DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Effects of JAK inhibition on bone loss and vascular inflammation in rheumatoid arthritis

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF CLINICAL MEDICINE

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1. Introduction

Rheumatoid arthritis (RA) is a chronic and systemic inflammatory condition, resulting in symmetrical polyarthritis and joint damage leading to physical disability. Studies indicate that patients with RA have an increased susceptibility to cardiovascular disease (CVD), which is the primary cause of mortality in this patient population. Compared to general population, these patients have nearly twice the risk of heart failure. Additionally, they also have a higher risk of atrial fibrillation, stroke and myocardial infarction [1]. Apart from the increased likelihood of CVD, individuals with RA are also susceptible to localized and systemic osteoporosis, which may lead to an increased risk of fragility fractures. The reduction in bone density throughout the body can lead to osteoporosis, which is characterized by deterioration of the bone's microstructure and reduced bone mass. Due to decreased bone formation and enhanced bone resorption, people with RA may develop generalized osteoporosis, periarticular bone loss, and marginal bone erosions [2]. The presence of chronic systemic inflammation associated with RA plays a significant role in the development of both bone loss and CVD. In the recent years, several new treatments have been introduced for the therapy of RA, with the latest of Janus kinase (JAK) inhibitors. These inhibitors are able to block the signaling pathway of various cytokines, hormones and growth factors, allowing the reduction of inflammation with a single synthetic compound. Inflammation is recognized as a contributing factor to the pathogenesis of atherosclerosis, and emerging evidence indicates that the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway also has a significant impact on bone metabolism and turnover [3].

The aim of this thesis is to understand the effects of JAK inhibition on the cardiovascular (CV) system and bone metabolism in RA.

2. Literature review

2.1. Epidemiology

The prevalence of RA in both the European and American populations ranges from 0.5% to 1%. The prevalence of RA is higher in women than in men, with a two to one ratio. The estimated lifetime risk of developing the disease is approximately 1.7% for men and 3.6% for women. The average age of onset of RA typically between 30 and 60 years [4]. RA can be associated with long-term adverse outcomes, like development of other chronic diseases (cardiovascular and respiratory disease, osteoporosis, malignancies), physical and work disability, psychiatric disorders, reduced quality of life and premature mortality [5-10]. RA therefore places a significant burden on affected individuals, society and healthcare systems.

2.2. Etiopathogenesis

2.2.1. Risk factors

The etiology of RA is influenced by genetic factors as well as lifestyle-related or environmental risk factors. It is well known that presence of cell surface antigens HLA-DR1 and HLA-DR4 is strongly associated with RA. The *HLA-DRB1*01* and *HLA-DRB1*04* genes encode a five amino acid sequence motif known as the "shared epitope," which is present in these antigens [11]. Another major genetic risk factor is variation in the protein tyrosine phosphatase gene (*PTPN22*), which may increase the risk of RA as well as other autoimmune diseases [12]. Other genetic loci, such as *CTLA4, IL23R, TRAF1, STAT4, PAD4* may also be associated with RA [13].

Environmental and lifestyle risk factors play a significant role in the pathogenesis of RA. Smoking has been found to enhance the citrullination of synovial proteins via peptidyl-arginine deiminase (PAD) enzymes, leading to elevated anti-citrullinated protein antibody (ACPA) levels [14]. Studies have reported a relationship between HLA-DR genes and smoking, suggesting a possible link between the citrullination of proteins in the lungs and T cell-mediated immune activation against them [15]. *HLADRB1, PTPN22* genes and smoking are

linked to the production of ACPA against α-enolase and vimentin, rather than that of anti-cyclic citrullinated peptide (anti-CCP) [16].

The gastrointestinal (GI) tract microbiome may also be an important pathogenic factor for RA. Various studies have reported a high prevalence of periodontitis in patients with RA, highlighting the association between these two conditions. Studies found that a periodontal pathogen, *Porphyromonas gingivalis,* increases citrullination through expression of PAD, leading to increased levels of ACPA, which binds to α-enolase [17, 18]. Dysbiosis and translocation of gut microbiome may also contribute to autoimmunity. Dysbiosis may lead to loss of barrier function and translocation of gut microbiome via the bloodstream, which may trigger the immune system [19-21]. Bacterial and viral antigens have been found to have similar amino-acid sequences to autoantigens, which can lead to immune cross-reactivity, called molecular mimicry. Gene-environmental interactions between Epstein-Barr virus and autoimmune diseases have also been reported [22]. In addition, elevated levels of certain Prevotella species have been found in the oral cavity of RA patients [23].

2.2.2. Pathophysiology

As discussed above, genetic and environmental triggers, as well as repeated activation of innate immunity, play a role in the pathogenesis of RA. Epigenetic modifications and posttranslational protein modifications may promote autoimmune responses. The PAD-catalysed citrullination of arginine, as described above, as well as carbamylation, acetylation and other protein modifications that generate neoepitopes of autologous proteins, may lead to the production of different autoantibodies, resulting in loss of immunological tolerance [24]. Namely, ACPA and rheumatoid factors (RF) are characteristic antibodies for RA. Altered antibody response to a number of citrullinated proteins induces ACPAs, whereas RF is produced against the Fc portion of immunoglobulins. Furthermore, autoantibodies are able to form immune complexes, that can activate the complement system [25-27]. Autoantibodies, including ACPAs, RFs and antibodies against carbamylated proteins can be present up to ten years before the onset of clinical arthritis [28]. Neoantigens produced by antigen-presenting cells (APCs) may activate MHC class II-dependent T cells, leading to differentiation of these T cells into effector, memory, and regulatory cells. This process may subsequently trigger the production of ACPAs in B lymphocytes. The immune activation is

followed by synovial inflammation. Neovascularization, which is a critical component of the inflammatory process in the synovium, has been demonstrated in RA patients. Due to increased vascular permeability and adhesion molecules, immune complexes accumulate in the joints and induce inflammation, causing inflammation that presumably contributes to sustained joint inflammation [29]. The synovium is infiltrated by leukocytes, like macrophages, T cells and B lymphocytes, causing an inflammatory cascade. $CD4^+$ T-lymphocytes enhance inflammation by activating synovial macrophages and enhance cartilage destruction and bone erosion through the production of interleukin-17 (IL-17) and tumor necrosis factor α (TNF-α). These inflammatory cytokines further stimulate the expression of Receptor Activator Nuclear Factor κB ligand (RANKL), which in turn leads to the activation of osteoclasts, ultimately resulting in bone resorption (Figure 1) [30]. Apart from generating autoantibodies, B cells also release various pro-inflammatory cytokines. These cytokines play a role in the differentiation and activation of T cells. B lymphocytes have been shown to regulate bone homeostasis by expressing RANKL, which increases osteoclast activity, however B cell precursors can produce osteoprotegerin (OPG), which is an inhibitor of osteoclast differentiation [31].

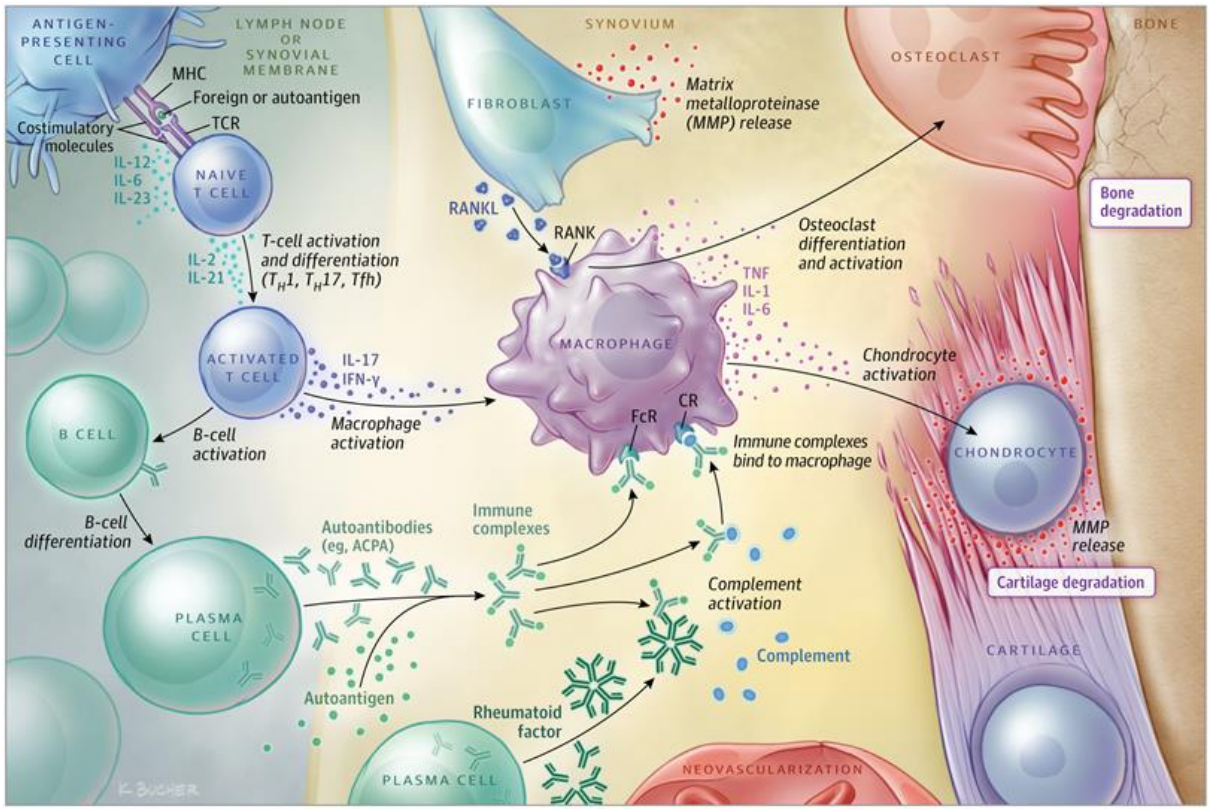

Figure 1. Pathogenesis of RA [32]

Macrophages are actively involved in the development of RA and exert their effects through various mechanisms. Synovial macrophages produce cytokines, such as IL-1, IL-6, IL-10, and TNF-α. These cytokines contribute to inflammation, stimulate fibroblast-like synoviocytes (FLS), activate B lymphocytes, and promote the formation of osteoclasts. Synovial macrophage infiltration correlates with radiological progression [33, 34] and ACPA can also enhance TNF-α production in macrophages [35]. FLSs play a pivotal role in the pathogenesis of RA by promoting synovial hyperplasia and producing enzymes, including matrix metalloproteinase 1 (MMP-1) and 13 (MMP-13), which are involved in tissue degradation and joint damage. They also secrete inflammatory cytokines and upregulate the expression of RANKL, which collectively contribute to the degradation of bone and cartilage in RA. Moreover, FLS cells are able to migrate from one joint to the other, which could explain the symmetrical distribution of joint inflammation observed in RA [36-38]. Various subsets of fibroblasts may have different roles and phenotypes. There are reports suggesting that synovial sub-lining FLSs are involved in the maintenance of inflammation, whereas lining FLSs are associated with cartilage and bone degradation in RA [39]. The synovium also contains neutrophils that generate reactive oxygen species (ROS), proteases and extracellular traps (NETs) consisting of released DNA complexes that contain citrullinated proteins [40].

2.3. Signs, symptoms and diagnostics

Clinically, RA is characterized by symmetric synovitis mainly affecting the wrists, metacarpophalangeal (MCP), and proximal interphalangeal (PIP) joints. However, other joints, such as metatarsophalangeal (MTP) joints, knees, elbows, shoulders and temporomandibular joints may also be affected. Patients with RA have fatigue, muscle pain and morning stiffness of joints, which often lasts for more than 30 minutes. Joints may be swollen, warm, tender to touch and passive joint movement may provoke pain. In long-standing disease, the joint capsule shrinks and characteristic joint deformities may develop (Figure 2). Furthermore, RA can also affect the cervical spine, leading to joint damage and subluxation of the atlantoaxial joint (AAS), which may lead to potentially life-threatening complications [41]. Extra-articular manifestations and chronic comorbidities may develop if inadequately treated. Some patients have fever, weight loss or chronic widespread pain and muscle weakness. Generalized bone

loss has also been observed in patients with RA, including osteoporosis, periarticular osteopenia, local bone erosions and periodontal bone loss. RA may lead to the emergence of skin manifestations and in some cases to the development of vasculitis. Patients with seropositive RA may develop rheumatoid nodules. Skin ulcers caused by vasculitis, neutrophilic infiltration or venous stasis may occur in RA patients. The most common respiratory manifestations linked to RA are interstitial lung disease (ILD) and pleural disease [42, 43]. Pericarditis and myocarditis are rare in RA, but due to accelerated atherosclerosis, the risk of CVD and the incidence of atrial fibrillation and heart failure are increased in RA patients [44-46].

Figure 2. Clinical manifestations of RA (Hamar A., Photograph of a patient with RA, 2023)

Early detection RA and initiation of treatment is critical to the management of the disease, but establishing a diagnosis of RA can be challenging. In 2010, the European Alliance of Associations for Rheumatology (EULAR) and the American College of Rheumatology (ACR) put forward revised classification criteria. These categories of criteria are divided into four groups: joint involvement, acute-phase reactants (CRP and/or ESR), serology (including RF

and/or ACPA), and duration of symptoms (<6 weeks or ≥6 weeks). A score of 6/10 is required to classify a patient as definite RA [47]. ACPA can be detected several years before disease onset and is positive in approximately 67% of RA patients, while RF is less specific but can be detected in up to 80% of RA patients. Both antibodies and higher acute-phase reactants are associated with worst radiographic progression and high disease activity [48, 49]. Inflammation of the joints in RA may cause cartilage erosion and bone degradation, leading to various radiographic changes, like joint space narrowing and bone erosions. Plain radiographs are able to detect marginal bone erosions, however in the first year of the disease, erosions of PIP and MCP joints can be found in only 15-30% of patients [50, 51]. MRI can detect bone marrow edema and early erosions [52, 53]. Ultrasonography is another tool to detect synovitis and erosions, and can also be used to assess bursae and ligaments [54].

2.4. Management

2.4.1. The treat-to-target approach

Early identification and initiation of treatment is crucial in managing patients with RA. Progression of joint damage may be prevented in early RA patients [55]. Unfortunately, there is no cure for RA, however with modern pharmacological and non-pharmacological interventions we are able to reduce inflammation, pain, decrease joint destruction and prevent long-term disability. The treatment strategy, in addition to the management approach, is extremely important as well. The treat-to-target (T2T) approach aims to achieve remission or low disease activity as treatment goal. The T2T strategy involves achieving a 50% improvement in DAS28 within three months and aiming to attain remission or low disease activity within six months. T2T also includes the followings: setting a therapeutic target, assessing the target, adapting medication if needed and shared-decision making with the patient. Current EULAR and ACR management guidelines recommend T2T approach, which involves symptomatic treatment and disease modification therapies as soon as possible [56].

2.4.2. Treatment options

Glucocorticoids (GCs) and non-steroidal anti-inflammatory drugs (NSAIDs) are frequently used in the management of RA. GCs are capable of rapidly lower inflammation in RA, but the cost/benefit ratio must be examined due to the increased risk of adverse effects. As a result, EULAR and ACR management guidelines recommend short-term low-dose GC therapy and GCs tapering as soon as possible. GCs should be considered only during flares or exacerbations, as well as upon starting a new DMARD therapy [57].

Disease-modifying antirheumatic drugs (DMARDs) represent the most efficacious therapies for the management of RA. They can be divided into three groups: conventional synthetic DMARDs (csDMARDs), biologic DMARDs (bDMARDs) and targeted synthetic DMARDs (tsDMARDs), such as JAK inhibitors. According to the 2021 ACR guideline, methotrexate is considered the primary choice of initial csDMARD therapy for treatment-naive RA patients, whether used in monotherapy or in combination with other csDMARDs. It is recognized as the gold standard in RA treatment [58]. When low disease activity or remission is not reached with first-line treatments, additional bDMARDs or tsDMARDs should be considered as second-line. The therapy should be switched to a different bDMARD/tsDMARD that operates through a distinct pathway if the treatment goal is not attained with the first bDMARD/tsDMARD [59]. Inhibition of TNF-α induced inflammatory response was the first attempt to find novel treatment methods in RA. TNF-α inhibitors (TNFi), including adalimumab, etanercept, infliximab, golimumab and certolizumab pegol have shown to decrease inflammation and radiological progression [60-63]. Abatacept, which suppresses T cell co-stimulation, has demonstrated comparable effectiveness to infliximab, while exhibiting a lower incidence of adverse events [64]. Rituximab, an anti-CD20+ monoclonal antibody, has shown efficacy in decreasing joint damage in patients who have not adequately responded to prior TNF-α inhibitors [65-67]. In the ADACTA trial, treatment with tocilizumab, an inhibitor of IL-6, demonstrated greater efficacy compared to adalimumab monotherapy in patients with RA who did not respond adequately to MTX therapy [68, 69]. The treatment of RA can also be effectively managed by Anakinra, which is a recombinant form of human IL-1 receptor antagonist [70].

2.4.3. JAK-STAT signaling pathway

Over fifty cytokines and growth factors utilize the JAK/STAT signaling pathway to transmit their signals from the cell surface to the nucleus [71, 72]. The JAK family contains four tyrosine kinases: JAK1, JAK2, JAK3 and non-receptor tyrosine kinase protein 2 (TYK2). Additionally, there are seven STAT proteins, such as STAT 1-4, STAT 5A/B and STAT 6 proteins. These molecules are transducers of cytokine signaling, utilizing various cellular processes, including hematopoiesis, lymphocyte proliferation, differentiation, migration, apoptosis and innate antiviral responses (Figure 3) [73]. The JAK/STAT signaling pathway can be activated by multiple cytokines, and the specific combination of JAKs and STATs that are activated depends on the ligand and receptor involved. Cytokines bind to their respective receptors, which are associated with JAKs. Upon binding, the receptors dimerize and activate JAKs by phosphorylation. The activated JAKs then phosphorylate specific tyrosine residues within the intracellular receptors, generating docking sites for the recruitment of STAT proteins. These phosphorylated STATs dissociate from the receptor docking sites and form dimers, that can translocate to the nucleus, where they bind to specific DNA sequences, resulting in activation of gene transcription. The SOCS family has been identified as inhibitors of this pathway [73].

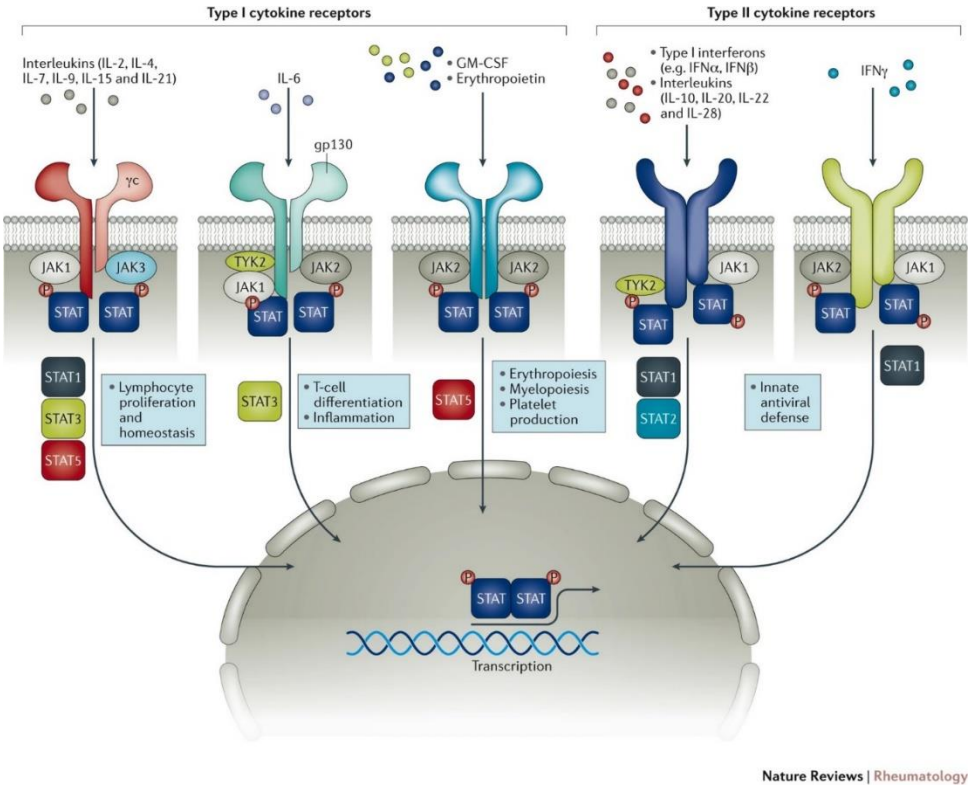

Figure 3. Overview of JAK-STAT signaling pathways [74]

The blockade of the JAK/STAT pathway presents a novel treatment approach in the therapy of RA, whereby JAK inhibitors (Jakinib) are able to stop this pathway and block the cytokines that use it. This provides an opportunity to regulate multiple cytokines simultaneously with a synthetic compound. Tofacitinib is an oral small-molecule JAK inhibitor that effectively targets JAK1 and JAK3, with lesser inhibitory effect on JAK2. JAK inhibitors have showed both safety and efficacy in patients with RA, with four Jakinibs, including tofacitinib, being approved for managing RA [59, 74-77]. Tofacitinib was the first Jakinib approved by both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for patients with moderate to severe RA, and can be given orally twice daily as monotherapy or in combination with MTX. Tofacitinib can inhibit the signaling pathways of numerous cytokines, including IL-12, members of the IL-6 family, IL-23, interferons, γ-chain cytokines, and others [74, 75]. Tofacitinib was reported to reduce gene expression of MMPs, CXCL10, CXCL13 and CCL2 chemokines in the synovium of RA, and decrease STAT1 and STAT3 phosphorylation [78]. It was showed that JAK inhibitor decreased osteoclast-mediated structural damage of joints in murine [79]. In clinical trials, tofacitinib prevented the progression of radiographic joint damage [79-84]. Adverse events of tofacitinib due to infections are similar to those of bDMARDs. The side effects that occurred most frequently were headache, nausea, upper respiratory tract infections, nasopharyngitis and diarrhea. In addition, patients receiving tofacitinib treatment had a higher incidence of Herpes zoster (HZ) than RA patients receiving other treatments [85].

Baricitinib is an orally administered tsDMARD, which inhibits both JAK1 and JAK2. It has been approved for the treatment of moderate to severe RA in monotherapy or in combination with MTX. 4 mg baricitinib daily showed improvement in clinical outcomes and decreased radiological progression. It has been reported that combination of baricitinib with csDMARDs resulted the most favorable ACR20 response rate. The most frequently reported adverse events were nausea, infections, lipid elevations, thrombocytosis and elevated risk of Herpes Zoster infection [86].

Both in monotherapy and in combination with csDMARD, upadacitinib, a selective JAK1 inhibitor, has demonstrated significant efficacy in patients who have shown an inadequate response to csDMARD or bDMARD. Recent meta-analysis shows that upadacitinib and baricitinib have better efficacy compared to tofacitinib and filgotinib. The most commonly reported adverse events in patients receiving upadacitinib treatment include UTIs, upper

respiratory tract infections, higher risk of Herpes Zoster infection, elevation of creatine phosphokinase (CPK) and transaminases [87, 88].

2.5. Accelerated atherosclerosis

Patients suffering from RA have a higher incidence of CV mortality and morbidity. Coronary atherosclerosis is the primary cause of death in RA, with a prevalence ranging from 10% to 30%, among the highest observed. Additionally, a meta-analysis revealed a 50% higher risk of CV mortality compared to the general population [89-92]. Traditional risk factors, such as smoking, physical inactivity, hypercholesterolemia, and hypertension, do not fully explain the increased CV morbidity and mortality rates observed in patients with RA [93, 94]. Women with RA have been observed to have a 2- to 3-fold higher risk of myocardial infarction, even in the absence of traditional coronary risk factors [95]. Studies have provided evidence for an increased prevalence of subclinical atherosclerosis in RA patients. Early signs of atherosclerosis, such as endothelial dysfunction and thickening of carotid artery intima-media (ccIMT), have been observed in patients with early RA. Importantly, effective treatment has shown promise in reversing these early signs of atherosclerosis [96].

Increased systemic inflammation may be the key mechanism connecting RA to increased CV risks and accelerated atherosclerosis. Studies have demonstrated that active inflammatory processes can lead to induction of thrombotic events and plaque rupture, ultimately resulting in vascular occlusion, cerebral stroke or myocardial infarction [97, 98]. Indeed, both RA and atherosclerosis share common pathophysiological features and underlying mechanisms. Genetic and environmental factors can contribute to endothelial dysfunction, a hallmark of both diseases. In RA and atherosclerosis, endothelial activation, collagen degradation, accumulation of inflammatory cells, and neovascularization are observed. The inflammatory cells involved, particularly monocytes/macrophages and T lymphocytes, play a crucial role in the pathogenesis. These cells produce pro-inflammatory cytokines like IL-6 and TNF-α, which not only enhance the infiltration of inflammatory cells into the intimal layer of blood vessels, but also stimulate the expression of MMPs, resulting in joint destruction and vascular extracellular matrix degradation. Therefore, the shared inflammatory processes contribute to the pathogenesis of both RA and atherosclerosis, linking

these two conditions at cellular and molecular level [45, 99, 100]. Elevated levels of adhesion molecules have been found in RA, and TNF-α has been identified as a factor that can upregulate the expression of these molecules. Elevated expression of adhesion molecules contributes to enhanced formation of atherosclerotic plaques [101]. IL-6 and TNF-α suppress the production of cyclooxygenase-1 and nitric oxide, resulting in endothelial dysfunction [102]. Increased oxidation of LDL-C by TNF-α has been showed to result in higher levels of oxLDL in RA [103, 104]. RA patients also have lower numbers of endothelial progenitor cells (EPC) and reduced EPC function. Elevated IL-6 and TNF-α levels were linked to a decreased number of EPCs, which may lead to endothelial dysfunction in RA [105-107]. TNF-α suppresses the degradation of asymmetric dimethylarginine (ADMA), a natural inhibitor of nitric oxide synthase, and also reduces the population of EPCs [106, 108].

Autoantibodies have been linked to CVD as well. ACPAs have been found in atherosclerotic plaques and are associated with increased coronary calcification and atherosclerosis regardless of traditional CV risk factors [109]. Association was found with anti-carbamylated proteins antibodies (anti-CarP) and endothelial dysfunction [110]. In addition, alterations in the composition of T cell subpopulations can be observed. Specifically, a group of CD4+/CD28− T cells have been linked to inflammation-induced vascular harm and endothelial dysfunction in RA [111, 112]. NETs may also contribute to atherosclerosis and RA via sustaining inflammation [113, 114].

RA patients have been found to have an abnormal profile of lipids. Inflammation has been observed to decrease low-density lipoprotein cholesterol (LDL-C) levels, levels of high-density lipoprotein cholesterol (HDL-C) and total cholesterol (TC), and also cause modifications in lipid structures and function [115]. Reduced levels of LDL-C have been associated with higher CV risks, while reductions in HDL-C levels lead to an elevated atherogenic index indicated by the TC/HDL-C ratio [116]. By influencing lipid oxidation, paraoxonases (PON) have a preventive function in vascular disorders, however PON-1 activity has been found to be reduced in rheumatoid arthritis. In addition to altered lipid metabolism, changes in glucose regulation and utilization have also been observed in RA. Increased insulin levels were linked to elevated levels of TNF-α, IL-6, CRP, and disease activity. TNF-α also hindered glucose uptake in muscles and stimulated lipolysis in adipocytes leading to an upsurge in the generation of pro-inflammatory cytokines [117].

2.6. Bone loss

RA has been associated with both generalized bone loss and localized inflammatory bone resorption. The prevalence of osteoporosis in RA is approximately 30%, which is twice as high as that observed in the general population [2, 81, 118-120]. RA patients have reduced bone mineral density (BMD), increased cortical porosity and an almost doubled risk for vertebral and hip fractures compared to healthy individuals [2, 118, 121]. Long disease duration, high disease activity and increased levels of bone biomarkers are additional factors that contribute to the risk of osteoporosis and fractures in RA [122-125]. However, recent studies showed that local and systemic bone loss may occur prior to the clinical onset [126].

2.6.1. RANK-RANKL system

Normal bone remodeling is maintained by the balance between the activities of osteoblasts and osteoclasts. Osteoblasts, originating from mesenchymal precursor cells, have a crucial role in synthesizing and mineralizing bone tissue. On the other hand, osteoclasts, derived from hematopoietic stem cells, contribute to bone resorption by secreting catalytic enzymes and acids, that degrade the bone matrix [127]. Bone turnover may be monitored by different bone biomarkers, such as osteocalcin (OC), procollagen type I N-propeptide (P1NP), C-terminal telopeptide (CTX), and cathepsin K (CATHK). OC, which is secreted solely by osteoblast, and P1NP, which is cleaved from procollagen type I and synthesized by osteoblasts and fibroblasts, are well known markers of bone formation. Meanwhile, CTX is released during enzymatic bone matrix degradation and CATHK is secreted by activated osteoaclasts, which makes them potential markers of bone resorption [81]. Osteoblasts regulate osteoclast differentiation and activation through RANK-RANKL system. RANKL regulates the formation and function of osteoclasts through its binding to RANK receptors found on both osteoclast precursors and mature cells, facilitated by macrophage colony-stimulating factor (M-CSF). RANKL binds to the RANK and enhances activation and differentiation of hematopoietic precursors of osteoclasts, leading to bone reabsorption. Osteoblasts, synovial fibroblasts, activated B cells and T lymphocytes are the main source of the production of RANKL [128, 129]. While RANKL promotes osteoclast proliferation, differentiation and maturation,

osteoprotegerin (OPG) represses this process and regulates osteoclastogenesis. OPG is a member of TNF receptor superfamily and acts as a soluble decoy receptor for RANKL. OPG is secreted by osteoblasts and inhibits osteoclast maturation and differentiation by blocking the interaction between RANKL and its receptor [130]. OPG-deficient mice developed severe osteoporosis due to excessive bone resorption, while transgenic overexpression of OPG leads to osteopetrosis in mice because they are unable to form osteoclasts [131]. OPG therefore plays an important role in the inhibition of bone resorption.

The disruption of the OPG/RANKL ratio directly impacts bone loss in inflammatory conditions. Inflammatory bone loss and bone resorption are linked to pro-inflammatory cytokines, including TNF-α, IL-1, IL-6, IL-17 and IL-23, as well as autoantibodies and the RANK-RANKL system. IL-6 and TNF-α have been reported to directly affect both bone degradation and formation in RA [132-135]. TNF-α promotes osteoclastogenesis, upregulates RANKL expression on osteoclasts and lymphocytes, and suppresses bone formation by inducing the production of DKK-1. Moreover, TNF-α may inhibits osteoblastogenesis and enhances osteoclast activity via increasing oxidative stress, leading to accelerated bone loss [136, 137]. In RA, treatment with TNF-α inhibitors has been reported to elevate levels of OC and P1NP, and reduce the levels of CTX and RANKL. Furthermore, TNF-α inhibitors increase the ratios of OPG/RANKL, P1NP/CTX and OC/CTX, promoting a favorable balance in bone turnover. Furthermore, TNF-α inhibitors suppress the levels DKK-1 [81]. IL-6 receptor inhibition directly reduces the formation of osteoclasts, which suggests that IL-6 contributes to bone resorption [138]. Altough, the role of IL-6 in bone remodeling in RA is contradictory: while IL-6 may inhibit osteoclastogenesis, it also promotes RANKL expression by osteoblasts, T lymphocytes, and fibroblasts, which increases osteoclast formation [139-142].

T lymphocytes (Th1, Th2, Th17, and Treg) also have a key role in modulating bone remodeling in RA, thus Treg, Th1 and Th2 cells inhibit osteoclastogenesis. Th1 and Th2 cells produce inhibitory cytokines (IFN-Y, IL-4), however, Th17 cells promote osteoclast differentiation by releasing RANKL, TNF-α and IL-17, as well as enhance RANKL and M-CSF production in osteoblasts and RANK expression in osteoclasts [143]. Activated B cells contribute to bone metabolism in RA by secreting RANKL. Through the production of RANKL, activated B cells promote the activation and differentiation of osteoclasts, leading to bone resorption and further contributing to the pathogenesis of bone loss in RA [144].

2.6.2. Wnt signaling pathway

Besides the RANK-RANKL system, the Wingless (Wnt) signaling pathway is implicated in the activation and differentiation of osteoblasts and osteoclasts, as well as other functions during embryogenesis [145-147]. Wnt signaling pathways regulate osteoblastogenesis, osteoblast maturation, OPG synthesis, hence bone formation by transmitting signals through cell surface receptors and activating transcription factors. Activation of the Wnt pathways stimulates osteoblastogenesis and induces the production of OPG in osteoblasts. OPG acts as a decoy receptor for RANKL, thereby inhibiting RANKL-induced osteoclastogenesis and bone resorption. There are two well-known endogenous inhibitors of the Wnt-β-catenin signaling pathway, sclerostin (SOST) and Dickkopf-related protein 1 (DKK1), which are able to bind to Wnt co-receptor low-density lipoprotein receptor-related protein 5 (LRP5) or 6 (LRP6) leading to decreased bone formation [145]. By increasing RANK/RANKL interactions, DKK1 promotes osteoclast differentiation [148, 149]. SOST and DKK-1 may affect each other directly, as DKK-1 inhibition reduces SOST levels in mice [119, 149, 150]. SOST also enhances bone resorption through an autologous effect on osteocyte RANKL production [151]. Studies have revealed that RA patients have increased RANKL and decreased OPG levels in their synovial fluids when compared to OA patients. Additionally, RA patients exhibit a decreased OPG/RANKL ratio [152]. Reports have demonstrated that RA patients with an elevated risk of erosions have higher DKK-1 levels in their synovial fluid and serum [153, 154].

2.6.3. Autoantibodies in bone loss

Autoantibodies in RA appears to have a significant impact on the mechanism of bone loss. RA patients with ACPA positivity exhibit more severe osteopenia, bone erosions, and more aggressive disease progression, both clinically and radiologically, compared to ACPA negative patients. The presence of ACPA may enhance osteoclast activation and differentiation prior to the onset of arthritis. ACPA may bind to citrullinated vimentin and induce the production of CXCL8 leading to osteoclast differentiation [155, 156]. The process of osteoclast differentiation can also be induced by immune complexes of ACPA and their targets [157, 158]. Citrullination of proteins by PAD is crucial for the differentiation of osteoclasts from macrophage precursors. It was showed that ACPAs increased

osteoclastogenesis on a PAD-dependent autocrine effect of IL-8, furthermore the inhibition of IL-8 decreased osteoclast differentiation. In vivo transfer of ACPAs to mice resulted in a significant bone loss, which was reversed by reparixin, an IL-8 receptor inhibitor [159]. Other autoantibodies, such as anti-carbamylated proteins, have been linked to lower systemic BMD in early arthritis patients [160].

2.7. Imaging methods for the assessment of vessels and bone

2.7.1. Ultrasound-based techniques

Ultrasound-based methods have been employed to identify underlying vascular abnormalities in patients with RA, which may not have presented clinically yet [161, 162]. Arterial endothelial dysfunction can be assessed noninvasively by an ultrasound-based method, which measures endothelium-dependent flow-mediated dilatation (FMD) of the arterial diameter caused by increased shear-stress. The thickening of the carotid artery's intima-media layer (IMT) and the existence of plaques in the carotid artery are identified as signs of CVD. The speed of arterial pressure waves can be measured using arterial pulse wave velocity (PWV). Studies have found association between PVW and coronary atherosclerosis [163]. According to studies, RA is associated with enlarged carotid IMT, abnormal arterial FMD, and increased PWV, all of which are indications of the subsequent development of CV events [161, 162, 164, 165].

2.7.2. ^{18}F-FDG-PET/CT

^{18}F-fluorodeoxyglucose-positron emission tomography/computed tomography (^{18}F-FDG-PET/CT) has the potential to detect tissue inflammation throughout the whole body simultaneously [166-169]. Hence, this imaging method may be utilized to evaluate both vascular and synovial inflammation within the same patient [167]. Numerous studies have explored the utilization of PET or PET/CT for assessing inflammation of joints and blood vessels in RA [170-178]. Patients with ankylosing spondylitis (AS), psoriatic arthritis (PsA), and RA have shown increased FDG uptake in the arterial wall [176, 178, 179] [180, 181]. Patients with RA

who are undergoing combination csDMARD [182], anti-TNF [170, 183-185], rituximab [186] or tocilizumab therapies [187] have been assessed using ^{18}F-FDG-PET/CT to monitor disease activity, joint damage, and predict treatment outcomes. PET/CT can be used to monitor the effects of statins on AS patients and determine inflammatory variability in atherosclerosis over time [172, 180]. PET imaging can also be utilized to analyze the structure of atherosclerotic plaques [175]. PET/CT can also detect and monitor vascular inflammation in large-vessel vasculitis [188-190]. Few studies have assessed synovial and vascular inflammation concurrently. However, a preliminary study conducted on psoriasis patients found detectable skin, joint, and subclinical vascular inflammation through FDG-PET/CT imaging [167]. In a cross-sectional study of 33 patients with RA, synovial and arterial FDG uptake correlated with each other, while another study showed that vascular inflammation correlated with sacroiliitis [178, 181].

2.7.3. DXA and QCT

The estimated prevalence of osteoporosis in patients with RA is around 30%. Dual-energy X-ray absorptiometry (DXA) is considered the standard method for assessing BMD at the femur and lumbar spine in osteoporosis. Peripheral quantitative computed tomography (QCT) allows for the measurement volumetric (3-dimensional) BMD, as well as the assessment of cortical and trabecular bone, in addition to bone microarchitecture. In contrast, DXA is limited to assessment of areal (2-dimensional) BMD. QCT is utilized for measuring BMD in peripheral areas of the body, such as legs or forearms [191-194]. In a previous research study, we conducted a comparison between DXA and QCT in both healthy individuals and patients with RA [195].

2.8. Aims

The aim of this thesis was to gain an understanding of the effects of JAK inhibition on vascular and bone status in patients with RA. We have performed a 12 months follow-up study to investigate the therapeutic effects of tofacitinib on bone metabolism and bone density, as well as aortic and joint inflammation. Although targeted therapies have shown potential benefits on bone remodeling, vascular and systemic inflammation, no PET-CT studies have included JAK inhibitors yet. Furthermore, no prospective studies have been conducted using PET/CT imaging to simultaneously assess both synovial and vascular inflammations in patients with RA.

Primary aims:

- To assess vascular and joint inflammation by ^{18}F-FDG-PET/CT imaging method
- To examine changes on bone metabolism
- To assess bone status and bone mineral density by DXA and QCT
- To assess vascular physiology by ultrasound
- To examine changes on disease activity

Secondary aims:

- To correlate bone mineral density and laboratory biomarkers
- To correlate vascular and synovial inflammation with each other
- To correlate vascular and synovial inflammation with bone biomarkers
- To correlate vascular and synovial inflammation with parameters of bone status
- To correlate vascular and synovial inflammation with disease activity
- To correlate vascular and synovial inflammation with parameters of vascular physiology

3. Patients and methods

This chapter is originally appeared in the published articles, on which the dissertation is based.

The study was conducted in the University of Debrecen, Institute of Internal Medicine, Department of Rheumatology and was designed as a randomized self-control trial in patients with RA. The study was an investigator-initiated phase II clinical study, sponsored by Pfizer. It was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonisation Guidelines and was approved by the Hungarian Scientific Research Council Ethical Committee (approval No. 56953-0/2015-EKL) and was registered in EudraCT (EudraCT number: 2015-002523-26). Written informed consent was obtained from each patient.

As this study was designed as a self-controlled therapeutic follow-up study, comparison was made between baseline and follow-up time points, and there was no control group included.

3.1. Patient characteristics

A total of 30 patients, consisting of 27 women and 3 men, were enrolled prior to tsDMARD therapy. They were selected based on having a DAS28-CRP score of ≥3.2, indicating moderately active disease. Patients ages 18-80 years, the mean age was 52.8±10.0 (range: 27-69) years and the mean disease duration was 7.7±5.0 (range: 1-21) years. The eligibility criteria involved a definitive diagnosis of RA in accordance with the 2010 EULAR/ACR classification criteria for RA [47]; patients had to have a DAS28 of at least 3.2 at baseline and clinical indication of targeted therapy. Enrolled patients were either treatment-naïve to tsDMARDs (n=16) or previously received maximum one biologic DMARD therapy (n=14). Tofacitinib therapy was initiated following the discontinuation of bDMARD treatment and an appropriate washout period. Patients were ineligible if they had acute or recent infection, any inflammatory disease apart from RA, chronic renal or liver failure, contraindications to JAK inhibition, current use of anti-osteoporotic drugs (bisphosphonates, calcitonin, teriparatide, denosumab), uncontrolled CVD or hypertension, and malignancy within the past 10 years.

None of the patients had known primary osteoporosis prior to the diagnosis of RA and altogether 10 patients in the 5 mg and 6 patients in the 10 mg subgroups had received vitamin D supplement therapy at the time of inclusion. However, the dose of vitamin D supplementation remained unchanged throughout the study. Although many of the patients may have been on corticosteroids previously, all patients had discontinued corticosteroid use for a minimum of three months before the study.

Patients were randomly assigned to receive tofacitinib twice daily (bid) in a 1:1 ratio, either at a dose of 5 mg or 10 mg. In addition to tofacitinib, all patients were also receiving concurrent csDMARD therapy, which included methotrexate (n=17), leflunomide (n=5), or sulfasalazine (n=3). The doses of the concomitant csDMARD therapies had remained stable for at least one year prior to the study, and no modifications in the doses were permitted throughout the duration of the study. Laboratory measurements and clinical evaluations were conducted at baseline, at month 6 and month 12, while FDG-PET/CT examinations were assessed at baseline and after 12 months. Eventually four patients (two on each arm) completed the 6-month follow-up but did not complete the treatment for the full one-year duration. Two patients discontinued due to treatment inefficacy; one had elevated transaminases; and one patient relocated abroad. The data analysis included only those patients who successfully completed the one-year treatment period. A total of fourteen patients had hypertension; two had diabetes mellitus; and seven were current smokers at the time of enrollment (Table 1).

Table 1. Patient characteristics

	Total	**Tofacitinib 10 mg**	**Tofacitinib 5 mg**	**p (10 vs. 5 mg)**
Number of patients (n)	30	15	15	
Disease duration (mean±SD) (range), years	7.7±5.0 (1-21)	7.1±4.9 (2-21)	6.3±4.7 (1-15)	0.121
Age (mean±SD) (range), years	52.8±10.0 (27-69)	53.3±8.8 (34-69)	52.3±11.4 (27-69)	0.763
Female sex, no. (%)	27 (90)	13 (87)	14 (93)	0.600
DAS28 (baseline) (mean±SD)	5.05±0.77	5.29±0.79	4.80±0.69	0.081
ACPA positivity, n (%)	24 (80)	11 (73)	13 (87)	0.651
RF positivity, n (%)	24 (80)	12 (80)	12 (80)	1.000
HAQ-DI (baseline) (mean±SD)	1.59 (0.50)	1.169 (0.592)	1.38 (0.58)	0.047
CRP (baseline) (mean±SD)	16.33 (18.90)	13.30 (9.72)	14.82 (14.85)	0.585
Fragility fracture history (n)	4	2	2	1.000
Patients with comorbidities (n) hypertension gout diabetes hypothyreosis anxiety previous malignacy	 15 3 2 5 2 2	 10 2 1 2 2 0	 5 1 1 3 0 2	-
DXA L2-4 osteopenia (T-score <-1)	10	3	7	0.245
DXA L2-4 osteoporosis (T-score <-2.5)	0	0	0	-
DXA femoral neck osteopenia (T-score <-1	8	4	4	1.000
DXA femoral neck osteoporosis (T-score <-2.5)	3	2	1	1.000
Concomittant use of DMARDs (n) MTX Leflunomide Sulfasalazine MTX + Sulfasalazine Leflunomide + Sulfasalazine	23 16 4 1 1 1	11 7 2 1 0 1	12 9 2 0 1 0	-
Previous use of GCs (n)	10	6	4	0.700
Previous biologics (n)	(n=15: TNFi [n=7], ABA [n=2], TOC [n=5], RTX [n=3], SYK [n=1])	(n=9: TNFi [n=6], ABA [n=2], TOC [n=1], RTX [n=2])	(n=6: TNFi [n=13], ABA [n=4], TOC [n=6], RTX [n=5], SYK [n=1])	-

3.2. Clinical assessments

Clinical evaluations were conducted at baseline, as well as at 6 and 12 months after initiating tofacitinib therapy. A thorough medical history was obtained using a questionnaire, which included inquiries about current smoking status, chest pain, hypertension, CVD, fragility fractures, and diabetes mellitus within the two years preceding the study (Table 1). This was followed by thorough physical examination and calculation of disease activity using DAS28-CRP (3 variables). The functional capacity of the patients was assessed using the Health Assessment Questionnaire (HAQ).

3.3. Laboratory measurements

Blood samples were collected from fasting patients at baseline, as well as at 6 and 12 months after initiating the therapy. After being drawn into ethylene-diamine-tetraacetate (EDTA)-treated tubes and promptly processed, the samples were divided into aliquots and stored at temperature of -70°C until they were ready to be utilized.

Serum levels of IgM rheumatoid factor (RF; normal: ≤ 50 IU/ml) and high sensitivity CRP (hsCRP; normal: ≤ 5mg/l) were quantitatively measured by nephelometry (Cobas Mira Plus, Roche Diagnostics, Basel, Switzerland), using RF and CRP reagents (both Dialab, Budapest, Hungary). The detection of ACPA (aCCP) autoantibodies in serum samples was performed using a second-generation Immunoscan-RA CCP2 ELISA test (Euro Diagnostica, Malmö, Sweden; normal: ≤25 IU/ml). The assay was conducted according to the instructions provided by the manufacturer.

Lipids, lipoproteins and apoproteins, including triglyceride (TG), low density lipoprotein-cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC), lipoprotein a [Lp(a)], apoprotein A (ApoA) and B (ApoB) were determined using routine laboratory methods.

Serum levels of 25-hydroxy-vitamin D3 (25OHVITD3; DiaSorin; normal: ≥75 nmol/l); phosphate (P; Roche Diagnostics; normal: 0.8-1.45 mmol/l); calcium (Ca; Roche Diagnostics; normal: 2.1-2.6 mmol/l); parathyroid hormone (PTH; Roche Diagnostics; normal: 1.6-6.9 pmol/l); sclerostin (SOST; Biomedica; median: 24.14 pmol/l); C-terminal collagen crosslinks

(CTX; Roche Diagnostics; normal: <0.57 µg/l), procollagen 1 N-terminal propeptide (P1NP; Roche Diagnostics; normal: <75 µg/l); cathepsin K (CATHK; Biomedica; median: 8.7 pmol/l), and osteocalcin (OC; Roche Diagnostics; normal: <41 µg/l) were determined by ELISA. Levels of Dickkopf-1 (DKK1; normal: 46.5-2225.7 pg/ml); osteoprotegerin (OPG; normal: 515.3-1964.2 pg/ml) and soluble RANKL (normal: <1067.4 pg/ml) were assessed by flow cytometry using a custom multiplex bead immunoassay kit (LEGENDplex, BioLegend) and analyzed by LEGENDplex software [196]. All measurements were carried out at three time points: at baseline, at 6 months after treatment initiation, and 12 months after treatment initiation.

3.4. Assessment of bone mineral density

In order to determine areal BMD of the hip bones and lumbar spine, DXA assessment was performed by a single technician during the study period, using the LUNAR Prodigy densitometer (GE-Lunar Corp., Madison, WI, USA). At our institute, the coefficient of variation of the technique was 0.8% as determined by measuring the anatomical spine phantom daily, and no machine drift was observed throughout the study. The short-term in vivo precision error for L2-L4 lumbar spine is 0.012 g/cm^2 (LSC = 0.034 g/cm^2 at 95% confidence level) and femur neck is 0.013 g/cm^2 (LSC = 0.035 g/cm2 at 95% confidence level). Single-slice quantitative computed tomography (QCT) of the ultra-distal region of the dominant forearm were performed in order to determine volumetric (3D) BMD, using a Stratec XCT-2000 instrument (Stratec Medizintechnik GmbH, Pforzheim, Germany). Distal sites at 4% of the radius length contain mainly trabecular bone. QCT is able to differentiate between trabecular and cortical bone. Cortical, trabecular and total BMD values obtained through QCT were reported in mg/cm³. The imaging acquisition was performed with a voxel size of 0.59 mm, and the analysis was conducted using XCT6.00B software (Stratec Medizintechnik GmbH, Pforzheim, Germany). The measuring mask was set to the radius, and a threshold density of 269 mg/mm³ was used to define trabecular bone.

3.5. ^{18}F-FDG-PET/CT assessments

All patients underwent ^{18}F-FDG-PET/CT (Philips Gemini TF) examination after fasting for at least 6 hours and serum glucose level check. The pre-scan glucose level was accepted as less than 7.2 mmol/l. Whole-body scans were performed using the AnyScan PC system (Mediso Medical Imaging Systems, Budapest, Hungary) from the base of the skull to the level of the knees. The scans were obtained two hours after intravenous administration of the 18F-FDG radiopharmaceutical (4.4 MBq/kg). Low-dose non-enhanced CT images were utilized to reconstruct axial, coronal, and sagittal attenuation-corrected and non-corrected PET images for interpretation. After a visual assessment of the PET and CT images, quantification of vascular inflammation was conducted. Two-dimensional circular regions of interest (ROIs) were drawn around the external aortic contour and merged into tube-like volumes of interest (VOIs) outlining 5 predefined aortic segments (ascending aorta, aortic arch, descending thoracic aorta, suprarenal, and infrarenal abdominal aorta) using dedicated analysis software (InterView FUSION, Mediso, Budapest, Hungary) to determine maximum (SUVmax) and mean standardized uptake values (SUVmean). The mean target-to-background ratio (TBRmean) and the maximum target-to-background ratio (TBRmax) are widely utilized parameters to globally assess vascular inflammation [172, 173, 197]. Aortic $TBR_{maxvasc}$ and $TBR_{meanvasc}$ values were obtained by dividing $SUV_{maxvasc}$ or $SUV_{meanvasc}$ values of the aortic segments by the SUV_{mean} value of the superior vena cava (blood pool) [166]. Thresholds for TBR have already been established [197]. The mean metabolic volumetric product (MVP_{mean}) was calculated by multiplying SUV_{mean} by VOI volume (cm^3) for each segment as previously reported [167]. To quantify synovial inflammation, SUV_{maxsyn} and $SUV_{meansyn}$ values were determined in VOIs placed with the help of the CT structural images around 5 predefined articular regions (hand/wrist, elbow, shoulder, hip and knee) on both sides, and liver $SUV_{meanliv}$ values were determined and used as reference values. Therefore, the degree of synovial inflammation was expressed as $SUV_{maxsyn}/SUV_{meanliv}$ ratios of each articular region ($SUV_{meansyn/liv}$). Finally mean (±SD) of the five $TBR_{maxvasc}$ and $TBR_{meanvasc}$ values in the five predefined aortic segments, as well as the mean (±SD) of the five $SUV_{meansyn}$ and $SUV_{meansyn/liv}$ values in the five articular regions, were calculated.

3.6. Assessment of vascular physiology

3.6.1. Flow-mediated vasodilation

Right arms of the patients were assessed by using high-resolution duplex ultrasonography equipped with a 5-10-MHz linear transducer and electrocardiogram (Hewlett-Packard Sonos 5500) gating. Longitudinal images were taken at ca. 4-7 cm proximally from the cubital fossa. The cuff was inflated for 5 minutes to maintain systolic blood pressure above 50 mmHg, followed by a rapid release to induce reactive hyperemia. Arterial diameter was initially measured at rest, and subsequently, changes in diameter were evaluated following a period of increased flow lasting 60 seconds [198]. Mean diameter was determined by calculating the average of three consecutive measurements synchronized with the R wave of the heart cycle [199, 200]. The percentage of change of the diameter at rest compared after flow presented as the change of FMD.

3.6.2. Carotid artery intima–media thickness

We determined carotid artery intima media thickness (ccIMT) by using duplex ultrasound (HP Sonos 5500) instrument with 5–10 MHz linear transducer. Both transverse and longitudinal section images were taken of the carotid artery in the end-diastolic phase. We performed 10 measurements on both sides of the patient and the mean value was calculated. According to the leading edge method ccIMT was presented as the distance between the first (lumen–intima border) and the second (media–adventitia border) echogenic line [198]. If no plaques were detected using the mediolateral transducer position, we proceeded to capture longitudinal images of the common carotid arteries, approximately 10 mm away from the carotid bulb.

3.6.3. Pulse wave velocity

Pulse wave velocity (PWV) is used to assess arterial stiffness. When the left ventricle contracts, it generates a pulse wave that propagates throughout the arterial tree. The rate at

which pressure waves move down the vessel is known as PWV and calculated by dividing the distance between the two places of the same artery by the pressure wave transit time. The increased speed of the pulse wave in the arteries is associated with arterial stiffness. The distance between two points on an artery segment can be measured directly or approximated using body height. To ensure consistent and reproducible results, it is necessary for the patient to rest in a quiet examination room for a minimum of 5 minutes prior to the assessment [201, 202].

The same investigator conducted FMD, IMT and PWV assessments at baseline and after 12 months of tofacitinib therapy.

3.7. Statistical analysis

We utilized various methods to perform statistical analysis through SPSS version 22.0 (IBM, Armonk, NY, USA) software. Continuous variables were expressed as mean ± SD, while categorical variables were expressed as percentages. Both parametric and non-parametric methods were employed. We evaluated the distribution of continuous variables using the Kolmogorov-Smirnov test. Wilcoxon tests and paired two-tailed t-tests were used to measure the statistical significance of continuous variables. Nominal variables, on the other hand, were compared through $\chi 2$ or Fisher's exact test, as appropriate. To assess correlations, Pearson's analysis was utilized. Regression analyses were conducted to investigate the independent associations between various parameters, such as inflammatory, clinical, bone and vascular, as independent variables and PET/CT parameters, as dependent variables. Additionally, univariable and multivariable regression analysis, employing the stepwise method, were utilized to examine the relationships between laboratory and clinical parameters (independent variables) and BMD as assessed by QCT and DXA (dependent variables). The β standardized linear coefficients showing linear correlations between two parameters were determined, and the B (+95% CI) regression coefficient indicated independent associations between dependent and independent variables during changes. Multivariate analysis of variance (MANOVA) was conducted to evaluate the impact of independent variables on two dependent variables simultaneously, while repeated measures analysis of variance (RM-ANOVA) was used to determine the further effects of various parameters including therapy

on 12-month changes of BMD and PET/CT parameters. In these analyses, partial η^2 is given as indicator of effect size, with values of 0.01 suggesting small, 0.06 medium and 0.14 large effect. The reliability of the vascular ultrasound measurements was tested by inter-item correlation and intra-class correlation (ICC). With respect to the PWV, IMT and FMD ultrasound tests, ICC=0.470; F-test value: 1.887; p=0.001. $P<0.05$ was considered significant in all tests. During data analysis for PET-CT parameters, we pooled the 5 mg and 10 mg bid arms because of the number of patients.

4. Results

4.1. Effects of tofacitinib treatment on bone metabolism in RA

4.1.1. Patient characteristics and clinical response to tofacitinib therapy

Four out of the thirty patients did not complete the study; two participants from each group withdrew after six months of therapy. The reasons for discontinuation varied, and included treatment inefficacy, elevated transaminase levels, and discontinuation of study visits. The analysis included a total of twenty-six patients, with thirteen from each group, who completed the study. Three patients, within the entire cohort, had femoral neck osteoporosis, while none had osteoporosis in the lumbar spine (L2-4 vertebrae). Eight patients had osteopenia in the femoral neck, and ten patients had osteopenia in the L2-4 region. Two patients in the 5 mg tofacitinib group had a history of vertebral fragility fractures prior to the study, but did not exhibit osteoporosis or osteopenia (Table 1).

JAK inhibition effectively reduced disease activity and systemic inflammation, as showed by the decrease in CRP levels in both groups. In the full cohort (n=26), DAS28 showed a significant decrease from 5.05±0.77 at baseline to 3.31±0.91 ($p<0.001$) after 6 months and to 3.32±1.12 ($p<0.001$) after 12 months of therapy. Additionally, DAS28 score significantly reduced after 6 months (3.23±0.54; $p<0.001$) and after 12 months (3.05±0.77; $p<0.001$) compared to baseline (4.80±0.69), in the 5 mg subset. In the 10 mg group, DAS28 score at baseline, after 6 and 12 months, was 5.29±0.79, 3.39±1.19 ($p<0.001$) and 3.58±1.36 ($p<0.001$), respectively (Table 2). In the full cohort of patients, tofacitinib therapy led to a reduction in CRP levels from 14.8±14.9 mg/l at baseline to 5.3±5.3 mg/l ($p<0.001$), following a 6-months period of therapy, and to 7.4±7.7 mg/l ($p=0.001$) after 12 months of treatment. The CRP levels significantly changed from 13.3±9.7 mg/l at baseline to 5.3±3.7 mg/l ($p=0.002$) at month 6, and to 7.1±4.0 mg/l ($p=0.022$) at month 12, in the 5 mg tofacitinib group. Furthermore, levels of CRP were 16.3±18.9 mg/l at baseline, 5.2±6.7 mg/l ($p=0.016$) after 6 months, and 7.7±10.3 mg/l ($p=0.014$) after 12 months, in the 10 mg group (Table 2). The HAQ disability index was used to assess the functional capacity of the patients. In the full cohort significant improvement was seen in HAQ from baseline to month 6, 1.38±0.58 to 1.02±0.67 ($p=0.001$),

and at month 12 to 1.02±0.71 (p=0.001). In the 10 mg group, HAQ value improved after 6 months (1.10 ± 0.74; p = 0.010) and after 12 months (1.15 ± 0.73; p = 0.005) compared to baseline (1.59±0.50). However, there was a non-significant trend towards improvement in HAQ score in the subset receiving 5 mg bid tofacitinib (Table 2).

Table 2. Effects of 1-year tofacitinib therapy on HAQ, DAS28 and CRP levels

Total patients	0	6	12	p 0-6	p 0-12	p 6-12
HAQ	1.38±0.58	1.02±0.67	1.02±0.71	**0.001**	**0.001**	0.978
DAS28	5.05±0.77	3.31±0.91	3.32±1.12	**<0.001**	**<0.001**	0.958
CRP	14.82±14.85	5.27±5.29	7.39±7.68	**<0.001**	**0.001**	**0.013**
2x5 mg						
HAQ	1.169±0.592	0.939±0.617	0.885±0.681	0.053	0.072	0.563
DAS28	4.80±0.69	3.23±0.54	3.05±0.77	**<0.001**	**<0.001**	0.405
CRP	13.30±9.72	5.32±3.69	7.11±3.99	**0.002**	**0.022**	0.099
2x10 mg						
HAQ	1.59±0.50	1.10±0.74	1.15±0.73	**0.010**	**0.005**	0.732
DAS28	5.29±0.79	3.39±1.19	3.58±1.36	**<0.001**	**<0.001**	0.505
CRP	16.33±18.90	5.21±6.67	7.67±10.32	**0.016**	**0,014**	0.077

4.1.2. Effects of tofacitinib therapy on bone loss and bone biomarkers

After 12 months of therapy, tofacitinib demonstrated that it may be able to prevent additional bone loss in RA patients. No significant changes were observed in the areal BMD of femoral neck (DXAFNBMD) and vertebrae of L2-4 (DXAL24BMD) over the one-year period in the full cohort and the subgroups receiving 5 mg or 10 mg tofacitinib (p =NS), determined by DXA. (Fig. 4A). Using QCT, there were no significant changes in the cortical (QCTCORTBMD), trabecular (QCTTRABBMD), and total (QCTTOTBMD) volumetric BMD between baseline and the 12-month time point in the full cohort, as well as in the 5 mg and 10 mg subsets (p=NS) (Fig.4B). One-year tofacitinib treatment resulted changes in areal BMD between −1.5% and 0.1% in the 5 mg subgroup, −0.2% and 1.4% in the 10 mg tofacitinib subset and −0.9% and 0.7% in the full cohort. Changes in volumetric BMD were between −8.1% and 8.2% in the 5 mg subset, −1.5% and 4.9% in the 10 mg subgroup and −4.9% and 6.6% in the full cohort.

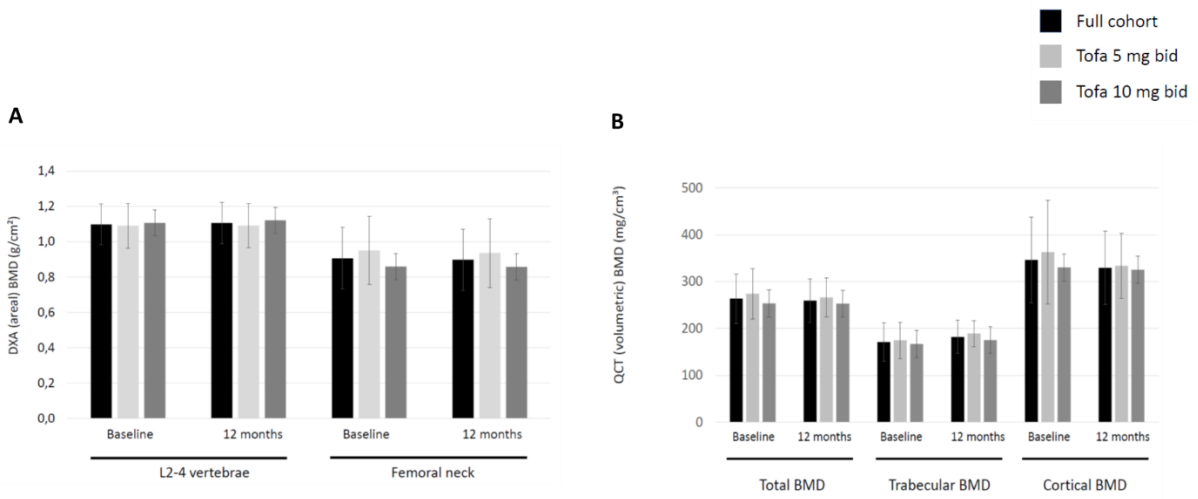

Figure 4. Effects of 12-months tofacitinib treatment on areal and volumetric BMD in full cohort and 5 mg, and 10 mg bid subsets. (A) Baseline and 12-month BMD of femoral neck and L2–4 vertebrae assessed by DXA. (B) Changes of cortical, trabecular and total volumetric BMD as determined by QCT.

According to bone biomarkers, we have measured 12 bone turnover markers. We have observed significant increase in the levels of OC from baseline to 6 months (p=0.013), but only non-significant enhance was seen at month 12 (Fig.5A). CTX levels significantly decreased from baseline to 6 months (p=0.009) and 12 months (p=0.003) (Fig.5B). Furthermore, levels of OPG also increased after 6 months (p=0.006) and 12 months of tofacitinib treatment (p=0.004) (Fig.5C), as well as levels of 25OHVITD3 from baseline to 6 months (p=0.017) and 12 months (p=0.009) (Fig.5D). With respect to the 5 mg subgroup, OC levels significantly increased after 6 months of therapy (p=0.027) (Fig.5A), as well as levels of OPG after 6 months (p=0.005) and 12 months (p=0.002) (Fig.5C). Additional, vitamin D3 levels significantly increased from baseline to 6 months (p=0.001) and 12 months (p=0.004) (Fig.5D). Moreover, in the 10 mg bid subset, levels of CTX decreased from baseline to 6 months (p=0.005) and 12 months (p=0.007) (Fig. 5B) and levels of OPG increased after 6 months (p=0.047) and 12 months (p=0.029) of therapy (Fig. 5C).

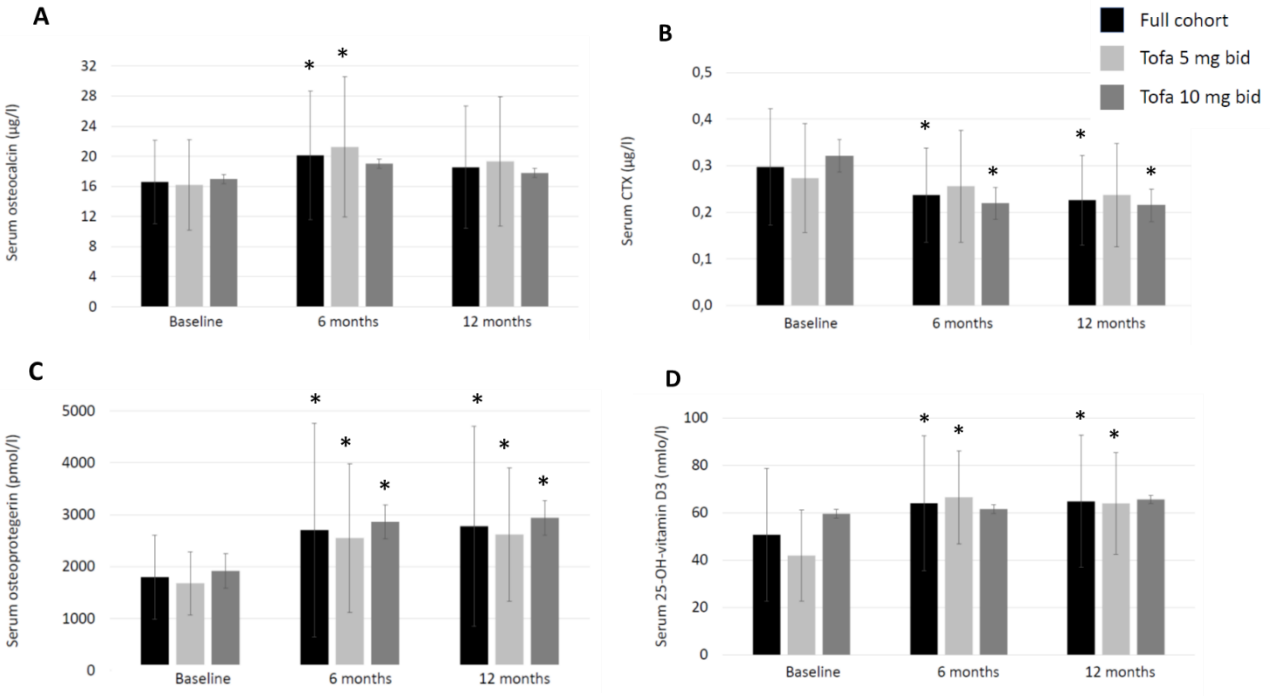

Figure 5. Effects of 12-months tofacitinib treatment on the levels of (A) OC, (B) CTX, (C) OPG, and (D) 25-hydroxy-vitamin D3 in the 5 mg and 10 mg subgroups and in the full cohort. (*p<0.05)

However, JAK inhibition did not change the levels of RANKL, SOST, DKK1, P1NP and PTH significantly, we have found favourable changes in the P1NP/CTX and OC/CTX ratios, but not in the OPG/RANKL ratio. We have found a significant increase in the P1NP/CTX ratio in total patients group from baseline to 6 months (p=0.002) and 12 months (p=0.001). OC/CTX ratios also increased from baseline to 6 months (p<0.001) and to 12 months (p<0.001). In the 5 mg subgroup P1NP/CTX ratios significantly increased after 6 months (p=0.023) and after 12 months (p=0.013) of tofacitinib therapy, however we did not find significant changes in the 10 mg subset. Additional, OC/CTX ratios in the 5 mg subset, significantly elevated from baseline to 6 months (p=0.010) and to 12 months (p=0.013). Moreover, in the 10 mg group, OC/CTX ratios also increased after 6 months (p=0.002) and after 12 months (p=0.005) of therapy (Table 3). When we compared the subgroups, we did not find any significant difference between them (Table 4).

Table 3. Effects of tofacitinib therapy on the levels of P1NP/CTX, OPG/RANKL, OC/CTX ratios

Total patients	0	6	12	p 0-6	p 0-12	p 6-12
P1NP/CTX	159.42±64.94	220.33±88.98	228.80±95.68	**0.002**	**0.001**	0.531
OPG/RANKL	18.06±13.94	20.17±12.32	16.99±9.98	0.468	0.680	0.177
OC/CTX	62.40±22.69	92.63±36.05	90.56±33.78	<0.001	<0.001	0.631
Tofacitinib 5 mg						
P1NP/CTX	159.95±68.21	219.66±14.33	233.57±88.33	0.023	0.013	0.524
OPG/RANKL	18.65±12.56	22.21±14.33	16.04±8.41	0.323	0.318	0.117
OC/CTX	64.76±21.46	89.03±28.38	88.17±26.42	0.010	0.015	0.892
Tofacitinib 10 mg						
P1NP/CTX	158.91±63.89	221.00±94.73	224.03±105.43	0.050	0.075	0.859
OPG/RANKL	17.47±15.62	18.14±10.00	17.95±11.56	0.888	0.916	0.945
OC/CTX	60.03±24.37	96.22±43.12	92.95±40.67	0.002	0.005	0.593

Table 4. Differences between the 5 mg bid and the 10 mg bid subsets of tofacitinib therapy

	2*5 mg tofacitinib	2*10 mg tofacitinib	p
P1NP/CTX_0	159.95±68.21	158.91±63.89	0.966
6	219.66±14.33	221.00±94.73	0.968
12	233.57±88.33	224.03±105.43	0.790
OPG/RANKL_0	18.65±12.56	17.47±15.62	0.810
6	22.21±14.33	18.14±10.00	0.374
12	16.04±8.41	17.95±11.56	0.608
OC/CTX_0	64.76±21.46	60.03±24.37	0.577
6	89.03±28.38	96.22±43.12	0.594
12	88.17±26.42	92.95±40.67	0.506

We have found correlations between laboratory biomarkers and bone mineral density parameters at baseline and after one-year of tofacitinib therapy. In the Pearson's correlation analysis negative correlation was observed between DXAL24BMD-0 and CTX-0, CTX-12, P1NP-12 and OC-12 ($p<0.005$) (Table 5). Moreover, DXAL24BMD-12 negatively correlated with RANKL-0, CTX-0, as well with P1NP-12 and CTX-12 ($p<0.05$) (Table 5). DXAFNBMD-0 showed inverse associations with OC-0, CTX-0, CTX-12, P1NP-12 and OC-12 ($p<0.05$) (Table 5). Similarly, DXAFNBMD-12 negatively correlated with CTX-0, OC-0, CTX-12, P1NP-12 and OC-12 ($p<0.05$) (Table 5). With respect to QCTTOTBMD-0 and QCTTRABBMD-0 both showed negative associations with PTH-12, however QCTCORTBMD-12 inversely correlated with RANKL-0 ($p<0.05$) (Table 5).

Table 5. Correlations between bone biomarkers and volumetric and areal BMD

Bone density	Bone biomarker	R value	p value
Bone imaging vs bone biomarkers			
DXAL24BMD-0	*OC-12*	-0.438	0.022
	CTX-0	-0.463	0.015
	CTX-12	-0.458	0.016
	P1NP-12	-0.477	0.012
DXAL24BMD-12	*CTX-0*	-0.474	0.013
	CTX-12	-0.392	0.043
	P1NP-12	-0.457	0.017
	RANKL-0	-0.390	0.045
DXAFNBMD-0	*OC-0*	-0.558	0.002
	OC-12	-0.463	0.015
	CTX-0	-0.555	0.003
	CTX-12	-0.450	0.018
	P1NP-12	-0.383	0.049
DXAFNBMD-12	*OC-0*	-0.536	0.004
	OC-12	-0.482	0.011
	CTX-0	-0.549	0.003
	CTX-12	-0.446	0.020
	P1NP-12	-0.382	0.049
QCTTOTBMD-0	*PTH-12*	-0.397	0.030
QCTTRABBMD-0	*PTH-12*	-0.375	0.041
QCTCORTBMD-12	*RANKL-0*	-0.398	0.029

The univariable regression analysis suggested that RANKL-0, CTX-0, CTX-12, and P1NP-12 may inversely determine DXAL24BMD-12 ($p<0.05$), while CTX-0 may be a negative determinant of DXAL24BMD-0 (Table 6). OC-0, CTX-0 and the age of patients were negative determinants of DXAFNBMD-0 and DXAFNBMD-12. In addition, P1NP-12 and OC-12 inversely correlated with DXAFNBMD-12 ($p<0.05$) (Table 6). QCTTRABBMD-0 by was negatively determined by DAS28-, QCTTOTBMD-12 by CRP-12, while QCTCORTBMD-12 by RANKL-0 and CRP-12 ($p<0.05$) (Table 6). The multivariable analysis confirmed negative associations between CTX-0 and DXAL24BMD-12, age and OC-0 with DXAFNBMD-0, age, OC-0 and CTX-0 with DXAFNBMD- 12, as well as RANKL-0 and CRP-12 with QCTCORTBMD- 12 ($p < 0.005$) (Table 6).

Table 6. Univariable and multivariable analysis of determinants of QCT and DXA parameters

Dependent variable	**Independent variable**	**Univariable regression analysis**				**Multivariable regression analysis**			
		β	**p**	**B**	**95% CI**	**β**	**p**	**B**	**95% CI**
DXAL24BMD-0	*CTX-0*	-0.463	0.015	-0.414	-0.741- -0.088				
DXAL24BMD-12	*CTX-0*	-0.474	0.013	-0.432	-0.763- -0.101	-0.474	0.013	-0.432	-0.763- -0.101
	CTX-12	-0.484	0.043	-0.392	-0.952- -0.016				
	P1NP-12	-0.457	0.017	-0.003	-0.006- -0.001				
	RANKL-0	-0.390	0.045	0	-0.001-0				
DXAFNBMD-0	*Age*	-0.531	0.004	-0.009	-0.025- -0.003	-0.522	0.001	-0.009	-0.012- -0.004
	OC-0	-0.558	0.002	-0.017	-0.027- -0.007	-0.550	<0.001	-0.017	-0.025- -0.008
	CTX-0	-0.555	0.003	-0.751	-1.215- -0.288				
DXAFNBMD-12	*Age*	-0.568	0.002	-0.010	-0.015- -0.004	-0.543	<0.001	-0.009	-0.013- -0.005
	OC-0	-0.536	0.004	-0.016	-0.027- -0.006	-0.345	0.030	-0.010	-0.020- -0.001
	OC-12	-0.482	0.011	-0.010	-0.017- -0.003				
	CTX-0	-0.549	0.003	-0.739	-1.202- -0.275	-0.312	0.048	-0.420	-0.836-0.005
	P1NP-12	-0.382	0.049	-0.004	-0.008-0				
QCTTOTBMD-0	-								
QCTTOTBMD-12	*CRP-12*	-0.389	0.033	-2.359	-4.519- -0.199				
QCTTRABBMD-0	*DAS28-0*	-0.389	0.034	-20.730	-39.730- -1.72				
QCTTRABBMD-12	-								
QCTCORTBMD-0	-								
QCTCORTBMD-12	*RANKL-0*	-0.398	0.029	-0.217	-0.410- -0.023	-0.364	0.031	-0.198	-0.377- -0.019
	CRP-12	-0.424	0.020	-4.305	-7.867- -0.743	-0.392	0.021	-3.983	-7.325- -0.641

RM-ANOVA analysis was conducted to examine the independent factors that contributed to the changes in volumetric and areal BMD data over a one-year period, with these BMD measurements serving as the dependent variables in the analysis. Significant effects on changes in DXAL24BMD over a one-year period were observed with tofacitinib therapy when combined with lower levels of CCP-0 or DKK1-0. Furthermore, lower CRP-0 or lower age combined with tofacitinib treatment were found to be significant determinants of 12-months changes in QCTCORTBMD ($p<0.05$) (Table 7).

Table 7. Significant results of general linear model (GLM) repeated measures analysis of variance (RM-ANOVA) test determining the effects of therapy and other independent variables on QCT and DXA parameters as dependent variables

Dependent variable	Effect	F	p	Partial η^2
DXAL24BMD 0-12	*treatment * lower CCP-0*	5.804	0.024	0.188
	*treatment * lower DKK1-0*	4.089	0.044	0.141
QCTCORTBMD 0-12	*treatment * lower age*	4.715	0.039	0.144
	*treatment * lower CRP-0*	4.774	0.037	0.146

4.2. Assessment of vascular and joint inflammation by PET/CT in associations with vascular and bone status

4.2.1. The effects of tofacitinib therapy on vascular and synovial inflammation

PWV and FMD showed no significant changes between baseline and after 12 months. In the subset of 5 mg bid group, we have observed a significant increase in carotid IMT after 12 months compared to baseline. However, we did not find significant changes in IMT from baseline to 12 months in the 10 mg subgroup.

Vascular and synovial inflammation was assessed by ^{18}F-FDG-PET/CT. One-year tofacitinib therapy resulted in a significant and simultaneous decrease in synovial and vascular inflammation as visualized by PET/CT (Figure 6 and 7).

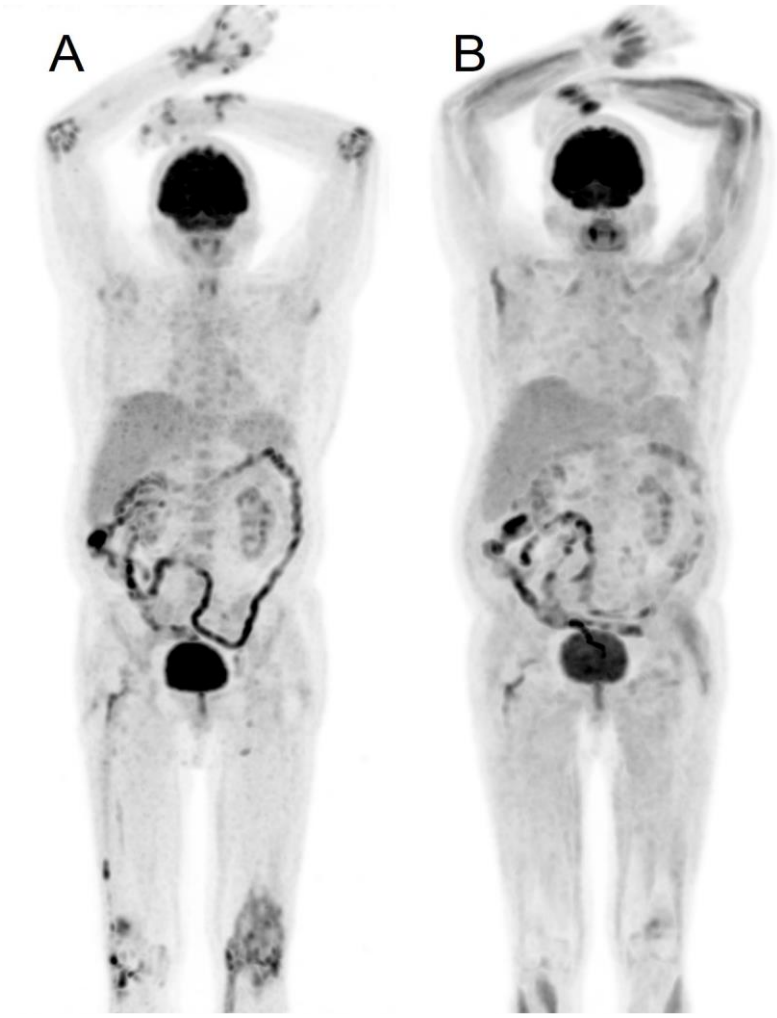

Figure 6. Representative image of joint inflammation visualized by ^{18}F-FDG-PET/CT at baseline and after tofacitinib treatment in a patient with RA. (A) Baseline PET/CT MIP image shows high levels of synovial activity in various joints (elbows, wrists, small hand joints, and knees bilaterally) (B) The FDG uptake shows a significant reduction after 12 months of therapy (MIP: multiple intensity projection)

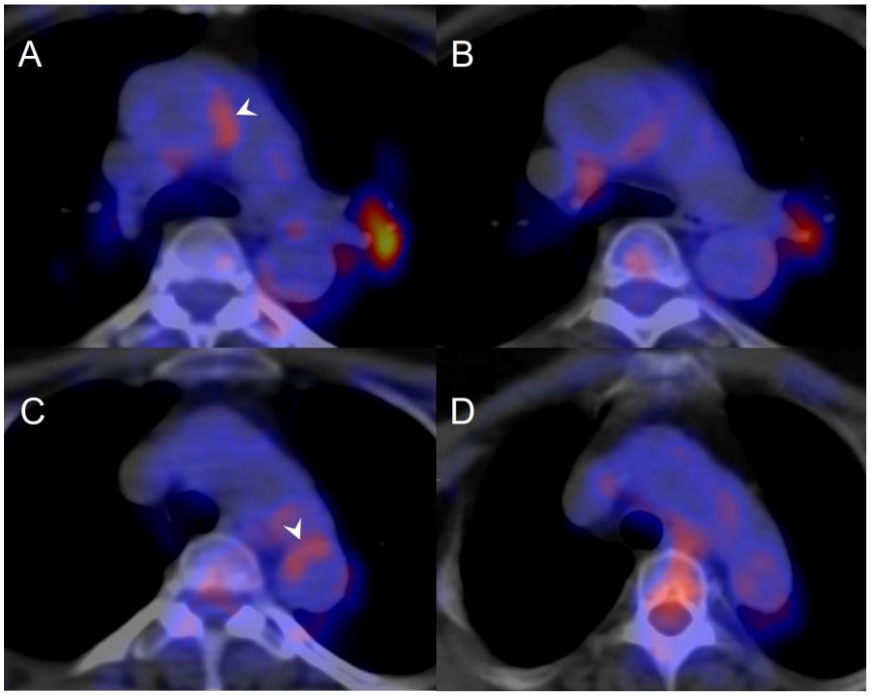

Figure 7. Representative image of vascular inflammation visualized by ^{18}F-FDG-PET/CT at baseline (A, C) and after 12 months of tofacitinib therapy (B, D) at the aortic arch.

The mean articular SUV-SYN significantly reduced after 12 months (2.55±0.50) of tofacitinib treatment compared to baseline (3.18±1.13; p=0.010) (Figure 8A). TBR-SYN mean showed a significant decrease from baseline (1.53±0.54) to 12 months (1.12±0.22; p=0.001) (Figure 8B). Aortic TBR-VASC max reduced from baseline (2.17±0.52) to 12 months (1.80±0.30; p<0.001) (Figure 8D). A non-significant tendency of reduction of TBR-VASC mean was observed over one year of tofacitinib treatment from baseline (1.29±0.29) to 12 months (1.20±0.20; p=0.170) (Figure 8C).

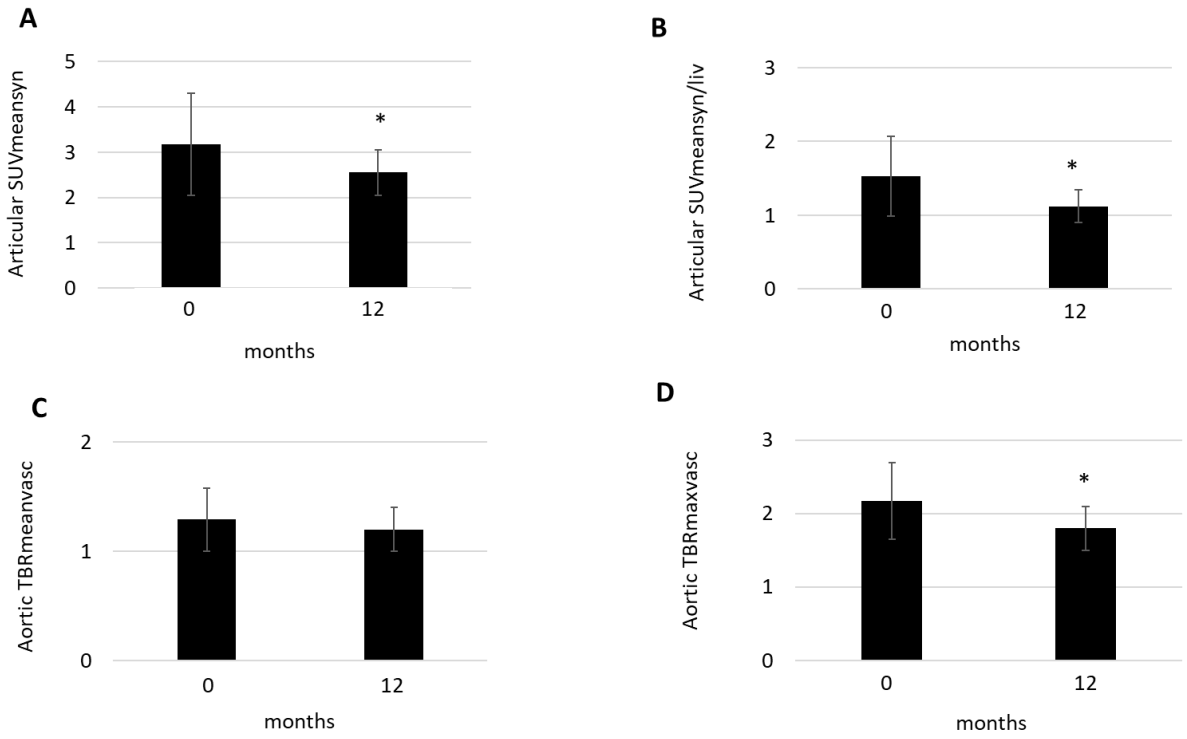

Figure 8. Effects of 12 months tofacitinib treatment on articular SUV-SYN mean (A), articular TBR-SYN mean (B), aortic TBR-VASC mean (C) and aortic TBR-VASC max (D) as measured by ^{18}F-FDG-PET/CT imaging (*P<0.05).

4.2.2. Statistical analysis of the effects of tofacitinib on vascular and synovial inflammation

No significant correlations were found between aortic TBR values and articular SUV/TBR. However, there were positive and significant correlations between articular TBR-SYN mean and SUV-SYN mean values with anti-CCP, RF, CRP, IMT, PWV, CTX, RANKL, Lp(a) and L2-4 BMD values determined by DXA ($p<0.05$) from baseline to 12 months of tofacitinib treatment. Aortic TBR-VASC max and TBR-VASC mean values exhibited variable and positive correlations with PWV, DAS28, P1NP, OC, ESR, and negative correlations with HAQ and L2-4 BMD ($p<0.05$) after 12 months of therapy compared to baseline. Synovial inflammation, as assessed by PET/CT, showed positive associations with CTX, Lp(a), CRP, PWV, IMT and inversely associated with DXA L2-4 BMD ($p<0.05$) after 12 months, in the univariable analysis (Table 8). Aortic inflammation was positively associated with OC, P1NP, DAS28, PWV and negatively with HAQ values (Table 8).

The multivariable analysis results confirmed previous findings showing an association between Lp(a) and synovial inflammation after 12 months. Additionally, vascular inflammation was found to be associated with HAQ, DAS28, and P1NP at various time points ($p<0.05$) (Table 8). We aimed to investigate the relationships between synovial inflammation measured by PET/CT and ultrasound-detected vascular pathophysiology, as covariates, with systemic inflammation and disease activity, as independent variables.

Table 8. Univariable and multivariable regression analyses of the associations between PET/CT as dependent variables and other parameters as independent variables

Dependent variable	Independent variable	Univariable analysis				Multivariable analysis			
		β	p	B	CI 95%	β	p	B	CI 95%
SUV-SYN$_{mean}$-12	*CRP-6*	0.499	0.030	0.042	0.005-0.080				
	CRP-12	0.529	0.020	0.038	0.007-0.070				
	LPA-0	0.671	0.001	0.001	0.001-0.002				
	LPA-6	0.676	0.001	0.002	0.001-0.003				
	LPA-12	0.683	0.001	0.002	0.001-0.003	0.453	0.001	0.001	0.001-0.002
	CTX-12	0.474	0.041	2.380	0.115-4.645				
	PWV-0	0.571	0.011	0.149	0.040-0.259				
TBR-SYN$_{mean}$-12	*LPA-0*	0.547	0.015	0	0-0.001				
	LPA-6	0.567	0.011	0.001	0-0.001				
	LPA-12	0.581	0.009	0.001	0-0.001	0.335	0.016	0	0-0.001
	IMT-12	0.467	0.044	0.668	0.021-1.315				
	DXAL24BMD-12	-0.518	0.023	-0.897	-0.197- -0.007				
	DXAL24TSC-0	-0.482	0.037	-0.102	-0.197- -0.007				
TBR-VASC$_{mean}$-12	*HAQ-6*	-0.457	0.049	-0.136	-0.271- -0.001	-0.542	<0.001	-0.161	-0.228--0.094
	DAS28-6	0.597	0.007	0.187	0.058-0.316	0.617	<0.001	0.194	0.123-0.265
	OC-0	0.531	0.019	0.017	0.003-0.031				
	P1NP-12	0.470	0.043	0.005	0-0.010	0.464	0.001	0.005	0.003-0.008
	PWV-0	0.526	0.021	0.069	0.009-0.098				
	PWV-12	0.546	0.016	0.069	0.014-0.113				
TBR-VASC$_{max}$-12	*HAQ-0*	-0.529	0.020	-0.291	-0.529- -0.052	-0.529	0.020	-0.291	-0.529--0.052
	HAQ-6	-0.471	0.042	-0.212	-0.415- -0.009				

In the MANOVA analysis DAS28, CRP, and ESR were found to significantly and independently determine both synovial inflammation and PWV or FMD after 12 months of tofacitinib therapy ($p<0.05$) (Table 9).

Table 9. Results of MANOVA analysis determining the effects of inflammatory markers as independent variables on 12-months vascular pathophysiology and PET/CT parameters as concurrent dependent variables.

Dependent variables	Independent variables	Effect	F	p	Partial η^2
Articular $SUV_{meansyn}$-12 AND FMD-12	*DAS28-0*	0.321	3.787	0.045	0.321
Articular $SUV_{meansyn}$-12 AND PWV-12	*CRP-12*	0.388	5.063	0.020	0.388
Articular $SUV_{meansyn/liv}$-12 AND FMD-12	*ESR-0*	0.338	4.092	0.037	0.338

RM-ANOVA analysis was utilized to assess the combined effects of tofacitinib therapy and additional factors on changes in PET/CT parameters over a one-year period. Significant 12-month changes in articular TBR-SYN mean and SUV-SYN mean were determined by the combination of treatment and higher baseline RANKL levels ($p<0.05$) (Table 10). Additionally, therapy in conjunction with elevated level of ESR or lower values of DXA L2-4 BMD were linked to more significant changes in TBR-VASC mean and TBR-VASC max over one-year period ($p<0.05$) (Table 10).

Table 10. Results of RM-ANOVA analysis determining the effects of tofacitinib therapy and other independent variables on 12 months changes in PET/CT parameters as dependent variables**.**

Dependent variable	Effect	F	p	Partial η^2
Articular $SUV_{meansyn}$ 0-12	*Treatment * RANKL-0*	4.619	0.046	0.214
Articular $SUV_{meansyn/liv}$ 0-12	*Treatment * RANKL-0*	11.777	0.002	0.409
Aortic $TBR_{meanvasc}$ 0-12	*Treatment * ESR-0*	9.899	0.006	0.368
	*Treatment * DXAL24BMD-0 (inv)*	5.485	0.032	0.244
	*Treatment * DXAL24TSC-0 (inv)*	4.726	0.044	0.218
Aortic $TBR_{maxvasc}$ 0-12	*Treatment * ESR-0*	7.535	0.014	0.307
	*Tretament * DXAL24BMD-0 (inv)*	4.826	0.042	0.221

5. Discussion

Effects of tofacitinib on bone metabolism in RA

Cardiovascular diseases and bone loss are major comorbidities in RA. Patients with RA are more prone to generalized osteoporosis and localized bone resorption [2, 81, 118, 119]. Various studies have shown that biological therapies may decrease the incidence of osteoporosis and periarticular erosions, which have both been linked to RA [203]. Studies have demonstrated that in RA, TNF-α inhibitors can raise serum levels of OC and P1NP, while reducing levels of CTX-I and RANKL, resulting in a favorable balance of bone remodeling [81]. Anti-TNF treatment was found to enhance the P1NP/CTX, OPG/RANKL, OC/CTX ratios, and reduce levels of DKK-1, resulting in increased bone formation [154, 204-206]. IL-6 inhibitor therapies significantly decreased levels of CTX-I, increased levels of P1NP and reduced the CTX-I/OC ratio [207, 208]. IL-6 inhibition has been observed to decrease DKK-1 levels and increase OPG/RANKL ratio, which suggests an improvement in bone remodeling [209]. In a prospective study rituximab therapy decreased synovial RANKL expression and increased serum OPG/RANKL ratio [210]. Several studies have also found that co-stimulation blockade inhibited osteoclast differentiation and maturation in murine models. In addition, it has been reported that abatacept inhibited RANKL and TNF-mediated osteoclastogenesis in vitro in a dose-dependent manner, even in the absence of T cells [211]. Improvement in bone biomarkers by biologics has been associated with decreased inflammatory markers, including CRP, and improvement of disease activity in RA [81]. TNF-α inhibitor therapy arrested generalized bone loss and improved or preserved BMD was found [212-214]. It was showed that RA was linked to low rate of hand BMD, and following TNF inhibitor treatment hand BMD remained stable over time [215]. Another study showed that TNF-α inhibitors in patients with RA or PsA effectively prevented bone loss at femoral neck and lumbar spine, as assessed by DXA, in the entire group [216]. IL-6 inhibitors also reduced radiological progression and bone loss in RA [217, 218]. The SAMURAI study's sub-analysis demonstrated that tocilizumab was more efficient in reducing structural joint damage progression in high-risk than in low-risk patients [219]. It has been reported that rituximab treatment improved BMD in RA patients [220]. JAK inhibitors, such as tofacitinib, have proven to be as effective and safe as biologics [59, 74-76]. Moreover, clinical trials have demonstrated that tofacitinib effectively inhibits

localized bone loss and reduces radiographic progression [82]. The potential of tofacitinib to inhibit joint damage was observed even when persistent inflammation was present [221]. JAK inhibition decreased RANKL expression and bone resorption in murine models, inhibited osteoclast differentiation, promoted osteoblast activity and stabilized Wnt-dependent bone formation [79, 222-224]. Baricitinib also reported to inhibit RANKL-mediated osteoclast activity [225]. Yokota et al. demonstrated that that JAK inhibitors reduced osteoclast formation induced by IL-6 and TNF-α. Additionally, their findings indicated that IL-6 and TNF-α-induced osteoclasts may differentiate through pathways, which are not dependent on RANKL [223].

Limited research has been conducted on the effects of tofacitinib on bone metabolism in arthritis. However, our study has shown that tofacitinib prevented the advancement of osteoporosis, as neither the areal nor the volumetric BMD changed over time. Additionally, there were improvements in clinical outcomes and a reduction in systemic inflammation with both doses. The measurement of areal and volumetric BMD was conducted through DEXA and QCT, which can evaluate trabecular and cortical bone loss and determine volumetric BMD in RA [195]. Unfortunately, we were unable to compare our BMD results with any other findings regarding JAK inhibition, as there have been no previous prospective investigations on the impact of tofacitinib on BMD alterations. The degree of areal bone loss at various sites varied from 0.2% to 1.5% in our study, after treatment with tofacitinib, in addition some sites even showed an increase in BMD after one year of therapy. However, annual bone loss determined by BMD in patients with RA was estimated between 2.5% and 3.9% [226]. In a recent study, long-term bDMARDs/tsDMARDs treatment of RA patients was found to have a protective effect on bone loss, thus BMD remained stable, while individuals on conventional therapy suffered significant bone loss. Unfortunately, this trial did not differentiate between the outcomes of patients treated with tofacitinib and those treated with biologics [227].

We have found that tofacitinib treatment enhanced bone formation markers, including OPG, OC and 25OHVITD3 levels, while decreased markers of bone resorption, such as CTX levels, leading to a positive balance of bone turnover. We have observed that the 10 mg dosage twice a day led to an elevation of levels of OPG and reduction in CTX levels, whereas these changes were not found in the 5 mg bid subset. Overall, we have found favourable changes in the P1NP/CTX and OC/CTX ratios, which suggest an improvement in bone remodeling balance. Tofacitinib has been reported to dampen the synthesis of RANKL and

increase the OPG/RANKL ratio in other trials, as well. Additionally, it has been shown to stabilize the anabolic Wnt proteins β-catenin and OC [79, 222, 224]. In a small study involving RA patients, tofacitinib treatment resulted in reduction of RANKL levels and the RANKL/OPG ratio, but there were no significant differences in the levels of OPG [228]. In murine models, it was observed that tofacitinib treatment increased both bone cortical and trabecular hardness, enhanced OPG/RANKL ratio and reduced the expression of RANKL. However, despite these effects, tofacitinib was unable to reverse the impact of inflammation on the trabecular and cortical bone structure, as well as on the mechanical properties in mice [222]. Bone formation may be promoted by tofacitinib through recruiting human mesenchymal stromal cells, inducing osteogenic differentiation and reducing osteoclast activity [229]. We have also found elevated 25OHVITD3 levels in response to tofacitinib treatment, which may be related to the ordinary improvement in functional capacity and physical activity of patients.

We found significant associations in correlation analyses between volumetric and areal BMD at baseline and after 12 months of tofacitinib therapy, and various bone turnover markers, including CTX, P1NP, RANKL, OC and PTH. DXA-measured areal BMD was negatively associated with CTX, P1NP, RANKL and OC, whereas QCT-measured volumetric BMD showed a negative association with RANKL and PT, but no significant correlations were observed with other biomarkers. The relationships between bone biomarkers including P1NP, CTX, OC, RANKL, and volumetric and areal BMD were supported by both univariable and multivariable regression models, indicating these markers may play a significant role in defining BMD measurements. Moreover, some baseline bone markers showed correlations with QCT and DXA BMD measurements after 12 months, suggesting their potential as predictors of BMD changes over the course of 12 months. In addition to bone biomarkers, CRP, DAS28, and age were found to be associated with BMD in both univariable and multivariable analyses. Age was found to be significantly associated with femoral neck BMD, but not lumbar spine BMD. Furthermore, age at baseline was identified as a predictor of femoral neck BMD after 12 months. CRP and DAS28 were found to be inversely linked with volumetric BMD, suggesting that tofacitinib treatment may be a primary factor in improving RA bone status. The RM-ANOVA analysis was utilized to assess the combined effects of tofacitinib therapy and other biomarkers on changes in BMD. The results of our study revealed that the effects of tofacitinib treatment on BMD changes over a one-year period were influenced by various factors. The combination of tofacitinib treatment with lower levels of DKK1 or anti-CCP antibody predicted

changes in DXA L2-4 vertebral BMD. On the other hand, the combination of therapy with lower age or levels of CRP predicted changes in QCT cortical BMD. These findings suggest that autoimmunity, age, as well as bone and inflammatory markers, may all play a role in modulating the effects of tofacitinib on BMD changes. We have found no significant differences between the subsets of patients receiving 5 mg or 10 mg of tofacitinib in relation to bone biomarker alterations or BMD. Due to potential safety concerns associated with the 10 mg bid dose of tofacitinib, it is not approved for the treatment of rheumatoid arthritis in the European Union. Therefore, the 5 mg bid dose of tofacitinib may be recommended as a suitable option for maintaining bone status in RA patients.

Effects of tofacitinib on the vasculature and joints in RA

RA is associated with an elevated risk of ischemic stroke, subclinical atherosclerosis, myocardial infarction, coronary calcification, arrhythmias and metabolic changes [98, 161, 230-233]. For many years, the risk of CVD in autoimmune conditions has been underestimated, although RA patients have almost two times greater risk of developing CVD compared to diabetes mellitus [234, 235]. Studies have shown that higher TNF-α and IL-6 levels are linked to an elevated risk of heart failure [96], and targeted therapies might have positive effects on cardiovascular outcomes and metabolism [161, 230, 236-238]. TNF-α inhibitors have been showed to decrease CV risk in patients with RA. They have showed improvement in dyslipidemia, insulin resistance, platelet activation, level of NT-proBNP, moreover, infliximab had an atheroprotective effect in monocytes [239-248]. In a study, tocilizumab therapy improved the pro-atherothrombotic profile of patients with RA by regulating inflammation, NETosis and modulating dyslipidemia and endothelial dysfunction [249]. The cardiovascular safety of TNF-α inhibitors and tocilizumab was found to be similar in the ENTRACTE trial [250]. The IL-1 receptor antagonist anakinra has shown positive effects on vascular and left ventricular function, peak aerobic capacity, and insulin resistance in RA patients with heart failure [251-253]. In addition, studies have suggested that bDMARDs may prevent the development of periarticular erosions and osteoporosis, and affect bone turnover in RA [59, 81, 119, 145, 204-206, 216]. Inhibition of TNF-α has been shown to lead to a decrease in bone resorption and an increase in bone formation [145].

Due to limited evidence on the effects of JAK inhibition on bone loss and CVD in RA, we conducted a one-year prospective study. The aim was to evaluate vascular and synovial inflammation using ^{18}F-FDG-PET/CT imaging, and to assess bone status and biomarkers in RA patients undergoing either 5mg or 10mg bid tofacitinib therapy. Our study was the first to simultaneously measure vascular and synovial inflammation in patients with RA receiving JAK inhibitor treatment. Our findings indicated that tofacitinib therapy effectively reduced inflammation (ESR, CRP) and disease activity. Additionally, it was found to improve quality of life based on the assessment of HAQ. Previously, FDG-PET/CT has been shown to be a valuable method for assessing disease activity in patients with RA receiving anti-inflammatory treatment [170]. Beckers et al. found significant associations between FDG uptake, disease activity and levels of CRP in RA [254]. Another study demonstrated the utility of ^{18}FDG-PET/CT in precisely and sensitively detecting presence of inflammation in the large joints of patients with RA. It may also provide an early assessment of the overall involvement of RA in the body [171]. Previous studies have reported that baseline SUVmax is associated with future damage of large joints [185]. Additionally, some researchers monitored changes in synovial inflammation, clinical effectiveness and prognosis in RA patients treated with csDMARD [182] or bDMARDs [183-187]. TBR assessment was effective in determining vessel wall inflammation and plaque composition in atherosclerosis. An increased inflammation of arterial wall was found in RA, moreover FDG uptake of arterial wall was associated with inflammatory markers, such as ESR, CRP and disease activity [172, 173, 175, 176]. Our study showed that JAK inhibition led to a significant reduction in mean synovial inflammation (SUV-SYN mean and TBR-SYN mean) and maximal aortic inflammation (TBR-VASC max) in five specific articular and aortic locations. However, no correlations were found between aortic TBR and articular SUV values when vascular and synovial inflammation were simultaneously evaluated using PET/CT. A prior small study revealed that ^{18}FDG-PET/CT could identify inflammation in the skin as well as subclinical vascular and synovial inflammation in patients with psoriasis [167]. Emami et al. performed a cross-sectional study and reported elevated arterial FDG uptake in patients with RA. They found an association between arterial and synovial FDG uptake, but no correlation was observed between levels of CRP and arterial or synovial FDG uptake [178]. However, in another study sacroiliitis was associated with higher arterial inflammation [179]. In our study, we observed significant associations between PET/CT parameters related to the joint or the aorta and inflammatory markers, including ESR, CRP and DAS28. While no significant

associations were found between aortic and synovial ^{18}FDG-PET/CT parameters, our study revealed correlations between vascular pathology assessed by ultrasound and PET/CT parameters. Specifically, PET/CT-measured synovial inflammation showed positive correlations with IMT and PWV. Furthermore, ESR and disease activity demonstrated variable correlations with aortic inflammation.

The MANOVA analysis revealed that acute phase reactants and disease activity determined both FMD or PWV and synovial inflammation. Additionally, PWV was found to be correlated with aortic inflammation. This suggests that systemic inflammation could contribute to synovial and vascular inflammation, and vascular pathophysiology. In fact, in RA, disease activity, CRP, and ESR are all major factors that contribute to vascular pathology [161, 162, 255]. Impaired vascular responsiveness has been reported in 86% of RA patients [256]. It has been showed that biologics may reduce the progression of altered IMT, PWV and FMD in rheumatoid arthritis [236]. Adalimumab, etanercept and infliximab therapies have demonstrated improvement in FMD, while rituximab, anakinra and tocilizumab therapies have shown to enhance endothelial function in RA patients [241].

Vascular and synovial inflammation detected by PET/CT were associated with BMD and bone biomarkers, as well as disease activity, vascular pathophysiology and systemic inflammation. In addition, baseline synovial SUVmax greater than 1.65 was identified as a predictive factor for the progression of joint destruction [185]. In our study, we observed that PET/CT-detected synovial and vascular inflammation is correlated not only with localized bone resorption, but also with generalized osteoporosis. The study revealed a correlation between synovial inflammation and RANKL and CTX, which are markers of bone resorption. Additionally, correlation was found between aortic inflammation and bone formation markers, such as OC and P1NP. Furthermore, we found that JAK inhibition, along with higher baseline levels of RANKL, led to changes in TBR-SYN mean and SUV-SYN mean in the RM-ANOVA analysis. Both aortic TBR and synovial SUV/TBR values were negatively correlated to lumbar spine BMD. As a result, systemic inflammation, as well as synovial and vascular inflammation, may contribute to bone loss in RA [161, 255, 257, 258]. There may be an association between atherosclerosis and bone loss, which is exacerbated by arthritis [257].

JAK inhibition has been linked to increased lipid levels, including mean HDL-C and LDL-C levels. Additionally, those who responded to treatment had higher LDL-C and HDL-C levels

than non-responders, and these changes were associated with lower levels of CRP [259]. However, these lipid changes did not have an impact on the atherogenic index or result in any cardiovascular consequences [161, 259]. In RA patients baricitinib caused similar alterations in HDL-C and LDL-C levels, and decreased level of Lp(a) was also found [260, 261]. In this study, the relationship between lipid levels (HDL-C, LDL-C, total cholesterol, and triglycerides) and PET/CT parameters was examined, but no significant association was found between lipids and synovial or aortic inflammation. However, a strong association was observed between Lp(a) and FDG uptake in the synovium, while no association was found in the aortic wall. Previously, Lp(a) has been associated with both rheumatoid arthritis and cardiovascular disease [165, 262-264]. We have previously discovered a link between Lp(a) and CRP [165], and biologics were also reported to decrease the synthesis of Lp(a) in RA patients [263, 264]. The mechanism behind the reduction of atherosclerosis caused by tofacitinib remains uncertain, and it is unclear whether the improvement is related to lipid metabolism [265]. In vitro studies have linked tofacitinib to lipid release from macrophages via reverse cholesterol transport and tofacitinib also reduced atherosclerosis and foam cell development in an animal model of atherosclerosis by increasing the expression of ABCA1, a critical molecule for efflux of HDL cholesterol, which could explain the lipid profile changes related to JAK inhibition therapy [266, 267]. It has also been found that tofacitinib improved HDL-C and LDL-C levels by reducing cholesterol ester catabolism [268]. The cardiovascular safety of tofacitinib was found to be comparable to that of TNF-α inhibitors [250, 269].

6. Summary

In summary, the treatment with tofacitinib effectively reduced both synovial and vascular inflammation simultaneously as determined by ^{18}F-FDG-PET/CT and attenuated the further development of bone loss in RA. Tofacitinib in both doses significantly decreased disease activity, improved clinical outcomes and decreased systemic inflammation. We have found that age, CTX, and OC were independent predictors of areal BMD, while CRP and RANKL were independent predictors of volumetric BMD. CRP, DKK-1, age and ACPA influenced the effects of tofacitinib therapy on BMD changes. Our findings indicate that CRP, IMT, PWV, CTX, RANKL, and Lp(a) could be considered as individual predictors of synovial inflammation. Additionally, HAQ, ESR, PWV, DAS28, OC and P1NP determined aortic FDG uptake. It appears that disease activity and systemic inflammation may play a role in influencing both vascular pathophysiology and synovial inflammation. ^{18}F-FDG-PET/CT may be suitable method for simultaneous assessment of vascular and synovial inflammation, as well as monitoring the effects of anti-rheumatic and other therapies on tissue inflammation.

To the best of our knowledge, this is the first prospective study conducted over one year to examine the effects of tofacitinib therapy on bone health and vascular pathophysiology in RA, in conjunction with disease activity, bone turnover markers, and inflammation. In addition, this may be the first study to evaluate the impact of 12 months of tofacitinib therapy on both aortic and synovial inflammation, as determined by ^{18}F-FDG-PET/CT. Our study contributes to the understanding of the effects of JAK inhibition in RA, but further research is needed to investigate the potential positive effects of tofacitinib and other JAK inhibitors on vascular and joint inflammation in RA.

Összefoglalás

Összefoglalásként a tofacitinib kezelés hatékonyan csökkentette a ^{18}F-FDG-PET/CT által mért szinoviális és vascularis gyulladást, valamint csökkentette a további csontvesztést RA-ban. A tofacitinib mindkét dózisban jelentősen csökkentette a betegség aktivitását, javította a klinikai tüneteket és mérsékelte a szisztémás gyulladást. Azt találtuk, hogy az életkor, a CTX és az OC független prediktorai voltak az areális BMD-nek, míg a CRP és a RANKL a volumetrikus BMD-nek. A CRP, a DKK-1, az életkor és az ACPA befolyásolta a tofacitinib terápia BMD változásra gyakorolt hatását. Eredményeink azt mutatják, hogy a CRP, az IMT, a PWV, a CTX, a RANKL és a Lp(a) a synovialis gyulladás egyéni prediktorainak tekinthetők. Emellett a HAQ, az ESR, a PWV, a DAS28, az OC és a P1NP meghatározták az aorta FDG-felvételét. Úgy tűnik, hogy a betegség aktivitása és a szisztémás gyulladás szerepet játszhat mind az érrendszeri patofiziológia, mind a synovialis gyulladás befolyásolásában. A ^{18}F-FDG-PET/CT pedig alkalmas módszer lehet az érrendszeri és a synovialis gyulladás egyidejű vizsgálatára, valamint az antireumatikus és egyéb terápiák szöveti gyulladásra gyakorolt hatásainak nyomon követésére.

Tudomásunk szerint ez az első olyan prospektív vizsgálat, amely egy éven keresztül vizsgálta a tofacitinib terápia csont- és érrendszerre gyakorolt hatásait RA-ban, a betegség aktivitásával, a csontanyagcsere markereivel és a gyulladással összefüggésben. Továbbá ez lehet az első olyan vizsgálat, amely 12 hónapon át vizsgálta ^{18}F-FDG-PET/CT segítségével a tofacitinib aortafali és ízületi gyulladásra gyakorolt hatásait. Tanulmányunk hozzájárul ahhoz, hogy jobban megértsük a JAK-gátlók használatának következményeit rheumatoid arthritisben, azonban további kutatásokra van szükség a tofacitinib és más JAK-inhibitorok vascularis és synovialis gyulladásra gyakorolt lehetséges egyéb pozitív hatásainak pontosabb feltérképezéséhez.

7. References

7.1. References

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7.2. List of publications

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1. **Hamar, A. B.**, Szekanecz, Z., Karancsiné Pusztai, A., Czókolyová, M., Végh, E., Pethő, Z., Bodnár, N., Gulyás, K., Horváth, Á., Soós, B., Bodoki, L., Bhattoa, H. P., Nagy, G., Tajti, G., Panyi, G., Szekanecz, É., Domján, A., Hódosi, K., Szántó, S., Szűcs, G., Szamosi, S.: Effects of one-year tofacitinib therapy on bone metabolism in rheumatoid arthritis.
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8. Abbrevations and keywords

Keywords: rheumatoid arthritis, JAK inhibition, tofacitinib, atherosclerosis, bone loss, PET-CT, inflammation, BMD, osteoporosis, cardiovascular risk, carotid ultrasound

The following table describes the significance of various abbreviations and acronyms used throughout the thesis.

^{18}F-FDG-PET/CT	^{18}F-fluorodeoxyglucose-positron emission tomography/computed tomography
25OHVITD3	25-hydroxy-vitamin D3
ABA	abatacept
ACP	antigen-presenting cell
ACPA	anti-citrullinated protein antibody
ACR	American College of Rheumatology
ADMA	asymmetric dimethylarginine
anti-CarP	anti-carbamylated proteins antibody
anti-CCP	anti-cyclic citrullinated peptide
ApoA	apoprotein A
ApoB	apoprotein B
AS	ankylosing spondylitis
bDMARD	biologic disease-modifying antirheumatic drug
BMD	bone mineral density
CATHK	cathepsin K
ccIMT	carotid artery intima-media thickness
CPK	creatine phosphokinase
CRP	C-reactive protein
CTX	C-terminal collagen crosslinks
CV	cardiovascular
DAS28	disease activity score 28
DKK1	Dickkopf-related protein 1
DMARD	disease-modifying antirheumatic drug
DXA	dual-energy X-ray absorptiometry
DXAFNBMD	femoral neck areal BMD determined by DXA
DXAL24BMD	L2–4 vertebral areal BMD determined by DXA
EPC	endothelial progenitor cell
ESR	erythrocyte sedimentation rate
EULAR	European Alliance of Associations for Rheumatology
FDA	U.S. Food and Drud Administration
FLS	fibroblast-like synoviocyte

FMD	flow-mediated dilatation
GC	glucocorticoid
HAQ	health assessment questionnaire
HDL-C	high-density lipoprotein cholesterol
HLA	human leukocyte antigen
IL	interleukin
JAK	Janus kinase
LDL-C	low-density lipoprotein cholesterol
Lp(a)	lipoprotein a
LRP	low-density lipoprotein receptor-related protein
MANOVA	multivariate analysis of variance
MCP	metacarpophalangeal
M-CSF	macrophage colony-stimulating factor
MHC	major hystocompatibility complex
MMP	matrix metalloproteinase
MTP	metatarsophalangeal
MTX	methotrexate
MVP	metabolic volumetric product
NET	neutrophil extracellular trap
NSAID	non-steroidal anti-inflammatory drug
OC	osteocalcin
OPG	osteoprotegerin
P1NP	procollagen 1 N-terminal propeptide
PAD	peptidylarginine deiminase
PIP	proximal interphalangeal
PON	paraoxonase
PsA	psoriatic arthritis
PWV	pulse wave velocity
QCT	quantitative computed tomography
QCTCORTBMD	cortical volumetric BMD determined by QCT
QCTTOTBMD	total volumetric BMD determined by QCT
QCTTRABBMD	trabecular volumetric BMD determined by QCT
RA	rheumatoid arthritis
RANK	Receptor Activator Nuclear Factor κB
RANKL	Receptor Activator Nuclear Factor κB ligand
RF	rheumatoid factor
RM-ANOVA	repeated measures analysis of variance
ROI	region of interest
RTX	rituximab
SOCS	suppressors of cytokine signaling
SOST	sclerostin
STAT	signal transducer and activator of transcription
SUV	standardized uptake value
SYK	spleen tyrosine kinase

T2T	treat-to-target
TBR	target-to-background ratio
TC	total cholesterol
TOC	tocilizumab
TNFi	TNF-α inhibitor
TNF-α	tumor necrosis factor α
tsDMARD	targeted synthetic disease-modifying antirheumatic drug
UTI	urinary tract infection
VOI	volume of interest

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