Intraarticular and Systemic Inflammatory Profiles May Identify Patients with Osteoarthritis

Bryan J. Heard, Marvin J. Fritzler, J. Preston Wiley, Jenelle McAllister, Liam Martin, Hani El-Gabalawy, David A. Hart, Cyril B. Frank, and Roman Krawetz

ABSTRACT. ***Objective.*** To determine whether cytokine/chemokine profiles from synovial fluid and sera discriminate mild/moderate osteoarthritis (OA) from normal and severe OA cohorts.

Methods. Multiplex technology was used to quantify expression levels for 42 cytokines in the synovial fluid of patients diagnosed with severe OA (n = 20) and mild/moderate OA (n = 12), as well as normal controls (n = 34). The same 42 cytokines were examined in serum samples of patients with severe OA (n = 26) and mild/moderate OA (n = 74) and normal individuals (n = 100). Treatment group comparisons followed by principal component analysis (PCA) and K-means clustering of the significantly different cytokines/chemokines revealed groupings of patients by physician diagnosis.

Results. Differences in cytokine/chemokine levels were found between control, mild/moderate OA, and severe OA synovial fluid samples, as well as between normal and mild/moderate OA serum samples, and between control and severe OA serum samples. No differences were observed between mild/moderate and severe OA serum samples. Visual groupings based on PCA were validated by K-means analysis, with the best results obtained from the comparison of normal and mild/moderate OA serum samples with 96% of normal and 93% of mild/moderate OA samples accurately identified.

Conclusion. Our study suggests that comparing the expression levels of cytokines/chemokines in synovial fluid and/or serum of patients with OA may have promise as a diagnostic platform to identify patients early in their disease course. This high-throughput low-cost assay may be able to provide clinicians with a diagnostic test to complement existing clinical and imaging modalities currently used to diagnose OA. (J Rheumatol First Release May 15 2013; doi:10.3899/jrheum.121204)

Key Indexing Terms:

CYTOKINES	OSTEOARTHRITIS	INFLAMMATION
LUMINEX	SYNOVIAL FLUID	SERUM

Osteoarthritis (OA) is emerging as the most common disease in the elderly as the global population ages. Following current trends, it has been suggested that OA will become the fourth leading cause of global disability by the year 2020[1,2]. OA is thought to be the result of a destabilization of the balance between synthesis and degradation of intraarticular cartilage, although the exact cause or mechanism remains to be determined. OA is a multifactorial disease that is not limited to destruction of cartilage surfaces of the joint. Indeed, all other joint tissues (synovium, bone, ligament, capsule, muscle) can also be affected by the disease state[3,4,5]. Most commonly, however, OA is associated with erosion of the cartilage surface of the joint, which presents as pain and loss of mobility, and the majority of research on OA has focused on cartilage and the factors that may regulate its destruction[6,7,8,9,10].

Many proteolytic enzymes, including several of the matrix metalloproteinases (MMP), have the ability to degrade the articular cartilage collagen scaffold[11,12,13] by recognizing specific sequences in extracellular matrix (ECM) proteins and then cleaving these proteins. A number of studies have used structural fragments of cartilage proteins as potential biomarkers of OA. Procollagen II C-propeptide upregulation, a precursor of collagen (COL) II that appears as a repair response to damaged cartilage, has been localized to the articular cartilage of patients diagnosed with early OA[14]. Studies have shown this upregulation is

From the McCaig Institute for Bone and Joint Health, the Department of Anatomy and Cell Biology, the Department of Surgery, the Department of Medicine, and the Sports Medicine Centre, Faculty of Kinesiology, University of Calgary, Calgary, Alberta; and the Arthritis Centre, University of Manitoba, Winnipeg, Manitoba, Canada.

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B.J. Heard, MSc; M.J. Fritzler, MD, PhD, McCaig Institute for Bone and Joint Health, Department of Medicine, University of Calgary; J.P. Wiley, MD; J. McAllister, MSc, McCaig Institute for Bone and Joint Health, Sports Medicine Centre, Faculty of Kinesiology; L. Martin, MD, McCaig Institute for Bone and Joint Health, Department of Medicine, University of Calgary; H. El-Gabalawy, MD, Arthritis Centre, University of Manitoba; D.A. Hart, PhD; C.B. Frank, MD, McCaig Institute for Bone and Joint Health, Department of Surgery, University of Calgary; R. Krawetz, PhD, McCaig Institute for Bone and Joint Health, Department of Surgery, Department of Anatomy and Cell Biology, University of Calgary.

Address correspondence to Dr. R. Krawetz, McCaig Institute for Bone and Joint Health, Faculty of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta T2N 4N1, Canada. E-mail: rkrawetz@ucalgary.ca

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detectable in patient synovial fluid and serum, highlighting it as a potential biomarker for early OA[5]. Conversely, proteolytic COL II breakdown is a key component in cartilage erosions observed in arthritic diseases. Fragments of COL II, or COL II epitopes, have been highlighted as potential OA biomarkers, generally all falling into one of 3 potential categories based on their origin from the native COL II molecule: cleavage neoepitopes, denaturation epitopes, or epitopes from the mature end of the molecule[15]. To date, however, no COL II/proCOL II early OA diagnostic assays have entered the clinical diagnostic market due to the variability of COL II epitopes within normal and arthritic patient populations. Other historical biomarkers such as cartilage oligomeric protein[16] and hyaluronan[17,18] have also been identified as early biomarkers, but have not enjoyed wide clinical adoption. One of the key limitations is the use of a single biomarker for a diagnostic assay, because baseline values can vary dramatically between individuals, and this approach can only be definitive in diseases where the product is completely differentially regulated (i.e., present vs absent) between normal and disease states, which has not been the case in OA.

Relevant to these approaches in biomarker development, several MMP have been implicated in arthritis, and in a number of gene expression studies messenger RNA levels of MMP have been shown to be regulated by inflammatory cytokines[19,20,21]. However, we recently published a study that suggests MMP expression levels are not sufficient to differentiate synovial fluid from normal individuals and from those with "early" OA[22].

Multiple cytokines have been demonstrated to inhibit ECM production by chondrocytes, thereby affecting both the synthesis and degradation of the cartilage. In patients with rheumatoid arthritis (RA), a distinct role for cytokines in the destructive pathway has been extensively characterized[23,24], but in OA the cytokines involved and their modes of action are less clear, although it was reported that proinflammatory cytokines are indeed expressed in OA synovial membranes and synovial fluids[25].

Cytokines are released from cells in response to specific signals and influence target cells by regulating (positively or negatively) gene expression. In general, most cytokines have a relatively short active lifetime and therefore interact with neighboring cells or a population of cells in a microenvironment, in both an autocrine and/or paracrine pathways. Networks of cytokine signaling are complex, with some cytokines being able to amplify their own message and inhibit the expression of competing pathways. In some cases, cells express soluble decoy receptors or receptor antagonists to specific cytokines [e.g., interleukin 1 (IL-1), IL-1 receptor antagonist protein] to attenuate these amplification cascades[26,27]. With specific relevance to OA and the balance between matrix synthesis and destruction, it is thought that proinflammatory cytokines such as IL-1α/β, and tumor necrosis factor-α (TNF-α) from the synovium induce the expression of MMP to remodel the cartilage surfaces, and that other growth factors (such as transforming growth factor-β) stimulate chondrocytes to produce matrix[28,29,30]. However, if a dramatic increase in the expression of cytokines occurs, synthesis is inhibited, leading to deregulation of this process, resulting in cartilage destruction within the joint microenvironment[29].

The field of biomarker research in OA has greatly expanded over the last decade. However, few, if any, definitive early markers of OA have been validated. We believe that using a multifactorial approach may shed light on potential diagnostic/prognostic biomarkers and possible underlying disease mechanisms. Multiplexing technology has been widely adopted for screening potential biomarkers in serum for many diseases[31] and is starting to be used in cell-based assays as well[32]. This high-throughput, low-volume (roughly 50–100 μl) platform facilitates evaluation of a large number of potential candidate molecules in OA joints and sera. Such analyses have the potential to identify a multicomponent phenotype that best discriminates between "normal" and OA patients. In this study we identified 5 candidate OA biomarkers in synovial fluids and 14 in serum.

MATERIALS AND METHODS

Normal group; synovial fluid. Inclusion criteria for control cadaveric donations [collected from the Southern Alberta Organ and Tissue Donation Program (SAOTDP)] were age ≥ 40 years, no history of arthritis, joint injury or surgery (including visual inspection of the cartilage surfaces during recovery), no prescription antiinflammatory medications, no comorbidities (such as diabetes/cancer), and synovial fluid availability within 4 h of death.

Serum. All controls were specifically questioned about a personal or family history of arthritis or autoimmune diseases, and were excluded if they had a personal or family history of RA, systemic lupus erythematosus, scleroderma, polymyositis, vasculitis, spondyloarthropathies, inflammatory bowel disease, diabetes mellitus type I, and/or thyroid disease.

Mild/moderate OA group; synovial fluid. Inclusion criteria included a diagnosis of mild/moderate OA (preradiographic, Outerbridge score < 2: no evidence of cartilage loss or exposed bone on any joint surface) based on arthroscopic examination performed by a certified orthopedic surgeon at the University of Calgary.

Serum. Inclusion criteria required a diagnosis of mild/moderate OA performed by a sports medicine physician at the University of Calgary based on clinical symptoms for ≥ 3 months with radiographic evidence of changes associated with OA.

Severe OA group; synovial fluid. Inclusion criteria included age ≥ 40 years; OA diagnosed by a rheumatologist at the University of Calgary based on the American College of Rheumatology criteria[33,34] including radiographic documentation; and no evidence of autoimmune disease or RA.

Serum. Inclusion criteria required radiographic evidence of OA of any compartment of the knee with collapsed or near-collapsed joint space of any compartment of the knee.

Our study protocol was approved by the University of Calgary Human Research Ethics Board and the Research Ethics Board of the University of Manitoba. All participants provided written consent to participate.

Sample handling. Synovial fluid from control cadaveric individuals was

obtained from the SAOTDP. Synovial fluid from patients with severe OA was aspirated from the knee joint by the attending rheumatologist using a conventional sterile technique; early OA synovial fluid samples were obtained by an orthopedic surgeon during arthroscopic surgery. All synovial fluid samples were collected without the use of lavage or any other diluting agent. Native synovial fluid samples were divided into aliquots, centrifuged at 3000 g for 15 min at 4°C, and stored in cryogenic vials at –80°C. For standardization of the protocol, all synovial fluid samples were subjected to only 1 freeze-thaw event prior to assessment.

Serum samples. Serum was collected by standard venipuncture with vacuum tubes from normal individuals at the University of Manitoba and from OA patients (mild/moderate and severe) at the University of Calgary. They were stored at –80°C until required for analysis.

Luminex multiplex array. Synovial fluid and serum aliquots were thawed on ice and 20 μl of fluid was diluted with Milliplex running buffer (Millipore). Sample analysis was performed (Eve Technologies) using the Milliplex MAP Human Cytokine/Chemokine Panel (Millipore) on the Luminex 100 platform (Luminex Corp.), according to the manufacturer's instructions and a previously published method[35]. All samples were assayed at least in duplicate and prepared standards were included in all runs.

Briefly, cytokine/chemokine-specific antibodies were precoated onto color-coded microparticles and dispensed into microtiter plates by the manufacturer. The standards and test samples were added to the microtiter wells and after standard incubation any unbound substances were washed away by vacuum filtration in an apparatus provided by the manufacturer. This was followed by the addition of biotinylated antibodies specific to the protein of interest to each well, and after standard incubation protocol any unbound biotinylated antibody was removed by vacuum washes as before. Streptavidin-PE antibodies were then added to each well, followed by a final wash to remove unbound streptavidin-PE. The microparticles were resuspended in Milliplex buffer and read using the Luminex 100 analyzer.

Statistical analysis. Statistical analysis was carried out with Stata 9.2 for Macintosh and Prism GraphPad 5 for Macintosh. Synovial fluid and serum samples were investigated separately to determine which had the greatest diagnostic value. Statistical analyses were completed in 4 steps: (1) each cytokine was examined in patient-group comparisons to acquire a panel of significant cytokines (potential biomarkers) within the synovial fluid and the serum; (2) principal components analysis (PCA) was applied to each panel of significant cytokines (synovial fluid and serum) to reduce the number of variables that define each patient from many to just 2 (the 2 principal components), the contributions of each cytokine to these principal components were reported; (3) patient-group comparisons were made using the principal components to determine whether the collective expression of significantly expressed cytokines was enough to statistically differentiate between patient groups; and (4) using K-means clustering, each patient was then investigated individually, as defined by their principal components, and the accuracy of our diagnostic test was determined.

Step 1: Synovial fluid cytokine expression treatment-group comparisons for normal (n = 34), mild/moderate OA (n = 12), and OA (n = 17), as well as the serum cytokine expression treatment-group comparison of normal (n = 100) and mild/moderate OA (n = 74) and severe OA (n = 26), were made using the Kruskal-Wallis test with Dunn's multiple comparisons test (significance accepted at $p \leq 0.05$). Step 2: Synovial fluid and serum PCA were performed using each specific panel of significantly expressed cytokines for each comparison in question and the weights of each of the cytokines on each component were reported. PCA is a data reduction algorithm that produces components that represent ideally weighted values that are calculated from the contributions of all cytokines investigated, while retaining much of the variability of the dataset. Normally in PCA, principal components (PC) are retained if their Eigen value is ≥ 1; however, in our study only the first 2 PC were investigated visually because they represented the majority of the dataset variability. Step 3: The PC generated from the panels of significantly expressed cytokines within synovial fluid and serum were investigated in patient-group comparisons using the Kruskal-Wallis test with Dunn's multiple comparison test (significance accepted at $p \leq 0.05$). Step 4: To mathematically verify the visual groupings of patients returned by PCA and thus determine the accuracy of the diagnostic test, K-means clustering was implemented using the PC responsible for the separation of treatment-group clusters as inputs. Briefly, K–means clustering is an unguided algorithm that separates data into a predetermined number of groups (k). This is accomplished by allocating each sample through an iterative process that calculates the sample's shortest distance between k randomly selected centroids (k = 2 centroids in our study). Distances are calculated from the multivariate data that can be associated with each sample. In our study, each patient sample was represented by its score(s) from the first and/or second PC.

RESULTS

Inflammatory profiles of significantly expressed cytokines within synovial fluid and serum samples from normal knee joints, patients with mild/moderate OA, and patients with severe OA were determined. Significant differences (Table 1A; total levels presented in Table 1B) in cytokine expression levels were observed between the patient-group comparisons within synovial fluid. We then examined serum samples collected from individuals with no history of joint disease/injury and compared these to samples of mild/moderate or severe knee OA. The inflammatory profile observed in the synovial fluid was not fully replicated; however, a unique discriminatory profile was observed (Table 1A, 1B). To determine whether the cytokines/chemokines of significance between normal and mild/moderate OA were valuable as biomarkers of disease, they were further examined by multivariate analysis.

Using the respective panels of significant cytokines/chemokines from patient groups, PCA was performed. When synovial fluid data from normal, mild/moderate OA, and OA cohorts were analyzed, 3 groupings of data were visualized (Figure 1A). However, when the patient cohorts were investigated in the serum only, 2 groups of data were discerned, because the mild/moderate OA and severe OA samples overlapped to represent a single OA group below the normal group (Figure 1B). Because there was no visual separation along the x axis (PC 1), but there was on the y axis (PC 2), only PC 2 was used as an input to K-means clustering for the serum data. The contribution of each cytokine to each PC is shown in Figure 2 (PC 1 and 2 for synovial fluid, and PC 2 for serum). Also shown in Figure 2, there were statistical differences upon investigation of the PC of each patient group within the synovial fluid and the serum. PC 1 of the synovial fluid returned statistical significance between all 3 treatment groups, while PC 2 showed that normal results were significantly different from the 2 OA cohorts. PC 2 of the serum showed that normal samples were significantly different from those of mild/moderate OA and severe OA. However, no statistical differences were found in any comparisons of mild/moderate and severe OA serum samples.

K-means clustering was used to assess the accuracy of

Table 1. A. Patient-group comparisons of cytokines and chemokines within synovial fluid samples and serum samples. Arrows represent significant changes in protein levels between groups. M/M-OA: mild/moderate osteoarthritis; S-OA: severe osteoarthritis. B. Means and standard deviations observed within serum and synovial fluid samples from all patient groups.

A

	Synovial Fluid Results			Serum Results		
Cytokine	Normal vs MM-OA	Normal vs S-OA	MM-OA vs S-OA	Normal vs MM-OA	Normal vs S-OA	MM-OA vs S-OA
EGF	-	-	↑	↓	↓	-
Eotaxin	-	-	↑	↑	↑	-
FGF-2(basic)	↓	-	-	-	-	-
Flt-3 Ligand	↓	↓	-	-	-	-
Fractalkine	-	-	-	↓	↓	-
G-CSF	-	↓	-	↓	↓	-
GM-CSF	↓	-	↑	-	-	-
GRO	-	-	↑	↓	↓	-
IFNalpha2	-	-	-	-	-	-
IFNgamma	-	-	-	-	-	-
IL-1alpha	↓	-	↑	-	-	-
IL-1beta	-	-	↑	↓	↓	-
IL-1ra	-	↑	↑	-	-	-
IL-2	-	-	-	↓	-	-
IL-3	-	-	-	-	-	-
IL-4	-	-	↑	↓	↓	-
IL-5	↑	-	-	↑	-	-
IL-6	-	↑	↑	↑	-	-
IL-7	-	-	-	-	-	-
IL-8	-	-	↑	↑	↑	-
IL-9	↓	-	-	↑	↑	-
IL-10	-	-	↑	↑	↑	-
IL-12(p40)	-	↑	↑	-	-	-
IL-12(p70)	-	↑	↑	-	-	-
IL-13	-	-	↑	-	-	-
IL-15	-	-	-	-	-	-
IL-17	-	-	-	-	-	-
IP-10	-	↑	↑	↑	-	-
MCP-1	-	↑	↑	↑	↑	-
MCP-3	-	↑	↑	↓	↓	-
MDC	↑	↑	-	-	-	-
MIP-1alpha	-	-	↑	-	-	-
MIP-1beta	-	↑	↑	↓	↓	-
PDGF-AA	-	↑	-	-	↑	-
PDGF-AB/BB	-	-	-	↓	↓	-
RANTES	-	-	-	↓	↓	-
CD40L	↓	-	-	↑	↑	-
sIL-2Ralpha	-	-	↑	-	-	-
TGFalpha	-	-	↑	↑	↑	-
TNFalpha	-	-	-	↑	↑	-
TNFbeta	-	-	-	-	-	-
VEGF	-	↑	↑	↑	↑	-

Format: First patient group **vs** Second patient group
↑ = Second is greater than First
↓ = Second is less than First

MM-OA = Mild/Moderate OA
S-OA = Severe OA

B

	Syovial Fluid						Serum					
	Normal		Mild/Moderate		Severe		Normal		Mild/Moderate		Severe	
Protein	Mean	STD (+/-)	Mean	STD (+/-)	Mean	STD (+/-)	Mean	STD (+/-)	Mean	STD (+/-)	Mean	STD (+/-)
egf	13	21	0	0	19	20	167	390	34	46	30	41
eotaxin	19	28	0	0	47	39	64	83	113	76	111	64
fgf2basic	259	415	29	40	40	31	-	-	-	-	-	-
flt3ligand	190	113	93	58	66	38	-	-	-	-	-	-
fractalkine	-	-	-	-	-	-	297	653	192	632	183	406
gcsf	137	226	15	15	66	228	20	42	13	21	11	12
gmcsf	35	132	0	0	12	13	-	-	-	-	-	-
gro	167	186	38	43	762	783	1611	1555	623	317	469	215
il1alpha	10	13	0	0	12	10	-	-	-	-	-	-
il1beta	2	3	0	0	5	6	24	114	11	34	6	14
il1ra	15	12	3	3	177	213	-	-	-	-	-	-
il2	-	-	-	-	-	-	20	103	17	60	8	12
il4	4	6	0	0	10	8	22	88	7	17	8	28
il5	0	0	0	0	0	0	1	9	2	6	1	0
il6	146	213	638	949	4747	6388	5	15	12	22	12	19
il8	37	28	7	6	714	1010	13	35	23	29	28	43
il9	3	3	0	0	3	3	3	24	3	12	1	1
il10	8	14	2	1	58	145	-	-	-	-	-	-
il12p40	29	46	0	0	82	67	-	-	-	-	-	-
il12p70	2	4	0	0	12	13	-	-	-	-	-	-
il13	2	4	0	0	6	6	-	-	-	-	-	-
ip10	1563	1617	572	283	8269	13418	109	102	153	120	137	86
mcp1	328	290	695	210	6462	6482	221	91	469	251	493	245
mcp3	27	22	0	0	125	118	33	44	23	34	17	15
mdc	207	119	1038	809	2953	3628	-	-	-	-	-	-
mip1alpha	31	51	0	0	103	98	-	-	-	-	-	-
mip1beta	23	36	2	3	142	92	141	193	68	121	56	52
pdgfaa	17	57	12	8	48	51	1862	2204	2668	6886	4353	11420
pdghabbb	-	-	-	-	-	-	16050	4523	11062	4889	11280	4928
rantes	-	-	-	-	-	-	1268	1609	768	243	765	215
cd40l	180	343	13	26	55	33	2280	948	6058	5143	4512	3491
sil2ralpha	16	11	5	5	8	8	-	-	-	-	-	-
tgfalpha	4	5	0	0	13	30	10	45	13	26	11	19
tnfalpha	-	-	-	-	-	-	5	20	9	16	6	5
vegf	89	127	56	51	307	264	40	69	99	132	110	155

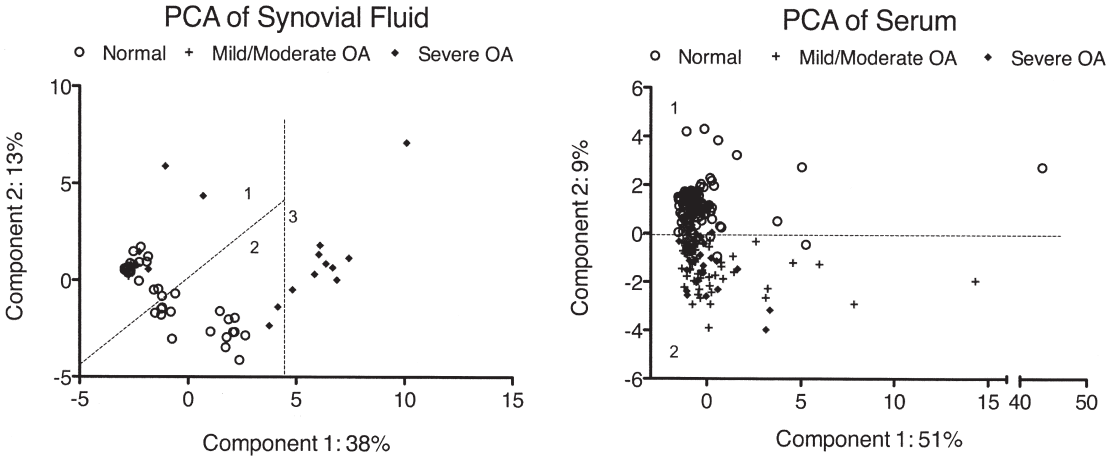

Figure 1. Cytokines and chemokines that displayed significant differences in protein levels between normal, mild/moderate osteoarthritis (OA), and severe OA synovial fluid or serum were analyzed by principal component analysis (PCA). Three distinct groups were observed in the synovial fluid data, and 2 distinct groups were observed within the serum data. In each of these plots the broken lines are intended to separate each group of data. Each group is labeled with a number that corresponds to the K-means clustering groups described in Table 2.

our diagnostic test by mathematically verifying the visual grouping of data returned by each PCA conducted (Table 2). The accuracy of the K-means algorithm has been represented as the percentage of subjects of a patient group who were allocated to each PCA group. The serum provided an interesting finding, as we were able to discriminate between normal and OA phenotypes with significant accuracy. Specifically, 96% of the normal samples, 93% of the mild/moderate OA samples and 82% of the severe OA samples were accurately allocated into the correct cluster. It is also important to note that in the comparison investigating normal, mild/moderate OA, and severe OA, a subset (18%) of severe OA patients presented with serum inflammatory profiles that were comparable to those of normal individuals (Figure 2C), this is consistent with the current literature that suggests inflammation normally subsides once the cartilage surface of the joint has been eroded[36].

DISCUSSION

This study demonstrates that multiplex analysis of synovial fluid and serum components has promise as a diagnostic approach to identify a mild/moderate OA phenotype based on the cytokine/chemokine expression in synovial fluid and serum samples, as well as the potential (with further study) to stratify patients with preradiographic and postradiographic OA based on cytokine/chemokine expression in synovial fluid. The reproducible, high-throughput assay that we describe can be easily standardized and performed, and the projected assay cost is < $100/patient. In addition, this diagnostic approach could complement the existing criteria for OA diagnosis, aid in the identification of patients in the early to mild/moderate stage of the disease, and be useful for inclusion criteria for clinical trials of therapies targeted for early to mild/moderate OA. However, the study as presented lacks a longitudinal component, which would strengthen the diagnostic and prognostic capabilities of this assay. Thus, we are assembling a number of longitudinal patient cohorts that will be followed up at regular intervals. Using these cohorts, we will also examine the effects of interventions (pharmaceutical, mechanical, etc.) on the expression of inflammatory biomarkers. However, our study provides the platform on which we can develop a novel and effective diagnostic for OA.

The contemporary clinical diagnosis of OA is based on symptoms of joint pain and swelling, and radiographic changes consistent with OA. These criteria typically identify patients with only moderate to severe OA, at a time when the disease is relatively advanced and treatment options are limited. It is generally accepted that managing patients in the early stages of OA will deliver the best possible outcomes; however, identifying these early individuals remains complicated. Noninvasive imaging such as magnetic resonance imaging and other novel diagnostic modalities are just now entering the clinical arena, but this approach is currently expensive, requires significant medical expertise, and requires access to the technology. Further, it is currently not known whether imaging at a single timepoint is sufficient to make a definitive diagnosis.

In our study, variations in a number of synovial fluid proteins have been identified [basic fibroblast growth factor (FGF-2), Fit-3 ligand, cluster of differentiation 40 ligand (CD40L), IL-5, and macrophage-derived chemokine (MDC)] as potential discriminators (biomarkers) of early OA; while identified serum biomarkers were epidermal growth factor, growth-related oncogene, methyl-accepting chemotaxis protein (MCP-1 and 3), RANTES, eotaxin,

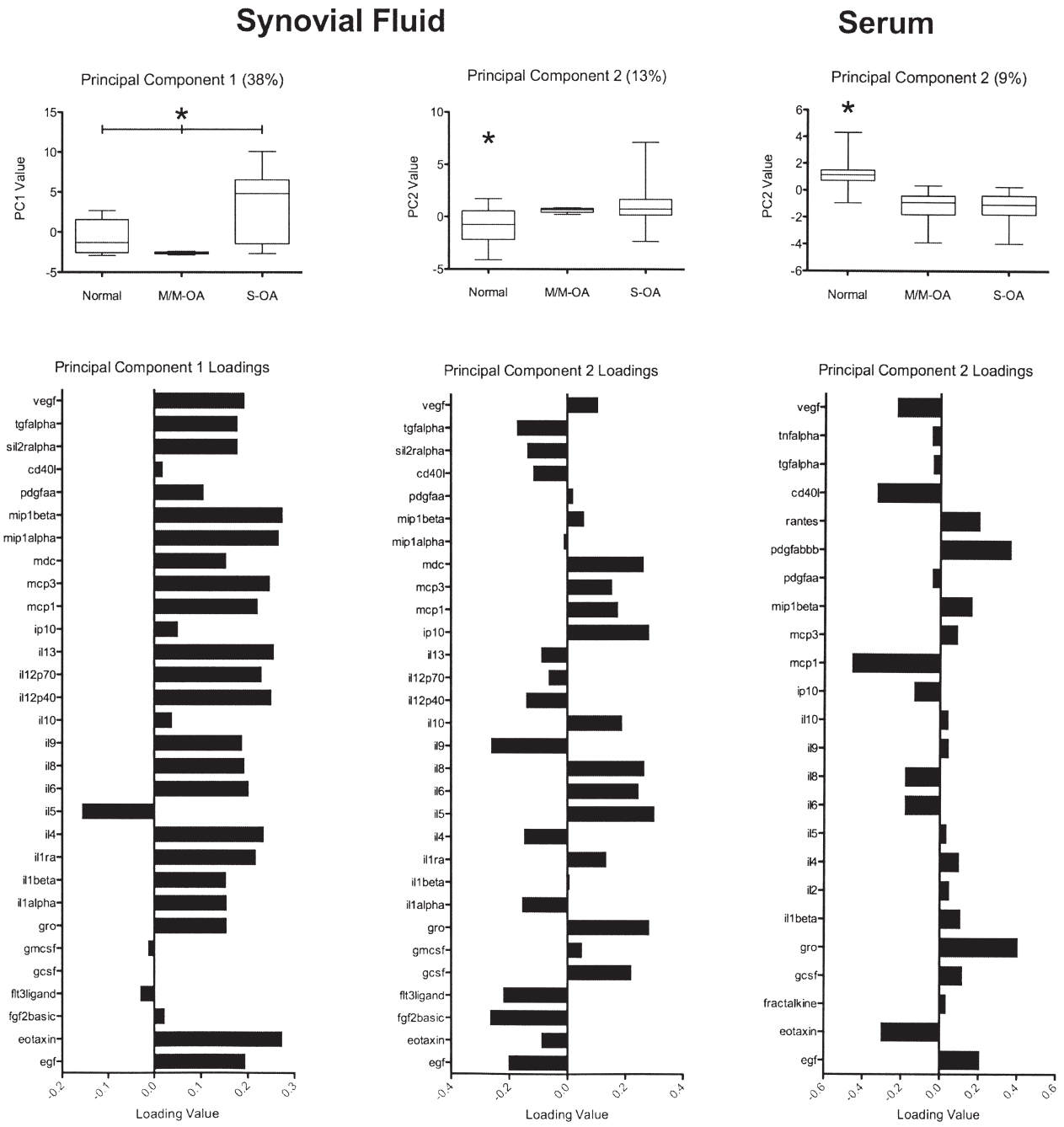

Figure 2. Contribution of each cytokine (factor loadings) to each principal component investigated and patient group comparisons of each principal component investigated. Synovial fluid factor loadings on principal components 1 and 2 are displayed in the bottom left and middle, with corresponding patient-group comparisons on the top left and middle. Factor loadings on principal component 2 of the serum are displayed on the bottom right, with corresponding patient-group comparisons shown directly above (on the top right). M/M-OA: mild/moderate osteoarthritis; S-OA: severe osteoarthritis; PC: principal component.

interferon-γ, IL-6, IL-8, IL-17, interferon-gamma inducible protein-10 (IP-10), platelet-derived growth factor AA/BB, CD40L, and vascular endothelial growth factor (VEGF). Interestingly, IL-6 and IL-1ra, reported by others to be potential biomarkers of OA[37,38,39,40,41], were not significantly different between normal and early OA synovial fluid samples, while IL-6 was identified in serum in our study. To date, many of the potential biomarkers identified by the multiplex technologies have not been extensively reported in OA or in preclinical models of OA. MCP-1 was shown to be the most influential cytokine in the separation of normal and OA samples. As well, a study reported that increased MCP-1 levels correlated with increased knee pain in patients with early joint disease in the absence of RA[42], and

Table 2. K-means clustering analysis.

Sample Investigated	Inputs	Treatment Group	Composition of K-means Grouping		
			Group 1	Group 2	Group 3
Synovial fluid	PC1, PC2	Normal	50	50	0
		Mild/moderate	100	0	0
		OA	35	12	53
Serum	PC2	Normal	96	4	—
		Mild/moderate	7	93	—
		OA	18	82	—

PC: principal component; OA: severe osteoarthritis.

the authors observed that patients with increased knee pain exhibited increased levels of MCP-1.

It is also notable that the expression of many cytokines [FGF-2, Fit-3 ligand, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1α, IL-9, and CD40L] was decreased in synovial fluid in mild/moderate OA knee joints compared to normal joints (Table 1), while only IL-5 and MDC were increased. Some evidence suggests FGF-2 may be chondroprotective within the joints[43], and it has been observed that MDC is increased within the synovial fluid and serum of patients with arthritis[44]. However, in a mouse model of arthritis, it was recently demonstrated that the absence of GM-CSF leads to decreased pain and cartilage damage[45]. The other factors (except IL-1α) have not been intensely studied in OA, but deserve further attention. When synovial fluid from patients with severe OA was compared to mild/moderate OA or normal synovial fluid, we observed an increase in the expression levels of multiple cytokines (as expected from previous reports). Specifically, IL-6, IL-1ra, IL-12 (p40 and p70), IP-10, MCP-1 and 3, and MIP-1β were all increased in severe OA samples compared to normal and mild/moderate synovial fluid. IL-12 has previously been observed to increase in autoimmune disorders, but not specifically in OA[46], while IP-10 levels have been shown to both increase and decrease in synovial fluid, plasma, and serum with OA progression and pain[47,48]. Therefore, the roles of these proteins in OA are not completely understood, but almost all these cytokines have been linked to pain[49,50,51]. This introduces the question of how many of these "biomarkers" are associated with osteoarthritic pain, and this should be addressed by looking for correlations with clinical pain scores. One cytokine that has been implicated in progression and pain in OA is TNF-α[52]; based on previous reports we expected to observe significant differences in TNF-α between our patient groups; however, this was not the case, as only minimal levels of TNF-α were detected within normal and severe OA synovial fluid samples, while mild/moderate OA samples displayed no expression. This suggests that either our test was not sensitive enough to detect TNF-α or that TNF-α may be expressed only in a subset of patients with OA. To test the assay further, we also assessed a number of RA synovial fluid samples and found high levels of TNF-α (data not shown), suggesting that TNF-α is not always expressed in OA (mild/moderate or severe).

In contrast to the synovial fluid cytokine profiles, we observed a number of upregulated and downregulated proteins in patients with OA compared to normal individuals. However, a number of proteins were conserved between synovial fluid and serum (up in OA: eotaxin, IL-6, IL-8, IL-10, MCP-1, TGF-α, and VEGF; down in OA: GM-CSF). While many of these proteins have been implicated in OA, the conservation of expression locally and systemically may suggest they play a role in regulating the process in OA from outside the joint.

Aside from correlating cytokines to pain and other patient outcome measures, groups have linked individual cytokine levels to radiography[52,53,54]. In serum samples, we did not identify any cytokines that were significantly different between mild/moderate and severe OA (while normal individuals had no radiographic changes), and in synovial fluid we identified a number of cytokines (n = 22) that increased with disease progression (Table 1). It is important to note that mild/moderate patients sampled for synovial fluid exhibited no radiographic evidence of OA. However, only a few of the 22 cytokines that increased with OA progression have been previously correlated to radiographic changes or risk of OA (based on protein and/or gene expression studies), IL-6 being the most studied. Our study suggests that additional proteins may correlate with disease severity; however, longitudinal studies must be carried out to prove this hypothesis.

Our study has limitations. First, a prospective study using cytokines as predictors of OA development would have been helpful. In our study, the cytokine profiles validated an established clinical diagnosis of OA. This is potentially a problem because diagnosis of OA, especially in the early stages, is a particular challenge. Accordingly, we termed our less severe cohort as mild/moderate because the clinical classification of "early OA" had not been achieved unequivocally. The second limitation is that our sample size, while adequate for the statistical methods described, could be increased to strengthen the power of the analysis for both synovial fluid and serum samples.

To overcome these limitations, we are currently recruiting and testing a larger, longitudinal patient cohort that will address the sample size issue, and allow us to perform repeated measures on the same population during their individual and potentially unique progression of disease.

A number of cytokines are differentially expressed in synovial fluid and sera from patients with mild/moderate OA compared to normal individuals and patients with severe OA. These discriminating factors (potential biomarkers) may indeed distinguish among patients with mild/moderate OA (balanced for age and sex) as well as normal individuals with no documented history of joint disease and no visible joint damage. These findings suggest that, with further study and validation, assessment of protein levels for cytokines and chemokines using the multiplex technology could contribute to development of diagnostics for mild/moderate and severe OA, as well as a potential approach to monitor the efficacy of interventions to control or reverse the disease course.

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