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In vitro leptin production by osteoarthritic synovial fibroblasts and chondrocytes: role of GILZ

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1. INTRODUCTION

1.1. Overview

Osteoarthritis (OA) is the most common joint disease in rheumatology and was initially considered as a degenerative disease due to aging and mechanical stress. OA is a prevalent disease with a significant impact on the quality of life, the ability to work and the public health cost. In French, "osteoarthritis" is named "*arthrose*" and comes from the greck word "*arthros*" meaning joint. In French terminology, "osteoarthritis" ("*arthrose*") is opposed to inflammatory arthritis [rheumatoid arthritis (RA), reactive arthritis …] ("*arthrite*"): "-ose" suffix is used for degenerative diseases, while the "-ite" suffix is reserved for inflammatory processes.

Progresses in molecular biology have modified the "degenerative" paradigm of OA: pro-inflammatory mediators were discovered in the synovial fluid. *In vitro*, these mediators have a catabolic and pro-inflammatory effect on chondrocytes, leading to a new paradigm in OA and to the development of the "inflammatory theory".

Obesity was a well-known local risk factor for weight-bearing joints. However, recent observations indicated that obesity was also a risk factor for non weight-bearing joints, such as hands. Obesity became not only a local risk factor but also a systemic one. Fat tissue produces pro-inflammatory cytokines and adipokines with a catabolic action on cartilage, leading to a new concept: the "metabolic theory" of OA.

Among adipokines, *in vivo* and *in vitro* observations have indicated that leptin had a major influence on OA process with catabolic and pro-inflammatory effects. A couple of years ago, our laboratory published that OA synovial fibroblasts themselves were able to spontaneously produce leptin [1].

Surprisingly, well-known anti-inflammatory glucocorticoids (GCs) strongly induced leptin secretion but also leptin receptor (Ob-R) expression in synovial fibroblasts.

1.2. Definition of osteoarthritis

1.2.1. Anatomy and radiology

OA is a joint disease. In the human body, joints can be classified according to their anatomy *(Figure 1)* [2-4]:

- the fibrous joint is a fibrous connective tissue that connects together two different bones. This articulation has no mobility. Sutures in skull or the joint between tibia and fibula in the ankle are two examples;
- the cartilaginous joint is a cartilaginous tissue that connects two different bones but with a little more mobility. Symphysis pubic or intervertebral joint are two cartilaginous joints;
- synovial joint is the most frequent joint. It presents a space between two bones ("articular cavity"), filled with a liquid ("synovial fluid") that is secreted by a membrane ("synovial membrane"). The articular cartilage covers the end of each bone and the articular capsule (a connective tissue) surrounds the joint. Knee and hip are two synovial joints. Articular cartilage has a major role, allowing a good mobility between the two bone structures. Articular cartilage has no direct vascularization or innervation and is composed of chondrocytes, surrounded by an extra-cellular matrix (ECM) of collagen and proteoglycans.

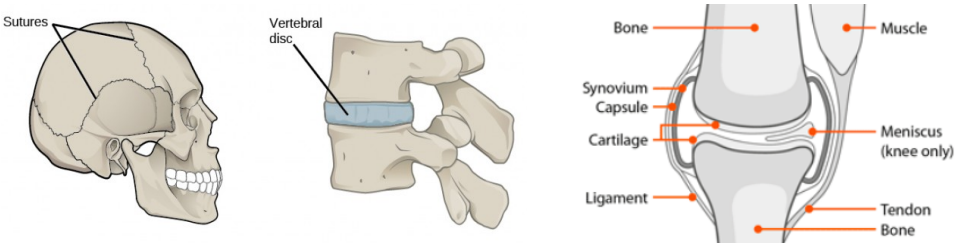

Figure 1. From left to right, fibrous, cartilaginous and synovial joints [2, 4].

In OA, the cartilage degradation leads to a progressive reduction of the joint space, which progresses to a direct contact between the two bone structures and finally to the joint destruction. There is also a densification of the subchondral bone and the formation of abnormal reactive new bone (osteophytosis) *(Figure 2 and 3)*.

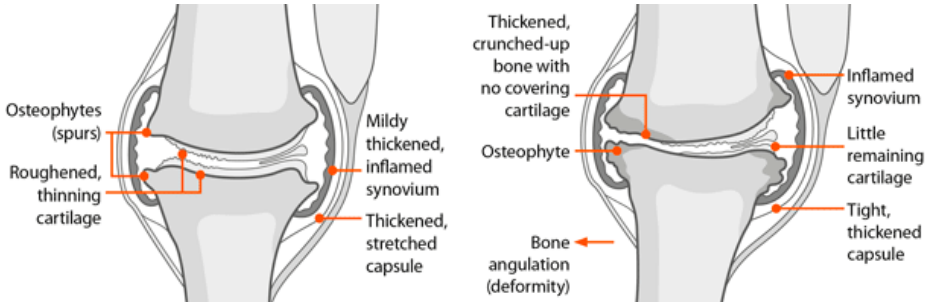

Figure 2. From left to right, moderate and severe knee OA [5].

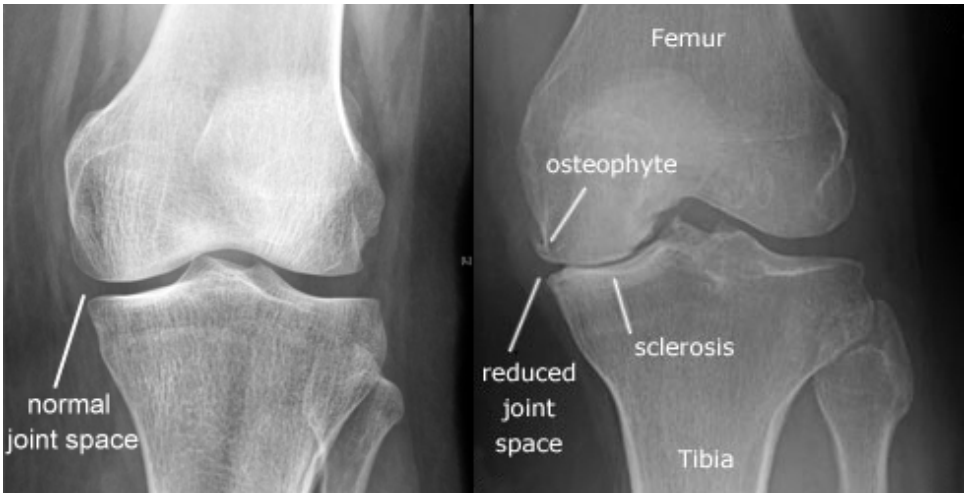

Figure 3. From left to right, normal X-ray and OA knee X-ray [6].

These lesions can be recognized on X-ray and are well described in the Kellgren and Lawrence classification, used to classify the severity of knee OA *(Figure 4)* [7]:

- grade 0: no radiographic feature of OA;
- grade 1: doubtful joint space narrowing and possible osteophytic lipping;
- grade 2: definite osteophyte and possible joint space narrowing on anteroposterior weight-bearing radiograph;
- grade 3: multiple osteophytes, definite joint space narrowing, sclerosis of the subchondral bone and possible bony deformity;
- grade 4: large osteophytes, marked joint space narrowing, severe sclerosis and definite bony deformity.

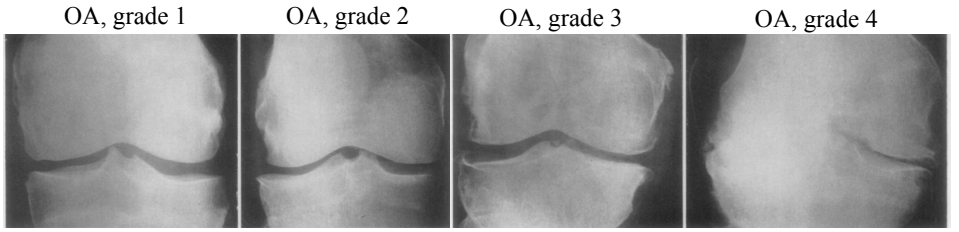

Figure 4. Kellgren and Lawrence classification [7]. OA: osteoarthritis.

Densification of the subchondral bone ("subchondral bone sclerosis") can be identified as a bone reaction that challenges the lack of cartilage protection. Osteophytosis is a bone spur on the margin of the bone that majors the articular surface and attempts to reduce the weight on the pre-existing cartilage [8]. Due to cartilage erosion and degradation, joint space narrowing appears. Later, subchondral bone degradation arises with subchondral cysts ("geodes") that are necrotic area with surrounding healing and bone reconstruction [8]. These radiographic lesions are not present in the early stage of the disease and are badly correlated to OA symptoms.

Magnetic resonance imaging (MRI) is a more accurate imaging technique, able to visualize cartilage defects, but also cartilage structure modifications during

the OA process [8]. Early lesions can be detected before the narrowing of joint space. MRI can also identify subchondral bone lesions before the appearance of sclerosis by the identification of bone oedema in the subchondral bone ("bone marrow lesions"). These bone marrow lesions are predictive of the radiographic progression in patients with knee OA and are associated with type II collagen degradation [9]. If cartilage is a key-lesion in OA, the synovial membrane (discussed later) is also early involved. MRI and ultrasounds early visualized the synovium membrane and the synovitis, another aspect of OA. Synovitis was detected with ultrasounds in 16.6 % of OA patients in a study partly realized in our department [10]. Of interest, two recent meta-analyses found no link between knee-OA pain and geodes or osteophytosis, and no or uncertain links with cartilage morphology [11, 12]. However, the pain was significantly associated with bone marrow lesions, synovitis and effusion.

OA is a prevalent disease. In USA, 12 % of the population between 25-74 years old are suffering from clinical OA symptoms [13]. Radiographic knee OA is found in 37 % [13], 19 % [14] or 28 % [15] of the United-States population according to the studies, while radiographic hand OA is present in 27 % [16]. Prevalence increases with age and OA is more prevalent in women [17]. In France, 4.7 % of men and 6.6 % of women are suffering from knee OA and 2.5 % and 1.9 % respectively are suffering from hip OA [18]. Each year in France, 4.6 millions patients have a medical appointment for an OA-related symptom [19].

OA has a significant impact on the quality of life. In a French survey including 10,412 OA patients, 80 % reported limitations in their activities of daily living and OA patients were significantly more limited than controls [20]. Moreover, OA leads to a higher dependence and social isolation in elderly patients [21, 22]. But the clinical impact was not only limited to the social life: only 14 % of working controls have limitations in their work compared to the 64 % of OA

patients still working [20]. Even if RA is usually considered as a more aggressive disease, the limitation in activities in OA patients is very close to the limitation in RA patients [23] and the economic cost of OA patients is seven times higher [24].

Moreover, OA has a significant weight in the total health expense. Direct costs contribute to 1.7 % of expenses for the French health insurance system (more than 1.6 billions €). From 1993 to 2003, there was a 156 % increase of the costs [19]. Prescriptions of drugs relating to OA symptoms have also increased (+54 %).

Accordingly, OA is now recognized as having a significant impact on the quality of life, the ability to work and the public health cost, involving a high proportion of the population.

1.2.2. Clinical symptomatology

Painful mobilization is the main symptom. For knee OA, according to the European League Against Rheumatism recommendations, three symptoms (persistent knee pain, limited morning stiffness and reduced function) and three signs (crepitus, restricted movement and bony enlargement) are the most useful elements recognized in OA [25]. When all these six elements are present, the probability to have radiographic knee OA rises up to 99 % [25]. The pain typically appears with activity and is reduced after immobilization. For most patients, clinical symptoms and anatomic lesions are slowly growing and accumulate with years. But other "phenotypes" are described. Acute flare can complicate the degenerative evolution, with painful joint swelling. This flare can be confounded with inflammatory, infectious or crystal diseases, but the synovial fluid analysis reveals a low synovial white blood cells count (< 2,000

white blood cells with < 25 % of neutrophils), few proteins (< 30 g/L) and normal glucose level, without bacteria or crystal deposition [26]. A third phenotype, less frequent, is the rapidly destructive OA, than can occur in 10 % of hip OA [27]. According to the initial description of Lequesne, more than 2 mm of narrowing in joint space and more than 50 % loss of joint space within a year support the diagnosis [28].

1.3. Osteoarthritis as a degenerative disease

1.3.1. Classification and epidemiology of osteoarthritis

For long, OA was considered as a degenerative cartilage disease. OA was initially considered as a consequence of [29]:

- an increased pressure on a normal joint due to an overload on weight-bearing joints or anatomic incongruence (hip dysplasia …);
- a physiological pressure on an abnormal joint, with genetic fragility of the cartilage matrix or metabolic abnormality (crystal diseases …).

Mitchell *et al.* proposed a pathogenic classification of OA [29] *(Table 1)*.

A. Abnormal concentrations of force on normal cartilage
Cartilage surface irregularities (*e.g.*, intra-articular fractures)
Misalignment of the joint (*e.g.*, leg length disparity, acetabular dysplasia, congenital hip dislocation)
Loss of ligamentous stability (*e.g.*, anterior cruciate ligament tear)
Loss of protective sensory feedback (*e.g.*, diabetic neuropathy)
Other causes (*e.g.*, obesity, occupational)
B. Normal concentrations of force on abnormal cartilage
Pre-existing arthritis (*e.g.*, RA)
Metabolic abnormalities (*e.g.*, crystal arthropathy)
Genetic (*e.g.*, generalized OA of hands)
C. Normal concentrations of force on normal cartilage supported by stiffened subchondral bone
Paget disease
D. Normal concentrations of force on normal cartilage supported by weakened subchondral bone
Avascular necrosis

Table 1. Classification of OA by etiologies [26, 29]. OA: osteoarthritis; RA: rheumatoid arthritis.

Next to its pathogenic classification, OA is also usually classified as primary or secondary. Secondary OA is caused by an underlying condition, including inflammatory disease, trauma and mechanical factor [26, 30] *(Table 2)*.

Primary OA
Secondary OA
Due to mechanical incongruity of joint, congenital or acquired (*e.g.*, acetabular dysplasia of hip or internal knee lesion)
Due to prior inflammatory disease (*e.g.*, RA)
Due to endocrine disorders (*e.g.*, diabetes, acromegaly)
Due to metabolic disorders (*e.g.*, monosodium urate and calcium pyrophosphate dihydrate crystals, hemochromatosis)
Miscellaneous (*e.g.*, avascular necrosis)

Table 2. Traditional classification of OA by cause [26, 30]. OA: osteoarthritis; RA: rheumatoid arthritis.

1.3.2. Classical risk factors of osteoarthritis

Several risks factors were identified and classified between the systemic or the local risk factors *(Figure 5)*. These factors major the risk to have abnormal cartilage or abnormal force on a normal cartilage.

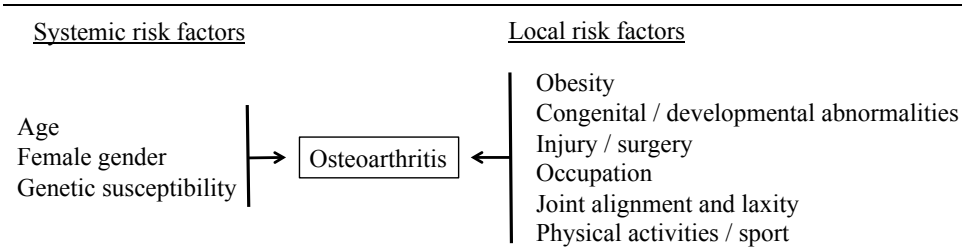

Figure 5. Systemic and local OA risk factors.

Among the systemic risk factors, age, female gender and genetic susceptibility are the most important. Age is one of the strongest risk factors for all joints *(Figure 6)* [17, 31]. Cartilage thinning, weak muscle strength, poor proprioception and oxidative damages are thought to be explanations. Women are more likely to develop OA than men and they also have more severe OA. The risk of OA is further increased at the time of menopause [32-36]. Genetics have also a significant involvement but probably more for hand and hip than for knee [37-39]. Heritability was between 50 and 65 % [37-39]. Other factors are sometimes evocated. Diet could have an influence: the Framingham study showed a higher OA progression when low vitamin C intake or when serum vitamin D levels are in the lowest and middle tertile [40, 41]. However, there is no difference in terms of incidence and confirmations studies are lacking.

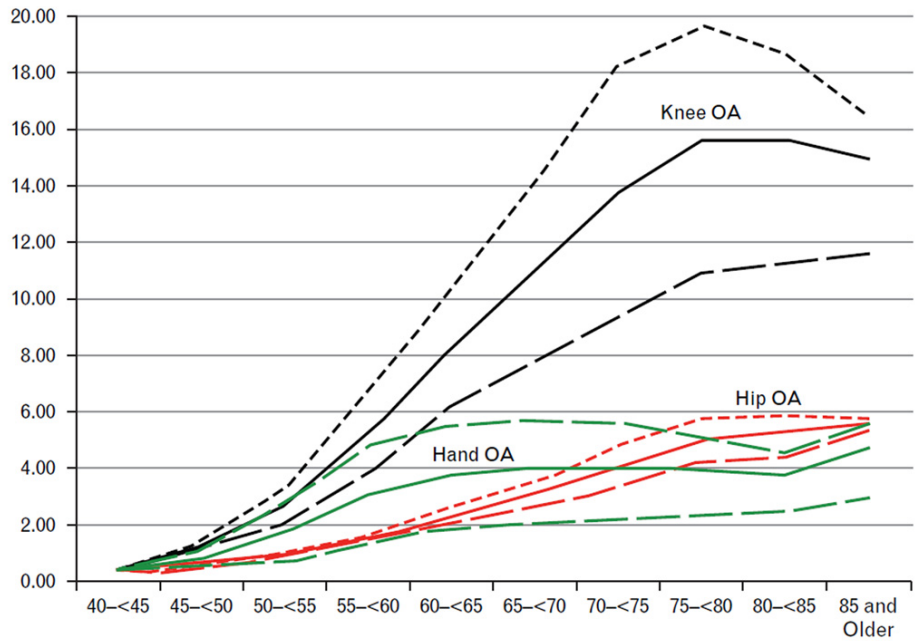

Figure 6. Incidence rates (/1000 person-year) of OA. Solid, all population; short dash line, women; long dash line, men [42]. OA: osteoarthritis.

Local events are also significant risks factors [26, 31]:

- obesity: obesity is a well-established risk factor in OA. Higher mechanical stress on a joint may explain the obesity-induced OA. Damages in OA weight-bearing joints, such as the knee, are correlated with the body mass index (BMI) ("mechanical" theory) [43]. Weight loss significantly lowers knee OA symptoms [44, 45], and weight gain from 20 to 50 years of age is associated with a higher risk of knee OA requiring arthroplasty [46]. A weight loss of 5 kg leads to a 50 % reduction in the risk to develop symptomatic OA [47]. Hip OA was also associated to the BMI [48, 49] but in a lower extend and association was not confirmed in another study [50]. In the Nurses' Health Study, women in the highest category of BMI at age of 18 years old had more than a five fold increased risk of total hip replacement at adulthood [51];
- congenital or developmental abnormalities: they can affect the shape of the joint and lead to a higher local weight [26]. Legg-Calvé-Perthes or congenital hip subluxations are associated with a higher hip OA incidence [52, 53]. Acetabular dysplasia, a more common condition, was associated with a higher risk of hip OA [54] and authors estimate that subclinical acetabular dysplasia could explain 25 % to 40 % of hip OA [53, 55];
- physical activities / sport: studies are controversial. There are evidences that people engaged in a high level of activity have a greater risk to develop knee OA [56, 57]. However, other studies did not find any correlation between moderate long distance running and OA in the absence of acute injury [58, 59];
- injury / surgery: injury of one of the joint structure can lead to higher OA incidence, such as trans-articular fracture, meniscal tear or anterior ligament injury [60, 61]. Prevalence of meniscal damage is higher among patients with radiographic knee OA than those without OA [62]. Meniscectomy is also a local OA risk factor and the amount of removed meniscus is an important predictor factor for OA development [63].

Minimally invasive procedures reduce the incidence of long-term OA changes of the knee compared with more invasive ones [63];

- occupation: some works are risk factors depending on the joint daily involved. As examples, farmers and cotton mill workers have higher incidence of hip and hand OA, respectively [64, 65]. Obese people whose work has entailed prolonged kneeling or squatting have a higher risk of OA [66]. However, if a recent meta-analysis confirms the correlation between occupational tasks and knee OA for men, evidences are less strong for women [67];
- joint alignment and laxity: abnormities in knee alignment lead to altered weight on the joint and local increased pressures. Several studies are in favor of a link between OA progression and abnormal anatomic alignment such as knees with varus alignment or knee valgus alignment at baseline [68, 69]. However, some authors speculated that abnormal alignment could rather indicate severity or progression than being linked with OA incidence [70, 71]. Knee laxity is also associated with OA development [72, 73] but authors enhanced that knee laxity could also be altered by the disease and be a consequence rather than a causative factor [71]. Limb length inequality has also been involved: inequality of more than 1 or 2 cm was associated with a higher OA incidence, pain and progression [74, 75].

1.4. Osteoarthritis as an inflammatory disease

1.4.1. Normal human cartilage

Articular cartilage is composed of a dense ECM and chondrocytes. Normal cartilage has a smooth surface without fissure. It is composed of four layers *(Figure 7)* [76-78]:

- the superficial layer, with flat and spindle-shaped cells. Cells and collagen fibrils are arranged parallel to the joint surface;
- the middle zone, with collagen organized obliquely. Chondrocytes are spherical;
- the deep zone, that provides the greatest resistance to compressive forces, with perpendicular collagen fibrils and the highest proteoglycan content. Chondrocytes are typically arranged in columns;
- the calcified cartilage, distinguished from the deep zone by the tidemark.

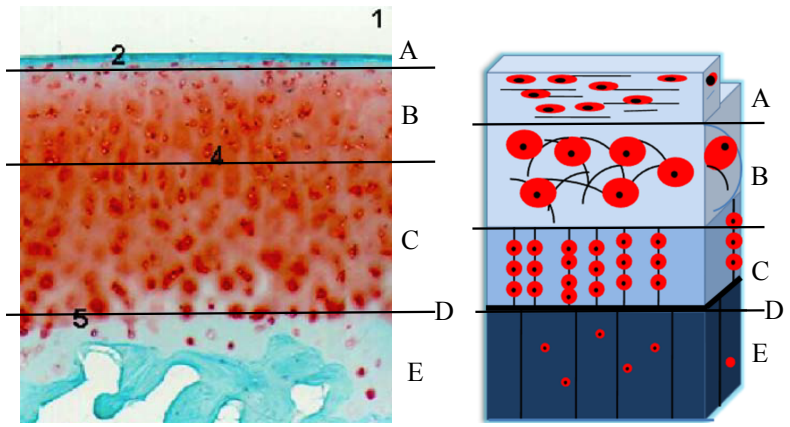

Figure 7. Normal human cartilage [77, 78]. Safranin O/fast green staining. A: superficial layer; B: middle zone; C: deep zone; D: tidemark; E: calcified cartilage. 1: smooth surface; 2: small and flat cells in the superficial zone; 4: safranin O coloration; 5: intact tidemark.

The ECM is principally composed of water, collagen and proteoglycans, with other non-collagenous proteins and glycoproteins present in lesser amounts *(Figure 8)* [76]. Collagen represents 60 % of the dry weight of cartilage. Type II collagen represents more than 90 % of the collagen in ECM, interacting with proteoglycans. Collagen types I, IV, V, VI, IX and XI are also present but contribute only for a minor proportion. Proteoglycans are glycosylated protein monomers and include aggrecan, decorin and fibromodulin. Aggrecan is the most abundant proteoglycan in human cartilage, interacting with hyaluronan to form large proteoglycan aggregates and providing to the cartilage its osmotic properties.

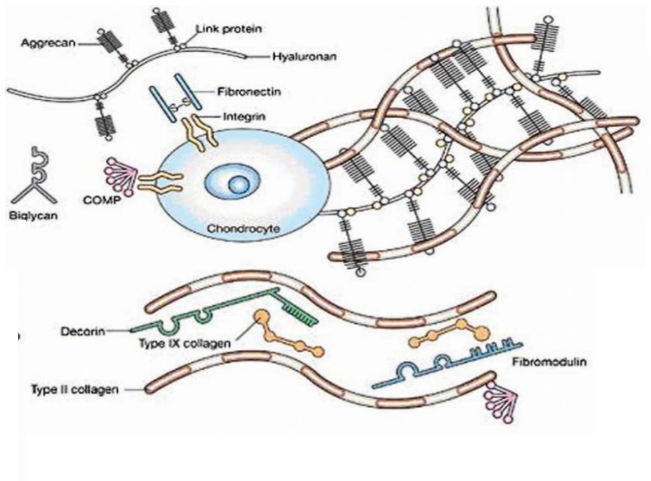

Figure 8. Extracellular matrix [76].

1.4.2. Osteoarthritic cartilage: characterization

OA was initially only considered as a consequence of increased pressure on the joint or genetic fragility of the cartilage matrix [79]. These degenerative injuries lead to cartilage degradation. Histological changes in OA are well described *(Figure 9)* [77]:

- in early OA, there is a loss of proteoglycans with reduction of the safranin O staining. Cells from the superficial zone become round and hypertrophic and then disappear. Middle and deep area show hyper cellularity, with multicellular clusters of hypertrophic chondrocytes;
- in advanced OA, a complete breakdown appears and cells disappear. The tissue is replaced by a fibro-cartilage with scar-like tissue. Cartilage becomes invaded by blood vessels from the subchondral bone that becomes thicker and more compact.

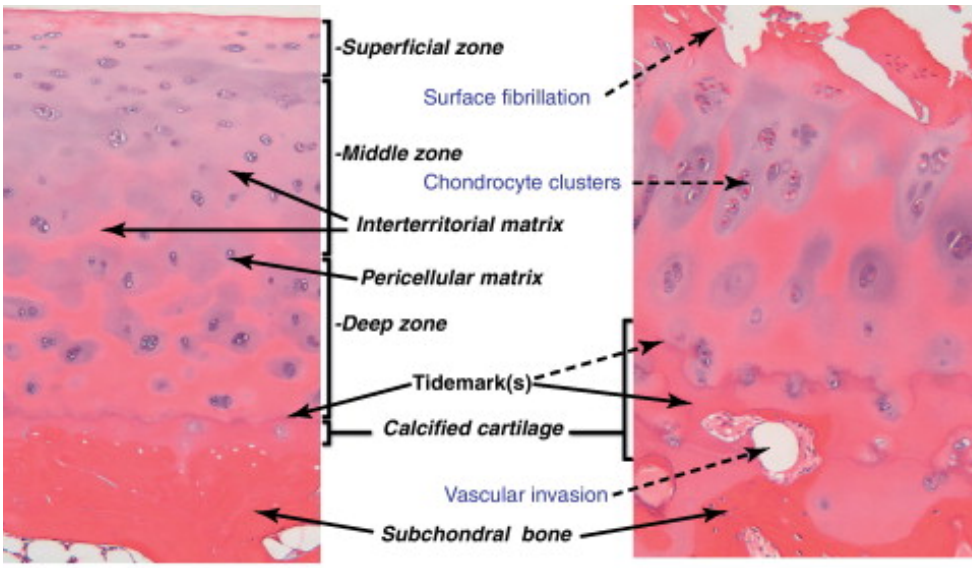

Figure 9. From left to right, normal and OA cartilage [80].

During OA process, there is a progressive shift from an anabolic to a catabolic activation. First step is characterized by the loss of collagen and proteoglycans from the ECM, with a "compensative reaction", chondrocyte proliferation and enhanced chondrocyte metabolism. Cell clusters appear. In later stages, the proliferating step fails and cartilage loss appears [81].

In early OA, there is a shift in the collagen distribution, with a loss of collagen type II production in the superficial layer and a shift towards collagen type I. In deep layers, a higher compensative production of collagen II is initially observed. An increased repair activity is detected in the early stage and is characterized by the acquisition of the "hypertrophic" phenotype [81]. In more advanced OA, loss of collagen type II and higher production of collagen type I is ongoing. Collagen type X expression progressively appears with OA aggravation. Collagen type X is absent in normal cartilage and is found in chondrocyte clusters, with hypertrophic phenotype.

1.4.3. Osteoarthritic cartilage: links with inflammation

If a degenerative hypothesis was supported for long, recent data are now in favor of a major inflammatory component. During OA process, chondrocytes acquire the capacity to produce pro-inflammatory and catabolic cytokines (positive deleterious feedback loop). Matrix metalloproteinases (MMP-1, -3, -8, -13) and pro-inflammatory cytokines [interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α)] are expressed in OA cartilage as confirmed by immunohistochemistry [82-84] and genes expression studies [77]. Secreted MMPs degrade the connective tissue matrix. IL-1β and TNF-α induce the synthesis of MMPs but also of cyclooxygenase type 2 (COX-2), nitric oxide (NO), IL-6, IL-8 … by chondrocytes leading to a catabolic response [85]. Expression of MMPs is associated with degenerative changes and the acquisition of a "hypertrophic" phenotype *(Figure 10)* [82-84]. Exposition of fragment of free type II collagen can be recognized by the hypertrophic chondrocyte after binding to discoidin domain receptor 2, leading to a higher MMP-13 secretion [86].

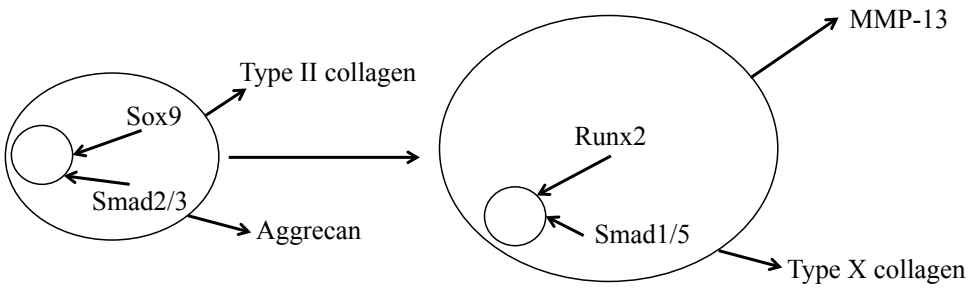

Figure 10. From left to right, normal and hypertrophic chondrocyte (adapted from [81]). The normal chondrocyte, governed by Sox9 and Smad2/3, produces aggrecan and type II collagen. The hypertrophic chondrocyte produces MMP-13 and type X collagen. Its metabolism is dependent on Runx2 and Smad1/5. The Smad pathway will be discussed in the next chapter. MMP: matrix metalloproteinase.

Degenerative and inflammatory hypotheses can be linked together thanks to several studies showing that chondrocytes can response to mechanical stress by increasing the production of inflammatory cytokines. *In vitro*, traumatic injuries lead to genes activation, with induction in chondrocytes of reactive oxygen species and up-regulation of MMP-13, ADAMTS-5 (a disintegrin and metalloprotease with thrombospondin motif type 5) and TNF-α [87-89]. However, non-injurious loading prevents the IL-1β-induced cartilage matrix degradation, supporting the idea that non-traumatic physical activity is protective [90].

Links between aging and OA could also be, in part, explained by the inflammatory hypothesis [79]. With aging, chondrocytes acquire a "senescence secretory" phenotype, with an increased IL-1β-induced MMP-13 production [91]. Moreover, advanced glycation end-products (AGEs) are produced by a non-enzymatic reaction and accumulate in cartilage with aging [79]. These AGEs can activate the production of the nuclear factor-κB (NF-κB), IL-6 and IL-8 [92, 93].

From a degenerative pathology, OA is now becoming an inflammatory disease. The aging and degenerative chondrocyte has an "inflammatory" phenotype.

1.4.4. Transforming growth factor-β pathway and osteoarthritis

Transforming growth factor-β (TGF-β) is a superfamily of growth factors, involved in cell migration, proliferation, differentiation and apoptosis. TGF-β binds TGF-β receptor and induces the phosphorylation of trans-membrane activin receptor-like kinases (ALKs). ALK activation leads to Smad phosphorylation [94, 95]. Classically, TGF-β binds to the type II receptor with activation of ALK5-Smad2/3 pathway (anabolic pathway) *(Figure 11)*. TGF-β can also tip the balance toward the ALK1-Smad1/5 pathway, which plays a major catabolic role in OA. Alternatively, TGF-β is able to activate mitogen-activated protein kinase pathway without interacting with Smad molecules ("non canonical pathway"). TGF-β and its receptor are expressed in the joint but their functions are tissue-dependent [94]. Endoglin is a TGF-β co-receptor that binds TGF-β with high affinity in the presence of the type II TGF-β receptor. In human chondrocytes, endoglin enhances TGF-β1-induced Smad1/5 phosphorylation and inhibits TGF-β1-induced Smad2 phosphorylation [96].

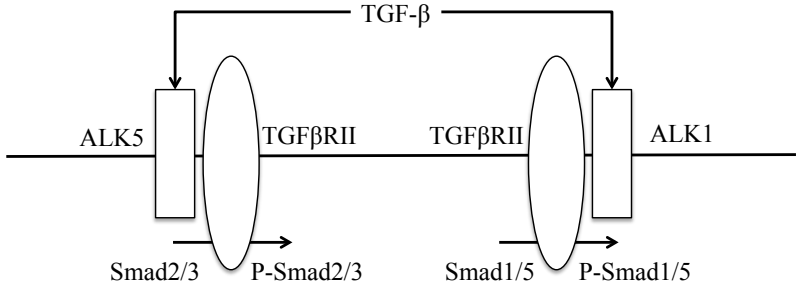

Figure 11. TGF-β pathway signaling (adapted from [97]). TGF: transforming growth factor; TGFβR: TGF-β receptor; ALK: activin receptor-like kinase.

The role of TGF-β in cartilage is controversial [98]. TGF-β1 favors OA but is essential for chondrogenesis. Its influence on MMPs expression is variable. This "dual" action can be explained by the modulation of the Smad pathway. A short TGF-β1 stimulation leads to ALK5-Smad2/3 activation and to an anabolic response in chondrocytes with induction of collagen II expression [98, 99]. However, continuous stimulation by TGF-β1 reduces ALK5-Smad2/3 pathway expression and negatively changes the ratio between ALK5 and ALK1, promoting an enhanced catabolic answer [98, 99].

TGF-β pathway can also be linked to chondrocyte senescence: chondrocyte responsiveness was different during aging. When stimulated with TGF-β1, chondrocytes from old donors do not produce more proteoglycans, while chondrocytes from younger patients increase their production [100]. Young chondrocytes coming from healthy joints produce collagen type II and expressed Sox9. Levels of TGF-β receptor type II, ALK5 and phospho-Smad2 are reduced with the increasing age of the donor and thereby with the acquisition of a hypertrophic phenotype, with MMP-13, collagen type X and ADAMTS-5 production and with Runx2 expression [81, 85, 99, 101, 102] *(Figure 12)*.

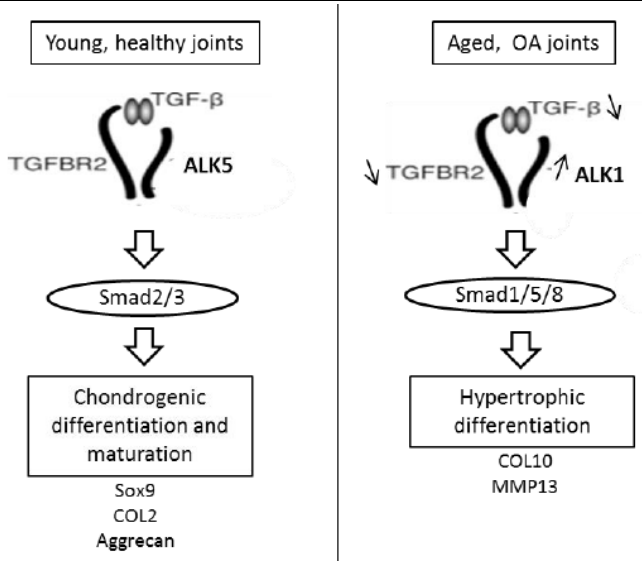

Figure 12. Difference between TGF-β pathway in young and older chondrocytes [98]. TGF: transforming growth factor; TGFβR: TGF-β receptor; ALK: activin receptor-like kinase; COL: collagen; MMP: matrix metalloproteinase.

ALK5/ALK1 ratio was reduced with age, leading the chondrocyte differentiation to a hypertrophic phenotype. ALK5-Smad2/3 can be therefore considered as a protective pathway in OA. According to *Figure 10*, Smad2/3 is associated with normal chondrocytes, while Smad1/5 is associated with hypertrophic chondrocytes. The shift in Smad balance related to the senescence could be the initial event that lead to chondrocyte hypertrophy and the first step leading to catabolic and pro-inflammatory events, with secondary irreversible and self-perpetuating cartilage damages [81].

TGF-β pathway seems differently modulated in bone compared to cartilage [103, 104]. Subchondral bone is involved in OA pathogenesis: increased subchondral bone activity predicts the cartilage loss and correlates with cartilage lesions progression [105]. Transgenic expression of active TGF-β1 in osteoblastic cells induces OA, whereas its inhibition in subchondral bone

attenuates the degeneration of articular cartilage [106]. Smad2/3 mediates this deleterious TGF-β contribution of bone in OA.

In the synovium, the situation is also different from the cartilage. Intra-articular injection of TGF-β leads to synovial fibrosis characterized by progressive synovial hyperplasia, fibroblasts proliferation and ECM deposition [107, 108]. Overexpression of Smad 7 strongly reduces the TGF-β-induced synovial thickness [107]. Smad7 antagonizes TGF-β-induced activation of Smad2/3, suggesting that synovial fibrosis induced by TGF-β is ALK5-Smad2/3-dependent.

In cartilage, ALK5-Smad2/3 is considered as a protective pathway. However, in bone and synovium, ALK5-Smad2/3 is moreover associated with deleterious effects: its inactivation reduces the OA-induction by subchondral bone and the synovial fibrosis.

1.4.5. Synovial membrane: from "bystander damage" to active pro-inflammatory actor

When many researches are focused on cartilage and peri-articular bone, others are in favor of a synovium involvement in OA. Synovium is a cellular membrane organized in two layers *(Figure 13)* [109, 110]: the intima in contact with the synovial fluid and the sub-intima, which is more external. The intima, a lining layer of 1-2 cells thickness, consists of macrophages and fibroblasts, while the sub-intima is relatively acellular, containing blood vessels, fat cells and fibroblasts, with few lymphocytes or macrophages in a collagenous ECM (collagens III, IV, V and VI with little type I collagen) [109, 110].

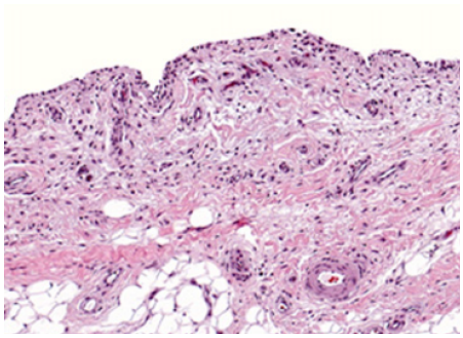

Figure 13. Synovial membrane: intima and subintima layer [111]. 20X. Haematoxylin / eosin staining.

There are two cell types in a normal synovium *(Figure 14)* [110]:

- 20 % of cells in the intima are macrophage-like synoviocytes ("type A synoviocytes") with a hematopoietic origin, derived from blood monocytes;
- 80 % are fibroblast-like synoviocytes ("type B synoviocytes", synovial fibroblasts) derived from a mesenchymal precursor. Synovial fibroblasts can differentiate into osteocytes, chondrocytes and adipocytes [112, 113] and synovium is recognized as a stem cells provider [114], with possible functions to repair the different tissues in the joint.

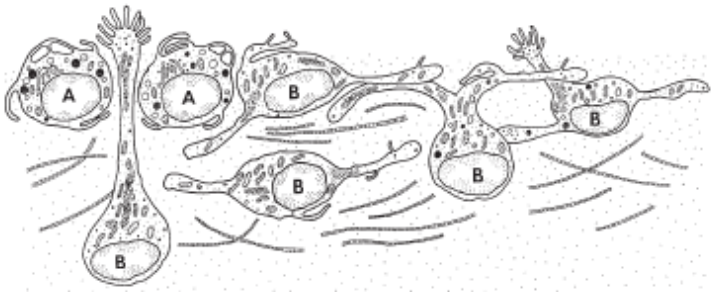

Figure 14. Localization and structure of two types of synoviocytes. Type A synoviocytes are located at the superficial layer of the synovial intima, while type B cells are present at various depths, frequently in the deeper layer of the synovial intima [110].

Synovial membrane produces the synovial fluid components (lubricin, hyaluronic acid …) that protect the articular cartilage surface. Synovial membrane also provides nutriments for cartilage metabolism. Therefore, the alteration of the synovial membrane in OA offers less protection and less food supply to the cartilage.

For long, synovitis was only visualized in late stages of OA and was considered as secondary to mechanic aggression of bone and cartilage fragments that were observed within the synovial membrane. However, several observations demonstrated that synovitis could appear even in the early stage of OA. Synovium can also acquire an "inflammatory" phenotype in OA with similar characteristics than those observed in RA (synovial lining and villous hyperplasia, infiltration by macrophages and lymphocytes, neoangiogenesis and fibrosis) *(Figure 15)* [111]. Synovitis can be visualized by MRI or ultra-sounds. Roemer *et al.* noted a synovitis in 95 % of knee joints with effusion and in 70 % of the knee joint without effusion [115]. Several works of our department confirmed these studies: synovitis were visualized with ultrasounds in a cohort of knee OA patients, with correlation between synovitis, effusion and clinical parameters suggestive of an inflammatory flare [10, 116]. Histologically-confirmed synovitis and angiogenesis were observed in synovial membrane when ultrasound-guided synovial biopsies were realized after intravenous injection of the microbubble contrast agent sulfur hexafluoride [117]. Further, several other studies have confirmed the correlation between synovitis area observed by MRI and specific histologic features of synovitis [118-120].

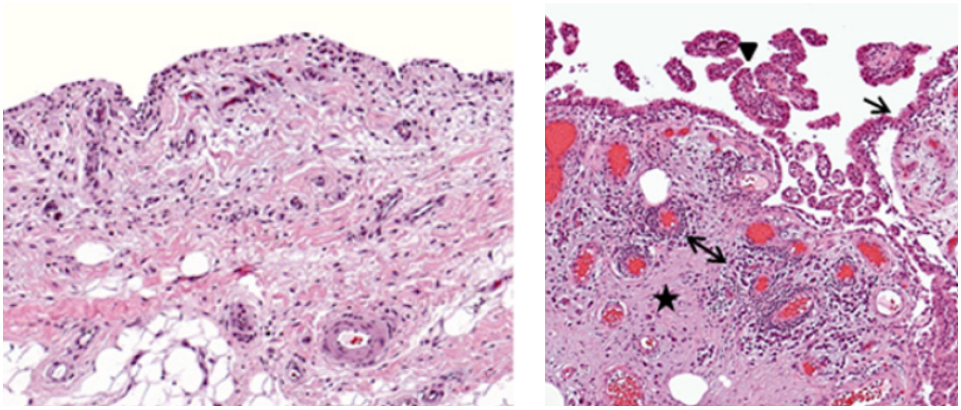

Figure 15. From left to right, normal and OA synovial membrane, with intimal hyperplasia (arrow), villous hyperplasia (arrowhead), fibrosis (star) and perivascular mononuclear cell infiltrates (double-headed arrow) [111]. 20X. Haematoxylin / eosin staining.

Synovitis is not only a "bystander damage" but is related to disease manifestations with correlation with pain, disease progression and disease stage [111]. MRI-defined synovitis is associated to knee OA pain in several studies and higher inflammation grade leads to a 9-fold higher risk to have painful knee OA [121, 122]. Similar correlations are published between knee symptoms and histologically-defined synovitis [123]. Ayral *et al.* report a correlation between synovitis and progression of cartilage erosion visualized in arthroscopy [124], with further confirmations with MRI and ultrasounds studies [10, 125]. Lastly, a higher histological grade of synovial inflammation is associated with advanced knee OA, evaluated by X-ray.

Several pathways can explain the development of synovitis *(Figure 16)*. Matrix fragments and products release can activate innate immune response through the recognition by Toll-like receptors (TLRs), activating NF-κB and promoting the production of pro-inflammatory mediators [126, 127]. TLRs are usually activated by bacteria to induce the innate immunity. These patterns are called "pathogen-associated molecular pattern" (PAMP) and are exogenous signals

inducing the first step of the immune system activation through its innate components. However, TLRs can also binds to non-infectious pattern as cartilage or matrix fragments, namely the endogenous "damage-associated molecular patterns" (DAMPS) molecules. Endogenous components of the human body can activate TLRs, without the intervention of any exogenous agent, and induce the innate immunity activation, leading to tissue damages. TLRs are expressed by a variety of cells, including macrophages, but are also expressed in synovial membrane and by synovial fibroblasts with subsequent production of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α …), recruiting immune cells and inducing catabolic reaction in chondrocytes [128]. Of interest, Sohn *et al.* recently published that plasma proteins present in the OA synovial fluid were able to activate synovial macrophages through TLR-4 [129]. TLRs are also expressed by chondrocytes: DAMPS can directly activate the cartilage through a similar mechanism that activates hypertrophic chondrocytes by collagen II fragments. Our laboratory identified TLR-4 on OA synovial fibroblasts and chondrocytes as a receptor involved in the secretion of pro-inflammatory mediators: blocking TLR-4 with TAK-242 inhibits the cytokines- (IL-6, IL-8, GRO-α, MCP-1) and MMPs- (MMP-1, -3, -13) induction by apolipoprotein-A1 and serum amyloid A protein (A-SAA) [130, 131].

Another pathway is the complement cascade. Complement pathway helps to clear pathogen agents but abnormal activation can lead to organ damages. Complement deposits were found in the synovium of OA patients [132] and increase of synovial complement components during acute OA flare was demonstrated [133]. Next to a local interaction with the complement pathway, our laboratory also demonstrated a systemic link with complement. We identified a complement fragment, C3f, as a serum biomarker of OA in a proteomic study: C3f was expressed at higher levels in serum of OA patients for all Kellgren and Lawrence scores compared to the controls and RA patients [134].

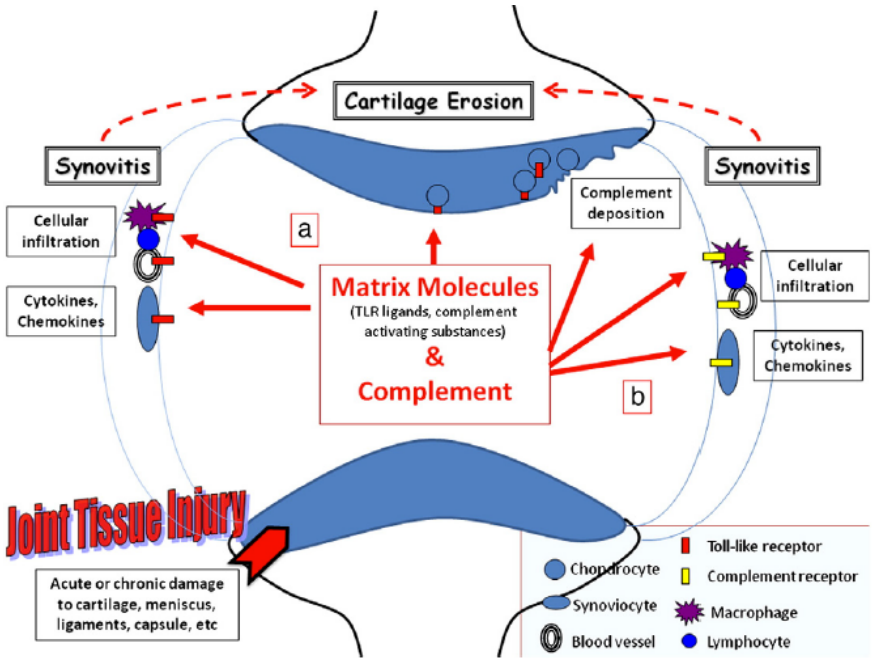

Figure 16. Toll-like receptor (a) and complement activation (b) in OA joint [111].

Activation of TLR and complements pathways leads to synovial inflammation. This synovial activation has an active influence on OA, with the production and the intra-articular release of pro-inflammatory and catabolic cytokines.

1.5. Osteoarthritis as a metabolic disease

1.5.1. Obesity: a systemic risk factor of osteoarthritis

We previously discussed obesity as a local risk factor by overloading the weight on joints. However, obesity is also a risk factor for non weight-bearing joints as it increases the risk of hand OA [135]. Further, obesity can also lead to OA, not only by mechanical factors, but also by metabolic factors.

Deleterious metabolic conditions, such as type 2 diabetes and metabolic syndrome (association of central obesity, high blood pressure, high triglyceride level, low high density lipoprotein cholesterol level and insulin resistance), are also associated with OA with a correlation independent of the BMI, suggesting once again a systemic link between metabolic conditions and OA [136]. Independently from the BMI, type 2 diabetes emerges as an independent risk predictor for arthroplasty [137] and both occurrence and progression of knee OA are related to the accumulation of the metabolic syndrome components [136]. Therapeutic interventions with anti-cholesterol drugs were also in favor of a metabolic involvement in OA: statin use is associated with reduced incidence and progression of knee OA [138], and peroxisome proliferator-activated receptor (PPAR) alpha agonist decreases inflammation and destructive response in OA cartilage [139]. Clinical evidence of involvement of fat in hand OA was sometimes discordant, with a positive correlation with fat mass in the Netherlands cohort [140], but no correlation with the BMI in the Scandinavian's one [141]. Meta-analysis is in favor of a moderate, but significant, association between BMI and hand OA [142]. Therefore, obesity is, additionally to the local and mechanical risk factor due to overweighted joints, also considered as a systemic and metabolic risk factor *(Figure 17)*.

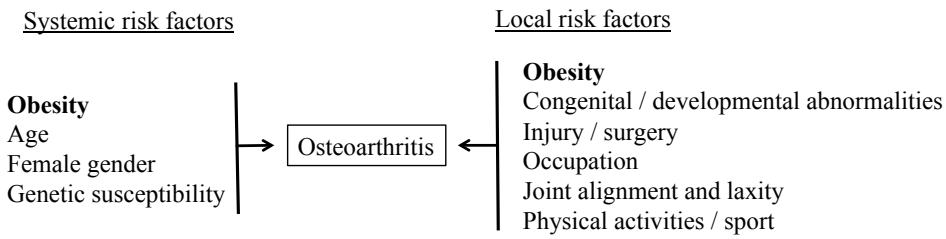

Figure 17. Systemic and local OA risk factors.

Further, obesity is known to be linked to several co-morbidities such as diabetes mellitus, heart disease, dyslipidemia, obstructive sleep apnea, polycystic ovary syndrome [143] … When OA was associated to different elements of the metabolic syndrome, Haara *et al.* also found an association between hand OA and cardiovascular death in men [144], and Jonsson *et al.* described an association between hand OA and carotid and coronary atherosclerosis in older women [145]. OA is therefore more than a joint disease due to mechanical stress; it is also the consequence of systemic metabolic abnormalities.

In addition to the total weight, fat distribution can also influence OA development. Upper body adiposity ("apple shape" or "central obesity") receives contribution from subcutaneous and visceral fat, while lower body adiposity ("pear shape") has mostly subcutaneous fat. The proportion between sub-cutaneous and visceral fat varies from one patient to another *(Figure 18)*. Visceral fat is more associated to insulin-resistance, metabolic syndrome and cardio-vascular events than subcutaneous fat [146]. Serum C-reactive protein is significantly increased when level of visceral fat is higher, while serum adiponectin (a cardio-protective adipokine) is reduced [146]. For OA, a similar observation was raised on a Netherlands cohort showing a correlation between hand OA and visceral adipose tissue but not with subcutaneous fat [140].

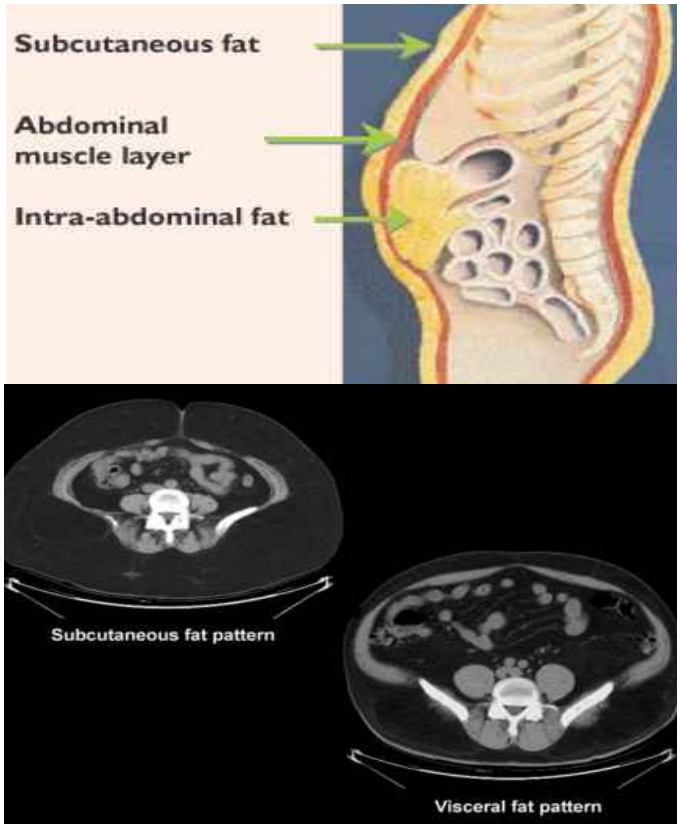

Figure 18. Drawing and CT-scan repartition between subcutaneous and visceral fat [147].

1.5.2. Metabolic pathways in osteoarthritis

Several pathways can explain the metabolic influence on OA in patients with metabolic syndrome *(Figure 19)* [148, 149]:

- the inflamed adipose tissue secretes adipokines, with a pro-inflammatory and catabolic action on cartilage. Adipokines will be reviewed later;
- the inflamed adipose tissue secretes pro-inflammatory cytokines (IL-1β, IL-6, TNF-α …), inducing a low grade inflammation;

- when exposed to high glucose levels, normal chondrocytes reduce their glucose intake but OA chondrocytes fail [150]. Larger amount of reactive oxygen species are produced, inducing oxidative stress and cartilage breakdown [150]. High glucose levels also induce MMP-1 and MMP-13 in OA chondrocytes *in vitro* [151] and lead to non-enzymatic glycation and the production of AGEs that accumulate in OA cartilage and induce inflammation [152, 153];
- abnormal lipid metabolism: the level of total fatty acid in the chondrocytes from OA patients increases with the cartilage damage [154] and there is a diminution of the cholesterol regulation genes in OA chondrocytes [155]. Moreover, oxidized low-density lipoprotein involved in atherosclerosis, can also promote inflammation in OA process [156]. Our laboratory enhanced the link between lipid metabolism and inflammation and proposed that the lipid metabolic profile was deregulated in the synovial fluid of OA patients: apolipoprotein-A1 was negatively correlated with MMP-1 and MMP-3 in the blood, but positively correlated with IL-6 and MMP-3 in the synovial fluid of OA patients [131]. Moreover, *in vitro*, apolipoprotein-A1 induced MMP-1, MMP-3 and IL-6 secretion in OA synovial fibroblasts and chondrocytes [131].

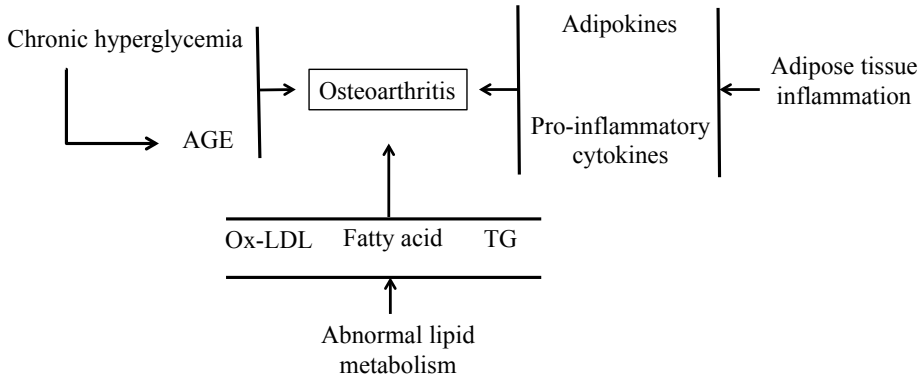

Figure 19. The metabolic component of OA (adapted from [148, 149]). AGE: advanced glycation end products; Ox-LDL: oxidized low-density lipoproteins; TG: triglyceride.

Next to the extra-articular fat, there is also an intra-articular fat tissue in the knee: the Hoffa's body. Hoffa's fat pad (infrapatellar fat pad) is an intra-capsular and extra-synovial adipose tissue structure in the knee joint *(Figure 20)*. It is able to contribute to intra-articular inflammation by producing cytokines, adipokines and lipid mediators [157, 158]. Hoffa's body is also a source of mesenchymal stem cell (MSCs), but comparison between normal and OA tissue are lacking [157]. In obese people, inflammation in the intra-articular fat tissue in MRI studies correlates with the severity of pain in knee OA [159]. Of interest, *in vitro*, infra-patellar fat is a more potent IL-6 producer than sub-cutaneous peri-articular fat [160].

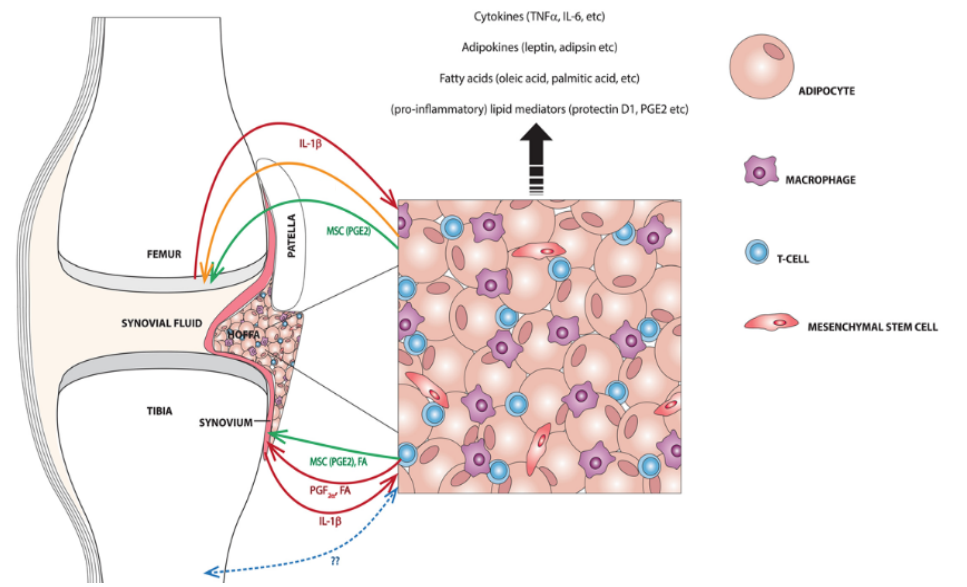

Figure 20. Infrapatellar fat pad and its relations with joint structure [157].

Among the different metabolic pathways involved, adipokine production by the adipose tissue is one of the strongest links between OA and the metabolic syndrome. Among the different adipokines, leptin levels are similar or higher in the synovial fluid compared to the serum of OA patients, suggesting an active and local production [161, 162]. Leptin is therefore of particular interest.

1.5.3. Osteoarthritis and leptin

Leptin is a 16 kilodalton adipocyte-derived protein involved in food intake and energy expenditure regulation. Leptin binds to its receptor, Ob-R, and activates intra-cellular signaling Janus kinase/signal transducers and activators of transcription, phospho-inositide 3-kinase, protein kinase C and mitogen-activated protein kinases [163]. Leptin acts as a negative feedback to reduce the energetic balance, by reducing food intake and majoring the energy expenditure. New roles of leptin are emerging, with implications in bone metabolism, endocrine functions, inflammation, immune response or even cancer signaling

(Figure 21) [164-167]. Although leptin was initially considered as an adipocyte-derived hormone, other cell lineages are leptin producers, as placental trophoblastic cells [168], bronchial epithelial cells [169], gastric cells [170] or bone marrow MSCs [171].

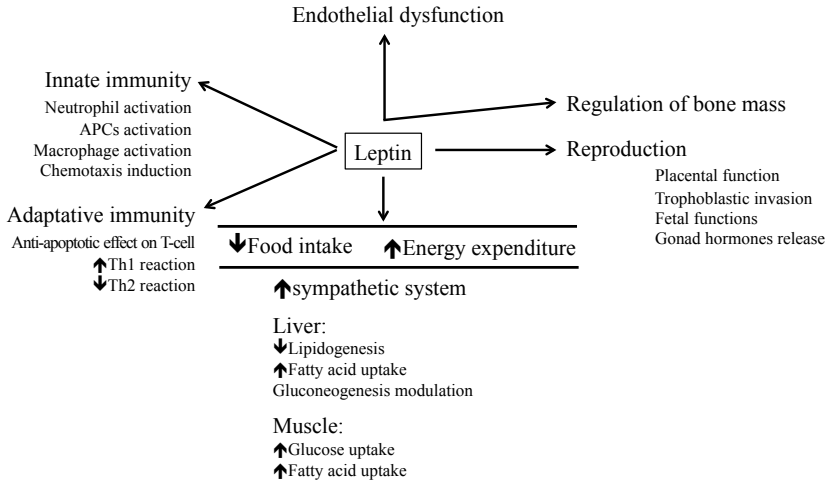

Figure 21. Pleiotropic function of leptin (adapted from [164-167]). APC: antigen-presenting cell.

Circulating leptin levels are positively correlated with the BMI (especially the fat mass). Obese peoples have increased levels of leptin [172]. In mice, leptin resistance or leptin deficiency leads to obesity, while exogenous injection of leptin inhibits food intake and reduces the size of body fat store [173]. Leptin deficiency is rare in human. In human common obesity, leptin levels are high and fall to reduce the body weight due to leptin resistance [174].

There are many links between leptin and OA. In two studies, leptin levels in the synovial fluid are increased compared to those in serum, suggesting a local production and not only a passive diffusion of leptin into the joint. On the contrary, other adipokines levels (adiponectin and resistin) in the synovial fluid are similar or reduced compared to those in serum [161, 162]. Leptin is usually

considered as detrimental in rheumatology: synovial leptin levels are higher in OA knee patients than in control subjects [175]. Serum leptin levels are also higher in patient knee OA [176, 177] but not in hand OA, while leptin levels are associated with hand-pain [178, 179]. Synovial levels are correlated with the radiologic severity of the knee disease [180], but also with knee and hip pain in OA patients [181]. Basal leptin levels in knee OA patients are associated with a higher cartilage loss by MRI and higher incidence of total knee replacement [182]. Moreover, glucosamine sulfate and exercise therapy reduce the serum leptin level in knee OA patients [183].

Intra-articular leptin injections in rats stimulate expression of MMPs and of aggrecanases ADAMTS-4 and -5 (demonstrating a catabolic activity), while anabolic factors are decreased [184]. *In vitro*, leptin shows evident pro-inflammatory properties with inducible NO synthase, COX-2, ADAMTS-4, -5, -9, MMP-9, MMP-13, IL-6 and IL-8 induction in cartilage *(Figure 22)* and IL-8 induction in synovial fibroblasts [162, 185-190]. Down-regulation of leptin leads to MMP-13 reduction in OA chondrocytes [191]. Of interest, leptin has also a significant catabolic effect on meniscus, a major component of the joint stability [192].

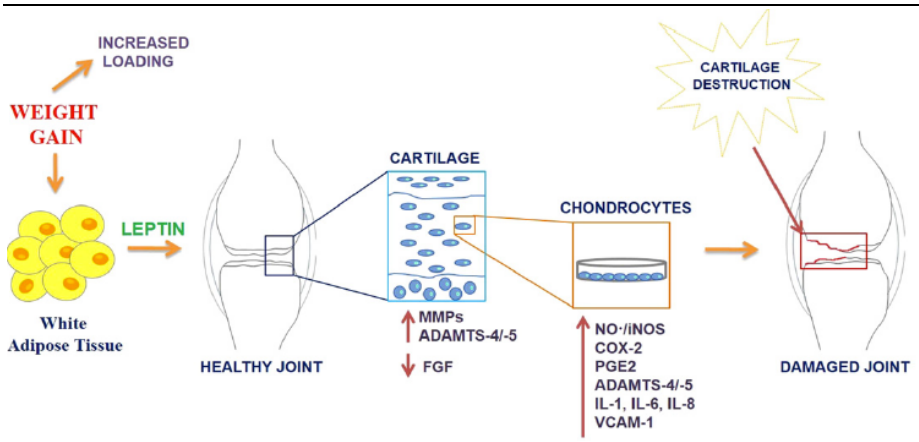

Figure 22. Action on leptin on cartilage and chondrocytes [186]. MMPs: matrix metalloproteinase proteins; ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs; FGF: fibroblast growth factor; iNOs: inducible nitric oxide synthase; COX: cyclooxygenase; PGE: prostaglandin E; IL: interleukin; VCAM: vascular cell adhesion molecule.

On the other hand, very few studies report an anabolic role of leptin enhancing TGF-β and insulin growth factor 1 production by chondrocytes after leptin injection in mice [175] or chondrogenesis stimulation [193]. Catabolic effect of leptin can perhaps trigger compensatory anabolic response in the early OA stage [186].

In 2009, Griffin *et al.* enhanced the link between OA and leptin: the incidence of knee OA is not increased in obese leptin-impaired mice (due to leptin or Ob-R deficiency) opposite to normal-weight mice control [194]. Then, without leptin, adiposity seems insufficient to induce knee OA. Regulation of leptin is tissue-dependent. In obese people, there is a hypothalamic resistance to leptin, leading to obesity, as explained earlier. But, in overweight people, in addition to higher intra-articular leptin levels, chondrocytes do not show leptin resistance but rather an increased response to leptin: negative leptin regulators "suppressor of cytokine signaling 3" (SOCS-3) and soluble OB-R (sOb-R) are decreased in obese patients with OA *(Figure 23)* [185, 195].

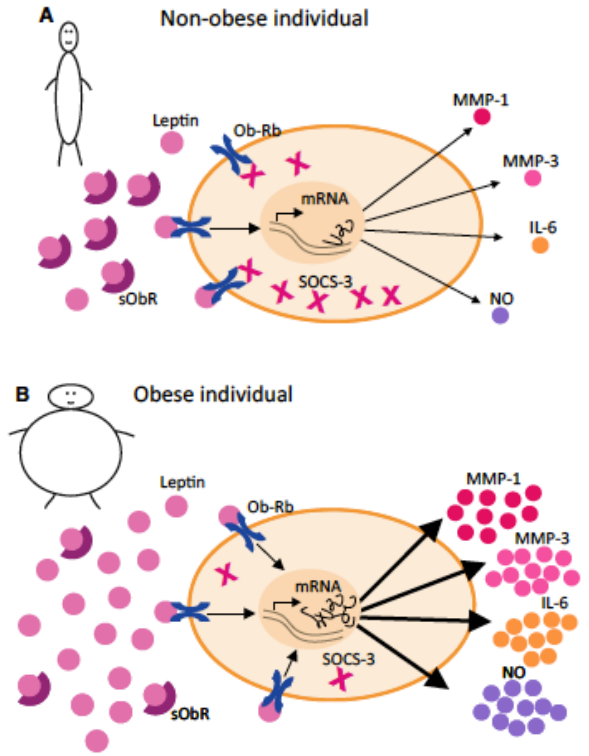

Figure 23. Leptin regulation in obese and non-obese patients. Leptin levels in synovial fluid are increased and the levels of negative regulators sObR and SOCS-3 are low in obese patients [185]. Ob-Rb: long form of the leptin receptor; sOb-R: soluble leptin receptor; MMP: matrix metalloproteinase proteins; IL: interleukin; NO: nitric oxide.

1.5.4. Osteoarthritis and other adipokines

Adipose tissue is an endocrine organ that produces quantities of different adipokines, chemokines, pro-inflammatory cytokines *(Figure 24)* …

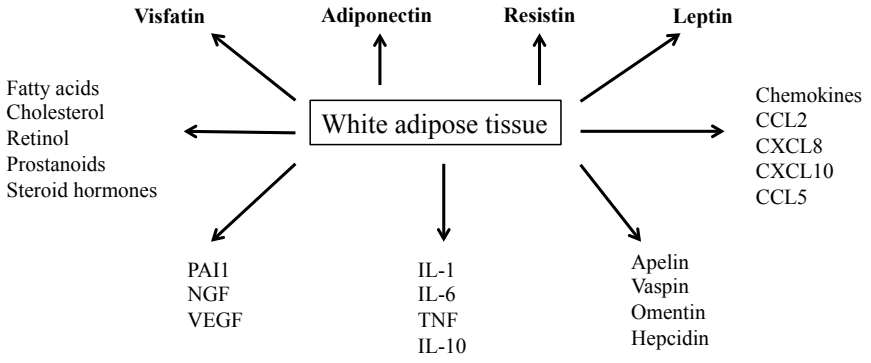

Figure 24. Adipose tissue as an endocrine organ (adapted from [196]). PAI: Plasminogen activator inhibitor; NGF: nerve growth factor; VEGF: vascular endothelial growth factor; IL: interleukin; TNF: tumor necrosis factor; CCL: CC chemokine ligand; CXCL: CXC chemokine ligand.

Next to leptin, other adipokines have been involved in the OA process: resistin, visfatin and adiponectin, but the literature presents various controversies.

Resistin is secreted by macrophages (more than by adipocytes) and is involved in insulin-resistance, sepsis and inflammatory diseases [197-200]. However, the association between resistin and insulin-resistance remains controversial [199, 201]. Serum levels of resistin are increased in obese patients [201-204] but all studies do not confirm this association [205, 206]. There are several evidences about resistin involvement in OA process. Synovial resistin levels are increased in patients after knee injury, a risk factor for post-traumatic OA [207]. Resistin levels are also correlated with IL-6, MMP-1 and MMP-3 synovial levels in knee OA patients [208] and are positively associated with the arthroscopic OA severity score and the Kellgren and Lawrence classification [209]. For hand OA, serum resistin levels were higher in radiographic than in non-radiographic patients or in controls, with a specific association with subchondral erosion [210]. Some studies are discordant: Filkova *et al.* do not find any difference in terms of serum resistin levels between 95 women with or without hand OA

[211]. Further, resistin is not associated with radiographic hand OA progression compared to adiponectin [135]. However, a recent meta-analysis confirms a global significant association between resistin levels and presence of OA [212]. Resistin has pro-inflammatory properties in mice by inducing synovial inflammation when injected in joints [213]. In humans, resistin enhances the up-regulation of IL-6, TNF-α, MMP-1 or MMP-13 in articular chondrocytes [207, 214].

Visfatin is significantly correlated with the BMI [215]. It has a pro-inflammatory action through the insulin receptor [216, 217]. Links between visfatin and OA are less described. Visfatin is released by all human OA tissue, mostly by the synovium [218]. It plays a catabolic role in OA and it is involved in cartilage destruction after chemical agent induction or surgery [219, 220]. *In vivo*, Duan *et al.* published higher levels of visfatin in the synovial fluid of OA patients compared to controls, with a positive correlation between the degradation of collagen and aggrecan and the resistin levels [221].

Adiponectin has insulin-sensitizing properties and serum levels are lower in obese patients [222, 223]. Adiponectin also demonstrates anti-inflammatory properties in atherosclerosis but its expression is higher in other chronic inflammatory diseases such as RA [224, 225]. Clinical studies about adiponectin do not show any obvious pathogenic influence on OA. Filkova *et al.* observed increased serum adiponectin levels in female with erosive hand OA [211], but there is no difference in synovial fluid adiponectin levels with the severity of knee OA or in serum levels with the severity of hand OA [178, 226]. Higher level of adiponectin is even associated with a lower risk for hand OA progression and with a decreased radiographic severity of knee OA [135, 227]. Two recent studies have however highlighted the role of adiponectin in OA: Gross et *al.* published an association between synovial fluid adiponectin, IL-6 and TGF-β1, while Francin *et al.* showed an association between adiponectin

and cartilage degradation by immunochemistry in OA human joints [228, 229]. Older studies described an association between serum adiponectin and two biomarkers of OA, the cartilage oligometric matrix protein COMP and MMP-3 [230]. Other authors also found a pro-inflammatory effect of adiponectin on human chondrocytes with increased MMP-3 expression and induction of NO synthase II or other pro-inflammatory cytokines [231, 232]. Adiponectin enhances IL-6 production in human synovial fibroblasts [233]. However, *in vitro* studies are also controversial. Adiponectin down-regulates the IL-1β-induced MMP-13 production in chondrocytes, in favor of a protective role in OA [234]. Francin *et al.* do not find any correlation between the chondrocyte phenotype and the expression of adiponectin, while correlation is positive for leptin [228].

In summary, they are many *in vivo* and *in vitro* evidences showing that adipokines are associated with OA pathogenesis, but studies are sometimes discordant. Involvement of a particular adipokine can never be systematically pointed out. Nevertheless, the deleterious role of leptin in OA seems the most described *in vivo* and *in vitro* compared to other adipokines.

1.5.5. Osteoarthritic synovial fibroblast and leptin

A few years ago, our laboratory published that human synovial fibroblasts from OA patients, without any adipogenic differentiation, were able *in vitro* to spontaneously produce leptin, hypothesizing that synovial fibroblasts themselves were able, concomitantly to systemic or joint-surrounding adipose tissue, to contribute to intra-articular or even systemic levels of leptin [1]. Leptin secretion and Ob-R expressions were surprisingly, but markedly, enhanced by GCs prednisolone, cortisone and dexamethasone [1]. Higher Ob-R expression could therefore lead to increased leptin responsiveness. Induction of leptin (a catabolic

and pro-inflammatory adipokine in OA process) by GCs (an anti-inflammatory agent) in OA cells needed therefore further investigations.

As previously described, TGF-β1 signaling depends on the ALK5-Smad2/3 pathway and / or the ALK1-Smad1/5 pathway. Davidson *et al.* showed an age-related shift in ALK1/ALK5 ratio in murine cartilage and a strong correlation between ALK1 and MMP-13 expression in human cartilage [235]. They suggested that dominant ALK1 signaling resulted in deviant chondrocyte behavior, contributing to age-related cartilage destruction and OA. Our laboratory demonstrated that TGF-β pathway was involved in prednisolone-induced leptin secretion through ALK1-Smad1/5 pathway (negatively involved in OA) and that TGF-β1 reduced the leptin secretion through Smad2/3 *(Figure 25)* [171].

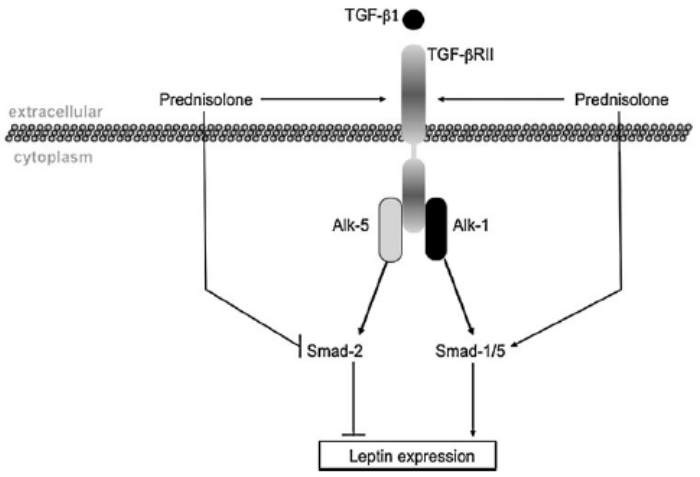

Figure 25. TGF-β pathway and leptin production [171]. TGF: transforming growth factor; TGF-βR: TGF-β receptor; ALK: activin receptor like kinase.

1.6. Treatment of osteoarthritis

OA is a prevalent disease with limited non-surgical treatment options. Symptomatic treatment with paracetamol is usually recommended in first-line, but efficacy is sometimes lacking: a significant but not clinically important effect was noted for knee pain, but not for spine OA pain [236]. Non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used, but remain limited because of cardiovascular, gastro-intestinal or renal toxicity, as noticed in the Osteoarthritis Research Society International 2014 recommendations *(Table 3)* [237]. GC intra-articular injections are considered as "appropriate" and will be reviewed later.

Viscosupplementation with hyaluronic acid only reaches the "uncertain" level for knee OA. New "symptomatic slow-acting drugs" were developed in OA (chondroitin, glucosamine …), but even if emerging, strong evidences of efficacy (even for symptomatic relief) are still lacking. Biological treatments were tried, but with limited or no significant effect for anti-TNF-α and anti-IL-1β therapies [238, 239]. Antibodies against nerve-growth factor in development seem efficient against the pain, even if cases of rapidly destructive OA were noticed [240]. In OA, there is still no validated medical curative treatment. The poor efficacy of drugs to modify disease evolution indicates that we probably miss the good targets. Recently, human genome wild expression has re-orientated the study of inflammatory mediators towards molecules such as IL-17 and TGF-β1, more likely to mimic the disease in OA models [241]. While waiting for new drugs fighting new targets, symptomatic pharmacological and non-pharmacological treatments remain usually used, up to the surgical time. The use of intra-articular GCs injection is still central. The evidence that GCs can induce leptin and Ob-R by synovial fibroblasts raises questions.

Non pharmacological interventions
Appropriate: balneotherapy (for multiple OA with co-morbidities), biomechanical interventions, walking stick (for only knee OA), exercise (land and water-based), strength training, self management, transcutaneous electrical nerve stimulation, weight management. **Uncertain:** acupuncture, crutcher, ultrasounds (for knee only OA). **Not appropriate**: neuromuscular electric stimulation, ultrasounds (for diffuse OA).
Pharmacological interventions
Appropriate: paracetamol (without co-morbidities), intra-articular corticosteroid, NSAID topical (for knee only OA), NSAID (individuals without co-morbidities), oral COX-2 inhibitors (individuals without co-morbidities; multiple OA with moderate co-morbidity risk), duloxetine (individuals without co-morbidities), capsaicin (for knee only OA without co-morbidities). **Uncertain:** glucosamine (for symptoms relief), chondroitin (for symptoms relief), hyaluronic acid (for knee only OA), opioid (oral), opioid (transdermal), NSAID topical (for diffuse OA), oral COX-2 inhibitors (knee only OA with moderate morbidity risk), avocado soybean unsaponifiables. **Not appropriate:** glucosamine (for disease modification), chondroitin (for disease modification), hyaluronic acid (for diffuse OA).

Table 3. Osteoarthritis Research Society International guidelines for the non-surgical treatment for knee OA (adapted from [237]).

1.7. Corticosteroids and osteoarthritis

1.7.1. Glucocorticoids and inflammation

GCs are the most potent anti-inflammatory drugs and they can activate genomic pathways, even if non-genomic actions are also described, but less characterized [242, 243]. They bind to the GC receptor (GR), an intra-cellular receptor, leading to GR activation. Consequently, the GC-GR complex down-regulates transcription of pro-inflammatory genes. This represents the « transrepression pathway », which is mainly anti-inflammatory *(Figure 26, left panel)*. Anti-inflammatory effects can be driven by a direct binding of the GC-GR complex to a glucocorticoid responsive element (GRE) on DNA enhancing the down-regulation of inflammatory cytokines (IL-6, IL-1 …), of COX-2 or of leuko-endothelial adhesive molecules transcription. GR-GC complex can also interact with transcription factors, such as NF-κb, that secondly interact with pro-inflammatory genes. The transrepression pathway is usually dependent on "monomeric GR" [242, 243].

Complex binding with GRE can also lead to the induction of gene transcription, mainly by involving metabolic genes but also by stimulating anti-inflammatory mediators (IL-10, Iκb …). This is the "transactivation pathway", depending on GR dimerization *(Figure 26, left panel)*. Typical genes controlled by the transactivation have a role in glucose and fat metabolism: glycerol-3-phosphate acyltransferase in energy and cholesterol metabolism; phosphoenolpyruvate carboxykinase in gluconeogenesis; stearoyl-CoA desaturase-1 in adipose tissue specification … [244]. They can be linked to the long-term metabolic adverse events of GCs.

However, this “dual” distinction between transactivation (attached to the metabolic properties of GCs) and transrepression (attached to the anti-inflammatory properties) is probably over-simplistic and some divergences exist. First, if transactivation pathway is linked to metabolic events, several anti-inflammatory genes can also be activated by GCs to transactivation, leading to an anti-inflammatory effect (annexin-1, sphingosine-1 phosphate …). Moreover, feedback inhibition of the hypothalamic-pituitary-adrenal axis and immunosuppression, two “non-metabolic” adverse events, could be transrepression-dependent [244]. Lastly, earlier works suggested that GR dimerization was necessary for the transactivation: a mutant version of GR, deficient in dimerization, was only able to activate the transrepression [245]. However, Presman *et al.* have recently challenged this dogma and found no correlation between the GR monomeric/dimeric state and transcriptional activity [246]. It can be resumed that metabolic events of GCs are mostly driven through the classical transactivation. In some cases, adverse events can however be driven through the transrepression of nGRE. Opposite, anti-inflammatory properties of GCs are mostly driven through transrepression, but also in some case through transactivation of anti-inflammatory genes. The reality is probably more complex than first expected *(Figure 26, right panel)*.

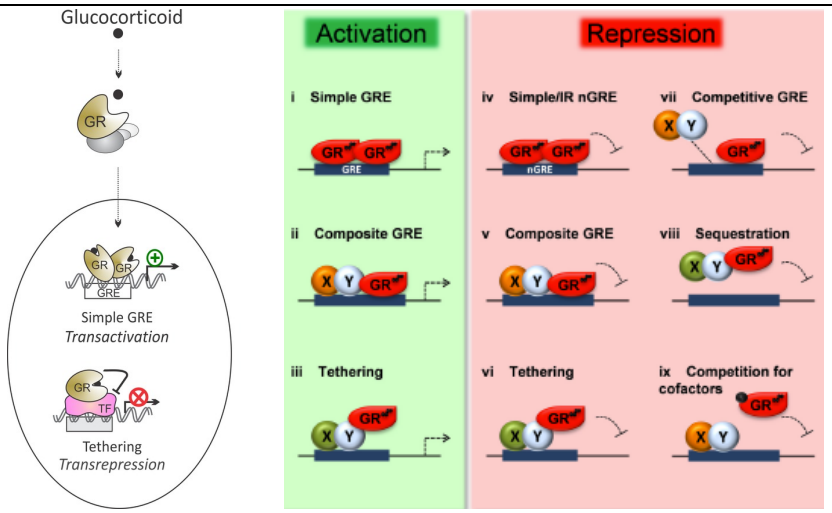

Figure 26. Left panel: "classical" distinction between transactivation and transrepression [247]. Right panel: more nuanced differences between gene activation or repression. Transactivation is mediated by: (i) binding of GR dimers to GRE; (ii) DNA binding of GR with another transcription factor (TF); (iii) binding of GR to a TF by a tethering mechanism. Transrepression can be achieved by: (iv) direct binding of GR dimers to GRE; (v) DNA-binding cross-talk with another TF; (vi) interference of monomeric GR with the transactivation activity of TFs by a tethering mechanism; (vii) competition for an overlapping binding site; (viii) sequestration of a DNA-bound TF; (ix) competition for binding cofactors with other DNA-bound TFs [247]. GRE: glucocorticoid responsive element; GR: glucocorticoid receptor.

GR is the native receptor of GCs. However, GCs can also act through the mineralocorticoid receptor (MR) [248]. Conversely mineralocorticoids, in addition to their native receptor MR, can act through the GR [249]. Mineralocorticoids are another class of corticosteroids involved in the salt and fluid balance in the kidney. Several other cells and organs express the MR and aldosterone can also have a physiological role in vascular, cardiac, ocular or cutaneous tissues [250]. In addition to regulation in water balance, other effects have been proved, linked to inflammation and metabolism *(Figure 27)*:

- association with pro-inflammatory effect and generation of oxidative stress [251];
- association with fibrosis [251], these three effects leading to endothelial dysfunction, hypertension and heart disease;

- possible implication in arthritis, as spironolactone (a MR antagonist) showed a positive effect on 21 patients with different inflammatory arthritis [252];
- association with the metabolic syndrome: recent works have also suggested that components of the metabolic syndrome were associated with abnormal aldosterone physiology [253] and that aldosterone is associated with the development of metabolic syndrome in humans [254, 255] and aggravation of glucose intolerance in rats [256].

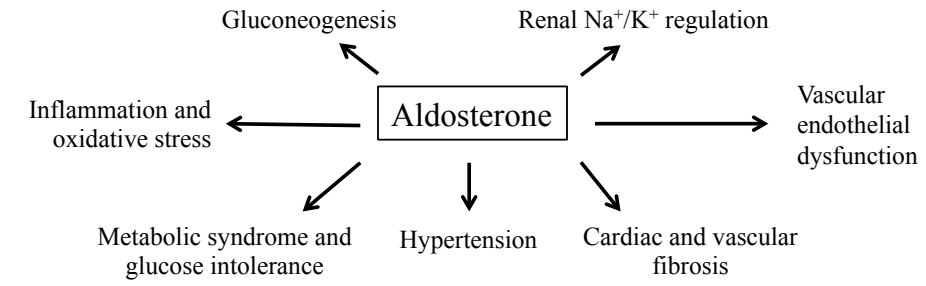

Figure 27. Different roles of aldosterone.

GCs act on adaptive and innate immune cells to down-regulate the production of pro-inflammatory cytokines, to up-regulate the production of anti-inflammatory cytokines and to re-orientate the inflammatory response toward an anti-inflammatory pathway. However, GCs may directly target articular cells such as synoviocytes and chondrocytes that also express GR [257]. Prednisone and cortisone, two chemical drugs, have only a weak affinity for GR. These drugs are made powerful thanks to the presence of 11β-hydroxysteroid deshydrogenase type 1 (11β-HSD-1) enzyme, that converts the hormonally inactive cortisone into the hormonally active cortisol *(Figure 28)* [258]. It also interconverts inactive prednisone into active prednisolone. 11β-HSD1 is expressed at high levels in the liver. Orally administered cortisone and prednisone are extensively converted to their active form on first-pass

metabolism through this organ. Individuals lacking 11β-HSD1 enzyme are unresponsive to high-dose cortisone acetate or prednisone, but maintain sensitivity to hydrocortisone (*e.g.* cortisol) and prednisolone. 11β-HSD1 is also expressed in human synovial tissue, permitting a local conversion into active forms when these GCs are directly injected in the joint [258].

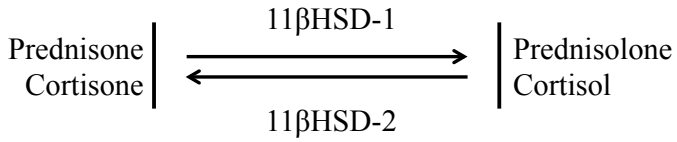

Figure 28. 11β-HSD1 and 11β-HSD2 enzymes. 11β-HSD1 enzyme converts hormonally inactive cortisone and prednisone with hormonally active cortisol and prednisolone. 11β-HSD2 is a powerful glucocorticoid inactivator expressed primarily in the kidney where it prevents cortisol binding to the mineralocorticoid receptor by inactivating it to cortisone (which itself has a very low affinity for the MR) [259]. 11β-HSD: 11β-hydroxysteroid deshydrogenase.

Leptin induction by GCs was already described in adipocytes and fat tissue. *In vitro,* dexamethasone induces leptin production by human adipocytes [172]. Oral administration of dexamethasone in healthy volunteers leads to higher circulating leptin levels and higher leptin expression in abdominal fat tissue [260]. Oral administration of prednisolone also induces a significant increase in circulating leptin levels in Crohn patients [261]. Expression of leptin and Ob-R by OA synovial fibroblasts under GC treatment raises new unexpected and interrogative questions in the OA process. Of interest, in addition to correlation with the metabolic syndrome, aldosterone is also linked to leptin, with an *in vitro* increase of leptin mRNA in brown adipose tissue after aldosterone exposure [262] and higher circulating leptin levels in patients with primary hyperaldosteronism [263].

1.7.2. Glucocorticoids and osteoarthritis

In the rheumatology field, GCs are widely used, especially to treat an inflammatory flare in rheumatic diseases. We previously described validated treatments of OA: curative treatments are lacking, viscosupplementation does not reach the higher proof level, NSAIDs are restricted to patients without co-morbidity risk and opioids induce respiratory failure and addiction. If symptomatology remains limited to one or a few joints, intra-articular injection of GC is a conventional treatment. From 1999 to 2013, a United-States register revealed that nearly 30 % of new knee-OA patients received at least one GC intra-articular injection during their follow-up, and the number of injection per patients is now increasing compared to previous data [264]. If intra-articular injections are frequently used [265] and validated by different Societies of Rheumatology, there is still no evidence about a real efficacy of GCs in clinical practice.

Cochrane's review in 2006 considered that the short-term benefit of intra-articular corticosteroid treatment in knee OA was well established (with no evidence for long term benefit) [266]. However, in the 2015 actualization of the Cochrane's review, the authors concluded that benefits of intra-articular corticosteroids after one to six weeks were finally unclear [267]. If intra-articular GC reduced the size of synovitis showed by MRI, other studies did not fond any difference between GC and a placebo injected in the joint (MRI and ultrasounds studies) [268-270]. Moreover, GCs did not reduce the need for total hip arthroplasty in rapidly destructive hip OA, a subset of hip OA [271]. Finally, in horses studies, intra- or peri-articular OA injection was even associated with a higher rate of musculoskeletal injuries [272].

In vitro, dexamethasone is efficient to reduce the cartilage degradation in IL-1β-induced injury, IL-1β being highly expressed in traumatic joint injury [273,

274]. However, there are some discrepancies in the literature and several studies do not confirm the positive action of GCs that was established in models of post-traumatic OA. Indeed, GCs induced chondrocytes death *in vitro* and reduced the ECM production by chondrocytes [275, 276]. Wyles *et al.* have shown that MSCs viability (from peri-articular tissue) was reduced when exposed to GCs, hypothesizing reduced regenerative capacities [277]. Moreover, while intra-articular GCs reduced macrophagic infiltration compared to placebo, no difference in the expression of MMPs was found in the synovial membrane [278]. A recent systematic review confirmed the time and dose dependent deleterious effect of GCs on articular cartilage. But, at low dose and during short time of culture, GCs might conserve their beneficial properties [279]. Effects of GCs could also depend on the activation and differential stage of cells targeted in joints [280]. Whether GCs are beneficial or deleterious is still an ongoing question that is probably dependent on OA sub-classification (post-traumatic, metabolic …).

1.7.3. Selective glucocorticoid receptor agonists

GCs are effective as anti-inflammatory agents but they display adverse events, especially metabolic *(Figure 29)*. These adverse events are relevant even when GCs are injected in a single joint: glycemic level is significantly higher several days after a GC injection in the knee, wrist or hand, with higher levels when the patient is insulin-dependent [281, 282].

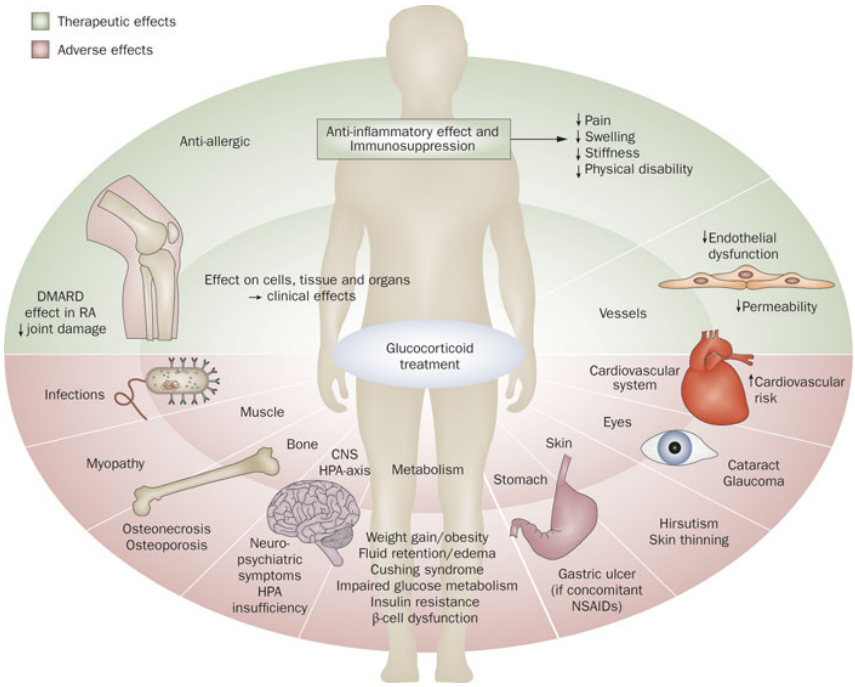

Figure 29. Systemic effects of GCs [283].

For several years, scientists have looked for selective GR agonists (SEGRAs) that offered better benefit/risk ratio than GCs [284]. Theses SEGRAs shared with GCs similar anti-inflammatory properties, but were responsible for less long-term side effects. They are GR agonists that bind the GR and prevent its dimerization. GR monomer is not able to bind to a classical GRE and only activates the transrepression pathway, preventing the transactivation of metabolic gene products [285]. However, as explained previously, distinction between transactivation and transrepression is probably less clear than first expected.

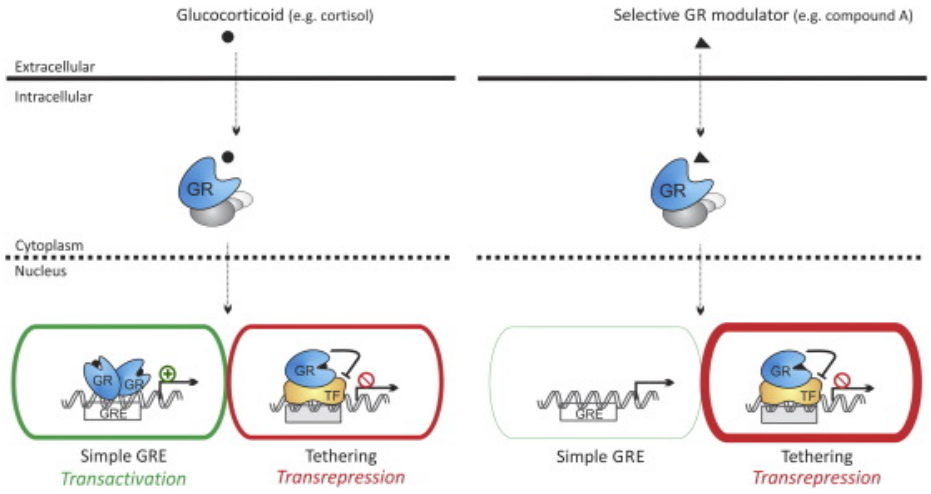

Figure 30. Principle of a selective GR modulator. Selective GR modulators differ from GCs in the way that upon binding to GR they trigger transrepression, but do not initiate transactivation [285]. GR: glucocorticoid receptor; GRE: glucocorticoid responsive element.

As examples, BOL-303242-X acts as a potent anti-inflammatory agent in various human ocular cell lines, with a better safety profile or ginsenoside Rg1 inhibits acute and chronic inflammation *in vivo*, without hyperglycemia or osteoporosis induction [286, 287]. Compound A (CpdA) is one of the most studied SEGRAs in rheumatology. CpdA, a plant-derived phenyl aziridine precursor, was found to reduce inflammation *in vitro* and in different inflammatory murine models, with a better safety profile in terms of hyperglycemia and bone loss [288-293]. In regards to this better benefit/risk ratio, there is no data in the literature about leptin production *in vitro* or *in vivo* when CpdA is used.

1.7.4. Glucocorticoids and signaling pathways: study of glucocorticoid-induced leucine zipper

Glucocorticoid-induced leucine zipper (GILZ) protein is an intra-cellular protein induced by GCs [294, 295], with a dose- and time-dependent increase [296]. GILZ is also induced by the mineralocorticoid aldosterone in the kidney or in the colon, with its involvement in ions and fluids transfers [297, 298]. Other studies also reported GILZ induction by progesterone [296] or by *Yersinia enterocolitica* and *Clostridium difficile* Toxin B in epithelial cells [299]. Different regulations by estrogens were noticed, with a down-regulation in human breast cancer but an up-regulation in lineage cancer cells [300]. Recent works have highlighted the role of GILZ as a main actor in the anti-inflammatory action of GCs, inhibiting NF-κB pathway in human macrophagic cells, in epithelial respiratory cells and in T lymphocytes [294, 301-303]. For example, in mice T-helper-1-mediated models of colitis (dinitrobenzene sulfonic acid-induced colitis), GILZ overexpression is protective and reduces NF-κB nuclear translocation, TNF-α and IL-1β production in CD4+ T lymphocytes of the lamina propria [304]. Also in mice, GILZ governs the therapeutic potential of MSCs by inducing a switch from pathogenic to regulatory Th17 cells in a mouse model of collagen-induced arthritis [305]. GILZ even displays other properties in depression [306], spermatogenesis [307] or presbyacousia [308].

The main molecular pathways are well known *(Figure 31)* [309]. GILZ, induced by GCs, directly interacts with NF-κB, inhibits NF-κB-dependent transcription and mediates anti-inflammatory and immunosuppressive effects. A protein complex converts Ras into its active GTP-bound form, leading to the activation of extracellular signal-regulated kinases-1/2 and Akt/protein kinase-B pathways. GILZ reduces the activation of Ras/Raf downstream targets, leading to inhibition of Ras- and Raf-dependent cell proliferation and Ras-induced transformation. Interestingly, GILZ expression could also be induced by

mineralocorticoids, *e.g.* in kidney with an involvement in epithelial channel induction through the MR [310].

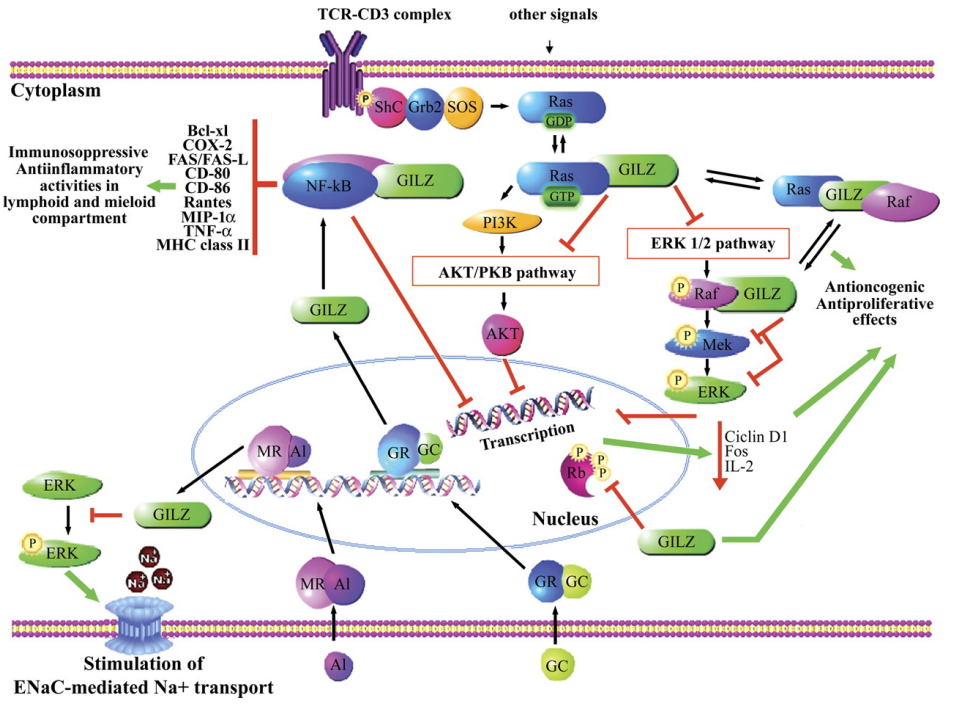

Figure 31. GILZ is a mediator of both anti-inflammatory and immunosuppressive glucocorticoid activities and aldosterone-induced epithelial sodium channel transport [295].

In rheumatology, Beaulieu *et al.* showed GILZ expression in the synovium of mice with collagen-induced arthritis, in the synovium of patients with active RA and in cultured RA synovial fibroblasts [311]. GILZ deletion *in vivo* with siRNA increased the clinical and histological severity of collagen-induced arthritis and increased the synovial expression of IL-1β and TNF-α [311], while GILZ overexpression reduced IL-6 and IL-8 levels. The anti-inflammatory effect of exogenous GILZ was confirmed in collagen-induced arthritis models, with a reduction of collagen-induced arthritis manifestation when GILZ was

overexpressed in mice joint, with a similar effect compared to dexamethasone [312].

However, GILZ is not always associated with an anti-inflammatory pattern, depending on the way of analysis (overexpression or invalidation), the type of cells and the studied model of inflammation. Deletion of GILZ (mice GILZ$^{-/-}$) did not confirm the central role of GILZ as an anti-inflammatory mediator of GCs in mice arthritis [312]. There was no effect of GILZ deletion on antigen-induced arthritis or K/BxN serum transfer arthritis and no reduction of exogenous glucocorticoid action on collagen-induced arthritis or cytokinemia [312]. In mice model of colitis, overexpression of GILZ reduced the susceptibility to Th1-model of colitis (dinitrobenzene sulfonic acid-induced colitis), but mice were more susceptible to develop Th2-mediated colitis (oxazolone-induced colitis) [304].

1.7.5. Glucocorticoid-induced leucine zipper and leptin

The central role of GILZ as an anti-inflammatory agent is therefore sparse and controversial and needs explorations, specifically in OA for which intra-articular injections of GCs are used for symptomatic treatment. Moreover, there are no data about the potential link between GILZ and leptin. However, there are several elements in the literature in favor of its existence.

Bone marrow MSCs have the potential to differentiate into different cell lineages, including adipocytes and osteoblasts, with a balance between the adipogenic and the osteogenic differentiation *(Figure 32)* [313]. Several studies demonstrated that GILZ can tip the balance of osteogenic and adipogenic differentiation of MSCs towards the osteogenic pathway [314].

Leptin has a similar effect than GILZ on the balance between adipogenic and osteogenic differentiation of MSCs. Leptin is produced by mature adipocytes and plays a negative feedback loop to reduce the body fat mass. This negative regulation can be indirect (reduction of the adipocyte function through modification of insulin action and through modulation of the sympathetic nervous system control) or direct, with a negative action on adipocyte lipid metabolism [173]. Negative effect is also explained by a reduction of adipogenesis, with inhibition of pre-adipocytes maturation [315, 316]. Thomas *et al.* published that leptin inhibited the MSCs differentiation to adipocytes [317]. Interestingly, leptin did not exert any anti-adipogenic effect on MSCs from osteoporosis patients conversely to its anti-adipogenic effect on control MSCs, due to impaired leptin cells response [318]. Further, substances like genistein, that induces adipogenesis in human synovial fibroblasts, inhibit leptin induction [1]. Leptin also acts on MSCs to enhance differentiation towards osteoblastic lineage [317]. Ob-R deficient mice have a reduced trabecular and cortical bone volume [319], while central leptin injection increased bone formation in leptin deficient mice [320].

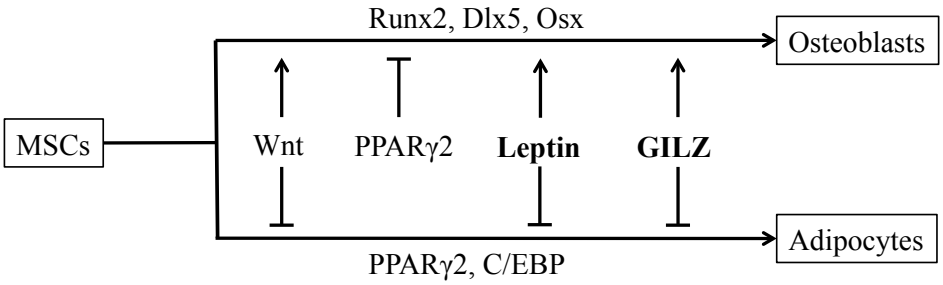

Figure 32. Adipogenic and osteogenic differentiation of MSCs (adapted from [321]). MSCs: mesenchymal stem cells; Dlx: distal-less homeobox; Osx: osterix; Wnt: wingless-type MMTV integration site family; GILZ: glucocorticoid-induced leucine zipper; PPAR: peroxisome proliferator-activated receptor; C/EBP: CCAAT/enhancer binding protein.

2. OBJECTIVES

1. Our laboratory previously showed that OA synovial fibroblasts were able to spontaneously produce leptin and express Ob-R *in vitro* [1]. Surprisingly, GC prednisolone strongly induced leptin secretion and Ob-R expression, without any adipogenic differentiation [1]. In OA process, GCs are used as symptomatic drugs with anti-inflammatories properties. But leptin has a detrimental role in OA, with a catabolic and pro-inflammatory action on the cartilage. This apparent contradiction needs further explorations.

We will study the molecular pathways involved in leptin secretion by OA synovial fibroblasts through the study of GILZ. GILZ protein is usually considered as a main actor of GCs anti-inflammatory action and is expressed by RA synovial fibroblasts [294, 301-303, 311]. However, several studies demonstrated that GILZ could also mediate GC's adverse events such as delayed epithelial repair or myopathy [322, 323]. GILZ is also involved in osteogenic and anti-adipogenic differentiation of MSCs [314], two biological processes also linked to leptin [317].

Recent studies have suggested that GILZ could not play a major role in the anti-inflammatory action of GCs in different mice models of arthritis [312]. We will position GILZ in the OA process and determine its involvement in the anti-inflammatory action of GCs and / or in the metabolic leptin secretion and Ob-R expression pathway.

2. Beside GCs, mineralocorticoids are another class of steroids. They can bind their own receptor, the MR, but can also bind the GR and can therefore have a potential action as leptin and Ob-R inducer [248]. Moreover, aldosterone has a greater "pro-inflammatory" profile than GCs and is linked to the metabolic syndrome and low-grade inflammation [251, 254, 255].

We will investigate the aldosterone's action on leptin secretion and GILZ expression. If mineralocorticoids are able to bind to the GR, GCs are also able to interact with the MR. Whether prednisolone-induced and aldosterone-induced leptin secretion is dependent on either the GR or MR will be investigated.

3. GCs have a well-known anti-inflammatory effect, but they can also induce long-term metabolic adverse events. SEGRAs have been developed to interact with the GR, inducing an anti-inflammatory effect but not the metabolic adverse events of GCs [284]. They offer a better safety profile in terms of hyperglycemia and bone loss compared to the commonly used GC treatment.

Considering the detrimental role played by leptin in OA and the better benefit/risk ratio of SEGRAs, we sought to determine whether SEGRA CpdA was a leptin or Ob-R inducer in human OA synovial fibroblasts, as observed with prednisolone. GILZ's modulation under CpdA stimulation will also be studied.

We previously reported that TGF-β pathway was involved in prednisolone-induced leptin secretion through ALK1-Smad1/5. Modulation of TGF-β pathway under CpdA stimulation will also be analyzed.

4. Synovial fibroblasts play a major role in OA. Under metabolic or mechanical stimulation, they acquire an inflammatory pattern and release pro-inflammatory and catabolic agents in the joint cavity. However, synovial fibroblasts are not the only cell lineage in the joint. Chondrocytes have a key role in the cartilage homeostasis by the secretion of fundamental ECM. They can be targeted by catabolic agents produced by the synovium but are also able to answer to metabolic, mechanical or aging stimuli by acquiring a hypertrophic phenotype and self-perpetuating the catabolic process by pro-inflammatory mediators secretion [82-84].

We will determine if leptin and Ob-R inductions are limited to the synovium or can also occur in OA chondrocytes. We will also study if prednisolone and aldosterone could also induce leptin and Ob-R through GR and/or MR interaction and if the SEGRA CpdA was a leptin and Ob-R inducer on these cells.

5. Leptin is the adipokine with the strongest evidence for its involvement in OA process. However, other adipokines are sometimes described for their role in OA, even if evidences are less obvious and sometimes controversial.

Next to leptin, we will study the *in vitro* secretion of adiponectin and resistin, two other adipokines with potential effects on OA pathogenesis, by human OA synovial fibroblasts and chondrocytes.

3. MATERIAL AND METHODS

3.1. Patients

Synovial tissue and cartilage were obtained from OA patients during knee or hip replacement surgery, respectively. The institutional review boards (Research Ethics Committee) of our hospital, Centre Hospitalier Universitaire de Liège, approved the study protocol and the use of verbal informed consent to allow research procedures on the tissues collected, as explained in the institutional information booklet written by the hospital and provided to each patient. Clinicians gave an oral statement about the research, informed the patients of authorizations obtained from the local Research Ethics Committee and answered any additional questions. Then, clinicians obtained verbal consent from the patient to participate in the research. All of the patients presented symptomatic knee or hip OA, without inflammatory disease or cancer. None of them had received oral or intra-articular glucocorticoids for at least one year.

For figures 33-43 and 51-55: n = 16 [the mean age was 71 (58-82) years, the mean BMI was 26.3 (22.2 - 32.7) kg/m^2 and 7 patients were female (44%)]. For figures 44-45: n = 23 [the mean age was 64 (47 - 85) years, the mean BMI was 28.8 (19.6 - 38.3) kg/m^2 and 6 patients were female (46%)]. For figures 46-50 and 58-59: n = 5 for synovial tissue [the mean age was 66 (56 - 83) years, the mean BMI was 23.7 (21.4 - 32.1) kg/m^2 and 3 patients were female (60%)] and n = 3 for cartilage [the mean age was 77 (58 - 81) years, the mean BMI was 26.3 (25.2 - 27.4) kg/m^2 and 1 patients was female (33%)].

3.2. Human synovial fibroblasts and human chondrocytes

Synovial fibroblasts were isolated from human synovial membrane [1, 324]: synovial membrane was cut and digested 16 h with 1 mg/mL collagenase (Sigma-Aldrich, USA). Chondrocytes were isolated from human cartilage [324]:

cartilage was cut in 1- to 2-mm^3 explants and digested at 37°C with gentle agitation successively by hyaluronidase (Sigma-Aldrich, USA) (0.5 mg/mL) for 30 min, pronase (Merck, Germany) (1 mg/mL) for 1 h and collagenase (Sigma-Aldrich, USA) (0.6-0.8 mg/mL) for 16 to 20 h.

After digestion, synovial fibroblasts and freshly isolated chondrocytes were filtered through a 70 μm nylon membrane (Falcon, USA) and washed four times. Cells were cultured in complete medium consisting of Dulbecco's Modified Eagle medium (DMEM) (Cambrex Bio Science, USA), with L-glutamine (2 mM), streptomycin (100 mg/mL) and penicillin (100 U/mL) (BioWhittaker, USA), and supplemented with 10% foetal bovine serum (FBS) (Lonza, Switzerland).

For experiments, $5x10^4$ synovial fibroblasts/well (0.5 mL of medium) or $2x10^5$ chondrocytes were plated in triplicates in 24-well plates (BD Biosciences, USA). Synovial fibroblasts were used at passage 3-10. Freshly isolated chondrocytes were used immediately or let to dedifferentiate.

3.3. Human pre-adipocytes

Pre-adipocytes are isolated from obese patients (BMI > 30 kg/m²) undergoing laparoscopic bariatric surgery according to [325]. Samples of visceral abdominal tissue from the greater omentum were harvested during surgery and processed immediately. Visceral adipose tissue samples were minced and digested with 1.5 mg/mL type I collagenase (Sigma-Aldrich, USA) in phosphate buffered saline (PBS) for 90 min at 37°C with manually shaking for 5–10 seconds every 15 min. To stop collagenase activity, FBS (10 %) was added. After centrifugation (400 g for 10 min), the floating mature adipocytes were removed and the stroma vascular fraction cell pellet was resuspended in erythrocyte lysis buffer for 10

min. After successive washes and a filtration through a 70 mm nylon cell strainer (BD Biosciences, USA), the stroma vascular fraction cells were suspended in PBS with 3 % FBS before being stained for flow cytometry. Flow cytometry was performed on a FACSAria (BD Biosciences, USA). The subpopulations of stroma vascular fraction were determined based on sideward scatter, CD45 and CD14 expression. Pre-adipocytes were sorted from the $CD45^{-}$ $CD14^{low}$ stroma vascular populations with a purity of > 90 % in all samples as described in [325].

3.4. Culture reagents

Cells were stimulated with

- corticosteroids: GC prednisolone, mineralocorticoid aldosterone, SEGRA CpdA (Sigma-Aldrich, USA; Santa Cruz Biotechnology, USA);
- receptor antagonists: GR antagonist mifepristone, MR antagonist eplerenone, MR antagonist spironolactone (Sigma-Aldrich, USA);
- TGF-β1 and TNF-α (Gibco-BRL, USA; Biosource, USA);
- MG132 (Alexis Corporation, Switzerland). Proteasome inhibitor MG132 (10 μM) was added in the medium the last 12h to enhance GILZ's visualization, when GILZ was analyzed with Western-blot.

3.5. Transfection with lentiviruses expressing glucocorticoid-induced leucine zipper and glucocorticoid receptor shRNA

Lentiviral vectors were generated by co-transfecting Lenti-X 293T cells (Clonetech, Belgium) with a pSPAX2 plasmid (Addgene, Plasmid #12260), a VSV-G-encoding vector, a GILZ (TSC22D3) shRNA plasmid

[#TRCN0000013793 (GILZ shRNA1), #TRCN0000364625 (GILZ shRNA2) or #TRCN0000369187 (GILZ shRNA3) (Sigma-Aldrich, USA)], a GR (NR3C1) shRNA plasmid [#TRCN0000245007 (GR shRNA1), # TRCN0000245003 (GR shRNA2) or # TRCN0000245004 (GR shRNA3) (Sigma-Aldrich, USA)], or with a non-target sequence-encoding plasmid (SHC002, Sigma, Belgium). At 72 h post-transfection, viral supernatants were collected, filtered and 100 times concentrated using ultracentrifugation. Lentiviral vectors were then titrated using a quantitative polymerase chain reaction (PCR) Lentivirus Titration Kit (LV900, ABM, USA). A total of 5 x 10^5 synovial fibroblasts/well were plated in 24-well plates (BD Biosciences, USA) and infected using lentivirus at multiplicity of infection (MOI) of 30, unless otherwise indicated in the text. After 72 h of incubation, the medium was removed and cells were stimulated.

3.6. Enzyme-linked immunosorbent assay

A commercially available sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, USA) was used to quantify adipokines (leptin, adiponectin, resistin), IL-6, IL-8, MMP-1 and MMP-3 in culture supernatants.

3.7. Western-blot

Total proteins extracts were collected, lysed, and total proteins separated by SDS-PAGE. Lysis buffer contained 25 mM Hepes, 150 mM NaCl, 0.5 % Triton X-100, 10 % glycerol, 1 mM dithiothreitol), phosphatase inhibitors (25 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM NaF) and complete protease inhibitor mixture (Roche Applied Science, USA). Different primary antibodies were used:

- receptors: Ob-R (B-3) (sc-8391), GR (41) (sc-136209) and MR (sc-11412) (Santa Cruz Biotechnology, USA);
- GILZ (sc-33780, Santa Cruz Biotechnology, USA);
- Smad pathway: phospho-Smad2 (S465/467; #3108), Smad2 (L16D3; #3103), phospho-Smad1/5 (S463/465; #9516), Smad1 (#9743) (Cell Signaling, USA);
- glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and α-tubulin (T6074) (Sigma-Aldrich, USA).

Incubation of membranes with primary antibodies, diluted 1/1000 in Tris-Buffered Saline with Tween (TBS-T) [20 mM Tris (pH 7.6), 500 mM NaCl, 0.2% Tween], was done at room temperature for 1–3 h or at 4°c overnight (supplemented with 10% milk powder) for phospho-Smad2 and phospho-Smad1/5. Western blots were revealed with 1/2000 diluted anti-mouse and anti-rabbit (Cell Signaling, USA) (room temperature, for 2h) and enhanced chemiluminescent reagents (Amersham Biosciences, UK). Western Blot band quantification was performed with image Studio Lite software.

3.8. Quantitative reverse transcription polymerase chain reaction

Total RNAs from synovial fibroblasts (either non-treated or treated with indicated concentrations of prednisolone or aldosterone for 5 days) were extracted and purified using a Nucleospin RNA Kit, rDNAse included (#740955, Macherey-Nagel, Germany). Next, cDNA was synthesized using reverse transcription of 1 µg of RNA (in each reaction) with a RevertAid H Minus First Strand cDNA Synthesis Kit (#K1632, Thermo Scientific, Belgium) according to the manufacturer's instructions. cDNA products were then amplified using PCR with the KAPA SYBR FAST detection system (#KK4611, Sopachem, Belgium). Real-time reverse transcription quantitative PCR (RT-

qPCR) experiments were run on a LightCycler 480 instrument (Roche, Belgium) and data were analyzed using LC480 software. For each gene, cDNA dilution curves were generated and used to calculate the individual real-time PCR efficiencies ($E = 10^{[-1/slope]}$). The $2^{-\Delta\Delta CT}$ method was then used to calculate the relative gene expression between non-treated (calibrator sample) and treated synovial fibroblasts. Input amounts were normalized to the *β2-microglobulin* endogenous control gene. All primers were purchased from Eurogentec (Belgium). The following primer sequences were used: *leptin*, 5'-AACCCTGTGCGGATTCTTGT-3' (forward) and 5'-TCTTGGACTTTTTGGATGGGC-3' (reverse); *GILZ*, 5'-GCACAATTTCTCCATCTCCTTCTT-3' (forward) and 5'-TCAGATGATTCTTCACCAGATCCA-3' (reverse); *β2-microglobulin*, 5'-TTTCATCCATCCGACATTGA-3' (forward) and 5'-CCAGTCCTTGCTGAAAGACA-3' (reverse).

3.9. Statistical analysis

Statistical analysis was performed by GraphPad Prism software (version 6) using an ordinary one-way ANOVA for multiple comparisons. Results were considered as significantly different when $p<0.05$. ImageStudioLite software version 5.2.5 was used to quantify Western Blot bands. Figures 33, 35-36, 38-45, 51-55 and 61-62: graphs represent averages of triplicates ± standard deviation (SD) from at least three independent experiments in different patients for ELISA and RT-qPCR experiments. Graphs represent averages of band quantifications ± SD from at least three independent experiments in different patients for Western-Blot experiments. One representative Western-Blot was shown next to the band quantification. Figure 34, 37, 46-50 and 56-59: graphs represent averages values of triplicates ± SD from one representative of at least three independent patients.

4. RESULTS

4.1. Glucocorticoid-induced leucine zipper expression in human osteoarthritic synovial fibroblasts

First, we investigated GILZ expression in OA synovial fibroblasts under GC prednisolone and mineralocorticoid aldosterone stimulation. Whether GILZ induction is dependent on the GR or the MR was also investigated.

4.1.1. Glucocorticoid-induced leucine zipper expression through the glucocorticoid receptor

Human OA synovial fibroblasts were stimulated for 5 days with prednisolone (1 μM) (a glucocorticoid) or aldosterone (1 or 10 μM) (a mineralocorticoid). Western blotting revealed that prednisolone and aldosterone induced GILZ expression in OA synovial fibroblasts *(Figure 33)*. GCs and mineralocorticoids have affinities for both GR and MR. Therefore, both receptors were investigated to determine which one was involved in GILZ expression. Cells were pre-incubated with a GR inhibitor [mifepristone (5 μM)] or MR inhibitors [eplerenone (5 μM) or spironolactone (5 μM)] and then stimulated for 5 days with prednisolone (1 μM) or aldosterone (1 or 10 μM). The GR inhibitor mifepristone strongly and significantly reduced both prednisolone- and aldosterone-induced GILZ protein expression *(Figure 33)*. In contrast, the MR inhibitors eplerenone and spironolactone did not significantly modulate either prednisolone- or aldosterone-induced GILZ expression *(Figure 33)*. These results suggest that GCs and mineralocorticoids are able to induce GILZ expression in OA synovial fibroblasts through GR but not MR.

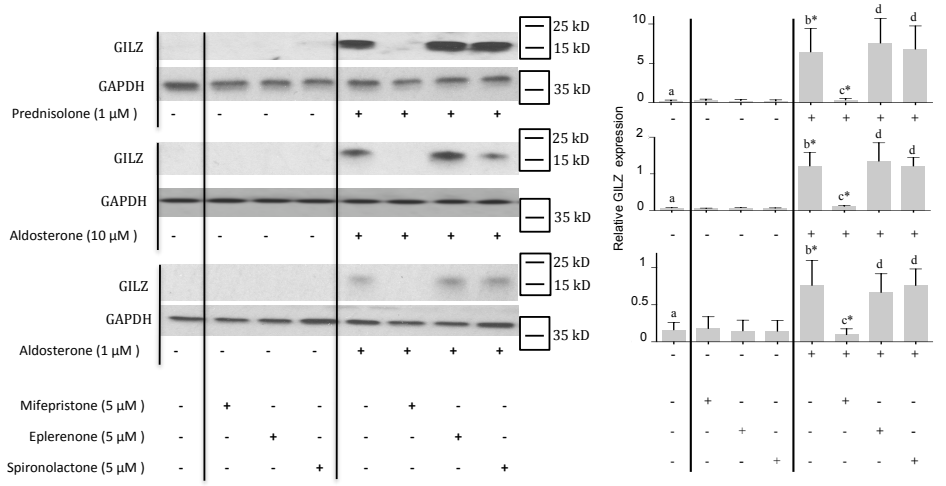

Figure 33. GILZ expression was induced by prednisolone and aldosterone through GR. Human OA synovial fibroblasts were pre-incubated or not for 1 h with a GR inhibitor (mifepristone) or MR inhibitors (eplerenone and spironolactone) and then stimulated for 5 days with prednisolone or aldosterone. GILZ and GAPDH in whole cell extracts were analyzed using Western blotting. Right panels are quantification of Western blot in left panels. Proteins were normalized to GAPDH levels. Each graph represent the mean +/- SD, n=4. Significance at $p<0.05$. b*, significantly different from a. c*, significantly different from b. d, not significantly different from b.

In order to ensure that GC and mineralocorticoids can have an action on synovial fibroblasts through their own receptor, we have checked the presence of GR and MR by Western-blot. GR and MR are expressed by OA synovial fibroblasts *(Figure 34)*. As expected, prednisolone induced a quick down-regulation of GR, with a reduction of GR expression already seen after a 1h-stimulation. Of interest, GR down-regulation was still persistent after 7 days. No down-regulation of MR was observed under prednisolone stimulation.

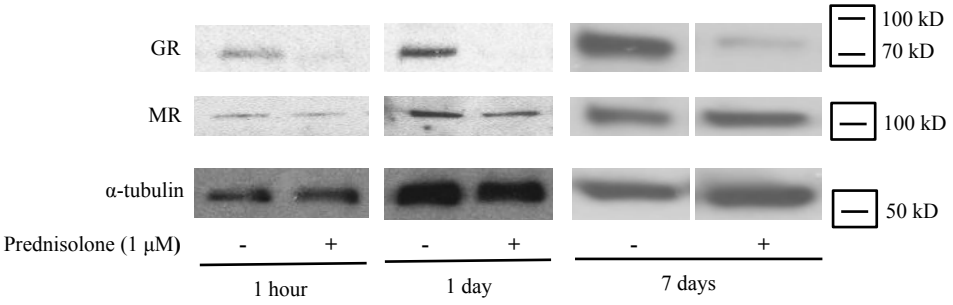

Figure 34. GR and MR are expressed by OA synovial fibroblasts. Human OA synovial fibroblasts were stimulated for 1h, 1 day or 7 days with prednisolone. GR, MR and α-tubulin in whole cell extracts were analyzed using Western blotting. This is a representative experiment from n=3.

To further confirm the involvement of GR in prednisolone- and/or aldosterone-induced GILZ expression, we performed a shRNA experiment to silence GR. Cells were infected with 3 different lentiviruses expressing GR shRNA or with a non-target control lentivirus. GR shRNA1, 2 and 3, but not the control shRNA, reduced endogenous GR expression *(Figure 35)*.

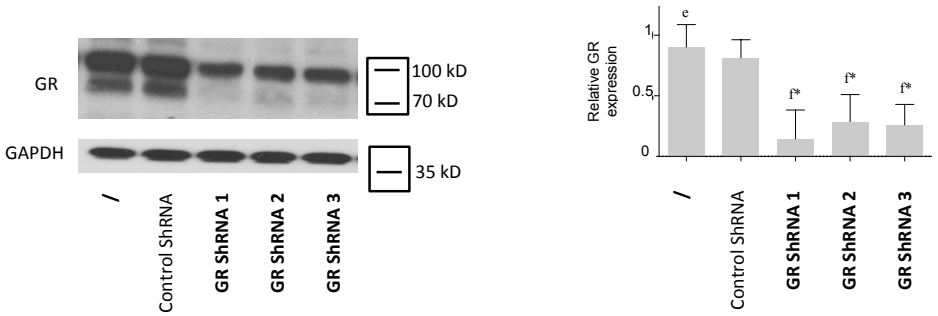

Figure 35. GR shRNA reduced GR expression. Synovial fibroblasts were infected with 3 different lentiviruses expressing GR shRNA or with a non-target control lentivirus. GR and GAPDH in whole cell extracts were analyzed using Western blotting. Right panels are quantification of Western blot in left panels. Proteins were normalized to GAPDH levels. Each graph represent the mean +/- SD, n=3. Significance at $p<0.05$. f*, significantly different from e.

The prednisolone- and aldosterone-induced GILZ expression levels were significantly reduced when GR was silenced *(Figure 36)*. The GR antagonist mifepristone was used as a positive control to show GR inactivation. Using GR silencing, we confirmed that the prednisolone- and aldosterone-induced GILZ expression was GR-dependent.

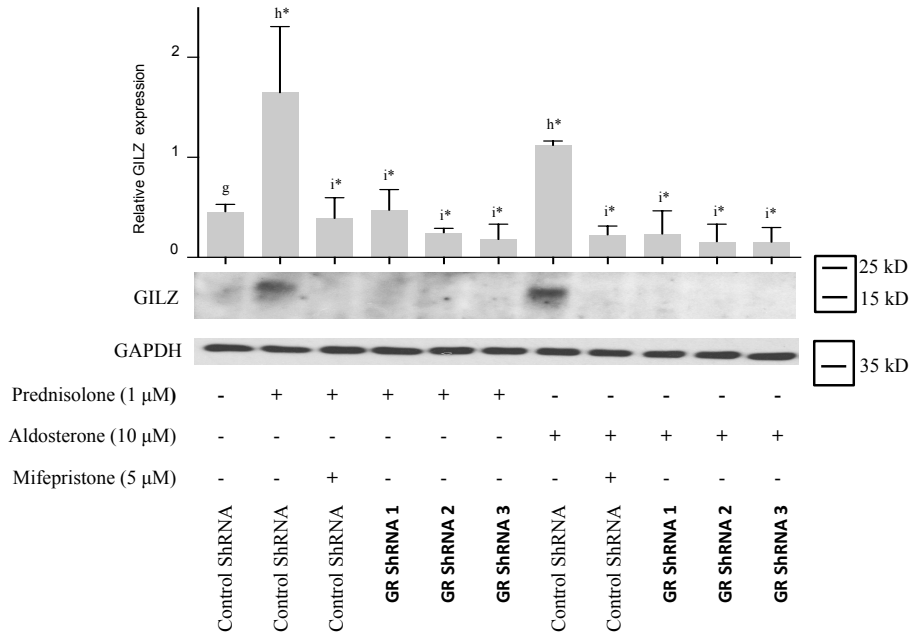

Figure 36. Influence of GR silencing on prednisolone and aldosterone-induced GILZ expression. Synovial fibroblasts were infected with 3 different lentiviruses expressing GR shRNA or with a non-target control lentivirus and stimulated or not for 5 days with prednisolone or aldosterone. GILZ and GAPDH in whole cell extracts were analyzed using Western blotting. Upper panels are quantification of Western blot in down panels. Proteins were normalized to GAPDH levels. Each graph represent the mean +/- SD, n=3. Significance at $p<0.05$. h*, significantly different from g. i*, significantly different from h.

In summary, using human OA synovial fibroblasts, we have shown that:

1) prednisolone and aldosterone induced GILZ expression by these cells;
2) mifepristone, but not eplerenone nor spironolactone, reduced prednisolone- and aldosterone-induced GILZ expression levels;

3) prednisolone- and aldosterone-induced GILZ expression levels were reduced when GR was silenced.

GILZ expression was induced by prednisolone and aldosterone through GR interaction in human OA synovial fibroblasts.

4.2. Modulation of leptin secretion in human osteoarthritic synovial fibroblasts

We will investigate similarities between leptin secretion and GILZ expression. Leptin secretion in OA synovial fibroblasts under GC prednisolone and mineralocorticoid aldosterone will be studied. Whether leptin induction is dependent on either the GR or MR will be investigated. TGF-β's and TNF-α's influence on GILZ and leptin will also be analyzed. Prednisolone and aldosterone are present in the human blood, but are also found in the synovial fluid at various concentrations for each corticoid. Dose-response experiments will be performed to determine the relevance of the *in vitro* effect.

Leptin secretion under prednisolone stimulation by OA synovial fibroblasts was time-dependent and leptin progressively accumulated in the cell culture supernatant *(Figure 37)*.

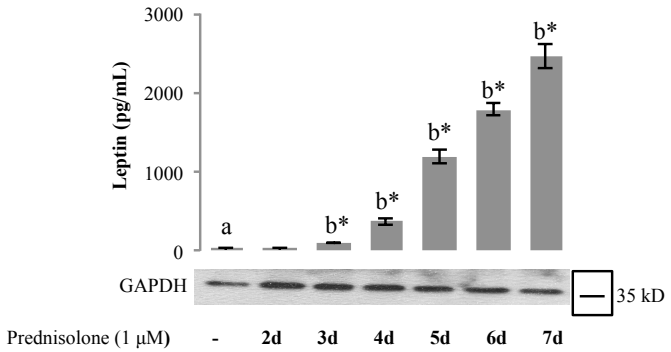

Figure 37. Leptin secretion under prednisolone stimulation is time-dependent in OA synovial fibroblasts. Human OA synovial fibroblasts were stimulated for 2-7 days with prednisolone. Leptin was measured in the cell culture supernatant using an ELISA. GAPDH in whole cell extracts was analyzed using Western blotting. Each graph represents the mean +/- SD. This is a representative experiment from n=3. Significance at $p<0.05$. b*, statistically different from a.

4.2.1. Leptin secretion and leptin receptor expression were induced by prednisolone and aldosterone through the glucocorticoid receptor

We first confirmed that prednisolone was able to enhance leptin secretion and Ob-R expression in synovial fibroblasts [*Figure 38 A*: b* *vs.* a]. Similarly to prednisolone, the mineralocorticoid aldosterone also induced leptin secretion and Ob-R protein expression at 1 µM (*Figure 38 B*: b* *vs.* a) and 10 µM (*Figure 38 C*: b* *vs.* a**)**. Moreover, using specific inhibitors [a GR inhibitor (mifepristone) and MR inhibitors (eplerenone and spironolactone)], we observed that the prednisolone- (*Figure 38 A*: c* *vs.* b*) and aldosterone-induced (*Figure 38 B and 38 C*: c* *vs.* b*) leptin secretion and Ob-R expression were GR-dependent, but not MR-dependent, as previously observed with GILZ. These results suggest that similar to GCs, mineralocorticoids are able to induce leptin secretion and Ob-R receptor expression in OA synovial fibroblasts through GR but not MR. The use of GR and MR antagonists did not modulate the endogenous level of leptin secretion and Ob-R expression.

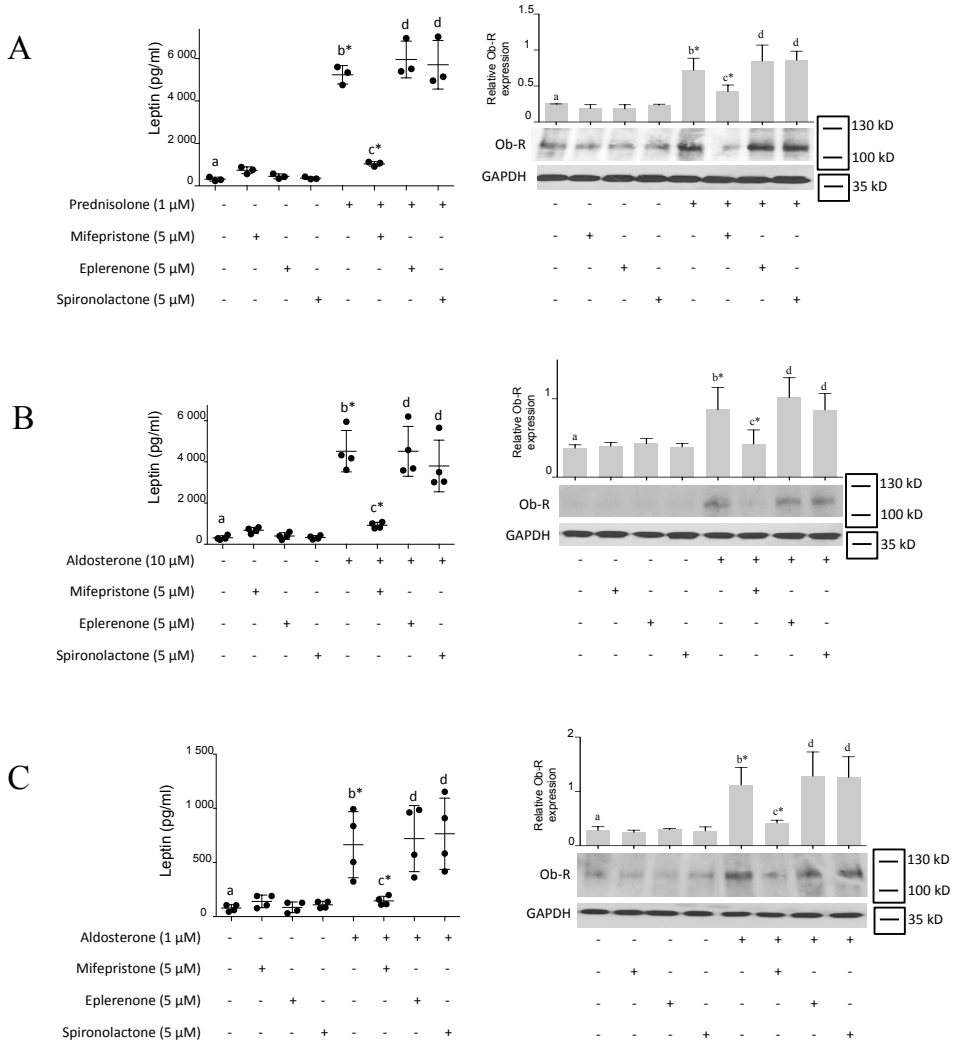

Figure 38. Leptin secretion and Ob-R expression were induced by prednisolone and aldosterone through GR signaling. Human OA synovial fibroblasts were pre-incubated or not for 1 h with a GR inhibitor (mifepristone) or MR inhibitors (eplerenone and spironolactone) and then stimulated for 5 days with prednisolone or aldosterone. Leptin was measured in the cell culture supernatant using an ELISA. Ob-R and GAPDH in whole cell extracts were analyzed using Western blotting. Upper panels on the right are quantification of Western blot in down panels. Proteins were normalized to GAPDH levels. Each graph represent the mean +/- SD, n=3 or 4. Significance at $p<0.05$. b*, significantly different from a. c*, significantly different from b. d, not significantly different from b.

Human OA synovial fibroblasts were then infected with 3 different lentiviruses expressing GR shRNA or with a non-target control lentivirus. The prednisolone- and aldosterone- inductions of leptin and Ob-R were abolished when GR was silenced *(Figure 39),* confirming the involvement of GR signaling in leptin and Ob-R induction.

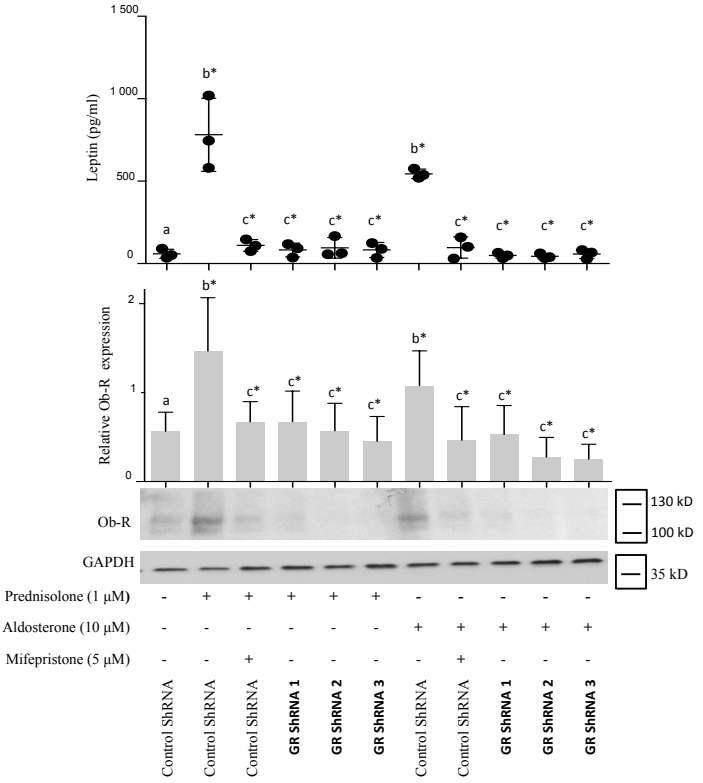

Figure 39. Influence of GR silencing on prednisolone- and aldosterone-induced leptin secretion and Ob-R expression. Synovial fibroblasts were infected with 3 different lentiviruses expressing GR shRNA or with a non-target control lentivirus and stimulated for 5 days with prednisolone or aldosterone. Leptin was measured in the cell culture supernatant using an ELISA. Ob-R and GAPDH were analyzed in whole cell extracts using Western blotting. Middle panel is quantification of Western blot in down panel. Proteins were normalized to GAPDH levels. Each graph represent the mean +/- SD, n=3. Significance at $p<0.05$. b*, significantly different from a. c*, significantly different from b*.

The leptin induction with prednisolone and with aldosterone was dose-dependent *(Figure 40)*. A dose response was observed not only for leptin secretion in the cell culture supernatant (measured by ELISA) *(Figure 40 A)* but also for leptin mRNA (measured by RT-qPCR) *(Figure 40 B)*. The leptin induction by prednisolone was significant for concentrations equal to or greater than 10 nM or 1000 nM for ELISA or RT-qPCR, respectively. Significance was not reached with 100 nM and 10 nM for RT-qPCR, although the mean value was higher than the control, but the variance was high. The induction was significant for aldosterone at concentrations of 100 nM and 1000 nM for ELISA and RT-qPCR, respectively.

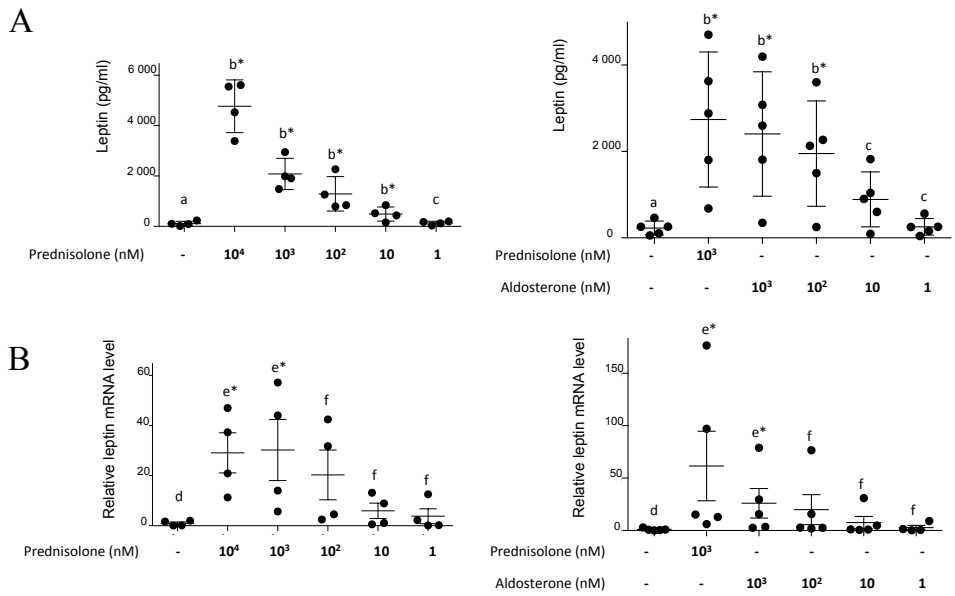

Figure 40. Prednisolone and aldosterone-induced leptin secretion was dose-dependent. Human OA synovial fibroblasts were stimulated for 5 days with increasing concentrations of prednisolone or aldosterone. Leptin secretion was measured in the cell culture supernatant using an ELISA, and leptin mRNA was measured using RT-qPCR. Each graph represent the mean +/- SD, n=4 or 5. Significance at $p<0.05$. b*, significantly different from a. c, not significantly different from a. e*, significantly different from d. f, not significantly different from d.

The Ob-R *(Figure 41)* and GILZ *(Figure 41* for Western-Blot and *Figure 42* for RT-qPCR*)* induction by prednisolone and aldosterone was also dose-dependent, with a significant induction for prednisolone and aldosterone at a concentration equal to or greater than 100 nM.

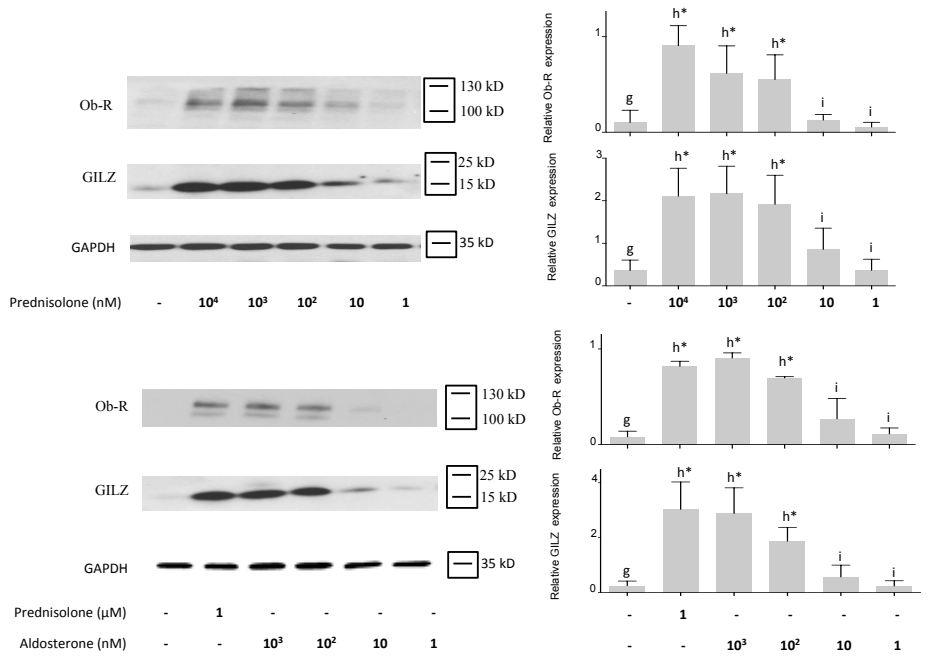

Figure 41. Prednisolone and aldosterone-induced Ob-R expression was dose-dependent. Human OA synovial fibroblasts were stimulated for 5 days with increasing concentrations of prednisolone or aldosterone. Ob-R, GILZ and GAPDH in whole cell extracts were analyzed using Western blotting. Right panels are quantification of Western blot in left panels. Proteins were normalized to GAPDH levels. Each graph represent the mean +/- SD, n=3. Significance at $p<0.05$. h*, significantly different from g. i, not significantly different from g.

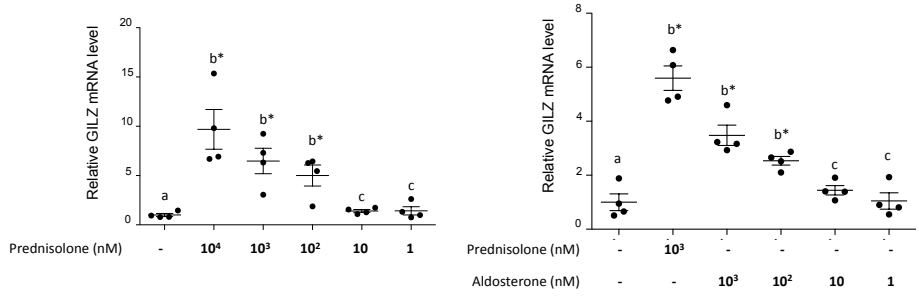

Figure 42. GILZ expression under prednisolone and aldosterone stimulation was dose-dependent. Human OA synovial fibroblasts were stimulated for 5 days with increasing concentrations of prednisolone or aldosterone. GILZ mRNA expression was measured by RT-qPCR. Each graph represents the mean +/- SD, n=4. Significance at $p<0.05$. b*, significantly different from a. c, not significantly different from a.

4.2.2. Influence of transforming growth factor-β and tumor necrosis factor-α on leptin secretion

We previously published that TGF-β1 did not induce leptin secretion and decreased glucocorticoid-induced leptin secretion [171]. We demonstrated here that TGF-β1 did not also induce GILZ expression and also decreased prednisolone-induced GILZ expression, which is in accordance with our previous results and supports a link between leptin and GILZ *(Figure 43)*.

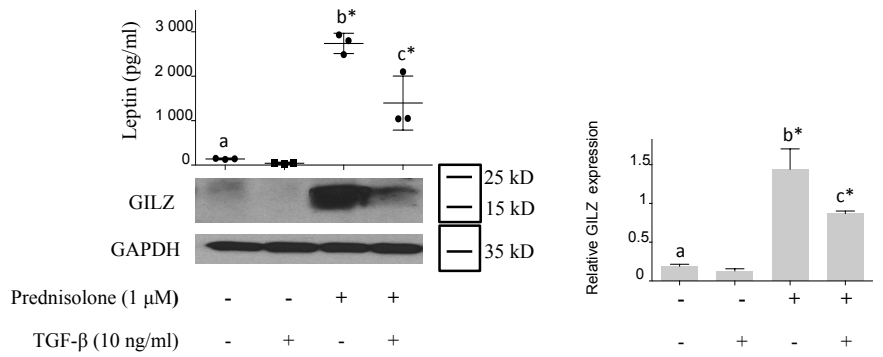

Figure 43. TGF-β1 decreased both prednisolone-induced leptin secretion and GILZ expression. Human OA synovial fibroblasts were stimulated for 5 days with prednisolone or TGF-β1. Leptin was measured in the cell culture supernatant using an ELISA. GILZ and GAPDH in whole cell extracts were analyzed using Western blotting. Right panels are quantification of Western blot in left panels. Proteins were normalized to GAPDH levels. Each graph represent the mean +/- SD, n=3. b*, significantly different from a. c*, significantly different from b*.

TNF-α significantly decreased the endogenous leptin secretion compared to the control (*Figure 44*: b *vs* a) but did not modify the prednisolone-induced leptin secretion (*Figure 45*: c *vs* b).

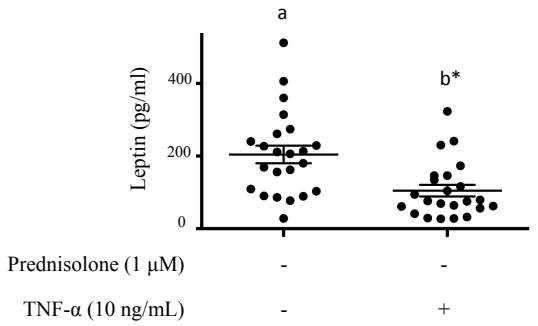

Figure 44. TNF-α decreased the endogenous leptin secretion. Human OA synovial fibroblasts were stimulated during 5 days with or without TNF-α. Leptin was measured in cell culture supernatant using an ELISA. Each graph represent the mean +/- SD, n=23. Significance at $p<0.05$. b*, statistically different from a.

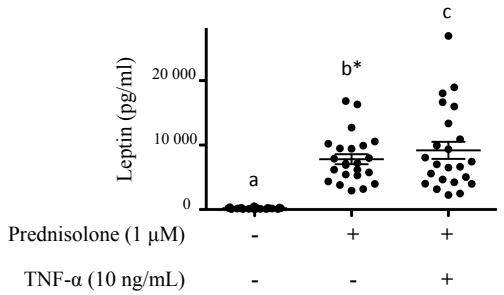

Figure 45. TNF-α did not modify the prednisolone-induced leptin secretion. Human OA synovial fibroblasts were stimulated during 5 days with prednisolone and/or TNF-α. Leptin was measured in cell culture supernatant using an ELISA. Each graph represent the mean +/- SD, n=23. Significance at $p<0.05$. b*, statistically different from a. c, not statistically different from b.

In summary, we have shown, using human OA synovial fibroblasts, that:

1) prednisolone and aldosterone induced leptin secretion by these cells;
2) mifepristone, but not eplerenone nor spironolactone, reduced prednisolone- and aldosterone-induced leptin expression levels;
3) prednisolone- and aldosterone-induced leptin expression levels were reduced when GR was silenced;
4) leptin, Ob-R and GILZ induction with prednisolone and with aldosterone were dose-dependent;
5) TGF-β1 reduced both prednisolone-induced leptin secretion and GILZ expression.

Leptin expression was induced by prednisolone and aldosterone through GR interaction in human OA synovial fibroblasts. Leptin exhibited modulations similar to those of GIZ in our experimental conditions.

4.3. The selective glucocorticoid receptor agonist Compound A

Considering the anti-inflammatory properties of GCs and the detrimental role of leptin in OA, we sought to determine whether SEGRAs (with a better risk-benefit ratio than classical GCs) could also reduce inflammation or induce leptin and Ob-R in human OA synovial fibroblasts. CpdA influence on GILZ will also be investigated.

4.3.1. Leptin secretion and leptin receptor expression

Human synovial fibroblasts were stimulated with prednisolone (1 μM) and / or CpdA (1 and 10 μM) for 7 days. In these experiments, CpdA, unlike prednisolone, could not induce leptin secretion or Ob-R protein expression *(Figure 46)*. Endogenous Ob-R expression was decreased when fibroblasts were stimulated with CpdA, suggesting lower leptin sensitivity for fibroblasts under CpdA stimulation. Moreover, CpdA was able to down-regulate prednisolone-induced leptin secretion and Ob-R protein expression in a dose-dependent manner.

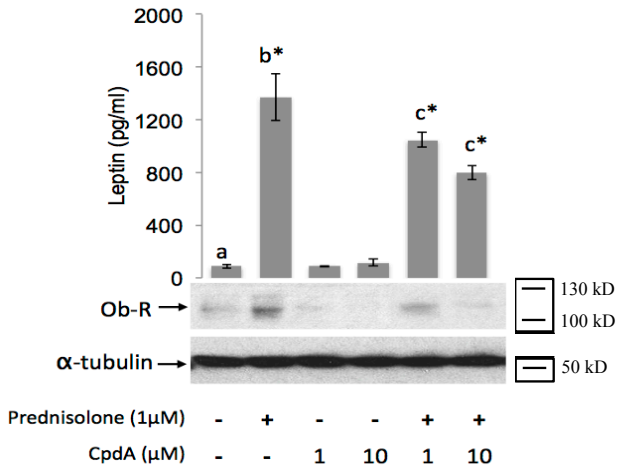

Figure 46. CpdA did not induce leptin nor Ob-R and reduced prednisolone-induced leptin and Ob-R. Human OA synovial fibroblasts were stimulated for 7 days with CpdA (1 and 10 μM) and/or prednisolone (1 μM). Leptin was measured in the cell culture supernatant using an ELISA. Ob-R and α-tubulin in whole cell extracts were analyzed using Western blotting. Each graph represents the mean +/- SD. This is a representative experiment from n=3. Significance at $p<0.05$. b*, significantly different from a. c*, significantly different from b.

CpdA did not induce leptin secretion or Ob-R protein expression and it down-regulated prednisolone-induced leptin secretion and Ob-R expression in human OA synovial fibroblasts.

4.3.2. Inflammatory parameters

If CpdA did not induce leptin secretion and Ob-R expression, opposite to prednisolone, its anti-inflammatory effect on OA cells must be assumed. Human OA synovial fibroblasts were pre-incubated for 1 h with prednisolone (1 μM) or CpdA (1, 5 or 10 μM) and then stimulated or not with TNF-α (10 ng/mL) for 12 h. CpdA significantly reduced endogenous and TNF-α-induced IL-6 secretion *(Figure 47)*.

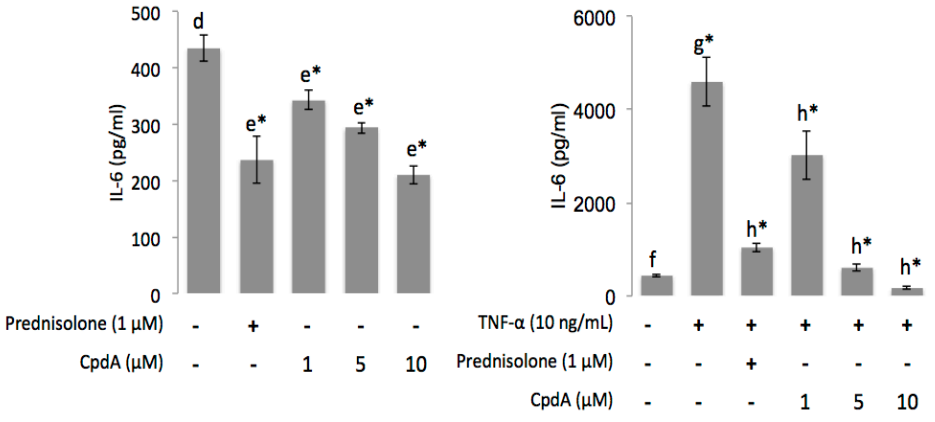

Figure 47. CpdA down-regulated endogenous and TNF-α-induced IL-6 secretion in OA synovial fibroblasts. Human OA synovial fibroblasts were pre-incubated 1h with prednisolone (1 µM) or CpdA (1 or 10 µM) and then stimulated with TNF-α (10 ng/mL) for 12h. IL-6 was measured in cell culture supernatant by ELISA. Each graph represents the mean +/- SD. This is a representative experiment from n=3. Significance at $p<0.05$. e*, significantly different from d. g*, significantly different from f. h*, significantly different from g.

TNF-α-induced IL-8, MMP-1 and MMP-3 secretions were also reduced under CpdA stimulation *(Figure 48)*.

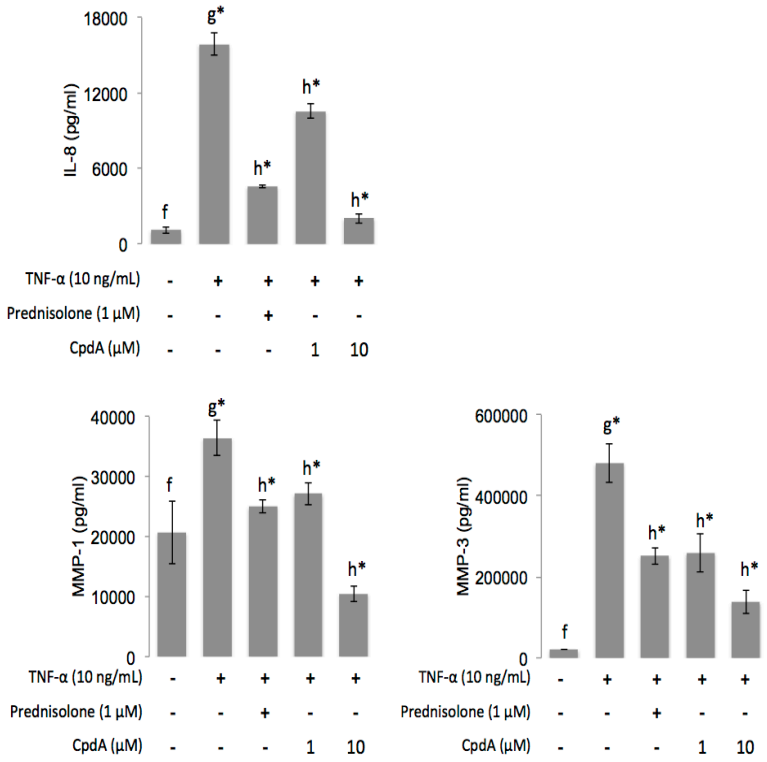

Figure 48. CpdA down-regulated TNF-α-induced IL-8, MMP-1 and MMP-3 secretion in OA synovial fibroblasts. Human OA synovial fibroblasts were pre-incubated 1h with prednisolone (1 µM) or CpdA (1 or 10 µM) and then stimulated with TNF-α (10 ng/mL) for 12h. IL-8, MMP-1 and MMP-3 were measured in cell culture supernatant using an ELISA. Each graph represents the mean +/- SD. This is a representative experiment from n=3. Significance at $p<0.05$. g*, significantly different from f. h*, significantly different from g.

These results suggest that CpdA owns anti-inflammatory properties similar to those of prednisolone and is able to decrease inflammation in human OA synovial fibroblasts.

4.3.3. Smad pathway

We previously reported that the TGF-β1 signaling pathway was involved via

Smad1/5 phosphorylation when prednisolone induced leptin expression in OA synovial fibroblasts [171]. Prednisolone was considered to induce leptin secretion by Smad1/5 phosphorylation. Because CpdA did not induce leptin secretion, we sought to address whether CpdA could also induce Smad1/5 phosphorylation as observed with prednisolone.

First, short-term stimulation was performed on human OA synovial fibroblasts with prednisolone (1 µM) and CpdA (1 and 5 µM) for 30 min, 1 h and 4 h *(Figure 49)*. TGF-β1 (10 ng/mL) was used as a positive control. TGF-β1 strongly induced Smad1/5 phosphorylation (30 min and 1 h) and Smad2 phosphorylation (30 min and 1 h). Prednisolone slightly induced Smad1/5 phosphorylation after 4 h, while CpdA did not increase, and further decreased, Smad1/5 phosphorylation after 4 h compared with the non-stimulated control. No phosphorylation of Smad2 was observed with prednisolone or CpdA, and no modification of Smad1, Smad2 or GAPDH protein levels was observed.

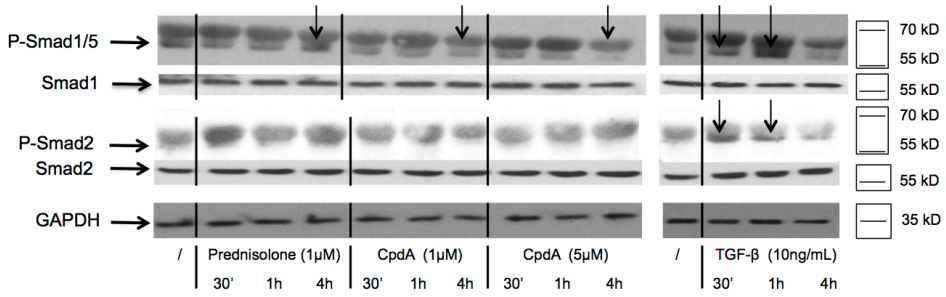

Figure 49. CpdA did not induce Smad1/5 phosphorylation. Human OA synovial fibroblasts were stimulated with prednisolone (1 mM) and CpdA (1 and 5 mM) for 30 min, 1 h, 4 h. TGF-β1 (10 ng/ml) was used as positive control. Phospho-Smad1/5, Smad1, phospho-Smad2, Smad2 and GAPDH expression levels were analyzed in total cell extracts by Western blot.

Second, synovial fibroblasts were stimulated with prednisolone (1 µM), CpdA (1 and 5 µM) and TGF-β1 (10 ng/mL) over a period of 7 days *(Figure 50)*. TGF-

β1 slightly but significantly induced Smad1/5 phosphorylation and strongly induced Smad2 phosphorylation. Prednisolone induced Smad1/5 phosphorylation but not Smad2 phosphorylation. CpdA did not induce Smad1/5 or Smad2 phosphorylation compared to the control. Moreover, CpdA significantly reduced Smad1/5 phosphorylation after stimulation with prednisolone.

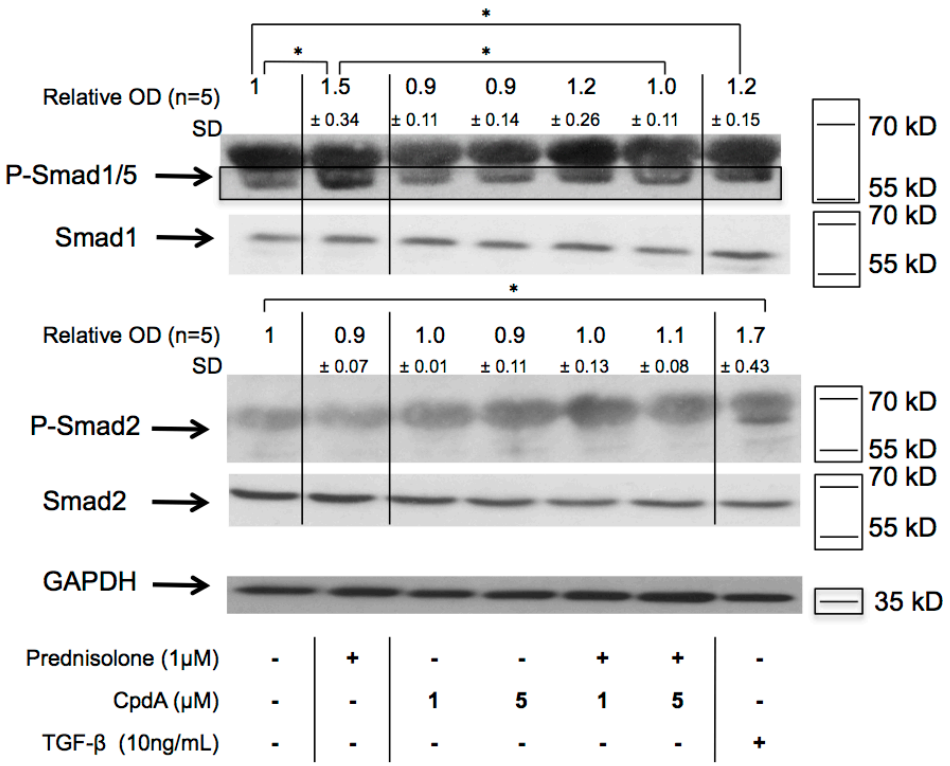

Figure 50. CpdA did not induce Smad1/5 phosphorylation. Human OA synovial fibroblasts were stimulated with prednisolone (1 mM) and CpdA (1 and 5 mM) for 7 days. TGF-β1 (10 ng/mL) was used as positive control. Phospho-Smad1/5, Smad1, phospho-Smad2, Smad2 and GAPDH expression levels were analyzed in cell extracts by Western blot. Band quantification represents the mean +/- SD of 5 different patients and are expressed relative to the non-stimulated control condition. Asterisks (*) indicate significantly different (p-value <0.05). This is a representative experiment from n=5. OD: optic density.

These results suggest that CpdA, unlike prednisolone, does not modulate the

Smad signaling pathway.

4.3.4. Glucocorticoid-induced leucine zipper expression

Selective glucocorticoid receptor agonist CpdA did not induce leptin secretion nor Ob-R expression. Of interest, CpdA did neither induce GILZ expression in OA synovial fibroblasts *(Figure 51)*.

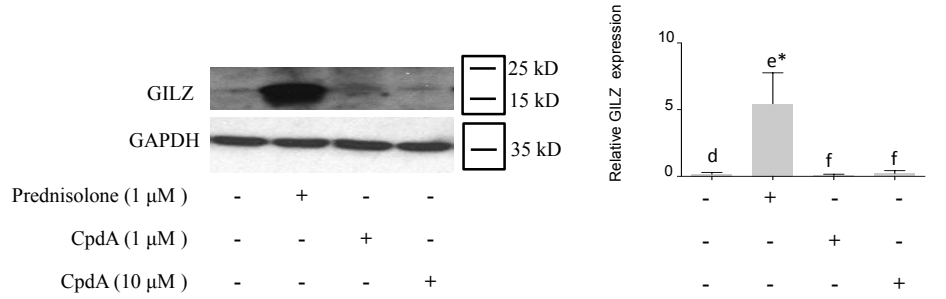

Figure 51. CpdA did not induce GILZ expression. Human OA synovial fibroblasts were stimulated for 5 days with prednisolone or CpdA. GILZ and GAPDH in whole cell extracts were analyzed using Western blotting. Right panels are quantification of Western blot in left panels. Proteins were normalized to GAPDH levels. Each graph represent the mean +/- SD, n=3. e*, significantly different from d. f, not significantly different from d.

In summary, we have shown, using human OA synovial fibroblasts, that:

1) CpdA did not induce leptin, or Ob-R, and reduced prednisolone-induced leptin and Ob-R;
2) CpdA down-regulated endogenous and TNF-α-induced IL-6 secretion and down-regulated TNF-α-induced IL-8, MMP-1 and MMP-3 secretions;
3) unlike prednisolone, CpdA did not induce Smad1/5 phosphorylation and further reduced prednisolone-induced Smad1/5 phosphorylation;
4) unlike prednisolone, CpdA did not induce GILZ expression.

Unlike prednisolone, CpdA did not induce leptin, Ob-R and GILZ expression, and did not impact the TGF-β pathway while exhibiting intact anti-inflammatory properties.

4.4. Role of glucocorticoid-induced leucine zipper in leptin and leptin receptor expression

4.4.1. Glucocorticoid-induced leucine zipper is involved in prednisolone- and aldosterone-induced leptin and leptin receptor expression

ShRNA experiments silencing GILZ expression were performed to determine whether GILZ was involved in prednisolone- and/or aldosterone-induced leptin secretion. Human OA synovial fibroblasts were infected with 3 different lentiviruses expressing GILZ shRNA or with a non-target control lentivirus. After 72 h of incubation, the medium was removed, and the cells were stimulated with prednisolone (1 μM) or aldosterone (1 or 10 μM). Upon prednisolone *(Figure 52 A)* and aldosterone *(Figure 52 B)* stimulation, GILZ shRNA reduced GILZ expression, as indicated by the experiments with 3 different types of shRNA. When GILZ was down-regulated, the prednisolone- and aldosterone-induced leptin secretion and Ob-R expression levels were significantly decreased compared to the controls.

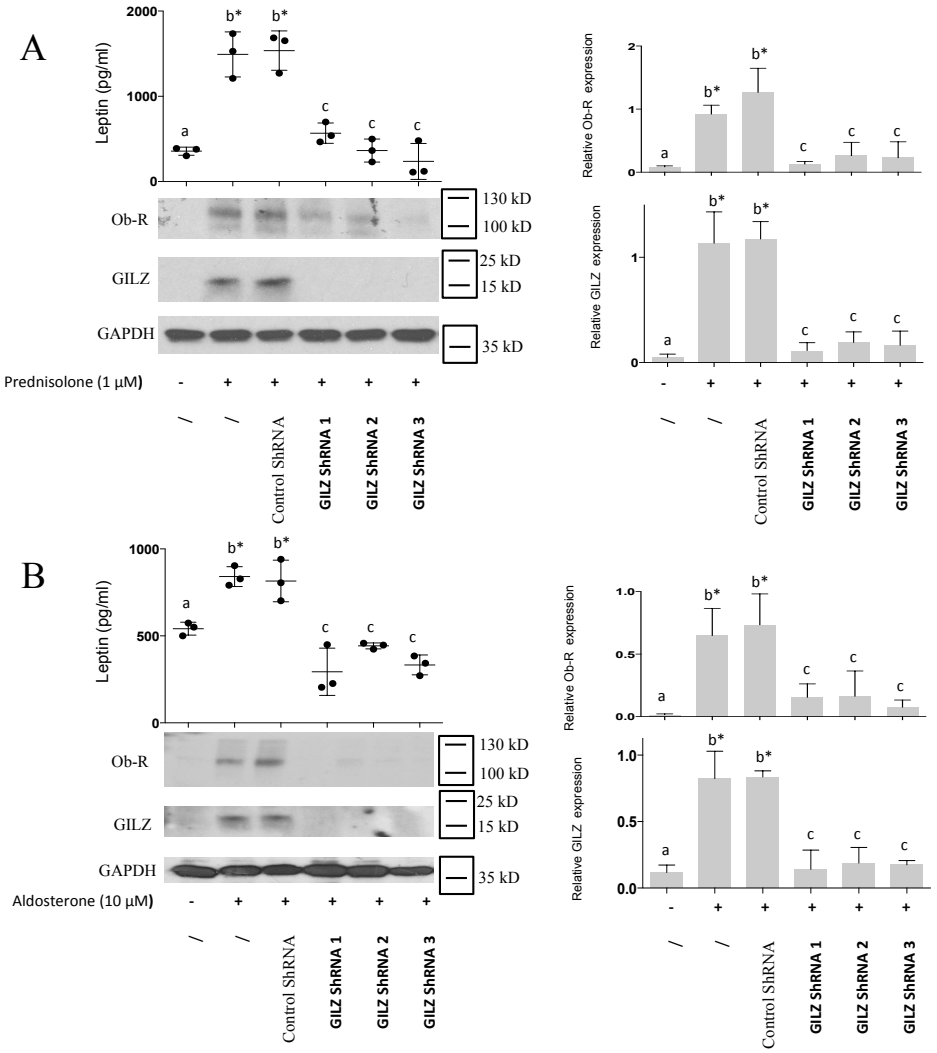

Figure 52. GILZ silencing inhibited prednisolone- and aldosterone-induced leptin secretion and Ob-R expression. Human OA synovial fibroblasts were infected with 3 different lentiviruses expressing GILZ shRNA or with a control lentivirus. After 72 h, cells were stimulated for 5 days with 1 µM prednisolone or 10 µM aldosterone. Leptin was measured in the cell culture supernatant using an ELISA. Ob-R, GILZ and GAPDH in whole cell extracts were analyzed using Western blotting. Right panels are quantification of Western blot in left panels. Proteins were normalized to GAPDH levels. Each graph represent the mean +/- SD, n=5. b*, significantly different from a. c, not significantly different from a.

The decrease in leptin and Ob-R expression was correlated with the shRNA MOI and with the degree of GILZ extinction *(Figure 53)*.

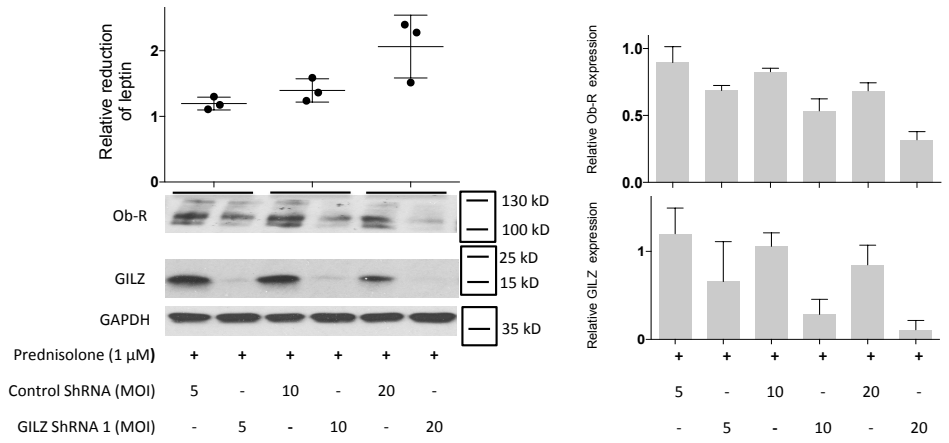

Figure 53. GILZ silencing inhibited prednisolone-induced leptin secretion and Ob-R expression. Human OA synovial fibroblasts were infected with a lentivirus expressing GILZ shRNA or with a control lentivirus. After 72 h, cells were stimulated for 5 days with 1 µM prednisolone. Leptin was measured in the cell culture supernatant using an ELISA. Ob-R, GILZ and GAPDH in whole cell extracts were analyzed using Western blotting. Right panels are quantification of Western blot in left panels. Proteins were normalized to GAPDH levels. Each graph represent the mean +/- SD, n=5.

GILZ silencing did not alter either the GR expression or the prednisolone-induced GR degradation *(Figure 54)*.

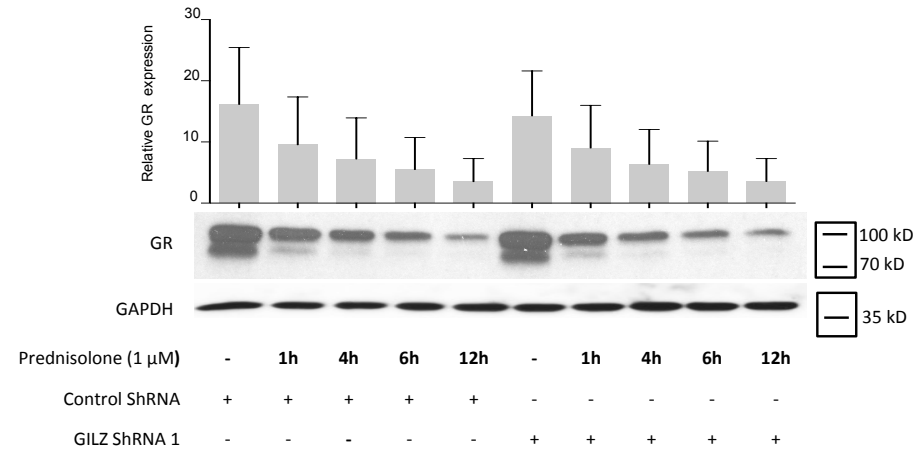

Figure 54. GILZ silencing did not alter the prednisolone-induced GR degradation. Human OA synovial fibroblasts were infected with a lentivirus expressing GILZ shRNA or with a control lentivirus. After 72 h, cells were stimulated for 1 h, 4 h, 6 h, 12 h with 1 µM prednisolone. Leptin was measured in the cell culture supernatant using an ELISA. GR and GAPDH in whole cell extracts were analyzed using Western blotting. Upper panel is quantification of Western blot in lower panel. Proteins were normalized to GAPDH levels. Each graph represent the mean +/- SD, n=3.

In summary, we have shown, using human OA synovial fibroblasts, that:

1) GILZ silencing inhibited prednisolone- and aldosterone-induced leptin secretion and Ob-R expression;
2) the decrease in leptin and Ob-R expression was correlated with the degree of GILZ extinction;
3) GILZ silencing did not alter either the GR expression or the prednisolone-induced GR degradation.

GILZ is involved in prednisolone- and aldosterone-leptin secretion and Ob-R expression in human OA synovial fibroblasts.

4.5. Role of glucocorticoid-induced leucine zipper in inflammation

Synovial fibroblasts spontaneously produced IL-6, IL-8 and MMP-1 pro-inflammatory molecules, and stimulation by TNF-α enhanced their secretions *(Figure 54 A, B, C)*. Human OA synovial fibroblasts were infected with 3 different lentiviruses expressing GILZ shRNA or with a non-target control lentivirus. Cells were pre-incubated *(Figure 55 D, E, F)* or not *(Figure 55 A, B, C)* for 1 h with prednisolone (1 μM) and then stimulated or not with TNF-α (10 ng/mL) for 12 h. GILZ-shRNA did not significantly alter the TNF-α-induced IL-6, IL-8 and MMP-1 production. Moreover, GILZ inhibition did not alter the capacity of prednisolone to reduce TNF-α-induced IL-6, IL-8 and MMP-1 production. These results suggest that GILZ is not an essential mediator of the anti-inflammatory actions of GCs in OA synovial fibroblasts.

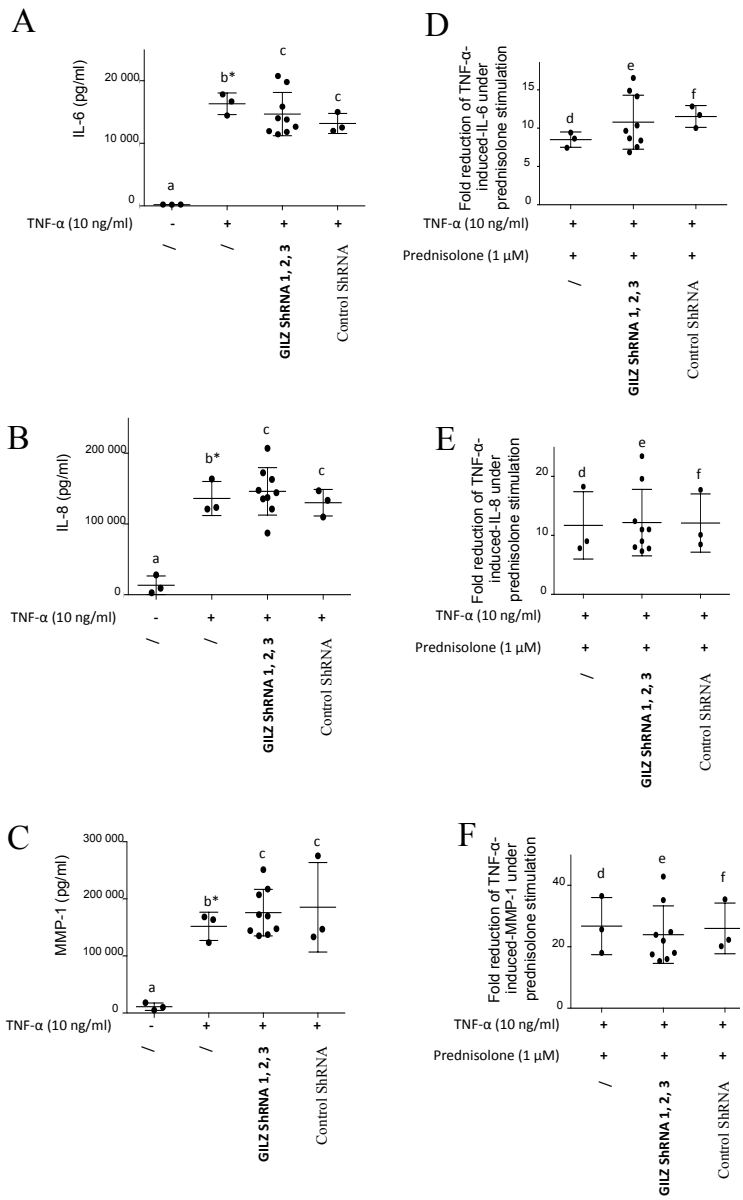

Figure 55. GILZ inhibition did not significantly alter either the TNF-α-induced IL-6, IL-8 and MMP-1 production or the capacity of prednisolone to reduce the TNF-α-induced IL-6, IL-8 and MMP-1 production. Human OA synovial fibroblasts were infected with lentiviruses expressing GILZ shRNA or with a control. Each graph represent the mean +/- SD, n=3. Fold reduction of IL-6, IL-8 and MMP-1 levels induced by TNF-α were measured by comparing levels in the presence or not of prednisolone (1 µM). Significance at $p<0.05$. b*, significantly different from a. c, not significantly different from b. e and f, not significantly different from d.

In summary, we have shown, using human OA synovial fibroblasts, that:

1) GILZ silencing did not reduce TNF-α-induced IL-6, IL-8 and MMP-1 secretions;
2) GILZ silencing did not alter the capacity of prednisone to reduce the TNF-α-induced IL-6, IL-8 and MMP-1 secretions.

It therefore appeared that in human OA synovial fibroblasts, GILZ was crucial for prednisolone- and aldosterone-induced leptin secretion and Ob-R expression (last section), but was not an essential mediator of the anti-inflammatory actions of prednisolone in these cells.

4.6. Secretion of leptin by chondrocytes

4.6.1. Dedifferentiated chondrocytes but not freshly isolated osteoarthritic chondrocytes produce leptin and express leptin receptor

OA chondrocytes were isolated from human cartilage from femoral head obtained during hip replacement surgery. Freshly isolated OA chondrocytes appeared as spherical non-adherent cells *(Figure 56, day 2)*. After 5 days, some fibroblastic cells started to appear. These fibroblastic cells, which correspond to dedifferentiated chondrocytes, tend to become predominant after 12 days of culture. Fresh isolated chondrocytes did not either produce leptin or express Ob-R, spontaneously or under prednisolone stimulation. With dedifferentiation and the acquisition of a fibroblastic shape, chondrocytes became able to produce leptin and express Ob-R *(Figure 56, day 12)*. Similar results were obtained when freshly isolated chondrocytes or dedifferentiated chondrocytes were separately stimulated with prednisolone during five days: there was no or only small amount of leptin secreted by freshly isolated chondrocytes, while there was high amount of endogenous and prednisolone-induced leptin by dedifferentiated chondrocytes *(data not shown)*.

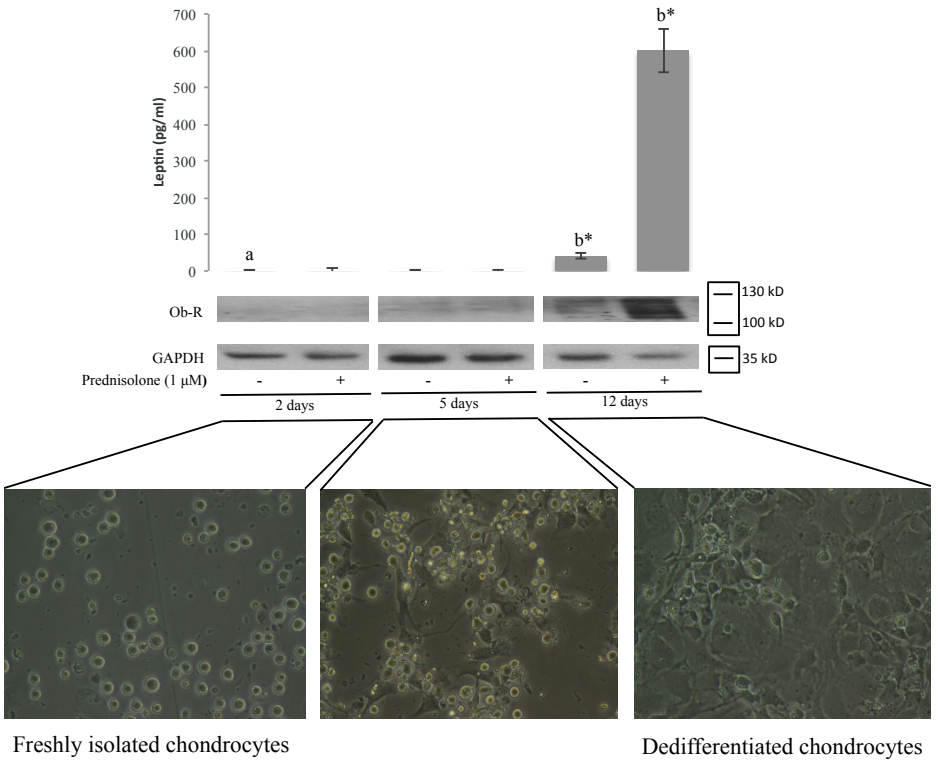

Figure 56. Leptin secretion and Ob-R expression during *in vitro* chondrocyte dedifferentiation. Freshly isolated chondrocytes were stimulated for 12 days with or without prednisolone. Leptin was measured in the cell culture supernatant using an ELISA. Ob-R and GAPDH in whole cell extracts were analyzed using Western blotting. Each graph represents the mean +/- SD. This is a representative experiment from n=3. Significance at $p<0.05$. b*, statistically different from a. 20X magnification.

As observed for OA synovial fibroblasts, the mineralocorticoid aldosterone also induced leptin secretion and Ob-R protein expression in dedifferentiated chondrocytes *(Figure 57)*. Moreover, using specific inhibitors [a GR inhibitor (mifepristone) and MR inhibitors (eplerenone and spironolactone)], we observed that the prednisolone- and aldosterone-induced leptin secretion and Ob-R expression were GR-dependent, but not MR-dependent. These results suggest that similar to prednisolone, aldosterone is able to induce leptin secretion and Ob-R receptor expression in OA dedifferentiated chondrocytes through GR but not MR.

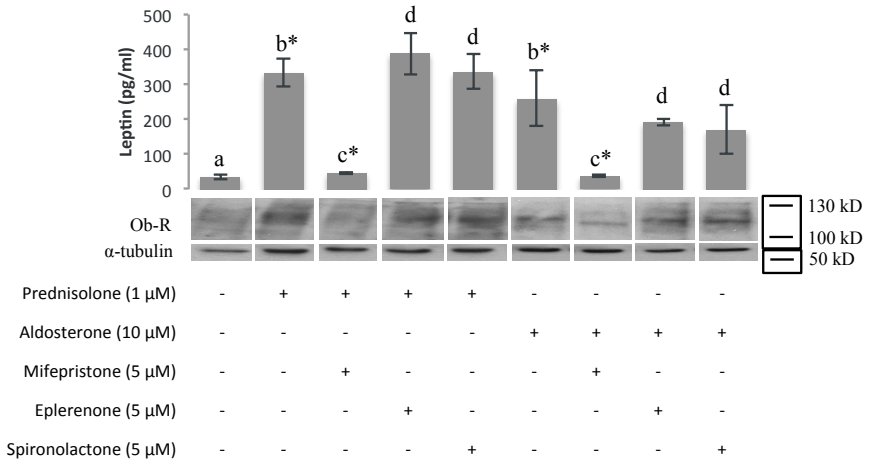

Figure 57. Leptin secretion and Ob-R expression were induced by prednisolone and aldosterone through GR signaling in dedifferentiated chondrocytes. Human OA dedifferentiated chondrocytes were pre-incubated or not for 1 h with a GR inhibitor (mifepristone) or MR inhibitors (eplerenone and spironolactone) and then stimulated for 5 days with prednisolone or aldosterone. Leptin was measured in the cell culture supernatant using an ELISA. Ob-R and GAPDH in whole cell extracts were analyzed using Western blotting. Each graph represents the mean +/- SD. This is a representative experiment from n=3. Significance at $p<0.05$. b*, statistically different from a. c*, statistically different from b. d, not statistically different from b.

We next analyzed the capacity of CpdA to stimulate the production of leptin in human OA dedifferentiated chondrocytes. After 7 days, prednisolone, but not CpdA, significantly induced leptin secretion *(Figure 58)*. However, CpdA remained able to reduce TNF-α-induced IL-6, IL-8, MMP-1 and MMP-3 secretions *(Figure 59)*.

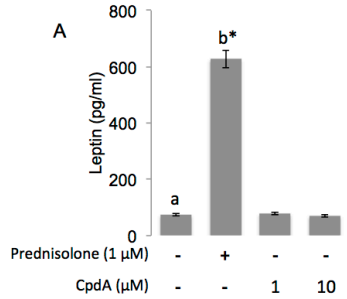

Figure 58. CpdA did not induce leptin secretion in OA chondrocytes. Human OA chondrocytes were stimulated with CpdA (1 or 10 µM) or prednisolone (1 µM) for 7 days. Leptin was measured in cell culture supernatant using an ELISA. Each graph represents the mean +/- SD. This is a representative experiment from n=3. Significance at $p<0.05$. b*, statistically different from a.

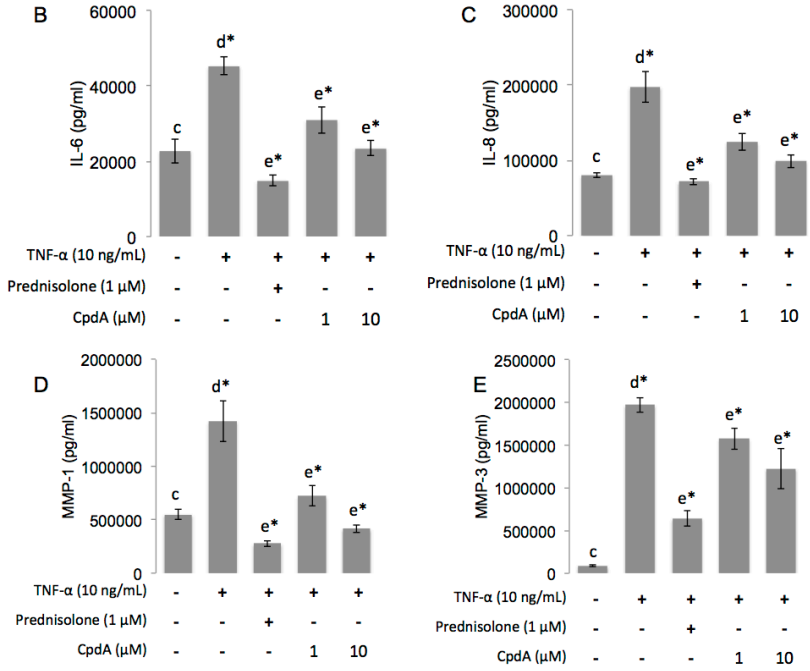

Figure 59. CpdA down-regulated TNF-α-induced IL-6, IL-8, MMP-1 and MMP-3 secretion in OA chondrocytes. Human OA chondrocytes were pre-incubated 1h with prednisolone (1 µM) or CpdA (1 or 10 µM) and then stimulated with TNF-α (10 ng/mL) for 12h. IL-6, IL-8, MMP-1 and MMP-3 were measured in cell culture supernatant using an ELISA. Each graph represents the mean +/- SD. This is a representative experiment from n=3. Significance at $p<0.05$. d*, statistically different from c. e*, statistically different from d.

In summary, we have shown, using human OA chondrocytes, that:

1) freshly isolated chondrocytes did not have either the capacity to produce leptin or Ob-R, spontaneously or under the influence of prednisolone;
2) with dedifferentiation occurring in culture after 12 days, chondrocytes acquired the capacity to spontaneously produce leptin and Ob-R, strongly stimulated in the presence of prednisolone or aldosterone;
3) mifepristone, but not eplerenone or spironolactone, reduced prednisolone- and aldosterone-induced leptin expression in human dedifferentiated OA chondrocytes, suggesting, as observed with human OA synovial fibroblasts, an action through the GR;
4) unlike prednisolone, CpdA was unable to induce leptin secretion in human dedifferentiated OA chondrocytes, but remained capable to down-regulate TNF-α-induced IL-6, IL-8, MMP-1 and MMP-3 secretions in the same cells.

Unlike freshly isolated human OA chondrocytes, only dedifferentiated chondrocytes were able to spontaneously produce leptin and express Ob-R. These expressions were strongly stimulated in the presence of prednisolone and of aldosterone impacting GR pathway. Compound A was unable to induce leptin and Ob-R expression in the same cells, while keeping its anti-inflammatory properties.

4.7. Additional results

4.7.1. Study of other adipokines: synovial fibroblasts and chondrocytes do not produce resistin or adiponectin *in vitro*

Fresh samples of synovial membrane were directly cut from the synovium explant *(Figure 60)*. The culture cell medium was analyzed after 1 and 3 days, without stimulation. There was a significant endogenous release of adiponectin (57 +/- 9 ng/mL after 1 day and 300 +/- 43 pg/mL after 3 days, per mg of synovium) (n=2). Significant endogenous resistin secretion (125 +/- 50 ng/mL) was observed with small cuts of fresh OA cartilage after 3 days (height 1mm, diameter 3mm) (n=2).

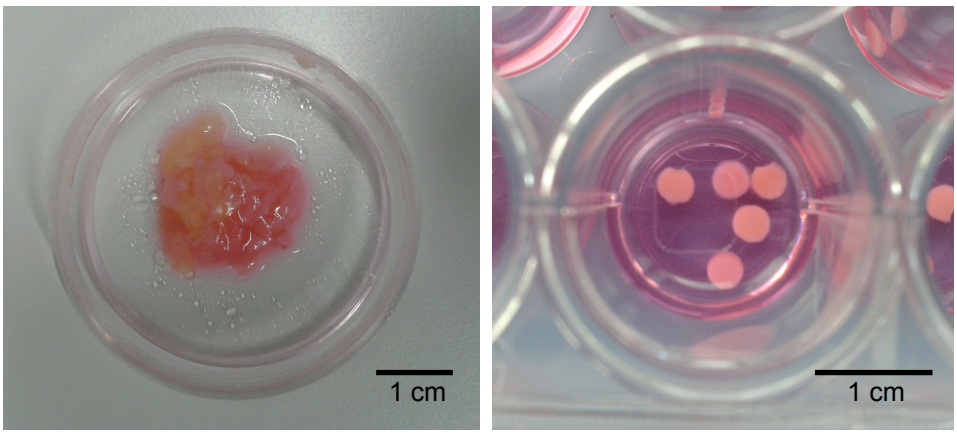

Figure 60. From left to right, fresh sample of OA synovium membrane and small cuts of fresh OA cartilage.

Chondrocytes and synoviocytes were isolated as explained earlier and stimulated or not with prednisolone (1 µM) during 7 days. As expected, endogenous leptin secretion was observed after stimulation by prednisolone in synovial fibroblasts and dedifferentiated chondrocytes. No endogenous or

prednisolone-induced adiponectin or resistin secretion was observed *(Figure 61)*. Of interest, CpdA did not either induce adiponectin or resistin *(data not shown)*.

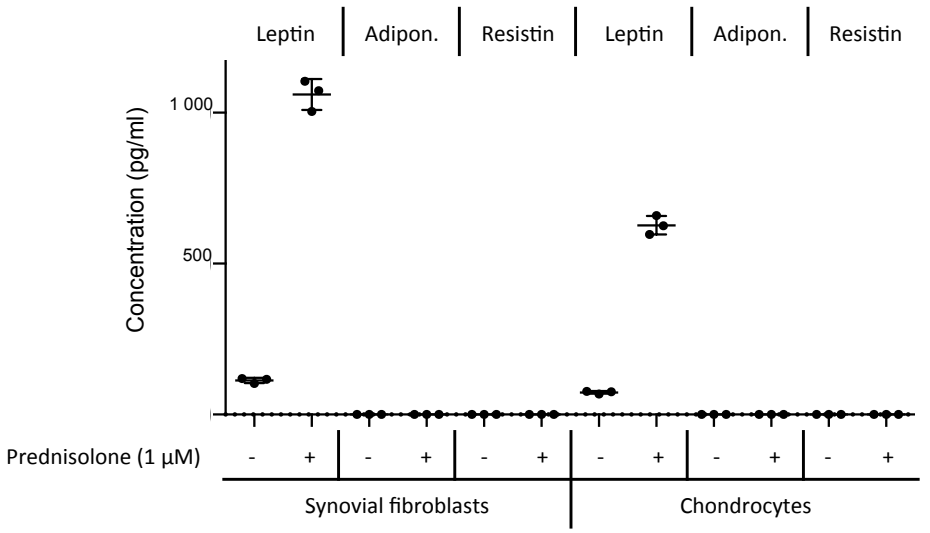

Figure 61. Synovial fibroblasts and chondrocytes produce leptin *in vitro* (spontaneously and under prednisolone secretion), but not adiponectin nor resistin. Human OA synovial fibroblasts and chondrocytes were stimulated or not with prednisolone for 7 days (1 µM). Leptin, adiponectin and resistin were measured in the cell culture supernatant using an ELISA. Each graph represent the mean +/- SD, n=3. Significance at $p<0.05$.

4.7.2. Study of leptin production by visceral pre-adipocytes

We have previously studied the leptin production by human OA synovial fibroblasts and chondrocytes. However, adipose cells are the first documented ones able to produce leptin. In order to compare our results with the fat tissue, we have analyzed the leptin secretion by visceral pre-adipocytes providing from human visceral fat tissue. Pre-adipocytes are fibroblastic cells, isolated from the stromal vascular fraction of adipose tissue, that can differentiate into adipocytes. Human visceral pre-adipocytes were stimulated during 5 days with GC

prednisolone or mineralocorticoid aldosterone. As described for synovial fibroblasts and for dedifferentiated chondrocytes, there was an *in vitro* significant endogenous secretion of leptin, enhanced with prednisolone and aldosterone stimulation.

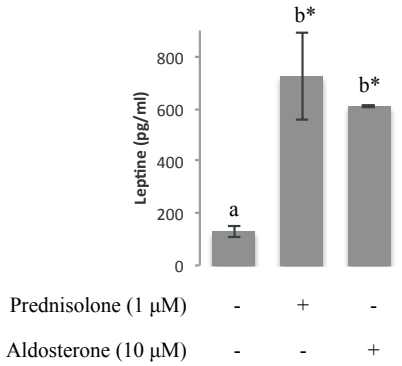

Figure 62. Spontaneous and corticoid-induced leptin secretion by human visceral pre-adipocytes. The cells were stimulated during 5 days with prednisolone or aldosterone. Leptin was measured in cell culture supernatant by ELISA. Each graph represent the mean +/- SD, n=3. Significance at $p<0.05$. b*, statistically different from a.

In summary, we have shown that:

1) fresh samples of OA synovium membrane and small cuts of fresh OA cartilage spontaneously released significant amounts of adiponectin and of resistin;
2) however, there was no spontaneous or prednisolone-induced production of adiponectin and of resistin neither in human OA synovial fibroblasts nor in dedifferentiated OA chondrocytes;
3) human visceral pre-adipocytes spontaneously produced leptin. Leptin secretion was stimulated by GC prednisolone and mineralocorticoid aldosterone.

4.8. Summary of the results

1. GILZ is expressed in human OA synovial fibroblasts: GC prednisolone and mineralocorticoid aldosterone induced its expression. A GR inhibitor, but not MR inhibitors, reduced the prednisolone- and aldosterone-induced GILZ expression levels, indicating that GILZ induction by prednisolone and aldosterone was GR-dependent. This was confirmed with GR-silencing experiments.

2. Leptin secretion and Ob-R expression were induced by GC prednisolone but also by mineralocorticoid aldosterone. A GR inhibitor, but not MR inhibitors, reduced the prednisolone- and aldosterone-induced leptin secretion and Ob-R expression levels, indicating that leptin and Ob-R induction by prednisolone and aldosterone were GR-dependent, as previously demonstrated for GILZ. This was also confirmed with GR-silencing experiments. Leptin, Ob-R and GILZ induction were dose-dependent. Additional links between leptin and GILZ was revealed with TGF-β1 experiments: TGF-β1 reduced both prednisolone-induced leptin secretion and GILZ expression.

3. The SEGRA CpdA did not induce either leptin secretion or Ob-R expression and could even reduce the prednisolone-induced leptin and Ob-R. Moreover, CpdA did not induce Smad1/5 phosphorylation that was previously involved in the prednisolone-induced leptin secretion in synovial fibroblasts. CpdA did not also induce GILZ expression. The SEGRA CpdA had, however, similar anti-inflammatory properties than prednisolone, down-regulating the endogenous and TNF-α-induced IL-6 secretion, as well as the TNF-α-induced IL-8, MMP-1 and MMP3 secretions.

4. GILZ silencing inhibited the prednisolone and aldosterone-induced leptin secretion and Ob-R expression, involving GILZ in the corticoid-induced leptin

secretion in human OA synovial fibroblasts. However, GILZ silencing did not reduce the TNF-α-induced IL-6, IL-8 and MMP-1 secretion and did not alter the capacity of prednisone to reduce the TNF-α-induced IL-6, IL-8 and MMP-1 secretions.

5. Freshly isolated chondrocytes did not either produce leptin or Ob-R, spontaneously or under prednisolone stimulation. *In vitro*, chondrocytes dedifferentiated and acquired a fibroblast-like shape. When dedifferentiated, they became able to produce leptin and express Ob-R. Dedifferentiated chondrocytes had the same profile than synovial fibroblasts: prednisolone and aldosterone induced leptin through GR, and CpdA did not induce leptin but reduces the inflammatory parameters. Data not shown also confirmed the GILZ involvement in leptin production in dedifferentiated chondrocytes, but not in the anti-inflammatory action of prednisolone.

6. *In vitro*, there is no endogenous or prednisolone-induced resistin or adiponectin secretion by osteoarthritis synovial fibroblasts.

5. DISCUSSION

5.1. Expression, modulation and function of glucocorticoid-induced leucine zipper in osteoarthritic synovial fibroblasts

In the rheumatology field, GCs are widely used, specifically to treat an inflammatory flare. In OA, intra-articular injections of GCs are efficient for symptomatic treatment [265] and remain widely used as symptomatic drugs with anti-inflammatory properties, but they also contribute to adverse events (diabetes, osteoporosis …). Moreover, GCs can enhance a catabolic reaction leading to the degradation of cartilage and also suppress matrix protein markers of chondrogenic differentiation [275, 276]. We previously showed that OA synovial fibroblasts were able to spontaneously produce leptin and express Ob-R *in vitro* [1]. Surprisingly, GC prednisolone strongly induced leptin secretion and Ob-R expression [1]. In OA, leptin plays a detrimental role with a catabolic and pro-inflammatory action on cartilage [186]. Leptin could therefore be part of the adverse events generated by GCs in OA. The fact that one of the most potent anti-inflammatory drugs could induce leptin secretion, a molecule with catabolic and pro-inflammatory actions on the cartilage, by constitutive cells of a joint themselves could raised questions about adequacy of the therapeutic choice. In order to better understand how leptin production is regulated by prednisolone, we decided to explore the expression of GILZ, a factor that could be involved in this apparent contradiction.

GILZ is an intracellular protein induced by GCs and is mainly found in immune cells [295, 326]. GILZ can also be dependent on mineralocorticoids, as observed in kidney cells [310]. In rheumatology, Beaulieu *et al.* observed that GILZ was expressed in the synovium of patients with RA and in cultured RA synovial fibroblasts. They also observed that GILZ expression was enhanced in the presence of dexamethasone and significantly reduced under mifepristone suggesting GR involvement [311]. Our work first extends the presence of GILZ

to human OA synovial fibroblasts and reports for the first time the ability of GC prednisolone, but also of mineralocorticoid aldosterone (discussed later), to induce GILZ expression (at the protein and the mRNA levels) in these cells. Both inductions were GR-dependent for the following reasons: a) a strong decrease of the effect observed by the use of mifepristone, a GR inhibitor; b) an absence of modulation by the use of eplerenone/spironolactone, two MR inhibitors; c) an abolishment of response when GR was silenced. MG132 reduces the degradation of ubiquitin-conjugated proteins and is a specific proteasome inhibitor. GILZ protein expression levels were enhanced by adding MG132 in the last 12 h of culture, indicating that GILZ degradation at least partially occurred by the proteasome pathway.

GILZ is primarily described as a mediator of the anti-inflammatory mechanism of GCs in immune-related cells [295, 326]. Overexpression of GILZ has a significant anti-inflammatory effect during the treatment of collagen-induced arthritis [311, 312]. However, in OA synovial fibroblasts, we have shown that GILZ depletion did not alter the TNF-α-induced production of the pro-inflammatory proteins IL-6, IL-8 and MMP-1, nor alter the ability of GCs to down-regulate their TNF-α-induced production. Our experimental data support the idea that GILZ is not significantly involved in the anti-inflammatory regulation of GCs in OA. Down-regulation of GILZ did not also modulate GR expression or down-regulation under prednisolone exposure. Our data have shown that ligated GR can therefore still lead to signal transduction in the absence of GILZ. Several authors also reported a lack of inflammatory variations when GILZ was modulated:

- in the K/BxN serum-transfer arthritis model, no significant difference was observed between wild type and GILZ$^{-/-}$ mice in clinical or histologic arthritis severity [312]. Lipopolysaccharide-induced production of the cytokines TNF-α, IL-6 and IL-1β, but also the inhibition of production of theses cytokines by dexamethasone was

unaffected by the absence of GILZ. In the collagen-induced arthritis model, the attenuation of arthritis by dexamethasone was similar in wild type and GILZ$^{-/-}$ mice and the *in vitro* effect of dexamethasone on TNF-α, IL-6 and IL-1β was similar than in controls [312];

- if GILZ overexpression significantly inhibited the production of key pro-inflammatory cytokines such as IL-6 and IL-8 by RA synovial fibroblasts, it was without any effect on a panel of other pro-inflammatory cytokines such as IL-1β, TNF-α or IL-12p70 produced by the same cells [311];
- experiences have also been performed using human umbilical venous endothelial cells (HUVECs) [327]. In the presence of TNF-α, leukocytes rolling and adhesion were significantly reduced in GILZ-transfected cells as compared to control-transfected cells. Overexpression of GILZ in HUVECS has also shown reduced expression of TNF-α-induced E-selectin, ICAM-1, CCL2, CXCL8 and IL-6 both at the mRNA levels (except for IL-6) and at the protein levels. However, there was no significant modification of these parameters when GILZ was silenced.

Involvement of GILZ in the anti-inflammatory process thus appears to be cell dependent, with differences between the study of endogenous GILZ (with silencing experiments) and exogenous GILZ (with overexpression). Taken together, our results do not support that, in human OA synovial fibroblasts, GILZ can reduce neither the TNF-α-induced production of several cytokines and pro-inflammatory mediators nor is required for GCs to exert their therapeutic effects. This lack of effect was also shown by others in human RA synovial fibroblasts, in murine dermal and synovial fibroblasts and in HUVECs [311, 312, 327].

If GILZ does not seem to be crucial for the anti-inflammatory properties of GCs in OA, we propose a new role for GILZ in OA synovial fibroblasts through its

contribution to the corticoid-induced leptin secretion and Ob-R expression (Figure 63).

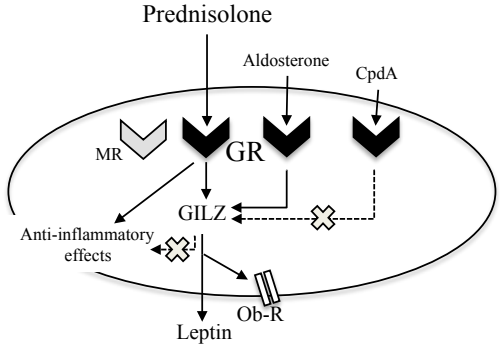

Figure 63. The absence of GILZ in OA synovial fibroblasts prevented corticoid-induced leptin secretion and Ob-R expression without modulating the anti-inflammatory properties of GCs. Prednisolone and aldosterone induced GILZ expression in OA synovial fibroblasts through GR but not MR, whereas CpdA did not. Similar effects on leptin secretion and Ob-R expression were observed. Thus, GILZ was involved in prednisolone- and aldosterone-induced leptin secretion and Ob-R expression. In addition, GILZ inhibition did not alter the anti-inflammatory action of prednisolone.

Indeed, we previously reported that human OA synovial fibroblasts could produce leptin (a pro-inflammatory adipokine involved in OA pathogeny) and its receptor, Ob-R, both spontaneously and after stimulation with GCs [1]. We have shown in this work that GILZ was crucial for these properties of GCs for the following reasons: a) GILZ induction by prednisolone corresponded to leptin and Ob-R induction by OA synovial fibroblasts; b) GILZ silencing abolished prednisolone-induced leptin and Ob-R expression, the decrease being correlated to the degree of GILZ extinction. The deleterious contribution of leptin in metabolic OA pathogeny [194] indicates that leptin and Ob-R expressions in synovial fibroblasts can contribute to the metabolic events generated by GCs. From a metabolic point of view, GILZ expression could be deleterious in OA because it induces leptin expression. Interestingly, GILZ, leptin and Ob-R protein expressions exhibit a close interplay when GILZ silencing significantly

reduced leptin and Ob-R expression. Moreover, we have shown that TGF-β1 decreased the GC-induced GILZ expression, similarly to leptin, confirming our previous results [171]. The links between GILZ and TGF-β will be discussed later. Other studies have already shown the involvement of GILZ in the adverse events of GCs: dexamethasone and GILZ inhibit the repair of respiratory epithelial cells [322]. In addition, the long-term used of glucocorticoids leads to anti-myogenic effects due to the presence of GILZ as an effector [323].

5.2. Influence of mineralocorticoids and the mineralocorticoid receptor on leptin secretion

In this work, we also determined the influence of mineralocorticoids (another class of corticosteroids) on leptin secretion in parallel to the study of GCs. Links between leptin and mineralocorticoids could be therefore suspected. Mineralocorticoid aldosterone is associated with pro-inflammatory effect and generation of oxidative stress [251], while GCs have a clear anti-inflammatory action. Aldosterone is also involved in the metabolic regulation: aldosterone is known for its bad metabolic profile (*i.e.*, an association with the development of metabolic syndrome in humans) and for its aggravation of glucose intolerance by high fructose alimentation in rats [256, 262]. In 2011, Brenner *et al.* reported the MR expression in the synovium of rats [328] that was confirmed in this work *in vitro* with human synovial fibroblasts. Of interest, while GR was quickly down-regulated under prednisolone stimulation, we have shown that there was no significant variation of the MR protein level with prednisolone or aldosterone (data not shown) stimulation. Leptin secretion in OA synovial fibroblasts under GC prednisolone and mineralocorticoid aldosterone will now be discussed, as well as whether leptin induction was dependent on either the GR or the MR.

In the present work, aldosterone was also a significant stimulator of leptin secretion, GILZ and Ob-R expressions. Aldosterone, as prednisolone, significantly induced leptin and Ob-R proteins and mRNA expression in human synovial fibroblasts. GILZ protein and mRNA was also induced under the same stimulating conditions. These results are in accordance with previous studies showing an *in vitro* increase in leptin mRNA in brown adipose tissue after aldosterone exposure and higher circulating leptin levels in patients with primary hyperaldosteronism [262, 263]. Aldosterone is also present in the synovial fluid of OA patients [329]. Therefore, aldosterone could induce leptin expression through a systemic or a local mechanism. Targeting the aldosterone pathway could be potentially considered as a treatment for OA.

GR is the native receptor of GCs. However, GCs can also act through the MR [248]. Conversely, mineralocorticoids, in addition to their native receptor MR, can act through the GR [249]. We described in the introduction the importance of the 11β-HSD1 enzyme to convert the hormonally inactive cortisone and prednisone into hormonally active cortisol and prednisolone [258]. On the other side, 11β-HSD2 enzyme is a powerful GC inactivator, expressed primarily in the kidney where it prevents cortisol / prednisolone binding to the MR by transforming it to cortisone / prednisone (which has a very low affinity for the MR) *(Figure 28)* [258]. In the synovium, both enzymes were expressed but immunohistochemistry showed that 11β-HSD1 expression occurred in both synovial fibroblasts and macrophages from RA patients, while 11β-HSD2 expression was restricted to synovial macrophages [259]. One unexpected important finding was the presence of considerable GCs metabolizing capacity (due to 11β-HSD1 expression) in the synovium from patients with OA, similar to that of RA [259]. Presence of abundant 11β-HSD1 activity on OA synovium, thus generating significant amounts of active GCs within the joint might explain why therapeutic GCs are so effective to damper flares of synovial inflammation.

Conversely, absence of 11β-HSD2 in synovial fibroblasts, the predominent cell type, opens the way to an hypothetical influence of GC on the MR.

Both aldosterone and prednisolone inductions were GR-dependent for the following reasons: a) a strong decrease of the effect observed by the use of the GR inhibitor mifepristone; b) an absence of modulation by the use of MR inhibitors eplerenone and spironolactone; c) a complete abolishment when GR was silenced. The observations mimicked those obtained when studying prednisolone and aldosterone-induced GILZ expression as previously discussed. Interestingly, GR inhibitor mifepristone down-regulated leptin levels in humans [330]. Lee *et al.* observed that in contrast to MR silencing, GR silencing blocked leptin induction by cortisol in human adipocytes [331]. Also as for prednisolone, leptin induction by aldosterone was dose-dependent at the protein and at the mRNA level.

The corticoid concentrations in the synovial fluid were not fully described. For GCs, 125 nM cortisol was measured in human OA synovial fluid [329]. Significant induction of leptin, Ob-R and GILZ was observed *in vitro* in this work at this concentration for prednisolone. Accordingly, a significant effect of GC on leptin production in an OA joint may be clinically relevant, even in the absence of any exogenous GC administration. However, the mineralocorticoid levels are 1000-fold decreased in biologic fluids compared to GC levels. Although 1 µM aldosterone is used in publications for mechanistic models [249], physiological concentrations are approximately 100 pM in OA synovial fluid [329], which is not sufficient to induce leptin or Ob-R expression in our *in vitro* model. However, the use of 1 and 10 µM aldosterone confirmed the focus of our mechanistic model on the role played by GILZ in leptin expression.

5.3. Influence of selective glucocorticoid receptor agonist Compound A on leptin secretion

In this work, we have observed that CpdA, a SEGRA, was unable to induce leptin secretion or Ob-R expression in human OA synovial fibroblasts after 5 days of stimulation. Endogenous Ob-R expression was decreased under CpdA treatment, which suggests attenuated leptin sensitivity when cells are stimulated with CpdA. Moreover, prednisolone-induced leptin secretion and Ob-R expression were down-regulated by CpdA in a dose-dependent manner. This decrease in expression could be due to direct inhibition by CpdA or competition between CpdA and prednisolone for the GR.

We have previously demonstrated that the TGF-β signaling pathway affected leptin expression, with a Smad balance in favor of Smad1/5 phosphorylation [171]. An increased Smad1/5:Smad2/3 ratio was associated with elevated MMP-13 expression in OA chondrocytes [81, 235]. In our work, we have observed that CpdA, unlike prednisolone, did not induce Smad1/5 phosphorylation; moreover, at higher doses, CpdA decreased prednisolone-induced Smad1/5 phosphorylation. The absence of leptin expression correlated with the absence of Smad1/5 phosphorylation under CpdA stimulation, and this result agrees with our previous publication, which correlated leptin expression with Smad1/5 phosphorylation under prednisolone treatment [171]. Indeed, in mouse models of inflammatory bowel disease, there is no significant change in TGF-β and Smad3/4 signaling in response to CpdA [289].

Anti-inflammatory properties of CpdA have been well characterized *in vitro* in RA synovial fibroblasts [292]. *In vivo*, inflammation was reduced by CpdA in murine models of collagen-induced arthritis [291]. In this work, we confirmed

similar anti-inflammatory properties of CpdA compared to prednisolone. Indeed, CpdA down-regulated endogenous and TNF-α-induced IL-6, IL-8, MMP-1 and MMP-3 levels. Our results reveal a new dissociative effect of CpdA in these cells. CpdA exhibited anti-inflammatory properties similar to those of prednisolone but, unlike prednisolone, CpdA did not induce leptin secretion. Due to the detrimental role of leptin in OA, this lack of expression highlights the potentially improved risk/benefit ratio of CpdA. Furthermore, the combined used of CpdA and classical GC therapy could reduce leptin expression and leptin sensitivity, although these *in vitro* results require *in vivo* confirmation.

Of interest, we have also shown in this work that CpdA did not induce GILZ expression. The absence of GILZ expression is consistent with our previous results, showing that CpdA did not induce leptin and Ob-R and exhibited similar anti-inflammatory effects than those of classical GCs. They are also in agreement with the observation aforementioned that GILZ silencing inhibited prednisolone-induced leptin secretion and Ob-R expression in the same cells. CpdA experiments are another way to support that GILZ is critical for GC-induced leptin and Ob-R expression. These results are in agreement with Drebert *et al.*, who also observed the absence of GILZ expression under CpdA treatment in colon cancer-derived myofibroblasts [332]. In this model, dexamethasone was able to inhibit the expression of hepatocyte growth factor and TGF-β whereas CpdA could not. Further, if dexamethasone and CpdA both decreased the expression of the chemokine CCL5 and the adhesion molecule ICAM, CpdA was unexpectedly unable to significantly repress gene expression levels of MCP-1, IL-1 β and TNF-α in the colon cancer-derived myofibroblasts, whereas it did in adenocarcinomic human alveolar epithelial cells [332] and in RA synovial fibroblasts [291] [292], thus emphasizing cell-specific mechanisms already discussed above. In a recent report, Gavrila *et al.* published that CpdA did not induce GILZ mRNA also in airway smooth muscle cell, confirming that CpdA had no transactivation potential by its failure to induce GILZ mRNA

[333]. Of interest, this report failed to see any preventive effect of mifepristone on CpdA response or any GRα translocation when CpdA was used [333]. They suggested that GRα could not play a role in response of CpdA stimulation. This observation contrasts with many other previous studies, but illustrates the high heterogeneity between different cell types.

GCs are necessary for osteogenic differentiation and enhancement of matrix mineralization, while CpdA failed to induce osteogenic differentiation of MSCs [334]. We have previously demonstrated that deprivation in the osteogenic medium (glycerophosphate, ascorbic acid, dexamethasone) of the GC component blocked the osteogenic differentiation of bone-marrow MSCs and of synovial fibroblasts [171], accompanied by a dramatic decrease in leptin secretion by these cells. Because leptin favors osteoblastic differentiation of MSCs [335] and CpdA did not induce leptin, our findings might explain why CpdA failed to induce osteogenic differentiation.

GCs exert their anti-inflammatory and immunosuppressive effects in human primarily via the cytosolic GR [242, 243] involved in the genomic regulation, through direct interactions with regulatory regions on DNA, the GRE elements. Direct binding of GR monomers to NF-κB or activator protein 2 prevents them from interacting with their binding site on DNA (transrepression) and induces the anti-inflammatory mechanisms. However, homodimers of the GR can also bind to GRE and stimulate gene activation (transactivation), which contributes mostly to the adverse effects of GCs. This balance between transactivation and transrepression is commonly accepted, although limitations have been discussed in the introduction. CpdA exhibits a dissociative action by stimulating GR transrepression activity (anti-inflammatory properties) without modulating any transactivation pathway connected to adverse events [291, 293, 336]. In this work, we have observed that CpdA maintained its anti-inflammatory properties but did not induce leptin secretion or Ob-R expression, unlike prednisolone. We

can therefore propose that leptin and Ob-R expression levels are governed by the transactivation pathway.

GCs can induce the expression of GILZ and dual specificity phosphatase 1 (DUSP1) proteins that share similar anti-inflammatory properties by inactivating MAP-kinases, overexpressing COX-2 or inhibiting NF-κB [336-338]. These inductions are two examples demonstrating that GC-induced transactivation can also lead to an anti-inflammatory effect and that the gate between transactivation and transrepression are not always closed. However, several discrepancies have been reported:

- first, involvement of GILZ as an anti-inflammatory mediator of GC is not entirely demonstrated. If the anti-inflammatory action of GILZ is well proved in immune cells, the situation is more complicated in mesenchymal cells. We have already detailed limitations found in the literature through the work of Ngo *et al* and of Cheng *et al* [312, 327]. Deletion of GILZ in mice arthritis did not confirm the central role of GILZ as an anti-inflammatory mediator of GC [312]. Our work confirmed this observation in OA synovial fibroblasts, with no significant effect of GILZ deletion in the TNF-α-induced pro-inflammatory cytokines secretion and in the capacity of prednisone to reduce these inductions;
- second, we have shown that GILZ was involved in prednisolone-induced leptin and Ob-R. According to the deleterious role of leptin in OA, GILZ expression can be considered as a metabolic adverse event of GCs (in addition to other adverse events such as myopathy or delayed epithelial repair [322, 323]). In OA synovial fibroblasts, GILZ-dependent leptin and Ob-R expressions could be therefore related to the transactivation pathway, especially if we consider that GILZ deletion does not influence the inflammatory response;
- finally, CpdA did not induce GILZ expression. We already outlined that

other authors noticed the absence of GILZ induction by CpdA [333, 339]. Another study supported this hypothesis with no induction of DUSP1 by the SEGRA CpdA [340]. However, other SEGRAs have shown different results in the literature: RU24858 and ORG 214007-0 can induce GILZ expression [341, 342]. There are probably many other differences in the mechanisms of action of SEGRAs, which could be tissue-dependent (immune *versus* mesenchymal cell). Of interest, many SEGRAs did not fully abolish the transactivation pathway, while CpdA has the most strongly dissociated profile [285, 293]. It could therefore explain why CpdA failed to induce leptin secretion and GILZ expression, but also DUSP1 expression.

5.4. Production of leptin by chondrocytes

Although the synovial fibroblast raises growing interest in the pathophysiology of OA, main events driving the process concern the chondrocytes. The chondrocytes have functional leptin receptors [343], but healthy cartilage does not express leptin while OA cartilage does [175]. Interestingly, leptin deficient or leptin receptor deficient mice do not develop knee OA despite extreme adiposity [194]. Moreover, obesity affects the cultured chondrocytes responsiveness to leptin, with a BMI-dependent effect of leptin for the expression of MMP-13 [195]. Recently, Vuolteenaho *et al.* noted that intra-articular leptin levels are increased, while its negative regulators SOCS-3 (in cartilage) and sOb-R (in the synovial fluid) are decreased in obese patients with OA [185].

We first demonstrated that freshly isolated chondrocytes did not produce leptin nor express Ob-R, even under prednisolone stimulation. Starting the culture, chondrocytes are non-adherent cells with a spherical shape and they spontaneously acquired morphological and biochemical alterations due to

dedifferentiation process. In monolayer culture, 12-14 days of culture later, the "dedifferentiated chondrocytes" acquired a fibroblast-like shape and the capacity to spontaneously produce leptin and express Ob-R, strongly stimulated in the presence of prednisolone. *In vitro* dedifferentiation was characterized for many years and was acquired by chondrocytes by cultivating them in monolayer, while 3-D culture keep them in a more differentiated pattern [344]. Dedifferentiation phenotype is classically characterized by the production of type I and III collagen, type III collagen expression being observed in the upper middle zone of OA cartilage [345]. Our laboratory characterized the *in vitro* dedifferentiated chondrocytes [346]: there was decreased expression of Sox 9 and collagen II (the major component of the healthy cartilage), while collagen I expression was higher. Moreover, dedifferentiated chondrocytes shared biochemical similarities with hypertrophic chondrocytes that are highly present in OA, especially in the early stage, with a higher expression of Runx-2. Dedifferentiated and hypertrophic chondrocytes are however distinct, because of different microscopic shape and the absence of collagen X in dedifferentiated chondrocytes [346].

Spontaneous production of leptin and Ob-R is the best at day 12-14, although prednisolone-induced leptin and Ob-R already slightly appeared at day 4. In dedifferentiated chondrocytes, leptin expression after prednisolone stimulation was dose-dependent and was confirmed at the protein and mRNA level. We also found that aldosterone was an inducer of leptin and Ob-R in these cells and that both inductions were GR-dependent for the following reasons: a) a strong decrease of the effect observed by the use of the GR inhibitor mifepristone; b) an absence of modulation by the use of MR inhibitors eplerenone/spironolactone. However, no GR silencing was performed with the dedifferentiated chondrocytes. Preliminary experiments recently demonstrated that GILZ silencing in dedifferentiated chondrocytes also inhibited leptin and Ob-R expression under prednisolone stimulation. We have also exposed

dedifferentiated chondrocytes to CpdA. Unlike prednisolone, CpdA was also unable to induce leptin secretion in human OA dedifferentiated chondrocytes. CpdA was however able to significantly reduce TNF-α-induced production of IL-6, IL-8, MMP-1 and MMP-3. Regarding to the spontaneous leptin production, to the prednisolone and aldosterone stimulation of leptin production, to GILZ implication and to CpdA effects, there were strong similarities between human OA synovial fibroblasts and human OA dedifferentiated chondrocytes.

As previously described, TGF-β1 signaling depends on the ALK5-Smad2/3 pathway (anabolic pathway) and / or the ALK1-Smad1/5 pathway (catabolic pathway). Davidson *et al.* showed an age-related shift in ALK1/ALK5 ratio in murine cartilage and a strong correlation between ALK1 and MMP-13 expression in human cartilage [235]. They suggested that dominant ALK1 signaling resulted in deviant chondrocyte behavior, contributing to age-related cartilage destruction and OA. Furthermore, Smad3-deficient mice have enhanced Smad-1/5/8 signaling and develop OA [347]. Our laboratory previously demonstrated that TGF-β pathway was involved in prednisolone-induced leptin secretion through ALK1-Smad1/5 pathway (involved in OA) and that TGF-β1 reduced the leptin secretion through Smad2/3 [171]. In the present work, we confirm this observation: CpdA does not induce leptin and does not modulate the Smad balance, while prednisolone does. Of interest, another work of our laboratory showed that dedifferentiated chondrocytes are associated to a predominant ALK1-Smad1/5 expression, while freshly isolated chondrocytes have higher expression of ALK5-Smad2/3 [346]. We showed that 12-14 days of dedifferentiation enhanced a shift in ALK1/ALK5 ratio by decreasing the expression of ALK5 and phospho-Smad2, accompanied by a gain of ALK1 and phospho-Smad1/5 expression. It therefore suggests that dedifferentiated chondrocytes rather signal *via* ALK1 « catabolic » pathway. Furthermore, Smad1 specific silencing experiments affected the capacity of dedifferentiated chondrocytes to produce leptin, spontaneously, and under prednisolone. This

observation is important because: a) the shift between the ALK5 to the ALK1 pathway during chondrocyte dedifferentiation is coherent with our results: there is no leptin produced by freshly isolated chondrocytes, while dedifferentiated chondrocytes (with an ALK1 dominance) produce leptin and expressed Ob-R; b) the shift in TGF-β pathway to ALK1-Smad1/5 observed in dedifferentiated chondrocytes is another similarity between the dedifferentiated chondrocyte and the hypertrophic one (higher ALK5/ALK1 ratio was associated to the hypertrophic phenotype). Taken together, *in vitro* dedifferentiation seems to be a good model to approach hypertrophic-like or OA-like phenotypes in a short time of culture.

5.5. Leptin is not the only pro-inflammatory cytokine induced by glucocorticoids

Leptin is not the only pro-inflammatory protein induced by GCs. GCs can also induce A-SAA, as observed in our laboratory [130]. A-SAA is an apolipoprotein playing a role in lipid metabolism. A-SAA is also an acute phase protein produced by the liver after stimulation with pro-inflammatory cytokines, which induces expression of pro-inflammatory cytokines and MMPs [348]. A-SAA was also detected in the synovium of RA patients and in RA human synovial fibroblasts [349]. Of interest, modulations were similar to those observed with leptin: prednisolone induced A-SAA expression in OA synovial fibroblasts and in dedifferentiated chondrocytes through the GR, while SEGRA CpdA did not [130]. As for leptin, prednisolone-induced A-SAA secretion was dependent of Smad1/5 pathway. Prednisolone-induced A-SAA secretion was also noticed in other cells, as macrophages, epithelial cells, smooth muscle cells or adipose tissue explants [350-352]. Interestingly, in hepatic cells, GCs alone were not able to induce A-SAA but needed a preliminary cytokine-driven induction [350, 351].

There are also other examples of GCs activation under inflammatory stimulation. We have previously discussed in the introduction the importance of the 11β-HSD1 enzyme to activate GCs in various tissues. Surprisingly, TNF-α and IL-1β increased the expression and the activity of 11β-HSD1 *in vitro*, leading to an enhanced local GC activity under pro-inflammatory activation [353]. If this activation probably corresponds to a "negative feedback" to reduce inflammation, it leads to deleterious effect in term of bone metabolism: the Wnt antagonist dickkopf-1 (DKK1) is secreted by synovial fibroblasts and is an actor of the bone remodeling in inflammatory arthritis, leading to the juxta-articular osteoporosis in RA [354]. DKK1 is up-regulated by GC, but also by TNF-α. Of interest, TNF-α did not directly up-regulated DKK1 expression, but indirectly up-regulate it through an increased expression of 11β-HSD1 and therefore an increased local GC metabolism [354].

5.6. Production of leptin and other adipokines by human osteoarthritic synovial fibroblasts

Adipose tissue is an endocrine organ that produces quantities of different adipokines, chemokines and pro-inflammatory cytokines. Next to leptin, other adipokines (resistin, adiponectin …) have been involved in OA process. For leptin, there are strong *in vitro* evidences of a pro-inflammatory and catabolic effect on the cartilage. Clinical studies are also in favor of leptin involvement in OA process. Moreover, leptin is the only adipokine for which there are similar or higher expression levels in the synovial fluid than in the serum of OA patients, suggesting an active intra-articular secretion and not only a passive diffusion [161, 162]. For the other adipokines, as discussed in the introduction, the literature presents various controversies. In this study, fresh synovial and cartilage explants released significant amount of resistin and adiponectin, but

theses samples were contaminated with synovial fluid, blood, intra-articular fat … and the release of adipokines can come from any component. When synoviocytes and chondrocytes are isolated and cultured *in vitro*, we could not observe any significant endogenous production of adiponectin or resistin, unlike leptin. Studying leptin was therefore relevant to analyze GC's action on articular cells. Koskinen *et al.* also reported significant release of resistin by cartilage samples after several washouts, with a correlation between the *in vitro* released and the synovial fluid concentration [208], but they did not perform cell culture.

Synovial fibroblasts are multipotent cells that have the ability to differentiate into adipocytes, especially when stimulated by PPAR-γ agonists [113]. A previous study from our laboratory realized red oil coloration in our culture lineage to ensure the absence of any adipogenic differentiation of synovial fibroblasts, spontaneously or under prednisolone stimulation [1]. Fat droplets were only visualized when cells were stimulated with PPAR-γ agonist and not with GCs. This rules out any contamination of our cultures by native adipocytes or by adipogenic transformation of the synoviocytes in the results observed. Of interest, stimulation with PPAR-γ agonists and presence of fat droplets were associated with higher adiponectin but less leptin secretion. Moreover, we can observe spontaneous leptin secretion in DMEM medium supplemented with charcoal-stripped FBS with no mixture of adipocyte-inducing substances. GC induction of leptin observed in our work is therefore independent of adipogenesis.

In animal studies, injections of lipopolysaccharide, TNF-α or IL-1β lead to higher blood leptin levels [355, 356]. However, in this *in vitro* study, TNF-α does not induce leptin secretion in OA synovial fibroblasts and even decreases it. In human studies, leptin levels are unchanged in RA patients when treated with anti-TNF-α infliximab or adalimumab [357, 358]. Moreover, systemic

leptin levels are usually not correlated with C-reactive protein or IL-6 [359, 360]. TNF-α does not also significantly modulate the prednisolone-induced leptin secretion. The absence of overall significant modulation does not reflect necessarily the reality because individual analyses of our results have shown that there was a significant higher leptin secretion for some patients when TNF-α was added to prednisolone stimulation. These individual observations are complementary to those previously described, with induction of hepatic SAA production by prednisolone with preliminary pro-inflammatory stimulus [350, 351] and with the induction of 11β-HSD1 by TNF-α and IL-1β [353].

5.7. Crosstalk between glucocorticoids and transforming growth factor-β signaling: a possible role for glucocorticoid-induced leucine zipper?

In this work, we demonstrate that GCs induce leptin through GILZ. Previously, our laboratory published that TGF-β pathway was involved in GC-induced leptin secretion through the ALK1-Smad1/5, while TGF-β1 it-self reduced the GC-induced leptin secretion but through ALK5-Smad2/3 [171]. Here, we add the information that the SEGRA CpdA does not induce leptin, does not induce the ALK1-Smad1/5 and does not modulate the Smad pathway. Of interest, CpdA does not either induce GILZ. Moreover, TGF-β1 reduces the GC-induced leptin but also the GC-induced GILZ expression. Links between GILZ and TGF-β pathway can be hypothesized.

The TGF-β and GC signaling pathway interact both positively and negatively in regulating a variety of physiologic and pathologic processes: GCs inhibit the TGF-β-induced expression of ECM proteins, such as fibronectin and collagen, and protease inhibitors, such as tissue inhibitor of metalloproteinases; GCs and TGF-β are both regulators of wound healing and fibrosis and they antagonistically regulate bone formation. It was found by Song *et al* that GR

inhibited TGF-β signaling by direct targeting the transcriptional activation function of Smad3, mediated by protein-protein interactions [361]. In 2006, Li *et al* observed that, in the absence of ligated GR, TGF-β-induced transactivation via Smad3 was markedly enhanced whereas, in the presence of ligated GR, repression of the TGF-β transactivation was enhanced [362]. It was thus the presence of ligated GR that determined whether transactivation or transrepression of TGF-β would occur.

In a mice model of intestinal inflammation, dexamethasone cooperates with TGF-β in FoxP3 induction and T-regulator lymphocytes differentiation in a GILZ-dependent manner [363]. More precisely, GILZ enhanced TGF-β signaling by binding to and promoting Smad2 phosphorylation leading to FoxP3 induction, ALK5 being required [363]. This is a new role for GILZ as a cross-talk between TGF-β signaling and stress hormones.

In our work, we also suggest that GILZ could play a cross-talk between GR and the TGF-β signaling in the prednisolone-induced leptin and Ob-R in human OA synovial fibroblasts and dedifferentiated chondrocytes. Experimental conditions that stimulate GILZ expression (prednisolone, aldosterone) increase ALK1-Smad1/5 and increase leptin secretion and Ob-R expression. Conversely, experimental conditions that decrease GILZ expression (TGF-β exposure in the presence of prednisolone) decrease also leptin secretion and Ob-R expression. Compound A, that does not affect GILZ, does not interfere with ALK1-Smad1/5 and does not modulate leptin secretion and Ob-R expression. Taken together, it appears that ALK1-Smad1/5 and ALK5-Smad2/3, respectively, activates and represses leptin secretion and Ob-B expression. TGF-β represses on leptin secretion and Ob-R expression. We can therefore propose that in the presence of ligated GR, TGF-β modulate the GC transactivation pathway via Smad1/5. That

situation is different to what was found previously described in literature with the plasminogen activator inhibitor as a target [362].

Keenan *et al* have studied the transactivation pathway in human bronchial epithelial cells and described another potential link between TGF-β and GILZ, very close to our findings [364]. They found that TGF-β deeply impaired the GC transactivation in these cells, with no induction of the transactivation effectors (ENaCα, IκBα, MKP-1 and GILZ), and with a down-regulation of the prednisolone-induced expression of these effectors [364]. Moreover, ALK5 inhibitor prevented the GC transactivation, hypothesizing that the ALK5-Smad2/3 pathway inhibited the transactivation (however through a Smad4-independent manner). This is coherent with our results, with no leptin and GILZ induction with TGF-β, and our previous result, with prednisolone-induced leptin inhibition by through ALK5-Smad2/3 [171]. It illustrates the complexity of the cross-talk between GR and TGF-β pathways. Other works will be however necessary to demonstrate the direct interaction between GILZ and Smad1/5 in these cells.

A manichean vision of TGF-β involvement in healthy cartilage and in OA cartilage appeared some years ago either in humans and in animals models. This vision was reminded in the introduction and also submitted to discussion according to our results. Simplistically, the good face of TGF-β is related to the use of the anabolic pathway ALK5-Smad2/3 by healthy young cartilage and correlates with the production of aggrecan, collagen type II and the use of the nuclear factor Sox-9. Conversely, the bad face of TGF-β is related to the use of the catabolic pathway ALK1-Smad1/5 by diseased and aged cartilage correlating with the production of MMP-13, collagen type X and using the nuclear factor Runx-2. Leptin production by synovial fibroblasts and by dedifferentiated chondrocytes can be added to this last pathway. However, this dual classification has some limitations. In a mouse model, it was found that after the anterior cruciate ligament was transected, MSC expressing

phosphorylated Smad2/3 were recruited to subchondral bone marrow leading to aberrant bone remodeling and OA progression [106]. After administration of a TGF-β receptor I-ALK5 inhibitor, after TGF-β receptor type II silencing or antibody-mediated neutralization of TGF-β in subchondral bone, OA severity was reduced [106]. In the synovium, the dual vision of a "good" TGF-β pathway was also controversial, by the gene analysis of human OA synovium that revealed the increase of TGF-β-response genes leading to synovial fibrosis, also under the activation of the Smad2/3 pathway [365, 366]. TGF-β pathway is differently involved in OA pathogenesis and is tissue-dependent. A therapeutic use of TGF-β in OA is therefore up to now difficult to elaborate in humans. Blocking the ALK5-Smad2/3 pathway could be interesting in the bone and in the synovium, but could be deleterious in the cartilage, for which the ALK5-Smad2/3 pathway is further associated to a normal phenotype. Injection of TGF-β in the joint of mice was already tried but led to osteophyte formation and synovial fibrosis [108, 367].

5.8. Position of glucocorticoid-induced leucine zipper in the osteoarthritic process

A few years ago, it was believed that GILZ was the factor responsible for the anti-inflammatory properties of GCs. Many publications later, it appeared that the situation is more complicated than expected. Transcending the classical paradigm of GCs (anti-inflammatory properties linked to the transrepression and adverse metabolic effects linked to the transactivation), GILZ itself is an exception because it depends on the transactivation. Moreover, in the paper of Ngo, Cheng and even Beaulieu, it became clear that the anti-inflammatory properties of GILZ are different according to the targeted cell types [311, 312, 327]. We demonstrated that, in human OA synovial fibroblasts and human OA dedifferentiated chondrocytes, GILZ could not reduce the spontaneous or TNF-

α-induced production of several cytokines and pro-inflammatory mediators nor was required for GCs to exert their therapeutic effects as shown in synovial fibroblasts coming from murine models of arthritis and in HUVECs [312, 327]. Cheng *et al.* concluded that "*rather than being essential to the action of GCs in arthritis, the expression of GILZ might represent a back-up pathway, wherein the effect of GILZ on transcription factors such as NF-kB and AP-1 operated in parallel with direct GR transrepression of these pathway. Given the lethal effects of inflammation in the absence of endogenous glucocorticoids, the existence of such redundancy in GC effects could confer a survival advantage*" [327]. In a similar reasoning, Ratman *et al.* also stated that "*a number of genes that are upregulated via the transactivation mechanism exhibit clear anti-inflammatory actions, for example GILZ and DUSP1, apparently, as an additional safety-switch to restore homeostasis*" [244]. Nevertheless, the inhibitory effect observed in response to local GILZ overexpression in collagen-induced arthritis suggests that further exploration of potential GC-mimicking therapeutic effects of GILZ is warranted. If GILZ is not necessary for the anti-inflammatory effect of GC in several cells, we propose a new role for GILZ in OA synovial fibroblast and dedifferentiated chondrocytes: GILZ is involved in the GC and mineralocorticoid-induced leptin secretion and Ob-R expression. As previously described, leptin induction is deleterious in the OA process and we can propose GILZ as a mediator of a new GC metabolic adverse events. In OA synovial fibroblast, GILZ (effector of the transactivation) is not necessary for the anti-inflammatory effect of GC and is involved in the corticoid-induced leptin secretion. A metabolic induction mediated by the transactivation: perhaps that the dogma is not ready to die.

6. CONCLUSIONS

Simplistic affirmations are probably never true and GILZ story is a good illustration. GILZ can mediate the anti-inflammatory properties of GCs but we know now with the study of GILZ that the anti-inflammatory properties of GCs may also occur through the GC/GR transactivation. Recent works have also highlighted the role of GILZ in the recovery of T-regulator lymphocyte either *in vitro* [363] or *in vivo* in the mouse model of collagen-induced arthritis driving the therapeutic potential of MSCs [368]. GILZ was also involved in the GC sensitivity through the modulation of the macrophage migration inhibitory factor [369]. GILZ overexpression or exogenous GILZ administration might therefore improve inflammation outcome and GC sensitivity. However, the absence of GILZ in mesenchymal cells does not lead to the variation of the inflammatory status and of the anti-inflammatory effect of GCs, suggesting major differences in different tissues but also between the endogenous or exogenous role of GILZ. In this work, down-regulation of endogenous GILZ does not modify the endogenous and TNF-α-induced secretion of IL-6, IL-8 and MMP-1 and neither the GC-induced down-regulation of these pro-inflammatory molecules in OA synovial fibroblasts.

We previously described in other sections the paradoxical expression of leptin and Ob-R (suggesting a higher leptin responsiveness) by GCs in synovial fibroblasts. Leptin is not the only pro-inflammatory molecule induced by GC in these cells, as our laboratory described a similar modulation with A-SAA [130]. Without endogenous GILZ, GCs lose their capacity to induce leptin secretion and Ob-R expression. Regarding to the deleterious involvement of leptin and its receptor in OA, leptin expression could be considered as a metabolic adverse event in rheumatology, with GILZ as an effector. This is a new role for GILZ in GC adverse events, as previously observed with the inhibition of the epithelial repair [322] and the GC-induced myopathy [323]. The SEGRA CpdA, a fully dissociated GR agonist, does not lead to leptin, OB-R or GILZ induction, positioning leptin induction in the transactivation pathway. If synovial

fibroblasts are the main cell type studies, similar results were obtained with human dedifferentiated chondrocytes. In addition to GCs, mineralocorticoids are also able to induce the leptin/Ob-R/GILZ pathway: these experiments provide new data on the GILZ and leptin modulation in OA synovial fibroblasts. Even if the *in vitro* dose-response experiments show no strong evidence in favor of a significant role in human, several drugs commonly used modulate the aldosterone pathway in human and could therefore have an influence on the leptin/Ob-R/GILZ pathway.

Further studies are necessary to clarify the physiological and the potential therapeutics role of the endogenous *versus* the exogenous GILZ. *In vivo* studies are planned to assess the clinical relevance of these findings and confirm *in vivo* the local, and even systemic, leptin secretion. More than 70 years after the first use of cortisone (called Compound E) in human to treat a refractory RA, and more than ever, the studies about GR agonists that differently modulate the down-stream pathway of classical GCs remain up to date and of the greatest therapeutic interest.

7. LIST OF ABBREVIATIONS

11β-HSD	11β-hydroxysteroid deshydrogenase
A-SAA	serum amyloid A
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
AGE	advanced glycation end product
ALK	activin receptor like kinase
BMI	body mass index
COX	cyclooxygenase
CpdA	compound A
DAMP	damage-associated molecular pattern
DMEM	Dulbecco's modified Eagle medium
ECM	extra-cellular matrix
ELISA	enzyme-linked immunosorbent assay
FBS	foetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GILZ	glucocorticoid-induced leucine zipper
GR	glucocorticoid receptor
GRE	glucocorticoid responsive element
HUVEC	human umbilical venous endothelial cell
IL	interleukin
MMP	matrix metalloproteinase
MOI	multiplicity of infection
MR	mineralocorticoid receptor
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
NF-κb	nuclear factor-kappa B
NSAID	non-steroidal anti-inflammatory drug
NO	nitric oxide
OA	osteoarthritis
Ob-R	leptin receptor
PAMP	pathogen-associated molecular pattern

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPAR	peroxisome proliferator-activated receptor
RA	rheumatoid arthritis
RT-qPCR	quantitative reverse transcription PCR
SD	standard deviation
SEGRA	selective glucocorticoid receptor agonist
sOb-R	soluble leptin receptor
TBS-T	tris-buffered saline with tween
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor

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9. APPENDICES