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RNA Sequencing Reveals Interacting Key Determinants of Osteoarthritis Acting in Subchondral Bone and Articular Cartilage: Identification of *IL11* and *CHADL* as Attractive Treatment Targets

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Objective. To identify key determinants of the interactive pathophysiologic processes in subchondral bone and cartilage in osteoarthritis (OA).

Methods. We performed RNA sequencing on macroscopically preserved and lesional OA subchondral bone from patients in the Research Arthritis and Articular Cartilage study who underwent joint replacement surgery due to OA (n = 24 sample pairs: 6 hips and 18 knees). Unsupervised hierarchical clustering and differential expression analyses were conducted. Results were combined with data on previously identified differentially expressed genes in cartilage (partly overlapping samples) as well as data on recently identified OA risk genes.

Results. We identified 1,569 genes that were significantly differentially expressed between lesional and preserved subchondral bone, including *CNTNAP2* (fold change [FC] 2.4, false discovery rate [FDR] 3.36×10^{-5}) and *STMN2* (FC 9.6, FDR 2.36×10^{-3}). Among these 1,569 genes, 305 were also differentially expressed, and with the same direction of effect, in cartilage, including the recently recognized OA susceptibility genes *IL11* and *CHADL*. Upon differential expression analysis with stratification for joint site, we identified 509 genes that were exclusively differentially expressed in subchondral bone of the knee, including *KLF11* and *WNT4*. These genes that were differentially expressed exclusively in the knee were enriched for involvement in epigenetic processes, characterized by, e.g., *HIST1H3J* and *HIST1H3H*.

Conclusion. *IL11* and *CHADL* were among the most consistently differentially expressed genes OA pathophysiology–related genes in both bone and cartilage. As these genes were recently also identified as robust OA risk genes, they classify as attractive therapeutic targets acting on 2 OA-relevant tissues.

INTRODUCTION

Osteoarthritis (OA) represents multiple subtypes of degenerative joint diseases, characterized by progressive and irreversible degeneration of articular cartilage and structural changes in subchondral bone. Globally, OA is a highly prevalent and disabling disease that results in high social and economic burdens to society (1). Yet, there is no proven therapy to prevent OA or slow its progression. Development of OA is dependent on multiple factors,

with both environmental and genetic components (2,3). To discover genes and underlying disease pathways, genetic investigations, such as large genome-wide association studies, have been performed, identifying compelling OA risk single-nucleotide polymorphisms (SNPs) (4–6). Functional follow-up studies involve exploring the expression patterns in disease-relevant tissues, behavior with pathophysiology, and/or expression quantitative trait locus (eQTL) or cis-eQTL analysis. To date, major efforts have been made to characterize pathophysiologic processes of OA in

articular cartilage. However, only a few studies have focused on OA pathophysiologic processes in the underlying bone (7,8).

In recent decades, there has been accumulating evidence that subchondral bone contributes to both onset and progression of OA (9–12). In healthy bone there is a balanced process between bone resorption and bone deposition, as a consequence of dynamic adaptation to mechanical load. In OA this balance is disturbed, which results in changes in the architecture of the subchondral trabecular bone, increased thickness of the subchondral bone plate, formation of new bony structures, called osteophytes, at the joint margins, and development of subchondral bone cysts (2,13,14). In addition, studies have shown an association between bone mineral density and OA development, which suggests that subchondral bone is involved in the early stages of OA (13,15). This was also suggested in studies of subchondral bone marrow lesions, showing these to be very early markers of OA (8,16).

In contrast to cartilage and despite its relevance, only a limited number of studies have focused on the characterization of OA disease processes at the gene expression level in subchondral bone. Chou et al (7) performed whole-genome expression profiling of non-OA and OA subchondral bone using microarray analysis, which led to identification of genes involved in pathways such as lipid metabolism and mineral metabolism. Kuttapitiya et al (8) used microarray analysis to identify genes involved in bone remodeling, pain sensitization, and matrix turnover that were differentially expressed between OA bone marrow lesional tissue and control tissue. However, both of these studies included samples from the knee only.

In the present study, we explored RNA sequencing data on preserved and lesional OA subchondral bone to identify genes that change with progression of OA. The samples used were obtained from the joints of patients in the Research Arthritis and Articular Cartilage (RAAK) study who underwent total joint replacement surgery due to OA. In total, we compared paired subchondral bone samples (preserved and lesional) from 24 OA patients from whom preserved and lesional cartilage was also collected. The results presented here contribute to further understanding of the ongoing OA process in the subchondral bone and provide insight into the pathophysiology of the disease in bone relative to cartilage.

MATERIALS AND METHODS

Sample description. The current investigation included 26 patients from the RAAK study who underwent joint replacement surgery due to OA. Macroscopically preserved and lesional OA subchondral bone was collected from the joints of these patients. Of note, classification of OA subchondral bone as preserved or lesional was based on classification of its overlying cartilage as preserved or lesional, as described previously (17). The results reported here were compared to the results of our earlier study of macroscopically preserved and lesional OA articular cartilage from 35 patients from the RAAK study (18). Fourteen of these 35 patients were included in the present study, as samples of both preserved and lesional subchondral bone and preserved and lesional articular cartilage were available. The sample size for the current study was determined using the R package ssize.fdr, version 1.2 (19), with parameters based on our previous similar analysis of articular cartilage (18) and a desired power of 0.8 (see Supplementary Figure 1, on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract). Since the parameters were based on cartilage, whereas bone is known to be more heterogeneous, we decided to include an excess of samples. The samples were either randomly selected or selected based on their overlap with the cartilage data. Informed consent was obtained from all participants in the RAAK study, and ethical approval for the RAAK study was granted by the medical ethics committee of Leiden University Medical Center (P08.239/P19.013).

RNA sequencing. RNA was isolated from subchondral bone using an RNeasy Mini kit (Qiagen). Paired-end 2×100–bp RNA sequencing (Illumina TruSeq RNA Library Prep Kit, Illumina HiSeq2000, and Illumina HiSeq4000) was performed. Strand-specific RNA sequencing libraries were generated, which yielded a mean of 20 million reads per sample. Data from both Illumina platforms were integrated and analyzed with the same in-house pipeline. RNA sequencing reads were aligned using GSNAP (20) against GRCh38, with default parameters. Read abundance per sample was estimated using HTSeq count, version 0.11.1 (21). Only uniquely mapping reads were used for estimating expression. The quality of the raw reads for RNA sequencing was checked using MultiQC, version 1.7 (22). The adaptors were clipped using Cutadapt version 1.1 (23), applying default settings (minimum overlap length of 3). To identify outliers, principal components analysis and hierarchical clustering of the samples were applied, and 1 extreme outlier was identified. A sensitivity analysis was performed, which showed that the outlier had a large effect on the results in the overall data set. Based on this, the outlier was removed from the data set. There was 1 sample without paired data, which was also removed from the data set. After removal of these samples, only 24 participants were included for further analysis. The RNA sequencing data are deposited at the European Genome-Phenome Archive (www.ega-archive.org; accession no. EGAS00001004476).

Cluster analysis. Prior to the cluster analysis, variance stabilizing transformation was performed on the data, and 1,000 genes were selected based on the highest coefficient of variation (24,25). To identify the optimal number of clusters in the unsupervised hierarchical clustering the silhouette width score approach was used, with a higher average silhouette width score indicating a more optimal number of clusters (26). Details on the cluster

analyses and the stability of cluster solutions have been reported previously (25).

Differential expression analysis and pathway enrichment. Differential expression analysis was performed on paired lesional and preserved subchondral bone samples, using the DESeq2 R package, version 1.24.0 (27). A general linear model assuming a negative binomial distribution was applied, followed by a paired Wald test between lesional and preserved OA samples, with the preserved samples set as a reference. The Benjamini-Hochberg method was used to correct for multiple testing, as indicated by the false discovery rate (FDR), with a significance cutoff value of 0.05. Gene enrichment was performed using the online functional annotation tool DAVID, selecting for the gene ontology (GO) terms Biological Processes (GOTERM_BP_DIRECT), Cellular Component (GOTERM_CC_DIRECT), and Molecular Function (GOTERM_MF_DIRECT) and for the Reactome Homo Sapiens (R-HSA) and the KEGG pathways (28). Moreover, the protein–protein interactions were analyzed using the online tool STRING, version 11.0 (29). An analysis summary scheme is shown in Figure 1.

Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) validation. Complementary DNA synthesis was performed with a Transcriptor First Strand cDNA Synthesis Kit (Roche), using 400 ng of RNA. We used qRT-PCR to quantitatively determine gene expression of *FRZB*, *CNTNAP2*, *STMN2*, *CHRDL2*, *POSTN*, and *ASPN*. Relative gene expression was evaluated using $-\Delta C_t$ values, with *GAPDH* and *SDHA* as internal controls. Generalized estimating equation analysis was performed to calculate the significance of differences between the lesional and preserved samples.

Comparison of subchondral bone and articular cartilage. The 1,569 genes that were significantly differentially expressed (by FDR) between preserved and lesional OA subchondral bone (24 paired samples) reported here were compared to the 2,387 genes that were significantly differentially expressed between preserved and lesional OA articular cartilage (35 paired samples) as determined in our earlier study (18). Genes that were significantly differentially expressed in both tissues were selected, and the directions of effect were explored.

RESULTS

Sample characteristics. To characterize the pathophysiologic process in subchondral bone with ongoing OA, we performed RNA sequencing on macroscopically preserved and lesional OA subchondral bone samples from patients in the RAAK study who underwent joint replacement surgery due to
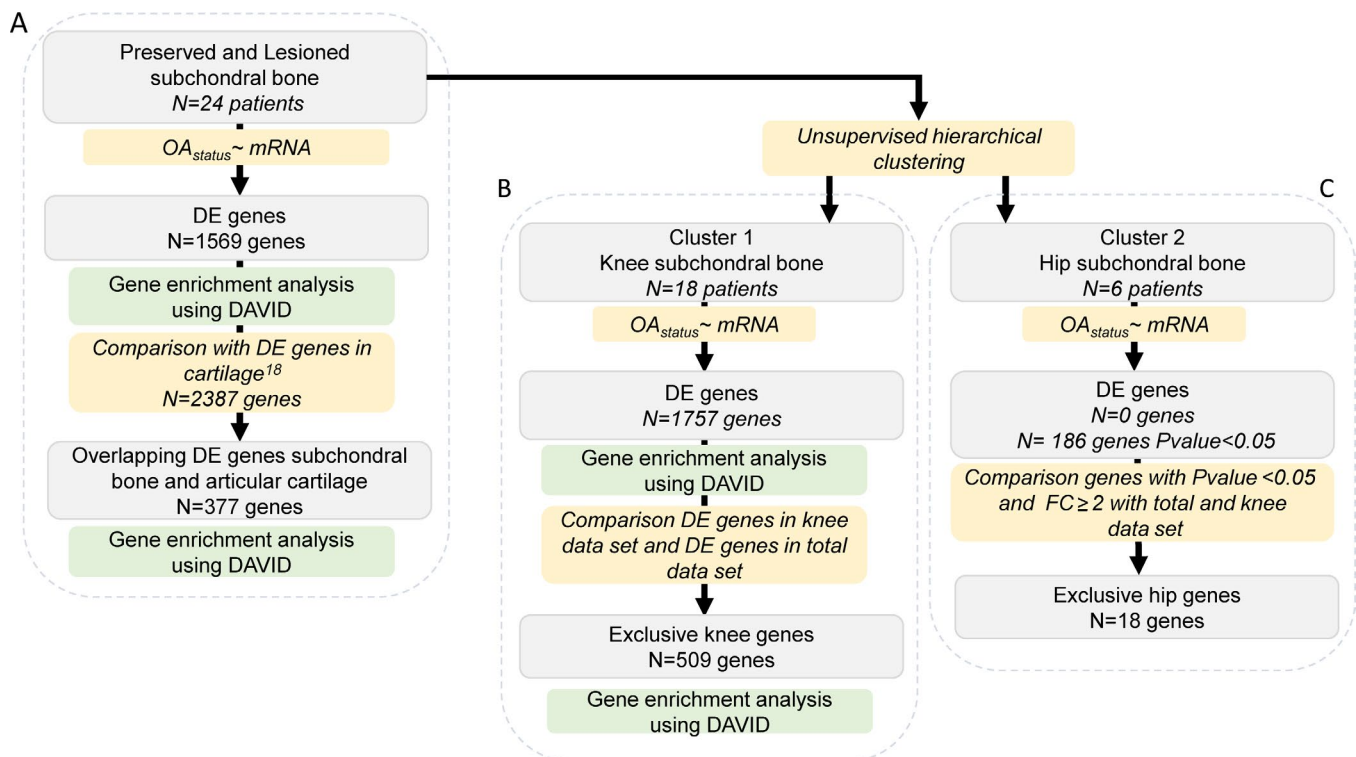
Figure 1. Overview of the study strategy. **A**, Determination of genes that were consistently differentially expressed (DE) between preserved and lesional osteoarthritis (OA) subchondral bone in the overall data set. **B**, Determination of genes that were differentially expressed in knee subchondral bone. **C**, Determination of genes that were differentially expressed in hip subchondral bone. Number of genes represents the significantly differentially expressed genes (according to the false discovery rate method), excluding those that were differentially expressed only in hip samples. FC = fold change.

OA. The RNA sequencing was performed on 24 paired samples (6 from hips and 18 from knees (Supplementary Table 1, on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract).

Prior to the differential expression analysis, we tested for possible contamination by cartilage tissue in the subchondral bone samples. We used RNA sequencing data on both tissue types from the same joint and evaluated the relative difference in expression levels of 3 cartilage-specific genes (*COL2A1*, *COMP*, and *CRTAC1*) and 3 bone-specific genes (*COL1A1*, *SPP1*, and *BGLAP*), as described previously (30). As shown in Supplementary Table 2 (http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract), we observed relatively low levels of cartilage-specific genes and high levels of bone-specific genes in the subchondral bone data set under study, suggesting no-to-minimal cross-contamination. Next, we explored whether the expression pattern in subchondral bone was associated with any baseline characteristics of the patients (Supplementary Table 1), by performing unsupervised hierarchical clustering. To include the most informative genes in the cluster analysis, 1,000 genes were selected based on the highest coefficient of variation in the

total data set (preserved and lesional; n = 24 pairs). As shown in Figure 2 and Supplementary Figure 2 (http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract), we identified 2 clusters. These appeared to be based on joint site, indicating an inherent difference between hip and knee subchondral bone.

Differential expression analysis and pathway enrichment. We first determined the genes that were consistently differentially expressed between preserved and lesional OA subchondral bone in the overall data set, to explore the most consistent OA pathways (Figure 1A). Upon differential expression analysis in the 24 sample pairs, we identified 1,569 genes that were genome-wide significantly differentially expressed between lesional and preserved OA subchondral bone tissue. Of these differentially expressed genes, 750 were up-regulated and 819 were down-regulated (Figure 3 and Supplementary Table 3, on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract). The most significantly down-regulated gene was *FRZB* (fold change [FC] 0.53, FDR 3.99 × 10^{-7}), encoding Frizzled-related protein, which is a well-known OA gene showing consistently lower expression in lesional relative
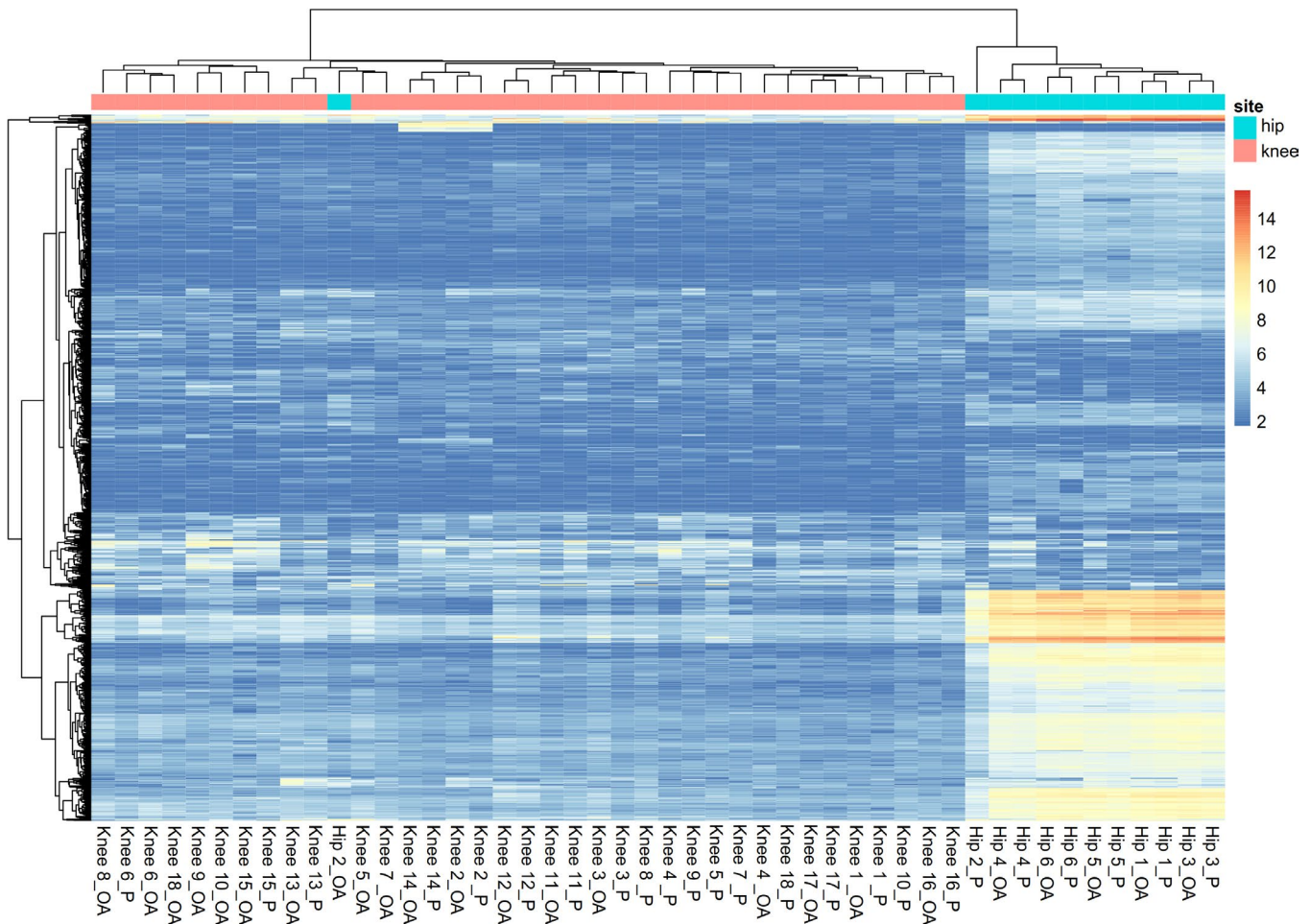
Figure 2. Cluster analysis using the 1,000 genes selected for their highest coefficient of variation. Two clusters based on joint site (knee or hip) were identified. OA = osteoarthritic; P = preserved.
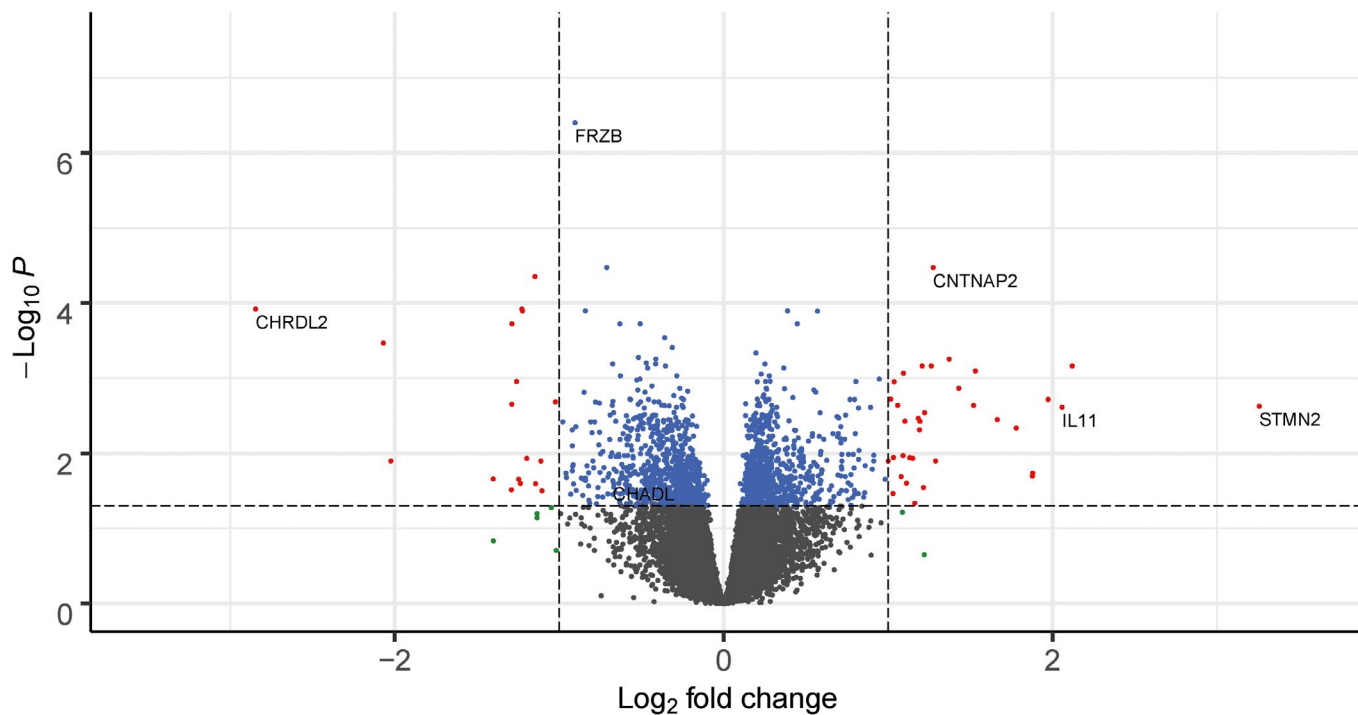
Figure 3. Volcano plot of differentially expressed genes in the subchondral bone. Blue dots represent genes that were significantly differentially expressed, red dots represent genes that were significantly differentially expressed and had an absolute fold change (FC) of ≥2, and green dots represent genes with an absolute FC of ≥2 that were not significantly differentially expressed.

to preserved OA articular cartilage (17,18). The most significantly up-regulated gene was *CNTNAP2* (FC 2.42, FDR 3.36 × 10^{-5}), encoding the contactin-associated protein-like 2 protein (CASPR2). Among the 1,569 differentially expressed genes, 53 had an absolute FC of ≥2 (35 up-regulated and 18 down-regulated). The most highly up-regulated gene was *STMN2* (FC 9.56, FDR 2.36 × 10^{-3}), encoding stathmin 2, while the most down-regulated gene was *CHRDL2* (FC 0.14, FDR 1.20 × 10^{-4}), encoding chordin-like protein 2.

Next, we explored whether the 1,569 significantly differentially expressed genes were enriched in relation to particular pathways or processes, using DAVID. The results demonstrated significantly enriched Gene Ontology (GO) terms regarding processes involved in translational and posttranslational processes, such as signal recognition particle–dependent cotranslational protein targeting to membrane (GO 0006614; 33 genes) (FDR 4.27 × 10^{-7}) and translational initiation (GO 0006413; 36 genes) (FDR 1.95 × 10^{-4}). These processes were both mainly characterized by ribosomal proteins such as *RPS24*, *RPS4X*, and *RPS18* (Supplementary Table 4, http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract). Gene enrichment analysis of the genes selected for the highest FC (FC ≥2; n = 53 genes) showed significant enrichment of processes regarding the extracellular matrix (GO 0005615; 16 genes) (FDR 1.19 × 10^{-5}), characterized by up-regulation of *WNT16* (FC 4.35, FDR 6.88 × 10^{-4}), *CRLF1* (FC 2.32, FDR 2.86 × 10^{-2}), and *OGN* (FC 3.43, FDR 4.62 × 10^{-3}), and the proteinaceous extracellular matrix (GO 0005578; 7 genes)

(FDR 4.50 × 10^{-2}), characterized by up-regulation of *POSTN* (FC 2.04, FDR 3.44 × 10^{-2}), *ASPN* (FC 3.17, FDR 3.56 × 10^{-3}), and *CTHRC1* (FC 2.15, FDR 3.75 × 10^{-3}) (Supplementary Table 5, http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract). To explore interactions between proteins encoded by the 53 differentially expressed genes with an FC of ≥2, we used the online tool STRING. We identified significant enrichment for protein–protein interactions among 22 of 44 proteins ($P = 3.20 × 10^{-9}$) (Figure 4).

Comparison of subchondral bone and articular cartilage. To investigate interacting OA pathophysiologic processes in subchondral bone and articular cartilage, we compared the differentially expressed genes identified in bone with our previously reported results on differentially expressed genes in articular cartilage (18) (Figure 1A) (24 sample pairs from bone and 35 from cartilage; 14 patients with available sample pairs from both bone and cartilage). This analysis revealed 337 genes that were differentially expressed in both subchondral bone and articular cartilage (Supplementary Figure 3, on the *Arthritis & Rheumatology* website at http://online library.wiley.com/doi/10.1002/art.41600/abstract). Of these 337 overlapping genes, the majority (305 genes) showed similar directions of effect in cartilage and bone (Supplementary Table 6, http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract), while 32 genes showed opposite directions of effect between the 2 tissue types (Supplementary Table 7, http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract). *ALX4*, encoding aristaless-like homeobox 4, was notable among the genes showing opposite
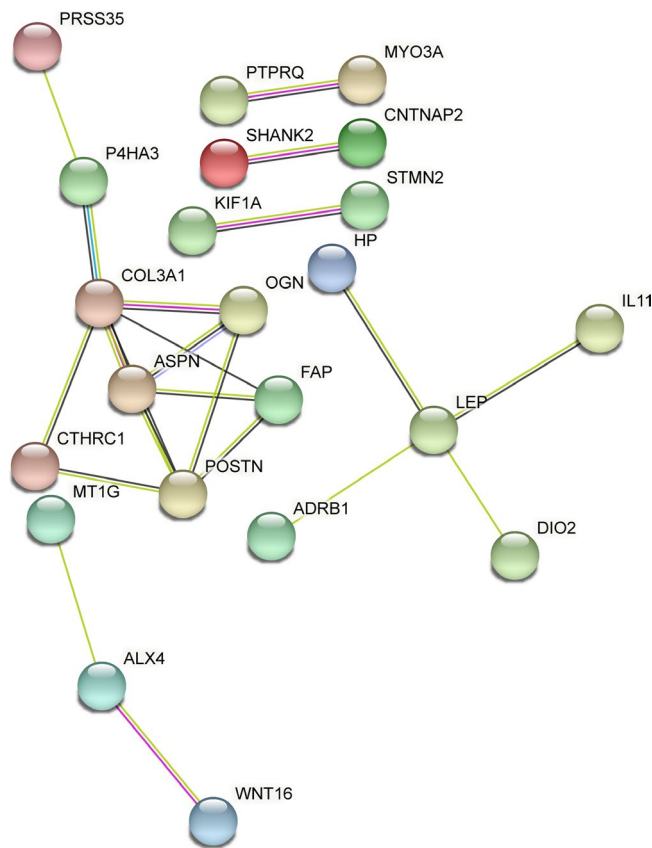
Figure 4. Protein–protein interaction network of proteins encoded by genes that showed an absolute fold change of ≥2 (n = 53 genes), created with the tool STRING.

directions of effect. *ALX4* is known to be involved in osteogenesis and was one of the most highly up-regulated genes in bone (Table 1). Among the 305 genes showing a similar direction of effect, 14 were among the top 25 genes with the highest FC in both tissues, such as *WNT16*, *IL11*, *CRLF1*, and *FRZB* (Table 1).

To explore common underlying pathways in subchondral bone and articular cartilage, we performed gene enrichment analysis with the 305 genes that showed similar directions of effect in cartilage and bone. We found significant enrichment for the GO terms extracellular region (GO 0005576; 36 genes) (FDR 4.56×10^{-3}), characterized by the expression of, for example, *COL6A3*, *FGF14*, and *GDF6*, proteinaceous extracellular matrix (GO 0005578; 17 genes) (FDR 7.98×10^{-3}), characterized by the expression of, for example, *CHADL*, *ADAMTS17*, and *SPOCK3,* and extracellular space (GO 0005615; 37 genes) (FDR 4.42×10^{-3}), characterized by the expression of, for example, *CD63*, *SPP1*, and *RELN* (Supplementary Table 8, on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract).

Differential expression analysis stratified by joint site. Since hip and knee samples showed different gene expression profiles in the cluster analysis (Figure 2), we repeated the differential expression analysis with stratification

by joint site to explore whether we could identify exclusive OA pathways that occur in subchondral bone of knees only or hips only. Differential expression analysis of the 18 knee sample pairs revealed 1,757 genes that were significantly differentially expressed (Figure 1B), of which 902 genes were up-regulated and 855 were down-regulated in lesional compared to preserved OA subchondral bone (Supplementary Table 9, http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract). Moreover, we identified 509 genes that were differentially expressed exclusively in the knee (Supplementary Table 10, http://online library.wiley.com/doi/10.1002/art.41600/abstract), i.e., these genes were not differentially expressed in an analysis of the total data set (Supplementary Table 3, http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract) or the hip data set (Supplementary Table 11, http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract). Enrichment analysis of these genes that were differentially expressed exclusively in the knee showed significant enrichment for processes involved in epigenetic regulation, such as nucleosome (GO 0000786; 20 genes) (FDR 1.81×10^{-9}), DNA methylation (R-HSA 5334118; 15 genes) (FDR 2.48×10^{-6}), and regulation of gene silencing (GO 0060968; 6 genes) (FDR 1.90×10^{-2}), all characterized by members of the histone H3 family, such as *HIST1H3J* and *HIST1H3H* (Supplementary Table 12, http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract).

Differential expression analysis using only the hip samples (6 pairs) did not reveal any genes that were significantly differentially expressed by the FDR method when comparing preserved and lesional subchondral bone. However, among the genes with a *P* value of <0.05 and an absolute FC of ≥2 (Supplementary Table 11), 18 genes appeared to be differentially expressed exclusively in the hip (Figure 1C); i.e., not differentially expressed in an analysis of the total data set (Supplementary Table 3) or the knee data set (Supplementary Table 9). Included among these genes with differential expression exclusively in the hip were *CALCR*, *LGR5*, and *COL2A1* (Supplementary Table 13, http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract).

Validation of differentially expressed genes. To validate and replicate the findings of the differential expression analysis performed using RNA sequencing, we used a set of 20 samples to conduct both technical replication (10 samples) and biologic replication (10 samples) by qRT-PCR. Validation analysis of 6 genes, *FRZB*, *CNTNAP2*, *STMN2*, *CHRDL2*, *POSTN*, and *ASPN*, showed significant differences between preserved and lesional subchondral bone, with directions of effect similar to those found by RNA sequencing. Replication analysis also showed significant differences, with the same directions of effects as shown by RNA sequencing (Supplementary Table 14, on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract).

Table 1. Genes classified as the top 25 genes based on the highest absolute FC in either bone or cartilage, 14 of which were among the top 25 with the highest FC in both tissues*

Ensemble ID/ gene name	Subchondral bone		Articular cartilage		Top 25 absolute FC	
	FC	FDR	FC	FDR	SB	AC
WNT16	4.35	6.88×10^{-4}	8.48	1.10×10^{-13}	x	x
IL11	4.16	2.44×10^{-3}	22.8	1.53×10^{-20}	x	x
GDF6	3.67	2.02×10^{-2}	1.58	3.19×10^{-2}	x	
OGN	3.43	4.62×10^{-3}	2.00	1.02×10^{-3}	x	
ASPN	3.17	3.56×10^{-3}	1.65	3.04×10^{-2}	x	
MYO3A	2.44	1.27×10^{-2}	2.25	1.16×10^{-4}	x	
CRLF1	2.32	2.86×10^{-2}	3.04	2.96×10^{-10}	x	x
GPR158	2.31	6.88×10^{-4}	2.73	3.63×10^{3}	x	x
PPP1R14C	2.19	1.14×10^{-2}	2.52	1.33×10^{-11}	x	
MT1G	2.16	2.50×10^{-2}	1.97	1.72×10^{-4}	x	
ALX4	2.08	2.30×10^{-3}	0.55	2.75×10^{-2}	x	
P4HA3	2.05	1.12×10^{-3}	1.84	1.49×10^{-5}	x	
FAP	2.05	1.14×10^{-2}	1.69	1.09×10^{-3}	x	
POSTN	2.04	3.44×10^{-2}	2.06	3.20×10^{-2}	x	
HOXB-AS1	2.00	1.27×10^{-2}	1.64	4.86×10^{-2}	x	
KIF20A	1.97	2.22×10^{-2}	1.59	4.44×10^{-2}	x	
TNFAIP	1.93	1.03×10^{-3}	3.58	2.48×10^{-8}	x	x
ERFE	1.87	1.63×10^{-2}	3.44	8.82×10^{-12}	x	x
PTGES	1.64	1.63×10^{-2}	3.06	3.61×10^{-12}		x
TNFRSF12A	1.50	2.31×10^{-2}	2.68	1.14×10^{-8}		x
WNT10B	1.49	3.25×10^{-2}	3.47	1.52×10^{-6}		x
COCH	1.46	4.21×10^{-2}	3.30	1.01×10^{-8}		x
CD55	1.46	2.48×10^{-2}	2.96	1.05×10^{-14}		x
P3H2	1.37	1.14×10^{-2}	3.23	4.71×10^{-18}		x
NGF	1.36	3.26×10^{-2}	4.91	2.53×10^{-14}		x
SPP1	1.36	4.81×10^{-2}	3.14	8.98×10^{-7}		x
NTRK3	0.70	3.56×10^{-3}	0.31	2.64×10^{-5}		x
LMO3	0.58	3.82×10^{-3}	0.28	1.67×10^{-5}		x
FRZB	0.53	3.99×10^{-7}	0.27	1.87×10^{-9}	x	x
RELN	0.53	2.56×10^{-2}	0.22	7.37×10^{-12}	x	x
SLC14A1	0.53	1.71×10^{-2}	0.51	7.05×10^{-6}	x	
CRISPLD1	0.51	1.84×10^{-2}	0.36	9.29×10^{-6}	x	x
ZNF385C	0.51	3.82×10^{-3}	0.43	2.30×10^{-6}	x	
HIF3A	0.49	2.07×10^{-3}	0.58	2.72×10^{-2}	x	
AL845331.2	0.46	3.16×10^{-2}	0.34	3.50×10^{-2}	x	x
GPC5	0.43	1.27×10^{-4}	0.36	1.47×10^{-8}	x	x
AC005165.1	0.43	1.20×10^{-4}	0.45	5.31×10^{-4}	x	
FGF14	0.41	1.89×10^{-4}	0.58	2.01×10^{-4}	x	
AC084816.1	0.38	2.20×10^{-2}	0.45	2.20×10^{-5}	x	
KIF1A	0.25	1.27×10^{-2}	0.37	8.64×10^{-8}	x	x
SPOCK3	0.24	3.41×10^{-4}	0.22	1.56×10^{-9}	x	x
CHRDL2	0.14	1.20×10^{-4}	0.13	7.07×10^{-9}	x	x

* FC = fold change; FDR = false discovery rate; SB = subchondral bone; AC = articular cartilage.

Differential expression of previously identified risk genes. In recent genome-wide association studies of hip and knee OA (5,6), 27 loci conferring risk to OA were identified (Table 2). To assess whether those OA susceptibility genes are also involved in OA pathophysiology in articular cartilage, subchondral bone, or both, we explored their expression levels and differential expression between lesional and preserved tissue in our data sets. As shown in Table 2, we identified 2 risk genes, *IL11* and *CHADL*, that were differentially expressed in both subchondral bone and articular cartilage. In addition, *IL11* showed both significant differential expression in knee subchondral bone (FC 4.07, FDR 7.00 × 10^{-3}) and a high FC (FC 4.77, $P = 4.43 \times 10^{-2}$) in hip subchondral bone. This indicates that, based on our data set, *IL11* has an effect in both tissues and at both joint sites, albeit not significant according to FDR in hip subchondral bone.

DISCUSSION

Differential expression analysis of gene expression levels in preserved and lesional OA subchondral bone (n = 24 paired samples) revealed 1,569 genes that were significantly differentially expressed, including *CNTNAP2* and *STMN2*. Upon comparing these 1,569 differentially expressed genes with the 2,387 genes with OA pathophysiology previously shown to be differentially expressed in cartilage, we found an overlap of 305 genes that had the same direction of effect. These 305 overlapping genes

Table 2. Expression levels and differential expression of OA risk genes identified in recent genome-wide association studies*

Gene	Bone, total data set			Cartilage, total data set		
	Expression†	FC, preserved vs. OA	FDR	Expression†	FC, preserved vs. OA	FDR
COL11a1	1	1.19	6.21×10^{-1}	1	1.07	7.59×10^{-1}
HDAC9	2	0.97	6.75×10^{-1}	1	0.59	9.10×10^{-6}
SMO	2	1.00	9.91×10^{-1}	1	0.69	7.85×10^{-5}
TNC	1	1.18	2.58×10^{-1}	1	1.41	1.09×10^{-2}
LMX1B	NE	NA	NA	3	0.99	9.80×10^{-1}
LTBP3	1	0.87	3.08×10^{-1}	1	1.08	6.95×10^{-1}
FAM101A (RFLNA)	4	0.99	9.77×10^{-1}	2	0.49	6.48×10^{-5}
IL11	3	4.16	2.44×10^{-3}	1	22.80	1.53×10^{-20}
ITIH1	NE	NA	NA	NE	NA	NA
FILIP1	2	0.84	7.07×10^{-2}	3	1.23	2.38×10^{-1}
RUNX2	1	1.07	4.75×10^{-1}	2	0.93	7.79×10^{-1}
ASTN2	4	0.87	2.42×10^{-1}	4	0.82	2.43×10^{-1}
SMAD3	1	0.93	3.74×10^{-1}	1	0.84	2.83×10^{-2}
HFE	3	1.01	9.07×10^{-1}	2	0.88	1.32×10^{-1}
CHADL	4	0.63	2.33×10^{-2}	1	0.63	1.29×10^{-1}
LTBP1	1	0.97	6.91×10^{-1}	1	1.15	1.70×10^{-1}
SBNO1	1	0.98	6.79×10^{-1}	1	1.10	3.94×10^{-1}
WWP2	1	0.82	2.47×10^{-1}	1	0.79	3.43×10^{-2}
GDF5	4	0.92	8.08×10^{-1}	1	1.23	3.09×10^{-1}
TGFB1	NE	NA	NA	NE	NA	NA
TNFSF15	4	1.23	2.42×10^{-1}	3	1.00	9.91×10^{-1}
FGF18	NE	NA	NA	2	1.58	9.51×10^{-4}
CTSK	1	1.41	3.23×10^{-1}	1	1.03	8.91×10^{-1}
DPEP1 (MBD1)	1	0.95	2.83×10^{-1}	1	0.96	6.20×10^{-1}
DIABLO	4	0.95	7.37×10^{-1}	3	1.08	6.46×10^{-1}
CRHR1	NE	NA	NA	4	0.62	5.10×10^{-1}
MAPT	3	0.61	3.47×10^{-2}	4	0.70	1.56×10^{-1}

* OA = osteoarthritis; FC = fold change; FDR = false discovery rate; NE = not expressed; NA = not applicable.
† In quartiles, with 1 being the highest expressed quartile and 4 being the lowest expressed quartile.

were enriched for processes related to the extracellular matrix, characterized by the expression of, among others, *COL6A3, GDF6,* and *SPP1.* Moreover, among the 305 overlapping genes were *IL11* and *CHADL* (Table 2 and Supplementary Table 6, on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract), which were previously identified as being OA risk genes (5,6). By applying hierarchical clustering on the overall RNA sequencing data set from subchondral bone, we observed 2 clusters based on joint site (knee and hip). When stratifying the analysis for joint site, we identified 1,757 genes that were differentially expressed between preserved and lesional knee OA bone, 509 of which were differentially expressed in the knee exclusively, including genes such as *WNT4* and *KLF11*. These OA genes that were differentially expressed exclusively in the knee were enriched for regulation of gene silencing by epigenetic processes such as DNA methylation and histone modification, characterized by genes such as *HIST1H3J* and *HIST1H3H*, as well as being enriched for other processes.

Among the 1,569 genes that were significantly differentially expressed between lesional and preserved OA subchondral bone using the FDR method in the complete data set, we identified *CNTNAP2* (FC 2.42, FDR 3.36×10^{-5}) and *STMN2* (FC 9.56, FDR 2.36×10^{-3}) as the most significantly up-regulated gene and the gene with the highest FC, respectively. *CNTNAP2*, encoding CASPR2, is known for its effect on cell–cell interactions in the

nervous system, synapse development, neural migration, and neural connectivity (31,32). Neither *CNTNAP2* nor its encoded protein were previously identified as being related to OA. *STMN2* also plays a role in the control of neuronal differentiation. Moreover, *STMN2* is expressed during osteogenesis, and it was previously shown to be highly up-regulated in OA bone marrow lesions as compared to control bone samples (8,33). In addition, we found other neural markers to be up-regulated in lesional compared to preserved OA subchondral bone, such as *NGF* and *THBS3* (Supplementary Table 3, http://onlinelibrary.wiley.com/doi/10.1002/art.41600/abstract). Based on these findings, we hypothesize that the formation of new neuronal structures in bone is increased with ongoing OA, which might suggest that OA-related pain originates from bone (8). However, functional follow-up research is needed to confirm this hypothesis.

The hierarchical clustering was done on the top 1,000 genes that showed the highest coefficient of variation between samples; hence, the clusters reflect particularly large differences. Based on the results observed here, it could thus be concluded that these highly variable genes reflect consistent differences between subchondral bone in the knee and subchondral bone in the hip, which was not previously seen in similar analyses of cartilage (25). Consequently, the fact that neither preserved and lesional samples from the same individual nor preserved samples or lesional samples as a group cluster together indicated that the 1,000

genes with the highest coefficient of variation are marking differences between knees and hips only. This does not rule out the relevance of the highly consistently differentially expressed genes reflecting OA subchondral bone pathology described here.

Upon differential expression analysis with stratification by joint site, we discovered 509 genes that were unique to the knee compared to the complete data set, which were significantly enriched for epigenetic processes such as DNA methylation, reflected by the expression of, among others, *HIST1H3J* and *HIST1H3H*. The significant enrichment of these epigenetic processes among the knee-exclusive genes indicates a change in epigenetics with ongoing knee OA, which is not seen with ongoing hip OA. This was also previously demonstrated in articular cartilage, where hip and knee methylation profiles clustered apart irrespective of OA status. However, this was characterized by the expression of different genes, such as the homeobox genes (34,35). We did not find FDR-significant genes when selecting the hip samples, which is likely due to the small sample size (n = 6 sample pairs). Nonetheless, we identified 18 genes that were exclusively differentially expressed in the hip based on the nominal *P* value and an absolute FC of ≥2, including genes such as *CALCR*, *LGR5*, and *COL2A1*. However, replication is needed to confirm our findings regarding these genes differentially expressed exclusively in the hip.

Given the accumulating awareness of cross-talk between articular cartilage and subchondral bone in OA (10,36), we compared RNA sequencing data from subchondral bone and from articular cartilage (24 sample pairs, and 35 sample pairs, respectively, with an overlap of 14 patients). Compared to the number of genes identified as being significantly differentially expressed between preserved and lesional OA articular cartilage based on FDR (n = 2,387 genes), we found fewer genes that were significantly differentially expressed by FDR between preserved and lesional OA subchondral bone (n = 1,569 genes). This difference might be due to the difference in sample size. However, it could also reflect the fact that bone as multicellular tissue is more heterogeneous. The relatively small overlap in genes that were differentially expressed in the same direction in both subchondral bone and cartilage (305 of 3,619; 8.43%) subchondral bone and cartilage suggests that there is a difference in OA pathophysiology between the 2 tissues.

To find genes that are most likely causal in OA, we explored 27 previously published genes with SNPs that were identified as being genome-wide significantly associated with OA (Table 2), suggesting that those genes have a more causal relationship to OA and making them attractive potential treatment targets (5,6). To examine whether the previously identified OA risk genes are involved in the OA pathophysiologic process in both cartilage and subchondral bone, we compared the expression levels and the differential expression between preserved and lesional samples (Table 2). We found that the OA risk genes *IL11* and *CHADL* were differentially expressed in both cartilage and subchondral bone and with the same direction of effect, thus making them attractive potential therapeutic targets with effects in both tissue types. *CHADL*, encoding chondroadherin-like protein, is involved in collagen binding and is a negative modulator of chondrocyte differentiation. The OA susceptibility allele rs117018441-T, located in an intron of *CHADL*, marks higher expression of *CHADL* compared to rs117018441-G in skeletal muscle and adipose tissue according to the Genotype-Tissue Expression Project (5,37). This may indicate that increased expression of *CHADL* has a negative regulatory role in both bone and cartilage and that inhibition of this gene could be a therapeutic strategy. However, when stratifying by joint site, we found *CHADL* to be differentially expressed specifically in the knee subchondral bone, suggesting that it is a treatment target for knee OA exclusively.

IL11, encoding interleukin-11 (IL-11), is known for its role in bone remodeling, and lack of IL-11 function is associated with impaired bone formation (38). Notably, *IL11* was recently proposed as a potential therapeutic target for OA in cartilage (6), since the OA risk allele rs4252548-T, a missense variant p.Arg112His, acts via reduced function of the IL-11 protein. As such, increasing IL-11 protein levels was proposed as a strategy for treatment of OA. In this study we have again shown that *IL11* is highly up-regulated in lesional versus preserved OA tissue in both subchondral bone and articular cartilage (FC 4.16 and 22.8, respectively). Taken together, these data indicate that reduced function of IL-11 predisposes to OA onset and that the up-regulation of *IL11* with OA pathophysiology could be considered an attempt of chondrocytes to enhance extracellular matrix integrity. Nonetheless, the consistent and considerable up-regulation of *IL11* in both subchondral bone and articular cartilage may not necessarily reflect a lack of potency to produce IL-11, unless translation of the protein is hampered. This requires further functional investigation, preferably in an in vitro model of OA. *CHADL* and *IL11* could both be highly suitable treatment targets with effects in both bone and cartilage. However, further functional research is needed to confirm the effects of these genes on bone and cartilage metabolism.

The classification of OA subchondral bone as preserved or lesional is derived from its overlying cartilage. We acknowledge that this ascertainment strategy is bound to introduce heterogeneity between samples. Nonetheless, we identified FDR-significant, and hence very consistent, differentially expressed genes. In other words, despite the fact that there may be heterogeneity in the preserved cartilage, we found consistent markers of the OA pathophysiologic process in subchondral bone.

To our knowledge, this is the first reported study of large-scale differential gene expression patterns in OA subchondral bone, performed using RNA sequencing in both hip and knee samples. We identified distinct differences in expression patterns between hips and knees. Moreover, we identified multiple genes that were previously demonstrated in OA articular cartilage, in addition to genes that were subchondral bone specific. These results will contribute to a better understanding of the pathophysiologic processes underlying the development of OA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Ms Tuerlings had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Tuerlings, Ramos, Coutinho de Almeida, Meulenbelt.

Acquisition of data. Tuerlings, van Hoolwerff, Houtman, Suchiman, Lakenberg, Mei, van der Linden, Nelissen, Ramos, Coutinho de Almeida, Meulenbelt.

Analysis and interpretation of data. Tuerlings, Ramos, Coutinho de Almeida, Meulenbelt.

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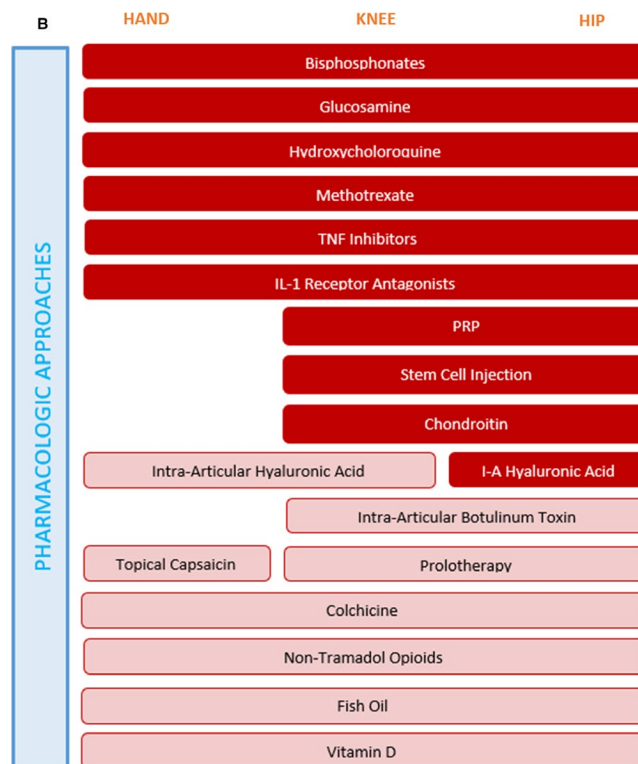
Errata

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In the article by Rodríguez-Carrio et al in the March 2021 issue of *Arthritis & Rheumatology* (Profiling of Serum Oxylipins During the Earliest Stages of Rheumatoid Arthritis [pages 401–413]), the academic degree listed for one of the authors, Dr. Roxana Coras, was incorrect and the institutional affiliation listed for two of the authors, Dr. Coras and Dr. Mónica Guma, was incomplete. The text in the title-page footnotes should have read "[2]Roxana Coras, MD, Mónica Guma, MD, PhD: University of California School of Medicine, San Diego, and Department of Medicine, Autonomous University of Barcelona, Barcelona, Spain."

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In the article by Kolasinski et al in the February 2020 issue of *Arthritis & Rheumatology* (2019 American College of Rheumatology/Arthritis Foundation Guideline for the Management of Osteoarthritis of the Hand, Hip, and Knee [pages 220–233]), there was an error in Figure 2B: Chondroitin should not have been included among the pharmacologic therapies recommended against for hand osteoarthritis. The corrected Figure 2B is shown below.

We regret the errors.