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Differential distribution of adipokines between serum and synovial fluid in patients with osteoarthritis. Contribution of joint tissues to their articular production

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Summary

Objective: To analyze the distribution of leptin, adiponectin and resistin between paired serum and synovial fluid (SF) samples of patients with osteoarthritis (OA) and to determine the potential sources of these adipokines in the joint. The active free form of leptin was also examined by evaluating the level of the soluble leptin receptor (sOb-R).

Methods: Levels of adipokines and sOb-R were measured by a sandwich enzyme-linked immunosorbent assay in serum and SF collected from OA patients. The levels of adipokines were also determined in conditioned media from cultured joint tissues (synovium, infrapatellar fat pad, meniscus, osteophyte, cartilage and bone).

Results: The adipokines exhibited different patterns of distribution between the joint and the circulating compartment. Serum levels of resistin and adiponectin exceeded those in the paired SF. Conversely, leptin SF concentrations were similar or higher than those measured in serum counterparts. Leptin and adiponectin in SF may derive from each joint tissue examined, whereas resistin was not detected in conditioned media of cultured explants. Synovium and infrapatellar fat pad were the major sources of adipokines, but osteophytes released also large amounts of leptin. The sOb-R deficiency found in SF further increased the difference in the bioactive leptin levels between serum and SF. A gender-specific difference was observed with women exhibiting the highest level of free leptin in the joint.

Conclusion: These data demonstrated that adipokines serum levels are not predictive values for SF determination. The joint cavity is a special space where each adipokine undergoes specific regulatory pathways, strengthening the hypothesis that adipokines may have local effects in the joint and may account for the high prevalence of OA in women.
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Introduction

Obesity is a chronic multi-factorial disease with increasing prevalence in most industrialized countries. It is associated with several other diseases such as diabetes mellitus, coronary artery disease, hypertension, and several forms of cancer. Obesity represents also a potent risk factor for the development and the progression of osteoarthritis (OA)[1−3]. The pathology of OA involves the whole joint in a degenerative process that includes focal and progressive articular cartilage loss with concomitant changes in the underlying bone and development of osteophytes. The overload effect may explain most of the increased risk for OA of the knee and hip among overweight persons. However, the risk factor for developing OA in non-weight-bearing joints, such as hands, was also shown to be associated with body mass index (BMI)[4,5]. In addition, if weight loss may prevent the onset of OA[6], Toda *et al.* found that the

loss of body fat is more closely related to symptomatic benefit than is the loss of body weight[7]. Taken together, these findings suggest that adipose factors may provide a metabolic link between obesity and OA. The potential role of adipose-derived proteins in OA is emphasized by the ability of the infrapatellar fat pad, which is an intra-articular tissue, to produce cytokines[8].

Adipose tissue expresses and secretes a large number of proteins that often share functional and structural properties of cytokines, and are therefore classified as adipokines[9]. These include leptin, resistin and adiponectin. In humans, circulating levels of both leptin and resistin are positively correlated with the BMI and fat mass[10−13], while adiponectin plasma concentration is decreased in obese individuals[13,14]. Leptin serves as a negative feedback signal for the central nervous system to inhibit food intake and stimulate energy expenditure[15−17]. Leptin exhibits also peripheral functions including the regulation of lipid metabolism[18], insulin secretion[15], reproductive functions[19], angiogenesis[20], and bone development[21]. Adiponectin increases insulin sensitivity by reducing circulating fatty acid concentration and triglyceride level in the liver and muscle[22,23]. Resistin is another adipose-derived hormone regulating insulin sensitivity seemingly opposite to adiponectin[24].

Leptin, adiponectin and resistin have been detected in the synovial fluid (SF) obtained from patients with OA[25,26].

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Schäffler *et al.* found a positive correlation between SF levels of resistin and of systemic markers of inflammation[26]. In experimental models, leptin may display pro- or anti-inflammatory effects in the joint depending on the immune response[27−29]. In humans, Bokarewa *et al.* suggested that intra-articular leptin may attenuate the erosive process in the joints during the course of rheumatoid arthritis[30]. In a previous study, we demonstrated that leptin expression was up-regulated in both osteophytes and cartilage obtained from patients with OA, especially in areas of matrix depletion, fibrillations, and chondrocytes clusters[25]. Leptin exhibited biological activity on chondrocytes with stimulation of anabolic functions through induction of growth factors synthesis[25] and was shown to increase the effects of pro-inflammatory cytokines[31,32].

Consequently, local changes in adipokines concentrations may have important pathophysiological implications on cartilage homeostasis. To add further insights, we examined first the distribution of leptin, adiponectin and resistin between paired SF and serum samples obtained from OA patients. Secondly, the soluble leptin receptor (sOb-R) was also sought to evaluate in both the joint space and the circulating compartment, the level of free leptin, the presumed biologically active form of this adipokine[33,34]. Finally, the possible articular sources of leptin, adiponectin and resistin were identified by measuring their concentrations in media of cultured joint tissues including synovium, infrapatellar fat pad, meniscus, osteophyte, cartilage and bone. These investigations indicated that adipokines exhibited different patterns of distribution between circulating and joint compartments, suggesting that the resulting imbalance between the synovial levels of adipokines known to have opposite biological effects in various diseases such as diabetes or inflammation, may have further implications in the development and the progression of OA.

Patients and methods

PATIENTS AND SAMPLES

Thirty-five patients (20 women and 15 men, age 50−83 years [mean age 68.4 years]) who required total knee arthroplasty were included in the present study. Knee OA was diagnosed from clinical and radiologic evaluation, based on the American College of Rheumatology criteria[35]. Because serum samples were not available from each patient, paired serum samples were obtained from 25 patients only. Collected serum and SF samples were centrifuged at 4000 g for 15 min and stored at −80°C. Specimens of synovium, infrapatellar fat pad, meniscus, osteophyte, cartilage and bone were collected from 12 OA patients. This human study was conducted in conformity with the declaration of Helsinki principles, and written informed consent was obtained from each participant.

EX VIVO CULTURE OF JOINT TISSUES

Synovium, infrapatellar fat pad, meniscus and osteophyte were washed in phosphate buffered saline (PBS) and then carefully dissected into small pieces (50−150 mg). One explant of each tissue was placed in each well of a 24-well plate. Disks of cartilage and bone (50−100 mg) were cut with a biopsy punch (6 mm) and two pieces were placed into each well of a 48-well plate. Explants in triplicate were first precultured for 2 days at 37°C in a humidified

atmosphere of 5% CO_2 in Dulbecco's Modified Eagle Medium/Nut Mix F12 (DMEM/Ham's F12 medium) supplemented with L-glutamine (2 mM), gentamycin (50 μg/ml), and amphotericin B (0.25 μg/ml). The tissue specimens were thereafter incubated in fresh medium and conditioned media were harvested on day 2 and stored at −20°C.

ASSAYS

The concentrations of leptin, resistin and adiponectin in SF and serum samples and in conditioned media of cultured explants, and the levels of sOb-R in biological fluids were determined in duplicate by a sandwich enzyme-linked immunosorbent assay (ELISA) using commercially available kits. The leptin, resistin, and adiponectin ELISA kits were purchased from R&D Systems (Lille, France) while that for sOb-R was obtained from BioVendor (Heidelberg, Germany). Dilution and spiking experiments were performed to validate the use of commercially available ELISA kits for SF samples. Samples were diluted as appropriate and paired samples were assayed in the same run. According to the manufacturers, the detection limits for leptin, resistin, adiponectin, and sOb-R assays were 7.8 pg/ml, 26 pg/ml, 250 pg/ml, and 800 pg/ml, respectively. The interassay coefficients of variation for the four assays were 4.4%, 8.4%, 7.8%, and 3.6%, respectively. Intraassay coefficients of variation were less than 5%.

STATISTICAL ANALYSIS

The results are shown as mean values (S.E.M.). The leptin/sOb-R ratio was used as an index of free leptin, and was determined after converting concentrations to μmol/ml. Statistical analysis was conducted with StatView for Windows, version 5.0 (SAS, Cary, NC). Differences between women and men were analyzed using the nonparametric Mann−Whitney *U* test. Comparisons of the levels between matched pairs of SF and serum samples were made by the nonparametric Wilcoxon signed rank test. The analysis of statistical correlation was performed by the Spearman test of rank correlation. A *P* value less than 0.05 was considered significant for differences and correlations.

Results

ADIPOKINES AND sOb-R DETERMINATION IN SF SAMPLES OBTAINED FROM OA PATIENTS

SF samples obtained from 35 OA patients (20 women and 15 men) were analyzed for leptin, adiponectin, resistin, and sOb-R. There was no significant difference between the female and male patients with respect to age or BMI (Table I).

The adipokines were detected in each SF tested with concentrations ranging from 0.80 to 72.31 ng/ml for leptin (mean [S.E.M.], 15.94 [2.42] ng/ml), from 0.62 to 7.01 μg/ml for adiponectin (mean [S.E.M.], 2.32 [0.29] μg/ml), and from 1.62 to 26.41 ng/ml for resistin (mean [S.E.M.], 7.26 [1.21] ng/ml). When gender-specific differences in these levels were examined, only SF level of leptin in the female group was found to be significantly higher than that in the male group (Table I).

Beside leptin, sOb-R could be determined in each SF sample. Mean [S.E.M.] level of the sOb-R was higher in women compared with men, but the difference did not reach statistical significance (Table I).

Table I
Concentrations of adipokines (leptin, adiponectin and resistin) and sOb-R in SF obtained from patients with OA

	Female	Male
n	20	15
Age (years)	71.05 (7.41)	65.80 (7.74)
BMI (kg/m^2)	28.20 (4.28)	30.13 (5.09)
Leptin (ng/ml)	20.77 (3.61)	9.49 (2.14)*
Adiponectin (μg/ml)	2.34 (0.37)	2.31 (0.47)
Resistin (ng/ml)	8.47 (1.75)	5.73 (1.58)
sOb-R (ng/ml)	7.33 (1.15)	4.85 (1.00)

Age and BMI are shown as mean values (SD). Concentrations values are expressed as mean values (s.e.m.). Comparisons between genders were performed using the Mann−Whitney *U* test and $P < 0.05$ was considered significant (*).

COMPARISON BETWEEN SERUM AND SF LEVELS

Similar analysis were performed with the paired samples of serum and SF collected at the same time from 25 patients with OA (14 women and 11 men). Our study revealed a significant difference between the two body fluid compartments with higher concentrations of resistin, adiponectin and sOb-R in serum samples than in the SF counterparts (Table II). By contrast, leptin concentrations in SF exceeded those in the paired serum, except for four patients (one woman and three men). Consistently, mean [s.e.m.] leptin level was significantly higher in SF obtained from female patients than that measured in paired serum specimens, while mean [s.e.m.] leptin level was similar in both biological fluids collected from male patients (Table II). Interestingly, the difference between SF and serum levels increased when the molar ratio leptin/sOb-R was used as an index of free leptin. As illustrated in Fig. 1, leptin in serum displayed a 1.5- and a 4.5-fold molar excess vs corresponding sOb-R in both male and female groups, respectively. These molar excess increased significantly in SF for both male and female OA patients (mean [s.e.m.], 6.22 [1.15] for men and 13.10 [2.14] for women) due mainly to the low SF sOb-R level in the joint space (Fig. 1). The free leptin level was higher in women than in men, but the difference reached statistical significance in serum only ($P = 0.0128$ for serum and $P = 0.0634$ for SF).

The calculated Spearman's rank correlation indicated that SF levels of adipokines significantly correlated with the corresponding values in paired serum samples, except for resistin in the female group (Table II). Adipokines levels in both compartments correlated more closely in the male group compared to the female group. A relationship between SF and serum levels was also found when

paired samples obtained from male OA patients were analyzed for sOb-R ($r = 0.75$, $P = 0.0339$). However, we failed to demonstrate any significant correlation for female patients.

PRODUCTION OF ADIPOKINES BY JOINT TISSUES OBTAINED FROM OA PATIENTS

To determine whether adipokines found in the SF may derive from articular tissues, we examined the production of leptin, adiponectin and resistin by cultured explants obtained from 12 patients. Specimens of synovium, infrapatellar fat pad, meniscus, osteophyte, cartilage and bone were cultured in serum-free medium for 48 h.

As illustrated in Fig. 2, each joint tissue studied including cartilage released adiponectin and leptin. Conversely, the conditioned media from *ex vivo* cultured specimens did not contain detectable amount of resistin. The production of leptin and adiponectin was quite variable depending on the tissue and ranged from 0.54 to 22.05 pg/mg of tissue for leptin and from 22.65 to 522.31 pg/mg of tissue for adiponectin. Synovium and infrapatellar fat pad have been shown to be the main sources of adipokines in the joint. Surprisingly, osteophytes released larger amounts of leptin than did synovium or infrapatellar fat pad, so that the leptin/adiponectin ratio was high compared to the other articular tissues [Fig. 2(c)].

Discussion

In order to better evaluate the local contribution of leptin, and several other adipokines, to the changes associated with OA, we explored the relation between SF and serum levels of adipokines in human OA, and the potential biological activity of leptin in the joint by determining the level of its soluble receptor in SF. Besides, we identified the articular sources of leptin, adiponectin and resistin.

Since females exhibited higher levels of serum leptin than males[36] and a larger predisposition toward OA[1,37], OA patients were separated by gender. No significant difference between males and females was observed with respect to age or BMI. Our data indicated that the three adipokines are present in the SF of OA patients with concentrations falling within the range of values determined by Schäffler *et al.* for adiponectin and resistin[26]. Except for adiponectin, adipokines concentrations were more elevated in SF from women compared with men, especially for leptin. This gender-specific difference cannot be only explained by the difference in serum level, since the SF/serum ratio was shown to be different between men and women (data not shown).

Table II
Levels of the adipokines (leptin, adiponectin and resistin) and sOb-R in paired samples of serum and SF from patients with OA: correlation between serum and SF levels

	Female (n = 14)			Male (n = 11)		
	Serum	SF	r	Serum	SF	r
Leptin (ng/ml)	14.39 (2.71)	17.57 (2.75)#	0.789 ($P = 0.0044$)	8.56 (2.66)*	9.73 (2.87)*	0.936 ($P = 0.0031$)
Adiponectin (μg/ml)	11.04 (1.62)	1.59 (0.25)#	0.559 ($P = 0.0437$)	8.68 (1.44)	2.23 (0.60)#	0.924 ($P = 0.0056$)
Resistin (ng/ml)	10.09 (0.98)	5.51 (1.45)#	−0.077 (NS)	14.01 (1.69)	3.67 (0.59)#	0.816 ($P = 0.0099$)
sOb-R (ng/ml)	17.38 (3.79)	7.81 (1.11)#	0.483 (NS)	14.47 (4.43)	4.34 (1.27)#	0.750 ($P = 0.0339$)

For serum and SF levels, results are expressed as mean values (s.e.m.). Statistical significance between matched pairs of SF and serum was determined using the Wilcoxon rank test (#). Comparisons between genders were performed using the Mann−Whitney *U* test (*). Correlations between SF levels and serum levels were calculated by Spearman's correlation analysis (*r*, correlation coefficient with *P* value of correlation). $P < 0.05$ was considered significant. NS: not significant ($P \geq 0.05$).
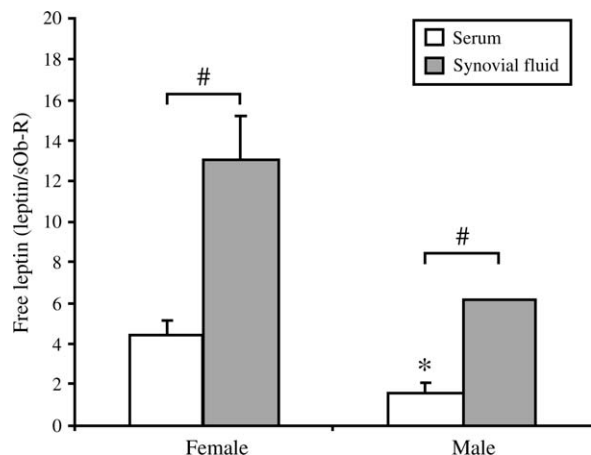
Fig. 1. Free leptin levels in paired samples of serum and SF obtained from female and male patients with OA. The molar ratio leptin/sOb-R was used as an index of free leptin. The Wilcoxon test was used to analyze difference in free leptin levels between serum and SF within the patient (#). The Mann–Whitney U test was used to test for statistically significant gender-specific difference in both body fluid compartments (*). $P < 0.05$ was considered significant.

When paired samples of SF and serum were examined, the adipokines exhibited different patterns of distribution between the joint and the circulating compartment. Serum levels of resistin and adiponectin exceeded those in the paired SF. Leptin contrasted with the other adipokines in that SF concentrations were higher than the serum counterparts in female patients, and were similar to serum corresponding values in male patients. These findings provide evidence for a specific local dysregulation of adipokines in the OA joint space and suggest that circulating levels of adipokines do not represent the situation in the joint.

Several works indicated that the soluble isoform of leptin receptor may determine the biological activity of this adipokine[33,34]. For the first time, sOb-R has been detected in SF obtained from patients with OA, but at a low level compared to serum values. The lower SF level of sOb-R in the female group compared to the male group, and the lack of any correlation between SF and serum levels in female OA patients suggest that sOb-R undergoes a specific regulation in female OA patients with either an increase of its expression in joint tissues or a proteolytic cleavage of the membrane-associated receptor, especially through metalloproteinases[38]. The high level of leptin associated with a decline in sOb-R level in the joint compartment led to a large rise in the SF bioavailable leptin. The presence of high level of bioactive leptin in the joint from OA patients may have pathophysiological implications, more especially as leptin was shown to increase nitric oxide production in chondrocytes stimulated with interleukin-1[32]. This free radical is known to interfere with chondrocytes functions resulting in loss of cartilage matrix through induction of apoptosis, activation of metalloproteinases or inhibition of proteoglycan and type II collagen synthesis[39]. The elevated level of bioactive free form of leptin in SF from female OA patients compared to male patients strengthens the hypothesis that obesity and female sex are both risk factors for OA that may be attributable to local rather than systemic biosynthesis of this adipokine[37].

The high SF level of leptin compared to serum may be due to the increased permeability of inflamed synovial membrane[40,41]. However, the SF level of resistin was low
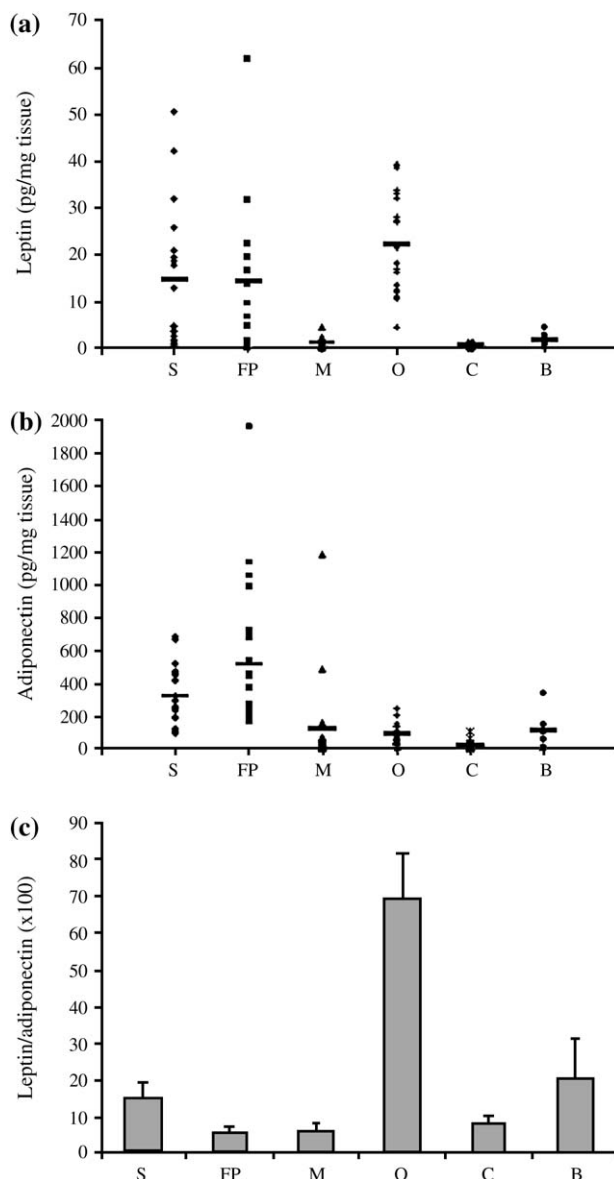

Fig. 2. Leptin (a) and adiponectin (b), and molar ratio leptin/adiponectin (c) produced by various cultured joint tissues obtained from 12 patients with OA. Explants of synovium (S), infrapatellar fat pad (FP), meniscus (M), osteophyte (O), cartilage (C) and bone (B) were precultured in serum-free medium for 48 h. Subsequently, the medium was changed and the release of adipokines was examined after a further 48 h incubation. Values are the mean of three paired replications for adipokines release by explants from the same individuals and the medians are indicated in (a) and (b).

compared to the corresponding value in serum. Since both adipokines have a similar molecular weight, the high SF/serum ratio of leptin suggests a local production in the joint. The present study indicated that various tissues obtained from OA-affected joints synthesized leptin. Synovium and infrapatellar fat pad, which has been recently found to be a source of cytokines in the knee[8], released large amounts of leptin. However, osteophytes was shown to represent the major source of intra-articular leptin while adiponectin production in osteophytes was similar to the other joint tissues. These data are in agreement with our previous study reporting a high level of expression of this adipokine

in osteophytes[25] and further support the contribution of leptin in the formation of these hypertrophic bone structures.

The present study revealed that the SF has low level of adiponectin compared to the serum counterpart. The transport of adiponectin across the synovial membrane barrier may be limited by its molecular weight (28 kDa), even more by its presence as two complexes in serum, as a hexamer and a higher order complex of between 12 and 18 subunits[42]. In fact, adiponectin found in SF may derive from joint tissues, mainly synovium and infrapatellar fat pad. We demonstrated for the first time that cartilage from OA-affected joints released adiponectin suggesting that, in addition to leptin, this adipokine may modulate chondrocyte functions. An imbalance between the rate of its intra-articular production and catabolism may explain the intra-articular deficiency of adiponectin. The weak correlation between SF level of adiponectin and the corresponding value in paired serum samples, especially in women, supports the view that adiponectin undergoes specific metabolic pathways in the joint.

As was shown for adiponectin, resistin was found in larger amount in serum than in SF. In male OA patients, there was a significant correlation between resistin levels in both compartments, indicating that the intra-articular resistin derived mostly from the blood. The SF/serum ratio of resistin levels was shown to be significantly higher in women than in men, and synovial resistin level failed to correlate with serum level in female OA patients. Taken together, these findings suggest that resistin is also produced locally in the joint from female OA patients. The lack of any detection for resistin in conditioned media of cultured explants may be due to a low sensitivity of the ELISA kit. SF levels of leptin and resistin were in the same order of magnitude while the minimum detectable dose of resistin was 8-fold higher than that of leptin. The high levels of resistin found in homogenized tissues of synovium and fat pad from OA-affected joints (data not shown) suggest that the experimental conditions used for *ex vivo* explants culture may be not suitable for resistin synthesis.

In conclusion, leptin, adiponectin and resistin exhibited different patterns of distribution between the synovial and the blood compartments. Circulating levels of adipokines do not represent the situation in the joint space, implying that adipokines may have local effects on articular tissues. Leptin contrasted with the other adipokines in that its level in the joint fluid exceeded that determined in serum, probably because of a local production. Additionally, the sOb-R deficiency observed in the SF led to a marked rise in the free form of leptin, increasing therefore the difference between serum and SF levels of the bioactive leptin. Resistin and adiponectin were found in SF at lower concentrations compared with the serum counterpart. Joint tissues including cartilage released larger amounts of adiponectin than leptin, indicating that adiponectin found in SF may result from specific regulatory pathways in the joint space. The present study showed that the excess of free leptin and the adiponectin deficiency usually found in obesity is also observed in OA-affected joints, and may account for the development of OA. In addition, the differing local turnover of adipokines between female and male OA patients may explain why the increased risk for OA of the knee among overweight persons is stronger in women than in men[1,37]. It remains to determine whether these adipokines in SF are not only markers of changes associated with OA, but are also involved in the pathogenesis or perpetuation of the disease process. More especially, a better understanding of the high level of leptin production in osteophytes may provide new insights on the role of this adipokine in the structural and biochemical changes found in OA. Besides, as the infrapatellar fat pad has been shown to be the primary source of adipokines in the joint, further investigations are needed to characterize the contribution of this intra-articular adipose tissue to joint destruction during OA.

Acknowledgments

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