quantified in a different centre). Consequently, the strong correspondence of the posterior probabilities for cluster assignment with an ab initio MOFA analysis of the independent validation data (figure 2c) further speaks to the strong transferability of the 7-gene classifier from this work.

Continuous spectrum of variation within synovium and cartilage tissues corresponds to patient clusters

Disease endpoints and discrete endotypes represent underlying processes that may be more sensitively captured by continuous axes of variation within the molecular data rather than categorical classifiers. Such an approach may help define disease trajectories earlier in the natural history of osteoarthritis. To evaluate this, we applied Multi-Omics Factor Analysis (MOFA),[3] an integrative method that can discover hidden factors that represent drivers of variability between samples or patients (latent factors) that is akin to a cross-data principal component analysis.

When analysing all tissues together, we identified latent factors (axes of variation) that were each predominantly related to one tissue only. MOFA detected 8 factors that together captured 45-63% of the variance in the RNA data in each tissue. The first factor captured 27% of the variance in the synovium and was strongly associated with pathways related to the complement cascade, ECM organisation, diseases of glycosylation, binding and uptake of ligands, ECM proteoglycans, membrane trafficking, and others (see Data availability). The second factor captured 24% of the variance in low-grade OA cartilage and was strongly associated with various immune system processes (chemokines binding chemokine receptors, interleukin signalling, cytokine signalling, and others) and the ECM (ECM organisation, collagen degradation, assembly of collagen fibrils and other multimeric structures, collagen formation, and others; see Data Availability). The association of the first two factors with immune system processes and the extracellular matrix is in keeping with the biological pathways identified to play an important role based on the clustering analysis.

For the comparison of consensus clustering and MOFA, we sought to capture more fine-grained within-tissue results in MOFA and thus carried out MOFA separately for each tissue. We found that the continuous axes of variation within low-grade OA cartilage and synovium correspond strongly with cluster assignment (figure 1f, online supplemental figure 3). This is consistent with variation within tissues being better captured as a continuous spectrum rather than as discrete clusters.

In low-grade OA cartilage, the first MOFA factor (i.e. the main axis of variation) explained 28% of variation in gene expression levels and demonstrated strong correspondence with the cluster assignment. In agreement with this, we found that the gene expression weights for this first factor and the log-fold-differences between clusters had very high correlation (Pearson $r=0.91$, $p<10^{-15}$; online supplemental figure 4a).

We would therefore expect that in each cartilage cluster, gene expression differences between samples with low versus high factor scores would mirror differences between the clusters. To test this, we compared the gene expression differences between subsets of the discrete cartilage clusters at a MOFA low-grade OA Factor 1 threshold of 0, which corresponded most closely to the cluster assignment, with consistent assignment for 84% of patients in cartilage-Cluster1 (38 of 45 with score >0) and 83% of patients in cartilage-Cluster2 (35 out of 42 with score <0). We analysed gene expression differences between the 38 and 7 samples in cartilage-Cluster1 with MOFA low-grade OA Factor 1 values above and below 0, respectively. Analogously, we analysed gene expression differences between the 7 and 35 samples in cartilage-Cluster2 with MOFA low-grade OA Factor 1 values above and below 0, respectively. Indeed, in both comparisons, we found that 85-92% of genes with

significant expression differences also showed significant expression differences between the clusters, and all but two genes in the expected direction (online supplemental figure 4b).

These findings were also recapitulated in synovium, for which MOFA Factors 1 and 2 also showed good correspondence to the division of samples into clusters (online supplemental figure 4c). Moreover, we found high correlation between the MOFA synovium Factor 2 RNA weights and log-fold-differences between synovium clusters (Pearson correlation $r=0.83$, $p<10^{-15}$; online supplemental figure 4c), as well as between MOFA synovium Factor 1 RNA weights and log-fold-differences between synovium sub-clusters (Pearson correlation $r>0.919$, $p<10^{-15}$; online supplemental figure 4c).

Associations between molecular clusters and clinical characteristics

To verify the robustness of the associations between clinical characteristics and low-grade OA cartilage clusters, we carried out sensitivity analyses explicitly adjusting for i) sex; ii) sex and age; iii) sex, age, and osteoarthritis joint; iv) sex, age, and BMI (based on knee osteoarthritis patients only, as we did not have BMI information for hip osteoarthritis patients). In addition, we also tested associations adjusting for sex and age, based on data from knee osteoarthritis patients only. In all these analyses, we observed qualitatively and quantitatively similar effects (online supplemental table 2). In fact, the associations with sex and prescription of proton pump inhibitors became stronger and more significant when sex and age were explicitly adjusted for (female sex: OR=5.14 and $p=0.0010$ versus unadjusted OR=4.12 and $p=0.0024$; prescription of proton pump inhibitors: OR=5.16 and $p=0.0034$ versus unadjusted OR=4.21 and $p=0.0040$). The association with sex also remained

significant in the analysis adjusting for sex, age, and osteoarthritis joint (OR=5.12, $p=0.00123$), and in the analysis adjusting for sex and age while using data from knee osteoarthritis patients only (OR=4.61, $p=0.00292$). In both of these analyses, the association with proton pump inhibitors was the same as in the sensitivity analysis adjusting for age and sex, as the medication analyses were already restricted to knee osteoarthritis patients only. When adjusting for age, sex, and BMI, the effect estimates for both sex and proton pump inhibitors were only slightly lower and retained nominal significance, although they were no longer significant after Bonferroni-correction (OR=3.94, $p=0.0087$ and OR=4.88, $p=0.00645$); we note that reduced significance could be due to a smaller number of patients and inclusion of an additional covariate in this analysis.

Several further clinical characteristics were associated with cluster assignment at nominal significance in the main analysis: patients in the high-inflammation cluster were more likely to be prescribed a higher number of drugs (unadjusted OR=1.21 per additional drug, $p=0.023$; adjusted for age and sex: OR=1.17 per additional drug, $p=0.086$) and to be older (unadjusted OR=1.06 per year, $p=0.036$; adjusted for sex: OR=1.06 per year, $p=0.013$). These findings support a mechanistic explanation of the established association between osteoarthritis, age, sex, multimorbidity and polypharmacy,[4, 5] although the direction of causation remains unclear.

For the association between cartilage clustering and prescription of proton pump inhibitors, we note that these drugs are often co-administered with non-steroidal anti-inflammatory drugs (NSAIDs) to reduce risk of gastrointestinal adverse effects due to NSAID use.[6] NSAIDs are very commonly used to relieve osteoarthritis pain, but can be bought without a

prescription in the United Kingdom (at low dose, which is often recommended to reduce the risk of adverse events).[7] Thus, it also possible that the association between cartilage clusters and proton pump inhibitors is due to confounding by NSAID use. As data on non-prescription medication use were not available in this study, we could not investigate this hypothesis further.

While we did not detect any significant associations between synovium clustering and clinical characteristics, we note that the smaller number of patients with synovium tissue reduced power to detect an association.

Clustering and MOFA sensitivity analyses based on samples from knee OA patients only

We carried out extensive sensitivity analyses to verify that the clustering and MOFA results were robust when restricted to knee OA patients only, and were not driven by cohort effects.

Clustering analyses

First, we checked that clustering was not associated with patient cohort (chi-square test, $p>0.99$ in synovium and low-grade OA cartilage), nor with batches samples were sent and sequenced (chi-square test, $p>0.96$ in synovium and low-grade OA cartilage).

Second, we re-ran clustering analyses for low-grade and high-grade OA cartilage using samples from knee OA patients only.

For high-grade cartilage, we again did not see robust clustering as in the main analysis (cluster consensus scores <0.8 , online supplemental figure 6a).

For low-grade cartilage, the analysis based on knee OA patients identified 2 clusters very similar to the main analysis: 91% (70/77) of samples were assigned to the same cluster (33 to Cluster 1, 37 to Cluster 2), while 7 samples were assigned to Cluster 2 in the sensitivity analysis, but Cluster 1 in the main analysis. Notably, all 7 patients had intermediate MOFA low-grade OA Factor 1 scores (online supplemental figure 6b), suggesting that the re-assignment may be due to a forced dichotomisation imposed on a continuous spectrum of variation within the tissue.

These results support the representation of the variation in low-grade OA cartilage as a continuous spectrum for improved sensitivity (notably, the MOFA results were highly similar for analyses based on all patients or knee OA patients only, see below). These results also show that the clustering in low-grade cartilage is qualitatively similar when based on knee OA patients only, and support the inclusion of samples from hip OA patients in the main analysis to increase sample size and boost power for improved robustness of clustering.

MOFA

First, we did not find any evidence for association between the patient cohorts and any of MOFA Factor 1 scores for low-grade OA cartilage, or MOFA Factor 1 or Factor 2 scores for synovium ($p > 0.96$ for cohort effect coefficients in all generalised linear models). As an example, online supplemental figure 7a shows similar distribution of MOFA low-grade Factor 1 scores for patients from all four cohorts.

Second, we re-ran the low-grade and high-grade OA cartilage analyses based on knee osteoarthritis patient samples only ($n=77$ and $n=85$, respectively). For each tissue, the results were highly similar to the analysis of all patients (gene expression weights for the

first MOFA factor and corresponding patient scores showed high correlation: all Pearson $r > 0.99$, $p < 10^{-15}$; online supplemental figures 7b-c).

Finally, we also re-ran the low-grade OA cartilage analysis based on patient samples from cohort 4 only ($n=50$, all knee OA). The results were also highly similar to the analysis of all 87 patients (gene expression weights for the first MOFA factor and corresponding patient scores showed high correlation: Pearson $r \geq 0.97$, $p < 10^{-15}$; online supplemental figure 7d).

Conclusions and note on cartilage scoring systems

These results show that the main spectrum of variation in low-grade OA cartilage as described in the main results is highly robust and not due to differences between cohorts. As detailed in the Methods, multiple normalisation steps were carried out to remove technical variation, including application of pSVA and regressing out effects of known sample collection and sequencing batches. This could also have removed differences due to different cartilage scoring systems used for different cohorts (online supplemental methods). As such differences cannot be distinguished from technical variation in the current data, future research will be needed to determine which cartilage scoring approach is most suited to collect samples that best reflect the inflammatory axis of variation.

MOFA sensitivity analyses of female and male patients separately, and analysis explicitly adjusting for sex and age

We carried out sensitivity analyses using MOFA to determine robustness of the results when separately analysing low-grade OA cartilage samples from female patients ($n=52$) and male patients ($n=35$). The results were highly similar to the analysis of all patients: the gene

expression weights for the first MOFA factor showed very high correlation (Pearson $r=0.99$, $p<10^{-15}$; online supplemental figure 8a). Similarly, the patient scores for the first MOFA factor in the sensitivity analyses were highly similar to the MOFA low-grade OA Factor 1 scores in the main analysis (Pearson $r=0.99$, $p<10^{-15}$; online supplemental figure 8a). Moreover, we applied MOFA to low-grade OA cartilage data after explicitly regressing out sex and age effects on expression of each individual gene using generalised linear models. Again, the results were highly similar to the main analysis: high correlation of gene weights for the first MOFA factors ($r=0.99$, $p<10^{-15}$; online supplemental figure 8b) and high correlation of patient scores for the first MOFA factors ($r=0.92$, $p<10^{-15}$; online supplemental figure 8b).

This provides evidence that the main spectrum of variation in low-grade OA cartilage as determined by MOFA is robust and does not merely reflect sex or age effects.

PAMR classifier construction based on low-grade cartilage samples from all patients

As a sensitivity analysis for the PAMR classifier, we also carried out classifier construction based on low-grade cartilage data from all 87 patients in the discovery set, using the same approach as in the main analysis. The resulting classifier contained the same seven genes, and yielded highly similar cluster assignment probabilities for all discovery samples from knee OA patients (Pearson correlation $r=0.999$, $p<10^{-10}$), and for all validation samples (Pearson correlation $r=0.998$, $p<10^{-10}$).

Supplemental methods

Patients

Cohorts 1, 2, 4 (knee osteoarthritis)

This work was approved by Oxford NHS REC C (10/H0606/20 and 15/SC/0132), and samples were collected under Human Tissue Authority license 12182, Sheffield Musculoskeletal Biobank, University of Sheffield, UK.

We confirmed that each patient had a joint replacement for osteoarthritis, with no history of significant knee surgery (apart from meniscectomy), knee fracture, or knee infection, and no malignancy within the previous 5 years. We further confirmed that each patient had not used glucocorticoids (systemic or intra-articular) within the previous 6 months, nor any other drug associated with immune modulation.

Cartilage samples from patients in cohort 1 were scored using the OARSI cartilage classification system.[8, 9] From each patient, we collected one sample with high OARSI grade demonstrating high-grade degeneration (“high-grade OA sample”), and one cartilage sample with low OARSI grade demonstrating low-grade degeneration or healthy tissue (“low-grade OA sample”).

Cartilage samples from patients in cohorts 2 and 4 were scored macroscopically using the International Cartilage Repair Society (ICRS) scoring system.[10] From each patient, we collected one sample with ICRS grade 3 or 4 demonstrating high-grade degeneration (“high-grade OA sample”), and one cartilage sample of ICRS grade 0 or 1 signifying healthy tissue or low-grade degeneration (“low-grade OA sample”). From patients in cohorts 2 and 4, we also collected synovial membrane from the suprapatellar region of the knee joint.

For patients in cohorts 1,2, and 4, we obtained information on clinical characteristics (age, height, weight, body mass index (BMI), American Society of Anaesthesiologists (ASA) grade [11]) from the electronic patient records.

For each patient, a list of drugs prescribed on the date of sample collection was also compiled from the electronic patient record and cross referenced with the patient medical history. Prescribed drugs were grouped by pharmacological mechanism into 58 categories by two clinical experts (AF & JMW; online supplemental table 4).

Cohort 3 (hip osteoarthritis)

Samples were collected under National Research Ethics approval reference 11/EE/0011, Cambridge Biomedical Research Centre Human Research Tissue Bank, Cambridge University Hospitals, UK.

We confirmed osteoarthritis disease status by examination of the excised femoral head. From each patient, we collected a cartilage sample showing a fibrillated or fissured surface signifying high-grade degeneration ("high-grade OA sample"), one cartilage sample showing a smooth shiny appearance signifying healthy tissue or low-grade degeneration ("low-grade OA sample").

Isolation of chondrocytes

Cohorts 1,2,4

Isolation of chondrocytes for patient cohorts 1 and 2 has been described previously,[12] and we followed the same protocol to isolate chondrocytes for patients in cohort 4. Briefly, osteochondral samples were transported in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) (Life Technologies) supplemented with 2mM glutamine (Life

Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies), 2.5 µg/ml amphotericin B (Sigma-Aldrich) and 50 µg/ml ascorbic acid (Sigma-Aldrich) (serum free media). We then took forward half of each sample for chondrocyte extraction. We removed cartilage from the bone, then dissected and washed twice in 1xPBS. The tissue was digested in 3 mg/ml collagenase type I (Sigma-Aldrich) in serum free media overnight at 37°C on a flatbed shaker. We passed the resulting cell suspension through a 70 µm cell strainer (Fisher Scientific), then centrifuged at 400g for 10 minutes. We then washed the cell pellet twice in serum free media and centrifuged at 400g for 10 minutes. Subsequently, we resuspended the resulting cell pellet in serum free media, counted cells using a haemocytometer, and checked viability using trypan blue exclusion (Invitrogen). The optimal cell number for spin column extraction from cells was between 4×10^6 and 1×10^7 . Finally, we pelleted and homogenised cells.

Cohort 3

The extraction of chondrocytes in the majority of these samples has previously been described,[13] with the remaining samples following the same protocol, which was based on the protocol for cohorts 1,2, and 4. Briefly, we minced each cartilage portion with a scalpel and placed in 20ml of Dulbecco's modified Eagle medium (Invitrogen) containing 10% foetal bovine serum (Invitrogen) and 6 mgml⁻¹ collagenase A (Sigma). We incubated the tissue culture flasks overnight to digest the cartilage pieces. We passed the resulting cell suspension through a 30 µm filter (Miltenyi) and centrifuged at 400g for 10 minutes. We then re-suspended the cell pellet in 1ml of PBS, counted cells using a haemocytometer mixed with trypan blue to determine cell viability.

Isolation of synoviocytes

We followed a previously established protocol to process synovial samples.[14] Briefly, synovial samples were transported in serum free media, as described above. We then dissected the synovial membrane from underlying tissue and trypsinised for 1 hour. This was followed by tissue digestion in 1mg/ml Collagenase Blend H (Sigma Aldrich) in serum free media overnight at 37°C on a flatbed shaker. We passed the resulting cell suspension through a 100 µm cell strainer (Fisher Scientific) and centrifuged at 400g for 10 minutes. We then washed the cell pellet twice in serum free media, centrifuged at 400g for 10 minutes, and re-suspended the resulting cell pellet in serum free media. We counted cells using a haemocytometer and checked the viability using trypan blue exclusion (Invitrogen). The optimal cell number for spin column extraction from cells was between 4×10^6 and 1×10^7 . Finally, we pelleted and homogenised cells.

RNA extraction

We used the Qiagen AllPrep RNA Mini Kit to extract RNA and followed manufacturer's instructions, with small variations for cohort 3 as previously described.[13] We froze samples at -80°C (cohorts 1, 2, 4) or -70°C (cohort 3) prior to assays.

RNA sequencing

As preparation for RNA sequencing, poly-A tailed RNA (mRNA) was purified from total RNA using Illumina's TruSeq RNA Sample Prep v2 kits. The mRNA was then fragmented using metal ion-catalyzed hydrolysis and a random-primed cDNA library was synthesised. We used the resulting double-strand cDNA as the input to a standard Illumina library prep, repairing ends to produce blunt ends by a combination of fill-in reactions and exonuclease activity.

This was followed by A-tailing to allow pooling of samples, adding an “A” base to the blunt ends and ligation to Illumina Paired-end Sequencing adapters containing unique index sequences. We used KAPA Hifi Polymerase for 10-cycle PCR amplification of libraries. We used a post-PCR Agilent Bioanalyzer to quantify samples, then carried out sample pooling and size-selection of pools using the LabChip XT Caliper. The multiplexed libraries were sequenced on the Illumina HiSeq 2000 for cohort 1 and HiSeq 4000 for cohorts 2-4 (75bp paired-ends). Sequenced data underwent initial analysis and quality control on reads as standard. The sequencing depth was similar across samples, with 90% of samples passing final quality control (see below) having 87.2-129.2 million reads.

We used samtools v1.3.1 [15] and biobambam v0.0.191 [16] to convert cram to fastq files after exclusion of reads that failed QC. We then used FastQC v0.11.5 to check sample quality [17] and excluded 9 samples accordingly (online supplemental table 5).

We used salmon 0.8.2 [18] to obtain transcript-level expression estimates, with --gcBias and --seqBias flags to account for potential biases, and supplying the GRCh38 cDNA assembly release 87 [downloaded from Ensembl: http://ftp.ensembl.org/pub/release-87/fasta/homo_sapiens/cdna/]. The transcript-level estimates were converted to gene-level scaled transcripts per million (TPM) estimates using tximport.[19] This yielded expression estimates for 39,037 genes based on Ensembl gene IDs.

We excluded 4 samples due to low mapping rate (<80%), 3 samples due to non-European ancestry recorded in the clinic, 18 samples due to low RIN (<5), 2 samples as duplicates, 8 samples due to abnormal gene read density plots (detected separately in cartilage and synovium for 3 cartilage and 5 synovium samples; all exclusions are listed in online supplemental table 5).

The resulting final gene expression dataset included 259 samples from 106 patients, with at most one sample per patient per tissue (table 1). For cartilage, we retained 15,249 genes that showed counts per million (CPM) of ≥ 1 in ≥ 40 samples. For synovium, we retained 16,004 genes that showed CPM ≥ 1 in ≥ 20 samples.

Sample clustering

We normalised RNA data from each tissue separately, using limma-voom [20] to remove heteroscedasticity from scaled TPM values. Especially when considering clustering within a particular tissue, technical effects can influence the results. Consequently, for RNA data, we applied pSVA [21] (designed specifically to preserve biological heterogeneity for clustering) to the voom-normalised data, separately within low-grade OA cartilage, high-grade OA cartilage, and synovium, using the RNA sequencing batches to remove technical variation (post-QC, 9 batches for low-grade OA cartilage, 10 batches for high-grade OA cartilage, 8 batches for synovium). After this step, we obtained residuals from linear regression of the post-pSVA-data on an artificial variable indicating RNA sequencing batches and RNA extraction batches (RNA extraction from paired samples always in the same batch; in total, 12 batches for low-grade OA cartilage, 13 for high-grade OA cartilage, 11 for synovium).

For each tissue, we applied ConsensusClusterPlus,[22] a consensus clustering method that splits samples into a discrete number of groups, so that samples within a group are more similar to each other than to samples outside the group. We used standard settings, with scaled gene expression or protein abundance values, a Euclidean distance between samples, and a hierarchical clustering algorithm (options innerLinkage="average", finalLinkage="average", corUse="complete.obs", clusterAlg="hc"). The maximum number of

clusters was 10, with 1000-fold re-sampling for 80% of samples and a fixed seed for reproducibility (options maxK=10, reps=1000, pItem=0.8, pFeature=1, seed=1262118388.71279). The final number of clusters was chosen based on the Consensus Cumulative Distribution Function plots, the Delta Area Plot, and a visual investigation of the Consensus Matrices, as advised in the manual. Results were confirmed via additional analysis using a distance metric based on Pearson correlation.

To further verify the results, we also carried out a sensitivity analysis of low-grade and high-grade OA cartilage clustering by restricting the analysis to samples from knee OA patients only (see below).

Differential gene expression between tissue clusters

To follow up the clustering results for low-grade OA cartilage and synovium, we tested gene differential expression between sets of samples based on cluster assignment (applying limma to the normalised expression values underlying the clustering, i.e. gene expression after voom, pSVA, and regression of batch covariates). The differential expression analysis was followed up by gene set enrichment analyses using SPIA and Goseq, with 8 gene differential expression FDR thresholds to assess robustness of the association (5%, 0.5%, 5×10^{-3} , ..., 5×10^{-7}). In each analysis, gene set association was defined at the 5% FDR threshold. As before for Goseq, genes with positive and negative log-fold-difference between clusters were analysed separately.

Associations between tissue clusters and clinical characteristics

We tested for association between low-grade OA cartilage dichotomous cluster assignment (high-inflammation cartilage-Cluster1 versus low-inflammation cartilage-Cluster2) and

clinical characteristics using a generalised linear model (via the glm function in R with option family="binomial"). We restricted the analysis to 9 drug categories, each with at least 20 patients who were also assigned a low-grade OA cartilage or synovium cluster (online supplemental table 2).

To identify the number of effective tests across clinical characteristics (age, height, weight, BMI, ASA grade, number of drugs taken) and the 9 drug categories, we calculated pairwise correlations across patients using pairwise complete observations. We then obtained and eigenvalues of the correlation matrix, and calculated the effective number of tests N_{eff} for clinical characteristics as

$$N_{eff} = N - \sum_{\lambda} I(\lambda > 1) * (\lambda - 1)$$

where N=16 is the number of characteristics tested, and λ denotes the eigenvalues. Across the Pearson and Spearman correlation matrices, we obtained $N_{eff} < 10.54$. We thus used a Bonferroni-corrected threshold of $p < 0.05/10.54 = 0.0047$ to define significance.

As a sensitivity analysis, we tested for association of low-grade OA cartilage dichotomous cluster assignment (high-inflammation cartilage-Cluster1 versus low-inflammation cartilage-Cluster2) using a generalised linear model (via the glm function in R with option family="binomial"), adjusting for i) sex; ii) sex and age; iii) sex, age, and osteoarthritis joint; iv) sex, age, and BMI (based on knee osteoarthritis patients only, as we did not have BMI information for hip osteoarthritis patients). We also tested associations adjusting for sex and age, using the same approach as above but based on data from knee osteoarthritis patients only.

For association with synovium cluster assignment, we carried out analogous tests for the two clusters, with the same Bonferroni-corrected significance threshold.

Multi-Omics Factor Analysis (MOFA) and correspondence to sample clustering

To test for patient heterogeneity using a method that can detect both discrete clustering and a continuous spectrum of variation, we used Multi-Omics Factor Analysis (MOFA).[3] MOFA can integrate data across tissues to discover hidden factors that represent drivers of variability between samples or patients. MOFA was run i) jointly on all RNA data; ii) on RNA data within each tissue. MOFA identifies a factor score for each sample or patient, calculates the variance explained by each factor in each tissue, calculates weights of genes on each factor from each tissue, carries out a gene set enrichment for each factor in each tissue based on gene weights. All analyses were restricted to genes with unique correspondence between Ensembl gene ID and gene name as above. The technical parameters applied were gaussian likelihoods, 5000 iterations, a maximum of 100 factors, and dropping factors that explain less than 5% during training, with fixed seed 20180613 for reproducibility.

We further investigated the correspondence between the discrete clusters identified by ConsensusClusterPlus and the spectrum of variation identified by the MOFA as follows.

We tested the gene expression differences between subsets of the discrete low-grade OA tissue clusters at a MOFA low-grade factor 1 threshold of 0, which corresponded most closely to the cluster assignment, with consistent assignment for 84% of patients in the first cluster (38 of 45 with score >0) and 83% of patients in the second cluster (35 out of 42 with score <0). We analysed gene expression differences between the 38 and 7 samples in cartilage-Cluster1 with MOFA Factor 1 values above and below 0, respectively. Analogously, we analysed gene expression differences between the 7 and 35 samples in cartilage-Cluster2 with MOFA Factor 1 values above and below 0, respectively. We applied limma to the normalised gene expression values underlying the clustering, i.e. gene expression after voom, pSVA, and regression of batch covariates.

Second, we calculated the Pearson correlation between gene weights from the RNA data on the MOFA Factor 1 and the log-fold differences between the two low-grade OA cartilage clusters using the `cor.test` function in R.

For synovium, we carried out a Pearson correlation analysis between the gene weights on the MOFA synovium Factors 1 and 2, and the log-fold differences between synovium-Cluster1 and synovium-Cluster2, as well as between the synovium subclusters synovium-Cluster1a and synovium-Cluster1b, and separately, synovium-Cluster2a and synovium-Cluster2b.

We also checked that the main axes of variation identified by MOFA were not associated with technical differences between patient cohorts as follows. We used generalised linear models to test for association between the four patient cohorts and each of MOFA Factor 1 scores for low-grade OA cartilage, and MOFA Factor 1 and Factor 2 scores for synovium.

Moreover, we re-ran the low-grade OA cartilage analysis based on patient samples from cohort 4 only (n=50), using the same settings for MOFA as in the main analysis. We then calculated the Pearson correlation between gene weights for the first MOFA factor with the results based on the analysis of all patients.

Finally, we re-ran the low-grade OA cartilage analysis i) separately based on samples for female (n=52) and male (n=35) patients; and ii) for all patients, after explicitly regressing out sex and age effects for each gene (using a linear model in R). Again, we used the same settings for MOFA as in the main analysis throughout. We calculated the Pearson correlation between gene weights for the first MOFA factor with the results of the main analysis, and the Pearson correlation between patient scores for the first MOFA factor with the results of the main analysis.

Construction of classifier to recapitulate heterogeneity within knee low-grade OA cartilage

First, we computed silhouette scores for each low-grade OA sample based on the clustering, to calculate how similar each sample is to samples in the same cluster versus the other cluster. As the clustering was based on all patients, we included all patients in the calculation of silhouette scores, but then restricted the construction of the classifier to knee OA patients only.

To calculate silhouette scores, for each sample i in cartilage-Cluster1, we obtained the average dis-similarity to other samples in cartilage-Cluster1 (written $j \in C_1, j \neq i$) as $a(i) = \frac{1}{|C_1|-1} (\sum_{j \in C_1, j \neq i} 1 - \text{sim}(i, j))$ where $\text{sim}(i, j)$ is the similarity between samples i and j in cartilage-Cluster1 computed by ConsensusCluster, and $|C_1|$ is the number of samples in cartilage-Cluster1. We then obtained the average dis-similarity of sample i to samples in

cartilage-Cluster2 as $b(i) = \frac{1}{|C_2|} (\sum_{j \in C_2} 1 - \text{sim}(i, j))$. The silhouette score for sample i was

then calculated as $\frac{b(i) - a(i)}{\max(a(i), b(i))}$. We proceeded analogously for samples in cartilage-Cluster2.

To train a classifier to recapitulate the low-grade OA cartilage cluster assignment for knee OA patients, we considered all samples from these patients with silhouette score >0.2 (removing 1 sample in low-grade OA cartilage-Cluster1, 2 samples in low-grade OA cartilage-Cluster2). We also restricted the analysis to 1,027 genes with high expression levels, obtained as median scaledTPM value of at least 5,000 across all knee low-grade OA cartilage samples. We applied pamr to the 1,027 genes and 74 samples, setting the seed to 20180927 for all random components. We applied the function `pamr.adaptthresh` (with options `ntries = 100`, `reduction.factor = 0.9`) to identify thresholding scales for the classifier training. We then used the `pamr.train` function to train a classifier, and the `pamr.cv` function to examine the classifier error rates in cross-fit validation within the training data. We identified the minimum error rate to be reached with a threshold of 5.87, yielding 7 genes and a cross-validation error rate of 0.08. We then used the `pamr.predict` function to predict cartilage-Cluster1 and cartilage-Cluster2 probabilities for all 77 knee low-grade OA samples, with an agnostic prior setting of 0.5 for both clusters (options `type="posterior"`, `prior=c(0.5,0.5)`). We then calculated Spearman correlations for the PAMR cluster probabilities and MOFA Factor 1 for knee low-grade OA cartilage to verify that the classifier recapitulated the continuous spectrum of variation.

We also carried out a sensitivity analysis by applying the above procedure to construct a classifier using samples from all patients in the discovery data.

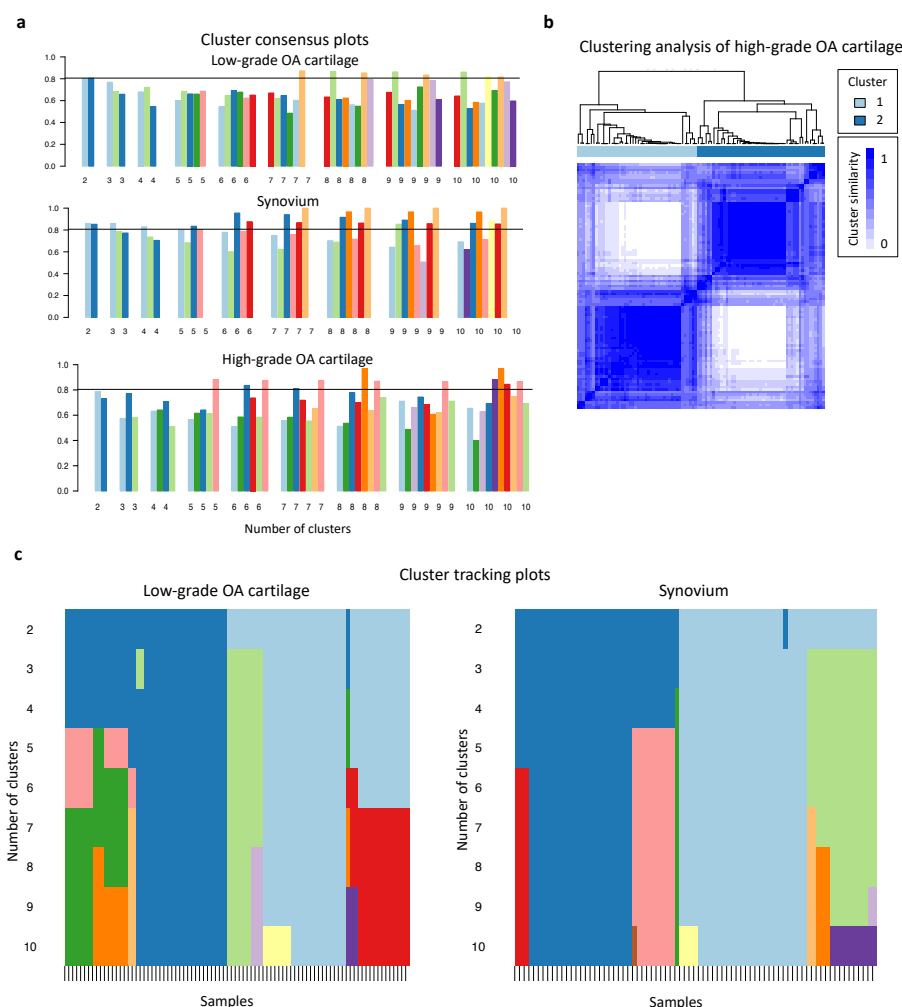
Validation of clustering classifier

We obtained RNA expression data from low-grade OA cartilage tissue of 60 knee osteoarthritis patients undergoing joint replacement (table 1), as described previously.[2] This dataset was completely independent of the discovery data, with patient recruitment, sample collection and RNA sequencing (Illumina HiSeq 2500) at different sites, transcript quantification using a different method (kallisto). We obtained the gene-level expression data from Github (file txi.RData on github.com/soulj/OAstratification, accessed 03/10/2018), with sample batch information provided in a separate file (patientDetails_all_withMed.csv). We then carried out further steps to harmonise data processing with our approach. First, we used tximport to transform the expression data to scaled transcript per million (scaledTPM) levels. We then applied the voom function in the limma R package to remove heteroscedasticity, followed by applying pSVA to remove batch effects (based on the known batches as listed in patient details). As for the discovery data, we calculated residuals from a linear regression of post-pSVA data on batches, and used these expression residuals as data post batch effect removal.

We applied the pamr.predict function to predict cartilage-Cluster1 and cartilage-Cluster2 probabilities for all 60 samples using the trained 7-gene classifier, with an agnostic prior setting of 0.5 for both clusters. We also applied MOFA (with the same parameters and options as above) to the data post batch effect removal. Finally, we calculated Spearman correlations for the PAMR cluster probabilities and MOFA factor 1.

The original publication also included a division of samples into 2 groups using non-negative matrix factorisation based on known biological networks. The group assignment was also provided on Github (file NetNMF_R2_L25.RData). This assignment was compared to a cluster assignment based on PAMR 7-gene classifier posterior probabilities.

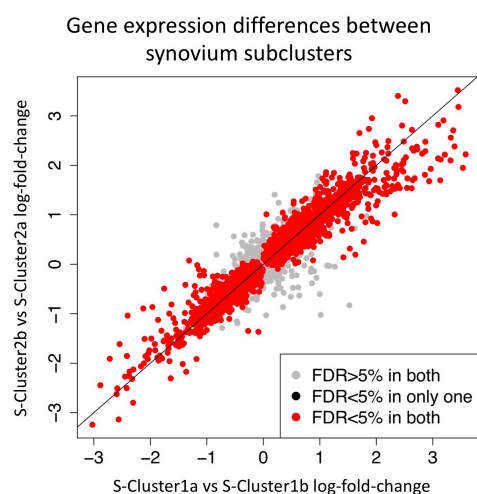
Supplemental figures



Supplemental figure 1. Cluster consensus and tracking plots for the clustering analysis of samples within tissues.

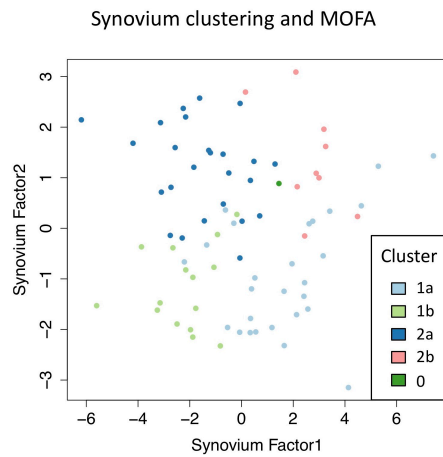
- a**, Cluster consensus plots for clustering in low-grade OA cartilage, synovium, and high-grade OA cartilage based on RNA data. The x-axis shows the number k of clusters, the y-axis the cluster consensus value (higher values showing stronger clustering). For clustering in low-grade OA cartilage and synovium, the cluster consensus value is above 0.8 for both clusters when $k=2$. For high-grade OA cartilage, the cluster consensus value is <0.8 for at least one cluster.

- b,** High-grade OA cartilage tissue samples from patients do not show a separation into two clusters by ConsensusCluster analysis based on RNA data (cluster consensus value <0.8).
- c,** Cluster tracking plots for low-grade OA cartilage and synovium samples based on RNA data. Each column is a sample, coloured by the cluster assignment when separating samples into $k=2, \dots, 10$ clusters (k values in rows).



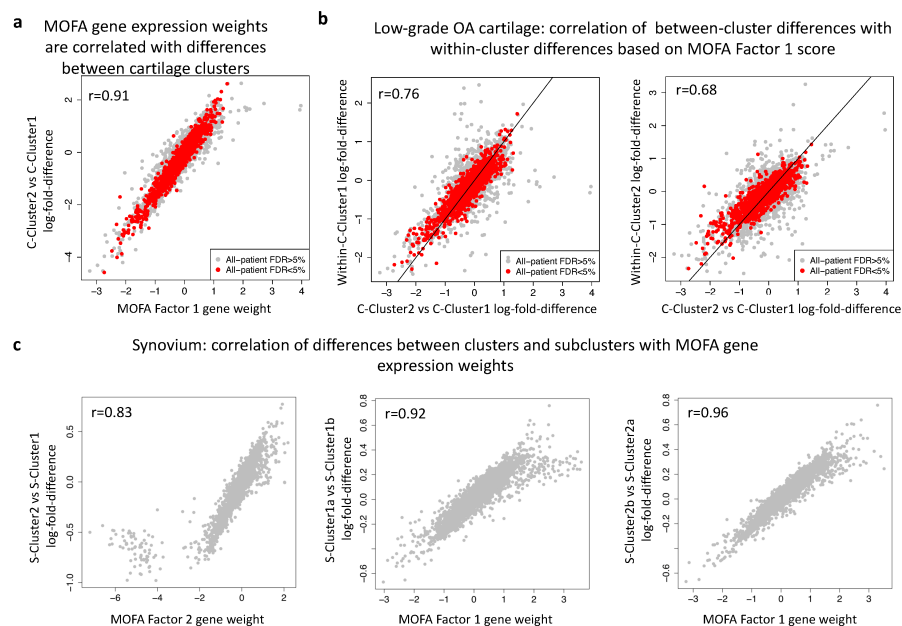
Supplemental figure 2. Gene expression differences between the synovium sub-clusters within each cluster are highly correlated.

The plot shows log-fold-differences of each gene in the comparison of sub-clusters within the larger (x-axis) and smaller (y-axis) cluster. Over 99% of the genes with significant differences between synovium-Cluster1a and synovium-Cluster1b also had directionally concordant differences between synovium-Cluster2b and synovium-Cluster2a, and over 80% were also significant at 5% FDR, and vice versa (i.e. genes with higher expression in synovium-Cluster1a compared to synovium-Cluster1b also had higher expression in synovium-Cluster2b compared to synovium-Cluster2a).



Supplemental figure 3. An analysis of synovium samples using Multi-Omics Factor Analysis (MOFA) identifies a continuous spectrum of variation between samples, which corresponds to the synovium clusters.

MOFA synovium Factor 1 captures differences between sub-clusters, while synovium factor 2 captures variation between clusters.



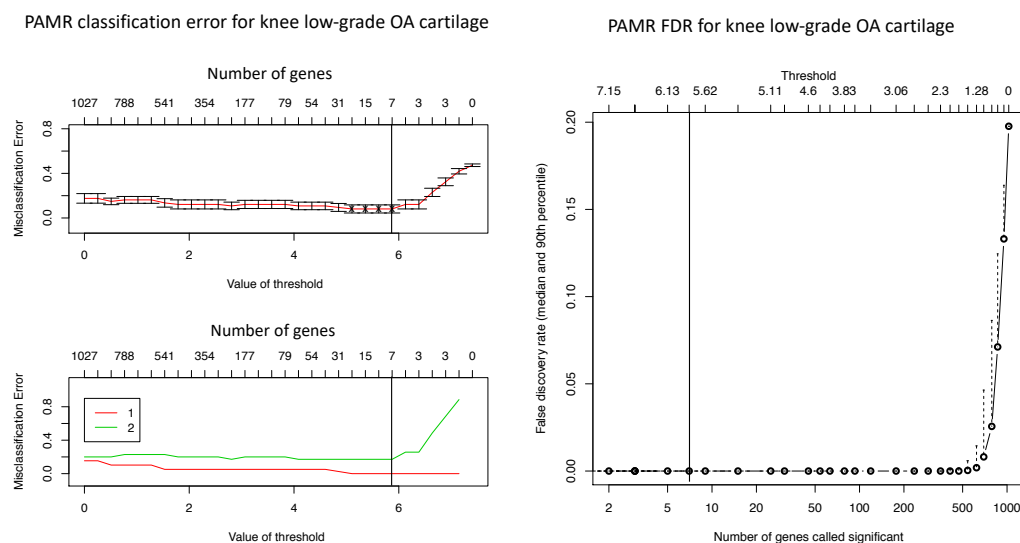
Supplemental figure 4. Multi-Omics factor analysis (MOFA) RNA gene weights are correlated with gene expression differences between tissue clusters.

- a**, Correlation between MOFA low-grade OA cartilage Factor 1 gene weights for RNA data and gene expression differences between low-grade OA cartilage clusters. Inset: Pearson correlation, $p < 10^{-15}$. Genes with significant differential expression between low-grade OA and high-grade OA cartilage are coloured red.
- b**, Gene expression differences between low-grade OA cartilage samples in the same clusters, divided by MOFA low-grade OA cartilage Factor 1 values >0 and <0 , are correlated with gene expression differences between clusters. Inset: Pearson correlation, $p < 10^{-10}$. Left shows results for low-grade OA cartilage-Cluster1, right for cartilage-Cluster2. Genes with significant differential expression between low-grade OA and high-grade OA cartilage are coloured red.

c, Correlation between MOFA synovium Factor 1 and 2 gene weights for RNA data and gene expression differences between synovium clusters and sub-clusters. Inset:

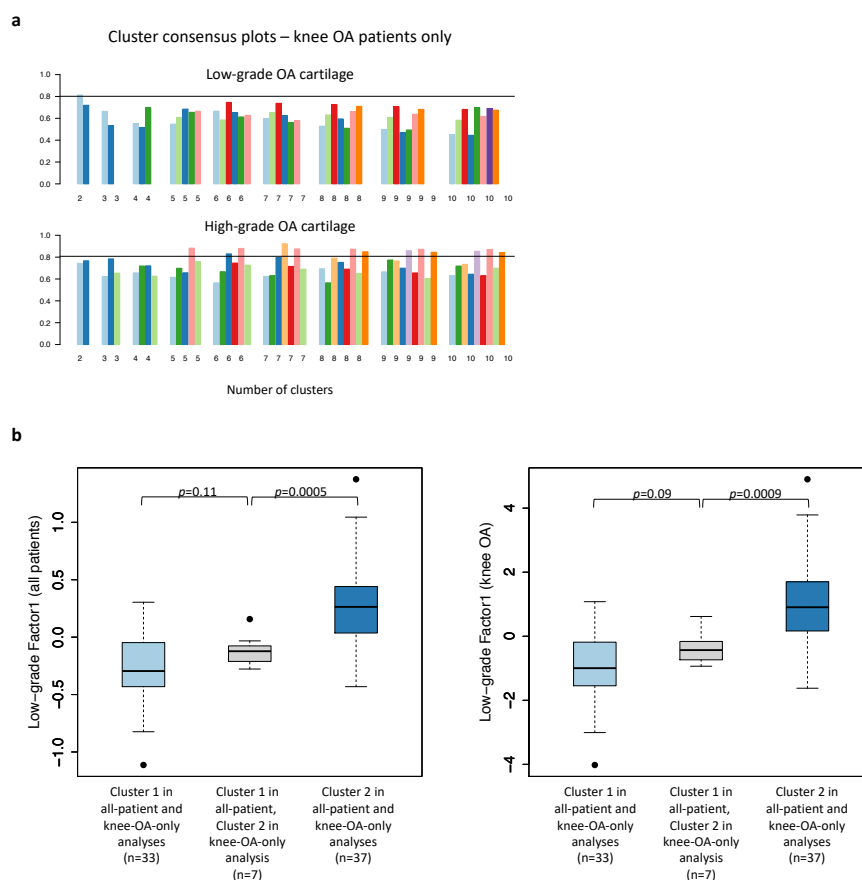
Pearson correlation, $p < 10^{-15}$.

C-Cluster: cartilage-Cluster; S-Cluster: synovium cluster



Supplemental figure 5. PAMR 7-gene knee low-grade OA cartilage classifier performance.

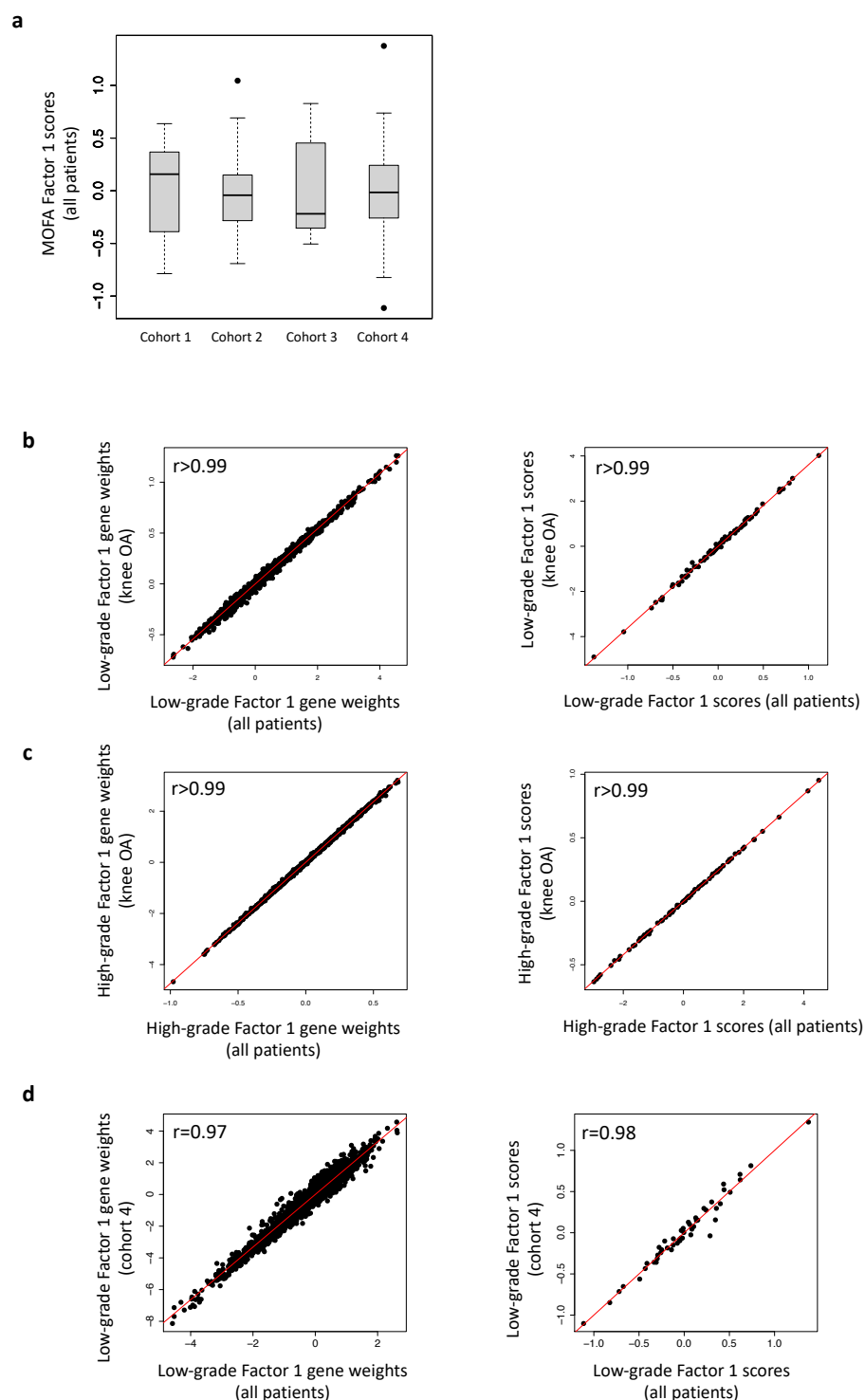
PAMR diagnostic plots for a classifier of knee low-grade OA cartilage based on RNA. Left: Sample classification error based on the PAMR internal threshold and the corresponding number of genes in the classifier. The top panel shows the overall error estimate, the bottom panel error rates separately for cartilage-Cluster1 and cartilage-Cluster2. The optimal selection as used in the paper included 7 genes and an internal threshold of 5.87 (vertical line). Right: False Discover Rate (FDR) for between-cluster differences for the genes in the classifier as calculated by PAMR. C-Cluster: low-grade OA cartilage-Cluster.



Supplemental figure 6. Qualitatively similar clustering analyses of low-grade and high-grade OA cartilage from knee osteoarthritis patients only.

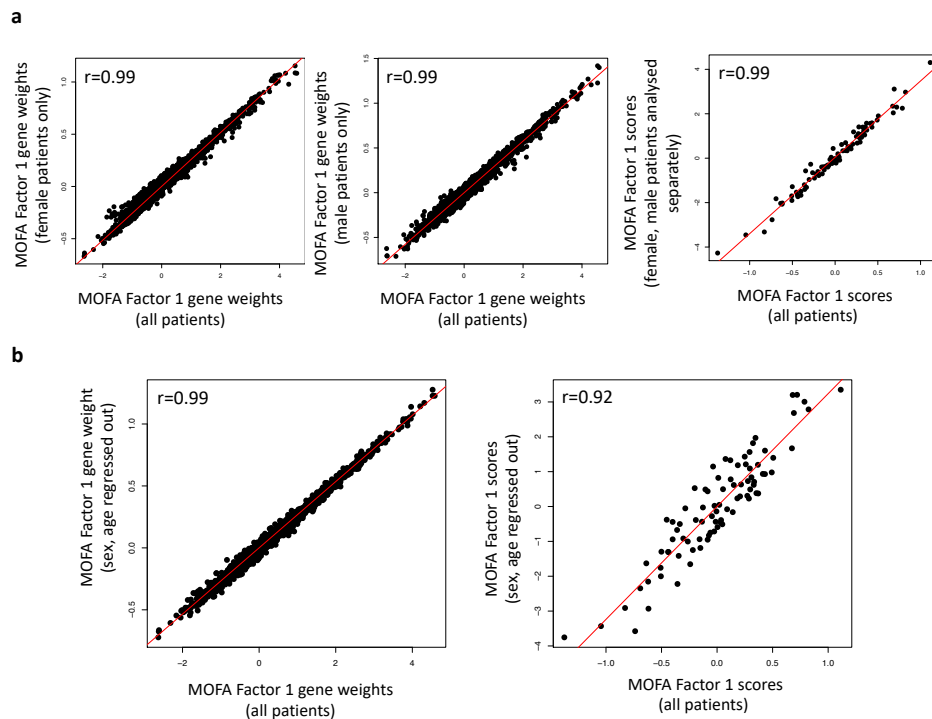
- a,** Cluster consensus plots for clustering in low-grade OA cartilage and high-grade OA cartilage samples from knee osteoarthritis patients only.
- b,** An analysis of knee OA patients only yields the same cluster assignment as the main analysis for 91% (70/77) patients, of whom 33 remain assigned to low-grade OA cartilage cluster 1 and 37 to low-grade OA cartilage cluster 2. The 4 patients re-assigned from cluster 1 to cluster 2 have intermediate MOFA low-grade Factor 1 scores both in the analysis of all patients (left) and the analysis of knee OA patients only (right), indicating the re-assignment may be due to forced dichotomisation of a

continuous spectrum of variation that is highly robust (see also online supplemental figures 7-8). *p*-values shown are from a Mann-Whitney U test.



Supplemental figure 7. MOFA sensitivity analyses based on data from knee osteoarthritis patients only show robustness of results.

- a,** MOFA low-grade OA cartilage Factor 1 scores do not show differences between patient cohorts (confirmed by a formal test using generalised linear models: $p>0.96$ for cohort effect coefficients).
- b,** A MOFA sensitivity analysis of low-grade OA cartilage samples from knee osteoarthritis patients only (n=77) yields result highly similar to the main analysis of all patient samples (n=87). Inset: Pearson correlation of gene weights or factor scores for MOFA low-grade OA cartilage Factor 1 as determined from the main analysis and this sensitivity analysis, $p<10^{-10}$.
- c,** A MOFA sensitivity analysis of high-grade OA cartilage samples from knee osteoarthritis patients only (n=85) yields result highly similar to the main analysis of all patient samples (n=95). Inset: Pearson correlation of gene weights or factor scores for MOFA high-grade OA cartilage Factor 1 as determined from the main analysis and this sensitivity analysis, $p<10^{-10}$.
- d,** A MOFA sensitivity analysis of low-grade OA cartilage samples from cohort 4 only (n=50) yields result highly similar to the main analysis of all patient samples (n=87). Inset: Pearson correlation of gene weights or factor scores for MOFA low-grade OA cartilage Factor 1 as determined from the main analysis and this sensitivity analysis, $p<10^{-10}$.



Supplemental figure 8. MOFA sensitivity analyses based on data from female and male patients separately, or based on regressing out sex and age effects, show robustness of results.

- a,** MOFA sensitivity analyses of low-grade OA cartilage samples from female patients only (n=52, left), male patients only (n=35, middle), or all patients with explicitly regressing out sex- and age-effects before MOFA (right): in all three, gene expression weights for the first MOFA factor are highly similar to the main analysis. Inset: Pearson correlation of gene weights for MOFA low-grade OA cartilage Factor 1 as determined from main and sensitivity analyses, $p<10^{-10}$.
- b,** For the same sensitivity analyses as in a, MOFA low-grade OA cartilage Factor 1 scores for patients are highly similar to the main analysis (left: separate analysis of n=52 female and n=35 male patients; right: explicitly regressing out sex and

age effects before applying MOFA). Inset: Pearson correlation of MOFA low-grade OA cartilage Factor 1 scores as determined from main and sensitivity analyses, $p < 10^{-10}$.

Supplemental tables

Supplemental table 1. Pathways associated with gene expression differences between low-grade OA cartilage clusters or between synovium clusters.

Supplemental table 2. Full association results between patient clinical characteristics and low-grade OA sample cluster assignment or synovium cluster assignment, including individual drugs assigned to drug classes.

The nine drug classes which were tested for association are shown (see **Methods**); for complete list of all drugs and assigned drug classes, see online supplemental table 4.

Supplemental table 3. Expression differences between low-grade OA cartilage clusters for genes highlighted in previous cartilage clustering analyses.

Supplemental table 4. Assignment of individual drugs to categories by pharmacological mechanism.

Supplemental table 5. List of all assayed patient tissue samples with detailed information including cohort, batch, and quality control exclusions.

Data available online

The RNA sequencing data reported in this paper have been deposited to the EGA (<https://www.ebi.ac.uk/ega/>; accession numbers EGAS00001002255, EGAD00001003355, EGAD00001003354, EGAD00001001331).

Data uploaded to <https://hmgubox.helmholtz-muenchen.de/d/f5be29c5123244359f73/>

MOFA analysis results

1. MOFA results: variance captured by each factor in each analysis. Fields highlighted where a factor captures at least 20% of the variance for a given tissue.
2. MOFA results: pathway associations of factors based on MOFA gene-set enrichment analysis using Reactome. The enrichment false discovery rate (FDR) is shown for each factor and tissue where that factor captures at least 20% of the variance.
3. MOFA gene weights for factor 1 in the low-grade OA cartilage analysis, and factors 1 and 2 in the synovium analysis. Legend see separate file.

PAMR analysis of patient stratification

4. For each patient, assignment of low-grade OA and synovium tissue samples to clusters, silhouette score for low-grade OA cluster assignment, PAMR posterior probability for low-grade OA clusters, and MOFA factor scores.
5. Construction of low-grade OA cartilage PAMR classifiers and application to replication data: R code. This file includes code for applying the classifier to any suitable low-grade OA cartilage gene expression data set (presuming scaled TPM

gene expression data, for which normalisation and batch effect removal have already been carried out).

6. Construction of low-grade OA cartilage PAMR classifiers and application to replication data: R data file.

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