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TITOLO TESI

INFRAPATELLAR FAT PAD FEATURES IN OSTEOARTHRITIS: A HISTOPATHOLOGICAL AND MOLECULAR STUDY

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RIASSUNTO

Introduzione e scopo dello studio: L'osteoartrosi del ginocchio (KOA) è la maggiore causa di disabilità e dolore nella popolazione anziana. L'obesità è considerata il fattore di rischio modificabile più comune per l'insorgenza e la progressione della KOA. Sebbene i meccanismi che collegano le due patologie sono poco chiari, dati recenti suggeriscono un ruolo significativo mediato dal tessuto adiposo infrapatellare (IFP). Lo scopo di questo lavoro è quello di descrivere le caratteristiche istomorfologiche dell' IFP in soggetti affetti da KOA e confrontarle con quelle ottenute da soggetti sani, utilizzando un sistema di 'score' istologico. Inoltre, abbiamo valutato l'espressione delle varie adipocitochine nell' IFP di soggetti con KOA.

Materiali e Metodi: Sono stati arruolati 28 soggetti ($BMI 35,5 \pm 5 \text{ kg / m}^2$) sottoposti a sostituzione totale del ginocchio per KOA. Sono stati utilizzati come controlli, campioni di IFP con membrana sinoviale adiacente prelevati da 8 donatori del programma "Donarsi alla Scienza" dell'Istituto di Anatomia Umana presso l'Università di Padova. L'aspetto microscopico dell' IFP è stato analizzato con metodi istologici e morfometrici. È stato anche effettuato l'esame istologico della membrana sinoviale adiacente all'IFP. Abbiamo determinato l'espressione di mRNA utilizzando real time PCR quantitativa per le seguenti adipocine (leptina, adiponectina, Peroxisome proliferator-activated receptor gamma, Fatty acids binding protein 4) e citochine (interleuchina 6 [IL-6], fattore di necrosi tumorale alfa [TNF- α], Monocyte chemoattractant protein-1 [MCP-1], vascular endothelial growth factor [VEGF]) nei campioni dell'IFP dei soggetti affetti da KOA.

Risultati: I campioni di IFP hanno evidenziato caratteristiche microscopiche simili al tessuto adiposo bianco. L'IFP è organizzato in lobuli adiposi separati da setti fibrosi. Non sono state rilevate differenze nel diametro medio del lobulo adiposo dell'IFP sia nei soggetti affetti da KOA che nei controlli. L' infiltrazione Mononucleare è presente in 22 soggetti con KOA, mentre non è stata osservata in nessun IFP dei controlli ($p = 0.001$). Il numero medio dei vasi e lo spessore dei setti interlobulari sono significativamente più alti nei soggetti con KOA rispetto ai controlli ($p < 0.0001$ e $p = 0,004$ rispettivamente). Il BMI correlava positivamente con lo spessore dei setti interlobulari nell' IFP ($p = 0.02$, $r = 0.42$). Inoltre, l'infiltrazione linfocitaria e l'iperplasia sinoviale erano più accentuate nel KOA rispetto ai controlli ($p < 0.001$ e $p = 0.001$ rispettivamente). La membrana sinoviale era anche più vascolarizzata e fibrotica rispetto ai controlli ($p < 0.001$ e $p = 0.002$ rispettivamente). Per quanto riguarda l'analisi molecolari, l' IFP mostrava un'espressione dei geni tipiche del tessuto adiposo infiammato come IL-6, TNF- α , MCP-1 e VEGF. Inoltre, abbiamo osservato una correlazione positiva tra l'angiogenesi sinoviale e l'espressione genica del VEGF nell' IFP ($p = 0.04$).

Conclusioni: Il nostro studio è il primo che descrive le caratteristiche istopatologiche dell' IFP in soggetti prevalentemente sovrappeso/obesi affetti da KOA. L'IFP aveva un fenotipo patologico caratterizzato dall'alterazione a livello della dimensione del lobulo, dello spessore dei setti interlobulari, del grado di vascolarizzazione ed dell'infiltrato infiammatorio. Queste alterazioni strutturali sono state associate ad un'inflammazione a livello della membrana sinoviale adiacente. Inoltre, abbiamo aggiunto un'altra prova dell'esistenza di una probabile crosstalk tra citochine prodotte dall' IFP e la membrana sinoviale.

ABSTRACT

Background and aims: Knee osteoarthritis (KOA) is a common cause of disability and pain in adults. Obesity is increasingly recognized as the primary modifiable risk factor for the onset and progression of KOA. The mechanisms that link the two conditions are still not fully clarified but recent data suggest an important role mediated by the infrapatellar fat pad (IFP). Accordingly, we aimed to determine the histomorphological characteristics of IFP in individuals affected by KOA and compare them to those obtained from lean healthy individuals using a histological scoring system. Moreover, we determined the expression of certain adipocytokines in IFP of KOA subjects.

Materials and Methods: We enrolled 28 subjects (BMI 35.5 ± 5 kg/m²) undergoing total knee replacement for OA. The controls were represented by IFP and adjacent synovial membrane specimens sampled from bodies or bodies part of 8 healthy subjects without history of osteoarthritis involved in the Body Donation Program ‘Donation to Science’ held by the University of Padova. The microscopic anatomy of IFP was analyzed through histological and morphometrical methods. The histology of the synovial membrane adjacent to the IFP was also analyzed. We determined mRNA expression using Quantitative real time PCR for adipokines (leptin, adiponectin, Peroxisome proliferator-activated receptor gamma, fatty acid binding protein4) and cytokines (Interleukin 6 [IL-6], Tumor necrosis factor alfa [TNF- α], Monocyte chemoattractant protein-1 [MCP-1], Vascular endothelial growth factor [VEGF]) in the IFP specimens of KOA subjects.

Results: All the evaluated IFPs showed microscopical characteristics similar to white adipose tissue. IFPs were organized in adipose lobuli separated by fibrous septa. No differences were detected in the mean diameter of the adipose lobuli of the IFP in KOA patients and controls. Mononuclear infiltration was present in 22 KOA patients while it was not observed in any of the IFP used as control ($p = 0.001$). The average number of vessels and the thickness of interlobular septa were significantly higher in KOA patients compared to controls ($p < 0.0001$ and $p = 0.004$ respectively). BMI correlated positively with the thickness of the interlobular septa in IFP ($p = 0.02$, $r = 0.42$). Concerning synovial membrane, the presence of lymphocytic infiltration and hyperplasia was statistically higher in KOA compared to controls ($p < 0.001$ and $p = 0.001$ respectively) and it was more vascularized and fibrotic compared to controls ($p < 0.001$ and $p = 0.002$ respectively). Furthermore, in IFP samples it was noticed an expression of genes typical of inflamed adipose tissue such as IL-6, TNF- α , MCP-1 and VEGF. Moreover, we observed a positive correlation between the number of blood vessels of KOA synovial membrane and mRNA expression of VEGF in IFP ($p = 0.04$).

Conclusion: Our study describes for the first time the histopathological characteristics of IFP in a large cohort of patients with KOA. IFP showed pathologic structural changes in the lobule dimension, interlobular septa, vascularization and inflammatory infiltrate. These changes were associated with synovial inflammation. Moreover, we added evidence about the existence of a probable crosstalk between cytokines produced by IFP and the synovial membrane.

INTRODUCTION

1. Adipose organ: Insights into the anatomy and physiology

In mammals the adipose tissues are organized in a multi-depot organ called the ‘adipose organ’ (1). Although the adipose organ is mainly involved in the store and release of fat in response to energy balance needs, it also has immune, endocrine, regenerative, mechanical, and thermal functions (2). The adipocytes represent the main parenchymal cells of the adipose organ. Traditionally, these adipocytes are divided in two cytotypes, white and brown adipocytes, with distinctive anatomical and functional features (1, 3). These cells are organized in two tissues, white adipose tissue (WAT) and brown adipose tissue (BAT).

1.1. White and brown adipose tissues

White adipocyte is a large spherical cell which stores lipids in the form of unilocular droplet that occupy about 90% of cell volume and thereby determine the cell size which ranges from 10 to over 200 microns in diameter (4, 5). The role of WAT is to store excess dietary fat in the form of triglycerides and to release free fatty acids (FFAs) in times of starvation or energy demand. WAT is distributed in two anatomical compartments of the body: visceral (VAT) and subcutaneous (SAT). VAT surrounds the inner organs and can be divided in omental, mesenteric, retroperitoneal (surrounding the kidney), gonadal (attached to the uterus and ovaries in females and epididymis and testis in men), and pericardial. It has been thoroughly confirmed that the adipocytes of visceral fat tissue are

more lipolytically active and thus contribute more to the FFA levels (6, 7). Particularly, visceral fat depots, including omental and mesenteric adipose tissue, represent a risk factor for the development of cardiovascular disease and type 2 diabetes mellitus. SAT store >80% of total body fat in the body. The most commonly defined and studied subcutaneous depots are the abdominal, gluteal and femoral (8).

Brown adipocytes are smaller than white adipocytes, have multilocular fat droplets, a high content of mitochondria, and a high level of expression of uncoupling protein 1 (UCP1) in the inner membrane of mitochondria. UCP1 is a critical player in allowing electrons to be released rather than stored, resulting in heat release (9, 10).

Most brown fat cells originate from precursor cells in the embryonic mesoderm that also give rise to skeletal muscle cells and a subpopulation of white adipocytes (11, 12). In rodents, BAT is found in the interscapular, perirenal, and periaortic regions. In human infants, similar to rodents, BAT depots mainly reside in the interscapular and perirenal area and this depot gradually disappears with increasing age (13, 14). Human adults mainly have BAT in the cervical, supraclavicular, axillary and paravertebral areas as demonstrated by cold-induced ¹⁸F-fluorodeoxyglucose (¹⁸F-FDG) uptake assessed by positron emission tomography scan (PET-CT) (15, 16). It is well established that BAT is the major site of nonshivering thermogenesis in human and rodent models (17, 18). In 1979, Rothwell and Stock first reported that BAT was also activated in rodents when they overeat as a mechanism to preserve energy balance and limit weight gain so-called diet-induced thermogenesis (19). In humans, heat production by BAT, despite its low amount, contributes up to 15% of total energy expenditure (20). Given the effect of BAT on

overall energy balance, today this tissue is increasingly regarded as a novel therapeutical target to counteract obesity and associated comorbidities.

1.2. Plasticity of adipose organ

In the last years it has been extensively studied a novel form of fat cells called beige adipocytes (or brite, brown-like, inducible brown). These are defined as UCP1- expressing, multilocular adipocytes that appear in white (or predominantly white) fat depots (21, 22). Clusters of beige adipocytes can develop in WAT in response to various stimuli as cold acclimation (young 1984) and chronic treatment with β 3-adrenergic receptor activators (22). Despite beige and brown share similar thermogenic ability they are considered distinct cell types since beige cells, at least those in the mouse subcutaneous depot, do not derive from the same embryonic precursors (Myf5 (encoding myogenic factor 5)-expressing) that give rise to brown adipocytes (12). The origin of beige adipocytes residing in WAT still not fully clarified. Some studies have shown that they originate from the trans-differentiation of mature white adipocytes (24, 25) while others support the idea that beige adipocytes arise by *de novo* differentiation from a precursor population (26) Taking into account the scarcity of BAT in adult humans, WAT to BAT conversion as future treatment for obesity may represent a promising strategy to tackle obesity (27)

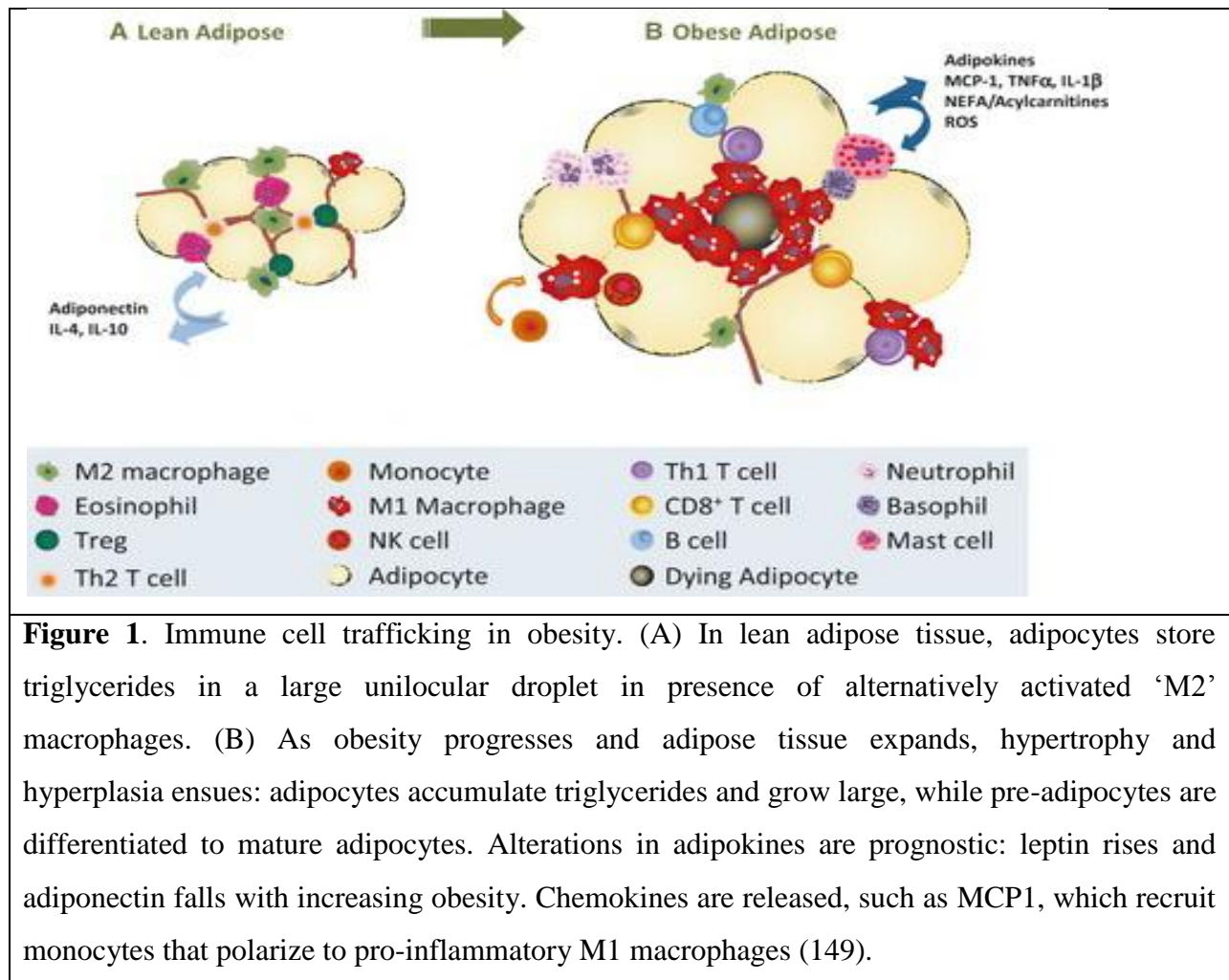
1.3. Adipose organ in obesity

Obesity is the epidemic of the 21st century. Owing to the modern sedentary lifestyle in both developed and rapidly developing countries, the prevalence of obesity has reached an alarming level, affecting over 500 million adults and 40 million children (28). Obesity is characterized by an abnormal and excess accumulation of adipose tissue in the body. Body mass index (BMI) is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m^2) and it is commonly used to define obesity in adults. The World Health Organization defined obesity by BMI greater than or equal to 30. Obesity is associated with higher mortality and an elevated risk to develop diseases, such as type 2 diabetes, cardiovascular disease, musculoskeletal disorders and several types of cancer (29,30). With the development of obesity adipose tissue becomes dysfunctional, mainly VAT which is responsible of the metabolic complications in obesity (31, 32). As individuals become obese, adipose tissue undergoes molecular and cellular alterations affecting systemic metabolism. Lipid overload leads to an increase in the volume and number of white adipocytes, mainly in visceral depots, that contribute to the adipocyte hyperlipolytic state characterized by an increase in the efflux of free fatty acids into circulation with consequent decrease of insulin sensitivity in skeletal muscle, liver and pancreas (33, 34). Moreover, the increased catabolism in mitochondria of hypertrophic adipocytes leads to oxidative stress and production of free radicals (35, 36). This is also associated with an increased endoplasmic reticulum stress due to an altered processing of excess of nutrients (37) and subsequently the hypertrophic adipocyte becomes dysfunctional and die (38).

It is well known that the adipose tissue produces and releases a variety of pro-inflammatory and anti-inflammatory factors, including the adipokines (proteins produced mainly by adipocytes). Most notable between them are leptin, adiponectin, resistin, and visfatin, as well as cytokines and chemokines not specific to adipocytes such as tumor necrosis factor- α (TNF- α), interleukins (ILs), monocyte chemoattractant protein-1 (MCP-1) and others that act in an autocrine, paracrine, or endocrine (39). The distressed hypertrophic adipocytes in obese adipose tissue produces abnormal amount of pro-inflammatory adipokines/cytokines causing recruitment of resident and circulating bone marrow-derived monocytes into the tissue (40, 41, 42). The majority of such activated adipose tissue macrophages (ATM) surround the dead adipocytes remnants forming distinctive figures called crown-like structures (CLS) (38). These macrophages forming the CLS have the main role to sequester and ingest adipocyte debris that is similar to a certain extent to those seen in foreign body tissue reaction. Moreover, WAT macrophages are subjected to phenotypic polarization in obesity, resulting in an increase in classically activated, pro-inflammatory M1 macrophages and reduction in alternatively activated, anti-inflammatory M2 macrophages (43).

Despite ATM are responsible for the major production of pro-inflammatory cytokines in obese adipose tissue, several cell types of the immune system and present in the stromal vascular fraction (SVF) are also involved as the mast cells, eosinophils, neutrophils and natural killer cells (44). Taken together, these phenomena induce a chronic inflammation of adipose tissue in obese states. Additionally, the failure of vasculature to expand in same proportion of adipocyte volume induces adipose tissue microhypoxia that

contributes to the inflammatory process within the tissue (45). The role played by the immune system in the inflammatory process of the obese adipose organ is summarized in figure 1.



1.4. Heterogeneity of adipose depots

Regional differences exist in adipose tissue morphological and functional characteristics. Different fat depots have distinct glycoprotein, adipokine, and paracrine secretion profiles and capacities to activate or respond to hormones. Several factors contribute to these differences as the ‘microenvironment’ and the different cell types present in the fat depots, particularly macrophages subtypes which have characteristics that vary among organs (46). Here below I’ll discuss briefly commonly studied local adipose depots that had gained an interest in the last years due to their crucial role in the crosstalk with the neighboring tissues.

1.4.1. Epicardial fat

Epicardial adipose tissue (EAT) is one of the local depots of visceral adipose tissue; it is situated between the myocardium and the visceral layer of the pericardium (47). EAT has a high energy metabolism that prevent large amount of lipid storage (48). Compared with other visceral adipose depots, epicardial fat is mainly composed of preadipocytes than mature adipocytes, has a great capacity for FFA release and uptake that manily serves as an energy source for the myocardium (49). The anatomical location of this pad suggests a paracrine or vasocrine crosstalk between the epicardial fat and the underlying myocardium and coronary circulation. Infact, human epicardial fat expresses abundant levels of UCP1 protein similar to BAT that suggest a thermogenic capacity that might serve as a heat provider to the myocardium during a drop of body temperature or during certain conditions of hypoxia or ischaemia (50). Moreover, studies have shown that

epicardial fat volume was considered as an independent risk factor for coronary artery disease (CAD) and therefore regarded as a potential marker of cardiovascular risk (51). Under physiological conditions it secretes anti-atherogenic adipokines as adiponectin that become downregulated in case of CAD or heart failure (52). Moreover, in patients affected by CAD, epicardial fat was highly infiltrated with pro-inflammatory macrophages M1 (53) associated with an upregulation and secretion of inflammatory cytokines (MCP-1, IL-6, IL-1 β , TNF- α) that pass to the adjacent myocardium and coronary arteries (54).

1.4.2. Mesenteric fat

Mesenteric fat had shown to play an important role in the pathogenesis of inflammatory bowel disease, particularly Chron's Disease (CD). Fat-wrapping, a hall mark of CD, is defined as a mesenteric WAT extension from the mesenteric attachment and partially covering the small and large intestinal circumference in association with loss of the bowel-mesentery-angle (55). The presence of a correlation between this fat and disease characteristics such as ulceration, stricture formation, transmural inflammation, altered wall thickness suggested a cross talk between mesenteric adipose tissue and CD (56). In a recent study, visceral fat area measured by computed tomography was found to correlate with the postoperative recurrence of CD (57). Research suggested that mesenteric adipose tissue observed in CD shares some features of those observed in obese states. Hypertrophy of the mesenteric WAT has been long recognized as a consistent

characteristic of surgical specimens in patients with CD (56). Histopathological examination of mesenteric fat obtained from CD patients is characterized by fibrosis, perivascular inflammation, intimal and medial thickening of vessels, and significant infiltration of inflammatory cells (58). Moreover, overexpression of pro-inflammatory adipocytokines as TNF- α and leptin in the mesenteric fat of CD subjects suggested a key role of adipose tissue in the intestinal inflammatory process (59, 60).

1.4.3. Thymus fat

The thymus grows rapidly during embryonic life and during childhood, and involutes gradually until old age. It has been reported that with increasing age, adipocytes constitute the majority of cells in the thymic space, while the number of thymocytes substantially decreases (61, 62). Research reports in the last years were interested in the angiogenic capacity of human thymus adipose tissue (TAT) (63). TAT obtained from adults undergoing cardiac surgery presented an upregulation of angiogenic gene vascular endothelial growth factor (VEGF) compared to SAT. In that study, adipogenic genes expression as peroxisome proliferator activated receptor-gamma (PPAR γ) and fatty acid binding protein 4 (FABP4) correlated positively with expression of VEGF, which suggest a relevant role for these genes in VEGF regulation (64). In a recent study, scientists went to evaluate the differentiative capacity of adipose of tissue stromal cells obtained from TAT and SAT of patients with myocardial ischemia (65). Compared to SAT, the stromal cells deriving from thymic fat showed higher adipogenic differentiative and proliferative

capacity that correlated also positively with aging. At the light of these results and in presence of a documented angiogenic capacity, TAT was considered a promising tissue that can find implication in regenerative medicine aimed to the regeneration of new vessels in the ischemic myocardium of elderly subjects.

2. Obesity as a risk factor of knee osteoarthritis

In the past osteoarthritis (OA) was defined as a degenerative disease mainly related to cartilage degradation. New evidence suggests considering OA as a whole joint disease (66). According to this, the 2010 EULAR recommendations for diagnosis of knee OA (KOA), defined OA as a common joint disorder showing focal cartilage loss, new bone formation and involvement of all joint tissues, including menisci, and synovial membrane (67).

Obesity and OA are represent major public health problems in developing and industrialized countries that share similarities as the chronic nature, major source of disability leading to increased risk of cardiovascular morbidity and mortality (68, 69). The knee is considered the main joint affected by OA (70) with a prevalence estimated between 10%-13% in people older than 16 years (71). Obesity is reported as the most conspicuous risk factor (72, 73) and it is of great interest since it is potentially modifiable. Concerning the relationship between obesity and KOA, the Framingham study of Felson et al. constituted milestone in this field. By assessing 1420 people for KOA, about 33% of study population developed radiographic features of KOA after

about 40 years of follow-up. In this large survey the risk of development of KOA was related to baseline obesity (74). Today, given the fact that the worldwide prevalence of obesity is continuously increasing, interest is growing to understand the link between these two conditions which may provide novel targets for the prevention and treatment of OA (75).

2.1. Pathophysiology of obesity and knee joint homeostasis

There is a debate how obesity contributes to the initiation and progression of KOA. The pathogenesis seems to be multifactorial and the candidate mechanisms for the contribution of obesity in joint damage include: biomechanical stresses due to increased joint loading in presence of a negative metabolic and inflammatory environment caused by metabolic risk factors and products of systemic and local adipose tissue.

2.1.1 Mechanical stress

In a recent study, Visser et al. in a cross-sectional cohort including 5002 participants with $BMI \geq 27 \text{ Kg/m}^2$, supported a prominent role of mechanical stress (weight, fat free mass, fat mass) in the pathogenesis of KOA (76). Elevated BMI results in an increased strain on medial and lateral parts of the knee (77). In addition, several studies reported that obese people have an impaired gait pattern due to kinematic changes, muscle weakness, joint misalignment and altered posture which consequently leads to abnormal joint loading (78, 79, 80).

Increased mechanical stress is associated with bone marrow lesions and thickening of cortical subchondral bone possibly through generating an inflammatory phenotype of osteoblasts (81). Moreover, activation of mechanoreceptors at surface of chondrocytes by compressive stress (82) induces the expression of catabolic phenotype characterized by decreased synthesis of the extracellular matrix and increased production of degradative matrix metalloproteinases (MMPs), aggrecanase ADAMTS-5 and expression of pro-inflammatory factors as IL -1 β , TNF- α , cyclooxygenase 2 (COX-2) and prostaglandin E₂ (PGE₂) (83). There is good evidence that cartilage loss occurs in the same regions of the joint as the changes in the subchondral bone, it is thought that there is a crosstalk between the stressed subchondral osteoblasts and the overlying chondrocytes (84).

2.1.2. Adipocytokines and systemic inflammation

Apart from mechanical factors, as mentioned previously, obesity is considered a chronic inflammatory disease characterized by the production by adipocytes of cytokines and adipokines that may act on different tissues throughout the body. Adipokines, including leptin, adiponectin and resistin are most commonly involved in the pathophysiology of KOA. Leptin being primarily secreted by adipocytes is known to be an important mediator of obesity and OA. First reports on animal models have shown that this peptide can mediate anabolic actions on chondrocytes by inducing mRNA and protein expression of insulin-like growth factor -1 (IGF-1) and transforming growth factor β (TGF- β) (85). Further studies have shown contradictory results when intra-articular injection of leptin in

rats resulted in an increased mRNA and protein expression of the following catabolic factors: as MMP-2, MMP-9, ADAMTS-4/5, cathepsin, and type II collagen (86). Vuolteenaho et al. reported that leptin can enhance nitric oxide (NO), PGE₂, IL-6, IL-8 production in OA human cartilage (87).

On human OA synovial fibroblast, leptin stimulated the expression of IL-6 and IL-8 (88, 89). Regarding subchondral osteoblasts leptin is involved in altering the secretion of alkaline phosphatase, TGF- β , osteocalcin and collagen type I by these cells and subsequently modifying their normal phenotype (90).

The role of adiponectin in OA pathogenesis has not been completely elucidated. After the first report that regarded this peptide as protective factor in OA (91), several studies have suggested an opposite role. Recently, adiponectin plasma levels have been found to positively correlate with clinical and radiological disease severity of KOA (92). This peptide has been detected in both synovial fluid and plasma of OA patients (93) and has demonstrated a pro-inflammatory role by inducing the production of MMPs, IL-6, and PGE₂ by chondrocytes and synovial fibroblasts (94).

Resistin is a dimeric protein produced by adipocytes and macrophages and it has been reported that its levels correlate positively with obesity, insulin resistance and inflammatory processes (95). Resistin may play an important role in initiating and promoting the development of OA and thus it may represent an important links between obesity related metabolic disorders and cartilage destruction (96). Injection of resistin into mice joints induces an arthritis-like condition characterized by leukocyte infiltration

of synovial tissues, hypertrophy of the synovial layer and pannus formation (97). In a recent meta-analysis, resistin expression in blood and synovial fluid was higher in OA subjects compared to controls and its level was related to disease prognosis (98).

2.2. Obesity-related metabolic disorders

It is well established that obesity is usually associated with metabolic disorders as insulin resistance, dyslipidemia and hypertension that tend to cluster into the so-called metabolic syndrome. These factors have demonstrated to induce a strong cumulative effect on early or end-stage KOA incidence (99, 100). The mechanisms by which these factors may alter normal knee joint homeostasis will be discussed separately in the following paragraph. (Figure 2)

2.2.1. Altered glucose metabolism

In the last years large interest is growing to define the relationship between OA and hyperglycemia and/or impaired glucose tolerance (IGT). Both Engstrom et al. and Hussain et al. showed an increased KOA risk in patients with hyperglycemia and IGT respectively (101, 100).

The relationship between diabetes and radiographic KOA progression was demonstrated in SEKOIA trial by Eymard et al. where type 2 diabetes in men was independently associated with greater annualized rates of joint space narrowing (102).

Few studies have tried to clarify the link between high blood glucose levels and KOA. In osteoarthritic chondrocytes high extracellular glucose concentration has shown to impair the downregulation of glucose transporter 1 (GLUT-1) and thus mediating cartilage destruction through the high production of reactive oxygen species (ROS) (103). In addition high extracellular glucose levels stimulated in osteoarthritic chondrocytes the expression of MMP-1 and MMP-13 (104). Locally the accumulation advanced glycation end-products (AGEs) resulted in an increased stiffness of collagen network (105) and in activation of the receptor of advanced glycation end products (RAGE) inducing the expression of pro-inflammatory mediators as COX-2, PGE₂, IL-6, IL-8, TNF- α and MMPs (106, 107, 108). Finally, diabetic peripheral neuropathy may represent an important risk factor for KOA due to altered proprioceptive acuity and decreased muscle strength (109).

2.2.2. Altered lipid metabolism

The involvement of altered lipid metabolism in the pathogenesis of OA was proposed by several studies. A characteristic of metabolic syndrome is the increased release of FFA from adipose tissue caused by impaired inhibition of intracellular lipolysis. High levels of FFA are found in joint tissues in OA (110). FFA can aggravate joint inflammation by inducing pro-inflammatory response in macrophages activating Toll-like receptors (TLRs-2/4) (111).

Furthermore, epidemiological studies have demonstrated that high density lipoprotein (HDL)-c is decreased in the serum of OA patients compared to non-OA individuals (112, 113).

Another fundamental feature of OA pathology is mediated by low density lipoprotein (LDL) and its oxidized form (Ox-LDL). Comparative analysis demonstrated that OA patients have higher serum LDL compared with age-, sex-, and BMI- matched healthy controls (114, 115). Synovial macrophages stimulated by Ox-LDL produced pro-inflammatory mediators as IL-6, IL-8, MCP-1 and growth factors as TGF- β (116).

2.2.3. Arterial hypertension

Hypertension, another common feature among morbidly obese population, has demonstrated to be an independent risk factor in the pathogenesis of KOA (100). This relation was related to vascular pathology since hypertension was proposed to reduce blood flow to subchondral bone and consequently impair nutrition and gas supply to the overlying cartilage and thus its degeneration (117). In addition, ischemic subchondral plate and due to bone loss might be susceptible for microcrack or micro-fracture at osteochondral junction under repetitive mechanical loading which contribute to the deterioration of cartilage degeneration (118).

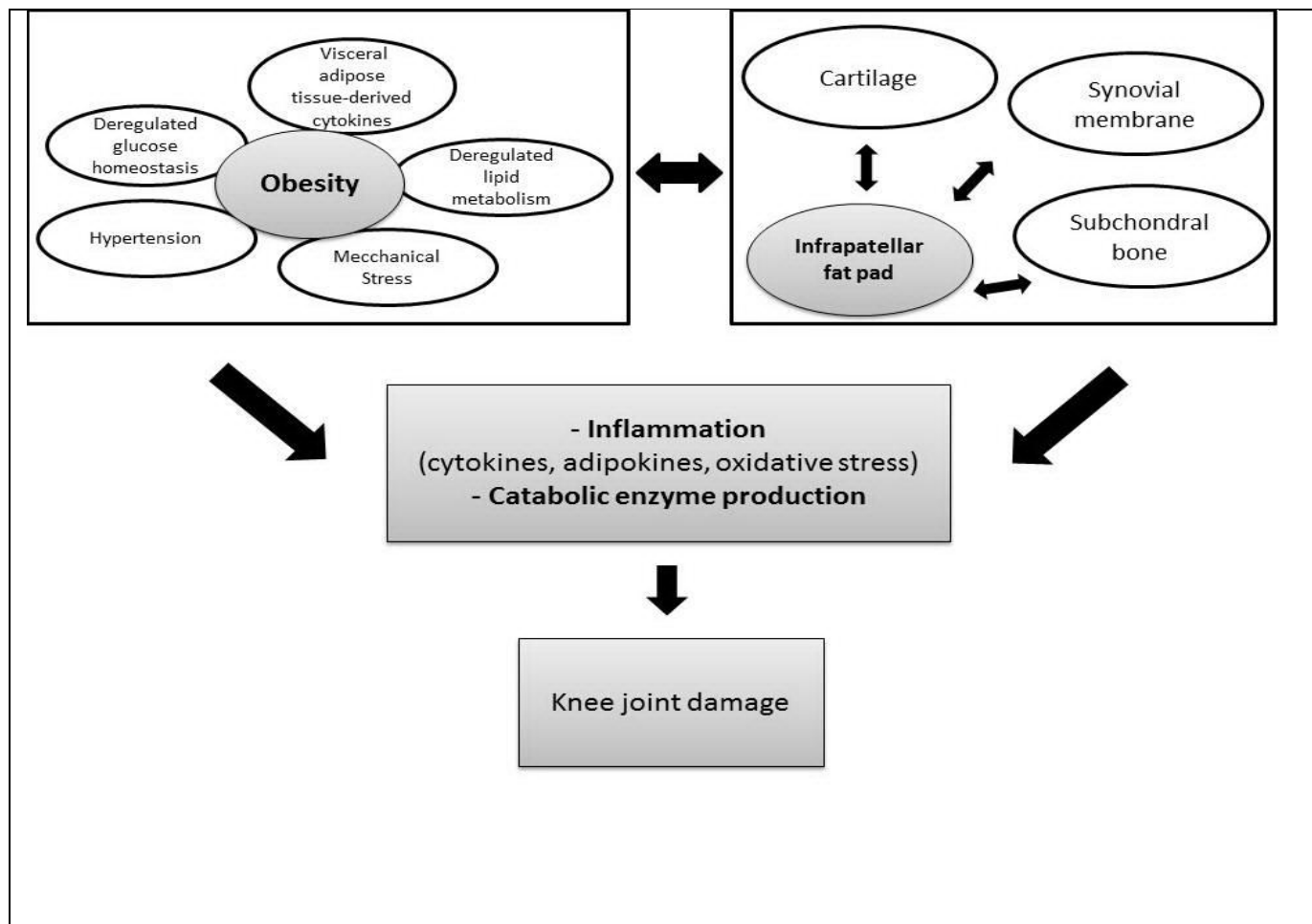


Figure 2. Multifactorial association between obesity and knee OA.

The mechanisms responsible for this link are thought to involve mechanical loading, hypertension, altered glucose and lipid metabolism. In addition increased visceral adiposity is responsible for a low-grade systemic inflammation state via the release of inflammatory adipocytokines. These stresses together with the pro-inflammatory role mediated by infrapatellar fat pad, which is intimately connected to the synovial membrane and presumably there is a cross-talk between it and the other tissue of the joint, result in local inflammation and production of catabolic enzymes in joint tissues and subsequently promoting articular damage.

3. Knee osteoarthritis: a focus on infrapatellar fat pad

The infrapatellar fat pad (IFP) or Hoffa fat pad of knee joint was firstly described by the german surgeon Albert Hoffa in 1904 (119). It is located under the patellar tendon filling the space between the patellar tendon, femoral condyles and tibial plateau. It is attached to the lower border of the inner non-articular surface of the patella, to the notch between the two condyles of the femur by running continuously with the infrapatellar plica posterosuperiorly, and to the periosteum of the tibia and the anterior part of the menisci. Moreover, this pad is intracapsular but extrasynovial, with synovial lining covering its posterior surface (120, 121).

The periphery of the IFP itself being described as highly vascularized is supplied by branches of the superior and inferior genicular artery while more centrally the vascular network become more limited (122).

From the microscopic point of view, IFP consists of white adipose tissue organized in lobuli that are delimited by thin connective septae. The IFP adipocytes are distributed with a large intercellular and covered by a thick fibrillary sheaths (123).

The exact role of IFP is still debated. For years knee fat pad has been thought to have mainly biomechanical functions filling the space between joint structures, adapting during the knee movement and contributing to the load bearing. In fact, seen its structural characteristics IFP could act as a plastic portion aimed at absorption of pressure variation during knee articular activity. Moreover, IFP is supposed to supply the blood to the

patella tendon and to be involved in the mechanisms inducing pain in KOA (124, 125, 126).

Recent evidences suggest that IFP might have not only a biomechanical but also a biochemical role in the pathogenesis of diseases such as OA through the secretion of inflammatory markers as cytokines and adipokines. The IFP is the main articular adipose tissue within the knee. Like all adipose depots, it is an endocrine organ that has shown to play a role the pathophysiology of KOA through the secretion of inflammatory mediators directly into knee joint. The first report about the inflammatory phenotype of IFP back 2003 when Ushiyama and colleagues showed in human OA IFP significant protein levels of IL-6, TNF- α , VEGF, and basic fibroblast growth factor (b FGF). Additionally, these inflammatory cytokines were also detected in the synovial fluid of the same subjects (127). When the SAT and IFP of the same subject were compared, IFP expressed higher levels of inflammatory cytokines as IL-6, sIL-6R, TNF- α , adipsin, visfatin and adiponectin (128, 129). Concerning, the role of excess of weight on the secretory profile of IFP, only TNF- α was found to correlate positively with BMI (128). It was hypothesized that these differences maybe also influenced by the presence of inflammatory cells in the IFP stromal fraction, in particular by the abundant number of pro-inflammatory macrophages (128). Moreover, in OA subjects it was demonstrated that the culture media of IFP adipocytes induced MMP-1 and MMP-13 expression in articular chondrocytes and leptin level correlated positively with the expression of both MMPs and cartilage collagen destruction (130). Concerning the synovial membrane, investigators have shown that IFP compared to SAT of subjects affected by KOA is able to induce a

stronger inflammatory response in the synovial membrane characterized by an increase in the levels of inflammatory markers in fibroblast-like synoviocytes (131). The contribution of IFP in KOA is summarized in figures 2 and 3.

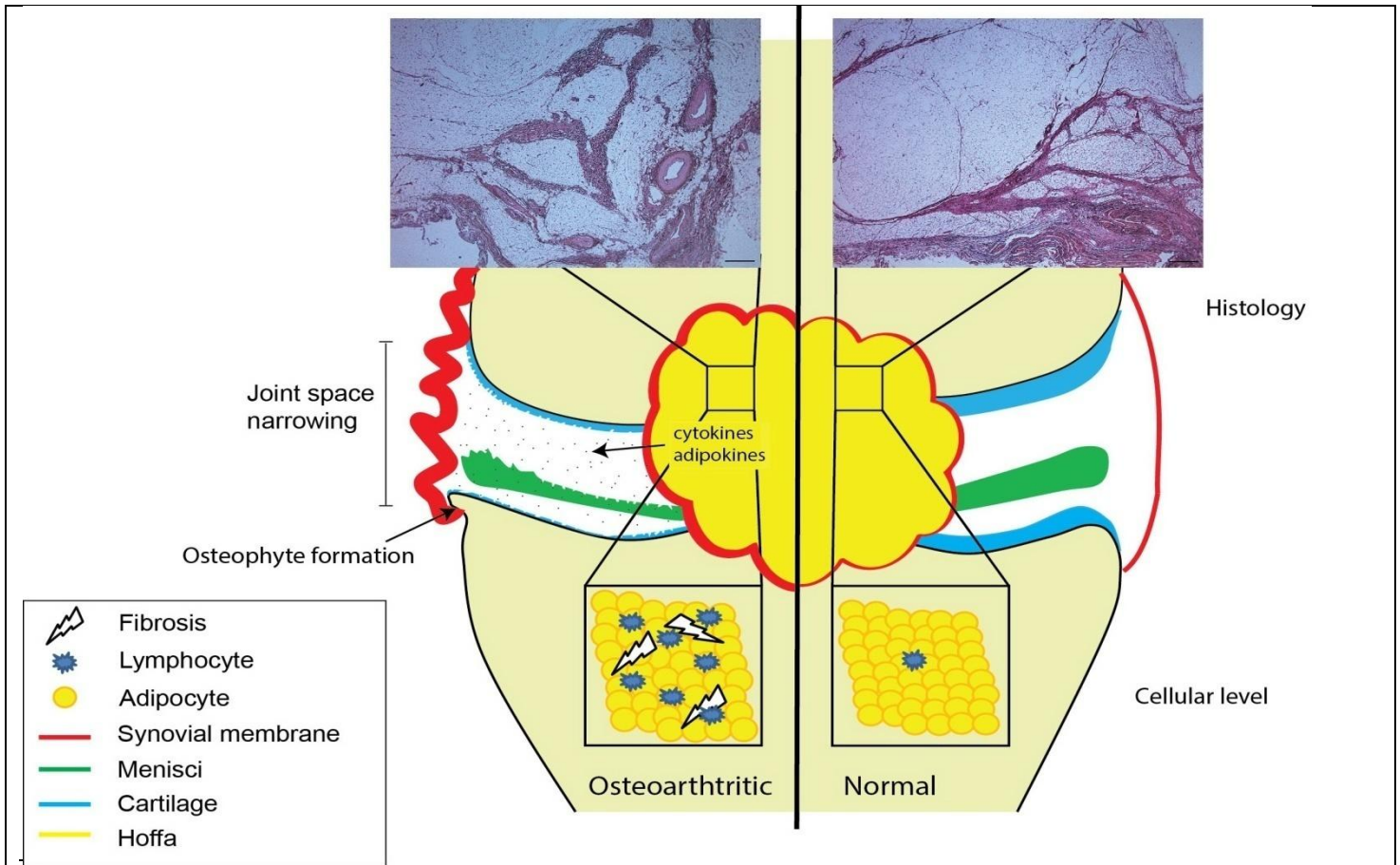


Figure 3. In the left part of the cartoon the classical OA features are showed: joint space narrowing, cartilage degradation, synovial membrane inflammation and hyperplasia, meniscus degeneration and osteophytes formation. Here, the role of IFP in OA as a source of cytokines and adipokines participating in the inflammatory process of the joint is highlighted. IFP structural changes are shown at cellular and histological levels in OA condition compare to the normality. In healthy individuals IFP showed no lymphocytic infiltration and absence of fibrosis as demonstrated by the histology picture (haematoxylin-eosin staining, taken from a subject involved in the Body Donation Program ‘Donation to Science’ of the held by the University of Padova without history of osteoarthritis)(123). On the contrary, the microscopic image (haematoxylin-eosin) of IFP of an OA patient undergoing total knee replacement showed lymphocytic infiltration and fibrosis.

AIMS OF THE STUDY

- Describe the histomorphological characteristics of infrapatellar fat pad and synovial membrane in mainly overweight/obese individuals and affected by knee osteoarthritis.
- Apply a ‘scoring’ system on the histopathologic features of infrapatellar fat pad.
- Determine the expression of different adipocytokines in infrapatellar fat pad of individuals with knee osteoarthritis and possible correlations with microscopic parameters of joint tissues.

MATERIALS AND METHODS

1. Patients and tissue collection

28 patients undergoing total knee replacement (TKR) for OA at both Orthopaedic Unit and Clinic of Padova University Hospital were enrolled in the study after providing written informed consent. The Local Ethical Committee approved the study protocol. For each patient the following clinical data were collected: age, sex, body mass index (BMI), and comorbidities. During the surgical procedure IFP together with adjacent synovial membrane were collected and processed as following: a part was fixed in 10% formalin and then paraffin-embedded for histochemical and immunohistochemical studies, and a part was frozen in liquid nitrogen and conserved at -80 °C for bio-molecular analysis.

The controls were represented by IFP and adjacent synovial membrane specimens sampled from bodies or bodies part of healthy subjects without history of osteoarthritis involved in the Body Donation Program 'Donation to Science' held by the University of Padova (123).

2. Microscopical study

Thick sections of 10 µm were obtained from the paraffin embedded samples, and were stained with haematoxylin-eosin (EE), Azan-Mallory, and Weigert-Van-Gieson stains.

The analysis of the samples was focused on the evaluation of both IFP and IFP adjacent synovial membrane. The microscopical characteristics of the IFP were scored according to the following parameters: presence of inflammatory cells, vascularity, adipose lobuli dimension and thickness of the interlobular septa. The presence of inflammatory cells was graded as follows: 0 = no presence of mononuclear cell infiltration; 1 = presence of perivascular mononuclear cell infiltration; 2 = perivascular and interstitial mononuclear cell infiltration. The vascularity was evaluated counting the number of vessels in 4 sections for each case independently from their diameter. The adipose lobuli dimension and the thickness of the interlobular septa were evaluated by morphometry.

IFP immunohistochemical stains were performed with anti-CD3 antibody (polyclonal rabbit antibody, Fitzgerald) to identify the presence of T-cells with a dilution of 1:500. The sections were incubated using the DAKO Autostainer System (EnVision™ FLEX, High pH). The EnVision™ FLEX reagents are intended for use on formalin-fixed, paraffin-embedded tissue section. Sections incubated without primary antibodies showed no immunoreactivity, confirming the specificity of the immunostaining.

The synovial membrane involvement was evaluated according to a synovial histopathological grading (132, 133) considering the following parameters: lymphocytic infiltration (0-3), synovial hyperplasia (0-2), vascularity (0-2), fibrosis (0-2), mucoid change (0-4), and presence of fragments of bone and cartilage (0-2). The grading characteristics were scored by an expert pathologist. All sections were observed under a DM4500-B light microscope (Leica Microsystems, Wetzlar, Germany) and recorded in full colour (24 bit) with a digital camera (DFC 480, Leica Microsystems).

3. Morphometry

After digitizing the images acquired from EE stained sections, the diameter of the adipocyte lobuli and the thickness of the interlobular septa were measured, using specific imaging software (Adobe Photoshop CS5, Adobe Systems Incorporated, USA). On the images acquired at 1.25 X primary magnification the septa were first manually identified. Subsequently, with a specific Programming Language Software (Matlab R2012b, The MathWorks, Inc., USA) the images were converted to 8-bit binary images. The thickness of interlobular septa and the dimension of adipose lobuli were then evaluated.

4. Quantitative real-time PCR

Total RNA was extracted from IFP using QIAMP mini kit (QIAGEN) following the manufacturer's protocol. First-strand cDNAs were synthesized from equal amounts of total RNA using random primers and M-MLV reverse transcriptase (Promega). Quantitative real time PCR (qRT-PCR) for adipokines (leptin, adiponectin, PPAR γ , FABP4), cytokines (IL-6, TNF- α , MCP-1 and VEGF) was performed using Sybr Green fluorophore. The changes in fluorescence at every cycle were monitored and a threshold cycle above background for each reaction was calculated. A melting curve analysis was performed to ensure a single amplified product for every reaction. All reactions were carried out in duplicate for each sample. 18S rRNA was constantly expressed under all experimental conditions and was then used as a reference gene for normalization. It was not possible to extract mRNA from IFP of cadavers. Primer sequences are listed in table (1).

Table 1. Primer sets used for quantitative real-time PCR (F= forward, R= Reverse)

TNF- α	F: TGCTTGTTCCCTCAGCCTCTT R: GGGCTACACGCTTGTC A
IL-6	F: TTCGGTACATCCTCGACG R: AAGCATCCATCTTTTTTCAGC
MCP-1	F: CCCCAGTCACCTGCTGTTAT R: TGGAATCCTGAACCCACTTC
PPAR γ 2	F: ACCCAGAAAGCGATTCCTTCA R: AGTGGTCTTCCATTACGGAGAGATC
Leptin	F: GTGCGGATTCTTGTGGCTTT R: GGAATGAAGTCCAAACCGGTG
Adiponectin	F: CCTGGTGAGAAGGGTGAGAA R: CAATCCCACACTGAATGCTG
FABP-4	F: GAAGTCAAGAGCACCATAACC R: CCACCACCAGTTTATCATCC
VEGF	F: TCACCARGCAGATTATGCGGA R: TGTTGTGCTGTAGGAAGCTCA
18 S	F: CTTGTCCCACCACTTGAAGG R: CACAGTCATTGCTCAGATCCA

5. Statistical analysis

Inter- and intra-reader reliability of histopathology scores were reported as a weighted kappa statistic. Shapiro-Wilk test was used to determine whether data were distributed normally. The data were shown as means \pm standard deviations (SD). Chi-square (χ^2) test or Fisher's exact test were performed to compare categorical and dichotomous data. Mann-Whitney test or Student's t-test were used to compare continuous variables. Spearman's or Pearson's correlations were performed to analyse associations between continues variables. One-way ANOVA or Kruskal-Wallis, with Tukey's post-hoc tests, were used to analyse categorical data. A $p < 0.05$ was considered significant. All analyses were performed with SPSS version 22.0.

RESULTS

1. Patients demographic and clinical characteristics

28 patients undergoing TKR were enrolled in the study. Specimens were collected from 8 cadavers as controls. The demographic and clinical data of OA and control subjects are outlined in Table 2. Females were 75% in the OA group, while 50% in the control group ($p = 0.047$). OA subjects were statistically younger ($p < 0.0001$) than controls (mean age \pm SD 68.9 ± 7.8 vs. 81.8 ± 4.9 years). BMI was statistically higher ($p = 0.0002$) in OA patients than controls (mean BMI \pm SD 30.5 ± 5.0 vs. 21.8 ± 2.3 Kg/m² respectively). Moreover, we aimed to assess possible relationship between the demographic data of OA patients and the correspondent inflammatory markers and histological features.

Table 2. Demographic and clinical data of OA patients and controls. * P < 0.05

	OA PATIENTS	CONTROLS	P
Number of patients	28	8	
Sex (female), number (%)	21 (75)	4 (50)	0.047*
Age, mean (SD), years	68.9 (7.8)	81.8 (4.9)	<0.0001*
BMI, mean (SD), Kg/m ²	30.5 (5.0)	21.8 (2.3)	0.0002
Comorbidities			
Hypertension, number (%)	17 (68%)	6 (60%)	0.382
Diabetes, number (%)	3 (12%)	3 (30%)	0.073
Hypercholesterolemia, number (%)	8 (32%)	3 (30%)	0.468

2. IFP histology and morphometry

Twenty seven IFP samples underwent histologic and morphologic analysis (1 sample was not analysed for technical reasons). All the evaluated IFPs showed microscopical characteristics similar to white adipose tissue. IFPs were organized in adipose lobuli separated by fibrous septa. The IFP histological characteristics are summarized in Table 3 and figure 4. No differences were detected in the mean diameter of the adipose lobuli of the IFP in OA patients and controls.

Table 3. IFP histopathologic scoring system. * P < 0.05

Hoffa histopathologic grading	OA patients (n =27)	Control (n =8)	P
Lymphocytic Infiltration, number (%)			0.001*
Grade 0	5 (18.5%)	8 (100%)	
Grade 1	8 (29.6%)	0 (0%)	
Grade 2	14 (51.9%)	0 (0%)	
Vascularity, mean (SD), number	34.91 (16.26)	11.81 (4.25)	<0.0001*
Thickness of the interlobular septa, mean (SD), mm	0.30 (0.08)	0.23 (0.03)	0.004 *
Diameter adipose lobuli, mean (SD)	1.09 (0.42)	1.15 (0.11)	0.141

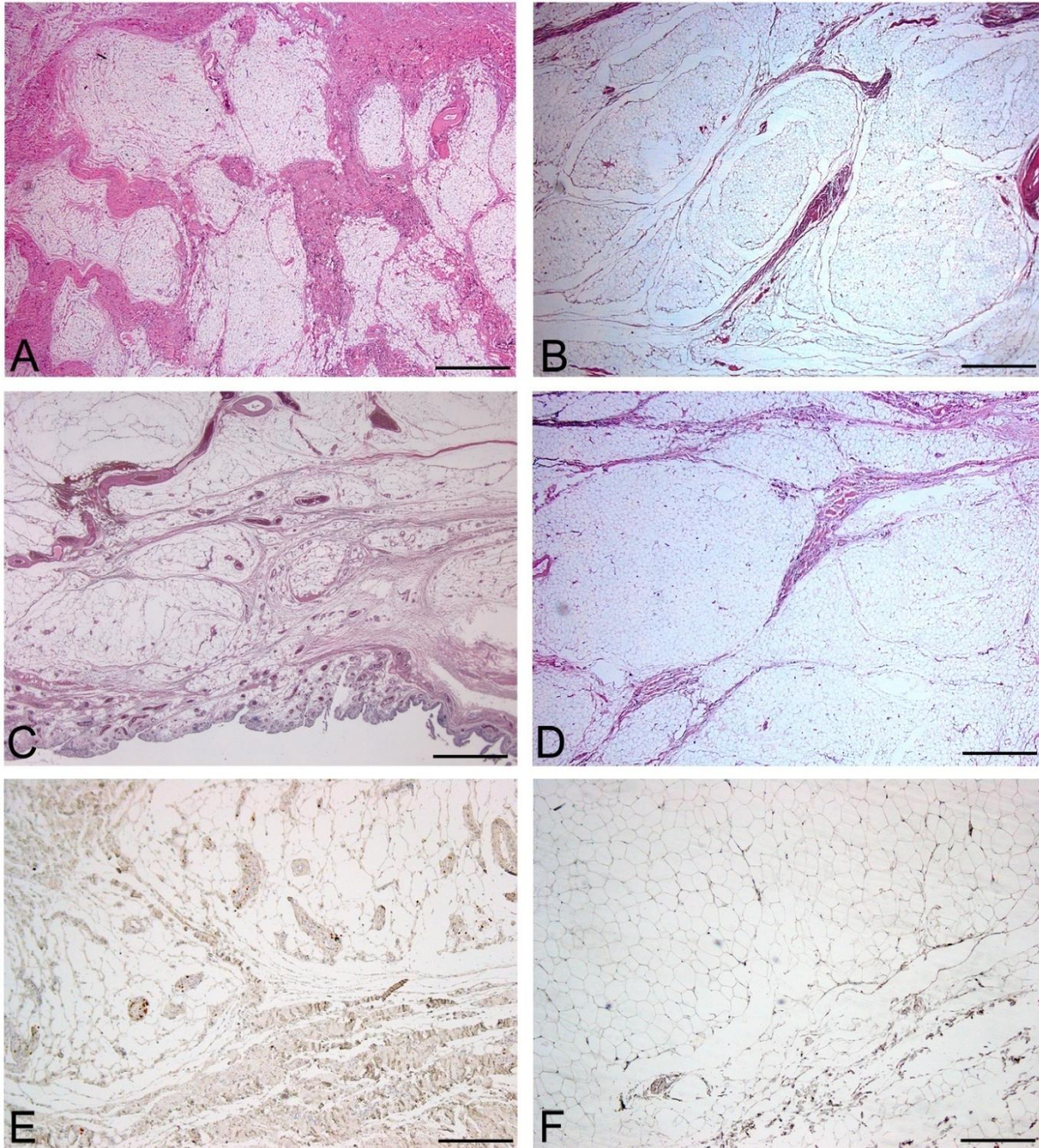


Figure 4. Microscopic appearance of the IFP in osteoarthritis (left column) and in control (right column), showing the adipose lobuli separated by thick fibrous septa in osteoarthritis (OA) (A) with respect of controls (B). In OA patients the vessels were more numerous (C) with respect of controls (D) and showed major mononuclear infiltration (E) with respect of controls (f). A-D, haematoxylin-eosin; E-F, immune-histochemistry for CD3. Scale bars: (A-D), 600 μm ; (E-F), 150 μm .

Mononuclear infiltration was present in 22 OA patients while it was not observed in any of the IFP used as control ($p = 0.001$). The average number of vessels and the thickness of interlobular septa were significantly higher in OA patients compared to controls ($p < 0.0001$ and $p = 0.004$ respectively). Notably, BMI correlated positively with the thickness of the interlobular septa in IFP ($p = 0.02$, $r = 0.42$). (Figure 5.)

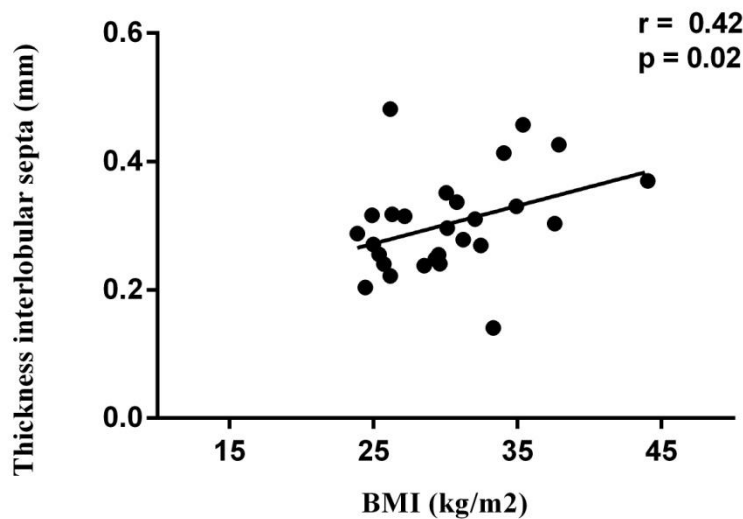


Figure 5. Correlation between BMI and thickness of interlobular septa in IFP of KOA subjects

3. Synovial membrane histopathological characteristics

Since the IFP adjacent synovial membrane was not present in 5 patients, the synovial membrane involvement was evaluated in 22 cases.

The presence of lymphocytic infiltration and hyperplasia was statistically higher in OA synovial membrane compared to controls ($p < 0.001$ and $p = 0.001$ respectively). In addition, synovial membrane of OA patients was more vascularized and fibrotic compared to controls ($p < 0.001$ and $p = 0.002$ respectively). No differences were found in mucoid change and detritus between OA patients and controls. The histological characteristics of IFP adjacent synovial membrane are described in Table 4 and figure 6.

Table 4. Synovitis score of synovial membrane adjacent to IFP. Data are expressed as number (%). * P < 0.05

Synovitis histopathologic grading	OA patients (n =22)	Control (n =8)	P
Lymphocytic Infiltration (0-3)			<0.001*
Grade 0	0 (0%)	6 (75%)	
Grade 1	5 (22.7%)	2 (25%)	
Grade 2	6 (27.3%)	0 (0%)	
Grade 3	11 (50%)	0 (0%)	
Vascularity (0-2)			<0.001*
Grade 0	0 (0%)	8 (100%)	
Grade 1	6 (27.3%)	0 (0%)	
Grade 2	16 (72.7%)	0 (0%)	
Detritus (0-2)			0.545
Grade 0	19 (86.4%)	8 (100%)	
Grade 1	2 (9.1%)	0 (0%)	
Grade 2	1 (4.5%)	0 (0%)	
Fibrosis (0-2)			0.002*
Grade 0	4 (18.2%)	7 (87.5%)	
Grade 1	10 (45.5%)	1 (12.5%)	
Grade 2	8 (36.4%)	0 (0%)	
Hyperplasia (0-2)			0.001*
Grade 0	3 (13.6%)	7 (87.5%)	
Grade 1	10 (45.5%)	1 (12.5%)	
Grade 2	9 (40.9%)	0 (0%)	
Mucoid change (0-4)			0.277
Grade 0	16 (72.7%)	7 (87.5%)	
Grade 1	0 (0%)	1 (12.5%)	
Grade 2	3 (13.6%)	0 (0%)	
Grade 3	1 (4.5%)	0 (0%)	
Grade 4	2 (9.1%)	0 (0%)	

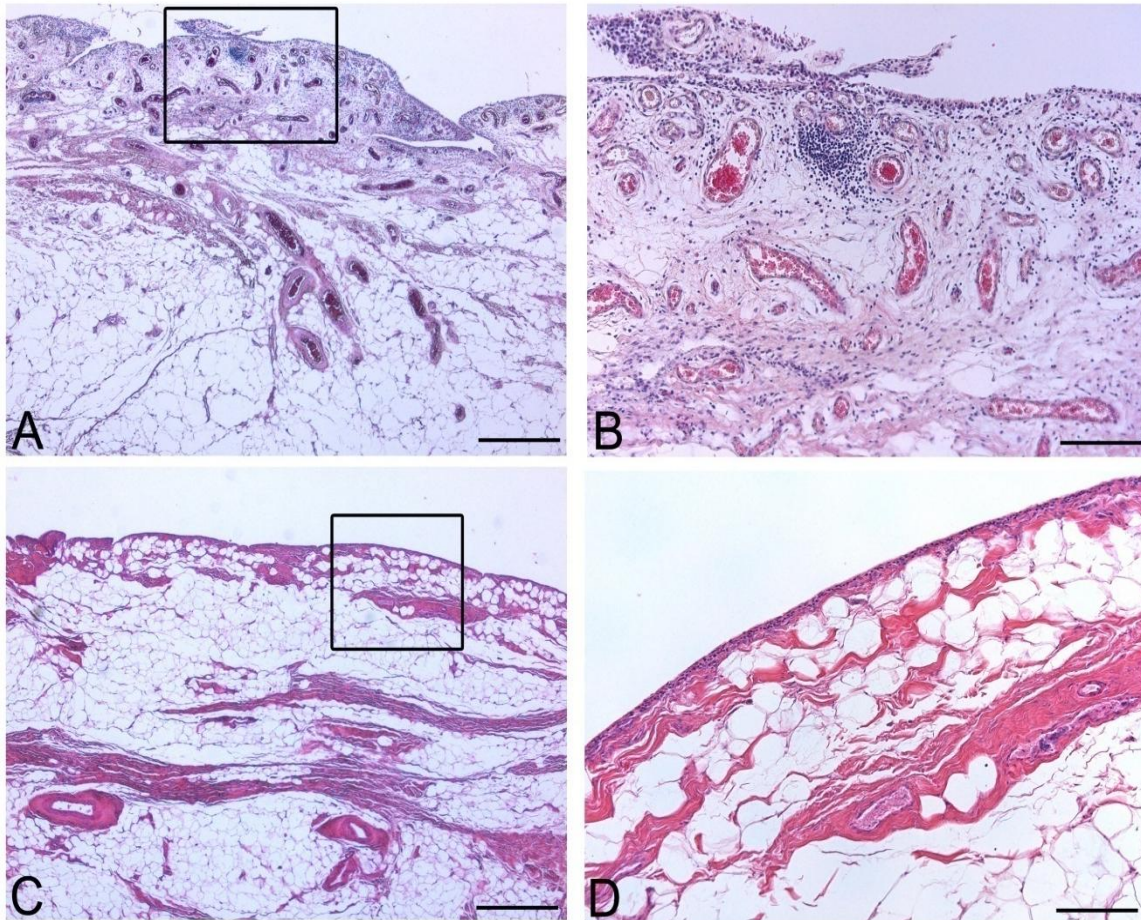


Figure 6. Microscopic appearance of the adjacent synovial membrane in osteoarthritis (first row) and in control (second row), showing the presence of lymphocytic infiltration and hyperplasia of the synovial membrane (A and B) with respect of the control (C-D). In OA patients the vessels were more numerous (b). A-D, haematoxylin-eosin; scale bars: (A-C), 600 μm ; (B-D), 150 μm

4. Correlations between gene expression and histological data

All the analyzed IFP samples from OA patients showed an expression of typical genes of white adipose tissue as well as leptin, adiponectin, PPAR γ , FABP4. Furthermore it was noticed an expression of genes typical of inflamed adipose tissue such as IL-6, TNF- α , MCP-1, in addition to VEGF (Figure 6.a).

We observed a positive correlation between the number of blood vessels of osteoarthritic synovial membrane and mRNA expression of VEGF in IFP ($p=0.04$) (figure 6.b)

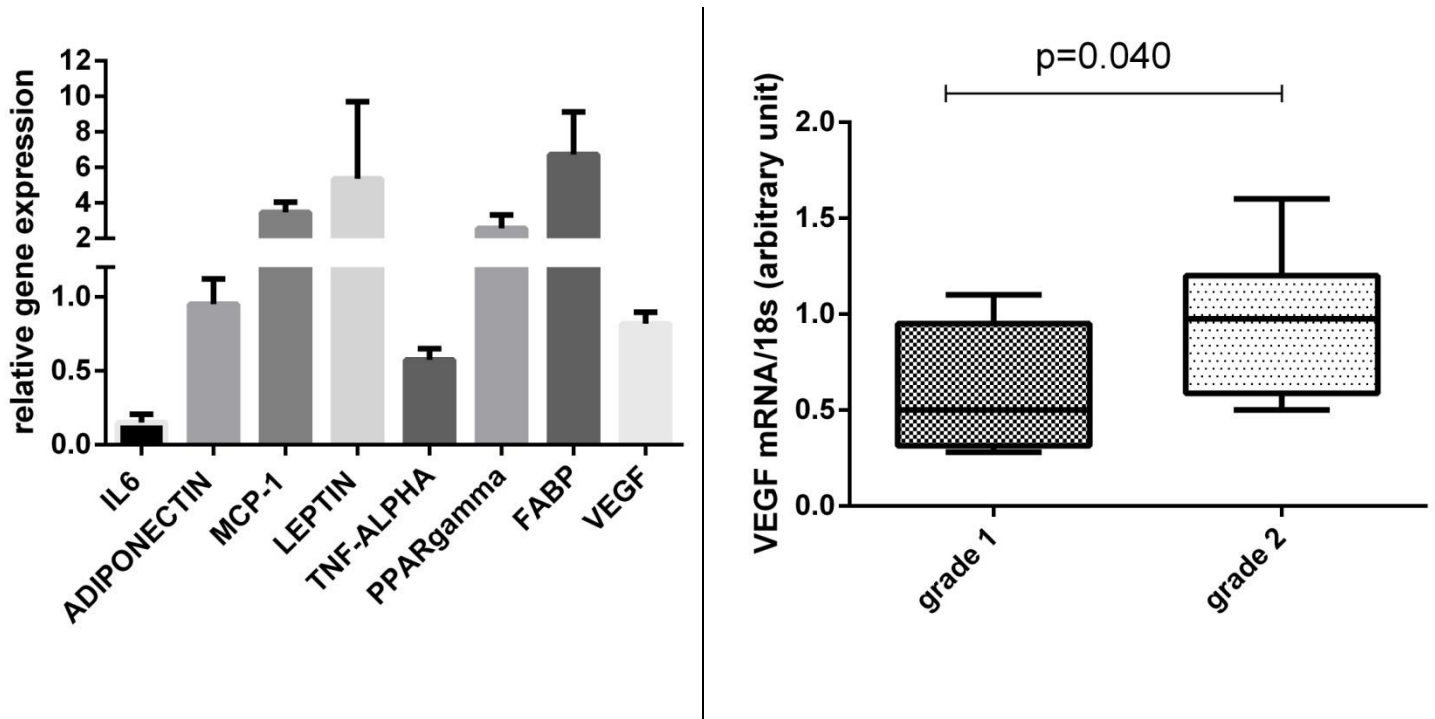


Figure 6. Relative gene expression is shown (a). While (b) shows the relationship between VEGF expression in IFP and grade of synovial hyperplasia in subjects with KOA.

DISCUSSION AND CONCLUSION

For many years the association linking obesity to osteoarthritis was attributed entirely for the increase of mechanical load. However, it is difficult to explain how BMI is associated with an increased risk of OA in non weight-bearing joints as the case of hand osteoarthritis (134). Moreover, in the Rotterdam cohort, a high BMI was associated with increased incidence and progression of OA of the knee but not of the hip (135). These findings suggest a role of additional factor that adds to the effect of mechanical stress and systemic low grade inflammation in overweight/obese subjects. In the case of knee the responsible factor may be IFP.

The function of the IFP is still debated, but it is thought to have both biomechanical and biological functions (125). Although its removal during total knee arthroplasty remains a matter of surgeon preference, recent study has suggested that its preservation is associated with improved outcome (136). In our study the IFP of subjects with OA showed pathological changes with presence of fibrosis, neoangiogenesis and inflammatory infiltration. In particular, IFP showed a statistically significant increase of the thickness of interlobular septa and a decrease of the adipose lobuli diameter compared to controls. Moreover, thickness of interlobular septa correlated positively with BMI of OA patients. In fact, as demonstrated by others, fibrosis was found in the IFP of subjects who underwent total knee arthroplasty and resection of a severely fibrotic IFP was

recommended since favourable effect has been reported following the surgical procedure (137).

On the other hand, adipose tissue fibrosis was also reported in obesity being associated with metabolic complications. Adipose tissue hypoxia initiates fibrosis which is further aggravated by chronic inflammation (138). Moreover, the presence of an inflammation stimulus in white adipose tissue may be responsible for an excessive synthesis of extracellular matrix components and subsequent interstitial deposition of fibrotic material. Henegar et al (2008) demonstrated that in white adipose tissue of obese subjects there is an increased expression of genes encoding extracellular matrix components together with an increased extracellular matrix (139). Fibrosis may represent an ubiquitous tissue response to an unresolved chronic inflammation (140). In the present study mononuclear infiltration was found in IFP of 81.5 % of OA patients, with perivascular and interstitial localization. This data partially agree with those reported by Maculé et al. (2005) that found chronic inflammatory infiltration of IFP in 35% of patients who underwent total knee arthroplasty (137). Jedrzejczyk et al. (1996) described the presence of subsynovial lymphocytic infiltration in IFP of normal subjects, especially in older persons (141). In the monoiodoacetate model of OA pain, histopathologic features of IFP similar to those observed in our OA study population were reported. These alterations included intercellular separation of adipocytes by fibrous tissue associated with hyperplastic synovial membrane (142). On the other hand, fibrosis has been reported also in other fat pads such as plantar fat pad (143). It has been hypothesized that these changes modify the ability of IFP to absorb physiological as well as pathological forces acting on knee joint and thus promoting and perpetuating joint

damage (142). Further studies could be addressed to define biomechanical models for the interpretation of the functional behaviour of IFP. In particular numerical models could be able to correlate healthy and degenerative conditions (144).

Moreover, when assessing the link between the inflammatory profile of IFP and its microscopic features of IFP itself or that of synovial membrane; we observed a positive correlation between VEGF expression in IFP and the vascularity score of synovial membrane. IFP in its anatomic proximity to synovial membrane and by releasing inflammatory cytokines has shown to be involved in the induction OA synovitis (131). VEGF is a potent angiogenic cytokine that has shown to play a role in OA (145, 146). This cytokine can also be produced by hypertrophic chondrocytes, macrophages and synovial fibroblasts (146, 147). The role of the synovium in OA pathology is becoming more evident. In conjunction with cartilage damage and bone alterations, the pathologic features of inflammation, hyperplasia, and extensive fibrosis are also often observed in the synovium of OA joints (148). These abnormalities although more pronounced in advanced OA, are present from the earliest stages of the OA process.

In conclusion, our study describes for the first time the histopathological characteristics of IFP in a large cohort of patients with KOA. IFP showed pathologic structural changes in the lobule dimension, interlobular septa, vascularization and inflammatory infiltrate. These changes were associated with synovial inflammation. Moreover, we added

evidence about the existence of a probable crosstalk between cytokines produced by IFP and the synovial membrane.

Our KOA subjects consisted mainly of overweight/obese subjects, thus we cannot exclude a possible effect of systemic factors or inflammatory markers on the IFP or synovial microscopic phenotype. Even if it seems challenging, further work should consider the metabolic inflammation which is a hall mark in obesity and the biomechanical aspect when assessing the role of IFP.

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