Impact of ionizing radiation on adipokine-induced inflammation in musculoskeletal diseases (MSD): Investigations in primary cells and MSD patients

Einfluss von ionisierender Strahlung auf die Adipokin-induzierte Entzündung bei muskuloskelettalen Erkrankungen (MSD): Untersuchungen in primären Zellen und in MSD Patienten

Vom Fachbereich Biologie der Technischen Universität Darmstadt zur Erlangung des akademischen Grades eines *Doctor rerum naturalium* genehmigte Dissertation von M.Sc. Kateryna Shreder aus Ulan-Ude

1. Referent: Prof. Marco Durante
2. Referent: Prof. Gerhard Thiel

Tag der Einreichung: 31.03.2017
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Für meine Eltern und Alex

Zusammenfassung

Rheumatoide Arthritis (RA) und Osteoarthritis (OA) sind die häufigsten muskuloskelettalen Erkrankungen (MSD). Beide Krankheitsbilder sind gekennzeichnet durch destruktive Veränderungen des Gelenkknorpels und des Knochens. Dies geht einher mit Schmerzen und zunehmender Beeinträchtigung alltäglicher Bewegungen. Die Produktion von entzündungsfördernden Zytokinen und matrixabbauenden Enzymen durch synoviale Zellen löst die Zerstörung von Knorpel und die Aktivierung von Osteoklasten aus. Außerdem wird der Aktivierungsstatus von RA synovialen Fibroblasten (RASF) unterstützt, was zur Aufrechterhaltung der Entzündung im arthritischen Gelenk führt. Neben Zytokinen sind ebenfalls Adipozyten-spezifische Proteine, Adipokine, dafür bekannt, die Expression von entzündungsfördernden Zytokinen in RASF zu induzieren.

Für die Therapie von MSD werden in der Regel entzündungshemmende Medikamente eingesetzt. Eine zusätzliche Schmerzlinderung kann durch die Behandlung von Patienten mit niedrig dosierter Strahlung erreicht werden. Dabei wird entweder lokale Photonenbestrahlung oder eine Ganzkörperbestrahlung mit Radon, angewendet. Die zugrunde liegenden zellulären und molekularen Mechanismen der entzündungshemmenden Wirkung von Radon sind weitgehend unbekannt.

In Rahmen dieser Arbeit wurde die Wirkung einer seriellen Radontherapie auf den Adipokin-Serumspiegel bei Patienten mit MSD (Patientenstudie RAD-ON01) untersucht. Darüber hinaus wurde untersucht, ob Röntgenstrahlung die Produktion von Adipokinen durch Adipozyten oder die Reaktion von synovialen Fibroblasten auf Adipokin-Behandlung beeinflusst, um zu verstehen, ob diese Mechanismen zur Entzündungsmilderung im arthritischen Gelenk führen können. Da die Zellen des Fettgewebes, Adipozyten, als Hauptproduzenten von Adipokinen beschrieben werden, wurde die Strahlungsantwort von humanen primären subkutanen Adipozyten *in vitro* untersucht.

In der vorliegenden Arbeit wurde zum ersten Mal eine signifikante Abnahme von Visfatin im Serum von MSD-Patienten nach serieller Radonbadbehandlung gezeigt. Darüber hinaus wurde eine positive Korrelation zwischen Visfatin-Serumspiegel und Schmerzdauer sowie Schmerzintensität festgestellt, was auf eine Hemmung der Entzündungsprozesse hindeutet. Außerdem zeigten die *in vitro* erzielten Ergebnisse, dass Röntgenstrahlung die Adipokin-induzierte Produktion von entzündungsfördernden und matrixabbauenden Faktoren (IL-6, MCP-1, MMP-1) durch RASF reduzieren kann, was potenziell zur Entzündungshemmung führt. Weiterhin konnte gezeigt werden, dass Röntgenstrahlung den Differenzierungsprozess von Adipozyten und die Adipokin-Produktion nicht beeinflusst, was auf einen relativ strahlenresistenten Phänotyp der Fettzellen hinweist. Darüber hinaus legen die *in vitro* Ergebnisse nahe, dass die reifen Adipozyten nicht die Hauptproduzenten von Adipokinen im arthritischen Gelenk sind.

Zusammenfassend wurde in der vorliegenden Arbeit gezeigt, dass die *in vitro* erzielten Ergebnisse mit den *in vivo* Untersuchungen in Patienten übereinstimmen. Dies weist darauf hin, dass ionisierende Strahlung den Entzündungsprozess in den Gelenken von MSD-Patienten beeinflussen kann. Die Erkenntnisse dieser Arbeit tragen zu einem besseren Verständnis von zellulären und molekularen Mechanismen im Zusammenhang mit der niedrig dosierten Strahlen- und Radontherapie bei.

Abstract

Rheumatoid arthritis (RA) and osteoarthritis (OA) are the most common musculoskeletal diseases (MSD) that affect the joints. Both diseases are characterized by the destructive changes of articular cartilage and bone, accompanied by increasing disability and pain. Despite the differences in the development of RA and OA, the inflammation process plays a decisive role in both cases. The key players in RA are synovial fibroblasts, which gain an aggressive behaviour and pro-inflammatory phenotype in the course of disease. The production of different pro-inflammatory cytokines and matrix-degrading enzymes by synovial cells not only triggers the destruction of cartilage and activation of bone-resorbing osteoclasts, but also sustain the activation status of rheumatoid arthritis synovial fibroblasts (RASF), contributing to the perpetuation of inflammation in arthritic joint. Besides cytokines, the adipokines, mostly produced by adipose tissue, are known to induce the expression of pro-inflammatory cytokines in RASF.

Chronic inflammatory diseases are usually treated with anti-inflammatory drugs. Besides medications, an additional pain relief is achieved by the treatment of patients with low-dose ionizing radiation, either as local photon irradiation or whole-body exposure to radon in radon baths or galleries. The anti-inflammatory effects of radon are used in the therapy of MSD since many years; however, the underlying cellular and molecular mechanisms are widely unknown.

Therefore, within the scope of this thesis, the effect of a serial radon-bath treatment on adipokine serum levels in MSD patients was investigated (RAD-ON01 study). In addition, it was examined whether X-ray radiation affects the ability of adipocytes to produce adipokines or influences the response of synovial fibroblasts to adipokines, leading to the attenuation of inflammation in arthritic joint.

In the present work, a significant decrease of adipokine visfatin in the serum of MSD patients after serial radon-bath treatment is shown for the first time. Furthermore, the positive correlation between visfatin serum levels and pain duration as well as pain intensity was determined, indicating an attenuation of inflammation. In addition, results obtained *in vitro* demonstrated the ability of X-ray radiation to reduce adipokine-induced production of pro-inflammatory and matrix-degrading factors (IL-6, MCP-1, MMP-1) by rheumatoid arthritis synovial fibroblasts, potentially inhibiting inflammation. Furthermore, the results of the present work showed that X-ray irradiation does not affect the differentiation process of adipocytes *in vitro* and their ability to produce adipokines, irrespective of the dose, indicating a radiation-resistant phenotype of adipose cells. In addition, the obtained results revealed that mature adipocytes are not the main source of adipokines in the arthritic joint.

Taken together, the results obtained *in vitro* are consistent with those obtained *in vivo*, indicating that ionizing radiation may affect the inflammation process in joints of MSD patients. The findings of this work contribute to a better understanding of cellular and molecular mechanisms related to the low-dose radio- (LD-RT) and radon therapy.

Contents

Abbreviations

C/EBP$_s$	CCAAT-enhancer-binding proteins
CAD	Coronary artery disease
CRP	C-reactive protein
CVD	Cardiovascular disease
DMARD	Disease-modifying antirheumatic drugs
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	Glucose transporter type 4
IBMX	3-isobutyl-1-methylxanthine
ICAM	Intercellular adhesion molecules
IGF-1	Insulin-like growth factor 1
IPFP	Infrapatellar fat pad
LD-RT	Low-dose radiotherapy
LET	Linear energy transfer
MCP-1	Monocyte chemoattractant protein 1
MMP	Matrix metalloproteinase
MS	Metabolic syndrome
MSD	Musculoskeletal diseases
MTX	Methotrexate
NO	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drugs
NSF	Normal synovial fibroblasts
OA	Osteoarthritis
OASF	Osteoarthritic synovial fibroblasts
OPG	Osteoprotegerin
PPARγ	Peroxisome proliferator-activated receptor gamma
qPCR	Quantitative real-time polymerase chain reaction
RA	Rheumatoid arthritis
RANKL	Receptor activator of NF-kB ligand
RASF	Rheumatoid arthritis synovial fibroblasts
RF	Rheumatoid factor
ROS	Reactive oxygen species
SF	Synovial fibroblasts
SGBS	Simpson-Golabi- Behmel syndrome
TNFα	Tumor necrosis factor alpha
VAS	Visual analogue scale
VCAM-1	Vascular cell adhesion protein 1
WAT	White adipose tissue

1 Introduction

In the present work, the impact of ionizing radiation on factors related to the changes in bone metabolism of patients suffering from musculoskeletal diseases (MSD) was investigated. Basic aspects of pathological processes related to MSD are explained in the following chapter. Furthermore, a short overview about cells and factors involved in these processes as well as about conventional therapies and the use of low-dose radiation in the treatment of MSD patients are highlighted in this chapter.

1.1 Anatomy of knee joints

The knee joint is the largest and one of the most complex joints in the body. The knee joint consist of three main bones: femur, tibia and patella (Fig. 1.1). Bone is a very dynamic organ that is continuously modulated. Two cell types are responsible for bone remodeling: bone-forming osteoblasts and bone-resorbing osteoclasts. Bone is well innervated and vascularized. The bone ends are encased with two types of cartilage: articular cartilage and meniscus (Fig. 1.1). The main function of the cartilage is to provide an even surface for the smooth moving of the bones. The only resident cells of cartilage are chondrocytes, which produce a large amount of collagenous extracellular matrix (ECM). ECM consists of approximately 80% water and 20% different types of collagen and proteoglycans [1]. The cartilage does not contain any nerves or blood vessels and is nourished by diffusion from synovial fluid [2].

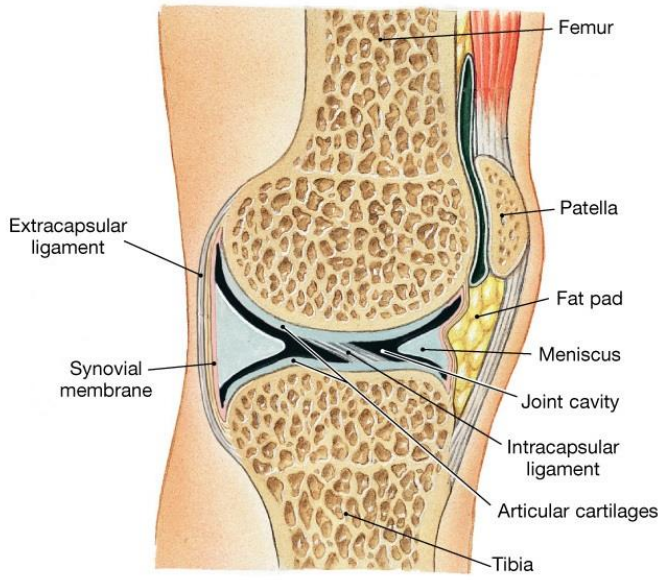

Fig. 1.1 Anatomy of knee joint (modified according to Courses by Dr. Robert Droual at Modesto J. College[1])

[1] Courses by Dr. Robert Droual at Modesto J. College, Chapter 8 – Articulations, http://droualb.faculty.mjc.edu/Lecture%20Notes/Unit%202/chapter_8_articulations%20with%20figures.htm [state: 15.03.2017]

The joint is surrounded by a joint cavity containing the synovial fluid, which enables the transport of nutritional substances and oxygen to the different joint tissues and the lubrication of articular surfaces [3]. The joint cavity is lined by a synovial membrane, which separates the synovial fluid from the joint tissues and supplies the joint with the nutrients (Fig. 1.1). There are two cell types of synovial membrane: synovial fibroblasts (SF) or type A cells and macrophages or Type B cells.

Other parts of the joints include ligaments, muscle and fat pads. Ligaments assure the joint stability and, together with the meniscus, the protection of articular capsule. Different groups of the joint muscles are responsible for the flexibility, extension and stability of the joint. Three fat pads were identified in the human knee joint. The infrapatellar fat pad (IPFP) is the largest articular fat pad, which is located intra-articular, but extra-synovial (Fig. 1.1). The IPFP fills the space behind the patellar tendon and inferior pole of the patella and is a highly innervated and vascularized joint tissue (Fig. 1.1) [4]. The physiological role of IPFP is not completely understood. However, facilitation of synovial fluid distribution and lubrication of knee joint are discussed to be the main functions of IPFP [5], [6].

1.2 Musculoskeletal diseases

Musculoskeletal diseases represent the highest cause of disability in Europe, and affect millions of people around the world [7]. They include degenerative and chronic inflammatory joint diseases (osteoarthritis, rheumatoid arthritis) and other bone diseases (osteoporosis). The majority of MSD is associated with chronic pain, joint damage and reducing mobility of patients. Rheumatoid arthritis (RA) and osteoarthritis (OA) are the most common MSD. Although the degradation of cartilage and bone, accompanied by intense pain and deformation of the joints, are common for both diseases, the pathogeneses of RA and OA are different. While typical changes occurring in the course of RA are caused by chronic inflammation, OA is considered as primary degenerative joint disease; the inflammation in case of OA is considered as a secondary course for disease development [8], [9]. Nevertheless, the underlying mechanisms of both RA and OA are not completely clarified. The development of both diseases is explained in detail below.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory, autoimmune disease, which is associated with progressive disability, systemic complications and even death [10]. The disease is characterized by synovial inflammation, hyperplasia, cell activation and invasive growth of synovium into cartilage, leading to joint destruction [11]. Although the exact cause of RA is still unknown, recent studies suggest that immune regulatory factors are involved in the disease [10], [12]. Thus, in previously published studies it was shown that more than 80% of patients suffering from RA carry the epitope of the HLA-DRB1*04 cluster [12]–[14]. These RA-associated HLA-DRB1 alleles encode in one of their hypervariable regions for an amino acid motif, which is known as a "shared epitope". The function of the HLA class II molecules is to present the antigens to T helper cells, which initiate an immune response. This leads to the activation of T-cells with subsequent production of disease-related pro-inflammatory cytokines. How the formation of RA is promoted by the "shared epitope" of the

HLA-DRB1 allele is not known [13], [15]. Additionally, the environmental factors such as smoking or infections could influence the development and severity of disease [16], [17].
A variety of different cell types, including synovial fibroblasts, macrophages, T cells and B cells, contributes to the inflammatory and destructive processes in RA [18]. Typical symptoms of RA such as chronic inflamed synovium, joint swelling and pain are the consequences of changed behaviour of synovia cells. The resulting joint damage usually occurs in the first year after disease outbreak and increased in course of disease [19].

Osteoarthritis

In contrast to RA, osteoarthritis (OA) is considered as primary degenerative joint disease. Although the initiating factors in OA are still unknown, an important role is attributed to the mechanical factors such as overweight or overuse; however, also genetic factors may contribute to the development of disease [8], [20]. Affected patients exhibit similar symptoms as RA patients, such as swelling joints, pain and reduced mobility.

The cartilage damage in OA is mediated by an increased release of matrix-degrading metalloproteases (MMPs), which are produced by chondrocytes [8], [21]. Chondrocytes are the only one cell type, residing in adult articular cartilage, and are capable to respond to any changes in surrounding conditions [8], [22]. In OA, this response is characterized by forming of chondrocyte clusters and hypertrophic proliferation. In the further course of the disease, the changes in composition and structure of the cartilage lead to an increased production of MMPs and other factors involved in cartilage degradation. As a consequence of disrupted cartilage integrity, the apoptosis rate of chondrocytes increased, resulting in the development of empty lacunas [23].

Although OA is described as a “non-inflammatory” disease, inflammation does play a role in the pathogenesis of this disease [3]. The increased level of pro-inflammatory cytokines in synovial fluid of the OA patients was reported in many studies [9], [24], [25]. In addition, it was shown that chondrocytes and osteoarthritic synovial fibroblasts (OASF) strongly respond to a stimulation with cytokines and adipokines by increasing production of MMPs [9], [26], [27]. Furthermore, it is known that chondrocytes support osteoclastogenesis by receptor activator of NF-kB ligand (RANKL) production [22], [28]. Thus, OA is a much more complex disease than “wear and tear” disorder. However, the question whether inflammation is a main trigger or a secondary cause of OA is still under investigation [8].

1.3 Role of synovial fibroblasts in musculoskeletal diseases

Synovial fibroblasts (also called fibroblast-like synoviocytes, SF) are cells of mesenchymal origin, and together with synovial macrophages are residing in synovial membrane [29]. In healthy joints, the physiological function of SF is the maintenance of matrix remodeling and supply of joint cavity with nutritive proteins and lubricating molecules such as hyaluronic acid and lubricin [29], [30]. In healthy individuals consisting of 1-3 layers of synovial fibroblasts, the synovial membrane becomes thickened under pathological conditions in RA and OA. Inflammation in the synovium is accompanied by infiltration of immune cells and aggressive proliferation of SF, leading to the hyperplasia of the synovial membrane. Although the inflammation in RA joints is more intense than in OA, typical inflammation-related changes of SF are present in both diseases [8], [31]. Since in the present work the synovial fibroblasts of

rheumatoid arthritis patients (RASF) were used as an investigation model, the pathogenic changes in these cells are described in detail below.

RASF in RA synovium possess an unique aggressive phenotype, which is a consequence of persistent cell activation [32], [33]. Multiple factors contribute to the activation of RASF (Fig. 1.2). However, the exact mechanisms are still unknown. Once activated, RASF actively participate in inflammation and cartilage destruction by secretion of pro-inflammatory cytokines (IL-6, IL-8, MCP-1), matrix-degrading enzymes (MMP-1, MMP-9), osteoclasts-activating factors (RANKL) and adhesion molecules (ICAM, VCAM) (Fig. 1.2) [32], [34], [35]. The latter are important for attachment to cartilage and invasion. Concurrently, the destruction of cartilage is assisted by increased secretion of MMPs. In addition, RASF contribute directly to the bone resorption via production of cathepsin K and L, enzymes involved in the degradation of collagens type I, II, IX, XI and proteoglycans [29], [36]. Moreover, RASF exhibit uncontrolled proliferation, lack of apoptosis and high migration potential. Both, reduced apoptosis and increased proliferation, lead to synovial hyperplasia, which induces the development of hypoxic conditions [37]. Local hypoxia induces proangiogenic and chemotactic factors in RASF, leading to the neoangiogenesis. This in turn contributes to the increased infiltration of immune cells in the joint. In addition, hypoxia increased unique long-distance migration potential of RASF, causing spreading of RA-mediated erosion and inflammation to the unaffected joints [37], [38].

Thus, synovial fibroblasts are the key effector cells in the development of RA. Targeting their aggressive destructive and inflammatory properties is a main task for the development of joint protective therapy of RA [37], [39].

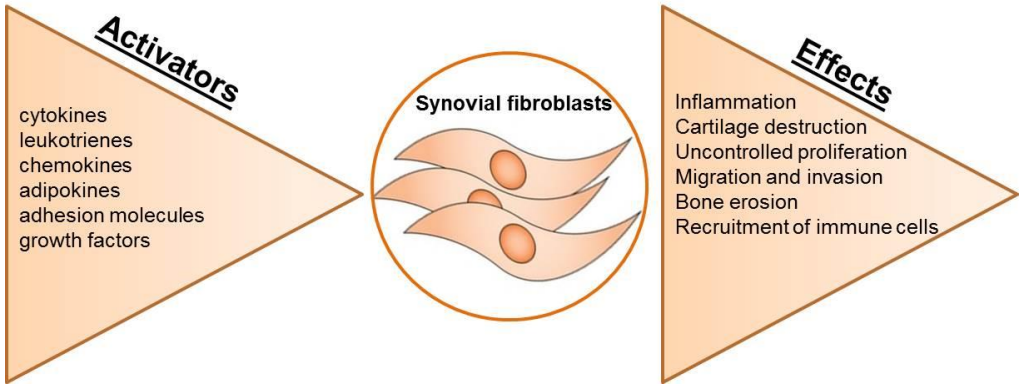

Fig. 1.2 Activating factors and contribution of RASF to the joint destruction in RA. Data from [8], [29], [39]

1.4 Role of adipose tissue in musculoskeletal diseases

Adipose tissue

Adipose tissue is a loose connective tissue with a primary role to store energy. However, recently, adipose tissue was discussed as an important endocrine organ, which is involved in the mediation of immune and inflammatory response [40]. Two types of adipose tissue are known: the white adipose tissue (WAT) and the brown adipose tissue, which have different functions and cellular composition [41]. In the present work, the cells of WAT were used as

an investigation model. Therefore, an overview about the main functions of WAT in physiological and pathological processes will be given below.

The adipose tissue produces several factors, which influence different biological processes, including energy homeostasis (lipid metabolism, insulin sensitivity, appetite, etc.), immune response, reproductive function, blood pressure and angiogenesis [42], [43]. The disturbance of the fine regulation of endocrine functions of adipose tissue leads to the chain reaction of pathological changes in cell composition as well as in expression and secretion of adipose tissue-specific factors. Altered secretion of adipokines, for instance, leads to the development of metabolic, cardiovascular, inflammatory and mammalian diseases [44]. The increased production of pro-inflammatory cytokines results in a chronic low-grade inflammation of adipose tissue. Inflamed fat tissue in turn can promote systemic inflammation and insulin resistance. Both insulin resistance and dysregulation of key adipokines represent a core of systemic metabolic disorders with a developing of various diseases as a consequence [45].

Metabolic syndrome (MS) is a group of risk factors for developing cardiovascular diseases (CVD), diabetes, etc. As mentioned above, the insulin resistance and the characteristic adipokine profile are the key components of MS. Moreover, body weight changes and increased blood pressure are associated with the development of MS [46], [47]. Several musculoskeletal diseases, including RA and OA, are associated with an increased prevalence of CVD [11], [48]. Many studies showed the occurrence of most components of MS in RA patients [49]. The relationship between adipose tissue, inflammation, metabolic syndrome and CVD is very complex. There is growing evidence that a changed secretome of adipose cells and a local inflammation within adipose tissue are the main contributors to insulin resistance and systemic inflammation, the main features of MS. However, the induction factor that caused these dramatic changes in adipose tissue remains unknown [50].

Infrapatellar fat pad

WAT is distributed throughout the body in several depots, which differ in their structural composition and biological function [51], [52]. The most widely investigated depots are subcutaneous (SAT) and visceral (VAT) fat tissues. Although it is known that adipose tissue produces a variety of inflammatory mediators, which can contribute to the destruction of cartilage and bone, the knowledge about one of the adipose tissue depots – infrapatellar fat pad (IPFP) – is still very limited [6]. The special location of IPFP, close to the synovial membrane and cartilage, has a special significance in musculoskeletal diseases, such as OA and RA.

Like all adipose tissue depots, IPFP is an endocrine organ, which secretes a variety of cytokines and adipokines [53]. It is very likely that the IPFP undergoes a series of cellular and molecular changes under pathological conditions such as inflammation. The increased production of IL-6, IL-8, TNFα as well as adipokines adiponectin, leptin and visfatin by IPFP in response to inflammatory stimuli was described in many studies [6], [54], [55]. The enhanced concentrations of these cytokines and adipokines were found in the synovial fluid of patients with MSD. Besides cytokines and adipokines, IPFP also secretes a large amount of lipids, whose primary function is to store and release energy [56]. However, a study of Gierman et al showed that the IPFP of OA patients exhibit more pro-inflammatory lipid profile

than IPFP from healthy donors [57]. In addition, a larger IPFP volume in patients with OA compared to healthy individuals was associated with more pronounced pain, suggesting that the IPFP could be a source of knee pain in patients with MSD [4].

Adipose tissue, including IPFP, consists to a larger extent of adipocytes. Other cell types of adipose tissue, called stromal vascular fraction (SVF), are stem cells, macrophages, lymphocytes, fibroblasts and endothelial cells. The balance between different cell types within adipose tissue is variable and depends on both location of the adipose tissue depot and surrounding conditions [56], [58], [59]. Since the crosstalk between IPFP and other tissues in the joint is known, an exposure to chronic inflammation under pathological conditions might induce the infiltration of immune cells, leading to inflammation of adipose tissue [54]. Considering the location of IPFP adjacent to the synovial membrane, it is conceivable that the inflammation-induced production of IPFP-secreted factors contributes to the perpetuation of inflammation and activation of SF in arthritic joint [53] (Fig. 1.3).

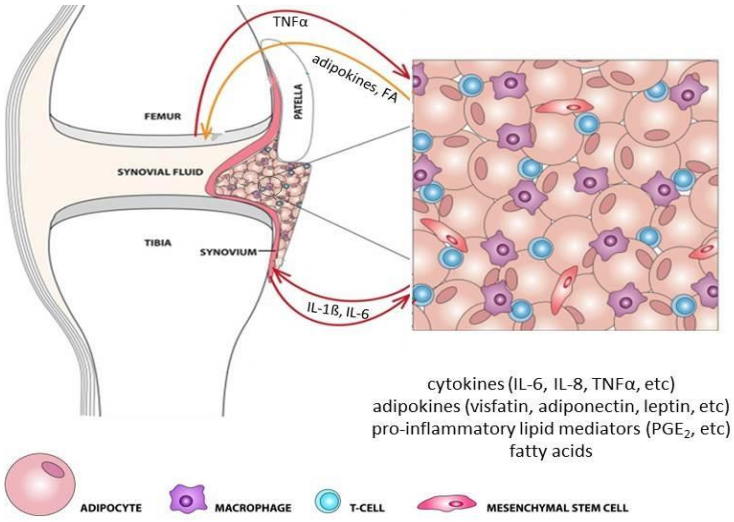

Fig. 1.3 Infrapatellar fat pad as a source of several pro-inflammatory factors. Modified according to [56]

Adipocytes

As mentioned above, adipocytes are the main cell type of adipose tissue. They differentiate from pluripotent mesenchymal stem cells and are primary responsible for the energy storage in form of triglycerides in their cellular lipid droplets [60]. However, adipocytes are now recognized as highly active secretory cells that play a role in whole body homeostasis, including inflammatory and immune responses [45], [61]. Adipocytes are major producers of adipokines such as adiponectin, leptin, visfatin and resistin. In addition, they secrete various pro-inflammatory cytokines, including IL-6, IL-8 and TNFα [62]. Under inflammatory conditions, the changes in the adipocyte metabolism initiate alterations in adipose tissue, leading to immune cell infiltration and increased production of pro-inflammatory factors, including adipokines [44], [60].

The differentiation process of adipocytes is complex and includes different steps of commitment, cell contact, mitosis, clonal expansion, growth arrest and maturation (Fig. 1.4) [43]. Although the cells undergo many steps on the way to mature adipocyte, the process can

be divided into two phases [63]. In the first phase – determination – the cell morphology does not change, but the pre-adipocytes loose the potential to differentiate into other cells [63]. The molecular mechanisms underlying this process are widely unknown [43]. In the second phase, called terminal determination, the pre-adipocytes acquire properties of mature adipocytes. The molecular mechanisms during this phase are characterized by a highly regulated interplay of stimulatory and inhibitory transcription factors [43], [63].

The critical factors for the complete and seamless adipocyte development are the peroxisome-proliferator-activated receptor gamma (PPARγ) and the CCAAT-enhancer-binding proteins (C/EBPs) [61], [63]. Hormones like insulin and glucocorticoids or isobutyl-methyl-xanthine (IBMX) trigger adipogenic differentiation by induction of C/EBPß and C/EBPγ. These in turn activate two isoforms of PPARγ, PPARγ1 and PPARγ2 as well as many other adipogenic genes. Upon ligand activation, PPARγ together with C/EBPα leads to the complete differentiation into mature adipocytes [61]. PPARγ is the key transcription factor, which is sole sufficient for adipogenesis. It was shown that only the presence of PPARγ is sufficient to induce adipocyte differentiation, but the differentiation cannot be induced in absence of PPARγ [63], [64]. At the late stages of differentiation, the adipocytes increase lipid accumulation and acquire sensitivity to insulin. In addition, the expression of late differentiation markers such as glucose transporter 4 (GLUT4), leptin, adiponectin and visfatin occurs (Fig. 1.4) [61].

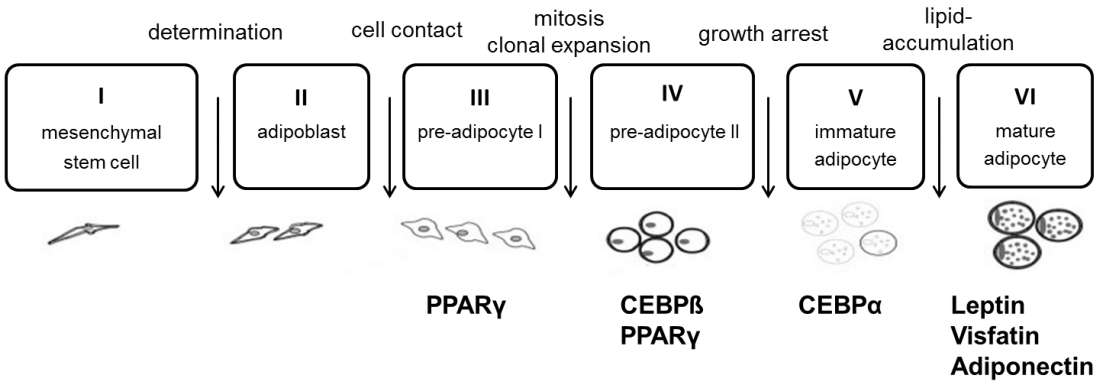

Fig. 1.4 Differentiation process of adipocytes. Cell development stages and gene expression in course of differentiation from MSC to mature adipocyte. Modified according to [43]

SGBS cells

Human primary adipocytes are widely used to investigate differentiation and metabolism of the fat cells. However, the usage of primary material is often associated with some limitations. The variations between donors and the isolation of the cells from different adipose tissue depots lead to different results and large variations in the studies and render the characterization of the cells more difficult. In addition, primary pre-adipocytes have a limited capacity for differentiation. Therefore, the different cell culture models were established in the past years. However, these investigation models are to a large extent of animal origin. Since it is known that the cellular composition and function of adipose tissue differ between species, the animal adipocyte models are not sufficient for investigation on human fat biology [65].

Due to mentioned limitations, the experiments of the present work were mostly performed with cells isolated from the subcutaneous fat tissue of a patient with Simpson-Golabi-Behmel syndrome (SGBS). The SGBS is a rare X-linked disorder, which is associated with pre- and postnatal overgrowth and increased risk of embryonal cancer [65], [66]. Although a mutation in Glypican 3 gene, which is known to be involved in the control of organ growth, is considered to be associated with this disease, the exact mechanism causing SGBS is not yet known [65]. The cell strain isolated from the subcutaneous adipose tissue of an infant with SGBS exhibit a high capacity for adipocyte differentiation. In addition, the morphology, the gene expression pattern and the biochemical function are very similar to that of human primary adipocytes [66], [67]. SGBS cells provide therefore an appropriate model for studies on various aspects of human adipose tissue [68]. However, it should be noted that the cells are obtained from the patient with a not characterized gene mutation, which could have an influence on some aspects of adipocyte biology [66].

1.5 Factors involved in the pathogenesis of musculoskeletal diseases

The production of various pro-inflammatory factors including cytokines, matrix-degrading enzymes and adipokines plays a central role in the pathogenesis of MSD [10]. The inflammatory process is accompanied by an increased secretion of cytokines such as IL-6, IL-8, TNFα, MCP-1 and IL-1ß. Furthermore, changes in the adipocyte metabolism lead to the altered production of adipokines such as visfatin, adiponectin, leptin and resistin [69]. The adipokines in turn induce the expression of pro-inflammatory cytokines and matrix-metal-proteinases in other cells of synovium, including synovial fibroblasts, chondrocytes and immune cells. This leads to perpetuation of inflammation and joint destruction. The key functions of pro-inflammatory factors involved in the pathogenesis of RA and OA are described below.

1.5.1 Cytokines

Cytokines are glycoproteins, which are responsible for the communication between immune cells [70]. Increased levels of cytokines such as IL-6, IL-8 and TNFα in serum and synovial fluid of patients with MSD were found to correlate with disease activity, radiographic joint damage and pain intensity [26], [70], [71]. Furthermore, the pro-inflammatory cytokines upregulate the expression of matrix-degrading and osteoclasts-activating factors and stimulate the production of reactive oxygen species (ROS), contributing to the disease progression [70], [72].

Interleukin 6 (IL-6) is one of the key cytokines involved in the pathogenesis of rheumatoid arthritis (RA) [10]. IL-6 drives the activation of local leukocytes, endothelial cells and synovial fibroblasts by inducing the production of various pro-inflammatory factors [10]. In addition, IL-6 upregulates the expression of MMP-1, MMP-3 and MMP-13 in chondrocytes, leading to cartilage destruction [26]. IL-6 also mediates systemic effects, which promote cognitive dysfunction and dysregulation of lipid and glucose metabolism, in particular insulin sensitivity and fatty acids oxidation [10], [73], [74]. Since the elevated circulating levels of IL-6 are associated not only with inflammation, but also with an increased risk of coronary artery disease (CAD) [73], the involvement of IL-6 in metabolic syndrome is conceivable. IL-6 is a cytokine, which is produced by a variety of different cell types. However, approximately 30% of circulating IL-6 is produced by adipose tissue [73].

Similar to IL-6, tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1ß) play the pivotal roles in the pathogenesis of rheumatoid arthritis. Both cytokines are produced by a variety of synovial cells, including synovial fibroblasts and immune cells [75]. TNFα and IL-1ß stimulate their own production and promote inflammation and cartilage destruction by inducing different pro-inflammatory cytokines, matrix-degrading factors and adhesion molecules in synovial cells [72]. In addition, TNFα directly mediates chemokine expression, adhesion and angiogenesis. Moreover, TNFα together with RANKL contributes to osteoclastogenesis [76].

The accumulation of immune cells in arthritic synovium is a hallmark for acute inflammation. Interleukin 8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) are the most important cytokines that mediate chemotaxis in arthritic joint [77]. IL-8 as a neutrophil chemoattractant is responsible for the increased number of neutrophils at the side of inflammation and is associated with joint swell and pain [77]. Beside chemotaxis, IL-8 stimulates both osteoclasts formation and activity, leading to the bone resorption [78]. The role of MCP-1 in MSD is the chemoattraction of macrophages in inflamed joint [77]. In addition, increased MCP-1 level causes insulin resistance in adipocytes and monocytes, leading to the development of metabolic disorders [69], [79]. Furthermore, both chemokines IL-8 and MCP-1 induce the formation of new blood vessels in arthritic synovium and are, therefore, important promoters of angiogenesis [77].

1.5.2 Matrix-degrading enzymes

The highly inflammatory microenvironment of arthritic synovium stimulates the synovial fibroblasts and chondrocytes to produce matrix metalloproteinases MMP-1, MMP-3, MMP-9 MMP-13 as well as cathepsins, the prominent mediators of cartilage and bone degradation [8], [80]. Collagenases MMP-1 and MMP-13 are responsible for the initial cleavage of collagens. MMP-1 is mostly produced by synovial fibroblasts, and MMP-13 – by chondrocytes. Besides collagen, MMP-13 cleaves aggrecan, a critical component of cartilage structure [81]. Other matrix enzymes such as MMP-3 (stromelysin 1) and MMP-9 (gelatinase B) degrade the fragments, remaining after initial collagen cleavage [10], [31]. Among them, cathepsins B, K and L are considered to be the main proteases in the degradation of bone matrix [82]. Cathepsins B and K degrade collagen types I and IV as well as fibronectin, whereas cathepsin L is responsible for cleavage of collagen types I, II, IX and XI, mediating both cartilage and bone destruction [36], [83]. The main producers of cathepsins in arthritic joint are synovial fibroblasts and osteoclasts [82], [83].

1.5.3 Adipokines

Adipokines are cytokine-like molecules, primarily produced by adipose tissue. They modulate a broad spectrum of physiological and pathophysiological processes, ranging from regulation of appetite and insulin sensitivity to inflammation and immune response [41], [84]. Imbalance in adipokine profile leads to changes in the metabolism of adipocytes and the whole adipose tissue, resulting in development of metabolic and other diseases as a consequence [45], [85]. Elevated levels of adipokines were detected in serum and synovial fluid of MSD patients [86]–[89]. In MSD, adipokines are considered to be the emerging modulators of inflammation and joint destruction, as they induce the production of a variety of pro-inflammatory cytokines, matrix-degrading and osteoclasts-activating factors in different

synovial cells [90]. The most investigated adipokines are adiponectin, visfatin, leptin and resistin [86].

Visfatin

Visfatin is an adipokine, mostly, but not exclusively produced by adipose tissue. Visfatin, also called nicotinamide phosphoribosyl transferase (NAMT), plays an important role in the regulation of many cellular processes because of its involvement in NAD synthesis [91], [92]. In degenerative joint diseases such as RA and OA, serum and synovial fluid levels of visfatin are elevated and correlate with degree of inflammation and disease activity [93], [94]. Visfatin promotes cytokine synthesis and increases motility of synovial fibroblasts, contributing to the synovial inflammation and spreading the disease to healthy joints [95], [96]. Moreover, visfatin contributes to vascular inflammation by inducing the production of pro-inflammatory cytokines by peripheral mononuclear cells and promoting macrophages survival [97], [98]. In addition, visfatin upregulates the expression and activity of MMPs and downregulates the expression of MMP-inhibitors, contributing to cartilage degradation [99]. Visfatin is considered to be a potential target for the therapy of RA and OA.

Adiponectin

Adiponectin is another well-studied adipokine, predominantly secreted by mature adipocytes [84], [86]. Besides its multiple functions in energy metabolism, adiponectin plays a role in bone metabolism, cardiovascular and immune systems [84], [100]. Although adiponectin was widely investigated in the last years, its specific functions in different physiological and pathological processes remain unclear. Several studies reported an anti-inflammatory and protective effects in obesity-related and vascular diseases [101]–[103]. By contrast, in degenerative joint diseases such as OA and RA, adiponectin supports inflammatory and matrix-degrading processes [100], [101]. Adiponectin was reported to induce the production of pro-inflammatory (IL-6, IL-8) and matrix-degrading (MMP-1, MMP-3) factors in synovial fibroblasts [27] and chondrocytes [104]–[107]. In addition, adiponectin contributes to leucocyte and monocyte infiltration by inducing the expression of adhesion molecules (VCAM-1) and chemoattractant factors (MCP-1) in chondrocytes [11], [108]. High serum and articular adiponectin levels are associated with the radiographic changes in patients with RA [27]. In addition, the synovial fluid level of adiponectin positively correlates with degenerative fragments of aggrecan in affected joints of patients with OA, suggesting its potential as a marker for disease progression [27], [86]. However, the findings about the association of adiponectin with clinical parameters such as disease severity or pain are contradictory [109]. The discrepancies between studies are more likely related to the different isoforms of adiponectin. In serum, adiponectin exists in three different forms – low molecular weight (LMW), middle molecular weight (MMW) and high molecular weight (HMW) – that differ in their biological activity [110], [111]. It was shown that HMW/MMW adiponectins have more pronounced pro-inflammatory effect on the synovial cells [111]–[113]. Many studies suggest adiponectin as an important target for the treatment of MSD. However, more research in this field is required to fully elucidate the function of this adipokine [114].

Leptin

Leptin is one of the most important adipokines, produced by adipose tissue. The main function of leptin is the regulation of body weight by inhibition of food intake and stimulation of energy expenditure [115]. Apart from this, leptin is involved in a variety of biological

processes such as glucose and lipid metabolism, blood pressure, reproduction, T cell function and bone metabolism [116], [117]. Several studies reported a close correlation between leptin levels and risk of aggressive course of RA [90], [118]. Leptin levels are elevated in serum and synovial fluid of MSD patients. Furthermore, leptin is known to induce IL-6 and IL-8 in synovial fibroblasts as well as IL-8 and adhesion molecules (VCAM-1) in chondrocytes [86], [106], [108]. In addition, leptin was shown to modulate the T-cell balance by driving T cell differentiation towards Th1 cells, exerting pro-inflammatory properties [101], [116], [119]. In OA, leptin is able to increase the production of osteoprotegerin (OPG) in monocytes and osteoblasts, promoting formation of osteophytes [86]. Although leptin is considered as an important target for therapy of MSD, current biological therapies such as TNFα-inhibitors do not affect leptin levels [90].

Resistin

Resistin is an adipocyte-derived adipokine with an important role in inflammation [120]. The known physiological functions of resistin are the regulation of metabolic and inflammatory processes as well as adipogenesis [114], [121]. High levels of resistin were found in serum and synovial fluid of RA and OA patients. In addition, resistin levels positively correlate with several markers of inflammation such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and TNFα [86], [120], [122]. Expression of resistin in peripheral blood mononuclear cells (PBMC) is upregulated by the pro-inflammatory cytokines IL-6, IL-1ß and TNFα. In turn, resistin induces the production of IL-6 and TNFα in macrophages [86], [123]. Moreover, the inhibition of TNFα in patients with RA results in a significant decrease of serum resistin levels in association with a reduced CRP level [86], [120]. Based on this, resistin is proposed as an important molecule in NF-kB activation and cytokine production by PBMC [120].

1.6 Therapy of musculoskeletal diseases

The most important goals for the treatment of patients suffering from musculoskeletal diseases (MSD) are to relieve pain, to stop or slow down inflammation and joint damage, and to improve joint mobility. Treatment of MSD includes usually medications, physical therapy and regular exercises. In addition, low-dose irradiation is used for the treatment of MSD.

1.6.1 Conventional medical treatment

In recent years, the prognosis for MSD patients has dramatically improved. The reason for that is the better understanding of disease pathogenesis and, therefore, the development of biologic therapies and the use of different treatment possibilities in combination [124]. There is a variety of medications for the treatment of MSD. Usually the disease-modifying-anti-rheumatic/osteoarthritic drugs (DMARDs/DMAODs) and the non-steroidal anti-inflammatory drugs (NSAIDs) are used for the treatment of MSD patients. NSAIDs reduce pain and inflammation by blocking cyclooxygenases (COX), which are associated with inflammation in arthritic joints [125]. DMARDs are used to control disease and to limit joint damage, resulting in the improved long-term outcome.

The important targets for medical therapy of MSD are cytokines, such as TNFα, IL-1ß and IL-6, which induce inflammation and contribute to joint destruction. Apart from them, also adipokines are in the focus of drug development, as they induce the production-of pro-

inflammatory cytokines in the joint. Recently, a variety of TNFα-blocking drugs were developed, while targeting of IL-1ß is still a challenge. Anakinra is the only approved antagonist for IL-1-receptor, which binds IL-1ß and, in the combination with methotrexate (MTX), provides significantly better clinical benefit than MTX alone [126]. Also an administration of tocilizumab, an antibody directed against IL-6, in combination with MTX results in the disease remission in approximately 30% of patients [127].

Bisphosphonates represent another drug class, which is successfully used in the therapy of MSD. Bisphosphonates are powerful inhibitors of bone resorption and are mostly used in the treatment of osteoporosis and OA [3], [128]. In their mechanism of action, bisphosphonates are internalized by osteoclasts at sites of bone resorption, leading to the disturbance of their cellular metabolism and finally to the apoptosis of osteoclasts [128], [129].

Although drug therapies are shown to be useful in different MSD and are often well tolerated by patients, adverse side effects (Tab. 1.1) often cannot be predicted. In addition, high costs and inconvenience of intravenous administration give an impetus for developing of new therapeutic possibilities [130].

Drug	Target	Side effects
Tocilizumab	IL-6	Infections, cardiac events, non-melanoma skin tumors, hematological disturbances
Rituximab	CD-20 protein	Severe infusion reactions, cardiac events
Anakinra	IL-1	Severe infusion reactions, headache, abdominal pain
Infliximab Adalimumab	TNFα	Increased risk for tuberculosis, infections, neutropenia, headache
Bisphosphonates	osteoclasts	Severe musculoskeletal pain, hypocalcemia, ocular inflammation, osteonecrosis

Tab. 1.1 Selected drugs for the therapy of MSD and their side effects. Data from [126], [130]–[132]

1.6.2 Low-dose radiotherapy

In addition to medical treatment, patients with several MSD can benefit from the application of low-dose ionizing radiation. There are two types of radiotherapy available: patients are exposed either locally (photon irradiation, LD-RT_p) or they receive a whole-body irradiation in radon galleries or radon baths (LD-RT_{radon}). LD-RT represents an effective and inexpensive alternative to the drug treatment. Although the risk for the side effects of LD-RT is low, radiation exposure is not recommended for younger patients and pregnant women, because of the risk for possible tumor induction as a long-term effect [133].

Photon irradiation is usually given in a fractionated regimen with a total dose of 6 Gy. The single dose is adjusted in consideration to the type and phase of disease, and is normally 0.5-1 Gy [133]. The largest group of patients, benefiting from LD-RT_p, are patients, suffering from degenerative joint disorders [133]. Clinical studies demonstrate significant pain relief and improved joint mobility in patients after LD-RT_p. However, the molecular and cellular mechanisms, underlying clinical effects, are not widely investigated. Several studies showed in animal models that low-dose ionizing radiation attenuate inflammation, leading to the improvement of clinical symptoms [134], [135]. The effects of low-dose radiation such as

reduction of pro-inflammatory cytokines (IL-6, TNFα), decrease in reactive oxygen species (ROS) and nitric oxide (NO), reduced infiltration of macrophages and increase in regulatory T-cell population were shown by a number of authors [135]–[138]. These findings suggest that low-dose ionizing radiation enhances immune functions and affects the signaling processes and cells involved in the development of inflammation [137].

In addition to LD-RT_p, radon treatment is used for the therapy of MSD. Low doses (0.2-0.5 mSv) of alpha radiation emitted by a noble gas radon (Rn-222) can be applied either in form of whole-body irradiation in radon galleries (radon concentration 20-160 kBq/m^3) or radon-enriched baths (radon concentration 0.3-3 kBq/L) [139]. The patients receive usually a series of 8-12 administrations, 20-60 min each [140], [141]. The penetration ability of alpha-particles is between 20 and 40 μm (water/tissue), and the residence time of radon in body is very short – about 15-30 min [142]. The investigations of radon solubility in different organs showed that the adipose tissue may be a major reservoir of radon [143], [144]. The estimated alpha dose given to the fat tissue after radon irradiation is much higher than to other organs [145].

LD-RT_{radon} has been reported to improve mobility of joints and to reduce pain and inflammation in patients with MSD [140], [146]. Although some clinical studies related to radon exposure were performed, underlying mechanisms are still widely unknown. However, Lange et al reported an increase in bone-forming factors (OPG) and decrease in bone-resorbing (RANKL) and inflammatory (TNFα) factors in patients after LD-RT_{radon} [141]. Also the decrease of anti-CCP antibodies (ACCP), which is close related to the bone destruction in RA, was shown in patients received LD-RT_{radon} [141], [146]. In addition, Rühle et al showed that LD-RT_{radon} caused immune modulations in peripheral blood of MSD patients, probably leading to the attenuation of inflammation [140]. The first findings on cellular and molecular levels indicate the ability of LD-RT_{radon} to influence osteo-immunological mechanisms, leading to the beneficial effects, such as improved joint mobility, pain relief and attenuation of inflammation. Combination of LD-RT_{radon} with physical exercises may even enhance the mobility improvement of MSD patients [142], [147].

1.7 Physical characteristics of ionizing radiation

Both photons (X-rays) and alpha particles (radon), discussed in the present work, are types of ionizing radiation. Ionizing radiation is a form of radiation with enough energy to remove electrons from atoms or molecules. The released energy (ΔEabs) per unit mass (Δm) is the dose (D), an important physical parameter for describing biological effects of ionizing radiation:

$$D = \frac{\Delta \mathrm{Eabs}}{\Delta \mathrm{m}} \left[Gy = \frac{J}{kg} \right]$$

When ionizing particles pass through the matter, they lose energy along the length of their track. The density of energy deposition in matter is called Linear Energy Transfer (LET). The LET depends on the type of radiation and is described as an energy loss (ΔE) per unit distance covered by the particle(Δds):

$$LET = \frac{\Delta \mathrm{E}}{\Delta \mathrm{ds}} \left[\frac{keV}{\mu m} \right]$$

Radiation with LET < 10 keV/μm is designated as sparsely ionizing radiation, and radiation with higher LET – as densely ionizing radiation.

The photon radiation (X-rays) is sparsely ionizing radiation. Photons interact with matter via photo- and Compton-effect. Free electrons, generated by ionization, transmit their energy to other electrons and ensure homogeneous dose distribution (Fig. 1.5). The energy deposition of photons decreases with increasing penetration depth.

Alpha particles, emitted by radon, are densely ionizing. The microscopic dose deposition pattern in this case is heterogeneous (Fig. 1.5). The probability for a cell to be traversed by one alpha particle is Poisson-distributed. At low doses, a fraction of cells does not receive radiation at all. Also, alpha particles have a very shot penetration ability, which depends on material density.

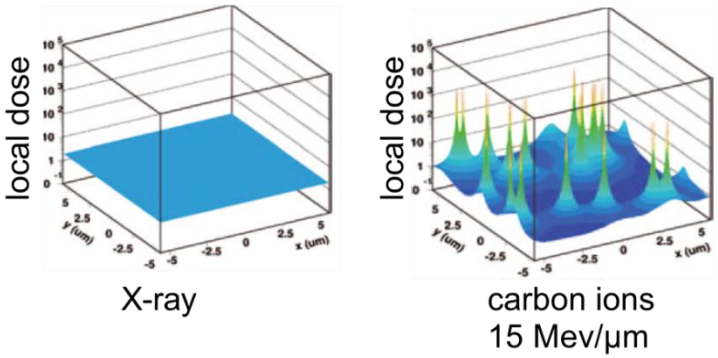

Fig. 1.5 Microscopic dose deposition of sparsely (X-ray) and densely (carbon ions) ionizing radiation. Presented are dose distributions of 2 Gy on the mammalian cell nuclei (modified according to [148]

The alpha-emitter radon (Rn-222) is a noble gas, which occurs naturally as a part of the radioactive decay chain of uranium. It has a half-life time of 3.8 days and is produced by an alpha decay of Rn-226 (Fig. 1.6). The total decay energy of radon and its decay products is approximately 19.2 MeV, and is mostly delivered by alpha-particles [149], [150]. Radon is incorporated via the epithelium tissues of lung and skin and remains in the body approximately 30 min [142]. Since radon is a noble gas, chemical reactions within the body do not occur. The energy deposition can have a biological effect.

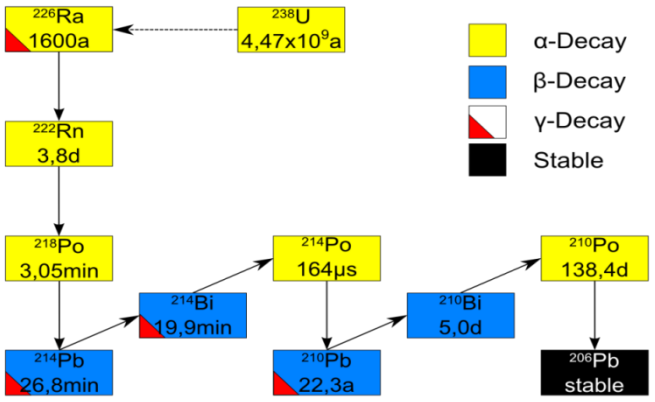

Fig. 1.6 Uranium decay chain. Adopted from [151]

1.8 Radiation biology

Radiation causes a variety of biochemical and biological alterations in biological matter such as cells or tissues. The most radiosensitive target is DNA. Radiation can induce DNA damage either directly, hitting DNA molecules, or indirectly via ionization of water molecules, which induce the formation of highly reactive water radicals. These free radicals in turn hit other molecules, e.g. DNA, lipids and proteins [152]. High LET radiation causes DNA damage mostly directly, whereas low LET radiation induces most of the damages via indirect way.

Besides damaging DNA and other cellular components, ionizing radiation has a variety of other effects, which affect cells and tissues. These effects are cell type and dose dependent, and include, inter alia, cell death, inducing of reactive oxygen species (ROS), changes in protein and gene expression as well as morphological and functional changes [153]–[155].

The main goal of tumor radiotherapy is the induction of tumor cell death and the activation of immune system. In contrast, the aim of low-dose radiation treatment is to modulate the immune response to attenuate a pre-existing inflammatory state. The beneficial ability of low-dose radiation to switch the phenotype of inflammatory cells to an anti-inflammatory without affecting their functionality is used, inter alia, for the therapy of degenerative joint diseases [138], [156].

1.9 Aims

Based on the current knowledge, in the present work, it was hypothesized that low-dose irradiation inhibits adipokine-induced inflammation in the joints of MSD patients. To obtain the best possible understanding of radiation effects on adipokine-induced inflammation, the investigations were performed *in vivo* and *in vitro*. To verify the hypothesis, following questions were addressed in this work:

- ***In vivo:*** Does radon treatment have an influence on the circulating levels of adipokines in MSD patients? The investigations were performed within the scope of RAD-ON01 study (see chapter 2.1);

- ***In vitro:***
  a) Do the adipokines adiponectin and visfatin have an influence on synovial fibroblasts? Thereby the synovial fibroblasts obtained from patients with rheumatoid arthritis and from healthy donors were used;
  b) If there is an effect on synovial fibroblasts, does irradiation of adipokine-stimulated synovial fibroblasts alters this effect? For this, the adipokine-stimulated synovial fibroblasts were irradiated with different doses of X-rays;

- ***In vitro:*** Since adipokines are mostly produced by adipose tissue, is the differentiation or activity of adipocytes influenced by radiation? For this purpose, the response of (pre)adipocytes to X-ray irradiation was investigated.

The data obtained within scope of this thesis should provide new insights on the molecular and cellular mechanisms related to low-dose radio- and radon therapy.

2 Methods

2.1 Patient study RAD-ON01

Study design

For this study, 100 patients were enrolled, who suffered from chronic degenerative musculoskeletal diseases (MSD) of spine and/or joints. The unblinding took place at the end of last follow-up (6 months after treatment start). The study was carried out in accordance with the recommendations of the ethical review committee of the Bavarian State Chamber of Physicians (Bayerische Landesärztekammer, Munich, Germany, ethical approval BLAK #12131). All patients have granted their written informed consent. Full study description is published by Ruehle et al [140].

Patients and radon treatment

In March 2013, 100 patients were separated in two groups of 50 patients each. The first group was treated with 100% radon water, the second group – with an even proportion of CO2 and radon water. The radon treatment, consisted of a series of 9 baths with duration of 20 min each over 3 weeks, was performed in the certified health resort Bad Steben, Germany. The temperature (34°C) and the humidity (60%) were controlled. The average activity of the radon containing baths was 1200 Bq/l. In the present work, in total 44 patients were analyzed.

The following inclusion criteria for patients were applied:

1. Age 18-75 years
2. Chronic degenerative MSD
3. Pain duration more than 1 year
4. Pain intensity VAS>4
5. Effective contraception
6. Accessibility for treatment and follow-up
7. Willingness to cooperate
8. Written consent
9. No participation in other studies (3 months before and during the participation in RAD - ON01 study)

The following exclusion criteria for patients were applied:

1. Pregnant or nursing women
2. Childbearing or procreative people, which are incapable to consequent contraception during the treatment
3. Heart failure >NYHA II or EF >40%
4. Not adjustable hypertonia >180/90 mmHg
5. Manifest, medicinal not adjustable hyperthyroidism
6. Acute inflammatory or consuming diseases
7. Lack of compliance with the protocol
8. Continuing misuse of drugs, alcohol or medicaments

9. Missing willingness to storage and handling of personal disease data in frame of study
10. Participation in other study at the same time

Clinical and diagnostic investigations

The patients were questioned and medically examined by a doctor before and then 6, 12, 18 and 30 weeks after treatment start. Pain duration was determined by evaluation of pain diaries filled out by patients. Peripheral blood was drawn at indicated time points, transported to the laboratory and possessed within 24 h. The serum samples were frozen at -80° C. In framework of this study, adipokines and marker of bone remodeling were measured in the blood serum using ELISA assays.

2.2 Cell culture

General conditions

Incubator conditions for all cell types were set to a relative humidity of 95%, 5% CO_2 and 37°C. Unless indicated otherwise, the medium change was performed two or three times a week. Detailed descriptions of the media composition see in the appendix (Tab. A.5). For cell count the automated cell counter TC20™ was used.

Primary human pre-adipocytes

Primary human pre-adipocytes were purchased from Lonza Ltd. These were isolated from subcutaneous fat of healthy donors. The cells were cultured in preadipocyte growth medium (Lonza Ltd.) until reaching the confluence. For the differentiation into adipocytes, appropriate supplements (Lonza Ltd.) including insulin, dexamethasone, 3-Isobutyl-1-methylxanthin (IBMX) and indomethacin were used. Medium was changed every second day. The first fat droplets were visible on day three after initiation of differentiation.

SGBS cells

Simpson-Golabi-Behmel syndrome (SGBS) pre-adipocytes were kindly provided by Prof. Dr. Wabitsch and Prof. Dr. Fischer-Posovszky, University Hospital Ulm. The SGBS cells were derived from subcutaneous fat of a patient with SGBS. Simpson-Golabi-Behmel syndrome is a rare congenital disorder, which is characterized by a pre- and postnatal overgrowth of several organs. Because of a high capacity for adipogenic differentiation, SGBS cells are an excellent tool for studies of adipocytes *in vitro* [68]. The protocol for cultivation and differentiation of cells, provided by Prof. Dr. Wabitsch, was strictly followed. Briefly summarized, the cells were grown in the DMEM/F12 medium, containing 10% FCS until reaching the confluence. To promote adipogenic differentiation, serum-free DMEM/F12 medium containing rosiglitazone, dexamethasone (DEX), IBMX, cortisol, transferrin, triiodotyronin (T3) and human insulin was added (day 0). After four days medium was replaced by medium containing cortisol, transferrin, triiodotyronin and human insulin (Fig. 2.1). The first fat droplets were visible on day three after initiation of differentiation. In order to perform experiments with undifferentiated pre-adipocytes, the growth medium containing 10% FCS without differentiation factors was used for cell cultivation.

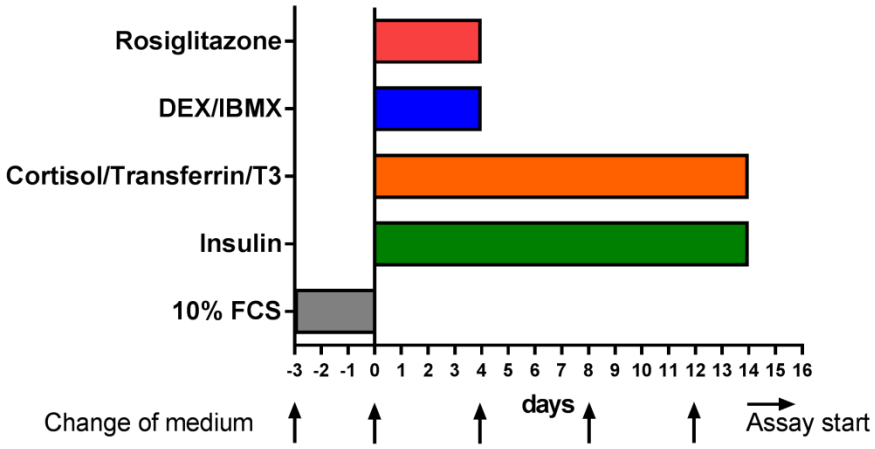

Fig. 2.1 Differentiation protocol for SGBS cells. Modified according to [68]

For the expansion of pre-adipocytes, different sizes of cell culture flasks and dishes were used. To achieve a high differentiation rate, primary human pre-adipocytes and SGBS pre-adipocytes were cultivated in Petri dishes (Ø 35 and 60 mm) or on chamber slides. For irradiation experiments, the pre-adipocytes were irradiated at confluence and differentiation medium was added immediately after irradiation (day 0).

Primary human synovial fibroblasts

Primary human normal synovial fibroblasts (NSF, obtained from knee joints of healthy donors) and rheumatoid arthritis synovial fibroblasts (RASF, obtained from knee joints of patients with rheumatoid arthritis) were purchased from Cell Applications. Furthermore, primary human synovial fibroblasts from two patients with rheumatoid arthritis were kindly provided by Prof. B. Suess and Dr. M. Saul, Technical University Darmstadt. Both cell types were cultivated identically in DMEM containing 10% FCS to reaching the confluence. Cultured synovial fibroblasts were used for experiments between passages 1-3, because in the higher passages the cell proliferation was decelerated.

2.3 Stimulation experiments

Dose- and time-dependent response of SF to visfatin stimulation

To evaluate the optimal concentration and duration for stimulation, the dose- and time-dependent effects of visfatin were investigated in a first step. Due to a shortage of NSF, experiments on dose and time-dependence were done only in RASF.

The cells were grown in CD35 dishes until reaching confluence. After rinsing the dishes with PBS, the cells were treated with different concentrations of human recombinant visfatin (100, 250, 500 and 1000 ng/ml, BioVendor) in serum-free DMEM. Unstimulated cells incubated in serum-free DMEM were served as control. Cell supernatants were collected and the cell number was determined at different time points (12, 24, 36 and 48 h). Dose- and time-dependent induction of cytokines IL-6 and IL-8 was detected using ELISA (eBioscience).

Adipokine stimulation of synovial fibroblasts for irradiation experiments

To examine the effect of X-ray irradiation on adipokine-induced release of inflammatory cytokines by human synovial fibroblasts, NSF and RASF were rinsed with PBS and then treated with human recombinant adiponectin (5 μg/ml, RayBiotech) or human recombinant visfatin (250 ng/ml, BioVendor). The treatment was performed in serum-free DMEM to avoid serum-dependent effects. The cells were irradiated with different doses of X-rays (0.5, 2 and 10 Gy) 24 h after the stimulation. Collection of the cell supernatants, isolation of RNA and the cell count were performed 24 h after irradiation (Fig. 2.2). Non-stimulated and non-irradiated NSF and RASF were used as controls. The medium was not changed during the experiment.

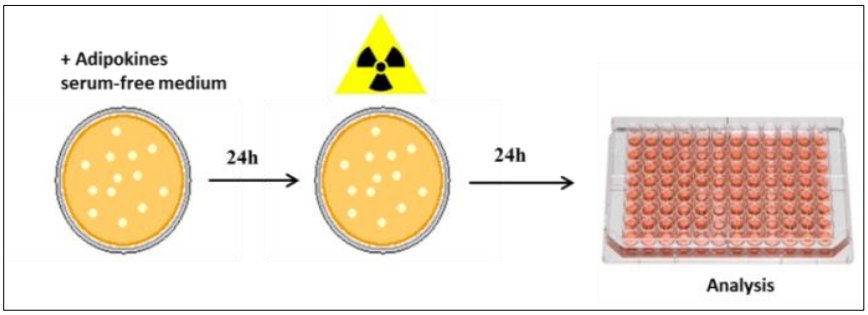

Fig. 2.2 Experimental setup for stimulation and irradiation of NSF and RASF

2.4 Irradiation experiments

All *in vitro* experiments were carried out with X-ray irradiation. Irradiation was performed on an X-ray tube (IV320-13, Seifert, Ahrensburg) with a tube voltage of 250 kV and a cathode current of 16 mA (Fig. 2.3). The cells were exposed at room temperature with an approximate dose rate of 1 Gy/min. The PTW-SN4 detector was used for dosimetry. The duration of total irradiation procedure including exposure and transport between incubator and the irradiation room was approximately 30 min. Non-irradiated cells (controls) were subjected to the same conditions as the irradiated samples (transport to the X-ray tube, room temperature during irradiation procedure etc.)

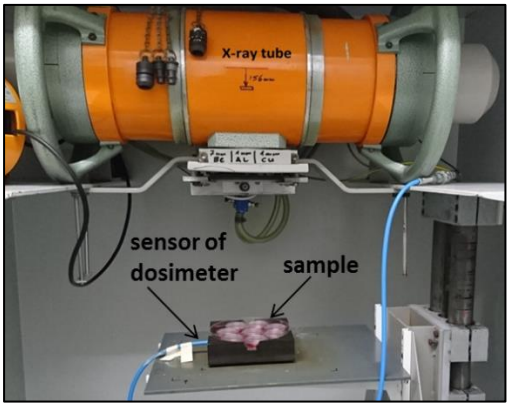

Fig. 2.3 X-ray irradiation of the samples. For the irradiation experiments samples and the sensor of dosimeter were placed below the exit window of X-ray tube

Clonogenic cell survival assay

The clonogenic survival assay is a common method to determine the reproductive ability (colony formation) of a single cell *in vitro*. Hence the cell survival curve shows the relationship between different doses of irradiation and the fraction of cells, which still retain their reproductive ability after treatment. To assess clonogenic survival, the cells were cultivated in T75 flasks, irradiated at subconfluence, trypsinized and seeded after appropriate dilutions (Tab. 2.1). After 14 days of cultivation, the cells were stained with methylene blue for 30 min and washed with distilled water. Colonies (> 50 cells) were counted using the light microscope. The experiments were done twice with three replicates for each dose and cell type.

Irradiation dose, Gy	RASF/75 cm2	NSF/75 cm2
0	100	100
	300	500
	500	1000
0.5	200	250
	500	500
	750	1000
1	500	500
	750	1000
	1000	1500
2	750	1000
	1000	1500
	1500	2000
5	1000	1500
	1500	2000
	2000	5000
10	1500	2000
	2000	5000
	5000	

Tab. 2.1 Seeding of synovial fibroblasts for clonogenic cell survival assay

2.5 Detection and analysis of adipocyte differentiation

Oil Red O staining of intracellular triglycerides

To determine triglyceride accumulation in adipocytes during differentiation, Oil Red O staining was used. Prior to staining, the cells were washed with PBS and fixed with 10% formalin for 30-60 min at room temperature. After removing the formalin and washing the cells with distilled water, 60% isopropanol was added for 2-5 min. Then the cells were stained with Oil Red O working solution (appendix, Tab. A.2) for 5 min at room temperature with slowly rotation. Finally, the cells were carefully washed with distilled water and the redly appearing lipids were directly visualized and imaged using a phase contrast microscope (Fig. 2.4).

DAPI staining

For visualization of the nuclei of adipocytes, DAPI staining was performed. For this, the cells were washed with distilled water after performing Oil Red O staining, and then DAPI working solution (1μg/ml) was added for 5 min. Afterwards, the cells were washed twice with PBS, few drops of Vectashield™ were added and the slides were covered with a glass cover slip. Blue appearing nuclei were detected at a wavelength of 340 nm.

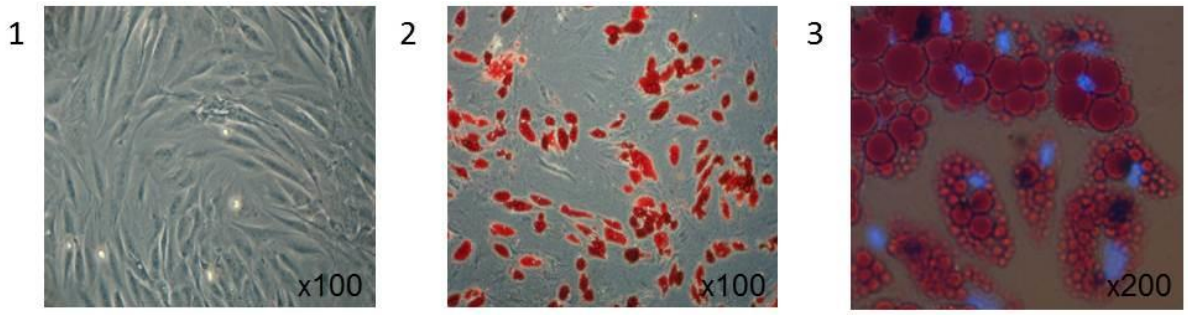

Fig. 2.4 Oil Red O and DAPI stained adipocytes. (1) Pre-adipocytes, (2) Mature adipocytes were stained with Oil Red O and (3) Mature adipocytes were stained with Oil red O and DAPI on day 20 after initiation of differentiation

Quantification of triglyceride accumulation

Lipid accumulation was quantified by the measurement of extracted Oil Red O. After staining with Oil Red O, the cells were carefully washed with distilled water and 4% triton X-100 (Sigma-Aldrich) in isopropanol was added to permeabilize the cells. The dishes were incubated 15 min at room temperature by gently shaking. Following Oil Red O extraction 100 μl per sample was transferred into a 96-well plate and the absorbance was measured at 540 nm using photometer ELx808 (Biotec).

Determination of adipocyte differentiation rate

To get an overview about the differentiation rate during the differentiation process, cells cultured on chamber slides were stained with Oil Red O and DAPI on defined days and 7 randomly selected areas per slide were analyzed. For this, all DAPI-stained cells and all Oil Red O- positive cells were counted and the differentiation rate was calculated as follows:

$$DR = \frac{n\,(Oil\ Red\ O)}{n\,(DAPI)} * 100\%$$

2.6 Enzyme-linked immunosorbent assay (ELISA)

To determine the released cytokine concentration of adipocytes and synovial fibroblasts in the cell supernatants, and the concentration of adipokines in the serum of patients, ELISA assays were performed. ELISA is an analytical biochemical assay, which is based on the specific interaction between antibody and corresponding antigen. The antibody binds antigen from the sample, which can then be photometrically detected by a color change of enzyme-coupled secondary antibody. The concentration of the dye is thereby proportional to the antigen concentration. Depending on the sensitivity of the ELISA (appendix, Tab. A.1) and cytokine concentration, the samples were used either undiluted or diluted according to the protocol. Manufacturer instructions were thereby strictly followed.

2.7 Quantitative real-time polymerase chain reaction (qPCR)

RNA isolation

RNA isolation was performed using RNeasy Mini Kit (Qiagen) according to the manufacturer instructions. The cells ($1x10^6$ - $1x10^7$) were trypsinized and centrifuged 8 min at 300 x g. The cell pellet was diluted in 350 μl buffer RLT and 350 μl 70% ethanol was added. Cell suspension was mixed and transferred directly into the RNeasy spin column and centrifuged 15 sec at 8000 x g. In the next step, the RNeasy spin column was washed with 700 μl of buffer RW1 15 sec at 8000 x g, followed by two washing steps with 500 μl of buffer RPE. In the last step, the RNeasy spin column was placed in a 1.5 ml collection tube, 50 μl RNase-free water was added and RNA was eluted by centrifugation 1 min at 8000 x g. The RNA concentration and purity were analyzed using Colibri Microvolume Spectrometer (Titertek Berthold). Because nucleic acids absorb light at 260 nm, and proteins and other contaminants – at 280 nm, the ratio 260/280 typically indicates purity of RNA or DNA. A 260/280 ratio of approximately 2.0 indicates a high purity of RNA, a ratio of approximately 1.8 is generally accepted as high purity for DNA.

cDNA synthesis by reverse transcription

The isolated RNA was converted to the complementary DNA (cDNA) using RevertAid™ First Strand cDNA Synthesis Kit (ThermoFisher Scientific) according to manufacturer instructions. For the cDNA synthesis, 1 μg of template RNA was used. Following reagents (Tab. 2.2) were added in a nuclease-free tube, mixed and incubated 60 min at 42°C. The reaction was stopped by heating at 70°C for 5 min. cDNA samples were stored at -80°C.

total RNA	1 µg
Oligo (dT)18 primer	1 µl
Water, nuclease-free	to 12 µl
5x Reaction Buffer	4 µl
RiboLock RNase Inhibitor (20 U/µl)	1 µl
10 mM dNTP Mix	2 µl
RevertAid M-MuLV RT (200 U/µl)	1 µl
Total volume	**20 µl**

Tab. 2.2 First strand cDNA synthesis mix

Quantitative real-time PCR (qPCR)

Real-time qPCR is a very sensitive method to increase extent of a target DNA sequence, which is directly related to the amount of fluorescence emitted from a reporter molecule. In this project, the SYBR® Green detection method was used. SYBR® Green dye binds to double-stranded DNA and the level of fluorescence increases only if it is bound to DNA. Measuring of fluorescence and thus quantification of the product is performed after each PCR cycle. Ct value shows thereby the cycle number, at which the fluorescence signal is over the baseline signal (threshold, Fig. 2.5A). Because SYBR® Green dye binds non-specifically any double strand DNA, including e.g. primer dimers, a melting curve analysis was performed to ensure that the target product was amplified. For this analysis, the temperature of reaction was increased to 95°C, whereas double-stranded DNA “melts” into single-stranded DNA, while incorporated dye dissociate from the DNA and the fluorescence level decreases. Changes in

fluorescence then are plotted against temperature for visualization of the melting dynamics (Fig. 2.5B).

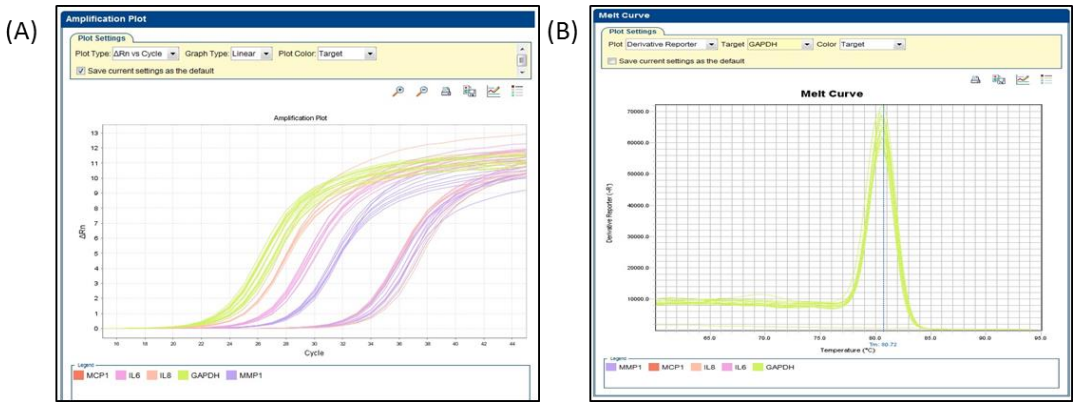

Fig. 2.5 A typical amplification plot and a melt curve of the SYBR® Green based qPCR. (A) Amplification of the target genes during one qPCR run. The normalized reporter value (ΔRn) is plotted against number of cycles (Ct value). (B) The melting curve of GAPDH. Change in fluorescence/ change in temperature (-R) is plotted against temperature

The qPCR reactions were performed using StepOnePlus real-time PCR System (Applied Biosystems), thereby comparative Ct (ΔΔCt) method with melting curve analysis was applied. It is a standard method to analyze relative changes in the gene expression. For performing ΔΔCt method, a suitable endogenous control and a reference sample are required. Comparison of the Ct value of a target gene with that of the endogenous control, which is constantly expressed in all samples, allows normalization of the gene expression level to the general amount of the cDNA. The expression in the samples is then compared to the expression in the reference sample. In this project, the housekeeping gene GAPDH was served as an endogenous control and the non-irradiated samples – as a reference. "QuantiTect SYBR® Green PCR Kit" and "QuntiTect Primer Assay" were purchased from Qiagen. The components of one PCR reaction and the cycling conditions are listed in Tab. 2.3 and Tab. 2.4.

Water, nuclease-free	18 µl
SYBR® Green MasterMix	25 µl
Primer (QuantiTect Primer Assay)	5 µl
cDNA	2 µl (100 ng)
Total volume	**50 µl**

Tab. 2.3 PCR reaction mix

Step	**Time**	**Temperature**	**Comments**
PCR initial activation step	15 min	95ºC	Activation of HotStarTaq DNA polymerase
3-step cycling:			
Denaturation	15 sec	94ºC	
Annealing	30 sec	55ºC	
Extension	30 sec	72ºC	Fluorescence data collection
Number of cycles	40		

Tab. 2.4 PCR cycling conditions

2.8 Statistical analysis

Statistical analysis was performed using two-tailed t-test after checking for normal distribution with D'Agostino and Pearson test. For the not normally distributed data, the Wilcoxon matched pairs signed rank test was used (GraphPad Prism 6). The results were estimated as significant for $p < 0.05$. For evaluating of relationships, Pearson's and Spearman's correlations were used for normal and non-normal distributions.

3 Results

Adipokines are cytokines, specifically produced by adipose tissue [157]. In this thesis, the impact of a radon treatment on adipokine serum levels in patients with MSD was investigated. Adipokines have been shown to contribute to the inflammation and joint destruction in RA and OA by induction of IL-6, IL-8 and MMP-1 in synovial fibroblasts. Thus, they are considered as potential targets for the therapy of rheumatoid arthritis and other MSD [158], [159]. Since in this thesis it was hypothesized that low-dose radiation affects the adipokine-induced inflammation in arthritic joints, the response of human synovial fibroblasts to adipokine stimulation and its modulation by radiation exposure were investigated (*in vitro*). To clarify whether ionizing radiation can affect the release of adipokines by cells of adipose tissue, the radiation response of adipocytes was investigated (*in vitro*).

3.1 Investigations of serum levels of adipokines in radon-treated patients (Patient study RAD-ON01)

The main goal of this prospective study (RAD-ON01) was to investigate a putative anti-inflammatory effect of radon exposure and the impact on the immune- and skeletal system of patients suffering from musculoskeletal diseases. To examine possible changes in the release of adipokines after radon spa treatment, the serum levels of visfatin, adiponectin, leptin and resistin have been measured in the serum of MSD patients. Furthermore, the relationship between adipokine levels and age, gender and pain duration (indicated by patients) was analyzed. The patient characteristics are summarized in Rühle et al [140].

Visfatin serum levels

Visfatin is an adipokine, which is closely related to synovial inflammation in the patients with MSD. Increased circulating visfatin levels have been found in patients with RA and OA [94], [160]. Since it is known that elevated visfatin levels positively correlate with disease activity, this adipokine is considered to be a therapeutic target for the rheumatoid arthritis and other MSD [99], [161].

As shown in Figure 3.1A, the results demonstrated a significant decrease of visfatin serum levels, which occurs 12 weeks after therapy start and still persist at the end of follow-up period (30 weeks). The mean level of visfatin before treatment (0 weeks) was 2.31 ng/ml (0.433 – 8.39 ng/ml, n=44) and at 30 weeks after treatment start – it was 1.26 ng/ml (0.265 – 4.65 ng/ml, n=44). Serum concentrations of visfatin were significantly higher in males than in females (Fig. 3.1B). No significant correlations between the visfatin levels and the age of the patients were found (Fig. 3.1C). Visfatin levels were significantly lower in the serum of patients, which have reported less pain at indicated time points (Fig. 3.1D).

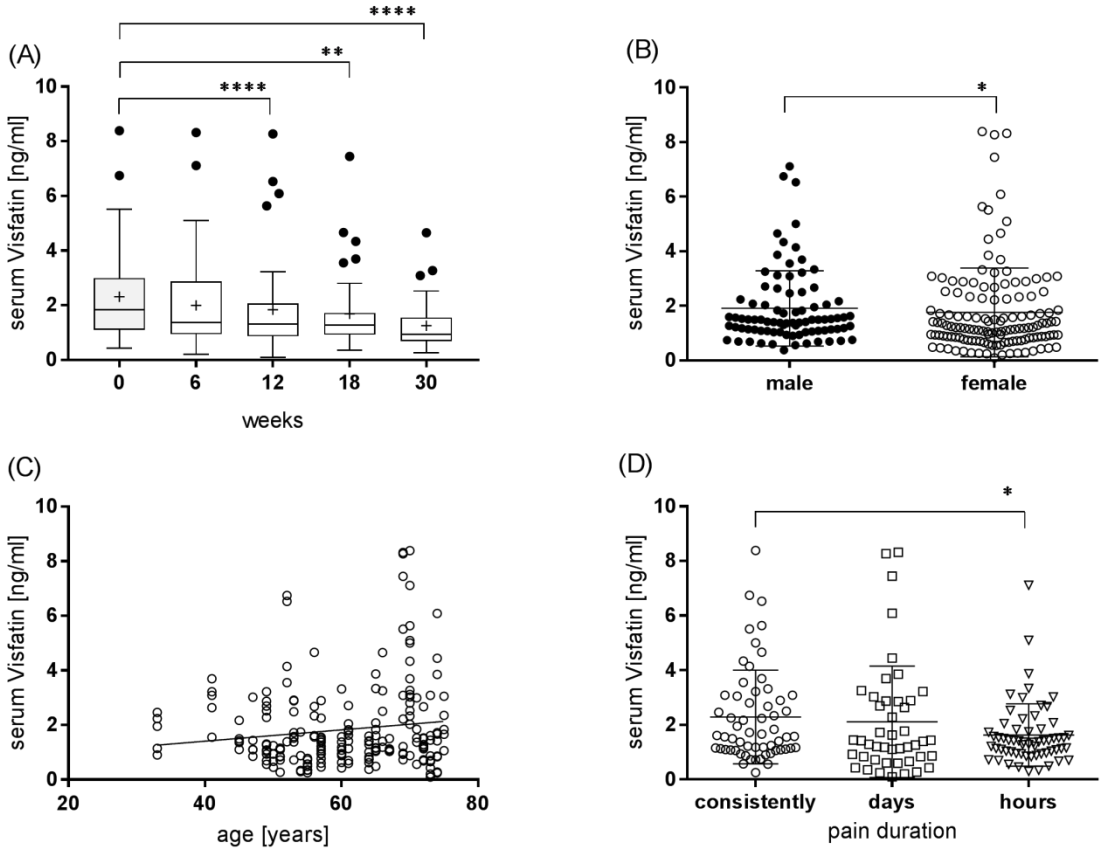

Fig. 3.1 Effect of radon treatment on the serum visfatin levels and the relationship between visfatin levels and gender, age and pain duration in patients with MSD. (A) The concentration of visfatin in serum was measured at the indicated weeks before (0 weeks) and after onset of the therapy (6-30 weeks). Boxplots show the median, Tukey whiskers (median ± 1.5 times interquartile range), mean (+) and outliers (•). (B) Comparison of visfatin serum concentrations between male and female patients. (C) Correlation of serum visfatin concentrations to the age of patients. (D) Association between serum visfatin levels and pain duration. N=44, (A) **p ≤ 0.01, ****p ≤ 0.0001, Wilcoxon matched-paired signed rank test. (B), (D) *p ≤ 0.05, Mann Whitney test

Adiponectin serum levels

Adiponectin is one of the most widely studied adipokines. Levels of adiponectin are higher in serum and synovial fluid of patients with RA in comparison to healthy donors. Moreover, they have been found to correlate with disease activity and rheumatoid factor RF [158], [162]. Adiponectin is suggested to support the inflammatory and joint destructive processes in RA.

The analysis of adiponectin showed no significant changes over 30 weeks (Fig. 3.2A). The mean level of adiponectin was 9.85 μg/ml (9.65 μg/ml at 0 weeks, 10.49 μg/ml at 30 weeks). Serum concentrations of adiponectin were significantly higher in females than in males (Fig. 3.2B). No significant correlations between adiponectin levels and age (Fig. 3.2C) or pain duration (Fig. 3.2D) were found.

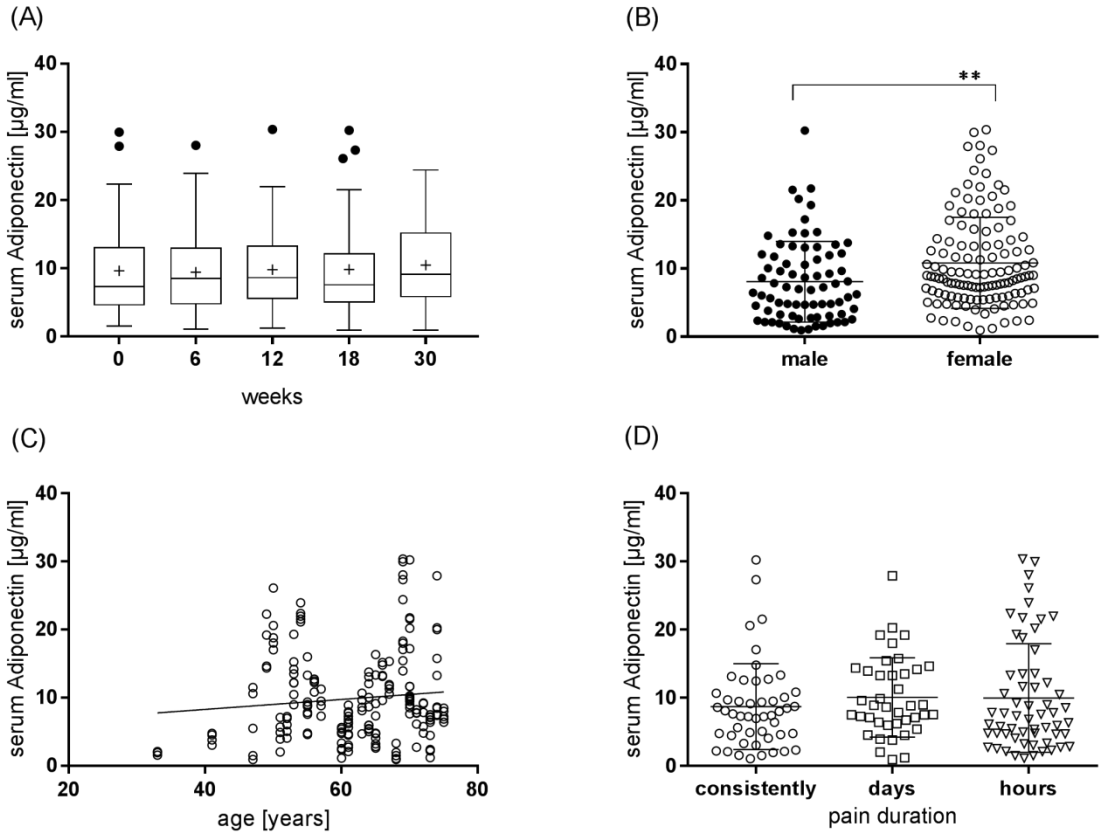

Fig. 3.2 Effect of radon treatment on the serum adiponectin levels and the relationship between adiponectin levels and gender, age and pain duration in patients with MSD. (A) The concentration of adiponectin in serum was measured at the indicated weeks before (0 weeks) and after onset of the therapy (6-30 weeks). Boxplots show the median, Tukey whiskers (median ± 1.5 times interquartile range), mean (+) and outliers (•). (B) Comparison of adiponectin serum concentrations between male and female patients. (C) Correlation of serum adiponectin concentrations to the age of patients. (D) Association between serum adiponectin levels and pain duration. N=39, **p ≤ 0.01 Mann Whitney test

Leptin and resistin serum levels

The adipokines leptin and resistin are generally considered as pro-inflammatory cytokines and play a potential role in MSD, in particular in RA. Serum levels of both adipokines were shown to be elevated in serum of RA patients [86], [94], [158]. However, their role in RA remains unclear.

The serum levels of leptin and resistin were not significantly changed after radon treatment (Fig. 3.3A, 3.4A). The mean level of leptin was 24.2 ng/ml over the follow-up period (22.28 ng/ml at 0 weeks, 23.08 ng/ml at 30 weeks), of resistin – 4.45 ng/ml (4.2 ng/ml at 0 weeks, 4.33 at 30 weeks). Serum concentrations of leptin were significantly higher in females than in males (Fig. 3.3B). In contrast, no gender-specific differences were found for resistin serum levels (Fig. 3.4B). No correlations were found between serum levels and age or pain duration neither for leptin, nor for resistin (Fig. 3.3C, D, 3.4C, D).

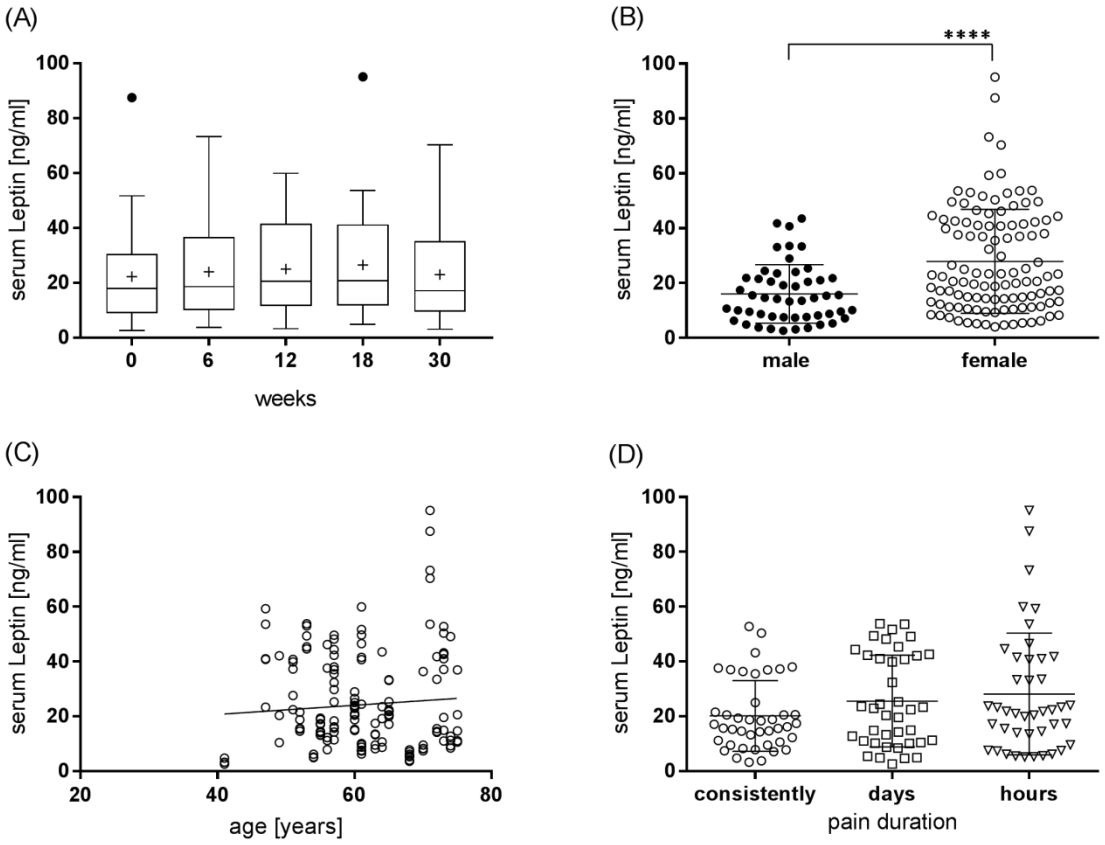

Fig. 3.3 Effect of radon treatment on the serum leptin levels and the relationship between leptin levels and gender, age and pain duration in patients with MSD. (A) The concentration of leptin in serum was measured at the indicated weeks before (0 weeks) and after onset of the therapy (6-30 weeks). Boxplots show the median, Tukey whiskers (median ± 1.5 times interquartile range), mean (+) and outliers (•). (B) Comparison of leptin serum concentrations between male and female patients. (C) Correlation of serum leptin concentrations to the age of patients. (D) Association between serum leptin levels and pain duration. N=32, ****p ≤ 0.0001 Mann Whitney test

3.2 Radiation-induced changes in human synovial fibroblasts

Rheumatoid arthritis synovial fibroblasts (RASF) play an essential role in the pathogenesis of rheumatoid arthritis. They contribute to inflammation and joint destruction by inducing the expression of pro-inflammatory and matrix-degrading factors in different cell types of synovia. Little is known about effects of ionizing radiation on human synovial fibroblasts. One of the aims of this thesis was to compare NSF and RASF in their response to radiation and to adipokine stimulation.

3.2.1 Proliferation of human synovial fibroblasts after irradiation

The effect of X-ray radiation on the proliferation of human synovial fibroblasts was investigated over a period of 28 days. Thereby NSF and RASF were compared. In the experiments presented here, the cell numbers of irradiated synovial fibroblasts, both NSF and RASF, were dose-dependent decreased compared to the control (Fig. 3.5). However, the changes were more pronounced for NSF compared to RASF. While the proliferation of 2 Gy-irradiated NSF has slowed down considerably from day 10 on, and proliferation of 10 Gy-irradiated NSF was completely inhibited over the all period of observation, the RASF showed only the slight trend towards reduced proliferation after irradiation.

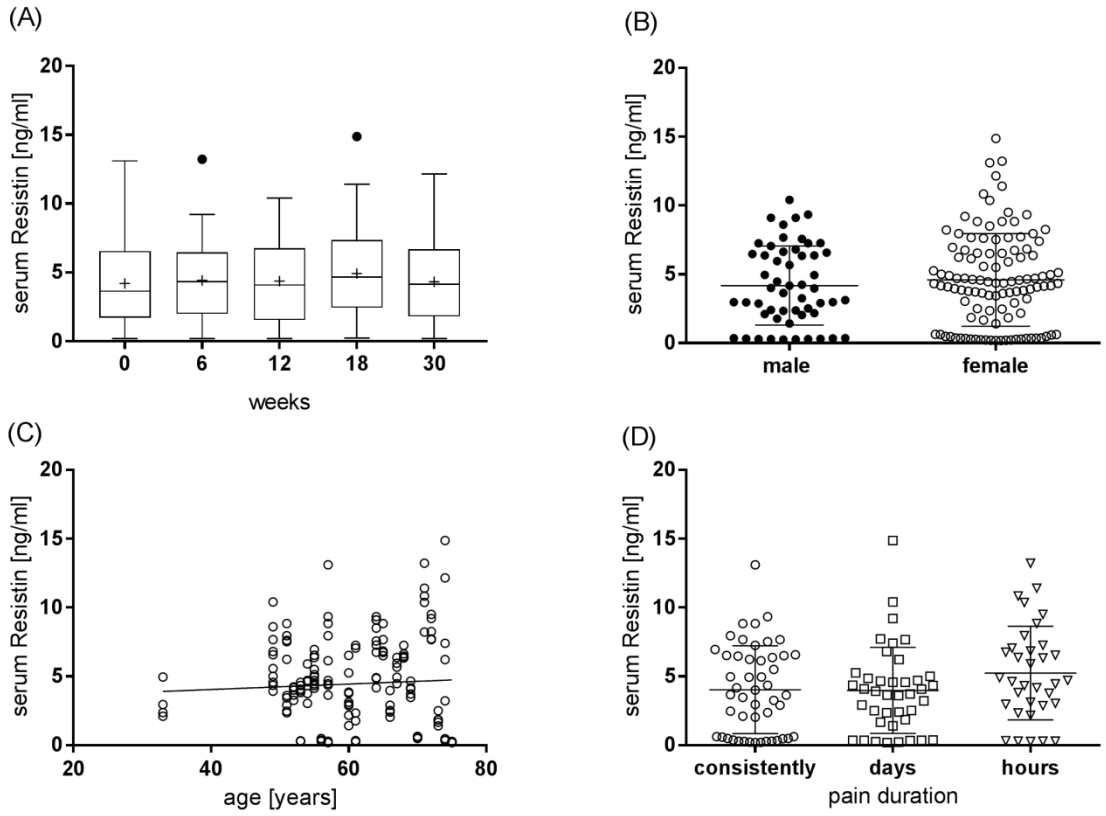

Fig. 3.4 Effect of radon treatment on the serum resistin levels and the relationship between leptin levels and gender, age and pain duration in patients with MSD. (A) The concentration of resistin in serum was measured at the indicated weeks before (0 weeks) and after onset of the therapy (6-30 weeks). Boxplots show the median, Tukey whiskers (median ± 1.5 times interquartile range), mean (+) and outliers (•). (B) Comparison of resistin serum concentrations between male and female patients. (C) Correlation of serum resistin concentrations to the age of patients. (D) Association between serum resistin levels and pain duration. N=33

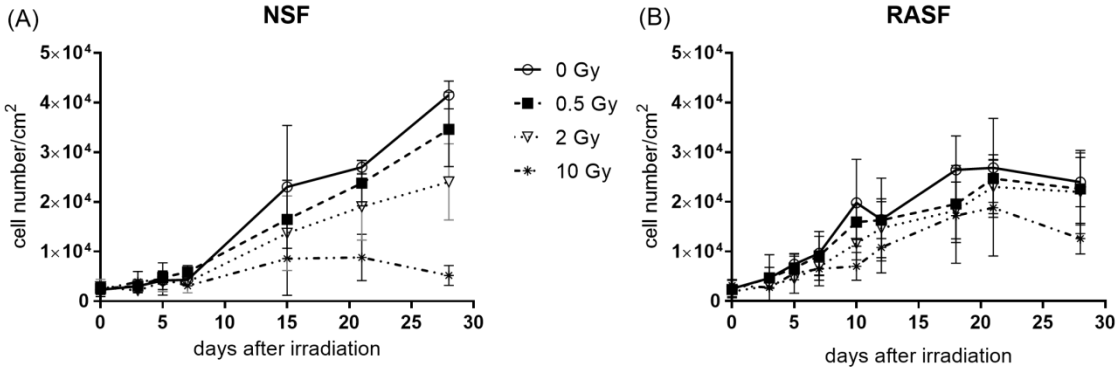

Fig. 3.5 Proliferation of human synovial fibroblasts after X-ray irradiation. (A) Synovial fibroblasts from healthy donors (NSF) and (B) from patients with rheumatoid arthritis (RASF) were irradiated with 0.5, 2 and 10 Gy X-rays and cultivated over 28 days. Cell counting was performed at regular intervals. Mean, SEM. N=3 for NSF, N=4 for RASF

3.2.2 Clonogenic survival of human synovial fibroblasts after irradiation

In addition to proliferation, clonogenic survival was determined to evaluate whether radiation affects an aggressive proliferative capacity of RASF, contributing to attenuation of inflammation in the joint.

As a first step, the plating efficiency of human synovial fibroblasts was measured. Although the cells were seeded at different cell densities (see chapter 2.4) and different types of culture flasks (T25 and T75) were used, the plating efficiency was found to be around 7% for NSF as well as for RASF. In the next step, the clonogenic cell survival assay was performed. The analysis of this assay showed that neither NSF nor RASF were able to form the colonies after irradiation (a colony is defined to consist of at least 50 daugter cells). Typical colonies were only found in the unirradiated samples (NSF) (Fig. 3.6-1). For the cells (NSF) irradiated with 0.5 Gy, few small (<50 cells) cell "crowds" were observed (Fig. 3.6-2). In samples irradiated with 1, 2, 5 or 10 Gy only single cells were present (Fig. 3.6-3).

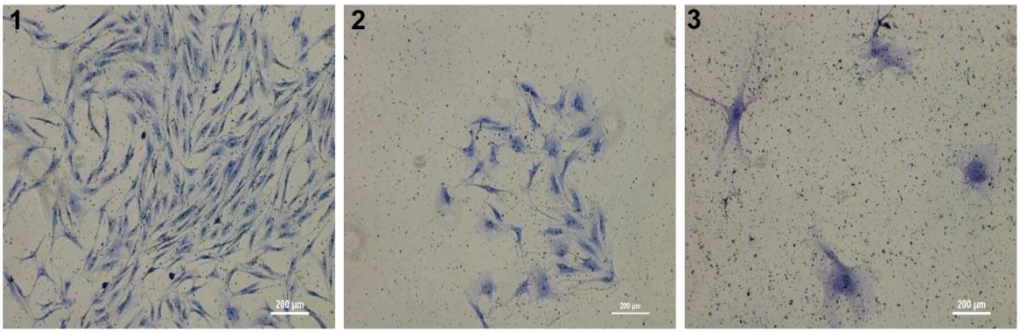

Fig. 3.6 Morphology of NSF as observed in the clonogenic cell survival assay. The cells were cultivated 14 days after irradiation with X-rays and stained with methylene blue. (1) Typical colony of NSF (control), (2) Cell "crowd" after exposure to 0.5 Gy X-rays and (3) Cells after irradiation with 5 Gy of X-rays

3.3 Radiation-induced changes in the response of human synovial fibroblasts to the adipokine stimulation

Adipokines such as adiponectin and visfatin are known to exert a pro-inflammatory influence on RASF. In order to investigate the effects of adipokines on NSF, and to compare them with the effects on RASF, both cell types (NSF and RASF) were treated with the corresponding recombinant adipokine for 48 h. The release and gene expression of the inflammatory cytokines (IL-6, IL-8, MCP-1) as well as matrix-degrading enzymes (MMP-1, MMP-3) were analyzed. In addition, release and gene expression of adiponectin, adiponectin receptor (AdipoR) and visfatin were examined.

To evaluate whether irradiation of human synovial fibroblast affects the adipokine-induced release of above-mentioned pro-inflammatory cytokines and matrix-degrading enzymes, NSF and RASF were treated with the corresponding adipokine 24 h prior to X-ray irradiation. Cell supernatants were collected 24 h after irradiation and release of IL-6, IL-8, MCP-1 and MMP-1 was analyzed. In addition, gene expression analysis of mentioned factors was performed to verify results obtained from ELISA assays (appendix, Fig. A.1, A.2).

3.3.1 Effect of adiponectin on human synovial fibroblasts

To evaluate the effects of adiponectin on human synovial fibroblasts, NSF and RASF were treated with 5 μg/ml adiponectin in serum-free medium for 48 h. Levels of IL-6, IL-8, MCP-1 as well as MMP-1 and MMP-3 were determined in cell supernatants and compared between NSF and RASF. Results obtained for the release were approved by gene expression analysis. The concentration of 5 μg/ml was chosen according to the literature. An additional experiment on time-dependent effects of adiponectin showed that the strongest induction of IL-6 is reached 48 h after treatment (appendix, Tab. A.8). Therefore, these parameters (5 μg/ml; 48 h) were chosen for subsequent experiments.

Adiponectin-induced changes in release and expression of pro-inflammatory factors by human synovial fibroblasts

As shown in Fig. 3.7, adiponectin increased the release of IL-6, IL-8 and MCP-1 in both cell types (NSF and RASF); however, the effect was stronger in RASF. For example, after treatment with 5 μg/ml adiponectin the IL-6 level in NSF was two- and in RASF - almost nine-fold increased (Fig. 3.7A). Similarly, IL-8 concentration was eight-fold higher in NSF and nineteen-fold higher in RASF after treatment with the same concentration of adiponectin (Fig. 3.7B). MCP-1 levels were two-fold (NSF) and three-fold (RASF) higher compared to the basal concentrations (Fig. 3.7C). Noteworthy, the basal levels of stated cytokines do not differ significantly between NSF (Mean ± SEM, pg/10^4 cells: 1.38 ± 0.5 (IL-6); 1.4 ± 0.09 (IL-8); 38.8 ± 6.5 (MCP-1)) and RASF (Mean ± SEM, pg/10^4 cells: 2.09 ± 1.09 (IL-6); 3.18 ± 2.1 (IL-8); 23.6 ± 8.4 (MCP-1)). Although clear differences in the release of IL-6 and IL-8 were observed in all samples after stimulation with adiponectin, the statistical significance could not be reached. Details on the changes in the cytokine release by NSF and RASF are listed in Tab. 3.1.

In contrast, the fold change of MMP-1 level was greater in NSF (nine-fold) than in RASF (six-fold, Fig. 3.8A) after stimulation with adiponectin. No marked differences were observed for MMP-3 concentrations in both cell types. With respect to the basal levels of matrix-degrading enzymes, RASF exhibited higher concentration of MMP-3 than NSF (Mean ± SEM, pg/10^4 cells: 60.7 ± 3.03 (NSF); 122.03 ± 16.9 (RASF)) and mostly the same level of MMP-1 (Mean ± SEM, pg/10^4 cells: 15.1 ± 9.8 (NSF); 26.7 ± 12.9 (RASF)).

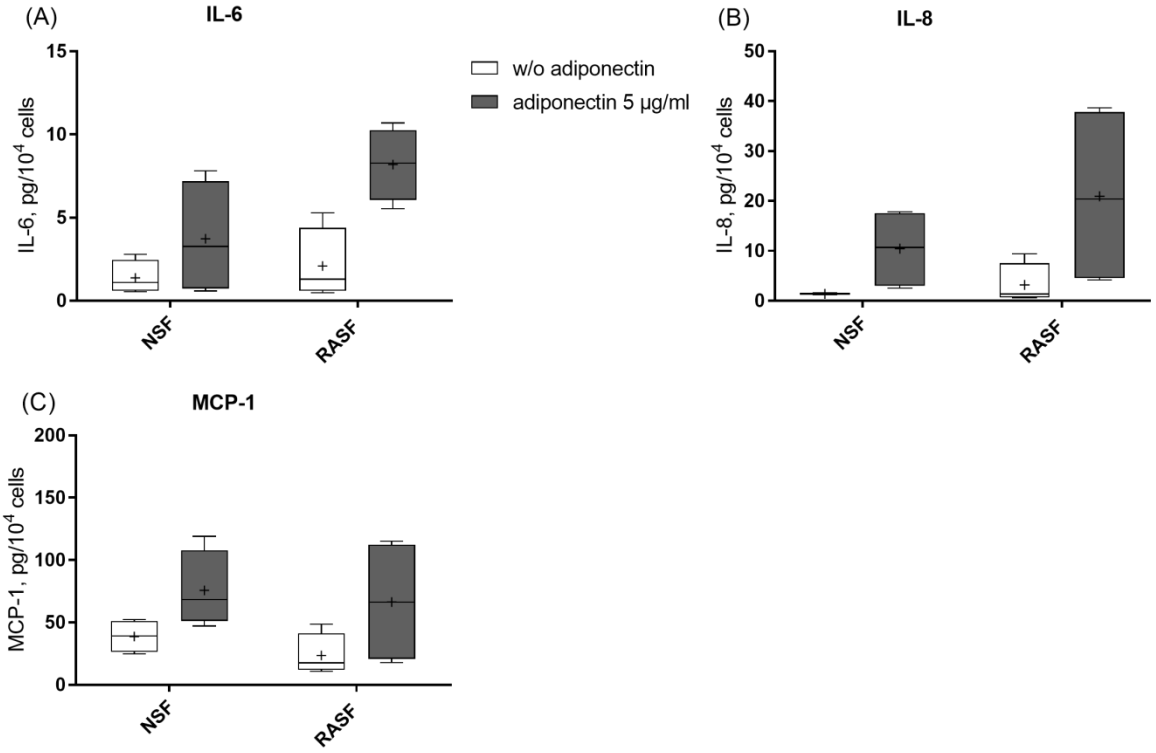

Fig. 3.7 Effect of adiponectin on cytokine release by human synovial fibroblasts. Synovial fibroblasts from healthy donors (NSF) and from patients with rheumatoid arthritis (RASF) were treated or not with 5 µg/ml adiponectin for 48 h. Amounts of (A) IL-6, (B) IL-8 and (C) MCP-1 in cell supernatants were measured using ELISA assay. Boxplots show the median, Tukey whiskers (median ± 1.5 times interquartile range) and mean (+). N=4

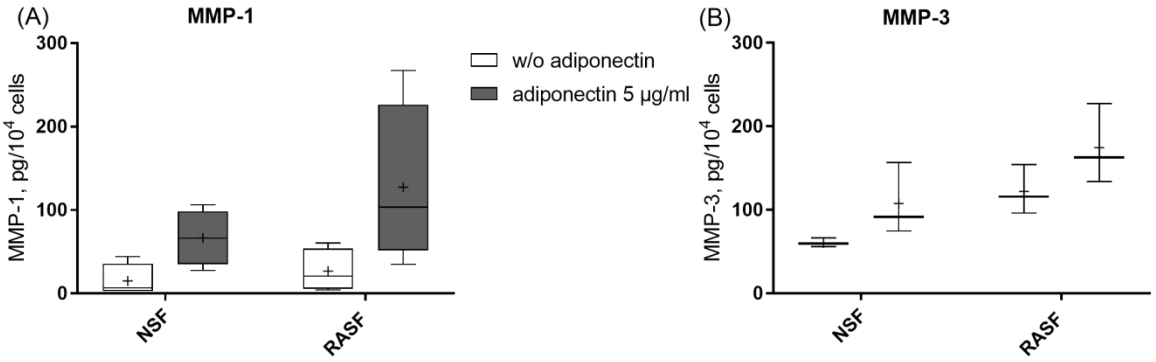

Fig. 3.8 Effect of adiponectin on MMP-1 and MMP-3 release by human synovial fibroblasts. Synovial fibroblasts from healthy donors (NSF) and from patients with rheumatoid arthritis (RASF) were treated or not with 5 µg/ml adiponectin for 48 h. Amounts of (A) MMP-1 and (B) MMP-3 in cell supernatants were measured using ELISA assay. Boxplots show the median, Tukey whiskers (median ± 1.5 times interquartile range) and mean (+). N=4 for MMP-1, N=3 for MMP-3

In addition to the analysis of the cytokine release in NSF and RASF, the gene expression profiles of both cell types were examined (Fig. 3.9). Considering the strong induction of cytokines IL-6, IL-8 and matrix-degrading enzyme MMP-1, the focus was set on the gene expression of these factors and the analysis was performed using quantitative real-time PCR. The results confirm the strong upregulation of genes, coding for IL-6, IL-8 and MMP-1. No significant differences were found for MCP-1 and MMP-3. The expression of adiponectin

receptor 1 (AdipoR) and visfatin remained as well unchanged. It is notable that the fold-changes of all analyzed genes were almost the same for NSF and RASF (Tab. 3.1).

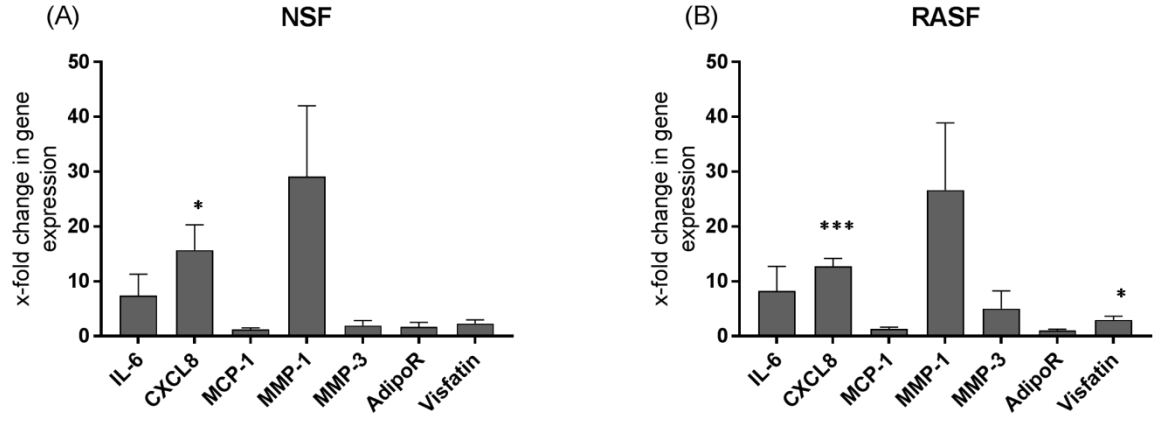

Fig. 3.9 Regulation of gene expression in human synovial fibroblasts by adiponectin. Expression of stated genes in (A) NSF and (B) RASF was analyzed using qPCR. The data are normalized to GAPDH gene expression and to the gene expression of untreated control. Bars indicate fold change induction after 48 h treatment with 5 µg/ml adiponectin. Mean, SEM. N=4, *p ≤ 0.05, ***p ≤ 0.001, two-tailed t-test

	NSF		RASF	
Name	**Fold change**	**Fold change**	**Fold change**	**Fold change**
	ELISA	**qPCR**	**ELISA**	**qPCR**
IL-6	2.29±0.6	7.4±3.85	9.14±4.5	8.25±4.49
IL-8 (CXCL8)	8.02±3.36	15.6±4.02	19.05±15.1	12.7±1.25
MCP-1	2.04±0.35	1.23±0.27	3.67±1.98	1.31±0.32
MMP-1	9.99±4.32	29.14±12.89	6.78±1.91	26.65±12.25
MMP-3	1.8±0.42	1.93±0.93	1.52±0.36	5.0±3.28
AdipoR		1.71±0.78		1.04±0.24
Visfatin		2.3±0.67		2.98±0.66

Tab. 3.1 Changes in cytokine and gene expression profiles of NSF and RASF after treatment with adiponectin. Mean ± SEM

3.3.2 Effect of visfatin on human synovial fibroblasts

Apart from investigations on adiponectin effects, the influence of another adipokine - visfatin - on human synovial fibroblasts was examined. For this, NSF and RASF were treated with 250 ng/ml of visfatin in serum-free medium for 48 h. Levels of IL-6, IL-8, MCP-1 and MMP-1 were determined in cell supernatants and compared between NSF and RASF. Results obtained for the release were approved by gene expression analysis. To choose the optimal dose and treatment duration, first, the dose- and time dependency of visfatin effects were investigated. Due to a shortage of NSF, experiments on dose and time-dependence were done only in RASF.

Dose- and time-dependent effects of visfatin on human synovial fibroblasts

To assess the right parameter for further experiments, RASF were treated with different concentrations of visfatin (100; 250; 500 and 1000 ng/ml) in serum-free medium. Dose- and

time-dependent production of IL-6 and IL-8 was evaluated in the cell supernatants at different time points (12, 24, 36 and 48 h) after treatment (Fig. 3.10). The results showed that the concentration of 250 ng/ml visfatin causes a strong induction of both cytokines and the highest effect on the cytokine release was observed 48 h after treatment. For example, the basal concentration of IL-6 was increased from 0.56 pg/10^4 cells to 310.2 pg/10^4 cells at 12 h and 1345.5 pg/10^4 cells at 48 h (Fig. 3.10A). Therefore, these parameters (250 ng/ml; 48 h) were chosen for all of the following experiments.

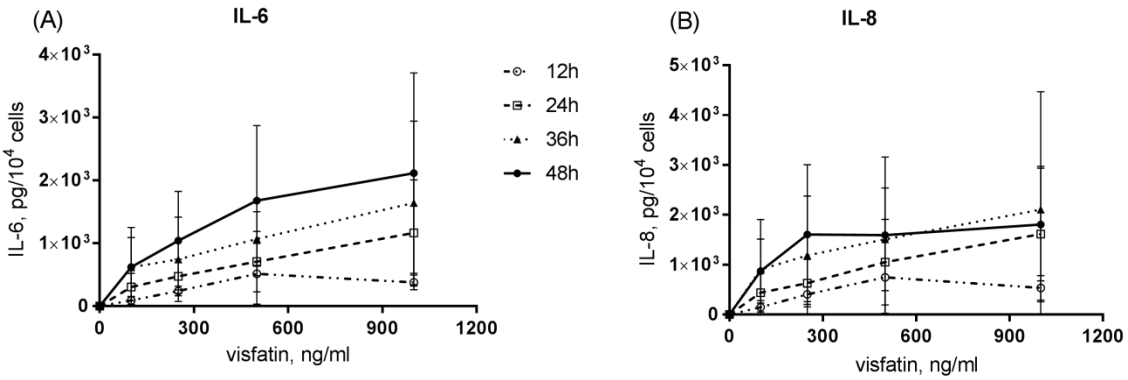

Fig. 3.10 Dose- and time-dependent effects of visfatin on synovial fibroblasts. Synovial fibroblasts from patients with rheumatoid arthritis (RASF) were treated or not with different concentrations of visfatin (100, 250, 500 and 1000 ng/ml). Amounts of (A) IL-6 and (B) IL-8 in cell supernatants were measured using ELISA assay at different time points (12, 24, 36, 48 h). Mean, SD. N=2, n=4

Visfatin-induced changes in release and expression of pro-inflammatory factors by human synovial fibroblasts

As depicted in Fig. 3.11, visfatin has a very strong influence on synovial fibroblasts. For example, after treatment with 250 ng/ml visfatin, the IL-6 level in NSF was increased from 1.48 ± 0.38 pg/10^4 cells to 89.09 ± 13.1 pg/10^4 cells and in RASF – from 1.05 ± 0.48 pg/10^4 cells to 110.47 ± 94.6 pg/10^4 cells (Fig. 3.11A). Similarly, MMP-1 levels increased from 9.8 ± 3.02 pg/10^4 cells to 161.1 ± 40.5 pg/10^4 cells (NSF) and from 9.8 ± 0.11 pg/10^4 cells to 151.0 ± 59.5 pg/10^4 cells (RASF) (Fig. 3.11D). Taken into account the wide variations between single experiments, the results suggest that the effect of visfatin with regard to induction of IL-6, IL-8, MCP-1 and MMP-1 is nearly the same on both NSF and RASF. Details on the changes in the cytokine release by NSF and RASF are listed in Tab. 3.2.

As a next step, the gene expression analysis of factors listed above was performed (Fig. 3.12). The results confirmed a strong visfatin-induced induction of genes coding for IL-6 (90-fold higher for NSF and 60-fold higher – for RASF) and IL-8 (278- and 245-fold respectively). Also levels of MCP-1, MMP-1 and MMP-3 were appreciably higher compared to the basal concentrations. However, the gene expression of adiponectin and visfatin remained unchanged. Similar to adiponectin-induced changes, the changes in the expression of all analyzed genes caused by visfatin treatment were almost the same for NSF and RASF (Tab. 3.2).

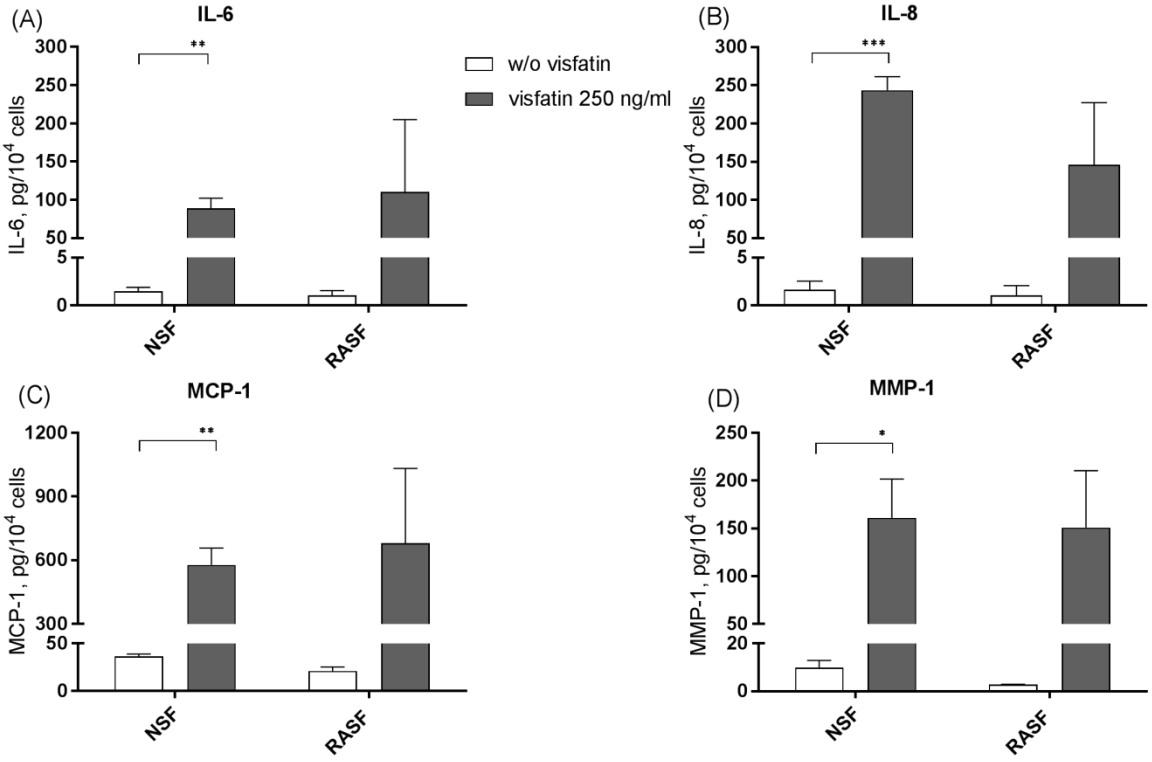

Fig. 3.11 Effect of visfatin on human synovial fibroblasts. Synovial fibroblasts from healthy donors (NSF) and from patients with rheumatoid arthritis (RASF) were treated or not with 250 ng/ml visfatin for 48 h. Amounts of (A) IL-6, (B) IL-8, (C) MCP-1 and (D) MMP-1 in cell supernatants were measured using ELISA assay. Mean, SEM. N=3, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, two-tailed t-test

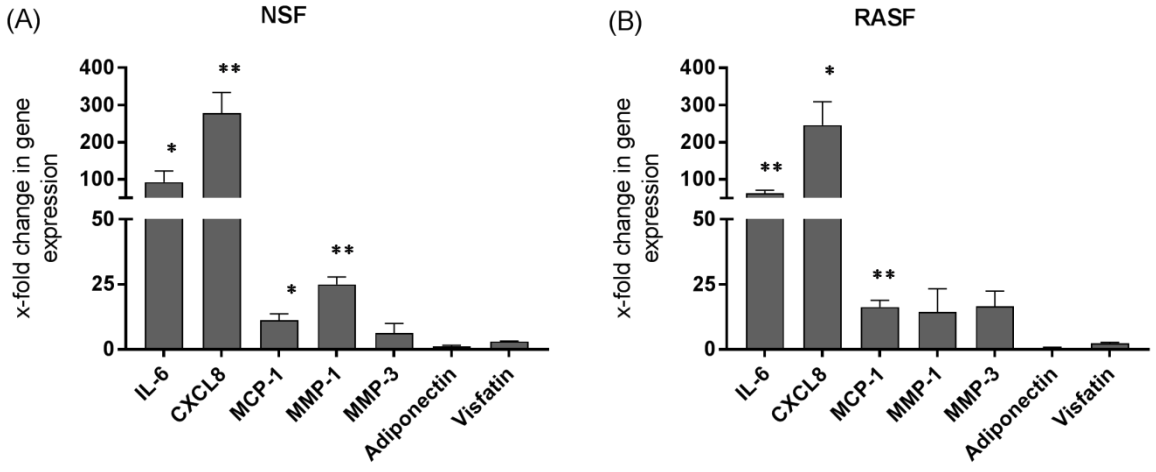

Fig. 3.12 Regulation of gene expression in human synovial fibroblasts by visfatin. Expression of stated genes in (A) NSF and (B) RASF was analyzed using qPCR. The data are normalized to GAPDH gene expression and to the gene expression of untreated control. Bars indicate fold change induction after 48 h treatment with 250 ng/ml visfatin. Mean, SEM. N=3, *p ≤ 0.05, **p ≤ 0.01, two-tailed t-test

	NSF		RASF	
Name	**Fold change**	**Fold change**	**Fold change**	**Fold change**
	ELISA	**qPCR**	**ELISA**	**qPCR**
IL-6	72.8±25.8	92.8±29.8	252.5±239.2	63.2±13.3
IL-8 (CXCL8)	142.8±37.6	278.2±55.2	324.39±179.79	245.7±63.0
MCP-1	15.84±1.73	11.2±2.4	41.02±27.2	16.25±2.6
MMP-1	16.8±1.29	24.9±2.8	43.96±21.6	14.47±8.8
MMP-3		6.35±3.6		16.66±5.7
Adiponectin		1.18±0.42		0.63±0.21
Visfatin		3.02±0.19		2.41±0.34

Tab. 3.2 Changes in cytokine and gene expression profiles of NSF and RASF after treatment with visfatin. Mean ± SEM

3.3.3 Radiation-induced changes in the response of human synovial fibroblasts to the adipokine treatment

RASF are characterized by a highly pro-inflammatory phenotype. A variety of cytokines and matrix-degrading enzymes produced by activated RASF mediates inflammation process in the joint, contributes to the destruction of cartilage and bone, recruits more inflammatory cells and enhances the inflammatory state of RASF [37]. Adipokines such as adiponectin or visfatin are known to increase the production of several cytokines including IL-6 and IL-8 by synovial fibroblasts. For the treatment of rheumatoid arthritis, targeting of the pro-inflammatory properties of this cells is therefore of special interest.

In this chapter, the impact of ionizing radiation on human synovial fibroblast with regard to production of pro-inflammatory cytokines IL-6, IL-8 and MCP-1 and matrix-degrading enzyme MMP-1 was investigated. The special focus was set on the radiation-induced changes in response of the cells to the treatment with adiponectin or visfatin.

In the Fig. 3.13 and 3.14, the effects of X-rays radiation on the adiponectin-induced release of IL-6, IL-8, MMP-1 and MCP-1 by human synovial fibroblasts are shown. Furthermore, Fig. 3.15 and 3.16 demonstrate the radiation-induced changes in the release of these factors in the visfatin-treated synovial fibroblasts. NSF and RASF were treated with the corresponding adipokine 24 h prior to irradiation with X-rays. Cell supernatants were collected 24 h after irradiation and amounts of above-mentioned factors were measured. Radiation effects on 1) cells pretreated with either adiponectin or visfatin and 2) untreated cells were determined and compared between NSF and RASF.

First, the radiation effects on the untreated cells were evaluated. Notably, the human synovial fibroblasts turned out not to be affected by irradiation with regard to the cytokine release. No significant changes for both NSF and RASF were detected, even after the exposure to 10 Gy X-rays. For example, the mean level of IL-6 for the unirradiated NSF was 1.38 ± 0.5 pg/10^4 cells and for 10 Gy irradiated cells - 1.31 ± 0.13 pg/10^4 (Fig.3.13A). Similarly, for RASF the IL-6 levels were 2.09 ± 1.09 pg/10^4 cells (control) and 1.49 ± 0.45 pg/10^4 cells (10 Gy) (Fig.3.13B). The same situation was observed for IL-8, MCP-1 and MMP-1 levels (Fig. 3.13C-D, 3.14A-D).

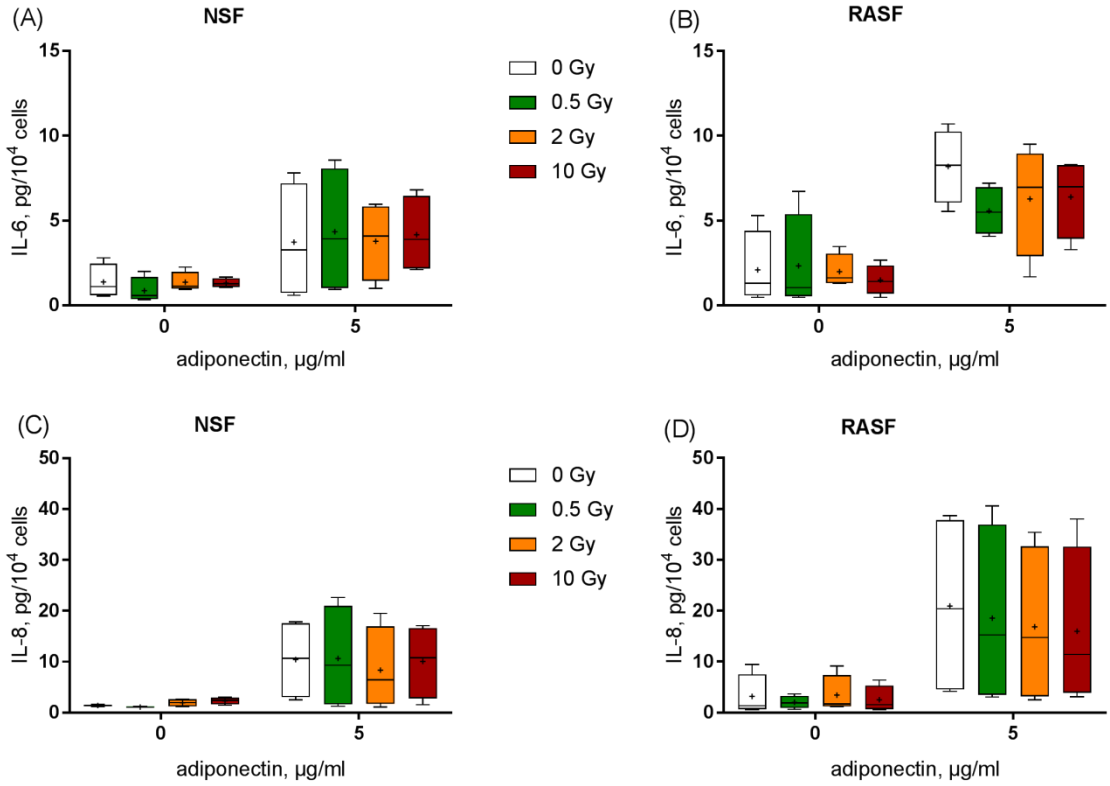

Fig. 3.13 Effect of X-ray irradiation on adiponectin-induced release of IL-6 and IL-8 by human synovial fibroblasts. Synovial fibroblasts from healthy donors (NSF) and from patients with rheumatoid arthritis (RASF) were treated or not with 5 µg/ml adiponectin for 24 h prior to irradiation with X-rays. Cell supernatants were collected 24 h after irradiation and amounts of (A-B) IL-6 and (C-D) IL-8 were measured using ELISA assay. Boxplots show the median, Tukey whiskers (median ± 1.5 times interquartile range) and mean (+). N=4

In contrast, the radiation-induced changes in the release of pro-inflammatory factors were observed in the adipokine-treated cells. Moreover, the radiation response of NSF differed from that of RASF. Interestingly, the release pattern of all measured cytokines in RASF was relatively similar. While adipokine-treated NSF mostly showed either unchanged (Fig. 3.13A, C) or slightly increased (Fig. 3.15A, 3.16C) levels of cytokines, trends towards a decrease in IL-6, IL-8, MCP-1 (Fig. 3.13B, D, 3.14D, 3.15B, D, 3.16D) and MMP-1 (Fig. 3.14B, 3.16B) production were observed in all stimulation experiments with RASF. Additionally performed gene expression analysis confirmed most of the results obtained for the release (appendix, Fig. A.1, A.2). However, trends towards reduced expression of pro-inflammatory factors were only observed in the adiponectin-treated RASF.

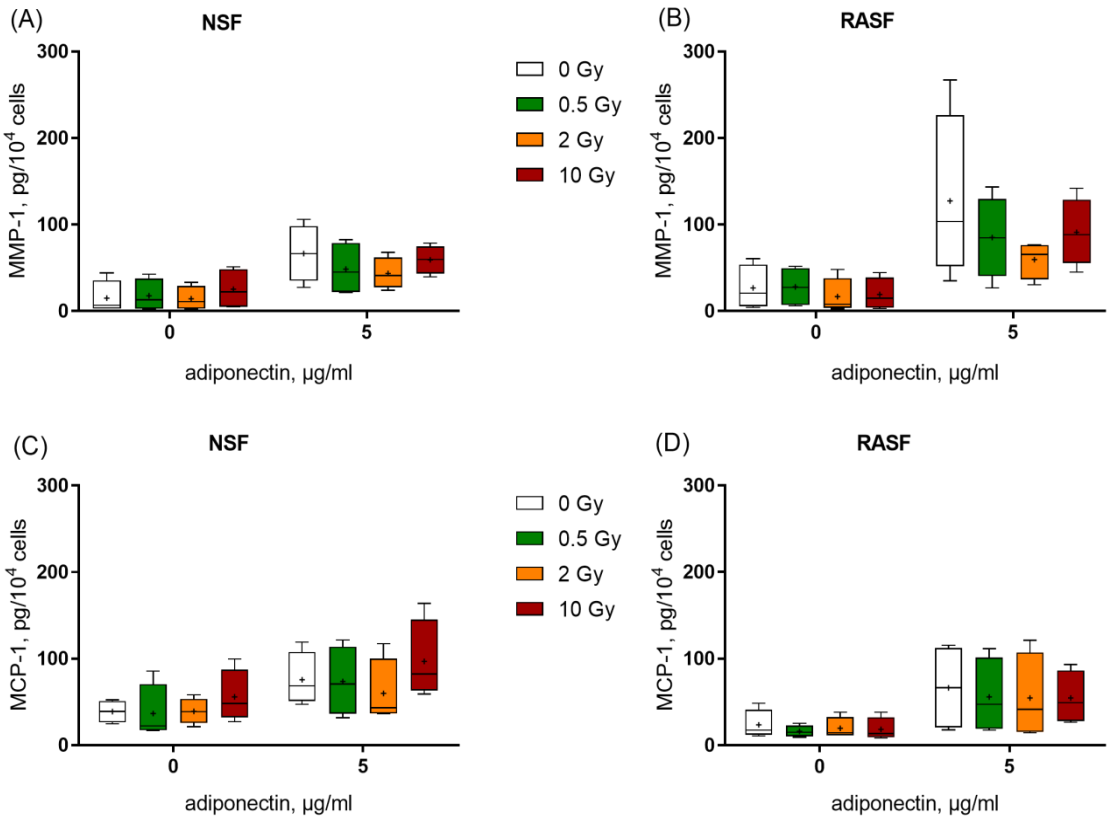

Fig. 3.14 Effect of X-ray irradiation on adiponectin-induced release of MMP-1 and MCP-1 by human synovial fibroblasts. Synovial fibroblasts from healthy donors (NSF) and from patients with rheumatoid arthritis (RASF) were treated or not with 5 µg/ml adiponectin for 24 h prior to irradiation with X-rays. Cell supernatants were collected 24 h after irradiation and amounts of (A-B) MMP-1 and (C-D) MCP-1 were measured using ELISA assay. Boxplots show the median, Tukey whiskers (median ± 1.5 times interquartile range) and mean (+). N=4

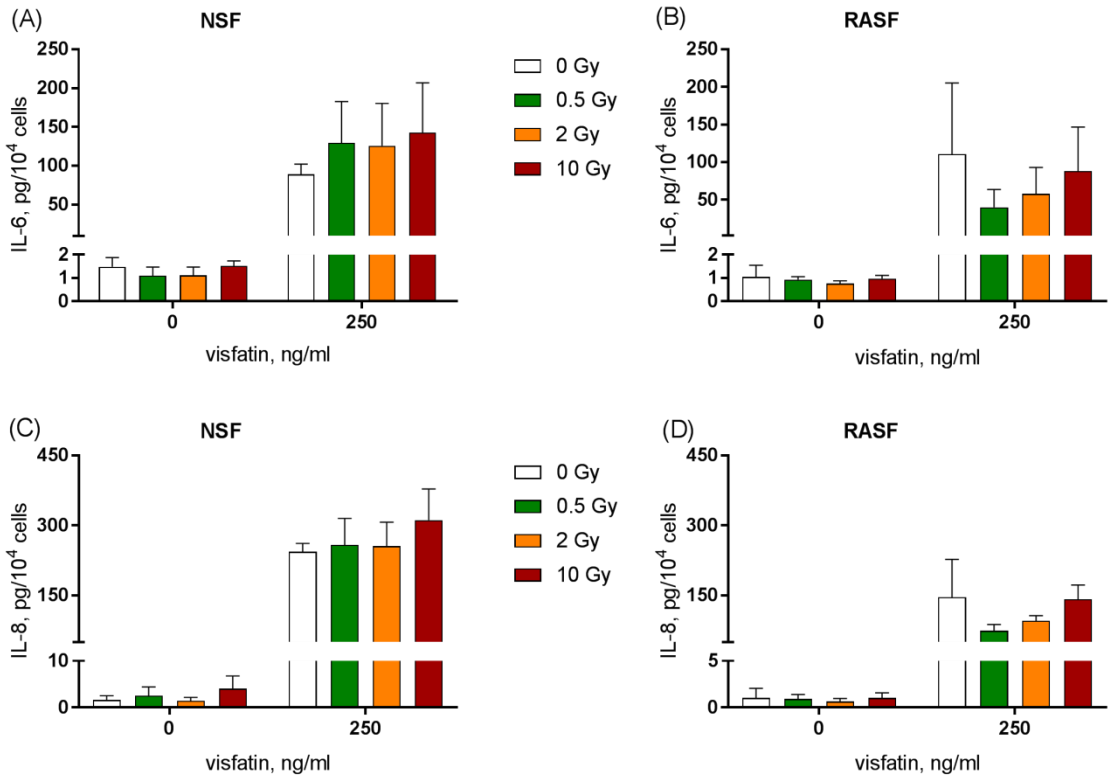

Fig. 3.15 Effect of X-ray irradiation on visfatin-induced release of IL-6 and IL-8 by human synovial fibroblasts. Synovial fibroblasts from healthy donors (NSF) and from patients with rheumatoid arthritis (RASF) were treated or not with 250 ng/ml visfatin for 24 h prior to irradiation with X-rays. Cell supernatants were collected 24 h after irradiation and amounts of (A-B) IL-6 and (C-D) IL-8 were measured using ELISA assay. Mean, SEM. N=3

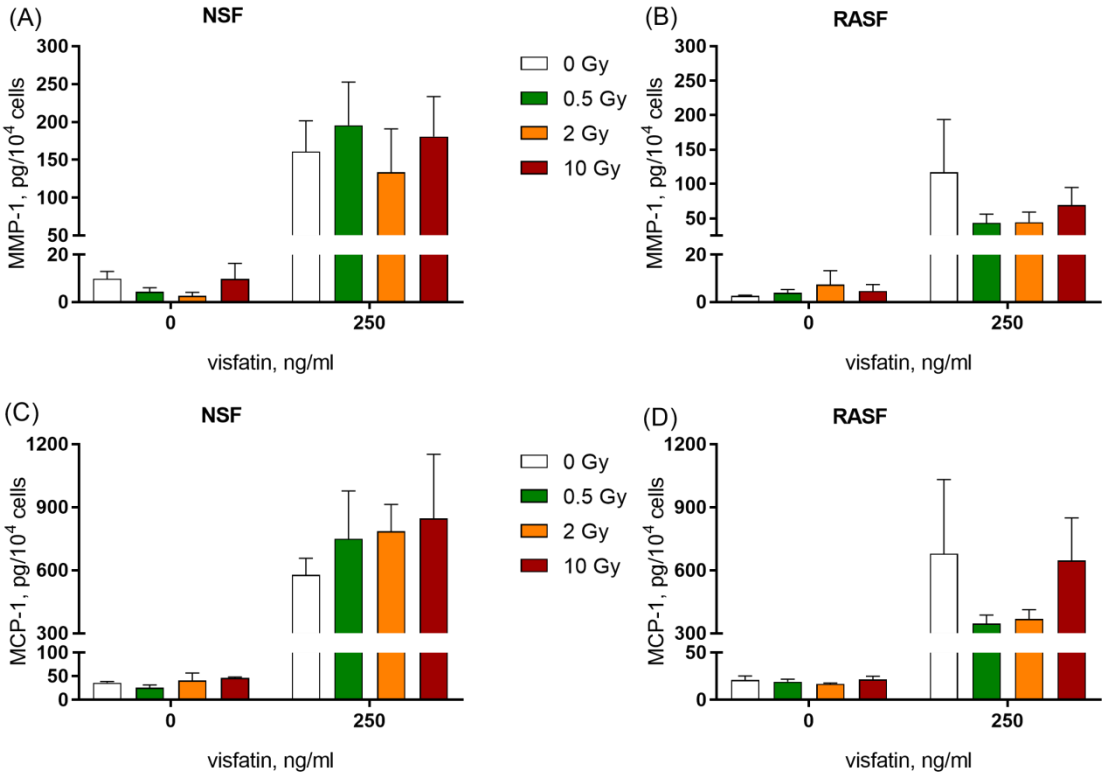

Fig. 3.16 Effect of X-ray irradiation on visfatin-induced release of MMP-1 and MCP-1 by human synovial fibroblasts. Synovial fibroblasts from healthy donors (NSF) and from patients with rheumatoid arthritis (RASF) were treated or not with 250 ng/ml visfatin for 24 h prior to irradiation with X-rays. Cell supernatants were collected 24 h after irradiation and amounts of (A-B) MMP-1 and (C-D) MCP-1 were measured using ELISA assay. Mean, SEM. N=3

3.4 Impact of ionizing radiation on differentiation of human adipocytes

Adipose tissue is a complex endocrine organ that produces a variety of immune and inflammatory mediators [43], [88]. Adipocytes, the dominant cell type of adipose tissue, are known to support inflammatory processes and cartilage degradation in RA by release of different cytokines and adipokines. The adipokines induce IL-6, IL-8 and MMP-1 in synovial fibroblasts [11], [163], [164] and are considered to be a potential target for the therapy of rheumatoid arthritis. As there are no data available about radiation response of adipocytes, one of the aims of this work was to investigate whether ionizing radiation can affect differentiation process of adipocytes or the adipokine release.

Due to ethical reasons, it was not possible to obtain pre-adipocytes from the infrapatellar fat pad (IPFP). The following experiments were mostly done with the cells isolated from the subcutaneous fat tissue of the patient with Simpson-Golabi-Behmel syndrome (SGBS, see chapter 1.4, 2.2). To avoid the potential differences in behavior of the cells after irradiation, additional experiments with the primary human adipocytes were performed.

3.4.1 Proliferation and differentiation of human (pre)adipocytes

In order to assess the radiation response of adipocytes, first, the proliferation capacity of irradiated pre-adipocytes compared to the unirradiated cells was evaluated. Radiation-

induced changes were monitored in primary- and in SGBS-pre-adipocytes over 21 days. As shown in the Fig. 3.17, the proliferation pattern of SGBS-pre-adipocytes was very similar to that of primary pre-adipocytes. It was established that the cell number of irradiated pre-adipocytes was in both cases dose dependent reduced (Fig. 3.17). The difference in the cell number between irradiated cells and controls was detectable from day 12 on for both the primary cells (Fig. 3.17A) and the SGBS-cells (Fig. 3.17B). It is notable that the irradiation with 0.5 Gy reduced the cell number of SGBS-cells compared to control, while did not affect that of primary cells. The strongly reduced cell number of pre-adipocytes exposed to 2 Gy X-rays was observed over the whole observation period in both cell types. No progress in the proliferation of the cells irradiated with 10 Gy was determined.

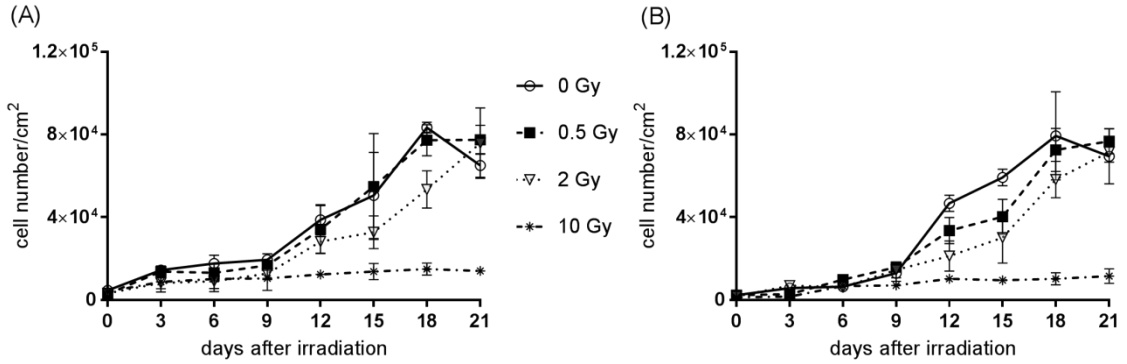

Fig. 3.17 Proliferation of human pre-adipocytes after X-ray irradiation. (A) Human primary subcutaneous pre-adipocytes and (B) SGBS pre-adipocytes were irradiated with 0.5, 2 and 10 Gy X-rays and cultivated over 21 days. Cell counting was performed at indicated days. Mean, SEM. N=3

To evaluate whether the ionizing radiation affects differentiation process from pre-adipocytes to adipocytes, the differentiation was initiated in the pre-adipocytes immediately after exposure to X-rays. For the primary cells, the amount of accumulated triglycerides was measured in the irradiated cells and compared to control. Thereby, a small dose dependent trend towards an increase of fat accumulation was detected 10 days after irradiation. This was also confirmed by triglyceride measurements on day 20. Furthermore, the fat amount was significantly higher in the cells exposed to the 10 Gy of X-rays compared to the controls (Fig. 3.18). To evaluate the effect of radiation on differentiation of SGSB-cells, these were cultivated on the chamber-slides and stained with Oil Red O and DAPI at indicated days. Then, the number of differentiated (Oil Red O+, DAPI+) cells and the total cell number (DAPI+) of one visual field were determined. In total, seven vision fields per slide were counted. The differentiation rate was calculated as a ratio of differentiated cells to the total cell number. As shown in the Fig. 3.19, the unirradiated SGBS-cells reached the differentiation rate about 60% already on day 7 after differentiation start and this rose to 80% until day 21. Further cultivation of cells did not result in an increase of differentiation rate and the curve achieved a plateau (Fig. 3.19). With regard to radiation effects on the differentiation process of SGBS-cells, a slight dose-dependent decrease of the differentiation rate was observed on day 7. However, this effect became weaker in the course of further cultivation. On day 21, the difference between unirradiated SGBS-cells and those exposed to 10 Gy X-rays was about 6%.

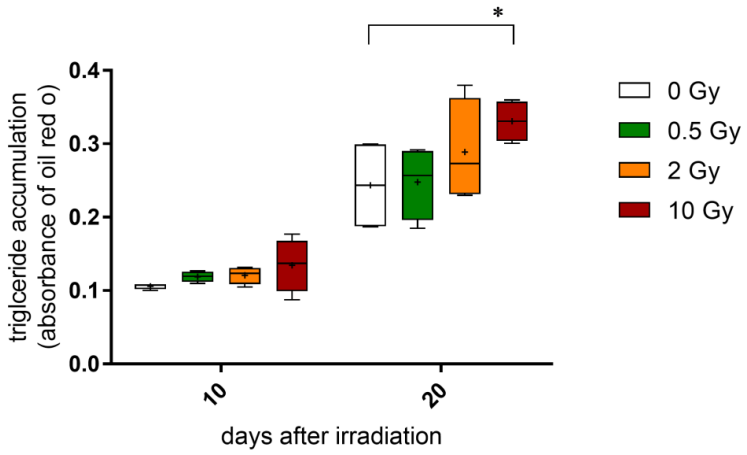

Fig. 3.18 Effect of X-ray irradiation on adipocyte differentiation. The quantification of triglyceride accumulation was performed in human primary subcutaneous adipocytes on day 10 and 20 after irradiation with X-rays. Boxplots show the median, Tukey whiskers (median ± 1.5 times interquartile range) and mean (+). N=4, *p ≤ 0.05, two-tailed t-test

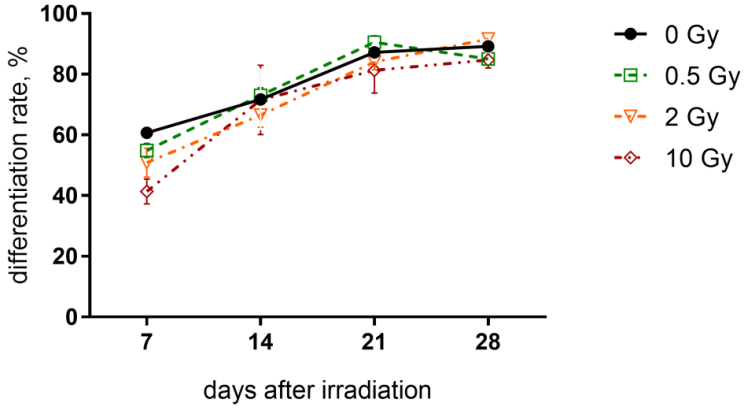

Fig. 3.19 Effect of X-ray irradiation on differentiation of SGBS-adipocytes. SGBS pre-adipocytes were irradiated with 0.5, 2 and 10 Gy X-rays and cultivated over 28 days. Differentiation rate was calculated at indicated days as a proportion between the differentiated and undifferentiated cells. Mean, SEM. N=3

3.4.2 Adipokine release and gene expression analysis of the adipogenic specific markers

In order to further investigate the impact of ionizing radiation on differentiation process of adipocytes, the release of adipokines was evaluated. For the primary adipocytes, the amount of adipokines was determined in cell supernatants 10 and 20 days after irradiation (medium change was not performed). Fig. 3.20A-B indicates progress in the released levels of leptin and adiponectin between 10 and 20 days, but irrespective of irradiation. With respect to the release of visfatin, the measured amount was under the detection limit 10 days after the start of differentiation and has dramatically increased until day 20. Moreover, cells exposed to 10 Gy X-rays exhibited a higher, even if not statistically significant, visfatin concentration than unirradiated cells (Fig. 3.20C).

For SGBS-cells, the amount of adipokines was measured in cell supernatants at indicated days 24 h after medium was changed. The levels of adiponectin and leptin were below the detection limit of ELISA Kits (1 ng/ml for leptin; 2 ng/ml for adiponectin). In view of very low amounts of these adipokines in cell supernatants of the primary adipocytes even after 10 cultivation days without medium change, this was expected. In contrast, visfatin concentrations were determined over the whole cultivation period from day 0 on (Fig. 3.21). These data indicate that SGBS-pre-adipocytes can release visfatin at the early stages of differentiation, which is not the case for primary pre-adipocytes. Owing to strong fluctuations between experiments, the radiation-mediated changes in visfatin release could not be recognized (Fig. 3.21B). Since the radiation-induced changes in the adipokine production by SGBS-adipocytes remained unclear due to variations in the release, the gene expression analysis was performed. Albeit the measured level of adiponectin expression was also under detection limit, the gene expression of leptin and visfatin expression could be evaluated. As shown in the Fig. 3.22, SGBS-adipocytes exhibit a trend to a dose dependent reduction of visfatin expression (Fig. 3.22 B), while for leptin expression no significant changes or trends were observed (Fig. 3.22A). The analysis of further adipogenic genes also reveals slight trends towards a reduced expression level of intermediate (PPARγ and C/EBPß, Fig. 3.23A,B) and late (C/EBPα, Fig. 3.23C) differentiation markers. Since the ability for differentiation depends, in particular, on the expression of these genes, the radiation-induced changes may have a crucial role for adipocyte development.

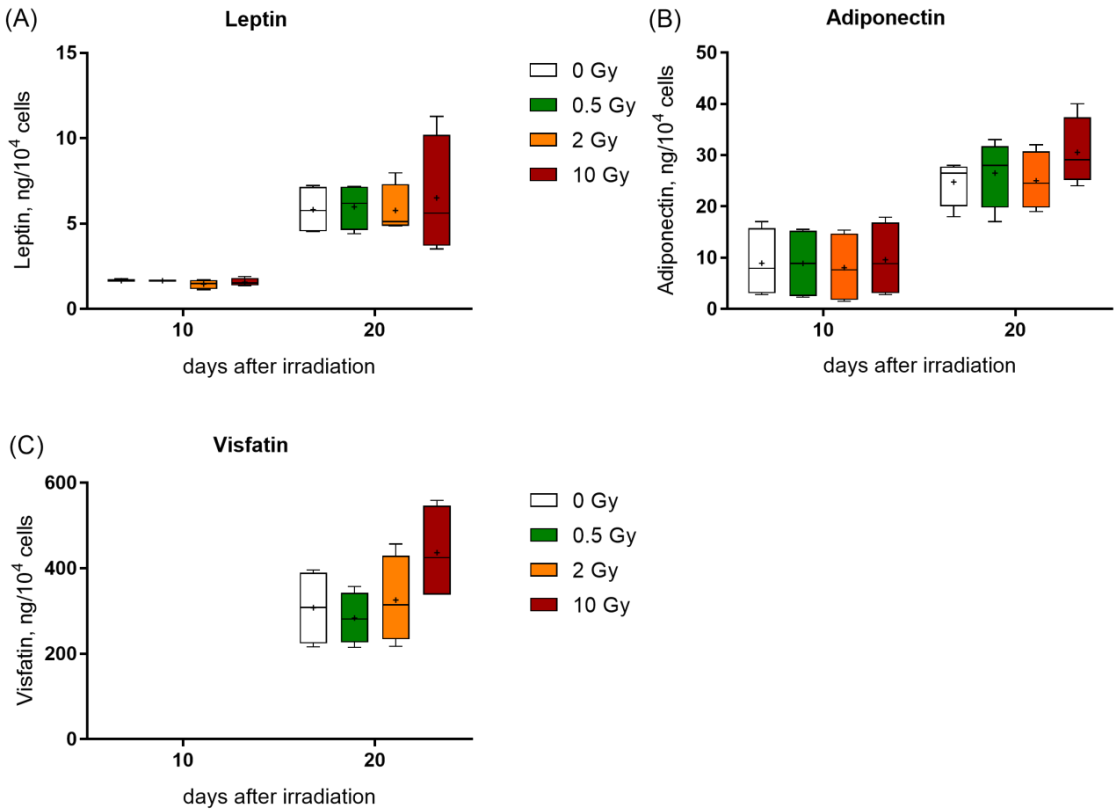

Fig. 3.20 Effect of X-ray irradiation on the adipokine release by human primary adipocytes. Human primary subcutaneous pre-adipocytes were irradiated with 0.5, 2 and 10 Gy of X-rays and differentiation was initiated. Cell supernatants were collected on day 10 and 20 after irradiation and amount of (A) leptin, (B) adiponectin and (C) visfatin were measured using ELISA assay. Boxplots show the median, Tukey whiskers (median ± 1.5 times interquartile range) and mean (+). N=4

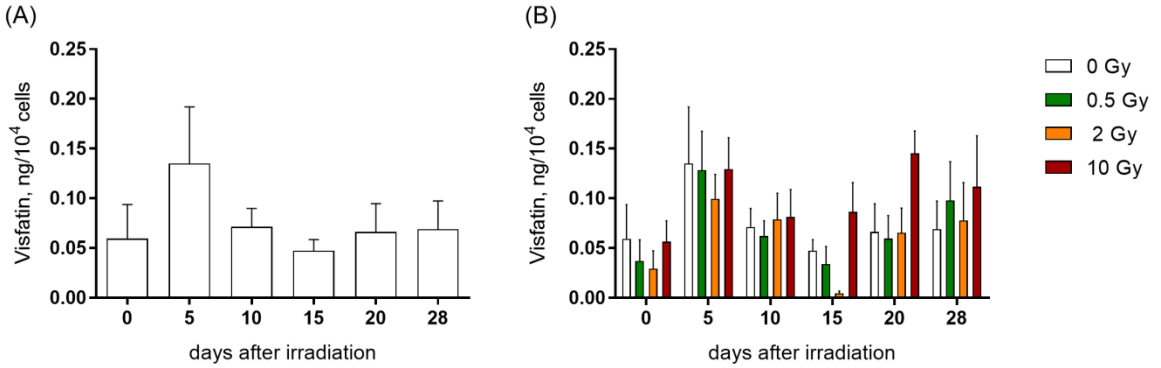

Fig. 3.21 Effect of X-ray irradiation on the visfatin release by SGBS-adipocytes. SGBS-pre-adipocytes were irradiated with 0.5, 2 and 10 Gy of X-rays and differentiation was initiated. Amount of visfatin in (A) unirradiated SGBS-cells (control) and (B) irradiated SGBS-cells was measured at the indicated days using ELISA assay. Mean, SEM. N=3

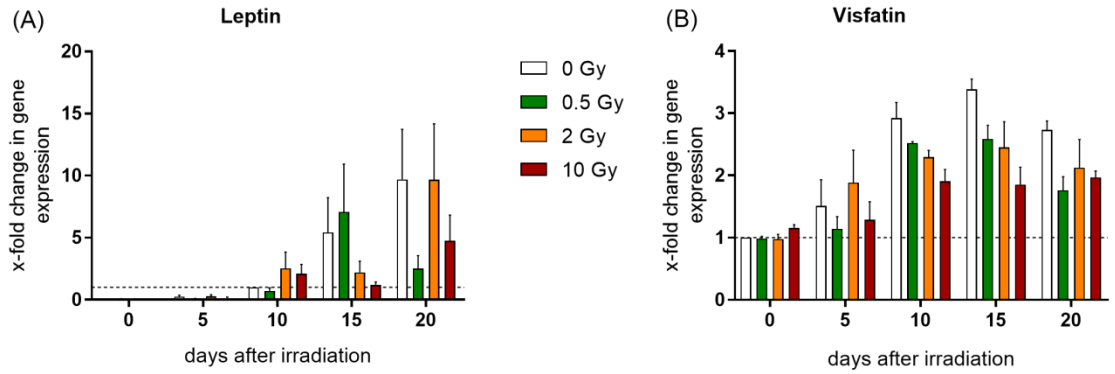

Fig. 3.22 Gene expression of adipokines leptin and visfatin in SGBS-adipocytes. SGBS-pre-adipocytes were irradiated with 0.5, 2 and 10 Gy of X-rays and differentiation was initiated. Expression of (A) Leptin and (B) Visfatin was analyzed at indicated days using qPCR. The data are normalized to GAPDH gene expression and to the gene expression on day 10 (for leptin) and on day 0 (for visfatin). Mean, SEM. N=3

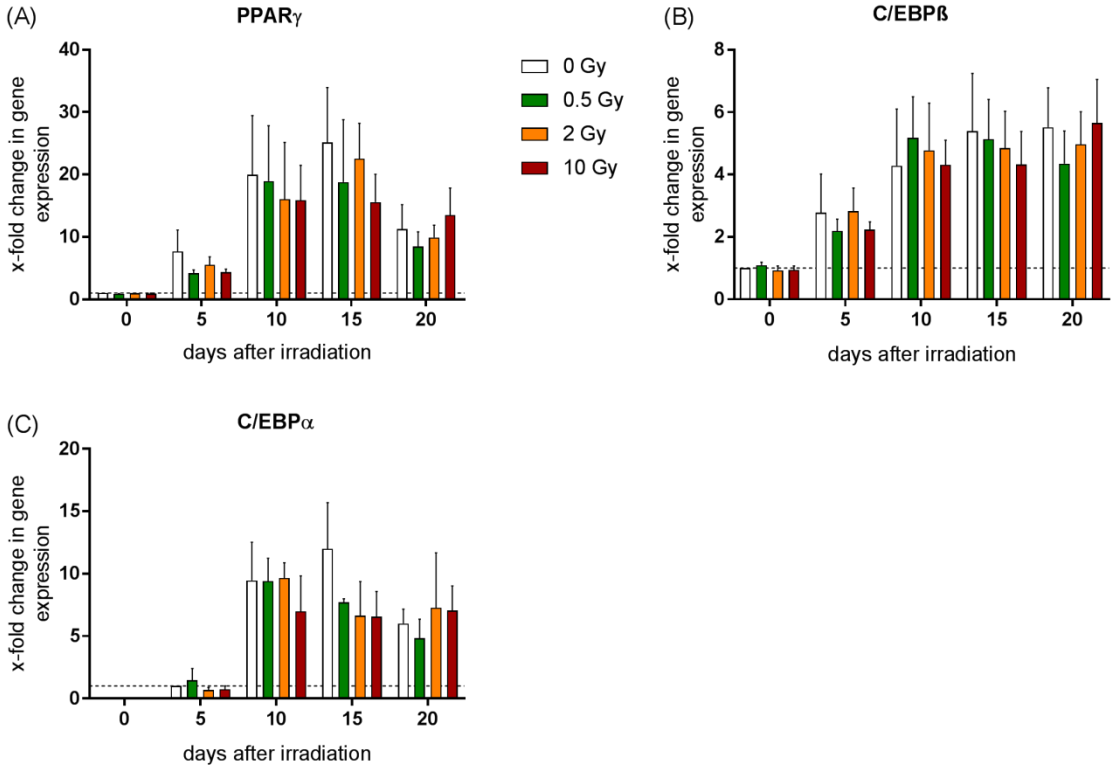

Fig. 3.23 Gene expression of selected differentiation markers in SGBS-adipocytes. SGBS-pre-adipocytes were irradiated with 0.5, 2 and 10 Gy of X-rays and differentiation was initiated. Gene expression of (A) PPAR$_\gamma$, (B) C/EBPß and (C) C/EBPα was analyzed at indicated days using qPCR. The data are normalized to GAPDH gene expression and to the gene expression on day 0 (for PPAR$_\gamma$ and C/EBPß) and on day 5 (for C/EBPα). Mean, SEM. N=3

3.4.3 Release of pro-inflammatory cytokines during differentiation

Besides other cell types of adipose tissue, adipocytes are known to produce TNFα, IL-6 and other pro-inflammatory cytokines [50], [165]. To determine whether ionizing radiation influences the cytokine release by adipocytes, the IL-6 and IL-8 levels were measured in cell supernatants of primary adipocytes and SGBS-cells following irradiation.

The levels of both cytokines were not significantly changed in cell supernatants of primary adipocytes collected 10 and 20 days after initiation of differentiation (medium change was not performed during cultivation) (Fig. 3.24). Thus, the mean level of IL-6 in the unirradiated cells was 95.2 ± 23 pg/10^4 cells on day 10 and 114.5 ± 15.3 pg/10^4 cells on day 20. Only the cells exposed to 10 Gy X-rays showed a slightly enhanced concentration of IL-6 (166.65 ± 27.8 pg/10^4 cells) 20 days after irradiation. Similar, no differences in the IL-8 levels of the controls between 10 and 20 days (126.7 ± 56.8 pg/10^4 cells and 173.7 ± 84.8 pg/10^4 cells, respectively) were observed. The slightly increased amounts of IL-8 were released by the cells exposed to higher doses of radiation (2 and 10 Gy).

The analysis of cytokine release by SGBS-adipocytes over 28 days indicated that the concentrations of IL-6 as well as IL-8 were decreasing in the course of differentiation (Fig. 3.25). It was therefore established that the SGBS-pre-adipocytes have more pronounced pro-

inflammatory profile than mature adipocytes. No apparent changes in the cytokine release of irradiated cells were seen when compared to the controls.

In addition, release of TNFα by primary adipocytes and SGBS-cells was investigated. However, the cytokine levels in either case were below the detection limit.

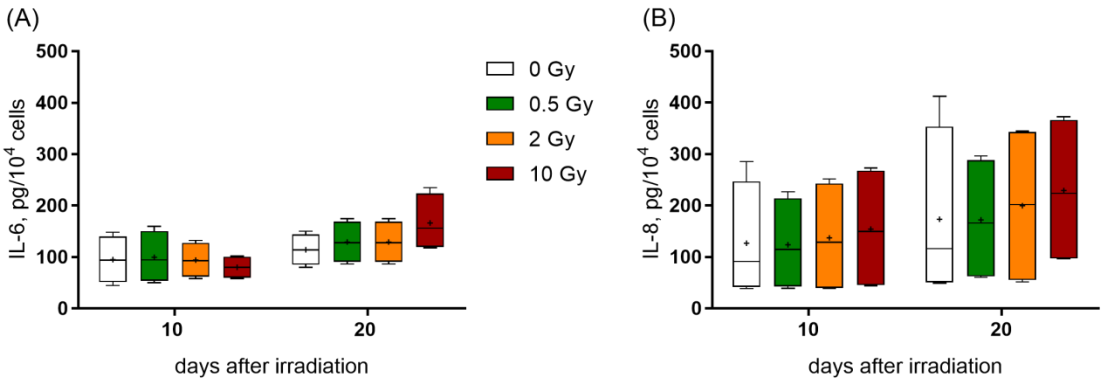

Fig. 3.24 Effect of X-ray irradiation on the release of IL-6 and IL-8 by human primary adipocytes. Human primary subcutaneous pre-adipocytes were irradiated with 0.5, 2 and 10 Gy of X-rays and differentiation was initiated. Cell supernatants were collected on day 10 and 20 after irradiation and amount of (A) IL-6 and (B) IL-8 and were measured using ELISA assay. Boxplots show the median, Tukey whiskers (median ± 1.5 times interquartile range) and mean (+). N=4

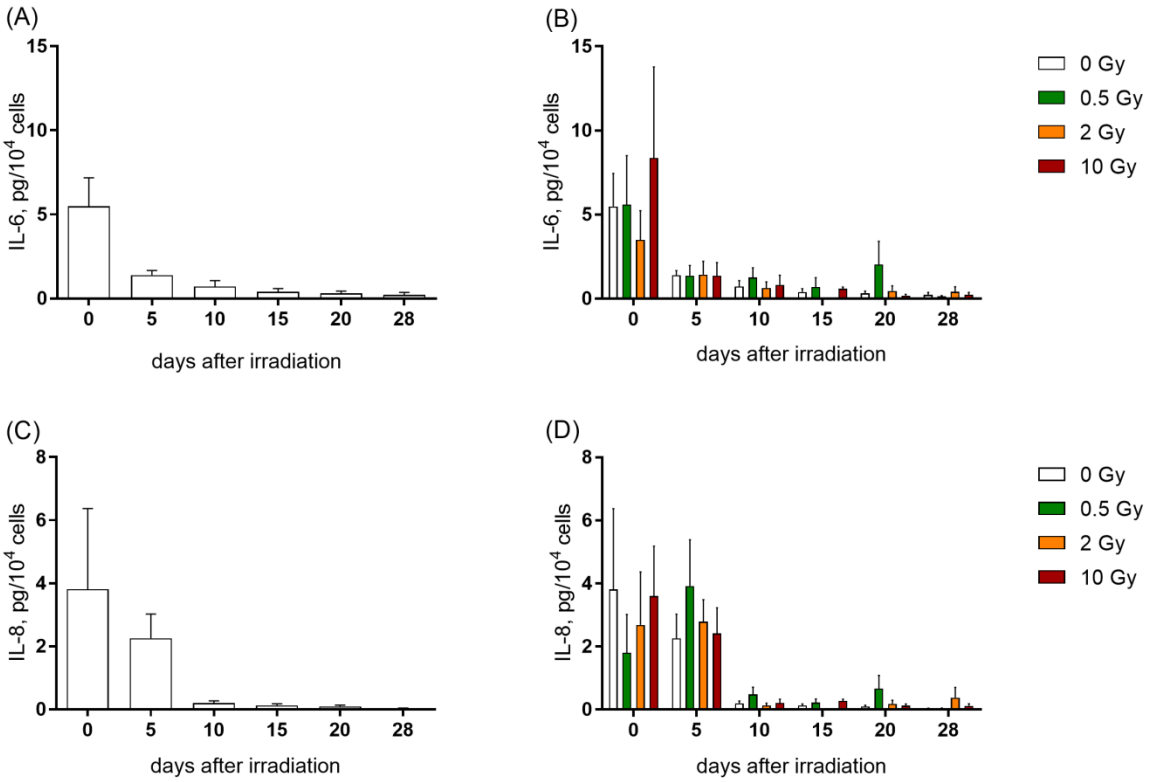

Fig. 3.25 Effect of X-ray irradiation on the IL-6 and IL-8 release by SGBS-adipocytes. SGBS-pre-adipocytes were irradiated with 0.5, 2 and 10 Gy of X-rays and differentiation was initiated. Amounts of (A-B) IL-6 and (C-D) IL-8 were measured at the indicated days using ELISA assay. (A) and (C) indicate release of IL-6 and IL-8 in unirradiated SGBS-cells. Mean, SEM. N=3

4 Discussion

In the present thesis, the impact of ionizing radiation on factors related to the changes in bone metabolism of patients with muskuloskeletal disoreders (MSD) was investigated. The special focus was set on adipokines, mostly secreted by adipocytes, which are known to contribute to the inflammation processes in the joint. Considering the predominantly pro-inflammatory properties of adipokines in rheumatoid arthritis (RA) and osteoarthritis (OA), and potential anti-inflammatory effects of low-dose ionizing irradiation, it was hypothesized that low-dose radiation inhibits the adipokine-induced inflammation in the joints of patients with MSD. To verify this hypothesis, the circulating levels of adipokines in patients with MSD after serial radon spa therapy were investigated. Furthermore, the radiation-mediated changes in the response of synovial fibroblasts to the adipokine treatment were examined *in vitro*. Thereby, synovial fibroblasts isolated from patients with RA (RASF) and from healthy donors (NSF) were used. In addition, it was assessed whether ionizing radiation influences the adipocyte development with regard to their differentiation and concomitant release of adipokines.

It should be noted that low doses of X-rays (photons) and alpha particles (radon exposure) that are used for the treatment of chronic inflammatory diseases are not in the same range. Accordingly, for *in vitro* experiments, doses of 0.5-10 Gy of X-ray were used, corresponding to the single or total doses applied during photon low-dose radiation therapy. For radon, the estimated total doses that patients receive during a radon treatment are given as effective doses, and are three orders of magnitude lower, ranging from 0.05 mSv to 2 mSv.

4.1 Impact of radon treatment on the adipokine serum levels of patients with musculoskeletal diseases (RAD-ON01)

Musculoskeletal diseases (MSD) are usually associated with chronic inflammation, which is accompanied by reduced mobility and pain. Elevated levels of various pro-inflammatory cytokines, joint destructive enzymes and adipokines were detected in serum and synovial fluid of patients with MSD [166], [167]. Adipokines, which are to a large extent secreted by adipose tissue, contribute to the inflammation process in the joint by inducing IL-6, IL-8 and MMP-1 in RA synovial fibroblasts [96], [163]. This in turn enhances the expression of RANKL, leading to an imbalanced OPG/RANKL ratio, the activation of bone destructive cells – osteoclasts, and finally to cartilage degradation and bone erosion [35], [168].

The objective of this part of the work was to assess whether radon treatment has an influence on the circulating levels of adipokines in patients suffering from MSD. The intension is to contribute with the obtained results to a better understanding of the pain-relieving and anti-inflammatory effects of radon therapy. In the framework of the RAD-ON01 study, the blood samples of 100 patients were taken prior to and in regular intervals after the start of the radon treatment (6, 12, 18, and 30 weeks after the first radon bath). The patients did not receive any anti-inflammatory drugs during the therapy and were examined by a medical doctor at indicated time points to measure pain and vascular parameters [140]. In the scope of this thesis, adipokine levels were measured in the serum of patients [169].

The main focus was set on the adipokines visfatin, adiponectin, leptin and resistin, as there is growing evidence that these adipokines play an important role in the development of degenerative joint diseases [101], [170]. Although many studies suggest the adipokines as the novel therapeutic targets, little is known about potential therapeutic actions. However, some studies showed that it may be possible to reduce adipokine serum levels using nonsteroidal anti-inflammatory drugs [171] or TNFα-inhibitors [120]. Popa et al, for instance, showed the significant reduction of adiponectin levels in serum of RA patients treated with TNFα-inhibitor infliximab, but only in patients, who additionally took the stable dosage of corticosteroids [172]. Administration of infliximab was also shown to decrease serum level of resistin in patients with RA [120]. In the present study, the effect of low-dose radon treatment on the circulating levels of adipokines in a large group of patients with MSD was shown for the first time.

In the present work, no significant changes for adiponectin, leptin and resistin (Fig. 3.2A, 3.3A, 3.4A) were observed. There are only two studies, published by Suman et al, providing information on the effects of ionizing radiation on the serum levels of adipokines. In these studies, levels of leptin and adiponectin were measured in serum of mice, irradiated either with heavy ions (1.6 Gy, ^{56}Fe, whole-body) or with γ-rays (2 Gy, ^{137}Cs, whole-body). While the adiponectin serum level remained unchanged, the serum concentration of leptin was found in both cases to be significantly higher 2 and 12 months after irradiation [173], [174]. The authors suggested that upregulation of leptin together with insulin-like growth factor 1 (IGF-1) could result in the activation of PI3K/Akt and JAK2 pathways, contributing to the pathophysiological consequences such as premature senescence and metabolic alterations [173], [174]. Considering the different dose and type of radiation as well as differences between species, it is not possible to compare the results presented here with that from Suman et al. However, radiation-induced increase of leptin, which is tightly associated with insulin metabolism, may represent an important link between radiotherapy and development of obesity in cancer survivors [175].

Since many functions of adipose tissue are co-regulated by gender and other systemic factors [176], the relationship between adipokine levels and age, gender and pain duration was analyzed in the work presented here. No correlations were found between serum levels of adiponectin, leptin and resistin and age of patients. With regard to gender-related differences, the mean levels of adiponectin and leptin were significantly lower in men than in women, whereas the mean level of resistin was comparable for both men and women (Fig. 3.2B, 3.3B, 3.4B). Although these data are in line with the other studies [87], [88], [177], the reason for the gender-related differences is not completely understood. According to Hellstroem et al and Ahonen et al, they may be caused by at least two different mechanisms. First, the fat tissue distribution differs between men and women; women have more subcutaneous fat tissue than men. Since adiponectin is mainly produced by the subcutaneous adipocytes, this would explain the difference in the serum levels [178]. Second, women may have a higher production rate of adipokines than men due to different hormone levels [177]. In addition, the results of the present work revealed no significant correlations between serum levels of adiponectin, leptin and resistin and pain duration, indicated by patients.

Since the expression of visfatin is highly increased during inflammation and correlates positively with several disease markers in patients with MSD [86], [93], [94] as well as in a

collagen-induced arthritis (CIA) mouse model [179], it is considered as a novel biomarker in chronic inflammatory diseases, including RA [84], [158], [180]. The ability of visfatin to induce a variety of pro-inflammatory and matrix-degrading factors in RASF and monocytes argues for its involvement in the pathogenesis of RA and other MSD [11], [96], [99]. In the present work it was found that the serum levels of visfatin were significantly reduced after radon therapy compared to baseline levels. Decrease of approximately 50 percent was detected already 12 weeks after therapy start, and was even more pronounced at the end of follow-up period (30 weeks) (Fig. 3.1A). This decrease was similar to the reduction of visfatin concentration, detected by Sglunda et al in serum of RA patients 3 months after the treatment with conventional synthetic disease modifying anti-rheumatic drugs (csDMARDs) [161], [169]. Furthermore, the authors demonstrated that a decrease in circulating visfatin predicted a decrease of disease activity after treatment, as assessed by disease activity score (DAS 28) [161]. The DAS 28 is considered as a quantitative assessment of disease activity and included, inter alia, pressure point measurements to evaluate pain and assessment of disease activity by patient, which is often based on the pain sensitivity. Besides the study of Sglunda et al, positive correlations of visfatin levels with osteoarthritic pain were reported by Bas et al [181]. Also in some other studies high visfatin levels were associated with pain and radiographic join damage [93], [182]. Therefore, the association between visfatin concentration and pain duration, shown in this thesis (Fig. 3.1D), provides an additional important indication for the existing relationship between pain and increased visfatin concentration in patients with MSD. Moreover, a positive correlation between serum concentrations of visfatin and pain perception, evaluated with the visual analog pain scale (VAS) in the framework of RAD-ON01 study [140], was found in the present work (Fig. 4.1). Although VAS is a scale for the measurement of subjective pain feeling, it is a required part of DAS 28 score and is frequently used in the pain treatment [183]. Thus, considering the long-lasting pain reduction for the majority of patients enrolled in the RAD-ON01 study [140], and the relationship between visfatin concentration and perception of pain duration (Fig. 3.1D) and intensity (Fig. 4.1), the decreased circulating visfatin levels after radon treatment could be related to the decrease of disease activity observed in the patients of this study.

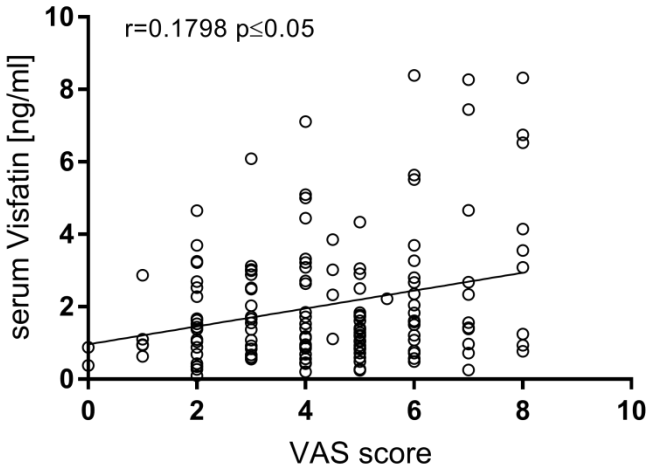

Fig. 4.1 Correlation analysis between VAS pain score and serum visfatin concentrations in patients with MSD before and after radon treatment [169]. N=44, Spearman's correlation analysis r=0.1798, *p ≤ 0.05

Furthermore, in the present work the serum levels of visfatin were higher in females than in males, but no age-related changes were detected (Fig. 3.1). The data about gender-dependent differences in the serum visfatin level are contradictory. While some studies reported that the visfatin level does not differ between genders [184], other studies demonstrated higher circulating concentrations of visfatin in women than in men [185].

Apart from this, Busso et al verified the key role of visfatin in RA by showing that inhibition of visfatin with a pharmacological substance APO866 leads to the significantly attenuated inflammation, reduced cartilage damage and disease severity in CIA mouse model [91], [179]. Radon therapy was also shown to decrease the serum levels of TNFα and RANKL, powerful promoters of inflammation and bone resorption, in patients with RA and OA [141].

In addition, it has been observed that RA and OA are associated with several components of metabolic syndrome, such as insulin resistance or changes in fat distribution and adipokine profile [47], [186]. Each of these components is an independent risk factor for developing cardiovascular diseases. Since MSD are closely associated with accelerated atherosclerosis [11], [40] and there is growing clinical evidence supporting a role of visfatin in development of cardiovascular diseases [97], it can be assumed that visfatin and other adipokines are involved in RA-associated metabolic syndrome. Novel treatment possibilities, which improve insulin sensitivity and normalize the adipokine levels in MSD patients, are therefore urgently needed. Also based on this, low-dose radon treatment could provide a therapeutic option for the therapy of RA and other musculoskeletal diseases.

4.2 Radiation response of adipokine-treated synovial fibroblasts

Besides chronic inflammation, musculoskeletal diseases such as RA and OA are characterized by prominent cartilage destruction and bone erosion. Rheumatoid arthritis synovial fibroblasts (RASF) are the dominant cell type, which promotes degenerative changes in the joint [18], [83]. In healthy joints, the main functions of SF are the maintenance of matrix remodeling and the supply of joint cavity and cartilage with the essential nourishing proteins and molecules [29], [80], [187]. In the course of RA, synovial fibroblasts acquire an aggressive phenotype, triggered by inflammation. Due to their invasive growth capacity, reduced ability to undergo apoptosis and high migration potential, RASF are often compared to cancer cells [32], [188].

In addition, RASF are known to produce a variety of pro-inflammatory cytokines, matrix-degrading enzymes and osteoclasts-activating factors, actively contributing to inflammation and destruction of cartilage and bone. [37], [188]. Cytokines such as IL-6, IL-8, MCP-1, together with other factors, play an important role in the self-activation of RASF as well as in the crosstalk between RASF and other cells in the joint. Besides cytokines, adipokines are known to induce the expression of pro-inflammatory and matrix-degrading factors in RASF, leading to perpetuation of inflammation and joint destruction [11]. Although RASF in the context of RA were widely investigated in the past, the activation mechanisms are not completely understood yet. There is also a lack of studies, clarifying the molecular differences between rheumatoid arthritis synovial fibroblasts (RASF) and synovial fibroblasts from healthy joints (NSF). Although some studies reveal significant differences between NSF and synovial fibroblasts from patients with osteoarthritis (OASF) [189], the latter are often used

as a control in the RASF investigations, while the data about NSF are rare. Due to ethical reasons, it is very difficult to obtain synovial fibroblasts from healthy individuals. That is why the knowledge about NSF is still limited.

For the treatment of patients suffering from MSD, usually pharmacological medication is used. Since the diseases such as RA or OA are still considered incurable, the aim of the treatment with drugs is to relieve pain and to prevent progression of the disease. However, the results obtained in the RAD-ON01 study as well as other studies suggest that additional pain relief can be achieved when the patients receive low-dose radiation therapy using photons [133] or radon spa treatment [140], [169], [190]. Although several studies showed the anti-inflammatory effects of low-dose radiation in arthritic joint [134], [135], [137], the radiation response of synovial fibroblasts was not investigated yet.

In the present work, *in vitro* examination of the effects of X-ray irradiation on NSF and RASF was performed. In addition, radiation-mediated changes in the adipokine-induced expression of inflammatory and bone destructive factors in NSF and RASF were investigated. For this purpose, as a first step, the response of synovial fibroblasts (NSF and RASF) to the adipokines adiponectin and visfatin was tested. It was found that the expression and release of pro-inflammatory cytokines such as IL-6, IL-8 and matrix-degrading enzyme MMP-1 in synovial fibroblasts were strongly induced by stimulation with both adipokines (Fig. 3.7-3.9, 3.11-3.12). In addition, a significant increase of MCP-1 expression was observed in SF after stimulation with visfatin, whereas after stimulation with adiponectin the level of MCP-1 remained unchanged (Fig. 3.9, 3.12).

Although clear differences in the release of IL-6, IL-8 and MMP-1 were observed in all samples after stimulation with adipokines, the statistical significance could not be reached. This is mostly due to the high variability between samples, which is most likely traced to interindividual donor differences. Furthermore, there is evidence that RASF exhibit stronger variability than NSF. It is important to note that the increase of all these factors in NSF was very similar to that in RASF, suggesting that the ability to respond to adipokine stimulation is not restricted to synovial fibroblasts of arthritic joints. Moreover, the basal level of these factors did not differ significantly between NSF and RASF. In previously published studies, an adiponectin- and visfatin-mediated enhancement of IL-6, IL-8 and MMP-1 in RASF was shown [105], [107], [186]. Similarly, it was reported that synovial fibroblasts from osteoarthritic joint (OASF) also respond to a stimulation with adipokines, albeit to a smaller extent than RASF [100], [164]. The response of NSF to adipokine stimulation was shown in this thesis for the first time. However, Ehling et al showed that also normal dermal fibroblasts are able to produce IL-6 after stimulation with adiponectin. According to this study, the capacity to exhibit the pro-inflammatory phenotype upon stimulation with adiponectin may be a general characteristic for cells of mesenchymal origin [164]. Hence, probably all synovial fibroblasts are able to exert pro-inflammatory properties, depending on the surrounding conditions.

The upregulation of cytokines such as IL-6, IL-8 as well as other key mediators in destructive arthritis (MMP-1, RANKL) is mediated by NF-kB, which is one of the most important regulators of inflammatory gene expression [191], [192]. Therefore, it can be assumed that adiponectin and visfatin activate the NF-kB-pathway in synovial fibroblasts, leading to the observed increased cytokine production. However, the synthesis of cytokines seems to be very

specific, since the up-regulation was observed for some (IL-6, IL-8, MMP-1), but not for all factors, mediated by NF-kB. Neither in the present work nor in other studies [164], the pro-inflammatory cytokines such as TNFα or IL-1ß were affected.

MCP-1 is another important factor involved in arthritic inflammation. The increased production of MCP-1 by synovial fibroblasts attracts peripheral monocytes and T cells to the site of inflammation [44], [187], [193]. As shown in Fig. 3.9 and 3.12, visfatin caused a higher increase of MCP-1 in RASF compared to adiponectin. One possible reason for this could be the activation of different pathways by adiponectin and visfatin. Different studies suggest an activation of the p38 MAP kinase pathway by visfatin [180]. Moreover, Shahrara et al showed that inhibition of p38 MAPK dramatically reduces MCP-1 expression, which results in the improvement of disease severity of arthritic mice [194]. In addition, Meier et al reported a significant decrease of IL-6 in visfatin-stimulated RASF only after inhibition of p38-MAPK, but not after inhibition of NF-kB signaling pathway [96]. Since also in some other studies the visfatin-induced upregulation of IL-6 and IL-8 was shown to be mediated by p38 MAPK, this pathway is more likely to be activated by visfatin. Data about mechanisms underlying the adiponectin-induced MCP-1 production are rare. Frommer et al suggested the p38 MAPK and PKC pathways to be involved in adiponectin-mediated signaling. However, although the inhibition of p38 MAPK in RASF reduced MCP-1 secretion, the observed differences were not significant [100].

Ionizing radiation modulates inflammatory processes. On the one hand, low doses of radiation (< 1 Gy) are considered to attenuate inflammatory processes and are therefore used for the therapy of MSD. On the other hand, high doses (> 1 Gy), applied for the cancer treatment, are able to initiate local and systemic inflammatory reactions [138]. Based on the results of the RAD-ON01 study, it was assumed that low-dose radiation can reduce the adipokine-induced production of pro-inflammatory cytokines by synovial fibroblasts.

To gain insights into the radiation response of synovial fibroblasts, the cells were exposed to different doses of X-rays (0.5, 2, 10 Gy). The proliferation capacity of synovial fibroblasts after X-ray irradiation was dose-dependently reduced. Furthermore, the results revealed that NSF are more sensitive to radiation exposure than RASF. Exposure to 0.5 Gy X-rays did not significantly affect the proliferation of NSF, while irradiation with 2 Gy reduced the cell growth by about 40 percent. The exposure to 10 Gy X-rays completely inhibited the proliferation of NSF (Fig. 3.5A). At present, it is the first investigation on the radiation response of synovial fibroblasts. However, similar radiation-mediated changes were detected in the proliferation of other cell types, such as osteoblasts or endothelial cells [195], [196]. A delayed proliferation is associated with the time needed for DNA repair. Depending on the severity of the radiation-induced DNA damage, the possible consequences for the cell include DNA repair, senescence, cell cycle arrest or apoptosis [197]. Thus, it can be assumed that decelerated proliferation after 0.5 and 2 Gy of X-rays is associated with DNA repair, while irradiation with 10 Gy most likely induced a senescence program in the cells. By contrast, RASF showed a higher resistance upon irradiation. Even the exposure to 10 Gy X-rays did not completely suppress the growth of the cells (Fig. 3.5B).

In addition, both cell types were incapable to form the colonies after irradiation. Moreover, RASF did not form colonies, even if the cells were not irradiated. However, very low plating

efficiencies of primary cells are well known and are probably caused by the lack of cell-cell contacts at low cell density, which is required for the clonogenic survival assay [198].

To further elucidate radiation-mediated changes in synovial fibroblasts, the production of major pro-inflammatory cytokines was investigated. Although in various cell types the changes in IL-6 and IL-8 expression were shown already a few hours after irradiation [199], [200], in the present study the cytokine production by unstimulated synovial fibroblasts was not affected by irradiation. Even the exposure to 10 Gy X-rays did not significantly change IL-6, IL-8 and MCP-1 levels of both NSF and RASF (Fig. 3.13 – 3.16). Similarly, neither NSF nor RASF showed the changes of MMP-1 synthesis after irradiation. Since the measurements were performed at the one time point only (48 h), it cannot be ruled out that changes in the cytokine response occur later.

Nevertheless, the treatment with adipokines changed the reaction of the cells to irradiation as can be seen on the changed cytokine production by irradiated synovial fibroblasts, pretreated with adipokines (Fig. 3.13 – 3.16). It is worth mentioning that the release of cytokines, which were highly upregulated after treatment with adipokines (IL-6, IL-8 and MMP-1), was reduced by exposure to X-rays. However, this response was different between NSF and RASF. In the adipokine-stimulated NSF, the production of the pro-inflammatory factors (IL-6, IL-8, MCP-1, MMP-1) was either unchanged or slightly increased. By contrast, the adipokine-pretreated RASF showed a slight, but consistent trend towards a decrease of cytokine production. In addition, this effect was more pronounced in visfatin-treated RASF in comparison to adiponectin-treated cells. Although statistical significance could not be reached due to pronounced fluctuations between single experiments, each of them revealed similar trends. As mentioned before, it is conceivable that adiponectin and visfatin activate different signaling pathways. It hence appears to be likely that ionizing radiation affects both p38 MAPK and NF-kB pathways, leading to the decreased expression of pro-inflammatory cytokines. However, this presumption is in conflict with a current knowledge about mentioned pathways in radiation response. Even though the role of p38 MAPK appears to variate between different cell types and stress factors, many groups reported the radiation-induced activation of this pathway [201], [202]. Similarly, the NF-kB activation in response to radiation is well known.

In previous studies, the ability of visfatin to induce reactive oxygen species (ROS) was shown. For example, Kim et al showed that visfatin enhances the expression of adhesions molecules ICAM-1 and VCAM-1 in endothelial cells through ROS-dependent activation of NF-kB pathway [203]. Low-dose radiation was shown to reduce significantly ROS level in stimulated murine macrophages [204]. However, data about the relationship between low-dose radiation and ROS level are contradictory. Thus, Large et al showed an elevated level of ROS in endothelial cell line following 0.5 Gy X-rays irradiation. These discrepancies suggest that the effects of low-dose radiation is dependent on the cell type or cellular environment, leading to various consequences [154]. Although there are no studies about ROS measurement in synovial fibroblasts, visfatin-induced expression of adhesion molecules is known [96]. Thus, it could be assumed that visfatin enhances the ROS level in synovial fibroblasts, leading to the activation of NF-kB and p38 MAPK pathways and finally to the overexpression of adhesion molecules and pro-inflammatory cytokines (Fig. 4.2). Low-dose radiation in turn could reduce the level of ROS and therefore attenuate their effect. However, the direct activation of p38 MAPK and NF-kB by visfatin cannot be ruled out. Therefore, it should be clarified whether

low-dose radiation affects a visfatin-induced production of ROS or rather NF-kB/p38 MAPK pathways directly.

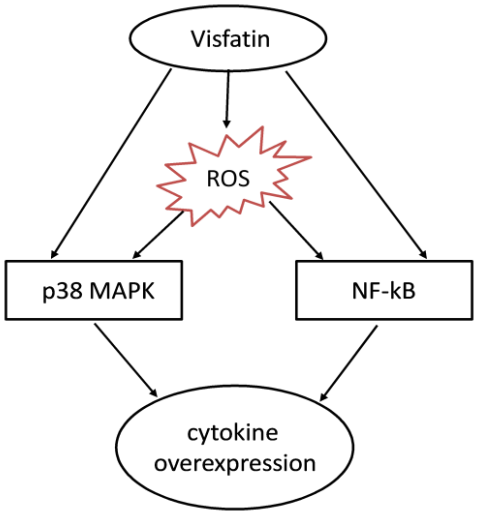

Fig. 4.2 Proposed signalling pathways and involvement of reactive oxygen species (ROS) in visfatin-mediated cytokine overexpression by RASF

Taken together, in the work presented here it was shown that the adipokines adiponectin and visfatin strongly induced the production of IL-6, IL-8, MCP-1 and MMP-1 in both NSF and RASF. Moreover, the impact of ionizing radiation on the adipokine-induced release of pro-inflammatory factors by synovial fibroblasts was shown for the first time. Thereby, it was determined that radiation can reduce the levels of IL-6, IL-8, MMP-1 and MCP-1 in the adipokine-treated RASF, but not in NSF. There is thus a need to clarify the molecular differences between NSF and RASF. In addition, it was found that irradiation did not affect the untreated synovial fibroblasts with regard to cytokine production. Therefore, the signaling pathways activated by adiponectin and visfatin should be investigated in more detail. The most likely candidates are, as mentioned before, p38 MAPK and NF-kB. It is also possible that part of the effects could be a consequence of increased oxidative stress caused by visfatin. It would therefore be reasonable to investigate the adipokine-mediated ROS production by synovial fibroblasts and the influence of ionizing radiation on it.

4.3 Radiation-mediated changes in human (pre)adipocytes during differentiation

The infrapatellar fat pad (IPFP) is a special depot of adipose tissue located in the knee joint, close to synovial layers and cartilage. Considering its location and the pro-inflammatory properties of adipose tissue (see chapter 1.4), the involvement of IPFP in the pathogenesis of degenerative joint diseases such as RA or OA is conceivable. Although it is assumed that the enhanced adipokine levels, detected in synovial fluid of patients with MSD [158], [205], originate from IPFP, only little is known about its role in the physiological and pathological processes.

In view of the important role of radiation therapy, the number of studies on radiation response of different tissues and cell types is constantly increasing. However, there are almost

no data available about effects of radiation exposure on adipose tissue or adipocytes. So far, two studies were published, which nonetheless provide controversial information. Poglio et al reported the loss of adipose tissue weight in mice after irradiation (7-10 Gy/^{137}Cs, whole-body irradiation) [206]. By contrast, Jo et al showed two times higher weights of adipose tissue in irradiated mice (5 Gy/^{137}Cs, whole-body irradiation) than in unirradiated mice [207]. Such differences could probably be related to the different time points, which were analyzed. Thus, in the study of Poglio et al the results are shown 7 days after irradiation and in the study of Jo et al – 8 months after irradiation. Nevertheless, both groups agreed on one point – ionizing radiation induces metabolic changes in the adipose tissue. Considering the lack of knowledge about radiation effects on adipocytes and the decrease of visfatin in serum of patients after low-dose radon treatment, the aim of this part of the thesis was to investigate radiation response of adipocytes with respect to the differentiation process and the ability to release adipokines.

Due to ethical reasons, it was not possible to obtain cells from human IPFP. Therefore, in this study, pre-adipocytes isolated from human subcutaneous adipose tissue were used. Moreover, the experiments were mostly done with SGBS-pre-adipocytes, which are characterized by a retained ability to adipogenic differentiation [68]. In order to verify whether the observed radiation response of SGBS-cells reflects the physiological behavior, additional experiments with human primary pre-adipocytes were conducted.

Initially, the sensitivity of pre-adipocytes to irradiation with respect to proliferation capacity was investigated. The results revealed that the proliferation of both SGBS- and primary pre-adipocytes after exposure to X-rays was dose-dependently reduced. However, only high doses of X-rays (2 and 10 Gy) caused a significant decrease of the cell number. Moreover, irradiation with 10 Gy completely inhibited the cell growth of SGBS-cells as well as of primary pre-adipocytes. Pre-adipocytes arise from mesenchymal stem cells and their proliferation pattern after irradiation was very similar to that of other cells of mesenchymal origin or mesenchymal stem cells itself [196], [208]. This observation is also in line with Jeong et al, who reported a significant decrease in the proliferation of adipose-derived stem cells obtained after irradiation of the pig [209].

To evaluate the radiation effects on differentiation process of adipocytes, the cells were irradiated as pre-adipocytes and then differentiation was initiated. The changes were monitored over a period of 20 - 28 days. The most prominent adipogenic marker is the ability to accumulate lipids. Therefore, the triglyceride accumulation and the differentiation rate during differentiation were examined in SGBS- and primary cells. The results revealed that primary adipocytes tend to increased lipid accumulation after irradiation. Moreover, exposure of the pre-adipocytes to 10 Gy of X-rays resulted in a significantly higher lipid content on day 20 after irradiation compared to unirradiated cells (Fig. 3.18). This is in line with Jo et al, who also reported increased size of irradiated adipocytes compared to controls [207]. Furthermore, some other studies considered the obesity development in the childhood leukemia survivors as a late effect of radiotherapy [210], [211]. However, although an increase in lipid accumulation after irradiation was detected, the differentiation rate did not differ between irradiated and unirradiated cells. The obtained results together with that of Jo et al suggest that high doses of radiation induce hypertrophy (cell size increase) rather than hyperplasia (cell number increase) of fat cells. At the earlier stages, however, the

differentiation rate was dose-dependently decreased (Fig. 3.19), which may reflect a delay of differentiation due to radiation-induced changes in the expression of genes responsible for maturation of adipocytes (Fig. 3.23).

The development and differentiation of adipocytes is subjected to a close regulation and controlling by a variety of growth- and transcription factors (Fig. 1.4). According to the literature, the crucial transcription factors for adipocyte development are peroxisome-proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer-binding proteins (C/EBPs). PPARγ and C/EBPα are necessary not only for induction of adipogenesis, but also for growth arrest, which is required for adipocyte maturation (Fig. 1.4) [61]. Together with C/EBPß, an earlier adipogenic marker, these genes determine the differentiation process of adipocytes. In the present work, the gene expression of PPARγ, C/EBPα and C/EBPß was found to be mostly unaffected by irradiation. However, the observed slight trends towards reduced expression (Fig. 3.23) could be associated with the delay at the beginning of differentiation mentioned before. In the late stages of differentiation, the mature adipocytes are characterized by the production of highly specific markers such as leptin, adiponectin and visfatin [43]. Although the analysis of gene expression of the mentioned markers met a number of technical difficulties, a slight, albeit not significant decrease in the visfatin expression was observed (Fig. 3.22B). This, however, was not supported by the investigation of visfatin release.

These results are in agreement with those published by Li et al [208] and Nicolay et al [212]. Both authors investigated the effects of ionizing radiation on the differentiation capacity of MSC, the adipocyte precursor cells. Li et al considered that irradiation with 2 and 4 Gy (photon radiation) did not significantly affect the differentiation process to adipocytes. However, for MSC exposed to 8 and 10 Gy, a reduced number of mature adipocytes was observed [208]. Furthermore, similar to the data presented here, Nicolay at al showed that expression of various adipogenic or osteogenic differentiation markers in irradiated MSC remained unchanged, suggesting that on transcriptional level the differentiation was not affected [212].

SGBS-cells constantly secreted visfatin during the whole observation period; however, the radiation-induced changes could not be established due to pronounced fluctuations (Fig. 3.21). In contrast to visfatin, the values of adiponectin and leptin were below detection limit. On the one hand, this could be explained by extremely low amounts of adipokines produced in 24 h. On the other hand, adipokine secretion by adipocytes is highly dependent on the level of insulin and other hormones [114]. Since the cells were starved in supplement-free medium 24 h prior to supernatant collection to avoid a contamination by adipokines from serum, it can be assumed that the cells had downregulated the adipokine production as a response to this stress.

For primary adipocytes, the release of adipokines was measured 10 and 20 days after irradiation without starvation of the cells (Fig. 3.20). The concentrations of adiponectin and leptin were higher on day 20 compared to day 10, which is explained by an increased adipokine secretion in the course of differentiation. However, no radiation-induced changes were observed. Only the cells exposed to 10 Gy X-rays showed a tendency towards higher adipokine release. Interestingly, the visfatin level was below the detection limit in the supernatants of primary adipocytes on day 10 and has dramatically increased until day 20

(Fig. 3.20C). This is different from SGBS-cells, which permanently secreted visfatin from the day 0 (pre-adipocytes) on (Fig. 3.21).

As the adipose tissue and adipocytes are also described to produce a variety of pro-inflammatory cytokines [165], [166], [213], radiation-mediated changes in the release of IL-6 and IL-8 during differentiation process of adipocytes were investigated. In primary adipocytes, the amounts of both cytokines did not differ between 10 and 20 cultivation days and were not significantly affected by irradiation (Fig. 3.24). Also in SGBS, the secretion of IL-6 and IL-8 was not changed by radiation; however, a continuous decrease in the course of differentiation was observed for both cytokines (Fig. 3.25). This data suggests that rather pre-adipocytes than mature adipocytes possess the pro-inflammatory properties. It is in accordance with Harkins et al, who reported higher IL-6 expression in mouse pre-adipocytes than in mature adipocytes [214].

Many studies suggested that adipose tissue is one of the main sources of pro-inflammatory cytokines. For example, Fried et al and Mohamed-Ali et al demonstrated that the adipose tissue releases a large amount of IL-6 [215], [216]. Moreover, Fried et al also reported that IL-6 was expressed to a large extent by the stromal vascular fraction (SVF) of adipose tissue; however, the exact cell type was not identified [216]. These data suggested that not adipocytes, but other cells of the adipose tissue may contribute to the enhanced concentration of IL-6 and IL-8 in the arthritic joint. However, it should be noted that in the present work and in most studies the experiments were done with subcutaneous adipose tissue or adipocytes. Although it has not been clarified yet whether the different fat depots have a different role in the physiological and pathological processes, some studies reported differences in the cytokine profile of different fat depots. For example, Distel et al demonstrated higher IL-6 release by IPFP than by subcutaneous adipose tissue [55]. Another example is provided by Klein-Wieringa et al, who found higher levels of IL-6, adiponectin and visfatin in the IPFP compared to subcutaneous fat tissue of OA patients [54]. These data indicate a more inflammatory phenotype of IPFP compared to other fat depots, which should be considered for further investigations.

In summary, in the present work the radiation response of human primary (pre)adipocytes was characterized for the first time. The potential of subcutaneous preadipocytes to differentiate into mature adipocytes seemed to be rather radiation-resistant. Even the high doses of X-rays did not significantly perturb adipogenic differentiation and the ability of adipocytes to produce adipokines. Furthermore, the results presented here showed that SGBS-cells exhibit mostly the same response to radiation as primary (pre)adipocytes.

5 Summary

In this thesis, a significant decrease of visfatin level in serum of patients after serial radon-baths treatment of patients with MSD (RAD-ON01 study) was shown for the first time. Furthermore, the amounts of visfatin were found to correlate with pain duration, indicated by patients, and with the VAS pain score. Considering the long-lasting pain relief in patients participating in this study [140] and the decrease of serum-TNFα, reported by Lange et al [141], the obtained results indicate that low-dose radon treatment attenuates the inflammation process in MSD patients. Furthermore, the *in vitro* investigations on effects of radiation on adipokine-treated synovial fibroblasts (SF), performed within scope of this thesis, supported the *in vivo* data. It was shown that the adipokine stimulation induced production of pro-inflammatory cytokines and matrix-degrading enzymes in both healthy (NSF) and rheumatoid arthritis SF (RASF), indicating the ability of all SF types to obtain an aggressive phenotype, depending on surrounding conditions. The ionizing radiation in turn reduced the adiponectin- and visfatin-induced production of pro-inflammatory cytokines (IL-6, IL-8, MCP-1) and matrix-degrading enzymes (MMP-1) in RASF.

Furthermore, in the present thesis, the radiation response of adipocytes was investigated for the first time. The results revealed that neither the ability of (pre)adipocytes to differentiate nor to produce adipokines was affected by ionizing radiation. Even the exposure to high doses of radiation (10 Gy) did not significantly change the gene expression of adipogenic markers. These results give inside into the regulation of the adipocyte development upon stress and the respective response. In addition, the obtained results suggest that adipocytes are probably not the main source of adipokines in the arthritic joint. However, it should be clarified whether fat cells, derived from other adipose tissue depots (esp. IPFP), exhibit the same radiation response as subcutaneous adipocytes used in this work.

To summarize the present work, the adipokines visfatin and adiponectin play an important role in the pathogenesis of MSD and presumably also in the RA-associated metabolic syndrome. Radiation exposure reduces adipokine-induced production of pro-inflammatory cytokines and matrix-degrading enzymes in RASF, supporting anti-inflammatory properties of low-dose radiation. These *in vitro* results are compliant with the *in vivo* observed decrease of serum visfatin levels in MSD patients after low-dose radon treatment, both indicating attenuation of inflammatory process in arthritic joint. Therefore, low-dose irradiation could provide a therapeutic option for the treatment of patients suffering from MSD. Ionizing radiation does not affect the differentiation process of adipocytes and their ability to produce adipokines, suggesting that adipocytes are not the main source of adipokines in the joint. The results obtained within the scope of this thesis contribute to a better understanding of the cellular and molecular mechanisms underlying clinical effects of low-dose radio- (LD-RT) and radon therapy.

Bibliography

[1] D. M. Salter, “The tissues we deal with: (II) Cartilage,” *Curr. Orthop.*, no. 12, pp. 251–257, 1998.

[2] A. J. S. Fox, A. Bedi, and S. A. Rodeo, “The basic science of articular cartilage: structure, composition, and function.,” *Sports Health*, vol. 1, no. 6, pp. 461–8, 2009.

[3] F. Bronner and M. Farach-Carson, *Bone and Osteoarthritis*, 4th ed. Springer-Verlag London Limited, 2007.

[4] S. M. Cowan, H. F. Hart, S. J. Warden, and K. M. Crossley, “Infrapatellar fat pad volume is greater in individuals with patellofemoral joint osteoarthritis and associated with pain,” *Rheumatol. Int.*, vol. 35, no. 8, pp. 1439–1442, 2015.

[5] M. A. MacConaill, “The movements of bones and joints,” *J Bone Jt. Surg Br*, vol. 32B, no. 2, pp. 244–252, 1950.

[6] S. Clockaerts *et al.*, “The infrapatellar fat pad should be considered as an active osteoarthritic joint tissue: A narrative review,” *Osteoarthr. Cartil.*, vol. 18, no. 7, pp. 876–882, 2010.

[7] B. Woolf, A. D.; Pfleger, “Burden of major musculoskeletal conditions. Bulletin of the World Health Organisation.,” vol. 9, no. 3, p. 2003, 2003.

[8] T. Pap and A. Korb-Pap, “Cartilage damage in osteoarthritis and rheumatoid arthritis--two unequal siblings.,” *Nat. Rev. Rheumatol.*, vol. 11, no. 10, pp. 606–15, 2015.

[9] J. Sokolove and C. M. Lepus, “Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations.,” *Ther. Adv. Musculoskelet. Dis.*, vol. 5, no. 2, pp. 77–94, 2013.

[10] I. McInnes, “The Pathogenesis of Rheumatoid Arthritis,” *N. Engl. J. Med.*, vol. 365, pp. 2205–19, 2011.

[11] V. Abella *et al.*, “Adipokines, metabolic syndrome and rheumatic diseases,” *J. Immunol. Res.*, vol. 2014, 2014.

[12] E. Choy, “Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis,” *Rheumatology.(Oxford)*, vol. 51 Suppl 5, no. 1462–0332 (Electronic), pp. v3-11, 2012.

[13] J. S. Smolen, D. Aletaha, M. Koeller, M. H. Weisman, and P. Emery, “New therapies for treatment of rheumatoid arthritis,” *Lancet*, vol. 370, no. 9602, pp. 1861–1874, 2007.

[14] P. K. Gregersen, J. Silver, and R. J. Winchester, “The Shared Epitope Hypothesis,” *Arthritis Rheum.*, vol. 30, no. 11, pp. 1205–1212, 1987.

[15] A. P. Cope, H. Schulze-Koops, and M. Aringer, “The central role of T cells in rheumatoid arthritis,” *Clin Exp Rheumatol*, vol. 25, no. 5 Suppl 46, pp. S4-11, 2007.

[16] L. Klareskog, L. Padyukov, and L. Alfredsson, “Smoking as a trigger for inflammatory rheumatic diseases,” pp. 49–54, 2007.

[17] M. T. Getts and S. D. Miller, “99th Dahlem Conference on Infection, Inflammation and Chronic Inflammatory Disorders: Triggering of autoimmune diseases by infections,” *Clin. Exp. Immunol.*, vol. 160, no. 1, pp. 15–21, 2010.

[18] S. Lefèvre *et al.*, “Synovial fibroblasts spread rheumatoid arthritis to unaffected joints.,” *Nat. Med.*, vol. 15, no. 12, pp. 1414–20, 2009.

[19] D. M. van der Heijde, “Joint erosions and patients with early rheumatoid arthritis,” *Br J Rheumatol*, vol. 34, no. 2, pp. 74–78, 1995.

[20] D. Felson, “Osteoarthritis as a disease of mechanics,” *Osteoarthr. Cartil.*, vol. 21, no. 1, pp. 10–15, 2013.

[21] L. Troeberg and H. Nagase, “Proteases involved in cartilage matrix degradation in osteoarthritis,” *Biochim Biophys Acta*, vol. 1824, no. 1, pp. 133–145, 2012.

[22] A. R. Sharma, S. Jagga, S. S. Lee, and J. S. Nam, “Interplay between cartilage and subchondral bone contributing to pathogenesis of osteoarthritis,” *Int. J. Mol. Sci.*, vol. 14, no. 10, pp. 19805–19830, 2013.

[23] B. Xia, D. Chen, J. Zhang, S. Hu, H. Jin, and P. Tong, “Osteoarthritis pathogenesis: a review of molecular mechanisms,” *Calcif Tissue Int*, vol. 95, no. 6, pp. 495–505, 2014.

[24] S. Kaneko, T. Satoh, J. Chiba, C. Ju, K. Inoue, and J. Kagawa, “Interleukin-6 and interleukin-8 levels in serum and synovial fluid of patients with osteoarthritis.,” *Cytokines. Cell. Mol. Ther.*, vol. 6, no. 2, pp. 71–9, 2000.

[25] M. N. Farahat, G. Yanni, R. Poston, and G. S. Panayi, “Cytokine expression in synovial membranes of patients with rheumatoid arthritis and osteoarthritis,” *Ann. Rheum. Dis.*, vol. 52, pp. 870–875, 1993.

[26] S. Nasi, A. So, C. Combes, M. Daudon, and N. Busso, “Interleukin-6 and chondrocyte mineralisation act in tandem to promote experimental osteoarthritis,” *Ann. Rheum. Dis.*, pp. 1–8, 2015.

[27] P. J. Francin *et al.*, “Association between adiponectin and cartilage degradation in human osteoarthritis,” *Osteoarthr. Cartil.*, vol. 22, no. 3, pp. 519–526, 2014.

[28] M. J. Martínez-Calatrava, I. Prieto-Potín, J. a Roman-Blas, L. Tardio, R. Largo, and G. Herrero-Beaumont, “RANKL synthesized by articular chondrocytes contributes to juxta-articular bone loss in chronic arthritis.,” *Arthritis Res. Ther.*, vol. 14, no. 3, p. R149, 2012.

[29] U. Müller-Ladner, “Cells of the synovium in rheumatoid arthritis - synovial fibroblasts.,” *Arthritis Res. Ther.*, vol. 9, no. 1, p. 203, 2007.

[30] K. Dasuri *et al.*, “The synovial proteome: analysis of fibroblast-like synoviocytes.,” *Arthritis Res. Ther.*, vol. 6, no. 2, pp. R161–R168, 2004.

[31] E. Hedbom and H. J. Hauselmann, “Molecular aspects of pathogenesis in osteoarthritis: the role of inflammation,” *Cell. Mol. Life Sci.*, vol. 59, no. 1, pp. 45–53, 2002.

[32] B. Bartok and G. S. Firestein, “Fibroblast-like synovioctyes: key effector cells in rheumatoid arthritis,” *Immunol Rev.*, vol. 233, no. 1, pp. 233–255, 2011.

[33] M. Frank-Bertoncelj and S. Gay, “The epigenome of synovial fibroblasts: an underestimated therapeutic target in rheumatoid arthritis.,” *Arthritis Res. Ther.*, vol. 16, no. 3, p. 117, 2014.

[34] H. Takayanagi, “New developments in osteoimmunology,” *Nat. Rev. Rheumatol.*, vol. 8, no. 11, pp. 684–689, 2012.

[35] X. Feng *et al.*, “Modulation of IL-6 induced RANKL expression in arthritic synovium by a transcription factor SOX5,” *Sci. Rep.*, vol. 6, no. August, p. 32001, 2016.

[36] A. Trabandt *et al.*, “Expression of the collagenolytic and Ras-induced cysteine proteinase cathepsin L and proliferation-associated oncogenes in synovial cells of MRL/I mice and patients with rheumatoid arthritis.,” *Matrix*, vol. 10, no. 6, pp. 349–361, 1990.

[37] E. Neumann, S. Lefèvre, B. Zimmermann, S. Gay, and U. Müller-Ladner, “Rheumatoid arthritis progression mediated by activated synovial fibroblasts,” *Trends Mol. Med.*, vol. 16, no. 10, pp. 458–468, 2010.

[38] M. A. Akhavani, L. Madden, I. Buysschaert, B. Sivakumar, N. Kang, and E. M. Paleolog, “Hypoxia upregulates angiogenesis and synovial cell migration in rheumatoid arthritis.,” *Arthritis Res Ther.*, vol. 11, no. 3, p. R64, 2009.

[39] M. Juarez, A. Filer, and C. D. Buckley, “Fibroblasts as therapeutic targets in rheumatoid arthritis and cancer,” *Swiss Med. Wkly.*, vol. 142, no. FEBRUARY, pp. 1–9, 2012.

[40] É. Toussirot *et al.*, “Serum adipokines and adipose tissue distribution in rheumatoid arthritis and ankylosing spondylitis. A comparative study,” *Front. Immunol.*, vol. 4, no. Article 453, pp. 1–10, 2013.

[41] C. Caruso, C. R. Balistreri, and G. Candore, “The role of adipose tissue and adipokines in obesity-related inflammatory diseases,” *Mediators Inflamm.*, vol. 2010, no. Article ID 802078, 2010.

[42] J. K. Sethi and A. J. Vidal-Puig, “Thematic review series: adipocyte biology. Adipose tissue function and plasticity orchestrate nutritional adaptation.,” *J. Lipid Res.*, vol. 48, no. 6, pp. 1253–62, 2007.

[43] A. Schäffler, U. Müller-Ladner, J. Schölmerich, and C. Büchler, “Role of adipose tissue as an inflammatory organ in human diseases,” *Endocr. Rev.*, vol. 27, no. 5, pp. 449–467, 2006.

[44] M. Fasshauer and M. Blüher, “Adipokines in health and disease,” *Trends Pharmacol. Sci.*, vol. 36, no. 7, pp. 461–470, 2015.

[45] A. T. Turer, J. A. Hill, J. K. Elmquist, and P. E. Scherer, “Adipose tissue biology and cardiomyopathy: Translational implications,” *Circ. Res.*, vol. 111, no. 12, pp. 1565–1577, 2012.

[46] M. Ozmen, O. Yersal, S. Ozturk, D. Solmaz, and M. H. Koseeoglu, “Prevalence of the metabolic syndrome in rheumatoid arthritis,” *Eur. J. Rheumatol.*, vol. 1, no. 1, pp. 1–4, 2014.

[47] G. Kerekes *et al.*, “Rheumatoid arthritis and metabolic syndrome,” *Nat. Rev. Rheumatol.*,

vol. 10, no. 11, pp. 691–696, 2014.

[48] S. Rostom, M. Mengat, R. Lahlou, S. Hari, R. Bahiri, and N. Hajjaj-Hassoun, “Metabolic syndrome in rheumatoid arthritis,” *Mediators Inflamm.*, vol. 2013, no. 2, 2013.

[49] V. da Cunha *et al.*, “Metabolic syndrome prevalence is increased in rheumatoid arthritis patients and is associated with disease activity.,” *Scand. J. Rheumatol.*, vol. 41, pp. 186–191, 2012.

[50] B. E. Wisse, “The inflammatory syndrome: the role of adipose tissue cytokines in metabolic disorders linked to obesity.,” *J. Am. Soc. Nephrol.*, vol. 15, no. 11, pp. 2792–800, 2004.

[51] B. Bjørndal, L. Burri, V. Staalesen, J. Skorve, and R. K. Berge, “Different adipose depots: Their role in the development of metabolic syndrome and mitochondrial response to hypolipidemic agents,” *J. Obes.*, vol. 2011, 2011.

[52] K. N. Frayn, F. Karpe, B. a Fielding, I. a Macdonald, and S. W. Coppack, “Integrative physiology of human adipose tissue.,” *Int. J. Obes. Relat. Metab. Disord.*, vol. 27, no. March, pp. 875–888, 2003.

[53] F. Eymard *et al.*, “Induction of an inflammatory and prodegradative phenotype in autologous fibroblast-like synoviocytes by the infrapatellar fat pad from patients with knee osteoarthritis,” *Arthritis Rheumatol.*, vol. 66, no. 8, pp. 2165–2174, 2014.

[54] I. R. Klein-Wieringa *et al.*, “The infrapatellar fat pad of patients with osteoarthritis has an inflammatory phenotype.,” *Ann. Rheum. Dis.*, vol. 70, no. 5, pp. 851–857, 2011.

[55] E. Distel, T. Cadoudal, S. Durant, A. Poignard, X. Chevalier, and C. Benelli, “The infrapatellar fat pad in knee osteoarthritis: An important source of interleukin-6 and its soluble receptor,” *Arthritis Rheum.*, vol. 60, no. 11, pp. 3374–3377, 2009.

[56] A. Ioan-Facsinay and M. Kloppenburg, “An emerging player in knee osteoarthritis: the infrapatellar fat pad.,” *Arthritis Res. Ther.*, vol. 15, no. 6, p. 225, 2013.

[57] L. M. Gierman *et al.*, “Metabolic profiling reveals differences in concentrations of oxylipins and fatty acids secreted by the infrapatellar fat pad of donors with end-stage osteoarthritis and normal donors,” *Arthritis Rheum.*, vol. 65, no. 10, pp. 2606–2614, 2013.

[58] M. Esteve Ràfols, “Adipose tissue: cell heterogeneity and functional diversity.,” *Endocrinol. y Nutr. órgano la Soc. Española Endocrinol. y Nutr.*, vol. 61, no. 2, pp. 100–112, 2014.

[59] S. Caspar-Bauguil *et al.*, “Adipose tissues as an ancestral immune organ: Site-specific change in obesity,” *FEBS Lett.*, vol. 579, no. 17, pp. 3487–3492, 2005.

[60] J. M. Rutkowski, J. H. Stern, and P. E. Scherer, “The cell biology of fat expansion,” *J. Cell Biol.*, vol. 208, no. 5, pp. 501–512, 2015.

[61] F. M. Gregoire, C. M. Smas, and H. S. Sul, “Understanding adipocyte differentiation.,” *Physiol. Rev.*, vol. 78, no. 3, pp. 783–809, 1998.

[62] S. Vielma, R. Klein, C. Levingston, and M. Young, “Adipocytes as immune regulatory

cells," *Int. Immunopharmacol*, vol. 16, no. 2, pp. 224–231, 2013.

[63] E. D. Rosen and O. a MacDougald, "Adipocyte differentiation from the inside out.," *Nat. Rev. Mol. Cell Biol.*, vol. 7, no. 12, pp. 885–96, 2006.

[64] P. Tontonoz, E. Hu, and B. M. Spiegelman, "Stimulation of adipogenesis in fibroblasts by PPAR-gamma2, a lipid-activated transcription factor," *Cell*, vol. 79, pp. 1147–1156, 1994.

[65] M. Wabitsch *et al.*, "Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation," *Int. J. Obes.*, vol. 25, no. 1, pp. 8–15, 2001.

[66] F. J. Ruiz-Ojeda, A. I. Ruperez, C. Gomez-Llorente, A. Gil, and C. M. Aguilera, "Cell models and their application for studying adipogenic differentiation in relation to obesity: A review," *Int. J. Mol. Sci.*, vol. 17, no. 1040, 2016.

[67] M. Wabitsch and R. Brenner, "Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation." .

[68] P. Fischer-Posovszky, F. Newell, M. Wabitsch, and H. Tornqvist, "Human SGBS Cells - a Unique Tool for Studies of Human Fat Cell Biology," *Obes. Facts*, vol. 1, pp. 184–189, 2008.

[69] U. Jung and M.-S. Choi, "Obesity and Its Metabolic Complications: The Role of Adipokines and the Relationship between Obesity, Inflammation, Insulin Resistance, Dyslipidemia and Nonalcoholic Fatty Liver Disease," *Int. J. Mol. Sci.*, vol. 15, no. 4, pp. 6184–6223, 2014.

[70] M. Imamura *et al.*, "Serum levels of proinflammatory cytokines in painful knee osteoarthritis and sensitization," *Int. J. Inflam.*, vol. 2015, 2015.

[71] M. Osiri, J. Wongpiyabovorn, Y. Sattayasomboon, and N. Thammacharoenrach, "Inflammatory cytokine levels, disease activity, and function of patients with rheumatoid arthritis treated with combined conventional disease-modifying antirheumatic drugs or biologics," *Clin. Rheumatol.*, vol. 35, no. 7, pp. 1673–1681, 2016.

[72] J.-P. Pelletier, J. Martel-Pelletier, and S. B. Abramson, "Osteoarthritis, an Inflammatory Disease. Potential Implication for the Selection of New Therapeutic Targets," *Arthritis Rheum.*, vol. 44, no. 6, pp. 1237–1247, 2001.

[73] M. Coelho, T. Oliveira, and R. Fernandes, "Biochemistry of adipose tissue: An endocrine organ," *Arch. Med. Sci.*, vol. 9, no. 2, pp. 191–200, 2013.

[74] A. L. Carey *et al.*, "Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase," *Diabetes*, vol. 55, pp. 2688–2697, 2006.

[75] P. Vasanthi, G. Nalini, and G. Rajasekhar, "Role of tumor necrosis factor-alpha in rheumatoid arthritis: A review," *APLAR J. Rheumatol.*, vol. 10, no. 4, pp. 270–274, 2007.

[76] I. B. McInnes, C. D. Buckley, and J. D. Isaacs, "Cytokines in Rheumatoid Arthritis - Shaping the Immunological Landscape," *Nat. Rev. Rheumatol.*, vol. 12, no. 1, pp. 63–

68, 2016.

[77] V. Slavic, A. Stankovi, and B. Kamenov, “The role of Interleukin-8 and Monocyte chemotactic protein-1 in rheumatoid arthritis,” vol. 12, no. 1, pp. 19–22, 2005.

[78] M. S. Bendre, D. C. Montague, T. Peery, N. S. Akel, D. Gaddy, and L. J. Suva, “Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease,” *Bone*, vol. 33, no. 1, pp. 28–37, 2003.

[79] G. Sommer *et al.*, “Visfatin is a positive regulator of MCP-1 in human adipocytes in vitro and in mice in vivo,” *Obesity (Silver Spring).*, vol. 18, no. 8, pp. 1486–1492, 2010.

[80] P. Bhattaram and U. Chandrasekharan, “The joint synovium: A critical determinant of articular cartilage fate in inflammatory joint diseases,” *Semin. Cell Dev. Biol.*, vol. 62, pp. 86–93, 2017.

[81] K. Huang and L. D. Wu, “Aggrecanase and aggrecan degradation in osteoarthritis: a review.,” *J. Int. Med. Res.*, vol. 36, no. 6, pp. 1149–1160, 2008.

[82] D. B. Logar, R. Komadina, J. Preželj, B. Ostanek, Z. Trošt, and J. Marc, “Expression of bone resorption genes in osteoarthritis and in osteoporosis,” *J. Bone Miner. Metab.*, vol. 25, no. 4, pp. 219–225, 2007.

[83] L. C. Huber, O. Distler, I. Tarner, R. E. Gay, S. Gay, and T. Pap, “Synovial fibroblasts: key players in rheumatoid arthritis.,” *Rheumatology (Oxford).*, vol. 45, no. 6, pp. 669–75, 2006.

[84] Y. Liu, C. Y. Song, S. S. Wu, Q. H. Liang, L. Q. Yuan, and E. Y. Liao, “Novel adipokines and bone metabolism,” *Int. J. Endocrinol.*, vol. 2013, no. ID 895045, 2013.

[85] Y. Deng and P. E. Scherer, “Adipokines as novel biomarkers of the metabolic syndrome,” *Ann. N. Y. Acad. Sci.*, vol. 1212, pp. E1–E19, 2010.

[86] E. Neumann, S. Junker, G. Schett, K. Frommer, and U. Müller-Ladner, “Adipokines in bone disease,” *Nat. Rev. Rheumatol.*, vol. 12, no. 5, pp. 296–302, 2016.

[87] T. Yoshino *et al.*, “Elevated serum levels of resistin, leptin, and adiponectin are associated with C-reactive protein and also other clinical conditions in rheumatoid arthritis.,” *Intern. Med.*, vol. 50, no. 4, pp. 269–275, 2011.

[88] T. N. de Boer *et al.*, “Serum adipokines in osteoarthritis; comparison with controls and relationship with local parameters of synovial inflammation and cartilage damage,” *Osteoarthr. Cartil.*, vol. 20, no. 8, pp. 846–853, 2012.

[89] N. Presle *et al.*, “Differential distribution of adipokines between serum and synovial fluid in patients with osteoarthritis. Contribution of joint tissues to their articular production,” *Osteoarthr. Cartil.*, vol. 14, no. 7, pp. 690–695, 2006.

[90] R. Gómez, J. Conde, M. Scotece, J. J. Gómez-Reino, F. Lago, and O. Gualillo, “What’s new in our understanding of the role of adipokines in rheumatic diseases?,” *Nat. Rev. Rheumatol.*, vol. 7, no. 9, pp. 528–536, 2011.

[91] J. P. Bao, W. P. Chen, and L. D. Wu, “Visfatin: a potential therapeutic target for

rheumatoid arthritis," *J. Int. Med. Res.*, vol. 37, no. 6, pp. 1655–1661, 2009.

[92] A. Rongvaux *et al.*, "Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAF biosynthesis," *Eur. J. Immunol.*, vol. 32, no. 11, pp. 3225–3234, 2002.

[93] Y. Rho *et al.*, "Adipocytokines Are Associated with Radiographic Joint Damage," *Arthritis Rheum.*, vol. 60, no. 7, pp. 1906–1914, 2009.

[94] M. Otero *et al.*, "Changes in plasma levels of fat-derived hormones adiponectin, leptin, resistin and visfatin in patients with rheumatoid arthritis," *Ann Rheum Dis*, vol. 65, pp. 1198–1201, 2006.

[95] M.-C. Laiguillon *et al.*, "Expression and function of visfatin (Nampt), an adipokine-enzyme involved in inflammatory pathways of osteoarthritis.," *Arthritis Res. Ther.*, vol. 16, no. 1, p. R38, 2014.

[96] F. M. P. Meier *et al.*, "Visfatin/pre-B-cell colony-enhancing factor (PBEF), a proinflammatory and cell motility-changing factor in rheumatoid arthritis," *J. Biol. Chem.*, vol. 287, no. 34, pp. 28378–28385, 2012.

[97] T. Romacho, C. F. Sánchez-Ferrer, and C. Peiró, "Review Article Visfatin / Nampt : An Adipokine with Cardiovascular Impact," *Mediators Inflamm.*, vol. 2013, no. ID 946427, 2013.

[98] Y. Li *et al.*, "Extracellular nampt promotes macrophage survival via a nonenzymatic interleukin-6/STAT3 signaling mechanism," *J. Biol. Chem.*, vol. 283, no. 50, pp. 34833–34843, 2008.

[99] F. Brentano *et al.*, "Pre-B cell colony-enhancing factor/visfatin, a new marker of inflammation in rheumatoid arthritis with proinflammatory and matrix-degrading activities," *Arthritis Rheum.*, vol. 56, no. 9, pp. 2829–2839, 2007.

[100] K. W. Frommer *et al.*, "Adiponectin-mediated changes in effector cells involved in the pathophysiology of rheumatoid arthritis," *Arthritis Rheum.*, vol. 62, no. 10, pp. 2886–2899, 2010.

[101] F. Lago, R. Gómez, J. Conde, J. J. Gómez Reino, F. Lago, and O. Gualillo, "Adipocytokines: emerging mediators of the immune response and inflammation," *Reumatol. Clin.*, vol. 5 Suppl 1, no. 12, pp. 6–12, 2009.

[102] N. Ouchi and K. Walsh, "Adiponectin as an anti-inflammatory factor," *Clin. Chim. Acta.*, vol. 380, no. 1–2, pp. 24–30, 2007.

[103] R. Shibata *et al.*, "Adiponectin protects against myocardial ischemia-reperfusion injury through AMPK- and COX-2-dependent mechanisms.," *Nat. Med.*, vol. 11, no. 10, pp. 1096–103, 2005.

[104] K. M. Tong *et al.*, "Adiponectin increases MMP-3 expression in human chondrocytes through adipor1 signaling pathway," *J. Cell. Biochem.*, vol. 112, no. 5, pp. 1431–1440, 2011.

[105] C.-H. Tang, Y.-C. Chiu, T.-W. Tan, R.-S. Yang, and W.-M. Fu, "Adiponectin Enhances IL-

6 Production in Human Synovial Fibroblast via an AdipoR1 Receptor, AMPK, p38, and NF- B Pathway," *J. Immunol.*, vol. 179, no. 8, pp. 5483–5492, 2007.

[106] R. Gomez, M. Scotece, J. Conde, J. J. Gomez-Reino, F. Lago, and O. Gualillo, "Adiponectin and leptin increase IL-8 production in human chondrocytes," *Ann. Rheum. Dis.*, vol. 70, no. 11, pp. 2052–2054, 2011.

[107] K. Kitahara, N. Kusunoki, T. Kakiuchi, T. Suguro, and S. Kawai, "Adiponectin stimulates IL-8 production by rheumatoid synovial fibroblasts," *Biochem. Biophys. Res. Commun.*, vol. 378, no. 2, pp. 218–223, 2009.

[108] J. Conde *et al.*, "Adiponectin and Leptin Induce VCAM-1 Expression in Human and Murine Chondrocytes," *PLoS One*, vol. 7, no. 12, pp. 1–7, 2012.

[109] O. Dikbas, M. Tosun, C. Bes, S. B. Tonuk, O. Y. Aksehirli, and M. Soy, "Serum levels of visfatin, resistin and adiponectin in patients with psoriatic arthritis and associations with disease severity," *Int. J. Rheum. Dis.*, vol. 19, no. 7, pp. 672–677, 2016.

[110] K. Robinson, J. Prins, and B. Venkatesh, "Clinical review: adiponectin biology and its role in inflammation and critical illness.," *Crit. Care*, vol. 15, no. 2, p. 221, 2011.

[111] E. Kontny, I. Janicka, U. Skalska, and W. Maslinski, "The effect of multimeric adiponectin isoforms and leptin on the function of rheumatoid fibroblast-like synoviocytes," *Scand. J. Rheumatol.*, vol. 44, no. 5, pp. 363–368, 2015.

[112] U. Skalska and E. Kontny, "Adiponectin Isoforms and Leptin Impact on Rheumatoid Adipose Mesenchymal Stem Cells Function," *Stem Cells Int.*, vol. 2016, no. July, 2016.

[113] K. W. Frommer *et al.*, "Adiponectin isoforms: a potential therapeutic target in rheumatoid arthritis?," *Ann. Rheum. Dis.*, vol. 71, no. 10, pp. 1724–32, 2012.

[114] R. Krysiak, G. Handzlik-Orlik, and B. Okopien, "The role of adipokines in connective tissue diseases," *Eur. J. Nutr.*, vol. 51, no. 5, pp. 513–528, 2012.

[115] A. La Cava and G. Matarese, "The weight of leptin in immunity," *Nat Rev Immunol*, vol. 4, no. 5, pp. 371–379, 2004.

[116] M. Otero *et al.*, "Towards a pro-inflammatory and immunomodulatory emerging role of leptin," *Rheumatology*, vol. 45, no. 8, pp. 944–950, 2006.

[117] V. Cirmanová, M. Bayer, L. Stárka, and K. Zajíčková, "The effect of leptin on bone - An evolving concept of action," *Physiol. Res.*, vol. 57, no. 1, pp. 143–151, 2008.

[118] B. Targonska-Stepniak, M. Majdan, and M. Dryglewska, "Leptin serum levels in rheumatoid arthritis patients: relation to disease duration and activity.," *Rheumatol. Int.*, vol. 28, pp. 585–591, 2008.

[119] G. M. Lord, G. Matarese, J. Howard, R. Baker, S. Bloom, and R. I. Lechler, "Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppresion," *Nature*, vol. 394, pp. 897–901, 1998.

[120] M. A. Gonzalez-Gay *et al.*, "Anti-TNF-[alpha] therapy modulates resistin in patients with rheumatoid arthritis," *Clin. Exp. Rheumatol.*, vol. 26, pp. 311–316, 2008.

[121] G. D. Norata, M. Ongari, K. Garlaschelli, S. Raselli, L. Grigore, and A. L. Catapano, "Plasma resistin levels correlate with determinants of the metabolic syndrome," *Eur. J. Endocrinol.*, vol. 156, no. 2, pp. 279–284, 2007.

[122] K. Migita *et al.*, "The serum levels of resistin in rheumatoid arthritis patients," *Clin Exp Rheumatol*, vol. 24, pp. 698–701, 2006.

[123] N. Silswal, A. K. Singh, B. Aruna, S. Mukhopadhyay, S. Ghosh, and N. Z. Ehtesham, "Human resistin stimulates the pro-inflammatory cytokines TNF-alpha and IL-12 in macrophages by NF-kB-dependent pathway," *Biochem. Biophys. Res. Commun.*, vol. 334, pp. 1092–1101, 2005.

[124] J. R. O'Dell *et al.*, "Therapies for active rheumatoid arthritis after methotrexate failure.," *N. Engl. J. Med.*, vol. 369, no. 4, pp. 307–18, 2013.

[125] K. Peterson *et al.*, "Drug Class Review: Nonsteroidal Antiinflammatory Drugs (NSAIDs)," no. November, p. 72, 2010.

[126] S. Cohen *et al.*, "Treatment of rheumatoid arthritis with anakinra, a recombinant human interleukin-1 receptor antagonist, in combination with methotrexate: Results of a twenty-four-week, multicenter, randomized, double-blind, placebo-controlled trial," *Arthritis Rheum.*, vol. 46, no. 3, pp. 614–624, 2002.

[127] P. Emery *et al.*, "IL-6 receptor inhibition with tocilizumab improves treatment outcomes in patients with rheumatoid arthritis refractory to anti-tumour necrosis factor biologicals: results from a 24-week multicentre randomised placebo-controlled trial.," *Ann. Rheum. Dis.*, vol. 67, no. 11, pp. 1516–23, 2008.

[128] R. Reyes-García, M. Muñoz-Torres, D. F. García, P. Mezquita-Raya, J. A. García Salcedo, and J. de Dios Luna, "Effects of alendronate treatment on serum levels of osteoprotegerin and total receptor activator of nuclear factor kappaB in women with postmenopausal osteoporosis.," *Menopause*, vol. 17, no. 1, pp. 140–4, 2010.

[129] M. Rogers *et al.*, "Molecular Mechanisms of Action of Bisphosphonates," *Bone*, vol. 24, no. 5, pp. 73–79, 1999.

[130] Z. Rosman, Y. Shoenfeld, and G. Zandman-Goddard, "Biologic therapy for autoimmune diseases: an update.," *BMC Med.*, vol. 11, no. 1, p. 88, 2013.

[131] K. Kennel and M. Drake, "Adverse Effects of Bisphosphonates : Implications for osteoporosis management," *Mayo Clin. Proc.*, vol. 55905, no. July, pp. 632–638, 2009.

[132] V. Strand, R. Kimberly, and J. D. Isaacs, "Biologic therapies in rheumatology: lessons learned, future directions," *Nat Rev Drug Discov*, vol. 6, no. 1, pp. 75–92, 2007.

[133] M. H. Seegenschmiedt, O. Micke, and R. Muecke, "Radiotherapy for non-malignant disorders: State of the art and update of the evidence-based practice guidelines," *Br. J. Radiol.*, vol. 88, no. 1051, pp. 1–11, 2015.

[134] S. Keller *et al.*, "Efficacy of low-dose radiotherapy in painful gonarthritis: experiences from a retrospective East German bicenter study.," *Radiat. Oncol.*, vol. 8, p. 29, 2013.

[135] A. Liebmann *et al.*, "Low-Dose X-Irradiation of Adjuvant-Induced Arthritis in Rats: Efficacy of Different Fractionation Schedules," *Strahlentherapie und Onkol.*, vol. 180,

no. 3, pp. 165–172, 2004.

[136] M. Tsukimoto, H. Nakatsukasa, K. Sugawara, K. Yamashita, and S. Kojima, “Repeated 0.5-Gy gamma irradiation attenuates experimental autoimmune encephalomyelitis with up-regulation of regulatory T cells and suppression of IL17 production,” *Radiat. Res.*, vol. 170, no. 4, pp. 429–436, 2009.

[137] E. J. Calabrese and V. Calabrese, “Reduction of arthritic symptoms by low dose radiation therapy (LD-RT) is associated with an anti-inflammatory phenotype.,” *Int. J. Radiat. Biol.*, vol. 89, no. 4, pp. 278–86, 2013.

[138] B. Frey, S. Hehlgans, F. Rödel, and U. S. Gaipl, “Modulation of inflammation by low and high doses of ionizing radiation: Implications for benign and malign diseases,” *Cancer Lett.*, vol. 368, no. 2, pp. 230–237, 2015.

[139] A. Falkenbach, J. Kovacs, A. Franke, K. Jörgens, and K. Ammer, “Radon therapy for the treatment of rheumatic diseases--review and meta-analysis of controlled clinical trials.,” *Rheumatol. Int.*, vol. 25, no. 3, pp. 205–210, 2005.

[140] P. F. Rühle *et al.*, “Modulation of the peripheral immune system after low-dose radon spa therapy: Detailed longitudinal immune monitoring of patients within the RAD-ON01 study,” *Autoimmunity*, vol. 50, no. 2, pp. 133–140, 2017.

[141] U. Lange *et al.*, “The impact of serial radon and hyperthermia exposure in a therapeutic adit on pivotal cytokines of bone metabolism in rheumatoid arthritis and osteoarthritis,” *Clin. Rheumatol.*, vol. 35, no. 11, pp. 2783–2788, 2016.

[142] Z. Zdrojewicz and J. J. Strzelczyk, “Radon Treatment Controversy,” *Dose-Response*, vol. 4, no. 2, pp. 106–118, 2006.

[143] E. Nussbaum and J. Hursch, “Radon Solubility in Rat Tissues,” *Science (80-.).*, vol. 125, no. 4, 1956.

[144] N. Harley and E. Robbins, “222-Rn alpha dose to organs other than the lung,” *Radiat. Prot. Dosimetry*, vol. 45, no. 1/4, pp. 619–622, 1992.

[145] A. Khursheed, “Doses to systemic tissues from radon gas,” *Radiat. Prot. Dosim. \r*, vol. 88, no. 2, pp. 171–181, 2000.

[146] A. Moder, C. Hufnagl, W. Hitzl, M. Jakab, and M. Ritter, “Radon-Therapy in Ankylosing Spondylitis reduces auto-Antibody titers,” *Open J. Mol. Integr. Physiol.*, vol. 1, no. 3, pp. 52–54, 2011.

[147] M. Winklmayr *et al.*, “Radon balneotherapy and physical activity for osteoporosis prevention: a randomized, placebo-controlled intervention study,” *Radiat. Environ. Biophys.*, vol. 54, no. 1, pp. 123–136, 2015.

[148] M. Scholz, “Dose response of biological systems to low- and high-LET radiation,” *Elsevier*, pp. 1–73, 2006.

[149] V. Chiste, M. . Be, and C. Dulieu, “Evaluation of decay data of radium-226 and its daughters,” *Int. Conf. Nucl. Data Sci. Technol.*, no. 2007, pp. 77–80, 2008.

[150] P. Deetjen, A. Falkenbach, D. Harder, H. Jöckel, A. Kaul, and H. Philipsborn, “Radon als

Heilmittel," *RADIZ-Informationsheft*, vol. 67, no. 25, p. 111, 2005.

[151] A. Maier *et al.*, "Experimental setup for radon exposure and first diffusion studies using gamma spectroscopy," *Nucl. Instruments Methods Phys. Res. Sect. B Beam Interact. with Mater. Atoms*, vol. 362, no. March 2016, pp. 187–193, 2015.

[152] M. Scholz, "Effects of Ion Radiation on Cells and Tissues," *Adv. Polym. Sci.*, vol. 162, 2003.

[153] G. Hildebrandt, L. Maggiorella, F. Rodel, V. Rodel, D. Willis, and K. R. Trott, "Mononuclear cell adhesion and cell adhesion molecule liberation after X-irradiation of activated endothelial cells in vitro," *Int J Radiat Biol*, vol. 78, no. 4, pp. 315–325, 2002.

[154] M. Large, S. Reichert, S. Hehlgans, C. Fournier, C. Rödel, and F. Rödel, "A non-linear detection of phospho-histone H2AX in EA.hy926 endothelial cells following low-dose X-irradiation is modulated by reactive oxygen species.," *Radiat. Oncol.*, vol. 9, no. 1, p. 80, 2014.

[155] T. K. Yeung, S. Lauk, R. H. Simmonds, J. W. Hopewell, and K. R. Trott, "Morphological and functional changes in the rat heart after X irradiation: strain differences.," *Radiat. Res.*, vol. 119, no. 3, pp. 489–499, 1989.

[156] R. Wunderlich *et al.*, "Low and moderate doses of ionizing radiation up to 2 Gy modulate transmigration and chemotaxis of activated macrophages, provoke an anti-inflammatory cytokine milieu, but do not impact upon viability and phagocytic function," *Clin. Exp. Immunol.*, vol. 179, no. 1, pp. 50–61, 2015.

[157] M. Khan and F. Joseph, "Adipose tissue and adipokines: the association with and application of adipokines in obesity.," *Scientifica (Cairo).*, vol. 2014, no. ID 328592, 2014.

[158] A. Del Prete, V. Salvi, and S. Sozzani, "Adipokines as potential biomarkers in rheumatoid arthritis," *Mediators Inflamm.*, vol. 2014, 2014.

[159] L. Šenolt, "Adipokines: role in local and systemic inflammation of rheumatic diseases," *Expert Rev. Clin. Immunol.*, vol. 13, no. 1, pp. 1–3, 2017.

[160] U. Syrbe *et al.*, "Serum adipokine levels in patients with ankylosing spondylitis and their relationship to clinical parameters and radiographic spinal progression," *Arthritis Rheumatol.*, vol. 67, no. 3, pp. 678–685, 2015.

[161] O. Sglunda *et al.*, "Decreased circulating visfatin is associated with improved disease activity in early rheumatoid arthritis: Data from the PERAC cohort," *PLoS One*, vol. 9, no. 7, pp. 1–5, 2014.

[162] X. Chen, J. Lu, J. Bao, J. Guo, J. Shi, and Y. Wang, "Adiponectin: A biomarker for rheumatoid arthritis?," *Cytokine Growth Factor Rev.*, vol. 24, no. 1, pp. 83–89, 2013.

[163] E. Neumann, K. W. Frommer, M. Vasile, and U. Müller-Ladner, "Adipocytokines as driving forces in rheumatoid arthritis and related inflammatory diseases?," *Arthritis Rheum.*, vol. 63, no. 5, pp. 1159–1169, 2011.

[164] A. Ehling *et al.*, "The potential of adiponectin in driving arthritis.," *J. Immunol.*, vol. 176, no. 7, pp. 4468–4478, 2006.

[165] L. Flower, R. Gray, J. Pinkney, and V. Mohamed-Ali, “Stimulation of interleukin-6 release by interleukin-1β from isolated human adipocytes,” *Cytokine*, vol. 21, no. 1, pp. 32–37, 2003.

[166] V. de S. Barbosa, J. Rego, and N. Antonio da Silva, “Possible role of adipokines in systemic lupus erythematosus and rheumatoid arthritis.,” *Rev. Bras. Reumatol.*, vol. 52, no. 2, pp. 278–287, 2012.

[167] G. R. Burmester, E. Feist, and T. Dörner, “Emerging cell and cytokine targets in rheumatoid arthritis.,” *Nat. Rev. Rheumatol.*, vol. 10, no. 2, pp. 77–88, 2014.

[168] M. Hashizume, N. Hayakawa, and M. Mihara, “IL-6 trans-signalling directly induces RANKL on fibroblast-like synovial cells and is involved in RANKL induction by TNF-alpha and IL-17,” *Rheumatology*, vol. 47, no. 11, pp. 1635–1640, 2008.

[169] A. Cucu, K. Shreder *et al.*, “Decrease of Markers related to Bone erosion in serum of Patients with Musculoskeletal Disorders after serial low-Dose radon spa Therapy,” *Front. Immunol.*, vol. 8, no. July, pp. 1–9, 2017.

[170] A. C. Bay-Jensen *et al.*, “Role of hormones in cartilage and joint metabolism: understanding an unhealthy metabolic phenotype in osteoarthritis,” *Menopause*, vol. 20, no. 5, pp. 578–586, 2013.

[171] R. B. Xiong, Q. Li, W. R. Wan, J. Q. Guo, B. De Luo, and L. Gan, “Effects and mechanisms of vitamin A and vitamin E on the levels of serum leptin and other related cytokines in rats with rheumatoid arthritis,” *Exp. Ther. Med.*, vol. 8, no. 2, pp. 499–504, 2014.

[172] C. Popa *et al.*, “Circulating leptin and adiponectin concentrations during tumor necrosis factor blockade in patients with active rheumatoid arthritis,” *J. Rheumatol.*, vol. 36, no. 4, pp. 724–730, 2009.

[173] S. Suman, S. Kumar, A. J. F. Jr, and K. Datta, “Space radiation exposure persistently increased leptin and IGF1 in serum and activated leptin-IGF1 signaling axis in mouse intestine,” *Nat. Publ. Gr.*, no. March, pp. 1–11, 2016.

[174] S. Suman, B. V. S. Kallakury, A. J. Fornace, and K. Datta, “Protracted upregulation of leptin and IGF1 is associated with activation of PI3K/Akt and JAK2 pathway in mouse intestine after ionizing radiation exposure,” *Int. J. Biol. Sci.*, vol. 11, no. 3, pp. 274–283, 2015.

[175] H. Greenlee, Z. Shi, C. L. Sardo Molmenti, A. Rundle, and W. Y. Tsai, “Trends in obesity prevalence in adults with a history of cancer: Results from the US National Health Interview Survey, 1997 to 2014,” *J. Clin. Oncol.*, vol. 34, no. 26, pp. 3133–3140, 2016.

[176] Y. Zhang, J. L. Zitsman, J. Hou, I. Fennoy, K. Guo, and R. L. Leibel, “Fat cell size and adipokine expression in relation to gender, depot and metabolic risk factors in morbidly obese adolescents,” vol. 22, no. 3, pp. 691–697, 2014.

[177] L. Hellström, H. Wahrenberg, K. Hruska, S. Reynisdottir, and P. Arner, “Mechanisms behind gender differences in circulating leptin levels,” *J. Intern. Med.*, vol. 247, no. 4, pp. 457–462, 2000.

[178] T. M. Ahonen, H. J. Kautiainen, S. M. Keinänen-Kiukaanniemi, E. A. Kumpusalo, and M. J. Vanhala, "Gender Difference Among Smoking, Adiponectin, and High-Sensitivity C-Reactive Protein," *Am. J. Prev. Med.*, vol. 35, no. 6, pp. 598–601, 2008.

[179] N. Busso *et al.*, "Pharmacological inhibition of nicotinamide phosphoribosyltransferase/visfatin enzymatic activity identifies a new inflammatory pathway linked to NAD," *PLoS One*, vol. 3, no. 5, 2008.

[180] A. R. Moschen *et al.*, "Visfatin, an Adipocytokine with Proinflammatory and Immunomodulating Properties," *J. Immunol.*, vol. 178, no. 3, pp. 1748–1758, 2007.

[181] S. Bas *et al.*, "Adipokines correlate with pain in lower limb osteoarthritis: Different associations in hip and knee," *Int. Orthop.*, vol. 38, no. 12, pp. 2577–2583, 2014.

[182] M. Almaghraby and A. Mahmoud, "Correlation of serum visfatin level with chest pain scoring as an indication of myocardial ischemia in chronic kidney disease patients," *Egypt. J. Intern. Med.*, vol. 26, no. 2, p. 45, 2014.

[183] J. Anderson *et al.*, "Rheumatoid arthritis disease activity measures: American College of Rheumatology recommendations for use in clinical practice," *Arthritis Care Res. (Hoboken).*, vol. 64, no. 5, pp. 640–647, 2012.

[184] M. Jurdana, A. Petelin, M. C. Bizjak, M. Bizjak, G. Biolo, and Z. Jenko-Praznikar, "Increased serum visfatin levels in obesity and its association with anthropometric/biochemical parameters, physical inactivity and nutrition," *ESPEN. J.*, vol. 8, no. 2, pp. 59–67, 2013.

[185] M. Walicka, W. Bik, and E. Marcinowska-suchowierska, "Gender dependent dimorphism in adipokines levels and its correlations with insulin resistance in extremely obese patients," *Postep. Nauk Med.*, vol. XXVI, no. 4, pp. 262–266, 2013.

[186] X. Wang, D. Hunter, J. Xu, and C. Ding, "Metabolic triggered inflammation in osteoarthritis," *Osteoarthr. Cartil.*, vol. 23, no. 1, pp. 22–30, 2015.

[187] A. M. Abeles and M. H. Pillinger, "The role of the synovial fibroblast in rheumatoid arthritis: Cartilage destruction and the regulation of matrix metalloproteinases," *Bull. NYU Hosp. Jt. Dis.*, vol. 64, no. 1–2, pp. 20–24, 2006.

[188] G. S. Firestein and N. Bottini, "Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors," *Nat. Rev. Rheumatol.*, vol. 9, no. 1, pp. 24–33, 2013.

[189] M. J. Del Rey *et al.*, "Transcriptome analysis reveals specific changes in osteoarthritis synovial fibroblasts," *Ann. Rheum. Dis.*, vol. 71, no. 2, pp. 275–280, 2012.

[190] G. Dischereit, N. Neumann, U. Müller-Ladner, B. Kürten, and U. Lange, "Einfluss einer seriellen niedrig-dosierten Radonstollen- Hyperthermie auf Schmerz , Krankheitsaktivität und zentrale Zytokine des Knochenmetabolismus bei ankyloslerender Spondylitis - eine Prospektivstudie," *Akt Rheumat*, no. 39, pp. 304–309, 2014.

[191] K. M. Porter, D. L. Epstein, and P. B. Liton, "Up-regulated expression of extracellular matrix remodeling genes in phagocytically challenged trabecular meshwork cells," *PLoS One*, vol. 7, no. 4, pp. 7–9, 2012.

[192] P. P. Tak and G. S. Firestein, “NF- κ B in defense and disease NF- κ B : a key role in inflammatory diseases,” vol. 107, no. 1, pp. 7–11, 2001.

[193] K. Hayashida, T. Nanki, H. Girschick, S. Yavuz, T. Ochi, and P. E. Lipsky, “Synovial stromal cells from rheumatoid arthritis patients attract monocytes by producing MCP-1 and IL-8.,” *Arthritis Res.*, vol. 3, no. 2, pp. 118–26, 2001.

[194] S. Shahrara *et al.*, “Inhibition of Monocyte Chemoattractant Protein-1 Ameliorates Rat Adjuvant-Induced Arthritis,” *J. Immunol.*, vol. 180, no. 5, pp. 3447–3456, 2008.

[195] I. Sofia Vala *et al.*, “Low doses of ionizing radiation promote tumor growth and metastasis by enhancing angiogenesis,” *PLoS One*, vol. 5, no. 6, 2010.

[196] T. J. Gal, T. Munoz-Antonia, C. Muro-Cacho, and D. W. Klotch, “Radiation Effects on Osteoblasts In Vitro,” *Arch Otolaryngol Head Neck Surg*, vol. 126, pp. 1124–1128, 2000.

[197] D. Eriksson and T. Stigbrand, “Radiation-induced cell death mechanisms,” *Tumor Biol.*, vol. 31, no. 4, pp. 363–372, 2010.

[198] N. Franken, H. M. Rodermond, J. Stap, J. Haveman, and C. van Bree, “Clonogenic assay of cells in vitro.,” *Nat. Protoc.*, vol. 1, no. 5, pp. 2315–2319, 2006.

[199] P. Fedorocko, A. Egyed, and A. Vacek, “Irradiation induces increased production of haemopoietic and proinflammatory cytokines in the mouse lung.,” *Int. J. Radiat. Biol.*, vol. 78, no. 4, pp. 305–13, 2002.

[200] C. Linard, A. Ropenga, M. C. Vozenin-Brotons, A. Chapel, and D. Mathe, “Abdominal irradiation increases inflammatory cytokine expression and activates NF-kappaB in rat ileal muscularis layer.,” *Am. J. Physiol. Gastrointest. Liver Physiol.*, vol. 285, no. 3, pp. G556-65, 2003.

[201] P. Dent, A. Yacoub, P. B. Fisher, M. P. Hagan, and S. Grant, “MAPK pathways in radiation responses.,” *Oncogene*, vol. 22, no. 37, pp. 5885–96, 2003.

[202] X. Wang *et al.*, “Involvement of the MKK6-p38gamma cascade in gamma-radiation-induced cell cycle arrest.,” *Mol. Cell. Biol.*, vol. 20, no. 13, pp. 4543–52, 2000.

[203] S. R. Kim *et al.*, “Visfatin enhances ICAM-1 and VCAM-1 expression through ROS-dependent NF-kappaB activation in endothelial cells,” *Biochim. Biophys. Acta - Mol. Cell Res.*, vol. 1783, no. 5, pp. 886–895, 2008.

[204] D. Schaue, B. Marples, and K. R. Trott, “The effects of low-dose X-irradiation on the oxidative burst in stimulated macrophages.,” *Int. J. Radiat. Biol.*, vol. 78, no. 7, pp. 567–76, 2002.

[205] T. Poonpet and S. Honsawek, “Adipokines: Biomarkers for osteoarthritis?,” *World J. Orthop.*, vol. 5, no. 3, p. 319, 2014.

[206] S. Poglio *et al.*, “Adipose tissue sensitivity to radiation exposure.,” *Am. J. Pathol.*, vol. 174, no. 1, pp. 44–53, 2009.

[207] S. K. Jo, M. A. Seol, H. R. Park, U. Jung, and C. Roh, “Ionising radiation triggers fat accumulation in white adipose tissue 4066,” *Int.J Radiat.Biol.*, vol. 87, no. 1362–3095 (Electronic), pp. 302–310, 2011.

[208] J. Li, D. L. W. Kwong, and G. C. F. Chan, “The effects of various irradiation doses on the growth and differentiation of marrow-derived human mesenchymal stromal cells,” *Pediatr. Transplant.*, vol. 11, no. 4, pp. 379–387, 2007.

[209] W. Jeong *et al.*, “Serial changes in the proliferation and differentiation of adipose-derived stem cells after ionizing radiation,” *Stem Cell Res. Ther.*, vol. 7, no. 1, p. 117, 2016.

[210] J. E. W. M. Van Dongen-Melman, A. C. S. Hokken-Koelega, K. Hahlen, A. De Groot, C. G. Tromp, and R. M. Egeler, “Obesity after successful treatment of acute lymphoblastic leukemia in childhood,” *Pediatr. Res.*, vol. 38, no. 1, pp. 86–90, 1995.

[211] G. T. Armstrong, M. Stovall, and L. L. Robinson, “Long-term effects of radiation exposure among adult survivors of Childhood cancer: Results from the CCSS,” *Radiat Res*, vol. 174, no. 6, pp. 840–850, 2011.

[212] N. H. Nicolay *et al.*, “Mesenchymal stem cells are resistant to carbon ion radiotherapy,” *Oncotarget*, vol. 6, no. 4, pp. 2076–2087, 2014.

[213] V. R. Sopasakis, I. Nagaev, and U. Smith, “Cytokine release from adipose tissue of nonobese individuals.,” *Int. J. Obes. (Lond).*, vol. 29, no. 9, pp. 1144–7, 2005.

[214] J. M. Harkins *et al.*, “Expression of interleukin-6 is greater in preadipocytes than in adipocytes of 3T3-L1 cells and C57BL/6J and ob/ob mice.,” *J. Nutr.*, vol. 134, no. 10, pp. 2673–2677, 2004.

[215] V. Mohamed-Ali *et al.*, “Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo,” *J. Clin. Endocrinol. Metab.*, vol. 82, no. 12, pp. 4196–4200, 1997.

[216] S. K. Fried, D. A. Bunkin, and A. S. Greenberg, “Omental and Subcutaneous Adipose Tissues of Obese Subjects Release Interleukin-6 : Depot Difference and Regulation by Glucocorticoid,” *J. Clin. Endocrinol. Metab.*, vol. 83, no. 3, pp. 847–850, 1998.

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Appendix

Tab. A.1 Kits for molecular biological and immunological analysis

Name	Manufacturer
Human Leptin ELISA Kit	TecoMedical Group
Human Leptin ELISA Kit	Life Technologies
Total Human Adiponectin ELISA	TecoMedical Group
Human Nampt (Visfatin/PBEF) ELISA Kit	Adipogen
Human Resistin ELISA Kit	Adipogen
Human sRANKL (total) ELISA	BioVendor
COMP® ELISA	Immundiagnostic Systems Ltd.
RayBio® Human MMP-1 ELISA Kit	RayBiotech
RayBio® Human MMP-3 ELISA Kit	RayBiotech
RayBio®Human MMP-13 ELISA Kit	RayBiotech
Human IL-6 ELISA Ready-SET-Go	eBioscience
Human IL-8 ELISA Ready-SET-Go	eBioscience
Human TNFα ELISA Ready-SET-Go	eBioscience
Human CCL2 (MCP-1) ELISA Ready-SET-Go	eBioscience
RNeasy Mini Kit	Qiagen
RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific
QuantiFast SYBR Green PCR Kit	Qiagen
Hs_LEP_1_SG_QuantiTect Primer Assay	Qiagen
Hs_ADIPOQ_va.1_SG_QuantiTect Primer Assay	Qiagen
Hs_NAMPT_1_SG_QuantiTect Primer Assay	Qiagen
Hs_PPARG_1_SG_QuantiTect Primer Assay	Qiagen
Hs_CEBPA_1_SG_QuantiTect Primer Assay	Qiagen
Hs_LEPR_1_SG_QuantiTect Primer Assay	Qiagen
Hs_ADIPOR1_1_SG_QuantiTect Primer Assay	Qiagen
Hs_SLC2A1_1_SG_QuantiTect Primer Assay	Qiagen
Hs_SLC2A4_1_SG_QuantiTect Primer Assay	Qiagen
Hs_MMP3_1_SG_QuantiTect Primer Assay	Qiagen
Hs_MMP1_1_1_SG_QuantiTect Primer Assay	Qiagen
Hs_MMP13_1_SG_QuantiTect Primer Assay	Qiagen
Hs_CXCL8_1_SG_QuantiTect Primer Assay	Qiagen
Hs_IL6_1_SG_QuantiTect Primer Assay	Qiagen
Hs_CCL2_1_SG_QuantiTect Primer Assay	Qiagen
Hs_GAPDH_1_SG_QuantiTect primer Assay	Qiagen

Tab. A.2 Chemicals

Name	Manufacturer
2-Mercaptoethanol	Sigma-Aldrich
Acrylamid Rotiphorese 30%	Roth
AEBSF, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride	Sigma-Aldrich
APS, amonium persulfate	Sigma-Aldrich
Basal medium & growth supplement for synovial fibroblasts	Cell Applications
Biotin	Sigma-Aldrich
BSA (bovine serum albumin)	
Cortisol	Sigma-Aldrich
DAPI	Serva
Dexamethasone	Sigma-Aldrich
DMEM	Merck
DMEM/F12	Invitrogen
DMSO (dimethyl sulfoxide)	Sigma-Aldrich
Ethanol	Merck
Fetal calf serum	Biochrom AG
Formaldehyde	Merk
Glycerol	Sigma-Aldrich
HEPES	Biochrom AG
Human insulin	Sigma-Aldrich
Human recombinant adiponectin	RayBiotech
Human recombinant visfatin	BioVendor
Human transferrin	Sigma-Aldrich
IBMX	Sigma-Aldrich
Isopropanol	Roth
L-Glutamine	Biochrom AG
Methanol	Roth
Methylene blue	Roth
Non-fat dry milk	Roth
Oil Red O	Sigma-Aldrich
Panthotenat	Sigma-Aldrich
PBS (phosphate buffered saline)	Merck
Penicillin/Streptomycin solution	Invitrogen
PGM (preadipocytes growth medium) + supplements	Lonza
Amersham ECL Prime Western Blotting Detection Reagent	GE Healthcare Life Sciences
Rosiglitazone	Cayman Chemicals
Rotiquant Coomassie Brilliant Blue Dye	Roth
SDS, Sodium dodecyl sulfate	Sigma-Aldrich
Sodium chloride	Merk
Sodium orthovanadate	Roth
TEMED	Sigma-Aldrich

Triiodothyronine (T3)	Sigma-Aldrich
Tris-Base	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Trypsin/EDTA	

Tab. A.3 Solutions

Substance	Concentration	Solvent
Cortisol	100 mM	EtOH
Dexamethasone	25 μM	EtOH
IBMX	250 mM	DMSO
Insulin	100 μM	10 mM HCl + 1% BSA
Oil Red O stock solution	3 mg/ml	Isopropanol, 99%
Oil red O working solution	3 ml	2 ml H2O
Panthotenat/Biotin	biotin 3.3 mM	DMEM/F12
	panthotenat 1.7 mM	
Rosiglitazone	10 mM	DMSO
Transferrin	1 mg/ml	DMEM/F12
Triiodothyronine (T3)	200 nM	H2O

Tab. A.4 Buffers

Name	Composition	Solvent
Blocking buffer	non-fat dry milk, 5%	TBST
Elektrophorese buffer	Tris-Base, 3.3 g	H2O, 1 L
	Glycine, 14.26 g	
	SDS, 1 g	
Resolving gel buffer	Tris-Base, 1.5 M	H2O
	SDS, 4% w/v	
	pH=8.8	
RIPA lysis buffer	Igepal-Buffer, 1%	H2O
	Sodium deoxycholate, 0.5%	
	Sodium fluoride, 50 mM	
	AEBSF, 1 mM	
	Aprotinin, 1 μg/ml	
	Sodium orthovanadate, 1 mM	
	SDS, 0.1%	

Sample buffer	Bromophenol blue, 0.035 g	
	Glycerol, 7 ml	
	SDS, 6 ml (10% v/v)	
	Tris-HCl, 1 ml (1 M, pH=6.7)	
	2-Mercaptoethanol, 1% v/v	
SDS running buffer	Tris-Base, 3.03 g	H2O, 1 L
	Glycine, 71.3 g	
	SDS, 1 g	
	pH=7.2-7.4	
Stacking gel buffer	Tris-Base, 0.5 M	H2O
	SDS, 4% w/v	
	pH=6.7	
TBST	Tris-HCl, 1% v/v, 1 M, pH=8.3	H2O
	NaCl, 5% v/v, 3 M	
	Tween 20, 0.05% v/v	
Transfer buffer	Glycine, 11.27 g	H2O, 800 ml
	Tris-HCl, 20 ml	
	SDS, 1 g	
	MeOH, 20% v/v	
Tris-HCl	Tris-Base, 1 M	H2O
	pH=8.3 (by HCl)	

Tab. A.5 Cell culture media

Pre-adipocytes growth medium (PGM2, Lonza)	
PBM2 (preadipocyte basal medium)	500 ml
FCS	2 ml
L-Glutamine	5 ml
GA-1000 (gentamicin)	0.5 ml
2x Adipocytes differentiation medium	(Lonza)
PGM2	100 ml
Human insulin	2 ml
Dexamethasone	0.2 ml
IBMX	0.2 ml
Indometacin	0.4 ml

SGBS media		
0F medium		
DMEM/F12	500 ml	
Panthotenat/Biotin	5 ml	
Penicillin/Streptomicin	5 ml	
Serum-containing medium (0F+10% FCS)		
0F medium	500 ml	
FCS	50 ml	
3 FC medium		
0F medium	10 ml	
Transferrin	100 μl	(0.01 mg/ml)
Insulin	2 μl	(20 nM)
Cortisol	10 μl	(100 nM)
Triiodothyronine (T3)	10 μl	(0.2 nM)
Quick-Diff medium		
0F medium	10 ml	
Transferrin	100 μl	(0.01 mg/ml)
Insulin	2 μl	(20 nM)
Cortisol	10 μl	(100 nM)
Triiodothyronine (T3)	10 μl	(0.2 nM)
Dexamethasone	2 μl	(20 nM)
IBMX	10 μl	(250 μM)
Rosiglitazone	2 μl	(2 μM)
Growth medium for RASF 0255		
DMEM	500 ml	
FCS (heat inactivated)	50 ml	
Penicillin/streptomycin	5 ml	
HEPES	5 ml	
Growth medium for NSF and RASF (Cell applications)		
Ready for use		

Tab. A.6 Consumables

Type	Product designation	Manufacturer
Cell culture flasks	T75, T25	BD Falcon
Cell scraper	S	TPP
Chamber slides	LabTek II	Lab-Tek Brand Products
Cover slips		Roth
Covers for qPCR	MicroAmp	Applied Biosystems
Cryo tubes	2 ml	Greiner
Multiwell plates for cell culture		TPP
Multiwell plates for immune assays		NUNC
Multiwell plates for qPCR	MicroAmp 0,1 ml	Applied Biosystems
Petri dishes	Nunclon 35 mm	NUNC
Reaction tubes	0.5-2 ml	Eppendorf
Reaction tubes	15-50 ml	TPP
Serological pipets (disposable)	2-50 ml	Sarstedt
Tips	SafeSeal professional	Biozym

Tab. A.7 Equipment and software

Type of device	Product designation	Manufacturer
Autoclave	EL	Tutthauer Systec
Cell counter	TC-20	Bio-Rad
Centrifuge	Multifuge 3 S-R	Thermo Scientific
Centrifuge	Fresco 17	Thermo Scientific
Dosimeter	SN54	PTW Freiburg
Electrophoresis Chamber	Miniprotean Tetra System	Bio-Rad
Freezer -80	MDF-U52V	Sanyo
Ice mashine	Flake-Line	Wessamat
Incubator	BBD-6220	Heraeus Instruments
Laminar flow bench	HeraSafe	Thermo Scientific
Liquid N_2 Tank	8213	Thermo Scientific
Microscope	BX61	Olympus
Microvolume spectrometer	Colibri	Titertek Berthold
pH meter	Calimatic 766	Knick
Photometer	ELx808	Biotec

Scales	ABJ-120-4NM	Kern
Ultrapure water system	Purelab Flex	ELGA/Veolia
Ultrasonic bath	Bioruptor UCD-200	Diagenode SA
Waterbath	VWB 6	VWR
Western Blot Imaging System	Fusion	Peqlab
Western Blot Transfer System	Trans-Blot®Turbo™Transfer System	Bio-Rad
X-ray tube	Isovolt DS1	Seifert

Type of software	Product designation	Developer
qPCR analysis	StepOne v 2.3	Applied Biosystems
Western Blot Imaging and Quantification	Fusion FX	Peqlab
ELISA analysis		
Data analysis	GraphPad Prism 6	GraphPad Software

Tab. A.8 Time-dependent effect of adiponectin on IL-6 release by RASF

Time, h	IL-6, pg/ml (Mean±SD, n=3)	
	w/o adiponectin	**adiponectin 5 μg/ml**
12	0.556±0.14	0.634±0.09
24	0.63±0.5	2.9±1.5
36	1.9±0.65	8.25±4.6
48	**1.97±0.36**	**9.5±2.5**

Effect of X-ray irradiation on adiponectin-induced expression of IL-6, IL-8, MCP-1, MMP-1 and MMP-3 by human synovial fibroblasts

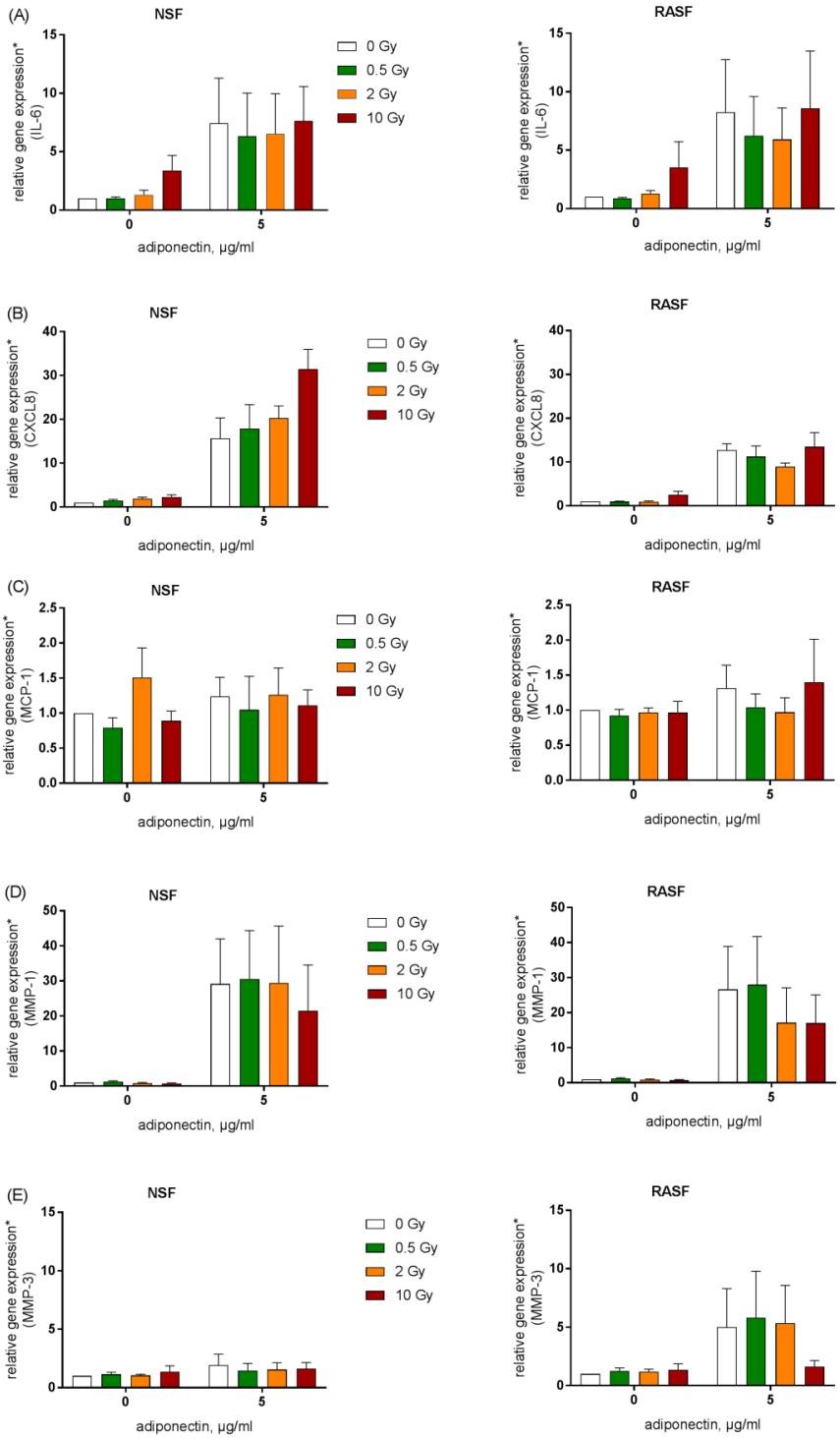

Fig. A.1 Effect of X-ray irradiation on adiponectin-induced expression of IL-6, IL-8, MCP-1, MMP-1 and MMP-3 by human synovial fibroblasts. Synovial fibroblasts from healthy donors (NSF) and from patients with rheumatoid arthritis (RASF) were treated or not with 5 µg/ml adiponectin for 24h prior to irradiation with X-rays. RNA was isolated 24h after irradiation and gene expression analysis of IL-6 (A), IL-8 (B), MCP-1 (C), MMP-1 (D) and MMP-3 (E) was performed. Mean, SEM. N=4

Effect of X-ray irradiation on visfatin-induced expression of IL-6, IL-8, MCP-1, MMP-1 and MMP-3 by human synovial fibroblasts

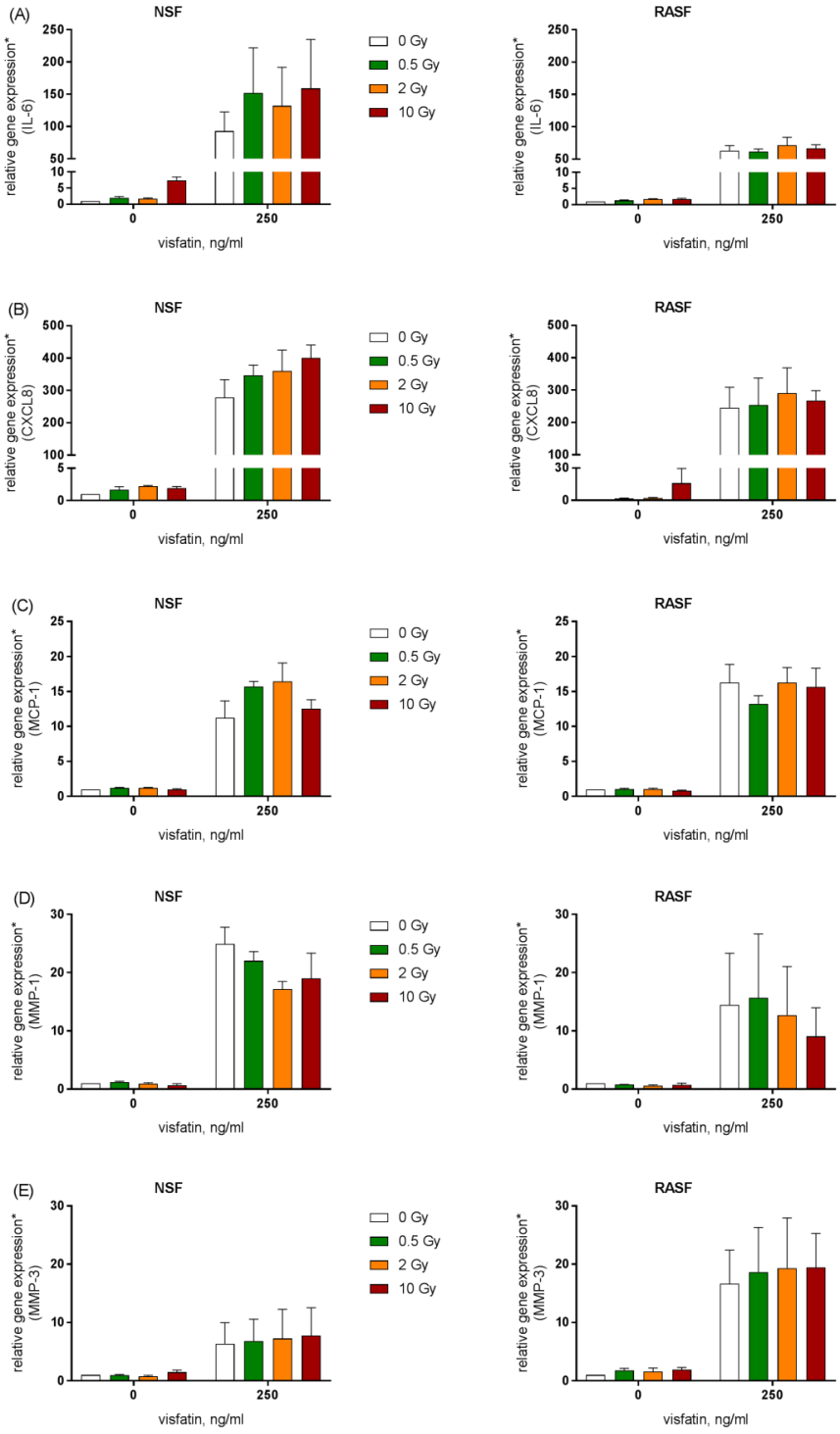

Fig. A.2 Effect of X-ray irradiation on visfatin-induced expression of IL-6, IL-8, MCP-1, MMP-1 and MMP-3 by human synovial fibroblasts. Synovial fibroblasts from healthy donors (NSF) and from patients with rheumatoid arthritis (RASF) were treated or not with 5 µg/ml adiponectin for 24h prior to irradiation with X-rays. RNA was isolated 24h after irradiation and gene expression analysis of IL-6 (A), IL-8 (B), MCP-1 (C), MMP-1 (D) and MMP-3 (E) was performed. Mean, SEM. N=3

Curriculum Vitae

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Publications

Peer-reviewed

A. Cucu*, **K. Shreder***, D. Kraft, P. Rühle, G. Klein, G.Thiel, B. Frey, U. Gaipl, C. Fournier. *Decrease of markers related to bone erosion in serum of patients with musculoskeletal disorders after serial low-dose radon spa therapy.* Frontiers of Immunology 2017; 8:882; doi: 10.3389/fimmu.2017.00882
* authors contributed equally to this work

Scientific reports

K. Shreder, A.Cucu, P.Rühle, U.Gaipl, M.Durante, C. Fournier. *Impact of Radon and low-dose X-ray exposure on adipokine induced inflammation in rheumatoid arthritis.* GSI Scientific Report 2015.

A.Groo, **K.Shreder**, T.Huhn, D.Kraft, H.Bönig, M.Durante, C.Fournier. *Differentiation of osteoblasts and adipocytes following irradiation.* GSI Scientific Report 2014.

Conference contributions

K. Shreder, A. Cucu, D. Kraft, S. Lehrian, C. Fournier. *Ionizing radiation inhibits adipokine induced inflammation in synovial fibroblasts and differentiation of osteoclasts.* European Workshop of Rheumatology Research (EWRR) 2017, Athens, Greece. Poster.

K. Shreder, A. Cucu, L. Deloch, B. Frey, U. Gaipl, M. Durante, C. Fournier. *Low-dose ionizing radiation inhibits adipokine induced inflammation in rheumatoid arthritis.* European Workshop of Rheumatology Research (EWRR) 2016, York, Great Britain. Poster.

K. Shreder, A. Cucu, P. Rühle, B. Frey, U. Gaipl, M. Durante, C. Fournier. *Low-dose ionizing radiation inhibits adipokine induced inflammation in rheumatoid arthritis.* 19th Annual Meeting of the Society for Biological Radiation Research (GBS) 2016, Erlangen, Germany. Poster.

K. Shreder, A. Groo, A. Maier, M. Seibert, M. Durante, C. Fournier. *Impact of low-dose radiation on differentiation of adipocytes and synovial fibroblasts.* The European Association for Cancer Research (EACR): Conference on Radiation Biology and Cancer 2015, Essen, Germany. Poster.

K. Shreder, A. Groo, M. Durante, C. Fournier. *Impact of low-dose radiation on differentiation of adipocytes and their role in bone remodeling.* 18th Annual Meeting of the Society for Biological Radiation Research (GBS) 2015, Dresden, Germany. Poster.

K. Shreder, A. Groo, M. Seibert, M. Durante, C. Fournier. *Impact of low-dose radiation on differentiation of adipocytes and synovial fibroblasts*. 17th Annual Meeting of the Society for Biological Radiation Research (GBS) 2014, Tuebingen, Germany. Poster.

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Ehrenwörtliche Erklärung

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Kateryna Shreder

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