Immunometabolism in osteoarthritis

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Chapter 1

Introduction immunometabolism in osteoarthritis

Osteoarthritis

Osteoarthritis (OA) is a heterogeneous joint disorder affecting mostly the hip, knee and hand joints. Clinical characteristics are pain, stiffness and disability, accompanied by cartilage loss and structural abnormalities of the joint such as osteophytes, joint space narrowing and bone sclerosis. Furthermore, soft tissue abnormalities, such as synovitis, and subchondral bone lesions can be present [1]. According to different sets of criteria, developed by the American College of Rheumatology (ACR), OA can be classified based on clinical and laboratory, clinical and radiographic or clinical criteria alone [2]. OA was long thought to be solely a chronic degenerative disease driven by cartilage loss, however, OA is a much more complex disease whereby inflammatory processes and all joint compartments, cartilage, bone and synovium, are involved [3, 4].

Pathophysiology of osteoarthritis

Articular cartilage consists of chondrocytes producing the extracellular matrix (ECM), containing collagen and proteoglycans. Normally chondrocytes maintain cartilage through normal anabolic (matrix-producing) and catabolic (matrix-degrading) activities, however, in OA this balance is disturbed. Chondrocytes proliferate and form clusters, leading to extensive matrix degradation and loss due to the production of proteases, such as matrix metalloproteinases and members of a disintegrin and metalloproteinase (ADAM) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families [5, 6]. Lying immediately beneath the cartilage is the subchondral bone, which provides mechanical support for the articular cartilage. However, during the course of OA, the subchondral bone undergoes changes, such as bone remodelling, tissue sclerosis, and the formation of osteophytes at the joint margins [3] and is a source of inflammatory mediators which can affect the cartilage and synovium [7].

In addition, the synovial tissue consists of a small layer of synoviocytes producing components of synovial fluid, such as hyaluronic acid and lubricin, essential for frictionless movement. In the OA joint, the production of these components are altered, which leads to adverse effects on the cartilage integrity [8]. Furthermore, synovial tissue inflammation is present in OA patients [9], characterized by synovial hyperplasia and influx of inflammatory cells. Although it is not completely known,

it is likely that cartilage degradation products induces synovial inflammation in OA, activating synoviocytes to produce pro-inflammatory mediators, such as TNFα and IL-1β, leading to immune cell attraction, such as macrophages and T cells. This will, conceivably, lead to a further phenotype switch of chondrocytes, and eventually a vicious circle of continued joint destruction [10]. As synovitis is associated with symptom severity and cartilage degradation [10], it is suggested that synovitis plays an important role in the pathophysiology of OA.

Obesity and osteoarthritis

Major risk factors for OA are gender and age [1, 11], however, the development and progression of OA are also associated with obesity [1, 11, 12]. The association of OA with obesity was thought to be solely due to mechanical stress caused by the increased or altered mechanical load on the joint. However, obesity is also a risk factor for non-weight-bearing joints such as the hand [12-14], indicating that mechanical factors alone cannot fully explain the association between obesity and OA. It is suggested that in addition to mechanical factors, systemic factors, such as low-grade inflammation, disturbed lipid metabolism and adipokines, contribute to the association between obesity and OA [11]. Both mechanical and systemic factors play a role in the association between OA and obesity, however, the relative contribution differs between weight-bearing and non-weight-bearing joints. In knee OA, mechanical stress is the most important underlying mechanism, whereas in hand OA systemic processes contribute the most [14].

Immunometabolism

Immunometabolism is an emerging field, focussing on the interplay between immunological and metabolic processes, both at a systemic and a cellular level. Immunometabolism on systemic level explores the link between immune cells and their effect on metabolic tissues, such as adipose tissue, which can affect whole-body metabolism. Immunometabolism on cellular level explores the intracellular metabolic pathways in immune cells that alter their function.

Systemic immunometabolism

Adipose tissue

Adipose tissue, or fat, is long thought to solely serve as energy depot, releasing fatty acids in times of energy demand and storing triglycerides (TGs) in periods of energy excess. However, it is also a highly active metabolic and endocrine organ as it can secrete various adipokines and cytokines, together called adipocytokines, which can affect whole-body metabolism [15-17]. Leptin, which was the first adipokine to be discovered is important as metabolic signal for the energy balance by inhibiting hunger [18]. Other adipokines such as adiponectin, resistin, and visfatin are involved in lipid and glucose homeostasis [18]. Cytokines secreted by the adipose tissue are mainly IL-6 and TNFα [18]. Adipokines mediate the crosstalk between adipose tissue and other metabolic organs and thereby affecting whole-body metabolism, however, they can also have an effect on the immune system. Adiponectin, depending on its molecular form can have a pro- or anti-inflammatory effect, while leptin and resistin are both thought to be pro-inflammatory [18-21].

Besides adipocytes, the adipose tissue contains a stromal vascular fraction, which consists of fibroblasts, progenitor cells, nerve cells, endothelial cells and immune cells. Among the immune cells present in adipose tissue, macrophages and T cells are most abundant, however, mast cells, natural killer (NK) cells and B cells can be found as well [22, 23].

Adipose tissue as inflammatory site

Obesity is associated with changes in the adipose tissue, which not only affects the adipocytes, but, also the immune cells present and their secretion profile (see figure 1). Adipocytes enlarge, which results in expansion of the adipose tissue, and results in hypoxia which causes adipocyte cell death [24-26]. This is accompanied by macrophage infiltration and formation of crown-like structures (CLS) by macrophages around dead or necrotic adipocytes [22, 27, 28]. This changes the polarization stage of the macrophages from an anti-inflammatory M1 macrophage to a pro-inflammatory M2 macrophage [29-31], contributing to the pro-inflammatory state of the adipose tissue. Together with macrophages, T cells are among the first cells infiltrating the adipose tissue [32-35]. T cells infiltrating the adipose tissue are mainly pro-inflammatory T helper 1 (Th1) cells and $CD8^+$ T cells, where they contribute to the pro-inflammatory state of the adipose tissue and overcome the anti-inflammatory effects of Th2 and T regulatory (Treg) cells [33, 36-38].

As macrophages and T cells are the most abundant cell types present in the adipose tissue most studies have focussed on these cells, however, other immune cells have also been implicated to be involved in adipose tissue inflammation. It has been shown that obesity leads to accumulation of pro-inflammatory cells such as neutrophils, mast cells and B cells, especially IgG-producing mature B cells, while a decrease in anti-inflammatory cells, such as eosinophils and innate lymphoid cells (ILCs) have been found [39], contributing to the pro-inflammatory state of the adipose tissue.

As the adipose tissue acquires a pro-inflammatory state, the secretion profile of the adipose tissue changes. While the lean state secretes adiponectin, in the obese state these levels decreases, while the levels of leptin, resistin, TNFα and IL-6 increases [18]. Furthermore, basal lipolysis, the breakdown of triglycerides and the release of lipids, in adipocytes is enhanced in the obese state [40]. All these factors contribute to a more pro-inflammatory status of the adipose tissue, which can affect whole-body metabolism, ultimately leading to insulin resistance.

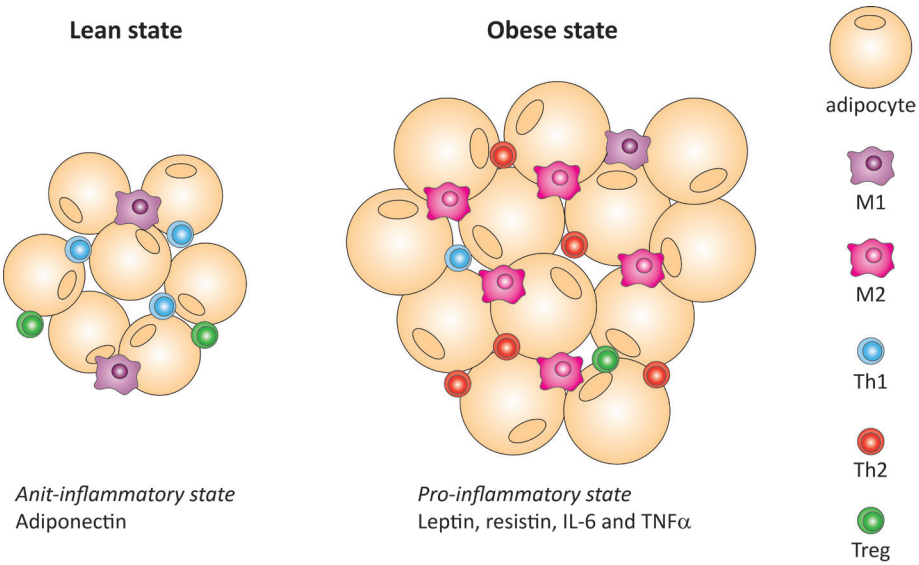

Figure 1. Schematic overview of adipose tissue in the lean and obese state.

Infrapatellar fat pad

The infrapatellar fat pad (IFP), also known as Hoffa's fat pad, is an adipose tissue organ located in the knee. It is intracapsularly and extrasynovially located and in close vicinity

to the synovium, cartilage and bone. Although the exact physiological function is unknown, it is thought that the IFP could involve shock absorption, promoting the free circulation of synovial fluid and protection of adjacent tissues [41, 42]. Furthermore, due to its location, it is considered to play a role in the pathophysiology of OA, through the secretion of soluble factors and the interaction with other joint tissues such as synovium, cartilage and possibly bone [41] (see figure 2).

Several studies have investigated whether the IFP would have a beneficial or detrimental role in knee OA. These studies suggest that the volume or maximal area of the IFP has a beneficial association with pain and structural abnormalities, such as joint space narrowing, osteophytes, cartilage volume and defects and bone marrow lesions [43-45], suggesting a protective role for size of the IFP in osteoarthritis. However, IFP signal intensity alterations on MRI, which may represent pathological changes such as inflammation and oedema in the IFP are associated with the development and progression of OA [46, 47].

Like other adipose tissues, the IFP of OA patients contains both adipocytes and stromal vascular fraction. The immune cells present in the stromal vascular fraction are mainly macrophages and T cells, although mast cells and B cells can be found as well [48]. Furthermore, the IFP is capable of secreting different adipocytokines, among which adiponectin, adipsin, leptin, resistin, visfatin and IL-6 [48-50]. Although the immune cell composition of the IFP is similar to synovium [51], it differs substantially from subcutaneous adipose tissue. The IFP contains more cellular infiltrate, the immune cell composition differs and it secretes higher levels of adipocytokines compared to subcutaneous adipose tissue [48-50, 52], suggesting that together with synovium, the IFP could play a role in the pathophysiological processes in the OA joint.

Whether or not the IFP is affected by obesity like other adipose tissues is largely unknown, although a few studies have investigated possible effects. Two magnetic resonance studies suggest that the size of the IFP is not influenced by obesity [43, 53], however, another study showed that the adipocytes from the IFP were larger in obese persons compared to lean persons and the IFP of obese persons showed more cellular infiltrate [54]. Furthermore, TNFα secretion by the IFP is higher in obese persons compared to lean subjects [48].

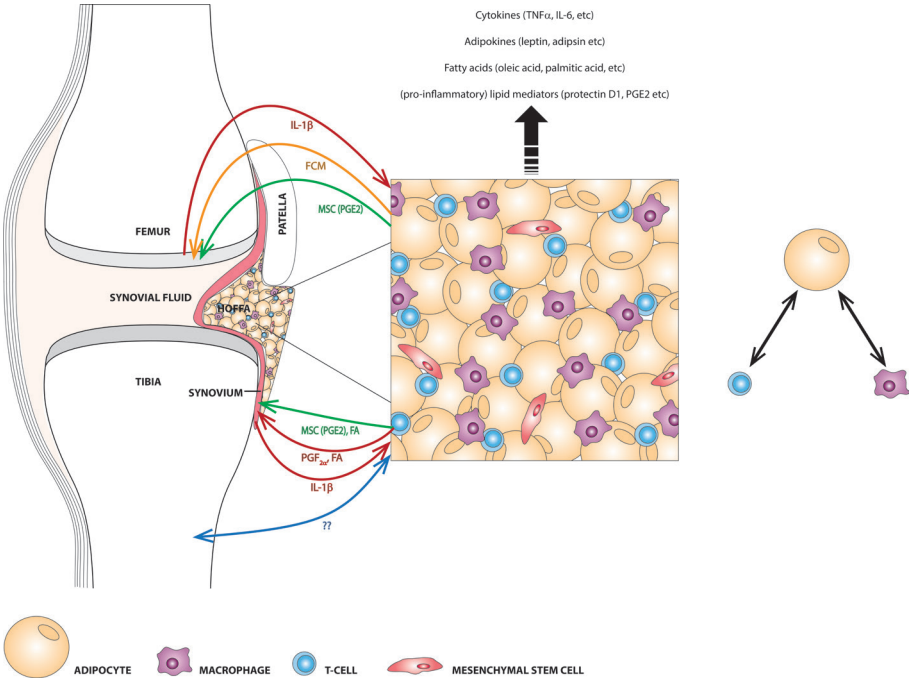

Figure 2. The infrapatellar fat pad and its interactions with other joint tissues.
Adapted from A. Ioan-Facsinay et al. Arthritis Res Ther 2013; 15 (6): 225

Cellular immunometabolism

Cellular metabolic pathways

In general, there are several cellular metabolic pathways active in the cell. These pathways can either be catabolic, obtaining energy and reducing power from nutrients or anabolic, producing new cell components through processes that require energy. Glycolysis converts glucose through several steps into pyruvate, which can then either be converted into lactate (generating 2 ATPs). Pyruvate can also enter the mitochondria where it is converted into acetyl-CoA and can enter the tricarboxylic acid (TCA) cycle (generating 36 ATPs). The TCA cycle generates NADH and FADH2, which will be oxidized by the electron transport chain (ETC) which will lead to ATP production by ATP synthase, also known as oxidative phosphorylation (OXPHOS). The TCA cycle can also be fuelled by glutamine metabolism or by acetyl-CoA derived from β-oxidation, the breakdown of fatty acids in the mitochondria. On the other hand, fatty acid synthesis converts citrate from the TCA cycle, which is transported into the cytosol, to fatty acids. Fatty acids can be converted to lipids such as triglycerides, phospholipids or cholesterol esters [55] (see figure 3). The

metabolic pathways utilized by immune cells and their function is intimately linked. Depending on activation status and function of the immune cell different metabolic configurations are used, however, the metabolic state can also undergo reprogramming and thereby change functional properties of the immune cell.

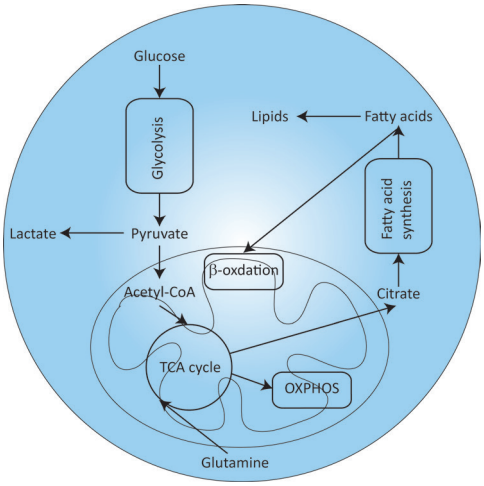

Figure 3. Major cellular metabolic pathways

T cell metabolism and function

T cell function and their metabolism are linked. Naïve or resting T cells have a different metabolic state when compared to activated T cells, and also T effector and Treg cells utilize different metabolic pathways.

Naïve or resting T cells have a relatively low metabolic rate aimed at energy production, rather than biosynthesis. They rely on the TCA cycle, linked to OXPHOS to generate ATP, fuelled by glucose, lipids or amino acids [56]. Activated T cells on the other hand have a high metabolic demand and are aimed at growth-promoting pathways and rely on glycolysis and OXPHOS [57, 58]. They have a high glycolytic rate, mediated by the upregulation of glucose transporter 1 (GLUT1) expression levels [56, 58-61]. Transgenic expression of GLUT1 or failure to elevate the expression of GLUT1 after antigenic stimulation affects the proliferation, survival and cytokine production, indicating that upregulated glucose metabolism is essential for T cell activation [56, 58-61]. Although fatty acids have been shown to influence proliferation of T cells, little is known about the dependence of T cell proliferation on fatty acid uptake. Recently, a study showed that full activation and

proliferation of T cells requires de novo fatty acid synthesis and fatty acid uptake [62]. In line with this, tissue resident memory $CD8^+$ T cells have been shown to take up fatty acids to fuel oxidative metabolism and failure to do so resulted in diminished persistence of these cells [63]. Furthermore, exogenous fatty acids fuelling β-oxidation during T cell activation promotes effector memory $CD4^+$ T cells [64].

Furthermore, T effector and Treg cells also differ in their usage of metabolic pathways. While T effector cells such as Th1, Th2 and Th17 express elevated levels of GLUT1 and rely on glycolysis, Treg cells do not have elevated expression levels of GLUT1 and rely on β-oxidation rather than glycolysis [56, 58, 65, 66]. Manipulation of either pathway has been shown to have an effect on both T effector cells and Tregs. Glycolysis will enhance T effector cells, but will inhibit Tregs, on the other hand lipid oxidation will promote the generation of Tregs, but suppress T effector cell function and survival [58, 65, 67].

T cells and obesity

In obese persons, the number, subsets and function of T cells seem to be altered although contradictory results are found. Total T cell numbers are elevated [68-70], however, diminished T cells numbers [71] have also been found in obese persons compared to lean persons. Decreased levels of $CD4^+$ T cells [71] and $CD8^+$ T cells [71, 72] have been found, however, also elevated levels of $CD4^+$ T cells [68-70] and normal levels for $CD8^+$ T cells [69, 70]. In addition, T cell function, such as proliferation and cytokine production is also altered in obese persons [68, 70, 73]. The mechanisms underlying these differences between obese and lean persons is unknown, however, free fatty acids levels in plasma are higher in obese persons compared to healthy persons [40, 74, 75], therefore, they could play an important role. There are several indications suggesting that fatty acids can modulate the immune response. Levels of several fatty acids are associated with levels of inflammatory markers in healthy individuals [76]. The type of fatty acids in the diet can influence the risk of development of inflammatory diseases [77-80] and modulate T cell function and phenotype *in vivo* [81-84]. Furthermore, *in vitro* studies suggest that high concentrations of fatty acids are toxic for the cell, while non-toxic concentrations are capable of inducing proliferation and cytokine production [85-90].

Aim of the thesis

Based on the two levels of immunometabolism, this thesis is divided into two parts. The first part focusses on the systemic level of immunometabolism, which explores the link between immune cells and their effect on metabolic tissues, such as adipose tissue. The IFP, an adipose tissue located in the knee, is thought to play a role in the pathophysiology of OA. However, inflammatory processes and obesity related features present in the IFP are unknown. The second part investigates the cellular level of immunometabolism, exploring the link between intracellular metabolic pathways of immune cells and their function. $CD4^+$ T cells and their function are intimately linked, and fatty acids have been described to affect these cells, however, how fatty acids can exert these effects is unknown. Therefore, the aims of the thesis are:

- To characterize the infrapatellar fat pad on the cellular and molecular level and determine its potential role in the pathophysiology of osteoarthritis
- To determine how fatty acids exert their effect on T cells

Outline of the thesis

Part 1 Systemic immunometabolism

OA and rheumatoid arthritis (RA) are both rheumatic diseases in which inflammation can be present, however, RA is in general associated with more inflammation in the synovium [91] and synovial fluid [92-94]. Whether the IFP participates in the inflammatory processes in the joint and thereby contributes to the disease pathogenesis is unknown. Therefore, in **chapter 2** we compared the IFP of OA and RA patients. IFP samples were obtained of OA patients and RA patients with secondary OA undergoing joint replacement surgery. The adipocytokine secretion profile of the adipose tissue (fat-conditioned medium) and adipocytes (adipocyte-conditioned medium) was determined by luminex. Furthermore, the immune cellular infiltrate was counted and the composition was investigated by flow cytometry.

Previously, $CD4^+$ T cells from the IFP of OA patients have been shown to secrete IL-6 directly *ex vivo* [51]. This has led us to the hypothesis that these cells recently have been activated which could suggest that these cells recognize adipose tissue antigens and could play a role in adipose tissue inflammation and thereby contribute to the pathogenesis of OA. Therefore, in **chapter 3,** we extensively characterized the IL-6^+ $CD4^+$ T cell population previously found in the IFP of OA patients. Using flow cytometry, we determined the expression levels of co-stimulatory molecules, activation markers and chemokine receptors. An in-house generated IL-6 capture complex was developed to perform TCRβ gene analysis to determine the clonality of these cells. The localization of the IL-6^+ $CD4^+$ T cells and the effect of adipocytes on $CD4^+$ T cells was determined.

Obesity is usually accompanied by adipose tissue inflammation, characterized by changes in adipocytes and inflammatory cells, however, the effect of obesity on the IFP is unclear. Therefore, in **chapter 4** we extensively investigated the cellular and molecular adipose tissue features typically associated with obesity and determined for all these features whether they associated with BMI, a measurement for obesity. First, the volume of IFP was determined with MRI and linear regression analysis were performed to determine whether the volume associated with BMI or other obesity-related features. Next, the IFP was obtained from OA patients undergoing joint replacement surgery and adipocyte volume and size was determined by light microscopy. The number of adipose tissue immune cells was determined by light microscopy and characterized by flow cytometry and luminex. As macrophages in the IFP expressed anti-inflammatory markers, while producing pro-inflammatory cytokines, we continued by characterizing these macrophages by flow cytometry.

Part 2 Cellular immunometabolism

In part two we focussed on cellular immunometabolism. As first step, we reviewed existing literature regarding the effect of fatty acids and lipid mediators, oxygenized fatty acids, on T cells and their function in **chapter 5**.

As metabolism and function of T cells is linked and fatty acids have been shown to enhance the proliferation of $CD4^+$ T cells, in **chapter 6**, we explored the possible mechanisms underlying this enhanced proliferation. Peripheral $CD4^+$ T cells were incubated with oleic acid for one day and proliferation was assessed after 4 days with incorporation of 3H-thymidine and cell trace violet staining. Functional

metabolic analysis was performed to determine the effect of OA treatment early on and after 24 hrs on the metabolism of $CD4^+$ T cells. Furthermore, by the usage of ^{13}C-OA metabolomics analysis was performed to determine whether oleic acid treatment influences any other metabolic pathway or whether it is used as substrate. In addition, ^{13}C-OA was used to determine the fate of oleic acid in the cell. To determine whether the formation of fatty acids is needed for enhanced proliferation with oleic acid, inhibition assays were performed with C75 and TOFA and proliferation was determined. Furthermore, the influence of oleic acid on signalling was assessed by calcium flux experiments and the degree of phosphorylation of ZAP70 with Western Blot.

Finally, in **chapter 7** and **8**, we provide a summary and discussion of our findings.

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