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**Regional Differences Between Perisynovial and Infrapatellar Adipose Tissue
Depots and Their Response to Class II and III Obesity in Patients with OA**

Natalia S. Harasymowicz, PhD

Nick D.Clement, PhD, FRCS (Ed)

Asim Azfer, PhD

Richard Burnett, FRCS (Ed)

Donald M. Salter, MD FRCPATH Professor

A. Hamish W.R. Simpson, DM(Oxon), MA(Cantab), FRCS (Eng & Ed),
Professor

Corresponding address

University of Edinburgh, Orthopaedics and Trauma Surgery, Chancellor's
Building, 49 Little France Crescent, EH16 4SB, Edinburgh, UK
tel. 004401312426295, fax. 04401312426644, corresponding email:
n.harasymowicz@gmail.com

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Response of Osteoarthritic Knee Adipose Tissue Deposits to Obesity

Abstract

Objective: Obesity is associated with an increased risk of developing Osteoarthritis (OA), which is postulated to be secondary to inflammation from adipose tissue (AT). There are peri-articular adipose depots in synovial joints, the association of this tissue with OA has not been extensively explored. The aim of this study was to investigate differences in local AT depots in knees with OA and changes related to class II and III obesity.

Methods: Synovium and the infrapatellar fat pad (IPFP) were collected during total knee replacement from 69 patients with end-stage OA. The histological changes, adiponectin, PPAR γ and TLR4 expression, and immune cell infiltration into AT, were investigated.

Results: IPFP and synovium AT depots differed significantly and were influenced by the patient's BMI. In obese patients' adipocytes from IPFP were significantly larger while in the synovium, there was marked fibrosis, macrophage infiltration and higher TLR4 expression identified in comparison to lean subjects. Adipose related markers: PPAR γ (in IPFP) and adiponectin and PPAR γ (in synovium) were expressed at lower levels in obese compared to lean subjects. Furthermore, there was an increased number of CD45⁺ hematopoietic cells, CD45⁺CD14⁺, and CD14⁺CD206⁺ macrophages in both tissues in obese patients.

Conclusions: These differences suggest that IPFP and synovium may contain two different white AT depots and supports the theory of inflammatory induced OA in class II and III obese patients. This warrant further investigation as a potentially reversible, or at least suppressible cause of OA in obese patients.

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The prevalence of obesity (Body Mass Index (BMI) of 30 or greater) has significantly increased within developed societies over the last decade. It has been estimated that more than 50% of US adults will be obese by 2030 (1). Obesity, characterized by an excess of white adipose tissue due to an imbalance between calories consumed and expended, is associated with an increased risk of a number of diseases including type 2 diabetes mellitus (T2DM), atherosclerosis (2), and some types of cancer (3). There is also a recognized association between obesity and osteoarthritis (OA) (4) with obese individuals having a higher risk of developing OA of both weight bearing and non-weight bearing joints.

Several mechanisms have been postulated by which obesity may initiate or accelerate the progression of OA. Biomechanical factors are likely to be important as weight bearing joints are overloaded, however, the increased incidence of OA in non-weight-bearing joints indicates that other factors are involved. It has been postulated that venous outlet obstruction in the subchondral bone might impair nutrient supply making articular cartilage more susceptible to damage (5). It is now becoming clear, however, that adipose tissue may create a systemic inflammatory milieu by releasing various cytokines and adipokines, which have the potential to damage articular cartilage directly (6). Furthermore, increasing BMI correlates with a higher content of free glycosaminoglycans (GAGs) in synovial fluid (7) and both hypercholesterolemia and hypertriglyceridemia have been hypothesized to interfere with cartilage metabolism (8).

White adipose tissue depots are predominantly present in subcutaneous sites as subcutaneous adipose tissue (SAT) or within the abdomen as omental/visceral adipose tissue (VAT). In a healthy subject of normal weight, the majority of adipose tissue (up to 80%) is represented by SAT and only 10-20% by VAT. These depots differ in a number of features such as adipocyte cell size, blood

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vessel density and immune cell content (9, 10). Adiponectin and peroxisome proliferator-activated receptor gamma (PPAR γ) are two important adipose tissue-related proteins. Adiponectin is a secretory protein which is postulated to have anti-atherogenic and anti-inflammatory properties (11). PPAR γ is a nuclear receptor crucial for the differentiation of mesenchymal stem cells into mature adipocytes and is important in fatty acids and glucose metabolism.

The healthy adult adipose tissue is dynamic and changes its size and function depending on incoming energy. Extracellular matrix (ECM) remodeling is essential to allow rapid adipose tissue responses to a nutrient deficiency or surplus. As such, there is constant matrix remodeling, with a balance between ECM production and degradation. In obesity, hypertrophic adipocytes which are unable to store additional triglycerides start a process of lipolysis with associated tissue fibrosis (12). These events lead to enhanced free fatty acid release. These free fatty acids may accumulate in other tissues such as the liver, skeletal muscle or joints with consequent organ lipotoxicity (13, 14).

Impaired adipocyte function in fat depots, in turn, leads to adipocyte necrosis which triggers an influx of pro-inflammatory cells, including hematopoietic cells such as macrophages, mast cells, and subtypes of T-cell (15). Significantly, up to 30% of upregulated genes in adipose tissue in obesity are related to macrophage function (16). Increased levels of circulating saturated fatty acids, potent agonists of Toll-like receptors (TLR) including TLR2 and TLR4, may also contribute to the production of a range of pro-inflammatory and pro-fibrosis mediators recognized as being potential targets in OA pathogenesis (17).

In synovial joints, adipose tissue is present in the sub-synoviocyte intimal layer and in periarticular depots such as the infrapatellar fat pad (IPFP) of the knee

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joint. The physiological role of these fat deposits and their origin are not well described. It has been suggested that IPFP is similar in type to that of SAT deposits (18) due to the fibrous tissue surrounding adipocytes, whereas others support a similarity of IPFP to VAT (19).

The aim of this study was to ascertain whether there were differences in the adipose tissue depots of knee joints of obese and non-obese patients who are undergoing arthroplasty for end stage OA, in respect to characteristics of SAT and VAT and their potential to act as sites of local pro-inflammatory mediator production that might contribute to OA progression.

Materials and Methods

Sample collection

Ethical approval was granted for the study by Lothian Research Ethics Committee and NHS Lothian Research and Development Management, allowing patients undergoing primary total knee replacement (TKR) surgery for OA to be consented (n=69) for the use of surgical discard tissue for this study. Patients were divided into a lean group (with BMI<25) referred to here as Lean and patients in class II and III obesity (with BMI of 35 or more) are referred to here as Obese. The analyzed samples were isolated by the same surgeon with the same incision technique of harvesting synovial punch from the suprapatellar synovial region and IPFP from the infrapatellar region. The synovial membrane was not separated from the adipose tissue. H&E sections for each patient were analyzed and samples with increased synovial membrane (SM) inflammation were not analyzed in this study. In general, samples from OA patients had no more than 10-15% of total SM per the whole tissue biopsy. Lower magnification and example

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of analyzed areas in histological assessments are presented in Supplementary Data.

Reagents:

All reagents were obtained from Sigma Aldrich (UK) unless otherwise stated. Antibodies for immunohistochemistry (IHC) were obtained from DAKO (UK) or otherwise stated. Flow Cytometry antibodies were obtained from ImmunoTool (Germany). Cell culture reagents were from Life Technologies (UK). Antibody concentration and negative controls summarized in Supplementary Data.

Histology, adipocyte area measurement, Picrosirius Red S staining

Tissues were fixed in 4% PFA, paraffin embedded and cut into 4 μm sections. Sections were deparaffinised, rehydrated and stained with H&E or Picrosirius Red S stain (PRS). Pictures from 10 different regions (representing 50-150 adipocytes) of each sample (n=12 per each group) were taken at a magnification 20x (for adipocyte size measurement) and 60x (for PRS) on a Nikon Eclipse E800 Microscope. Adipocyte size was then assessed by measuring the longest (a) and the shortest (b) diameters and using the mathematical formula $P = \pi \left(\frac{a}{2} \times \frac{b}{2} \right)$ for the area of an ellipse to calculate the area of the cell. For quantification of fibrosis pictures of PRS stained sections were converted into an 8-bit image with the exclusion of the background and the percentage area of fibrosis was calculated. For adipocyte size and area of fibrosis measurements ImageJ software was used.

Immunohistochemistry

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IHC was performed by incubating sections (n=10 per each group) with the primary antibody (anti-hCD45, anti-hCD206 R&D System, anti-hTLR4, Bioss) followed by a secondary antibody conjugated with HRP. Chromogenic substrate 3,3'-Diaminobenzidine (DAB) was used to develop color. Counterstaining was performed with Harris Haematoxylin.

Tissue explant culture and ELISA assays

Tissues (n=5 per each group) were cut into 1-3mm³ fragments, weighed and cultured for 24 hours in Iscove's Modified Dulbecco Medium (IMDM) supplemented with 10% Fetal Bovine Serum (FBS) and Pen/Strep. Explants in duplicates were then cultivated for 3 days in serum-free IMDM with Pen/Strep thereafter supernatants were collected and total adiponectin levels in the media assessed using Adiponectin/Arcp30 Quantikine ELISA kit (R&D System). The aforementioned kit allowed analysis of Total Adiponectin. The concentration of the protein produced was adjusted for the weight of the wet tissue explant (mg).

Western Blotting

Tissue samples (n=7 for each group) were snap frozen in liquid nitrogen, homogenized and protein concentration was measured by the Bradford Assay (Bio-Rad). 25µg of protein from each sample was subjected to 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membrane (Immobilon-FL, Millipore, MA) using Bio-Rad Wet Transfer system. Blots were blocked by Odyssey Blocking buffer (1:1 with PBS) and incubated with the appropriate antibody cocktail (mouse anti-hAdiponectin 1:500 Peprotech and rabbit anti hGAPDH 1:10000 Sigma Aldrich, or rabbit anti-hPPARG 1:1000, Santa Cruz and mouse anti-hβ-actin 1:10000, Bioss) diluted in Odyssey Blocking buffer (1:1 with PBST) overnight at 4°C. Immunoblots were

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visualized by incubation with fluorescent dye-conjugated secondary antibodies cocktail (anti-rabbit IRDYE800CW and anti-mouse IRDYE680RD both 1:10000, LiCor) and scanned under infrared Western Blot detection system Odyssey FC® Imaging System. For semi-quantitative analysis of protein expression densitometry of bands on Western Blots were performed using Image Studio Lite 3.1 software. Denaturing conditions allowed the analysis of Total Adiponectin in the investigated blots.

RNA isolation, cDNA synthesis, and Real-Time qPCR

RNA from IPFP and synovium samples (n=7 per each group) was obtained using a combined Qiazol and Qiagen Mini RNA isolation kit technique. RNA stability was established by gel electrophoresis and 2 bands (28sRNA and 18sRNA) were detected. RNA purity was assessed by NanoDrop absorbance measurement with 260/280 and 260/230 ratio >1.9. 1 µg of RNA was treated with DNase I (Invitrogen) and reverse transcribed by iSCRIPT (Bio-Rad) as per the suppliers' manual. Real Time PCR was conducted on Lightcycler 96 (Roche). 10ng of cDNA was analyzed. The primer concentration was 10µM. The reactions were performed in triplicates for each analyzed gene. Reactions using SYBRGreen chemistry were also subjected to melting curve analysis. Each gene amplification curve and efficiency was validated and ranged from 95-105%. Target gene expression was normalized with the reference gene β 2-microglobulin (β 2M). Relative mRNA expression was assessed by $-\Delta\Delta$ CT method with the lowest Ct value served as a calibrator (Livak et al method (20)). The lean sample value was used as the reference level and set at 1 in order to analyze fold change.

Used primer sequences:

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ADIPOQ Forward GCA TTC AGT GTG GGA TTG GA Reverse TAA AGC
GAA TGG GCA TGT TG product size 80kb;

PPAR γ Forward GCT GTG CAG GAG ATC ACA GA Reverse GGG CTC CAT
AAA GTC ACC AA product size 225kb;

TLR4 Forward AGC TGT ACC GCC TTC TCA GC Reverse CCT GCC AAT
TGC ATC CTG TA product size 152kb;

β 2M Forward TGT GCT CGC GCT ACT CTC TC Reverse CCA TTC TCT GCT
GGA TGA CG product size 90kb.

Flow cytometry

The stromovascular fraction (SVF) from each tissue explant was obtained as follows: 1-3 g of tissue was intensively washed with sterile PBS and minced into 1-2 mm³ pieces and then incubated for 1.5 h at 37°C in 2 mg/ml of collagenase I and II in 0.5% BSA in Hanks' Balanced Salt Solution (HBSS) with Ca²⁺ and Mg²⁺. The cell fraction was filtered and lysed by Red Cell Lysis Buffer (Millipore). Cells were then incubated for 30 min at 4°C with anti-hCD-45, anti-hCD14, and anti-hCD206, antibody or isotype control (1:100 dilution, ImmunoTool) and TLR-4 (1:100 dilution Biolegend) in the dark and analyzed by flow cytometry (Beckman Coulter XL). The gating strategy is shown in Supplementary Data.

Statistical analyses

All statistical analysis was performed using GraphPad (Prism 6.0, USA). Mann-Whitney U test and Wilcoxon matched paired test were used to compare linear variables between groups and Spearman rho correlation was used to assess the

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association between non-parametric linear variables. A Mann-Whitney U test was performed for linear variables between groups (lean versus obese) and a Wilcoxon matched paired analysis was used to assess differences in linear variables between anatomical sites in the same group (IPFP versus synovium from the same donor). A p-value of <0.05 was considered to indicate statistical significance.

Results

1. Obesity is associated with changes in adipocyte size and fibrosis in knee joint fat depots.

Histomorphometric analysis demonstrated differences in adipocyte size and fibrosis between knee joint fat depots in lean and obese individuals (Figure 1). The median size of adipocytes in the synovial adipose tissue of lean individuals ($5.52 \mu\text{m}^2$ IQR 3.41 to 8.21) was greater ($p=0.003$) than that of adipocytes in the IPFP ($4.89 \mu\text{m}^2$ IQR 3.00 to 7.87). This difference was reversed in obese patients where the median adipocyte size of adipocytes in the synovial adipose tissue ($5.75 \mu\text{m}^2$ IQR 3.29 to 9.03) was less than that of adipocytes in the IPFP ($6.54 \mu\text{m}^2$ IQR 3.82 to 9.00) ($p<0.0001$). Furthermore, there was no significant difference in the median adipocyte size in the synovium of lean and obese subjects ($p=0.17$) while in the IPFP, the mean adipocyte size was significantly higher in obese compared to lean patients ($p<0.0001$).

Analysis of pericellular fibrosis showed no differences in the synovial tissue compared to IPFP in lean individuals. Similarly, there was no difference seen between IPFP and synovium of obese individuals. However, there was a significantly higher area ($p<0.005$) of matrix deposition in the synovial adipose tissue of obese ($24.66\pm5.62\%$) compared to lean individuals ($14.44\pm5.21\%$)

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whereas, there was no significant difference observed in the IPFP (obese $20.91 \pm 8.1\%$, lean $16.89 \pm 3.35\%$). The mean adipocyte fibrosis was normalized to median adipocyte size in each patient to adjust for adipocyte size difference.

2. Obesity is associated with changes in adipose tissue-related genes and protein expression in knee joint fat depots.

Real Time quantitative PCR and WB analysis showed differential expression of adipose tissue-related genes and proteins in the synovium and IPFP in lean and obese individuals (Table 1 for qRT PCR and Figure 2 for WB and ELISA).

The expression of adiponectin gene (ADIPOQ) and protein was not different in paired synovium and IPFP samples from lean subjects but was significantly lower in synovium compared to IPFP from obese individuals ($p=0.02$ for qRT-PCR, $p=0.04$ for ELISA and $p=0.03$ for WB). Furthermore, synovium from lean patients expressed significantly higher adiponectin/ADIPOQ ($p=0.04$ for qRT-PCR, $p=0.03$ for ELISA and $p=0.004$ for WB) than obese subjects but there was no such difference in lean versus obese IPFP (Table 1 for qRT PCR and Figure 2 for WB and ELISA).

PPAR γ gene and protein expression analysis showed significantly lower expression in the synovium than in IPFP in lean ($p=0.02$ for qRT-PCR, $p=0.01$ for WB) but not obese patients. PPAR γ was also expressed at a significantly lower level in obese patients, both in IPFP ($p=0.02$ qRT-PCR, $p=0.03$ for WB) and synovium ($p=0.04$ qRT-PCR, $p=0.03$ for WB) when compared to lean individuals (Table 1 for qRT PCR and Figure 2 for WB).

3. CD45⁺ hematopoietic cells number and macrophage content of knee joint synovium and IPFP is increased with obesity.

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IHC and flow cytometry analyses showed that the number of CD45⁺ hematopoietic cells in the synovial adipose tissue of lean individuals ($18.7 \pm 6.9\%$) was greater ($p=0.03$) than IPFP ($15.02 \pm 6.7\%$) (Figure 3). CD45⁺ hematopoietic cells content was elevated in both IPFP ($19.4 \pm 3.4\%$) and synovium ($26.6 \pm 8.7\%$) in obese compared to lean subjects ($p=0.04$ for IPFP, $p=0.004$ synovium) but there was no difference between IPFP and synovium in obese subjects.

Flow cytometry profiling showed that frequency of CD45⁺CD14⁺ total macrophages ($8.2 \pm 3.5\%$ for IPFP, $8.84 \pm 2.15\%$ for synovium) and CD14⁺CD206⁺ M2-type macrophages ($6.8 \pm 2.2\%$ for IPFP and $5.7 \pm 1.6\%$ for synovium) was not different between synovium and IPFP of lean individuals (Figure 4). However, there was a greater percentage of CD45⁺CD14⁺ total macrophages ($10.6 \pm 4.1\%$ for IPFP and $12.5 \pm 2.8\%$ for synovium) and CD14⁺CD206⁺ M2-type macrophages ($8.9 \pm 3.2\%$ for IPFP and $9.5 \pm 2.6\%$ for synovium) in obese individuals both in IPFP ($p=0.02$) and synovium ($p=0.0001$) for both cell types. CD45⁺CD14⁺ total macrophages and CD14⁺CD206⁺ M2-type macrophages content correlated significantly with BMI score as summarized in Figure 4.

4. TLR4 expression is increased in the synovium in obesity and correlates with BMI.

Real Time PCR analysis showed that the TLR4 gene expression was not different in synovium and IPFP of lean individuals (Table 1). There was a significantly higher expression of TLR4 in the synovium of obese compared to lean subjects ($p=0.03$) however, no difference in TLR4 expression was seen in IPFP of lean and obese individuals or between obese IPFP and synovium. Flow cytometry analysis showed that the TLR4⁺ cell content did not differ between synovium ($4.7 \pm 2.3\%$) and IPFP ($5.5 \pm 4.7\%$) of lean individuals but was significantly ($p=0.009$) higher in the synovium ($9.6 \pm 3.7\%$) of obese compared to lean subjects (Figure 5A). TLR4⁺

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cell content correlated with BMI score (Figure 5C). There was no significant difference between lean ($5.54 \pm 4.73\%$) and obese IPFP ($9.0 \pm 3.81\%$) TLR4⁺ cell content and no correlation of TLR4⁺ cells with BMI in IPFP (Figure 5B).

Discussion

Obesity is a strong risk factor for the development of OA. The association of OA in obese patients in both load bearing and non-load bearing joints suggests that excessive or abnormal loading of joints does not explain this relationship entirely. As such a systemic/metabolic hypothesis has developed which supports the hypothesis that metabolic factors related to obesity act directly or indirectly on chondrocytes leading to the increased risk of developing OA (21). In the current study, we assessed whether there were any differences in the intraarticular fat deposits within the knee joint (namely synovium and IPFP) of lean and obese individuals undergoing arthroplasty which may indicate a local, in addition to a systemic, contribution development and progression of OA. We found that in lean individuals, the adipocytes were larger in synovial adipose tissue compared to IPFP. There was no difference in pericellular fibrosis or adiponectin expression but PPAR γ expression was significantly lower in synovium compared to IPFP in these patients. A significantly higher content of CD45⁺ hematopoietic cells was identified in synovium compared to IPFP but no differences in macrophage number (characterized by CD45⁺CD14⁺ and CD14⁺CD206⁺ expression) and TLR4⁺ expression in the fat deposits were seen in lean individuals. In obese subjects, adipocyte size was larger in IPFP and pericellular fibrosis was greater in synovial fat in comparison to lean individuals. Adiponectin and PPAR γ expression was lower while TLR4 expression was greater in synovium when compared to IPFP of these patients. In both IPFP and synovium of obese patients, there was a significantly higher content of CD45⁺ hematopoietic cells,

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CD45⁺CD14⁺ total macrophages, and CD14⁺CD206⁺ M2-type macrophages compared to lean subjects with OA.

A previous study identified the presence of CD86 (M1 type marker) and CD206 (M2 type marker) by IHC and Flow Cytometry in the IPFP, but did not find the influence of BMI on their number (22). A Recent study also found no differences in immune cell population between paired IPFP and synovium, however, these authors investigated individuals with mean BMI of 28.9 (no more than 32) and this may explain the contrasting results to the current study (23).

Studies of fat deposits in obesity indicate that, with weight gain, adipocytes in subcutaneous adipose tissue (SAT) increase in size significantly more than those in visceral adipose tissue (VAT) (9). The current study has demonstrated that in obese patients, adipocytes in the IPFP significantly increase in size whilst there is no change in the size of adipocytes in the synovium.

Fibrosis, a hallmark of metabolically dysfunctional white adipose with resident adipocytes being surrounded by a network of extracellular matrix is typical for the visceral fat sampled from obese patients (24). The current study, which has demonstrated an increase in adipocyte size in IPFP and pericellular fibrosis in synovial fat, supports the theory that synovial and IPFP adipose tissue depots may each represent a different subtype of white adipose tissue. The synovial adipose tissue demonstrates characteristics akin to those of VAT whereas the IPFP is more in keeping with SAT. Conversely, other authors have suggested a pro-fibrotic role of the IPFP, which is independent of the BMI index of the patient (25). The fibrosis and collagen deposition could have been quantified further in this study by using Picosirius Red staining with polarized light to evaluate birefringence.

Adiponectin has been postulated to be both pro-catabolic (26, 27) and pro-anabolic (28) in OA. As such the influence of adiponectin on the OA pathogenetic

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pathway is not clear. The adiponectin concentration has been reported to be elevated in the serum of OA patients compared to healthy individuals (29), however, levels in synovial fluid are significantly lower than those of peripheral blood in OA (30). However, adiponectin levels in synovial fluid have been shown to correlate with proteoglycan catabolism [35]. The IPFP has been shown to produce more adiponectin than that of subcutaneous depot fat from the same donor (6, 31) consistent with the observation of differential expression and production of adiponectin by IPFP and synovium in the current study. On the other hand, we did not find that adiponectin expression by IPFP correlated negatively with BMI of OA patients as suggested by other authors (32).

Differential expression of adiponectin has also been reported in various kinds of adipose tissue depots. SAT produces more adiponectin than VAT (33, 34) and during weight gain, a significant downregulation of adiponectin production in VAT but not in SAT has been reported (35). We observed a decrease in adiponectin gene expression and ex-vivo protein production by synovium but not in the IPFP of obese individuals. Although in the present study synovial membrane was not dissected from the adjacent adipose tissue, we hypothesize that the results obtained may indicate a difference in the origin of the two fatty tissue depots analyzed.

PPAR γ is a ligand-activated transcription factor that plays a key role in lipid homeostasis. Our data indicate that PPAR γ expression was significantly lower in both IPFP and synovium of obese patients. Such changes are likely to have a proinflammatory/pro-catabolic effect in the joint as PPAR γ activation in adipose tissue is associated with beneficial effects on the expression and secretion of a range of factors, including adiponectin by adipocytes and suppression of production of inflammatory mediators such as resistin, IL-6, and TNF α by macrophages (36). Notably, mice with a cartilage-specific PPAR γ knock-out

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develop spontaneous OA (37) indicating a role for this transcription factor in the regulation of chondrocyte function including inhibition of pro-catabolic pathways by supporting autophagy (38). PPAR γ agonists have potent anti-inflammatory and anti-catabolic activity when applied to articular chondrocytes and synovial fibroblasts (39) and have been shown to have some degree of efficacy in instability-induced OA models in guinea pigs and dogs (40, 41). Although effects of these agents have not yet been shown in human studies, the lower expression of PPAR γ in the tissues from the knee joint of obese patients indicate that targeted therapy for this subgroup of OA patients might be beneficial.

Leukocyte numbers increase with obesity, both in the circulation and in local adipose tissue depots and are correlated with such pro-inflammatory diseases as T2DM (42) and liver steatosis (43). Our flow cytometry and IHC data indicate that the frequency of hematopoietic cells was significantly higher in the synovium in comparison to the IPFP in lean OA patients with a further increase in CD45+ cells being seen with obesity suggesting a more pro-inflammatory profile of both tissues. The main hematopoietic cells present in adipose tissue are macrophages which are postulated to play an important role in obesity-related pro-inflammation and organ fibrosis (44). In the current study, we observed for the first time, an increased frequency of CD14+CD206+ M2-type macrophages in both knee joint synovial and IPFP deposits of obese patients with OA, which correlated significantly with BMI. CD206 is considered to be an anti-inflammatory macrophage marker. However, M2-type macrophages also play a role in pro-fibrotic processes during wound healing and are an important source of pro-fibrotic factor TGF- β (45). This is the very interesting future goal and limitation of the current study to determine TGF- β expression in fatty tissue deposits in obesity driven OA.

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The expression of TLR4 and the number of TLR4 positive cells expression were significantly increased in the knee joint synovium in obese individuals in comparison to lean patients in the current study. In keeping with this, our IHC analysis of TLR4 indicated that it was expressed at a significantly higher level in the synovial adipose tissue of obese patients compared to lean ones. In humans, TLR4 expression is upregulated in obese human VAT but not in SAT (46) consistent with our premise that synovial fat deposits in the knee have characteristics of VAT. TLR4 plays a crucial role the accumulation of macrophages in adipose tissue of obese patients (47). TLR4 ligands activate fibroblasts and promote their differentiation into collagen-producing cells (48). Moreover, in adipose tissue, it is proposed that interactions between adipocytes and macrophages through TLR4/MD-2 aggravates adipose tissue inflammation (49) and possibly by such mechanisms could potentiate OA disease progression.

The analysis of the end-stage OA, the exclusion of patients with a BMI of between 30 and 35 and no separation of synovial membrane from adipose tissue are potential limitations of this study. However, recent studies and observations suggest that BMI is not a perfect index. People classified as obese with BMI index of 30 have been shown to have the same or even a better health profile compared to those with normal weight (50). A further limitation was the effect of comorbidities, such as T2DM, which given the number of patients and subgroups it was not possible to evaluate fully in the current study.

Conclusion.

The findings of this study show that there are significant differences between synovial and IPFP adipose tissue in patients undergoing joint arthroplasty for OA which support the theory that synovial fat has characteristics of VAT whilst IPFP is more akin to SAT. Obesity is associated with changes in these fat depots, with

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synovial fat in particular showing changes typical of VAT, which is likely to be associated with a pro-inflammatory and catabolic phenotype. Nevertheless, there is an increased number of CD45⁺ hematopoietic cells and CD45⁺CD14⁺ and CD14⁺CD206⁺ macrophages in both adipose tissue depots in obese patients which supports the idea that in addition to biomechanical factors, local inflammatory produced mediators probably contribute to OA in obesity. Targeting this adipose dependent inflammation by novel therapies, such as PPAR γ agonists, may have a benefit and delay OA progression in obese patients.

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Tables:

Table 1 Quantitative PCR analysis of ADIPOQ, PPARG and TLR4 gene expression in IPFP and synovium obtained from OA patients (n=7 for each group). $\beta 2M$ was used to normalize gene expression. $-\Delta\Delta CT$ method was used to investigate fold change in gene expression. The control values (from lean IPFP patients) were expressed as 1 to indicate a precise fold change value ($\pm SEM$) for each gene of interest. p values < 0.05 were considered significant and indicated (a) for p<0.05 in paired analysis (b) p<0.05 in obese vs lean analysis, NS- not significant.

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Figures:

Figure 1 Representative H&E (A) and Picrosirius Red S (B) staining of adipose tissue depots from IPFP and synovium (n=10 for each group). Differences per the group and anatomical location are illustrated for median cell size (C). Fibrosis percentage normalized to the adipocyte size (D). The mean percentage/median μm^2 adipocyte for group \pm SD are presented. p values<0.05 indicated as *, p values<0.001 indicated as ***, NS-not significant.

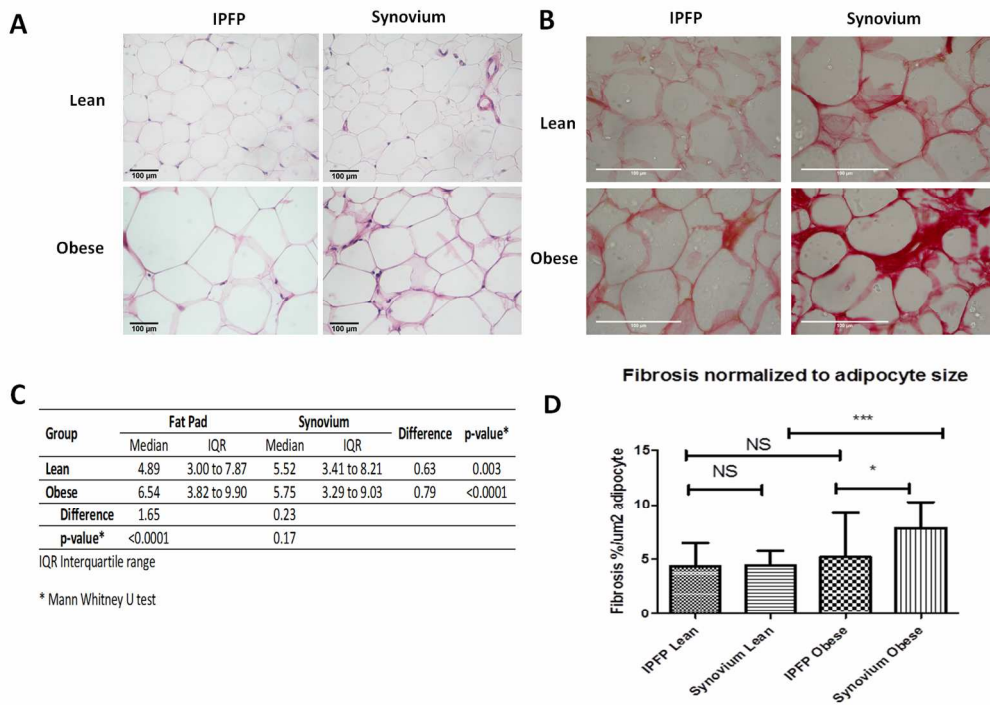
Figure 2 A representative WB blot of Adiponectin (A) and PPARG (B) protein expression in IPFP and synovium of OA patients. Semi-quantitative WB analysis of Total Adiponectin (C) and PPAR γ (D) expression in IPFP and synovium from OA patients. Densitometry data is presented as the mean value of the ratios of Adiponectin to GAPDH and PPAR γ to β -actin expression for each group \pm SD (n=7 per each group). Ex vivo adiponectin release by IPFP and synovium from OA patients (C). p values < 0.05 were considered significant and indicated (a) for p<0.05 in paired analysis (b) p<0.05 in obese vs lean analysis, NS- not significant.

Figure 3 CD45⁺ hematopoietic cells expression in IPFP and synovium from OA patients. Flow cytometry (A) and IHC (B) analysis presented. Stromovascular (SVF) fractions from IPFP and synovium were analyzed according to CD45⁺ expression. The isotype control was analyzed at the same time with the sample of interest (Gating strategy shown in Supplementary Data). The mean percentage of hematopoietic cells for the groups \pm SD is shown (A). p value<0.05 indicated as *, p value<0.001 indicated as *** NS- not significant. Representative IHC staining for CD45 marker in IPFP and synovium from Lean and Obese (B) OA patients. Scale bars represents 250 μm (outer pictures) and 50 μm (inner pictures).

Response of Osteoarthritic Knee Adipose Tissue Deposits to Obesity

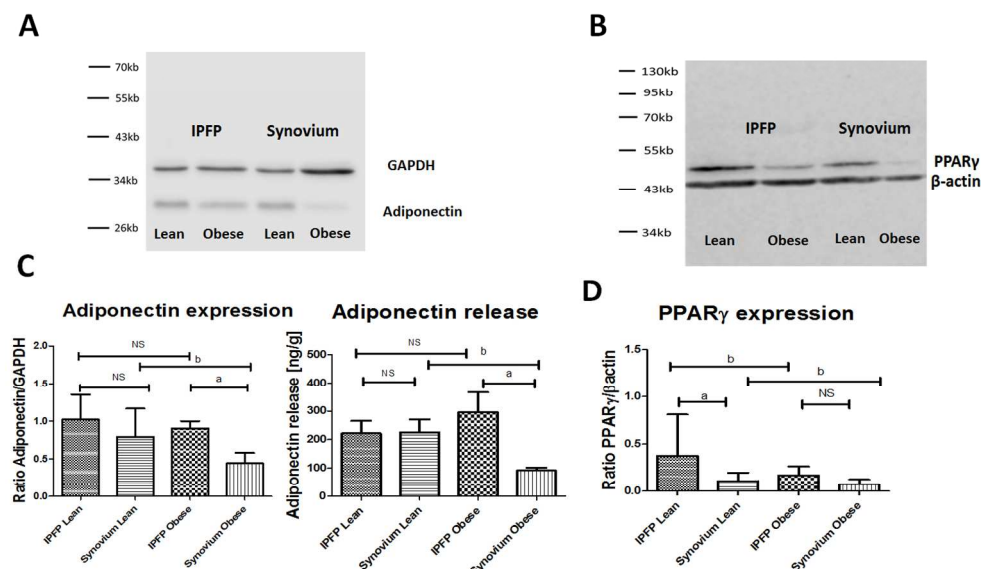
Figure 4 Percentage of CD45⁺CD14⁺, CD14⁺CD206⁺ in SVF fraction of paired synovium and IPFP from lean and obese OA patients (A, B). Mean percentages for the group \pm SD were presented (A,B) n=10 (Lean) and n=14 (Obese) in CD45⁺CD14⁺ detection, n=10 (Lean) n=14 (Obese) in CD14⁺CD206⁺ detection. Correlation of percentage of CD45⁺CD14⁺ and CD14⁺CD206⁺ with BMI score in all obtained tissues synovium (n=33) and IPFP (n=26) (A,B). p-value <0.05 indicated as *, p value<0.005 indicated as **, p value<0.001 indicated as *** NS- not significant. Representative IHC staining for CD206 in IPFP and synovium from Lean and Obese OA patients (C).

Figure 5 Percentage of TLR4⁺ cells in SVF fraction of paired synovium and IPFP from lean and obese OA patients was shown (A). Mean percentages for the group \pm SD n=5 (Lean) and n=5 (Obese) OA patients were presented. Correlation of percentage of TLR4 with BMI score in all obtained tissues IPFP (n=15) (B) and synovium (n=15) (C). p-value <0.05 indicated as *. NS- not significant. Representative IHC staining shown (D).



Representative H&E (A) and Picrosirius Red S (B) staining of adipose tissue depots from IPFP and synovium (n=10 for each group). Differences per the group and anatomical location are illustrated for median cell size (C). Fibrosis percentage normalized to the adipocyte size (D). The mean percentage/median um2 adipocyte for group \pm SD are presented. p values<0.05 indicated as *, p values<0.001 indicated as ***, NS-not significant.

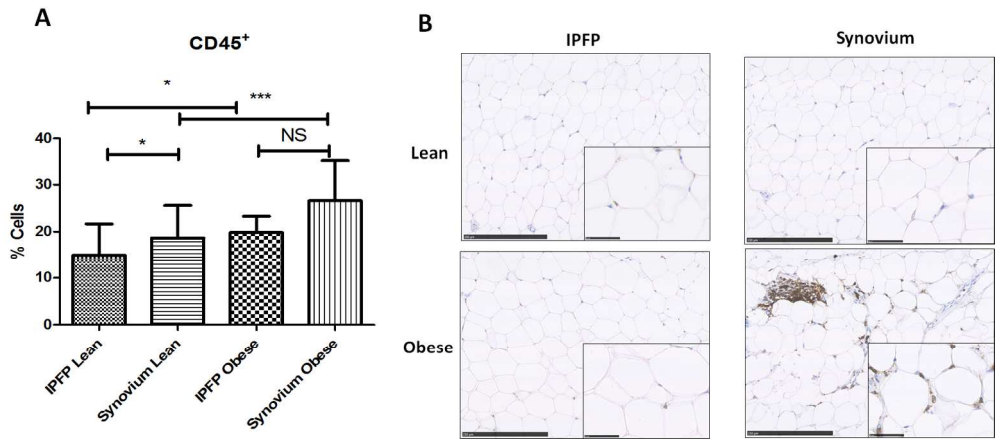
Figure 1
147x106mm (300 x 300 DPI)



A representative WB blot of Adiponectin (A) and PPAR γ (B) protein expression in IPFP and synovium of OA patients. Semi-quantitative WB analysis of Total Adiponectin (C) and PPAR γ (D) expression in IPFP and synovium from OA patients. Densitometry data is presented as the mean value of the ratios of Adiponectin to GAPDH and PPAR γ to β -actin expression for each group \pm SD ($n=7$ per each group). Ex vivo adiponectin release by IPFP and synovium from OA patients (C). p values < 0.05 were considered significant and indicated (a) for $p<0.05$ in paired analysis (b) $p<0.05$ in obese vs lean analysis, NS- not significant.

Figure 2

130x76mm (300 x 300 DPI)

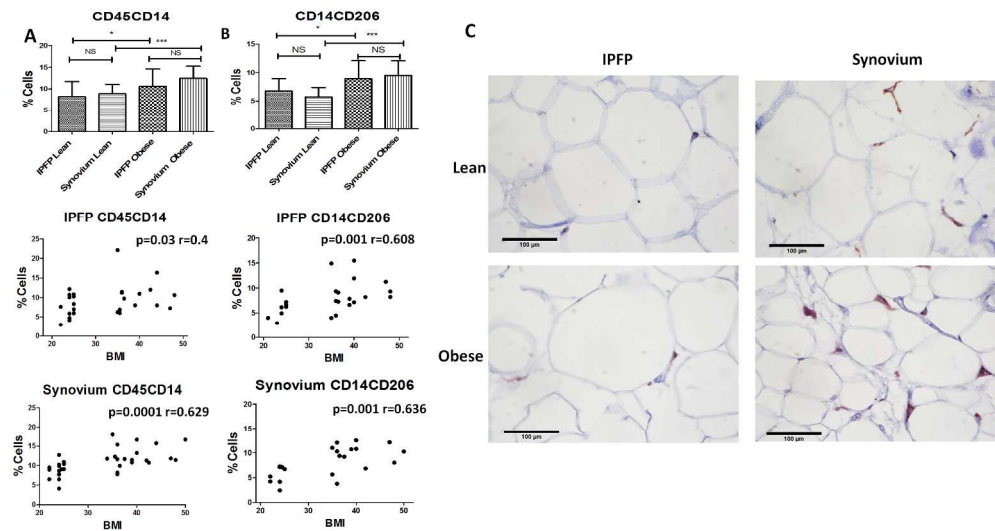


CD45+ hematopoietic cells expression in IPFP and synovium from OA patients. Flow cytometry (A) and IHC (B) analysis presented. Stromovascular (SVF) fractions from IPFP and synovium were analyzed according to CD45+ expression. The isotype control was analyzed at the same time with the sample of interest (Gating strategy shown in Supplementary Data). The mean percentage of hematopoietic cells for the groups \pm SD is shown (A). p value<0.05 indicated as *, p value<0.001 indicated as *** NS- not significant. Representative IHC staining for CD45 marker in IPFP and synovium from Lean and Obese (B) OA patients. Scale bars represents 250um (outer pictures) and 50um (inner pictures).

Figure 3

163x73mm (300 x 300 DPI)

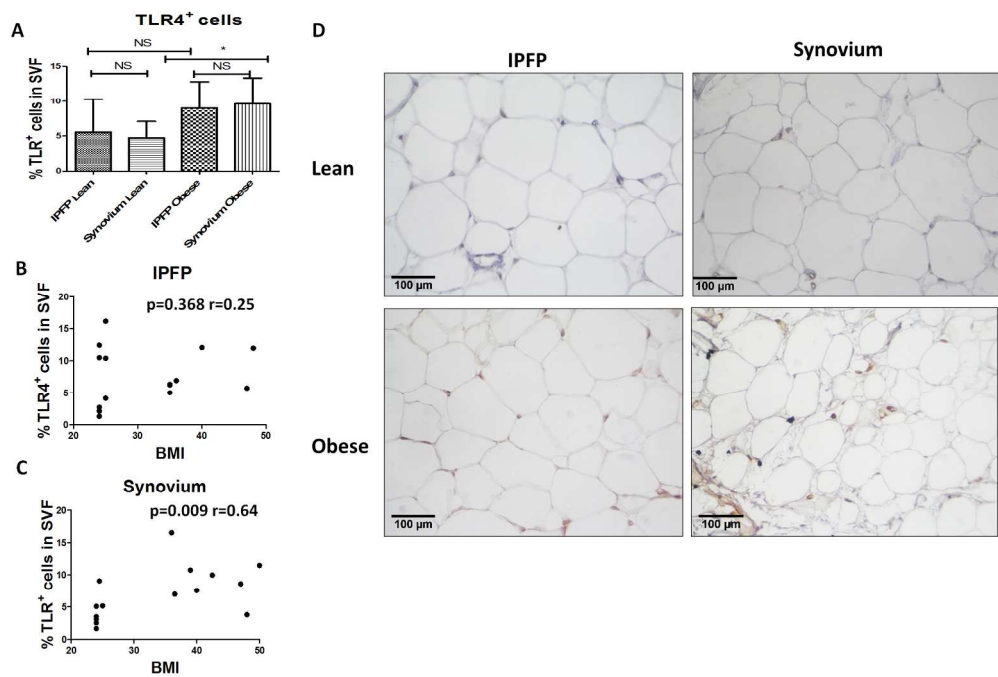
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Percentage of CD45+CD14+, CD14+CD206+ in SVF fraction of paired synovium and IPFP from lean and obese OA patients (A, B). Mean percentages for the group \pm SD were presented (A,B) $n=10$ (Lean) and $n=14$ (Obese) in CD45+CD14+ detection, $n=10$ (Lean) $n=14$ (Obese) in CD14+CD206+ detection. Correlation of percentage of CD45+CD14+ and CD14+CD206+ with BMI score in all obtained tissues synovium ($n=33$) and IPFP ($n=26$) (A,B). p -value <0.05 indicated as *, p value <0.005 indicated as **, p value <0.001 indicated as *** NS- not significant. Representative IHC staining for CD206 in IPFP and synovium from Lean and Obese OA patients (C).

Figure 4

258x138mm (300 x 300 DPI)



Percentage of TLR4+ cells in SVF fraction of paired synovium and IPFP from lean and obese OA patients was shown (A). Mean percentages for the group \pm SD $n=5$ (Lean) and $n=5$ (Obese) OA patients were presented. Correlation of percentage of TLR4 with BMI score in all obtained tissues IPFP ($n=15$) (B) and synovium ($n=15$) (C). p -value <0.05 indicated as *. NS- not significant. Representative IHC staining shown (D).

206x141mm (300 x 300 DPI)

Accepted

Table 1 ADIPOQ, PPARG and TLR4 gene expression analysis by Real Time qPCR in IPFP and synovium from Lean and Obese Patients with OA *

Fold Change (versus Lean IPFP)		ADIPOQ	PPARG	TLR4
IPFP	Lean	1 (± 0.75)	1 (± 0.43)	1 (± 0.13)
	Obese	0.74 (± 0.56) ^{a,NS}	0.35 (± 0.9) ^{a,b}	1.01 (± 0.12) ^{NS,NS}
Synovium	Lean	0.41 (± 0.51) ^{NS,b}	0.25 (± 0.53) ^{a,b}	1.85 (± 0.13) ^{NS,b}
	Obese	0.09 (± 0.04) ^{a,b}	0.06 (± 0.62) ^{a,b}	3.65 (± 0.14) ^{NS,b}

* a – $p < 0.05$ in paired analysis (tissues from the same donor), b – $p < 0.05$ unpaired analysis (in obese vs lean donor), NS-non significant