Microarray Analysis of the Infrapatellar Fat Pad in Knee Osteoarthritis: Relationship with Joint Inflammation

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ABSTRACT. ***Objective.*** To examine differences in genes involved in fat metabolism, energy homeostasis, adipogenesis, and inflammation between endstage and early-stage knee osteoarthritis (OA) infrapatellar fat pads (IFP).

Methods. Twenty-nine endstage and 5 early-stage primary OA IFP were harvested at knee surgery. Total RNA was extracted, labeled, and hybridized to whole-genome expression arrays. Unsupervised analysis of all samples using principal components analysis or 2-way hierarchical clustering showed groupings based on tissue source and disease. Statistical testing identified sets of genes that displayed differences between the 2 fat types. Western blot analysis was performed for protein expression of an identified gene of interest.

Results. The 29 IFP demonstrated an elevation in the expression of adipokines such as adiponectin and leptin. A statistically significant increased expression was seen for genes of adipogenesis, such as peroxisome proliferator-activated receptor-γ (PPAR-γ), diacylglycerol acyltransferase 2 (DGAT2), cluster of differentiation (CD36), and thyroid hormone responsive spot (THRSP) in the severe OA fat pads as compared to the controls. A subset of 5 patients in the endstage OA group were consistently similar in gene expression to early OA tissue. Protein expression of PPAR-γ2 was 5.4-fold and PPAR-γ1 was 1.4-fold greater in endstage versus early OA tissue.

Conclusion. Endstage OA fat pads demonstrated a significant upregulation of genes for fat metabolism and energy homeostasis and a mixed result for inflammatory cytokines. (J Rheumatol First Release July 15 2011; doi:10.3899/jrheum.101302)

Key Indexing Terms:
OSTEOARTHRITIS KNEE INFRAPATELLAR FAT PAD GENETICS

Osteoarthritis (OA) is characterized by the progressive destruction of articular cartilage, along with alterations of the subchondral bone[1]. Excessive production of a number of different proinflammatory mediators produces a low-grade intraarticular inflammation with synovitis[2,3]. Although the exact pathogenesis of OA is not fully understood, it is widely accepted that metabolic factors, in addition to hereditary, developmental, and mechanical influences, play an important role in disease progression[4,5,6,7].

Visceral white adipose tissue has traditionally been viewed as a passive energy storage site; however, it has become increasingly recognized as an active endocrine organ producing various inflammatory cytokines and adipokines that act in an endocrine, autocrine, and paracrine manner[8]. Examples include interleukin 1ß (IL-1ß) and IL-6 as well as tumor necrosis factor-α (TNF-α), and the hormones leptin (LEP), adiponectin (ADIPOQ), and resistin[9]. These adipokines regulate systemic metabolic functions including hemostasis, glucose and lipid metabolism, reproductive functions, blood pressure regulation, insulin sensitivity, and bone formation, as well as angiogenesis[10]. In addition, these hormones are known to modulate the intraarticular inflammatory process[11] and may play a key role in the initiation and progression of articular cartilage degeneration.

The knee is the joint most commonly affected by OA. The infrapatellar fat pad (IFP) of the knee, also known as Hoffa's fat pad, is intracapsular and extrasynovial in structure, and it is thought to have primarily a biomechanical function of absorbing forces generated in the knee[12]. The role of the fat pad in the joint inflammatory process of OA is not well understood. Some have shown that this adipose tissue is capable of releasing growth factors [e.g., vascular endothelial growth factor and inflammatory cytokines (e.g., IL-6), as well as adipokines (leptin and adiponectin)][13,14]. The limitations of these studies are that they did not use a control group, and 1 study included only 5 fat pad samples from OA knees, and the other only 12 samples. Although these studies present important findings, without a reference group of normal or early OA

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samples, it cannot be concluded that this effect is necessarily associated with OA. A recent study by Distel, *et al* used real-time quantitative polymerase chain reaction (qPCR) and ELISA to analyze the inflammatory profile of the knee fat pad and compared it to a reference sample of subcutaneous fat taken from the ipsilateral thigh. They found that the levels of gene expression of IL-6 and its soluble receptor were elevated in the fat pad compared to the subcutaneous fat taken from this endstage OA population[15]. Again, this study did not compare its findings to that of a control early-stage OA population and studied only 11 obese female patients.

The primary objective of our study was to examine the difference in expression patterns for genes involved in fat metabolism, energy homeostasis, adipogenesis, and joint inflammation between the endstage and early-stage knee OA IFP using microarray analysis. We hypothesized that the IFP of the patients with endstage knee OA would show an increased expression of genes involved in fat and energy metabolism and joint inflammation.

MATERIALS AND METHODS

We studied 29 consecutive patients who presented with a diagnosis of endstage primary knee OA at the Toronto Western Hospital during 2009. They comprised our experimental group. Our reference group consisted of 5 patients with early-stage primary OA who presented for consultation with a diagnosis of a degenerative meniscal tear. All patients with early OA in this group had only a partial-thickness cartilage defect in any compartment of the knee, further defined by a grade 1 or 2 lesion by the Outerbridge classification[16]. A grade 1 lesion is softening and swelling of the cartilage, while a grade 2 lesion is a partial-thickness defect with fissures on the surface that do not reach the subchondral bone or exceed 1.5 cm in diameter[16]. Patients gave their consent to participate to a study nurse not involved in the patients' medical care. The study protocol was approved by the Human Subject Review Committee. All patients were of Caucasian ethnicity.

Demographic data of age, sex, comorbidity, medications, and body mass index (BMI) were collected. Waist circumference was measured over the bare skin of the abdomen at the point of the smallest diameter between the iliac crest and costal margin[17]. All measurements were taken by a single person using a stiff measuring tape and taken in duplicate and averaged together for the final values. All patients with endstage OA were taking nonsteroidal anti-inflammatory drugs (NSAID) that were stopped 10 days prior to surgery and tissue sample collection. Narcotic medication was permitted to be taken until the day of surgery. One patient with early-stage OA was taking NSAID therapy and it was also stopped 10 days prior to surgery. All others were taking oral acetaminophen as needed.

Continuous data are reported with means and SD and compared with the Mann-Whitney U test, while binary data are presented with frequencies and compared with the Fisher's exact test. Statistical comparisons of clinical data were performed with SPSS version 13.0 (SPSS, Chicago, IL, USA). All reported p values are 2-tailed with an α of 0.05 unless otherwise specified.

Tissue samples and analysis. For the endstage OA group, IFP samples were collected from patients at the time of knee replacement surgery under sterile conditions. Similar samples were collected from patients with early-stage OA at the time of arthroscopic knee surgery for debridement of meniscal tears. All patients were fasting 12–16 h prior to sample collection. Fat samples were immediately placed in a solution of RNAlater (Ambion, Austin, TX, USA) for preservation until analyzed.

Isolation of total RNA. Fat samples were weighed in sterile 1.5 ml tubes and homogenized in solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 7.2 µl/ml ß-mercaptoethanol). TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was then added to each sample and total RNA was isolated following the manufacturer's protocol. Samples were transferred to an RNeasy Mini column (Qiagen, Valencia, CA, USA) for sample cleanup and treated with DNase (Qiagen) for 15 min at room temperature. Quality and quantity of the isolated RNA were determined by running samples on the Agilent Bioanalyzer 2100 and Nanodrop, respectively.

Microarray analysis. Total RNA was reverse transcribed to produce first single-strand, then second-strand cDNA following manufacturer's instructions (Illumina TotalPrep Amplification kit; Ambion). Samples were column-purified and run on the Nanodrop spectrophotometer for quantification. An appropriate quantity of cRNA was then aliquoted and hybridized to the Illumina whole-genome expression array overnight at 58°C with rocking. Microarrays were then washed and scanned, and the resulting images quantified.

Microarray image analysis. All files were loaded into GeneSpring (version 11.0.1, Agilent, Mississauga, ON, Canada) for analysis. Background subtracted probe intensities were converted to $\log_2$, then normalized using Genespring's quantile normalization. Further median-based normalization was applied for each probe across all samples. Initial filtering was done to remove probes that were low-expressing or did not express across all samples. A 2-way, unsupervised hierarchical clustering algorithm using average linkage rules and a Pearson centered similarity metric was used to assess any general, overall trends in the data. A standard t-test was used to find significance, with a Benjamin and Hochberg multiple testing correction using a false discovery cutoff of 0.05 with a 2-fold cutoff in normalized average expression. Hierarchical clustering was used to assess overall variability between the groups on a probe-to-probe basis.

Real-time PCR. Triplicate reactions were carried out on each sample of cDNA generated from total RNA. Reactions were carried out as per manufacturer's instructions using the full velocity SYBR Green qPCR Master Mix (Agilent). PCR primer sequences used were leptin: sense: GTG TGA GCA GTG AGT TAC, antisense: TAG GTG GTT GTG AGG AT; adiponectin: sense: GGA AGG ACT ACT ACT CAA TG; antisense: CAG CAC TTA GAG ATG GA; PPARG: sense: AGA CAT TCC ATT CAC AAG A; antisense: CAG ACA CGA CAT TCA AT; DGAT2: sense: ACA GAA GTG AGC AAG AAG; antisense: GAC CAC GAT GAT GAT AG; THRSP: sense: CCA ATG ATG AGA GCA GAA; antisense: GTG AAT AGC AAC AGA CAT; ACOT1: sense: TCA ACA GAA ATC GCA TCA; antisense: CTC ATT AGC ATA GAA CTC A; FABP5: sense: GGG AGA GAA GTT TGA AGA A; antisense: GAG TAC AGG TGA CAT TG; and CD36: sense: GTT GGA GAC CTG CTT ATC; antisense: TGC TGT TCA TCA TCA CT. T-tests between the patients with endstage OA and the patients with early-stage OA were validated at the $p < 0.05$ level. A t-test did not show significance for fatty acid binding protein (FABP5) between the endstage and early-stage OA patients ($p < 0.14$; Table 1).

Total protein isolation. IFP were washed with ice-cold phosphate buffered saline and homogenized using a Polytron homogenizer in an extraction buffer

Table 1. Expression of specific mRNA ratio (endstage OA/early-stage OA) detected by microarray and qPCR analysis.

Gene	Microarray	Q-PCR
Leptin	10	69.8
Adiponectin	11	6.4
PPAR-γ	6	6.7
FABP5	3.5	3.1
DGAT	7.1	17.7
CD36	3.6	23.4
THRSP	7.3	27.2

q-PCR: quantitative polymerase chain reaction; PPAR: peroxisome proliferator-activated receptor; FABP: fatty acid binding protein; DGAT: diacylglycerol acyltransferase; CD: cluster of differentiation; THRSP: thyroid hormone responsive spot.

(100 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaCl, and 1% Triton X-100) in the presence of protease inhibitors (Roche). Tissue homogenates were centrifuged (13,000 × g, 30 min, 4°C) and the resulting infranatants were then collected and total protein concentration determined by Lowry assay (BioRad, Mississauga, ON, Canada).

Western blot analysis. One hundred milligrams of each sample were run on SDS-PAGE and probed with antibodies directed against peroxisome proliferator-activated receptor (PPAR-γ; Cell Signaling Technology, Danvers, MA, USA) and GAPDH (Epitope Biotech, Burnaby, BC, Canada) overnight at 4°C. The appropriate secondary antibodies were used and proteins visualized by incubating blots in ECL reagent (Perkin Elmer, Waltham, MA, USA), then imaged on a digital fluorescent imager (Cell Biosciences, Santa Clara, CA, USA). Relative quantities of PPAR-γ were determined by measuring the band intensities from both control and disease patient samples. These were then normalized for protein loading by dividing through by the intensities of GAPDH in each of the samples.

RESULTS

In our sample of 29 patients with endstage OA, we had 14 men (48%). The mean age of our cohort was 66.9 years (range 44–85 yrs, SD 9.6) and the mean BMI was 33.1 kg/m^2 (range 21.5–49.3 kg/m^2, SD 6.3). There were 2 men (40%) among the 5 patients with early OA. The mean age of the patients with early OA was 42.6 years (range 35–50 yrs, SD 12.4) and the mean BMI was 34.1 kg/m^2 (range 26.4–39.6 kg/m^2, SD 6.4; Table 2).

A total of 48,803 probes are present on the Illumina HT-12_V3_0_R2 human whole-genome chip. After filtering to remove probes indicating low or absent expression, 26,776 probes were included for subsequent statistical testing. The result of a 2-way hierarchical clustering of these probes is given in Figure 1. The patients with early OA group together (blue branches of tree), and the patients with endstage OA (red bars) group together. Five of the patients with endstage OA form a branch with the patients with early OA.

In total, 3562 probes were classified by t-test as significantly differentially expressed between the early and endstage OA fat pads, at a false discovery rate of $p < 0.05$. Of those probes, 1613 were found to have greater than a 2-fold difference in normalized expression value between the 2 groups. To visualize this difference on an individual probe and sample level, a 2-way hierarchical clustering was performed (Figure 2). The patients with early OA clearly group together with an evident pattern of probes, either overexpressed or underexpressed, relative to the endstage OA tissue. Interestingly, the 5 endstage OA samples that grouped with the patients with early OA previously in an unsupervised analysis (Figure 1) still appear more similar to the patients with early OA.

Through our searches, we noted that there were a number of genes involved in fat metabolism, energy homeostasis, and adipogenesis that were upregulated in the endstage OA tissue. PPAR-γ, an adipogenic transcription factor, was found to be upregulated by 6-fold in the endstage OA IFP as compared to early OA. The elevated gene expression was consistent with the increased protein expression of PPAR-γ2 at 5.4-fold and PPAR-γ1 at 1.4-fold greater than early-stage OA (Figure 3).

Two FABP related to PPAR-γ, FABP4 and FABP5, were found to be upregulated 2.8-fold and 3.5-fold, respectively, in the endstage OA tissue. Diacylglycerol acyltransferase 2 (DGAT2) was 7.1-fold upregulated in the endstage OA tissue. CD36, a scavenger receptor transcriptionally induced by PPAR-γ[18], functions as a cell-surface protein for various cell types and is 3.6-fold overexpressed in the endstage OA subjects. Of the cell death-inducing DNA fragmentation factor-α-like effector (CIDE) family of genes, CIDE-A and CIDE-C were significantly upregulated 6.5 times and > 5 times, respectively, in the endstage OA tissue.

Other lipid and energy storage-related genes that were upregulated in the endstage OA tissue are given in Table 3.

A number of proteins are known to be secreted from various adipocyte deposits in the body and may affect obesity-related diseases such as OA. Of these, we observed a significant 11-fold increase in ADIPOQ gene expression in the endstage OA tissue as compared to early OA. Another adipokine, LEP, is also well known to be present in the synovial fluid of patients with knee OA[19]. In our study, LEP was 10-fold overexpressed in the fat pads of endstage OA tissue but did not achieve our significance threshold ($p < 0.07$). Leptin receptor, however, was significant and was 9-fold overexpressed in the patients with endstage OA as compared to the patients with early-stage OA.

Table 2. Demographic data among the endstage osteoarthritis (OA) infrapatellar fat pad group, patients with endstage OA whose genetic profile mimicked the patients with early-stage OA (like early-stage), and patients with early-stage OA. Early-stage OA is defined as Outerbridge grade 1 or 2 on arthroscopic inspection[16].

Characteristics	Endstage OA, n = 24	Like Early-stage OA, n = 5	Early-stage OA, n = 5
Mean age, yrs (SD)	67.4 (9.3)	64.8 (14.5)	42.6 (12.4)
Men, n (%)	11 (46)	2 (40)	2 (40)
Mean body mass index, kg/m^2 (SD)	33.5 (6.5)	31.2 (5.7)	34.1 (6.4)
Percentage obese by BMI	70.8	40	60
Mean waist circumference, cm (SD)	106.7 (12.0)	103.5 (8.6)	104.8 (11.4)
Percentage obese by waist circumference*	88	75	80
DM/HTN/HCL, n	5/14/8	1/2/1	1/0/1

* Obese defined as ≥ 90 cm (females), ≥ 100 cm (males). DM: diabetes; HTN: hypertension; HCL: hypercholesterolemia.

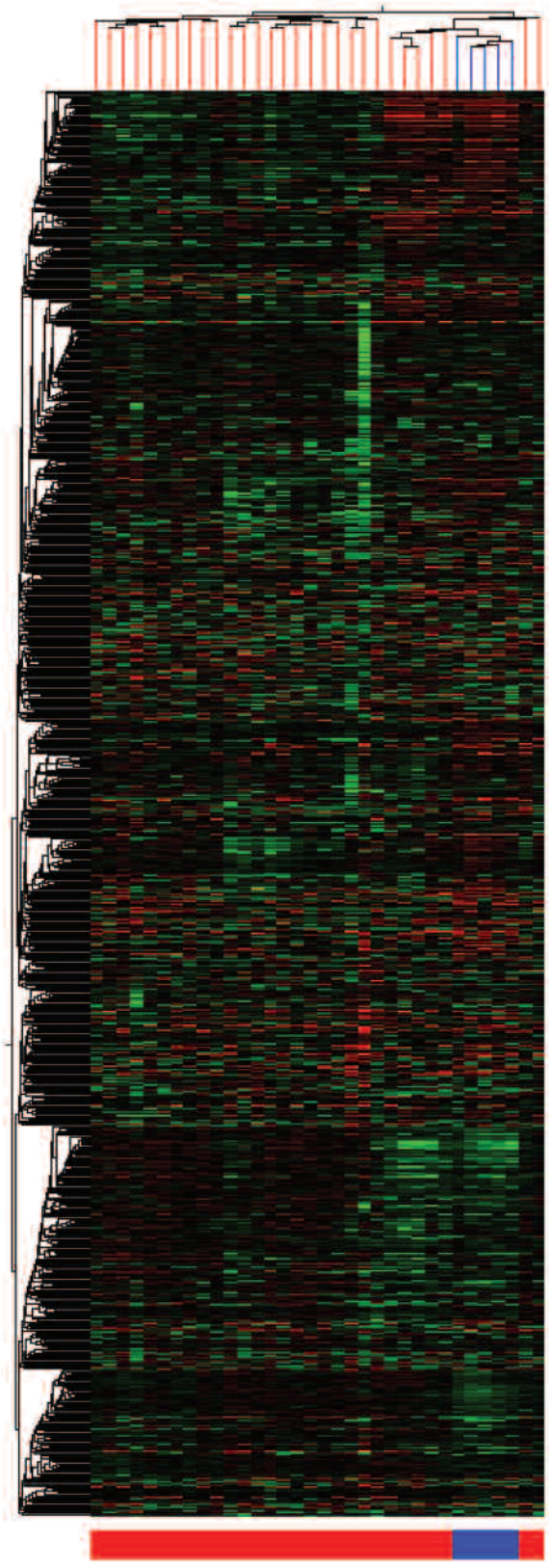

Figure 1. Unsupervised analysis comparing probe expression between diseased fat pads and normal fat pads.

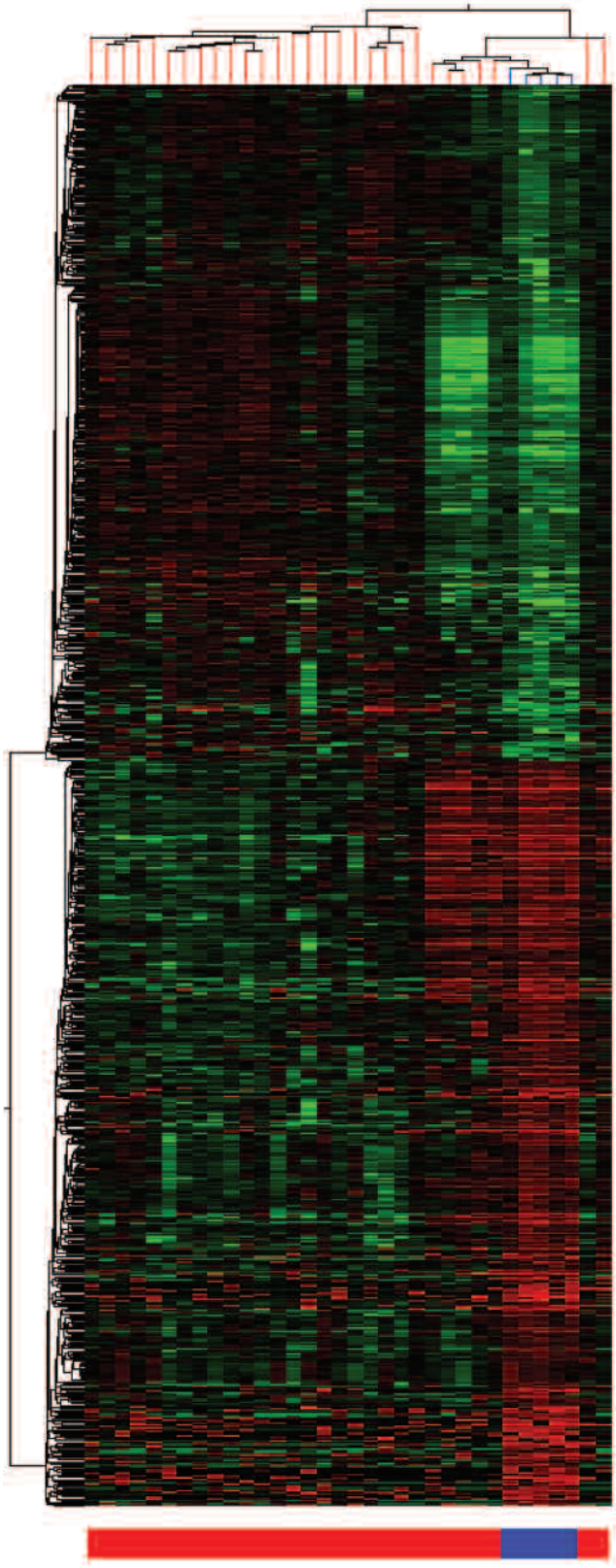

Figure 2. Two-way hierarchical clustering comparing probe expression between diseased fat pads and normal fat pads.

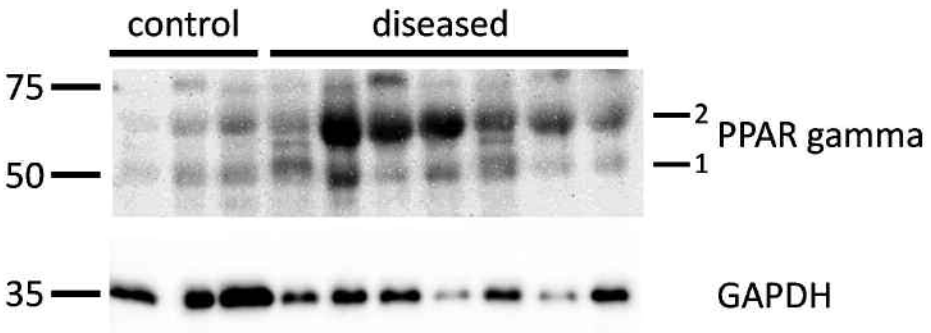

Figure 3. Western blots of peroxisome proliferator-activated receptor (PPAR-γ) and GAPDH expression in control and diseased fat pads. Total protein was extracted from control and diseased fat pads. Equal amounts of total protein (100 µg) were loaded per lane and run on a denaturing SDS-polyacrylamide gel. Proteins were transferred to a PVDF membrane and probed for PPAR-γ and visualized by chemiluminescence. Samples were also probed for GAPDH expression as a reference protein.

Table 3. Genes upregulated in 29 endstage infrapatellar fat pads as compared to 5 patients with early-stage OA.

Adipokines/Inflammatory Genes	Fold Increase	Lipid Metabolism Genes	Fold Increase*
Adiponectin	11×*	Fatty acid binding protein 4	2.8×
Leptin	10×	Fatty acid binding protein 5	3.5×
Leptin receptor	9×*	Diacylglycerol acyltransferase 2	7.1×
Vascular cell adhesion molecule	–7×*	Peroxisome proliferator activated receptor	6×
Connective tissue growth factor	–3×*	CD36	3.6×
Prostaglandin E receptor 3	10×*	Cell death-inducing DNA fragmentation effector (CIDE)	
ADAMTS5	1.7×*	Family:	
		CIDE-A	6.5×
		CIDE-C	> 5×

* Statistically significant difference between groups. "–" Denotes downregulation. CD: cluster of differentiation.

We also evaluated for the gene expression for various inflammatory cytokines. We found upregulation of prostaglandin E receptor 3 (10-fold) and the matrix metalloproteinases (MMP)9 and MMP15, and downregulation of MMP17 in endstage OA tissue. A disintegrin-like and metalloprotease with thrombospondin type 1 motifs 5 (ADAMTS5) was also found to be upregulated 1.7× in endstage OA tissue. MMP and ADAMTS5 facilitate the turnover of extracellular matrix molecules, including the breakdown of collagens[20]. Other inflammatory genes demonstrating a statistically significant downregulation include vascular cell adhesion molecule-1 (VCAM1; 7-fold), connective tissue growth factor (CTGF; 3-fold), and cluster of differentiation 44 (3-fold).

DISCUSSION

Few authors have shown upregulation of genes and protein expression for various inflammatory cytokines from the IFP[13,14,15]. Our study is possibly the first to use microarray analysis for a broad genetic profile and the first to use a reference group of early OA tissue. We found that the endstage OA fat pads demonstrated a significant upregulation of genes for fat metabolism and energy homeostasis and a mixed result for inflammatory cytokines.

Considering the intraarticular location of the IFP and the known active metabolic properties of adipose tissue, it is likely the IFP is involved in the pathophysiology of knee OA. The upregulation of genes we found in patients with endstage OA may be primary, and independent of the joint inflammation, or it may be secondary to the joint inflammation and OA process. We and others believe that the genes are likely turned on secondary to infiltration of the adipose tissue by local immune cells and cytokines present in the joint[21]. As a result of the OA, monocytes and lymphocytes that have passed into the joint from the systemic circulation infiltrate the IFP and regulate gene expression. Further, inflammatory mediators such as IL-1ß and TNF-α are known to be released into the synovial fluid locally by cartilage and synovium and may stimulate catabolic genes within the IFP[22]. Fat pad volume is believed to increase in response to local inflammation and substance P-induced vasodilation and immune cell extravasation[21,23].

The adipokines ADIPOQ and LEP were both over-expressed in the endstage OA tissue (11-fold and 7-fold, respectively); however, only ADIPOQ reached statistical significance. One group of investigators has shown decreased gene and protein expression of LEP from the IFP relative to a

control sample of subcutaneous fat[14]. Although ADIPOQ appears to have a protective role in obesity-associated illnesses such as type II diabetes and atherosclerosis[24], within the knee joint its role remains unclear. Some have suggested it may have an antiinflammatory role through downregulation of IL-1ß levels, thereby decreasing MMP13 levels[25]. Others suggest a proinflammatory effect through increased production of NOS2, MMP3, and MMP9[26,27]. This difference between an antiinflammatory effect at the vascular level and a proinflammatory effect in the synovial joint may be related to the concentration of the hormone present[26]. In rheumatoid arthritis, synovial fluid levels of ADIPOQ have been shown to be higher than those seen in OA[28].

Fat pad gene expression of MMP9 and MMP15 in our study further supports their roles in joint inflammation in knee OA. In contrast, CTGF and VCAM1, cytokines that are known to promote chondrocyte proliferation[29] and inflammatory cell aggregation[30], respectively, were both found to be underexpressed in diseased fat pads. Moreover, VCAM1 has been suggested as a predictor of severe, endstage knee OA in a longitudinal study[30].

We found a significant upregulation of the PPAR-γ gene and protein expression in the endstage OA fat pads as compared to the early-stage OA. One other group reported a decreased gene expression of PPAR-γ in 11 IFP samples as compared to a reference of subcutaneous fat in obese women patients[15]. PPAR-γ is a nutritional sensor that acts as a transcription factor and regulates the expression of a number of target genes[31]. Ligand binding of the PPAR-γ receptor site is known to improve insulin sensitivity and stimulate lipid storage in adipocytes[32]. Recent literature has also suggested that PPAR-γ activators may have a protective effect in arthritis[33,34]. Binding of the receptor site with the thiazolidinediones (TZD) class of medications has been shown to decrease cartilage lesions in a dog model[34] and also to inhibit major inflammatory signaling pathways, reducing the production of mediators of chondrocyte death in arthritis[33]. PPAR-γ also appears to be expressed by other major cell populations within synovial joints, such as chondrocytes, synoviocytes, fibroblasts, and endothelial cells[35,36]. The upregulation of PPAR-γ we found in the fat pads may be related to the increased expression of ADIPOQ, because others have suggested a direct correlation between PPAR-γ activity and ADIPOQ expression[37]. Further work is needed to evaluate whether PPAR-γ binding may ameliorate joint inflammation and patient symptoms in OA.

We found that endstage OA tissue samples from 5 patients consistently demonstrated a gene profile most consistent with early-stage OA tissue, which we have termed "like early-stage OA". We found no identifiable differences for these unique 5 patients as compared to the remainder of the group of patients with endstage OA in age, sex, BMI, or metabolic abnormalities (Table 2). In particular, there were no differences in the use of insulin-sensitizing medications (e.g., TZD) between groups. This finding of a heterogeneous group contributes to understanding of the relationship between genetic profile and phenotypic presentation of OA.

Our report has several strengths. We are the first to use a reference group of early OA fat pads. Our sampling of patients included both men and women and a representation of non-obese patients. We used microarray analysis that allows discovery of novel gene expression differences between late and early-stage OA tissue.

A potential limitation of our report is the small sample size for the early OA group. It should also be noted that our early-stage OA group does not represent a normal, or non-OA, knee tissue group.

In IFP from knees with endstage OA microarray analysis showed a significant upregulation of genes for fat metabolism and energy homeostasis and a mixed result for inflammatory cytokines. In particular, we found an increased expression of the PPAR-γ gene and protein from the endstage OA IFP[33]. Interestingly, many of the genes upregulated in the disease IFP have relationships to the components of the metabolic syndrome and may suggest a metabolic pathway connecting these chronic diseases[38]. Future work should be directed toward understanding the combined effects of all mediators released from the fat pad and knee cartilage cells.

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