

Identification of the Molecular Response of Articular Cartilage to Injury, by Microarray Screening

Wnt-16 Expression and Signaling After Injury and in Osteoarthritis

Francesco Dell'Accio,¹ Cosimo De Bari,² Noha M. Eltawil,¹ Paul Vanhummelen,³
and Costantino Pitzalis¹

Objective. To characterize the molecular response of adult human articular cartilage to acute mechanical injury.

Methods. An established *ex vivo* model was used to compare gene expression of adult human articular cartilage explants 24 hours after mechanical injury with that of uninjured controls by microarray analysis of gene expression. Confirmation for selected genes was obtained by real-time polymerase chain reaction and immunohistochemical analysis. Expression of selected genes was also investigated in preserved and osteoarthritic (OA) cartilage.

Results. Six hundred ninety genes were significantly regulated at least 2-fold following mechanical injury. They included genes previously reported to be differentially expressed in OA versus normal cartilage or having allelic variants genetically linked to OA. Significant functional clusters included genes associated with wound healing, developmental processes, and skeletal development. The transforming growth

factor β , fibroblast growth factor, and Wnt pathways were modulated. A systematic analysis of the Wnt signaling pathway revealed up-regulation of Wnt-16, down-regulation of FRZB, up-regulation of Wnt target genes, and nuclear localization of β -catenin in injured cartilage. In addition, in OA, Wnt-16 and β -catenin were barely detectable in preserved cartilage areas, but were dramatically up-regulated in areas of the same joint with moderate to severe OA damage.

Conclusion. Our findings indicate that mechanical injury to adult human articular cartilage results in the activation of a signaling response, with reactivation of morphogenetic pathways. Therapeutic targeting of such pathways may improve current protocols of joint surface defect repair and/or prevent the evolution of such lesions into posttraumatic OA.

Joint surface defects in weight-bearing joints are frequent (1) and, when they acquire a chronic symptomatic course, often require surgical treatment for symptom relief and in an attempt to avoid possible evolution toward osteoarthritis (OA) (2). The most common surgical techniques for joint resurfacing include microfracture and autologous chondrocyte implantation (ACI) (3). Although both techniques are efficacious in a large number of patients, neither of the two is optimal because microfracture often results in nondurable, largely fibrocartilaginous repair tissue (4,5), whereas ACI has a number of shortcomings due to the autologous nature of the cell preparation, including high costs, problems with reproducibility, and a lack of consistency of results (3,4,6,7).

The factors that lead to the chronic evolution of joint surface defects are not known. The natural history of acute full-thickness joint surface defects is not suffi-

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¹Francesco Dell'Accio, MD, PhD, Noha M. Eltawil, MSc, Costantino Pitzalis, MD, PhD, FRCP: Barts and The London School of Medicine and Dentistry, London, UK; ²Cosimo De Bari, MD, PhD: University of Aberdeen, Aberdeen, UK; ³Paul Vanhummelen, PhD: Flanders Interuniversity Institute for Biotechnology (VIB) Micro-Array Facility, Leuven, Belgium.

Address correspondence and reprint requests to Francesco Dell'Accio, MD, PhD, Centre for Experimental Medicine and Rheumatology, William Harvey Research Institute, Queen Mary's School of Medicine and Dentistry, II Floor John Vane Building, Charterhouse Square, London EC1M 6BQ, UK. E-mail: f.dellaccio@qmul.ac.uk.

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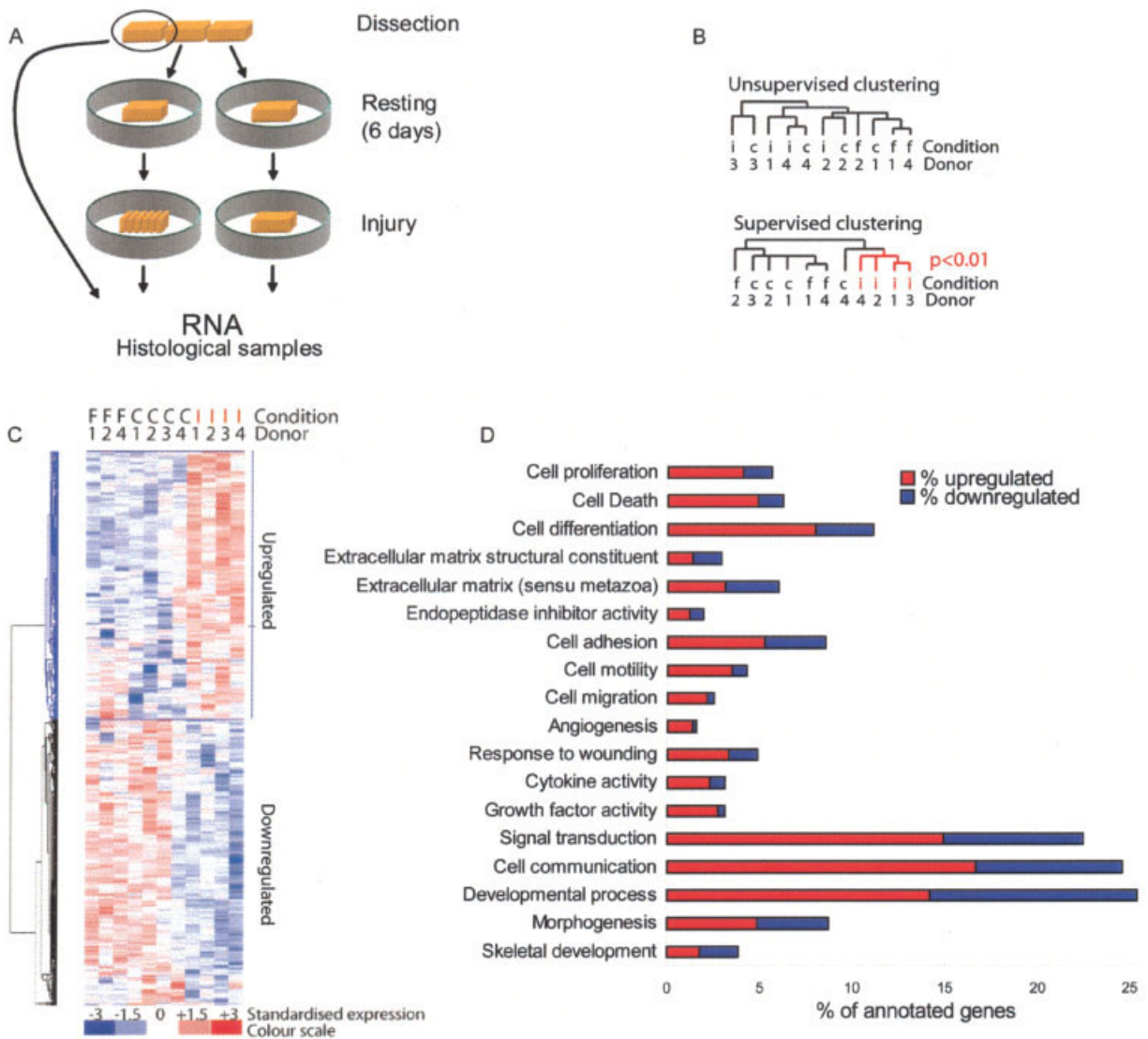


Figure 1. Genome-wide differential expression screening for genes modulated by mechanical injury to adult human articular cartilage. **A**, Ex vivo model of mechanical injury to adult human articular cartilage, as described in Materials and Methods. **B**, Dendrogram resulting from unsupervised clustering and supervised clustering of freshly dissected (f), control (c), and injured (i) samples from 4 different donors (donors 1–4). Injured samples clustered significantly in supervised clustering analysis. **C**, Hierarchical clustering of genes up-regulated or down-regulated ≥ 2 -fold 24 hours after injury to adult human articular cartilage explants in vitro. Red indicates expression levels higher than the mean of all samples, white indicates mean expression, and blue indicates expression levels lower than the mean. **D**, Representative clusters of genes differentially expressed following injury to adult human articular cartilage.

ciently understood; however, the results of longitudinal clinical studies indicate that, in young individuals, acute full-thickness joint surface defects exhibit the potential for intrinsic repair (8,9). In 2 independent, long-term, prospective studies, acute traumatic chondral lesions in young athletes had a good to excellent clinical outcome in 78% of cases in the absence of specific surgical

treatments (8,9). Likewise, spontaneous repair of relatively small experimental full-thickness joint surface defects in animal models has been reported (10).

Given this evidence, and the presence of progenitor cells (11–15) in adult joints, we hypothesized that injured articular cartilage may elicit a molecular response to injury, which together with other factors,

including comorbidity, age, and body weight, may lead either to healing or to the development of posttraumatic OA. We recently reported that mechanical injury to adult human articular cartilage in vitro resulted in up-regulation of bone morphogenetic protein 2 (BMP-2), down-regulation of messenger RNA (mRNA) for the Wnt inhibitor FRZB, and in the activation of the respective downstream signaling pathways (16). Allelic polymorphisms of FRZB (17) and BMP-2 (18) are associated with OA, thereby suggesting a functional role of the respective signaling pathways in adult joint homeostasis. In addition, previous studies have demonstrated the release of signaling molecules, including fibroblast growth factor 2 (FGF-2) (19) and BMP-2 (20), from injured adult articular cartilage. Taken together, these data support the hypothesis that injured articular cartilage can be a source of signaling molecules and that the response to injury might participate in determining whether the outcome of a joint surface defect is repair or posttraumatic OA.

Capitalizing on our preliminary findings (16), in this study we used a validated *ex vivo* cartilage injury model (16,19) and microarray analysis of gene expression to investigate the early molecular response of adult articular cartilage to acute mechanical injury at the whole genome level. We report the regulation of signaling molecules and related pathways. Notably, we identified a striking regulation of Wnt-16 after injury and in OA. We therefore focused our analysis on the Wnt signaling pathway, which is well known to play a role in the homeostasis of skeletal tissue.

MATERIALS AND METHODS

Ex vivo cartilage injury model and tissue culture.

Well-preserved cartilage samples (Mankin score <5) (21) were obtained from a total of 8 patients (5 men and 3 women, with a mean \pm SD age of 68 ± 7 years). Seven patients had undergone total knee replacement for unicompartmental OA (e.g., lateral condyle in genu varum), and 1 patient (a 53-year-old man) had undergone limb amputation because of a traffic accident and did not have OA. Our model of cartilage injury (16) is summarized in Figure 1A. One rectangular cartilage explant was dissected and divided into 3 equal parts of 6×6 mm each. One part was immediately snap-frozen in liquid nitrogen for total RNA extraction (within 30 seconds after dissection; preserving 1 full-thickness slice for histologic analysis as described below). The remaining 2 explants were maintained in culture as described previously (16). After 6 days, the medium was replaced, and 1 of each pair of adjacent samples was cut at 1-mm intervals using a scalpel. The other explant of each pair was left uninjured (Figure 1A). After 24 hours, the explants were used for RNA extraction, and 1 aliquot was processed for histologic and immunohistochemical

analyses. The peripheral part (1 mm) of each explant was discarded to eliminate the portion of cartilage that was injured during dissection before the culture. Explants from the 4 donors with the lowest and most homogenous Mankin scores across the explants and the best RNA quality were selected for microarray analysis of gene expression. Donor 1 (a 66-year-old man), donor 3 (a 67-year-old woman), and donor 4 (a 73-year-old man) underwent total knee replacement for OA, and donor 2 (a 53-year-old man) underwent knee amputation and did not have OA. Donors 1, 2, and 4 all had Mankin scores of 0, and donor 3 had a Mankin score of 1. Not enough well-preserved cartilage from donor 3 was available, and therefore, no freshly dissected explant was included for this donor.

Total RNA extraction. Each frozen explant was pulverized using a mortar and pestle prechilled in liquid nitrogen, suspended in 4 ml of TRIzol reagent (Invitrogen Life Technologies, Paisley, UK), and homogenized using a Polytron homogenizer. The suspension was incubated on ice for 20 minutes and spun at 10,000g for 10 minutes to eliminate debris. Chloroform (800 μ l) was added. The tubes were shaken for 30 seconds, incubated on ice for 2 minutes, and centrifuged at 10,000g for 15 minutes. The aqueous phase was moved to a clean tube. An equal volume of phenol solution saturated with 0.1M citrate buffer (pH 4.3) (Sigma-Aldrich, Gillingham, UK) was added to each tube, mixed thoroughly, and incubated on ice for 30 minutes. Chloroform-isoamyl alcohol 24:1 (2 ml) was added to each tube, mixed thoroughly, incubated on ice for 2 minutes, and centrifuged at 10,000g for 15 minutes. The aqueous phase was then moved to a clean tube. Extraction with phenol and separation with chloroform-isoamyl alcohol were repeated, as described above, 3 times. The aqueous phase obtained after the third separation was collected, and RNA was precipitated with isopropanol (1 hour at 14,000g at 4°C), washed with 70% ethanol in water, dried, and resuspended in water according to standard protocols. RNA yield was assessed by spectrophotometry using a NanoDrop spectrophotometer (Labtech, Ringmer, UK). RNA quality was assessed by MOPS-formaldehyde-EDTA 1.4% agarose gel electrophoresis or, routinely, in 1% Tris-borate-EDTA agarose gel electrophoresis (see Supplementary Figure 1, available on the *Arthritis & Rheumatism* Web site at <http://www.mrw.interscience.wiley.com/suppmat/0004-3591/suppmat/>).

Histologic analysis and immunostaining. To determine the Mankin score (21), a full-thickness slice of each explant was fixed in buffered 4% paraformaldehyde (PFA), embedded in paraffin, sectioned, and stained with hematoxylin and eosin or Safranin O according to standard protocols. Each sample was scored independently by 2 investigators who were blinded with regard to the origin of the sample. The part of the score that referred to the tidemark was omitted. Immunostaining for β -catenin was performed as previously described (16). Immunostaining for Wnt-16 and β -catenin was performed as follows. Paraffin sections were deparaffinized and rehydrated, postfixed with 4% buffered PFA, and then washed in phosphate buffered saline. Sections were then equilibrated in 0.02% HCl for 15 minutes, digested in 0.60 mg/ml of pepsin (Sigma-Aldrich) in 0.02% HCl for 45 minutes at 37°C, washed in water, and allowed to air dry for 20 minutes. The sections were postfixed in 4% PFA for 10 minutes at room temperature, washed twice with Tris buffered saline-0.05% Tween 20

(TBST), and incubated with 10% H₂O₂ for 15 minutes. The sections were blocked in blocking solution (Dako, Ely, UK) for 1 hour at room temperature, blotted, and incubated overnight at 4°C with a mouse anti-human Wnt-16 monoclonal antibody (BD PharMingen, Oxford, UK) diluted 1:200 or β -catenin (BD Transduction Laboratories, Oxford, UK) at a final concentration of 1 μ g/ml. Sections were then washed twice in TBST, and incubated for 1 hour with biotinylated rabbit anti-mouse Ig (Dako) diluted 1:200. After washing twice in TBST, sections were incubated for 30 minutes with StreptABComplex/HRP (Dako). Liquid DAB Substrate Chromogen System (Dako) was used as peroxidase substrate. As negative controls, we used isotype-matched nonimmune Ig instead of the primary antibody, and for Wnt-16, we confirmed specificity by preabsorbing primary antibody with a blocking peptide (Wnt-16-transfected cell lysate), according to standard protocols.

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR and quantitative real-time RT-PCR were performed as described previously (16). Gene expression was calculated using a standard curve and was normalized to the expression of the housekeeping gene β -actin. Primer sequences were as follows: for Wnt-16, forward 5'-AAAGAAATGTTTCCCTGCC-3' and reverse 5'-GAC-ATTTCCATGGGTTTGC-3'; and for β -actin, forward 5'-CACGCTGCTTCCAGCTC-3' and reverse 5'-CACAG-GACTCCATGCCAG-3'.

Microarray analysis. Explants from the 4 donors (3 men and 1 woman, with a mean age of 64.7 years) with the lowest Mankin scores, best RNA quality, and most uniform samples were selected. One of these donors was a patient who underwent limb amputation after a traffic accident and did not have OA. Total RNA was controlled for integrity and purity using an Agilent Bioanalyzer and a NanoDrop spectrophotometer, respectively. All samples were of similar RNA quality with limited signs of degradation. In terms of purity, all RNA absorption ratios at 260:280 nm and 260:230 nm were >1.8. Because of the limited availability of total RNA, 2 rounds of amplification were necessary for generation of the probes. RNA amplification was performed by *in vitro* transcription (IVT) using a 2-cycle complementary DNA synthesis kit, according to the recommendations of the manufacturer (Affymetrix, High Wycombe, UK). Probes were biotin-labeled during the second IVT reaction using the Affymetrix IVT labeling kit. The probes were purified and analyzed again for yield (30–120 μ g) and purity (260:280 nm and 260:230 nm >1.8). Twenty micrograms of the resulting antisense RNA was fragmented with alkaline hydrolysis and resuspended with control spikes in 300 μ l of hybridization buffer (Eukaryotic Hybridization Control Kit; Affymetrix).

Human Genome U133 Plus 2.0 array (Affymetrix) is a single array representing ~47,000 transcripts. The gene array chips were hybridized in a rotisserie oven at 45°C and washed and stained in GeneChip Fluidics Stations 400 and 450 (Affymetrix) using the EukGE-WS2v4 protocol. A GeneChip Scanner 3000 (Affymetrix) was used for scanning, and image analysis was performed with GCOS (Affymetrix). Data preprocessing, including normalization, modeling, and “presence/absence” calls, was determined with MAS5 software (Affymetrix). The target value was set at 100.

Statistical analysis and hierarchical clustering. Gene expression values normalized and modeled with MAS5 were log-transformed. Gene expression in injured versus rested control samples were compared using DNA Chip Analyzer (dChip) software (<http://biosun1.harvard.edu/complab/dchip/>) (22), which uses Student's paired *t*-test. The false discovery rate was estimated after 50 permutations, as previously described (23,24). Clustering analysis was performed using dChip software, according to principles described by Golub et al (23) and Eisen et al (25). The gene list used for unsupervised sample clustering included expression levels from all genes displaying a high variance across the samples to eliminate the “noise” of genes that were neither up- nor down-regulated.

In detail, we filtered genes having a “present” call in $\geq 20\%$ of the samples, with an SD of the log-transformed expression levels of ≥ 0.8 , and with log-transformed expression levels of ≥ 4.32 in $>50\%$ of the samples. Conversely, for supervised clustering of functional families of genes, we used a gene list including all genes regulated ≥ 2 -fold in injured versus uninjured samples ($P \leq 0.05$ by Student's paired *t*-test). For pathway and functional category classification of the differentially expressed genes, we used the annotations publicly available from the National Center for Biotechnology Information LocusLink database (<http://www.ncbi.nlm.nih.gov/LocusLink/>), which classifies a gene according to molecular function, biologic process, and cellular component using Gene Ontology annotations (<http://www.geneontology.org/>). Significant functional clusters within the differentially expressed genes were then identified as described elsewhere (26,27). In Figure 3, the WNT pathway was traced using GenMAPP (available at <http://www.genmapp.org>).

RESULTS

Microarray analysis and unsupervised clustering of the samples. Gene expression in all explants from the 4 selected donors was compared using Affymetrix U133 Plus 2.0 chips, which cover the whole human genome with 54,613 probe sets. Fifty-two percent of all of the probes had a “present” call in $\geq 20\%$ of the samples, including injured, control, and freshly dissected samples. The remaining probes were excluded from further analysis. To investigate how gene expression varied across the samples, we performed unsupervised sample clustering. In this analysis, samples were grouped according to their expression profile based on all genes, whether or not the genes were differentially expressed in the experimental (injured) versus the control (rested, uninjured) group. Unsupervised clustering did not identify significant clusters of samples ($P \leq 0.01$) (Figure 1B), thus ruling out a bias that could affect further analysis.

Analysis of differential gene expression and supervised clustering of the samples. We then compared gene expression in injured versus uninjured explants. Eight hundred thirty redundant probes representing 690 nonredundant transcripts were significantly up-regulated

Table 1. Representative overrepresented gene clusters*

Cluster name and clustering statistics	Representative gene	Accession no.	Fold change	<i>P</i>
Skeletal development (20 genes, <i>P</i> = 0.000000)	Osteoprotegerin	NM_002546	7.8	0.036
	C-type lectin domain family 3, member A	BE872674	-4.2	0.006
Cell death (27 genes, <i>P</i> = 0.0003 in the "up-regulated" gene sublist)	Serine/threonine kinase 17a	NM_004760	2.6	0.012
	TNF (ligand) superfamily, member 13	AF114013	-2.1	0.008
Regulation of cell proliferation (14 genes, <i>P</i> = 0.0008 in the "up-regulated" gene sublist)	CDK inhibitor 2B	AW444761	17.8	0.015
	IGF-1	AI972496	-3.2	0.021
Cell differentiation (41 genes, <i>P</i> = 0.000056 in the "up-regulated gene" sublist)	IGFBP-3	BF340228	6.8	0.008
	Zinc finger and BTB domain-containing 16	AI833064	-3.8	0.010
Extracellular matrix structural constituent (7 genes, <i>P</i> = 0.000000)	Tenascin XB	BE044614	3.6	0.014
	COL4A5	AW052179	-4.9	0.039
Extracellular matrix (16 genes, sensu metazoa; <i>P</i> = 0.000000)	Transglutaminase 2	BC003551	5.8	0.016
	EGF-containing fibulin-like extracellular matrix protein 1	NM_004105	-2.7	0.007
Serine-type endopeptidase inhibitor activity (8 genes, <i>P</i> = 0.0003 in the "up-regulated" gene sublist)	Serpin peptidase inhibitor, clade E	NM_000602	10.0	0.001
	Kallmann syndrome 1 sequence	NM_000216	-2.5	0.000
Cell adhesion (44 genes, <i>P</i> = 0.0002)	$\alpha 5$ integrin	NM_002205	3.5	0.031
	COL16A1	NM_001856	-2.3	0.032
Cell motility (22 genes, <i>P</i> = 0.00026) and cell migration (13 genes, <i>P</i> = 0.000121)	PA, urokinase receptor	AY029180	6.3	0.009
	Myosin regulatory light chain-interacting protein	AW292746	-3.9	0.005
Angiogenesis (7 genes, <i>P</i> = 0.000037 in the "up-regulated" gene sublist)	VEGF	H95344	3.1	0.008
Response to wounding (17 genes, <i>P</i> = 0.000007) and wound healing (<i>P</i> = 0.0001 in the "up-regulated" gene sublist)	Serpin peptidase inhibitor, clade E, member 1	NM_000602	10.1	0.001
	Complement factor H	N92818	-3.5	0.001
Cytokine activity (16 genes, <i>P</i> = 0.000257, and 12 genes, <i>P</i> = 0.000005 in the "up-regulated" gene sublist) and growth factor activity (<i>P</i> = 0.000001 and 0.000000 in the "up-regulated" gene sublist)	β A inhibin	MI3436	4.8	0.030
	TNF (ligand) superfamily, member 13b	AF134715	-2.3	0.022
Signal transduction (78 genes, <i>P</i> = 0.001 in the "up-regulated" gene sublist) and cell communication (87 genes, <i>P</i> = 0.000116 in the "up-regulated" gene sublist)	TGF β 2	NM_003238	3.0	0.023
	KIAA0789 gene product	NM_014653	-4.4	0.004
Developmental process (131 genes, <i>P</i> = 0.000001) and anatomical structure morphogenesis (42 genes, <i>P</i> = 0.000154)	BMP-2	NM_001200	4.0	0.023
	Homeobox A13	BG289306	-4.1	0.003

* Genes that were differentially expressed ≥ 2 -fold in injured versus control explants were used for hierarchical clustering and functional clustering using DNA Chip Analyzer software. Representative up-regulated and down-regulated genes are listed for each cluster. Largely overlapping clusters or subclusters have been grouped. TNF = tumor necrosis factor; CDK = cyclin-dependent kinase; IGF-1 = insulin-like growth factor 1; IGFBP-3 = IGF binding protein 3; EGF = epidermal growth factor; PA = plasminogen activator; VEGF = vascular endothelial growth factor; TGF β 2 = transforming growth factor β 2; BMP-2 = bone morphogenetic protein 2.

or down-regulated (regulated ≥ 2 -fold) ($P \leq 0.05$). The estimated false discovery rate was 8.4% (70 probes) after 50 permutations. Of the 690 probes, 335 were up-regulated and 355 down-regulated.

To test whether dissection of the explants from the femoral condyles and exposure to in vitro culture conditions represented a significant bias in the differential gene expression of injured versus control samples,

we used supervised sample clustering. Supervised clustering groups the samples according to the similarity of their gene expression, limited to genes differentially expressed in the experimental (injured) and control (rested) samples. If the dissection of the samples from the condyles and the exposure to culture conditions represented a significant bias in the comparison, we would expect the freshly dissected samples (snap-frozen

in liquid nitrogen for RNA extraction within 30 seconds after dissection) to cluster separately from the controls (rested for 6 days and left uninjured). Instead, this analysis revealed significant clustering of the injured samples, but no separation between the control (rested) samples and the freshly dissected samples (Figures 1B and C), thereby ruling out this bias.

Clustering of differentially expressed genes. Hierarchical gene clustering algorithms group genes according to the similarity of their expression pattern across samples (23,25). Using these algorithms, dChip software performs clustering of genes sharing a common function, pathway, or gene family, according to gene annotations. Figure 1D shows a selection of statistically significant clusters that, based on the literature, are functionally relevant in the context of cartilage biology and wound healing. Table 1 lists representative genes and statistics for selected clusters. Significant clusters included genes known to be relevant to cell proliferation and cell death (see Supplementary Table 1, available on the *Arthritis & Rheumatism* Web site at <http://www.mrw.interscience.wiley.com/suppmat/0004-3591/suppmat/>); however, there was no indication of whether the overall transcriptional regulation of these genes would support cell death or proliferation at this time point and under these experimental conditions.

Among the regulated matrix molecules, collagens constituted a significant cluster. All 3 chains of type VI collagen were up-regulated, whereas type IV, type XII, and type XVI collagen were down-regulated, in the injured samples. Several genes involved in the synthesis and metabolism of proteoglycans and their posttranscriptional modifications were up-regulated (see Supplementary Table 2, available on the *Arthritis & Rheumatism* Web site at <http://www.mrw.interscience.wiley.com/suppmat/0004-3591/suppmat/>). The regulation of genes involved in the catabolism of extracellular matrix components (listed in Supplementary Table 2) did not form a statistically significant cluster. Although scattered genes involved in inflammatory responses were regulated (listed in Supplementary Table 2), these genes did not represent a statistically significant cluster. A striking finding was a marked overrepresentation and statistically significant clustering of genes encoding signaling molecules or known to be involved in cell communication, morphogenesis, and developmental processes including skeletal development, and genes known to play a role in the response to wounding and wound healing (Figure 1D).

We then investigated whether the identified gene regulation was specific to these experimental conditions

or whether it could also be detected in a pathologic process affecting articular cartilage, such as OA. We cross-referenced our data with published microarray data comparing gene expression of intact versus damaged regions of human OA cartilage (28) or of OA cartilage versus normal cartilage in patients (29) or in a rat model of OA (30). This comparison revealed that ~30% of the genes reported to be differentially regulated in OA cartilage compared with normal or preserved cartilage were also regulated in our data set (Figure 2). Interestingly, the greatest overlap was found in the genes that are differentially regulated in early OA compared with normal cartilage, and the smallest overlaps were seen in the genes that are differentially expressed in severe OA versus normal cartilage and in the genes that are differentially expressed in the rat model of experimental OA (30).

If failure of the response to cartilage injury were a pathogenetic element in the development of OA, we would also expect at least a portion of the genes having polymorphisms associated with OA to be regulated after cartilage injury. To test this hypothesis, we selected from the literature genes reported to have polymorphisms associated with OA. Forty-eight percent of these genes were significantly regulated in our microarray (Table 2).

Regulation of morphogenetic pathways following injury. Interestingly, several signaling pathways involved in skeletal development, including the FGF, transforming growth factor β (TGF β), and Wnt pathways, were regulated in our data set (for a complete list of genes associated with these clusters, see Supplementary Table 3, available on the *Arthritis & Rheumatism* Web site at

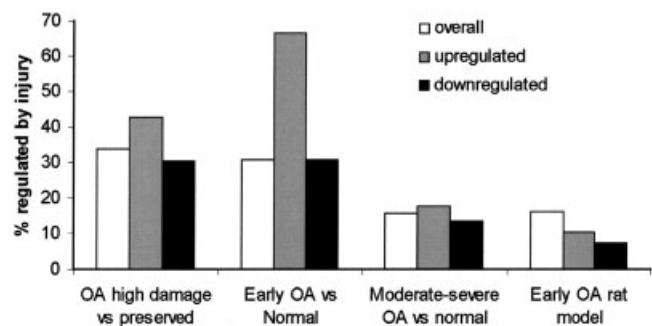


Figure 2. Percentages of genes regulated in osteoarthritic (OA) cartilage that were also regulated by injury in the present study. Genes included were from published data sets from previous studies comparing gene expression in OA cartilage with high levels of damage versus preserved cartilage from the same joint (28), early OA cartilage versus normal cartilage (29), moderate to severe OA cartilage versus normal cartilage (29), and OA cartilage from the destabilized knee versus normal cartilage from the sham-operated knee in a rat model (30).

<http://www.mrw.interscience.wiley.com/suppmat/0004-3591/suppmat/>). We further focused on the Wnt pathway (Figure 3) because we previously reported down-regulation of the Wnt inhibitor FRZB after injury *in vitro* and *in vivo* (16), and loss of function mutations of FRZB are genetically linked to OA (17).

The down-regulation of FRZB mRNA (16) was confirmed. Notably, the ligand Wnt-16, which is known to signal through the β -catenin-dependent pathway (31), was the only Wnt ligand to be statistically significantly regulated at the mRNA level. It was expressed at the lower limits of detection by microarray hybridization in control and freshly dissected cartilage explants and was up-regulated >20 times upon injury. Known transcriptional target genes of the β -catenin-dependent Wnt signaling pathway, such as osteoprotegerin (32), c-Myc (33), cyclin D1 (34), and axin 2 (35), were up-regulated consistently with net activation of canonical Wnt signaling. (For a more comprehensive list of genes in the Wnt

signaling pathway and for details on expression levels and statistics, see Supplementary Table 3.)

To validate the up-regulation of Wnt-16 in a larger number of samples, we used quantitative real-time RT-PCR and immunohistochemistry. In 8 pairs of samples, each from an individual donor, Wnt-16 mRNA was barely detectable in control explants and was reproducibly up-regulated in injured samples 24 hours after injury (Figure 3B). This up-regulation was also confirmed at the protein level by immunohistochemistry analysis in 3 independent pairs of explants (Figure 4A). Wnt-16 protein was undetectable in control explants and was intensely stained in the injured explants. Wnt-16 was also undetectable or at the lower limit of detection at the mRNA and protein levels in 3 cartilage samples from preserved areas of joints with OA, but was dramatically up-regulated in samples from areas of the same joints with moderate to severe OA, with a territorial and interterritorial expression pattern (Figure 4), confirming

Table 2. Regulation of genes with allelic polymorphisms associated with osteoarthritis*

Gene symbol	Gene	Fold change	<i>P</i>	Ref.
ANKH	Ankylosis, progressive homolog	2.7	0.000	18
BMP2	Bone morphogenetic protein 2	4.6	0.023	18
CILP	Cartilage intermediate-layer protein/nucleotide pyrophosphohydrolase	-1.5	0.019	47
CIRBP	Cold-inducible RNA binding protein	-1.4	0.014	47
DUSP1	Dual specificity phosphatase 1	1.3	0.312	47
EIF1	Eukaryotic translation initiation factor 1	-1.8	0.166	47
EIF4A1	Eukaryotic translation initiation factor 4A, isoform 1	1.6	0.005	18
ER1	Estrogen receptor 1	-3.1	0.004	17
FRP	Frizzled-related protein	-2.5	0.031	18
GRK6	G protein-coupled receptor kinase 6	4.1	0.155	47
H3F3B	H3 histone, family 3B (H3.3B)	1.4	0.011	47
HIST2H2AA	Histone 2, H2AA	-1.4	0.035	48
IL1R1	Interleukin-1 receptor type I	-1.1	0.308	49
LRCH1	Leucine-rich repeats and calponin homology domain-containing 1	1.9	0.336	18
NCOR2	Nuclear receptor corepressor 2	1.0	0.963	47
RHOB	Ras homolog gene family, member B	1.1	0.715	47
S100B	S100 calcium binding protein, β (neural)	-1.3	0.075	18
SERPINA3	Serpin peptidase inhibitor, clade A (α -1 antiproteinase, antitrypsin), member 3	1.1	0.804	47
TM2D3	TM2 domain-containing 3	-1.5	0.002	18
TNFRSF11B	Osteoprotegerin	12.2	0.036	50
ASPEN	Asporin	-2.1	0.044	18
ADAM12	ADAM metallopeptidase domain 12 (meltrin α)	3.5	0.060	18
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	15.4	0.072	18
CLEC3B	C-type lectin domain family 3, member B	-1.4	0.219	18
VDR	Vitamin D (1,25-dihydroxyvitamin D ₃) receptor	-2.1	0.132	

* Of the 25 selected genes found in previous studies to have allelic polymorphisms associated with osteoarthritis, 12 (48%) were significantly regulated, regardless of the fold change of the average of the means, in the injury model used in the present study. Five genes (20%) were up-regulated, and 7 (28%) were down-regulated.

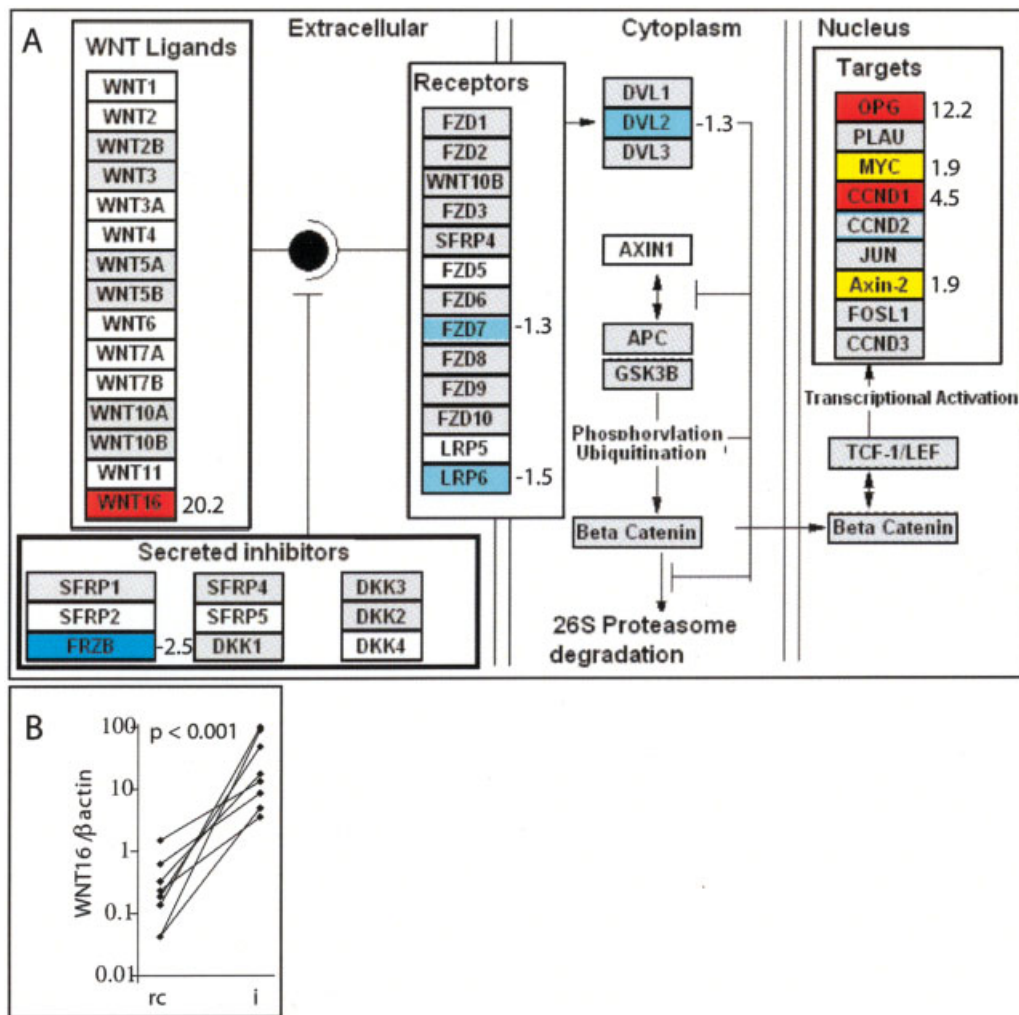


Figure 3. Modulation of Wnt signaling after cartilage injury. **A**, Gene regulation within the canonical Wnt pathway. The Wnt pathway was drawn using Gene Map Annotator and Pathway Profiler (<http://www.genmapp.org>), and the microarray expression data set was used to analyze differential gene expression in injured versus control explants. White boxes indicate genes detectable in $<20\%$ of all samples. Gray boxes indicate genes that were detected in $\geq 20\%$ of all samples but with no significant difference between injured and control samples. Red and yellow boxes indicate genes that were significantly ($P \leq 0.05$) up-regulated in the injured samples ≥ 2 -fold or 1–2-fold, respectively. Dark blue and light blue boxes indicate genes that were significantly down-regulated in the injured explants ≥ 2 -fold or 1–2-fold, respectively. The numbers to the right of the boxes indicate the average fold change over the paired control. sFRP-4 = secreted Frizzled receptor protein 4; LRP-5 = low-density lipoprotein receptor–related protein 5; APC = antigen-presenting cell; GSK-3 β = glycogen synthase kinase 3 β ; OPG = osteoprotegerin; TCF-1 = T cell factor 1; LEF = lymphoid enhancer factor. **B**, Quantitative real-time reverse transcriptase–polymerase chain reaction for Wnt-16 mRNA in 8 independent pairs of rested control (rc) and injured (i) adult human articular cartilage explants. Results were normalized to the housekeeping gene β -actin. The P value is for the comparison of the rested control versus the injured explants. Color figure can be viewed in the online issue, which is available at <http://www.arthritisrheum.org>.

that Wnt-16 up-regulation also takes place in vivo in cartilage pathology.

Accumulation and nuclear localization of β -catenin is an indication of activation of the canonical Wnt pathway (36). In the rested control samples, most

cells stained negative for β -catenin, and 46% stained only weakly positive, with a prevalently cytoplasmic pattern. In the injured samples, 86% of all cells stained strongly positive with striking nuclear staining (Figure 4). In the preserved cartilage areas of OA joints,

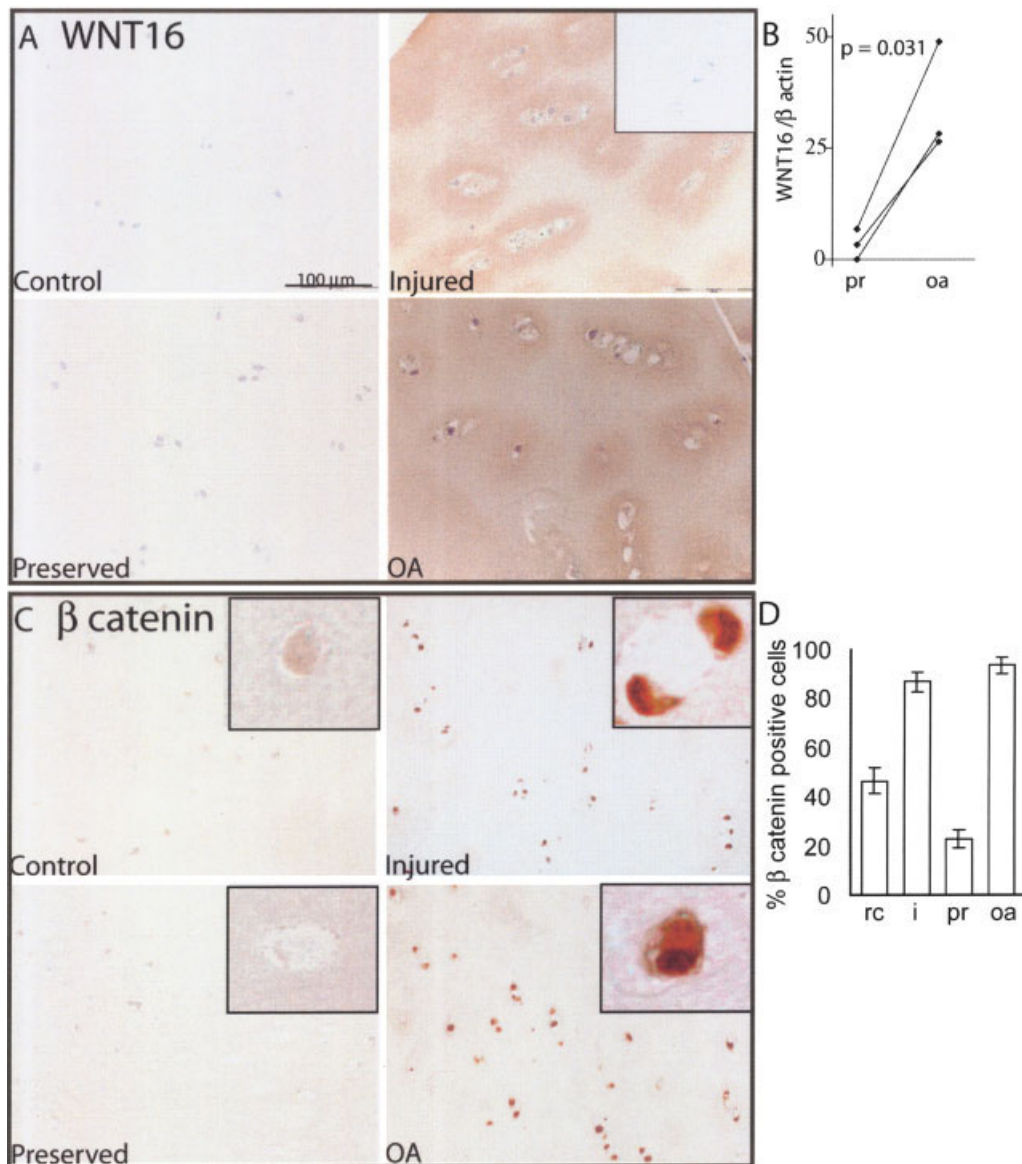


Figure 4. Wnt signaling in injury and osteoarthritis (OA). **A** and **C**, Immunohistochemical analysis of Wnt-16 (**A**) and β -catenin (**C**) in a rested control explant, an injured explant, a freshly dissected cartilage sample from a preserved joint area, and a cartilage sample from a damaged area of a knee with OA. **Inset** in **A** shows the negative control for immunostaining (primary antibody preabsorbed with Wnt-16). **Insets** in **C** are higher-magnification views. **B**, Quantitative real-time reverse transcriptase-polymerase chain reaction for Wnt-16 mRNA in preserved (pr) cartilage areas or areas with severe OA from 3 independent joints. Results were normalized to the housekeeping gene β -actin. The *P* value is for the comparison of the preserved area and the osteoarthritic area. **D**, Relative quantitation of β -catenin-positive cells in rested controls (rc), injured samples (i), freshly dissected samples from preserved joint areas, and samples from areas with severe OA. Positive cells were counted, regardless of the intensity of the staining, which was very faint in the control and preserved samples, and regardless of the nuclear/cytoplasmic pattern, which was strikingly nuclear in the injured and OA samples. Bars show the mean \pm SD. Color figure can be viewed in the online issue, which is available at <http://www.arthritisrheum.org>.

β -catenin was weakly detectable in 23% of the cells and undetectable in most cells. In severely damaged areas of the same joints, 94% of the cells showed strong nuclear

and cytoplasmic staining (Figure 4). These data suggest activation of the canonical Wnt pathway associated with injury in vitro and OA lesions in vivo.

DISCUSSION

The outcome of traumatic joint surface injuries is determined by the persistence of injurious factors, patient-related factors, inflammation, and the efficiency of the repair mechanisms. Our knowledge of the molecular mechanisms set into action by the joint surface in response to injury is limited. Yet the response to injury is of interest in rheumatology because its components may regulate the pathologic events leading to posttraumatic OA or the reparative events, or both. Indeed, injury to the articular cartilage results in up-regulation of “catabolic” molecules such as interleukin-1 (37) and metalloproteinases (38), as well as “anabolic” genes such as BMP-2 (16,20) and FGF-2 (19).

In this study, we used microarray analysis to characterize the early response of adult articular cartilage to injury. This analysis revealed regulation of genes encoding signaling molecules, including members of the Wnt, TGF β , and FGF families of morphogens. Wnt-16 was the only Wnt ligand to be regulated in our assay. Wnt-16 up-regulation was associated with down-regulation of the Wnt inhibitor FRZB, nuclear accumulation of β -catenin, and up-regulation of several Wnt target genes. We showed that similar events also take place in OA cartilage, thereby confirming that such molecular regulation is not limited to our *in vitro* model of acute injury, but also occurs *in vivo* in OA.

In our system, the duration of the “resting” period allowed for the response to dissection to subside before reinjury limited the genes that could be detected to those that are regulated transiently (details available upon request from the corresponding author). This could be one reason for the poor representation of some genes expected to be regulated by injury, such as those associated with cell proliferation (39) or matrix remodeling (38), as was the case for ADAMTS-5 and matrix metalloproteinase 13 (MMP-13). Regulation of genes associated with events that are spatially restricted, such as cell death (39), may be either undetectable or difficult to interpret, since microarray data represent the average of all gene expression within the entire explant.

Genes associated with cell signaling, response to injury, and wound healing were represented in statistically significant clusters. These molecules included members of the Wnt, FGF, and TGF β families. Our experimental setup did not allow for an investigation of the function of the regulation of such pleiotropic molecules *in vivo* in the context of joint surface repair or the development of OA. However, since regeneration often recapitulates organ morphogenesis (40), one could spec-

ulate that such genes, which are known to be involved in embryonic skeletal morphogenesis, may play a role in the activation of repair and in the morphogenesis of repair tissue.

We previously reported that activation of the canonical Wnt pathway using LiCl treatment of cartilage explants resulted in up-regulation of aggrecan and COL2A1 mRNA (16), suggesting an anabolic response in that model. However, Wnt proteins are pleiotropic signaling molecules, and their function is highly context dependent. For instance, activation of canonical Wnt signaling suppresses chondrogenesis in some systems (41), but promotes it under other experimental conditions (42,43). Given the lack of *in vitro* models of cartilage regeneration, the function of Wnt-16 in this context needs to be investigated *in vivo*, where adult articular cartilage interacts with other tissue within the joint, in suitable animal models of joint surface injury and OA. In an elegant study, Diarra et al (44) recently showed that modulation of Wnt signaling changes the rheumatoid arthritis-like erosive outcome in a murine model of inflammatory arthritis into a nonerosive OA-like outcome with osteophyte formation, thereby stressing the role of this signaling pathway in the remodeling of adult skeletal tissues.

Tight modulation of the Wnt signaling pathway is required for regeneration processes in regenerating organisms (45) and in wound healing in mammals (46). Therefore, timely and controlled activation of the canonical Wnt pathway following injury may represent one mechanism of activating a repair response, but deregulation of this activation may be ineffective or may even contribute to further joint damage. It is also possible that molecular events triggered by mechanical injury, including activation of the canonical Wnt pathway, may play a role in cartilage breakdown and in the development of posttraumatic OA, as suggested by some previous studies (17,44). Testing these hypotheses in suitable animal models of joint surface injury and repair is currently a major effort in our laboratory.

The finding that Wnt-16, which is known to play a redundant role in mouse embryonic joint morphogenesis with Wnt-4 and Wnt-9A (31), is the only Wnt ligand regulated in this experimental system raises the important biologic issue of the specificity and/or redundancy of the different Wnt ligands. The identification of the interaction and hierarchy of these signaling molecules in the context of cartilage homeostasis also needs to be investigated in order to optimally target the respective pathways to promote repair or to stop further joint

surface breakdown, thereby preventing the development of posttraumatic OA.

AUTHOR CONTRIBUTIONS

Dr. Dell'Accio 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 design. Dell'Accio, De Bari, Vanhummelen, Pitzalis.

Acquisition of data. Dell'Accio, Eltawil, Vanhummelen.

Analysis and interpretation of data. Dell'Accio, De Bari, Vanhummelen, Pitzalis.

Manuscript preparation. Dell'Accio, De Bari, Vanhummelen, Pitzalis.

Statistical analysis. Dell'Accio, Eltawil.

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