INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by cartilage and bone destruction, infiltration of lymphocytes into synovial tissue, hyperproliferation of synovial fibroblasts, which produce matrix-degrading enzymes and proinflammatory cytokines. Synovial fibroblasts are important propagator of RA. They migrate to distant sites and may thereby spread arthritis to unaffected joints ***(Lowin et al., 2015).***

The prevalence of RA in Western countries is in the range of 0.5% to 1%.There are regional and ethnic variations in the prevalence of RA. A high prevalence of 5–6% has been reported in Native American populations, while low prevalence was in people from China and Japan (0.2%-0.3%). These findings reflect the fact that genetic and environmental factors contribute to RA ***(Gibofsky, 2012; Smolen et al., 2018).***

Mechanisms of synovium inflammation are still unclear and often lead to progressive joint destruction and deformation. Cells of the adaptive immunity pathway were thought to be the principal actors of RA pathogenesis. However, an increasing body of evidence suggests that cells of the innate immunity and resident articular cells are involved in RA pathogenesis ***(Baillet et al., 2010).***

Calprotectin is a member of the S100 protein family. It is a heterocomplex of myeloid-related proteins (MRP-8/MRP-14). It is also known as S100A8/A9, the L1 protein

or calgranulin A and B ***(Kang et al., 2014).*** It is a calcium-binding cytosolic protein which presents in regenerative cells such as neutrophils, monocytes, macrophages, epithelial, and endothelial cells ***(Guo et al., 2016).***

It acts not only as a chemoattractant, but also as a pro-inflammatory factor that exerts cytokine-like activities. For example, it can bind to Toll like receptor 4 (TLR-4) or the advanced glycation end product receptor to activate the intracellular signaling pathways including the mitogen-activated protein kinase and nuclear factor-kappa B (NF-κB) pathways ***(Zheng et al., 2014).***

It is one of the damage-associated molecular pattern molecules (DAMPs) highly up-regulated in various autoimmune disorders such as juvenile idiopathic arthritis (JIA), reactive arthritis, acute gouty arthritis and systemic lupus erythematosis (SLE) ***(Hammer et al., 2011; Guo et al., 2016).***

Synovial fluid proteomic analysis revealed that proteins of calprotectin heterocomplex (S100A8 & S100A9) were the most abundant proteins in RA synovial fluid and significantly higher in RA than osteoarthritis (OA) and other inflammatory arthritides such as ankylosing spondylitis, SLE and pseudogout ***(Baillet et al., 2010).*** Calprotectin has a molecular weight of only 36.5 kDa and may diffuse from inflamed joints into the blood circulation, where it can be measured in serum. A significant correlation was found between calprotectin levels in serum and synovial fluid ***(Kang et al., 2014).***

It is a sensitive biomarker of RA disease activity. It was found that serum calprotectin levels stratified disease activity in the categories defined by the respective values of the disease activity scale-28 (DAS28), simple disease activity index (SDAI), and clinical disease activity index (CDAI) composite indices (remission, low, moderate, and high activity) because calprotectin is a proinflammatory biomarker released by activated phagocytes such as monocytes and granulocytes and directly reflects inflammation in the synovium and synovial fluid ***(García-Arias et al., 2013; Inciarto-Mundo et al., 2016).***

Anti-TNF-α therapy has been demonstrated to reduce both the number of infiltrating calprotectin expressing macrophages in synovial tissue and serum calprotectin levels. These findings suggest that calprotectin can monitor the treatment outcome in RA patients ***(Angel et al., 2012; Nordal et al., 2017).***

In the last 15 years musculoskeletal ultrasound (MSUS) has gained an increasingly important role in the evaluation and treatment monitoring of RA. B-mode or grey scale (GS) scanning allows direct visualizing of the morphology and quantity (hypertrophy) of the synovial tissue. Doppler techniques such as power Doppler (PD), color Doppler and spectral Doppler identify in real time the increased synovial micro-vascular blood flow. These parameters correlate with the level of disease activity ***(Micu and Fodor, 2015)***.

Clinical examination might lack sensitivity in patients with mild synovitis, be limited in patients with established deformities, and be overestimated in patients with concomitant fibromyalgia or other comorbidities. Therefore, identifying novel, sensitive serum biomarkers of RA disease activity remains challenging. Numerous studies have shown that the sensitivity of ultrasound examination is superior to that of clinical examination, including the determination of subclinical synovitis ***(Hurnakova et al., 2015).***

Calprotectin is associated with joint damage and is an indicator of radiographic progression in patients with RA. Circulating calprotectin levels elevate with disease activity and decrease after successful treatment. Several studies suggested that serum level of calprotectin is significantly associated with US examination of disease activity in RA patients ***(Hammer et al., 2010; Hurnakova et al., 2017; Nordal et al., 2017).***

Aim of the Work

This cross sectional study was proposed to study the role of calprotectin in rheumatoid arthritis by measuring its serum level in RA patients and evaluating its use as a marker of disease activity and severity assessed clinically, radiologically and by musculoskeletal ultrasound.

CALPROTECTIN

Calprotectin was first described in 1980 ***(Fagerhol et al., 1980).*** It was named calprotectin because it is a calcium binding protein with anti-microbial properties ***(Chatzikonstantinou et al., 2016).*** Calprotectin is also referred as S100A8/S100A9, MRP8/MRP14, calgranulin A and calgranulin B, L1 antigen and cystic fibrosis antigen ***(Dhas et al., 2012).***

Physical structure

Calprotectin is a calcium (Ca2+), zinc (Zn2+) and manganese (Mn2+) binding heterocomplex molecule. Its molecular weight is 36.5 kDa with 2 heavy and 1 light non-covalently linked chains ***(Herrera et al., 2016).*** It consists of S100A8 and S100A9 subunits, which are 8.3 kDa and 13.3 kDa respectively. S100A8 and S100A9 are composed of 93 and 114 amino acids respectively.S100A8 constitutes the lighter chain of calprotectin and is designated as L1L and S100A9 as L1H which constitutes the heavy chain. S100A8 is the active subunit of S100A8/S100A9 and S100A9 acts as the regulatory subunit preventing early degradation of S100A8 ***(Dhas et al., 2012, Zheng et al., 2014).*** Human S100A8 and S100A9 form heterodimers and even higher oligomeric forms. A tetramer of S100A8 andS100A9 was described (Figure 1). Tetramer formation was strictly

dependent on the presence of calcium and in the absence of calcium the heterodimer is the preferred form of human S100A8and S100A9 ***(Ehrchen et al., 2009).***

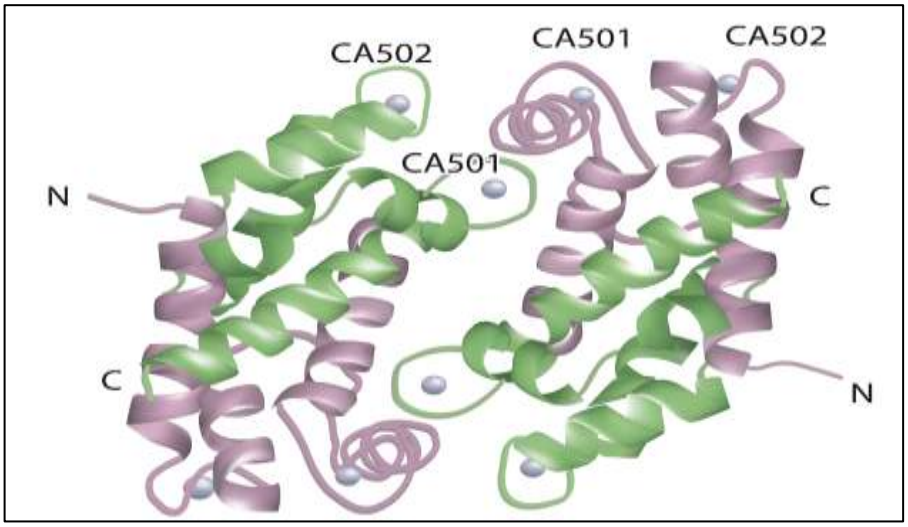

Figure (1): The heterotetramer structure of calprotectin.S100A8 chains (upper left); S100A9 chains (upper right); CA501 and CA502 sites where Ca2+ ions (spheres) bind. C and N denote protein termini ***(Herrera et al., 2016).***

Secretion and release of calprotectin

Calprotectin is primarily expressed in innate immune cells particularly in neutrophils constituting approximately 40%-60% of the cytosolic proteins in these cells ***(Cesaro et al., 2012).*** It is also found in monocytes, keratinocytes, muscle tissue and infiltrating tissue macrophages ***(Dhas et al., 2012).*** It can be induced from endothelial and epithelial cells ***(Chen et al., 2015).*** It is found in body fluids, including serum, urine, seminal fluid, plasma, saliva, sputum, cerebrospinal fluid and in feces and abscess fluid ***(Donato et al., 2013).***

Calprotectin release from monocytes can be induced by granulocyte monocyte colony stimulating factor (GM-CSF), tumor necrotic factor- alpha (TNFα), interluckin-1β (IL-1β) and lipopolysaccharides (LPS). Likewise, calprotectin was shown to be secreted by neutrophils stimulated with LPS, TNFα, and IL-1β ***(Tardif et al., 2015).*** Chemoattractants such as complement5-a (C5a) cause its rapid release from neutrophils ***(Goyette and Geczy, 2011).***

Activation of protein kinase C by proinflammatory stimuli and elevation of intracellular Ca2+ following contact with activated endothelium, collagen, or fibronectin can also stimulate calprotectin release from phagocytes ***(Goyette and Geczy, 2011).***

Mechanism of secretion

Calprotectin is not secreted via the classical Golgi-associated pathway. It is released during inflammatory events by two mechanisms, either after cell death or via an active non classical secretary mechanism (alternative pathway) ***(Zheng et al., 2014).*** However, there is good evidence that active non classical secretion is the major physiological source for extracellular calprotectin ***(Ehrchen et al., 2009).***

The alternative secretory pathway is an energy dependent process and it is dependent on a functional

microtubule network ***(Ehrchen et al., 2009).*** Activation of neutrophils leads to the translocation of calprotectin heterodimers and homodimers (S100A8 and S100A9) from the cytosol to the cytoskeleton and membrane. Secretion of calprotectin is dependent on Reactive Oxygen Species (ROS) production and K^+ fluxes from ATP-sensitive K^+ channels. The majority of secreted calprotectin is found in soluble form, but some is associated with large vesicles ***(Tardif et al., 2015).***

Another mechanism of secretion is through neutrophil necrosis. This might contribute significantly to the high concentrations found in acute inflammatory lesions or chronic conditions where neutrophil infiltration is significant, such as cystic fibrosis and rheumatoid arthritis ***(Tardif et al., 2015).***

Mechanism of action

Receptors of calprotectin

Calprotectin is one of the Damage Associated Molecular Patterns (DAMPs). It binds to diverse cell-surface proteins. It binds to Toll like receptor 4 (TLR-4) ***(Fassl et al., 2015).*** It also binds to CD36 and the receptor for advanced glycation end products (RAGE) ***(Wang et al., 2014).*** The soluble calprotectin binds to the cell surface of endothelial cells by interacting with specific binding sites

such as heparin sulfate proteoglycans and novel carboxylated glycans ***(Stephan and Nolan, 2016).***

Calprotectin binding to TLR-4 or RAGE leads to activation of intracellular signaling pathways including the mitogen-activated protein kinase (MAPK) and NF-κB pathways ***(Schiopu and Cotoi, 2013, Stephan and Nolan, 2016).*** Calprotectin binding to TLR4 triggers myeloid differentiation primary response protein 88(MyD88) mediated TLR-4 signaling. MyD88-mediated signaling occurs mainly at the plasma membrane and stimulates the recruitment and the activation by phosphorylation of IL-1R-associated kinases (IRAKs) leading to NF-kB activation and secretion of pro-inflammatory cytokines such as TNF and IL-17. Calprotectin-TLR-4 interaction has been shown to be involved in the pathogenesis of systemic infections, autoimmune diseases, malignancy, and acute coronary syndrome ***(Schiopu and Cotoi, 2013, Moltini et al., 2016).*** RAGE activation by calprotectin or other ligands leads to further enhancement of calprotectin production, creating a putative positive feedback loop in chronic inflammation ***(Schiopu and Cotoi, 2013).***

Functions of calprotectin

Calprotectin is involved in the pathophysiology of various pathological conditions such as rheumatic diseases,

inflammatory bowel diseases, cardiovascular diseases, cystic fibrosis, cancer and infections ***(Dahs et al., 2012).*** Calprotectin as one of the DAMPs, it acts as an amplifier of inflammation, autoimmunity, infection and cancer. It easily diffuses to extracellular fluids so it is a prominent biomarker in multiple diseases ***(Ehrchen et al., 2009; Dahs et al., 2012).***

i. Proinflammatory effect

The cytoplasmic calprotectin translocates to the membrane following phagocyte activation and promotes formation and stabilization of microtubules and enhances tubulin polymerization in neutrophils. Interactions with cytoskeletal components are calcium dependent process and are important for migration, degranulation, phagocytosis of activated monocytes and neutrophils ***(Donato et al., 2013).***

Calprotectin promotes neutrophil and monocyte recruitment by activating the microvascular endothelium and by stimulating phagocyte macrophage-1 antigen (Mac-1) expression, affinity and binding to intercellular adhesion molecule 1 (ICAM-1), fibronectin, and fibrinogen ***(Schiopu and Cotoi, 2013).***

Calprotectin in neutrophils binds fatty acids particularly polyunsaturated fatty acids, such as arachidonic

acid (AA) and contribute to their uptake and release. Zinc regulates this process. The arachidonic acid-calprotectin complex is internalized by nearby cells to generate eicosanoids, which contribute to initiation and regulation of inflammatory responses. Calprotectin also serves as a scaffold for Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and arachidonic acid binding. It also facilitates NADPH oxidase activation to increase reactive oxygen species (ROS) generation ***(Perera et al., 2010).***

Calprotectin binds heparin sulphate proteoglycans and carboxylated glycans on endothelial cells and triggers endothelial activation, characterized by enhanced production of inflammatory cytokines, chemokines, increased expression of adhesion molecules and increased platelet aggregation at the surface of the endothelium ***(Schiopu and Cotoi, 2013).*** It facilitates arachidonic acid uptake by endothelial cells ***(Stephan and Nolan, 2016).*** It also stabilizes and protects leukotrienes from non-enzymatic hydrolysis thus increasing the availability of bioactive leukotrienes ***(Donato et al., 2013).*** Extended calprotectin exposure leads to endothelial cell dysfunction and increased endothelial permeability. These effects are partly mediated by RAGE and exacerbated by hyperglycemia ***(Schiopu and Cotoi, 2013).***

Calprotectin promotes endothelial apoptosis via caspase-dependent and independent mechanisms ***(Donato et al., 2013).*** Additionally, endothelial cells treated with high concentrations of calprotectin were shown to down regulate antiapoptotic genes and genes responsible for the integrity of the endovascular monolayer ***(Schiopu and Cotoi, 2013).*** This facilitates entry of monocytes and lipids into the arterial wall ***(Donato et al., 2013).*** Low concentrations of calprotectin promote proliferation and tube formation of vascular endothelial cells (neovascularization). This finding provides a new explanation for neovascularization development in inflammation and tumor ***(Li et al., 2012).***

Calprotectin is a prominent biomarker in inflammatory diseases. Its correlation with inflammatory bowel disease (IBD) is extensively studied. IBD and irritable bowel syndrome share many symptoms. While irritable bowel syndrome is a functional bowel disorder for which no specific treatment is available, the range of effective therapies for IBD is evolving rapidly. Accurate diagnosis of IBD is therefore essential. Fecal and serum calprotectin are elevated in inflammatory bowel disease patients (IBD) ***(Caviglia et al., 2014).*** Fecal calprotectin has adequate sensitivity and specificity to identify patients with IBD from irritable bowel syndrome (IBS). Fecal calprotectin levels correlate with disease activity and response to treatment. A rising fecal calprotectin can predict an imminent clinical relapse of IBD allowing prompt initiation of treatment ***(Walsham and Sherwood, 2016).***

Several experimental and clinical studies demonstrated that calprotectin is a promising clinical biomarker in cardiovascular diseases (CVD). It correlates with the extent of subclinical carotid and coronary artery disease. In addition, it increases rapidly in plasma during myocardial ischemia. It was found that plasma calprotectin levels increase before the classical markers of myocardial injury such as troponin T or creatine kinase in myocardial infarction patients ***(Schiopu and Cotoi, 2013).*** A role of calprotectin in the pathophysiology of atherosclerosis is proposed because it was found abundant in foam cells in atherosclerotic lesions. In addition, it can promote endothelial cell activation and damage. This could facilitate entry of monocytes and lipids into the artery wall. It also influences cardiomyocyte contractility by causing RAGE-dependent decreases in Ca2+ flux ***(Donato et al., 2013).***

Diabetes mellitus, obesity, smoking and hyperlipidemia are traditional cardiovascular risk factors that have been associated with increased levels of plasma calprotectin. The correlation between calprotectin and diabetes mellitus is the most extensively studied. Several clinical data showed elevated plasma calprotectin levels in patients with type 2 diabetes or impaired glucose tolerance compared with non-diabetic controls. Hyperglycemia induces the production of reactive oxygen species (ROS) in endothelial cells leading to increase calprotectin secretion ***(Pedersen et al., 2014; Cotoi et al., 2013).***

In cystic fibrosis (CF), calprotectin was found in broncho-alveolar lavage fluid, sputum and serum of patients with CF. Reid et al reported that serum calprotectin is sensitive to change with treatment and predicts time of re-exacerbation. They also found that stable patients with higher serum calprotectin at baseline would exacerbate sooner and have more rapid lung function decline ***(Reid et al., 2015).***

ii. Apoptosis and tumourgenesis

Calprotectin induces apoptosis of numerous cell lines tumor cells including colon carcinoma cells, breast cancer cells and neuroblastoma cells, gastric cancer cells, HaCaT keratinocytes, EL-4 lymphoma cells, colon carcinoma cells, and normal cells like human dermal microvascular endothelial cell line (HMEC-1) cells ***(Kwon et al., 2013; Zheng et al., 2014).***

Apoptosis inducing activity of calprotectin involves inhibition of antiapoptotic genes and activation of p53, Bak, Bax, caspase-3 and caspase-9. This indicates that apoptotic activity of calprotectin is via activation of mitochondrial apoptotic pathway ***(Dhas et al., 2012).*** Calprotectin can also induce cell death by chelating nutrient zinc from extracellular fluid ***(Yousefi et al., 2007).***