DOI:10.14753/SE.2023.2780

Examination of osteoclasts in rheumatoid and psoriatic arthritis

Ph.D. thesis

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Budapest

2022

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LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
anti-CCP	anti-cyclic citrullinated peptide autoantibody
ATCC	American Type Culture Collection
AV	annexin V
BMM	bone marrow macrophage cell culture
BP	biological process
BSA	bovine serum albumin
CASPAR	Classification Criteria for Psoriatic Arthritis
CC	cellular component
CD	cluster of differentiation
CD14	cluster of differentiation 14
c-Fms	colony-stimulating factor 1 receptor
CNS	central nervous system
CRP	C-reactive protein
DAS	disease activity scores
DE	differential expression
DMARDs	disease modifying anti-rheumatoid drugs
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMA	European Medicines Agency
ER	endoplasmic reticulum
ESR	erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
EV	extracellular vesicle
FBS	fetal bovine serum
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
GMP	granulocyte-macrophage progenitor cell
GO	gene ontology

GSEA	Gene Set Enrichment Analysis
HPLC	high-performance liquid chromatography
HR	hazard ratio
IL	interleukin
ISEV	International Society for Extracellular Vesicles
JAK	Janus-associated kinase
JIA	juvenile idiopathic arthritis
lEV	large-sized extracellular vesicle
M-CSF	macrophage colony stimulating factor
mEV	medium-sized extracellular vesicle
MF	molecular function
MHC	major histocompatibility complex
MFI	mean fluorescence intensity
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NSAIDs	non-steroidal anti-inflammatory drugs
OCL	osteoclast
OPG	osteoprotegerin
PBMC	peripheral blood mononuclear cell
PBS	Phosphate-Buffered Saline
PCA	principal component analysis
PCR	polymerase chain reaction
PE	phycoerythrin
PFP	platelet-free plasma
PsA	psoriatic arthritis
RA	rheumatoid arthritis
RANK	receptor activator of nuclear factor κB
RANKL	RANK ligand
RF	rheumatoid factor
rh	recombinant human
RNA	ribonucleic acid
RVG	rabies virus glycoprotein

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SEC	size exclusion chromatography
sEV	small-sized extracellular vesicle
SLE	systemic lupus erythematosus
TCA cycle	tricarboxylic acid cycle
TFA	trifluoroacetic acid
TNF	tumor necrosis factor
TNFi	TNF-α inhibitors
TRAF	TNF receptor associated factor
TRAP	tartrate-resistant acid phosphatase
V-ATPase	vacuolar-type H^+-ATPase

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1. INTRODUCTION

1.1 Rheumatoid arthritis and psoriatic arthritis

Both rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are chronic, immune-mediated disorders associated with arthritis and the inflammation of joints. The prevalence of RA is around 0.5-1.0% in developed countries and is higher in women compared to men [1]. The prevalence of PsA ranges between 0.16-0.25% and affects 20-30% of patients with psoriasis [2,3].

RA and PsA have some similarities in immunopathogenesis and symptoms as well, although there are also differences between them. In both diseases innate and adaptive immune responses are also involved, however, activated T cells and macrophages play a key role in the pathophysiology of PsA [4], and T cells and B cells of RA [5].

In RA fibroblasts, leukocytes and endothelial cells are the characteristic cellular elements of inflammation by producing inflammatory mediators, angiogenetic factors and enzymes. B-cells secrete auntoantibodies (RF, anti-CCP), while the ratio of T-cells is changed, therefore the ratio of inflammatory cytokines produced by T-cells is also changed. As inflammation progresses, cartilage and bone tissue are also damaged because of proteolytic enzymes, activation of the complement system, autoantibodies and soluble inflammatory factors [6–8].

In the patogenesis of PsA macrophages, granulocytes and T-cells with altered function play a significant role. IL-23 is mostly produced by macrophages, NK-cells and T-cells, and as a result, Th17 cells produce IL-17. This cytokine with other synergistic cytokines can activate osteoclasts and synovial fibroblasts in the joint, triggers increased recycling of epidermal cells in the skin, and triggers cytokine production by keratinocytes [9].

The role of inflammatory cytokines is important in both diseases. IL-1, IL-6, and TNF-α (tumor necrosis factor) cytokines are the most significant cytokines in RA, and IL-17, IL-23, and TNF-α are in PsA [10]. IL-6 stimulates osteoclasts RANK-L

dependently [11], IL-17A inducates RANK-L and prostaglandin syntesis [12,13], IL-23 triggers the maturation of T-helper 17 cells, which stimulates osteoclastogenesis through IL-17 and RANK-L expression [14], while TNF-α increases RANK and c-Fms (colony-stimulating factor 1 receptor) expression, so it also has an osteoclastogenic effect [15].

Predisposing genetic factors are also important in both diseases. For example the presence of HLA-B27 and HLA-DR4 alleles increases the risk of PsA, while the presence of the HLA-DRB1 allele and single nucleotide polymorphism of the PTPN22 gene was identified to be associated with the susceptibility to RA [16,17].

Several co-morbidities are associated with immune-mediated inflammatory diseases, also with RA and PsA. In the case of some cardiovascular diseases, the hazard ratio (HR) was increased in RA (HR=1.39) and PsA (HR=1.24) as well [18]. The risk of some malignancies [19,20], non-alcoholic fatty liver disease (NAFLD) [21], depression, and anxiety co-morbidities is also increased in patients suffering from RA or PsA [22].

Disease-modifying anti-rheumatoid drugs (DMARDs), eg. methotrexate and leflunomide are effective first-line medications in the treatment of both RA and PsA [16], as well as non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids [23]. Biological therapeutical drugs that are targeting specific molecules of the inflammatory pathways are being used from the early 21st century in parenteral administration. TNF-α inhibitors (TNFi), like etanercept, infliximab, adalimumab, golimumab, and certolizumab-pegol drugs are used in both RA and PsA [24,25]. Ustekinumab [26], which targets IL-12/IL-23 cytokines, and secukinumab [27], which targets IL-17 cytokines are used in the treatment of PsA. An IL-1 receptor antagonist - anakinra [28] -, a T-cell activation inhibitor - abatacept [29] -, an inhibitor of CD20, which is expressed by B-cells - rituximab [30] -, and an IL-6 receptor inhibitor - tocilizumab [31] - are biological therapeutical drugs approved for the treatment of RA. Small molecule non-biological agents, which target intracellular signaling pathways, and are administered orally are the newest drugs in this field, and being used since last decade. The specific inhibitor of Janus-associated kinases (JAK) 1 and 3, tofacitinib was first approved for the treatment of moderate-to-severe RA, then the indications were expanded to PsA and some other immune-mediated diseases also [32]. Apremilast, which inhibits the PDE-4 enzyme is used in the treatment of PsA [33] (**Table 1**).

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Table 1 Some therapeutical agents, which are approved and used for the treatment of RA and/or PsA. (Own figure)

<table>
<tr><th>Rheumatoid arthritis</th><th>Psoriatic arthritis</th></tr>
<tr><td colspan="2">DMARDs</td></tr>
<tr><td colspan="2">methotrexate, leflunomide</td></tr>
<tr><td colspan="2">NSAIDs, corticosteroids</td></tr>
<tr><td colspan="2">Biological therapy</td></tr>
<tr><td colspan="2">TNF-α inhibitors; etanercept, infliximab, adalimumab, golimumab, certolizumab-pegol</td></tr>
<tr><td>IL-1 R inhibitor; anakinra</td><td rowspan="2">IL-12/IL-23p40 inhibitor; ustekinumab</td></tr>
<tr><td>T-cell activation inhibitor; abatacept</td></tr>
<tr><td>CD20 inhibitor; rituximab</td><td rowspan="2">IL-17A inhibitor; secukinumab</td></tr>
<tr><td>IL-6 R inhibitor; tocilizumab</td></tr>
<tr><td colspan="2">Small molecule non-biological agents</td></tr>
<tr><td colspan="2">tofacitinib, upadacitinib</td></tr>
<tr><td>baricitinib, filgotinib</td><td>apremilast</td></tr>
</table>

1.2 Osteoclasts

Activation of the bone-resorbing cells, the osteoclasts are increased in both RA and PsA inflammatory arthropathies, resulting in local and systemic bone loss. Therefore investigation of osteoclasts in these diseases is important for a better understanding of the pathomechanism of RA and PsA.

In vertebrates, bone tissue has several basic, essential functions in maintaining the body's homeostasis. It provides mechanical stability and framework of the body, participates in the mechanism of displacement, protects internal organs - the central nervous system and chest organs -, borders the bone marrow where hematopoiesis occurs, and stores minerals and fat, thereby regulating the homeostasis of minerals [34]. In addition bone tissue also has significant effects on immune homeostasis [35]. Bone building and bone breakdown take place simultaneously for a lifetime, and bone tissue is

completely renewed every 7-10 years [36]. Two types of cells are involved in the constant remodeling of bone tissue; osteoclasts break down bone, while osteoblasts build new bone [37].

Osteoclasts are formed from bone marrow-derived hematopoietic progenitor cells which are committed in granulocyte/monocyte/macrophage direction and express CD14 [38]. The presence of macrophage colony-stimulating factor (M-CSF) produced by osteoblasts is essential for the differentiation and activation of osteoclasts [39]. Osteoblasts promote osteoclastogenesis not only by secretion of soluble cytokines but also by direct cell-cell contact. The integrant membrane protein on the surface of osteoblasts, receptor activator of nuclear factor κB ligand (RANKL), binds to the receptor activator of nuclear factor κB (RANK), which is expressed on many progenitors of osteoclasts and the osteoclasts themselves. The RANK-RANKL signaling is the most important factor in osteoclast differentiation, but other soluble cytokines and T-cells are also essential [40,41]. Osteoprotegerin (OPG) is a soluble factor released by osteoblasts, which binds RANKL and prevents the binding of RANK to RANKL [42]. In the presence of M-CSF osteoclast precursor cells are formed from granulocyte-macrophage progenitor (GMP) cells. Osteoclast precursor cells differentiate into mononuclear prefusion osteoclasts in the presence of M-CSF and RANKL, which then fuse into multinuclear osteoclasts [43]. In addition to promoting differentiation, RANKL is also essential in the activation and survival of osteoclasts [44,45] (**Figure 1**). *In vitro* circumstances using M-CSF and RANKL recombinant growth factors and adherent conditions are also required for osteoclastogenesis [46].

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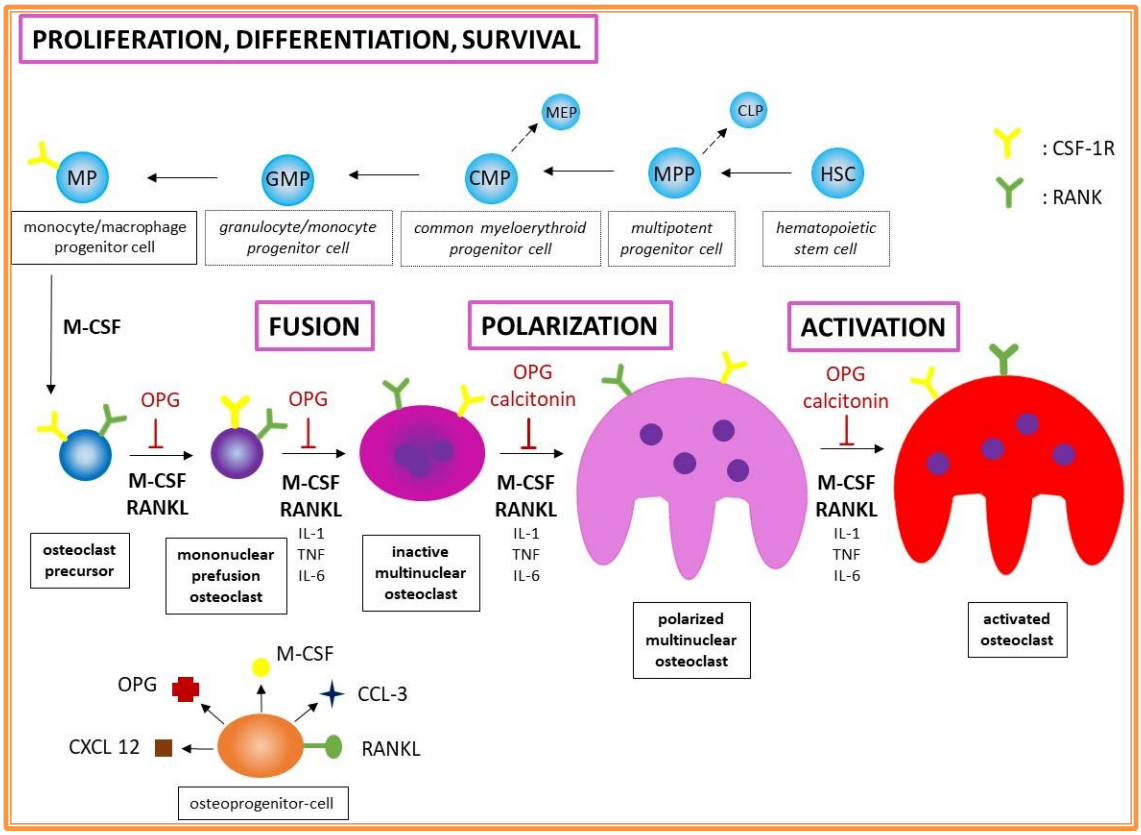

Figure 1 The process of osteoclast differentiation and its positive and negative regulators. (Own figure)

The activation of osteoclasts is decreased in osteopetrosis, while the number of osteoclast precursors is elevated during estrogen deficiency leading to osteoporosis as well as bone metastasis of tumors and inflammatory diseases. Hence the close connection between the bone tissue and the immune system, inflammatory procedure fosters pathological bone destruction via osteoclast activation. During inflammation, the amount of pro-inflammatory TNF and RANKL produced by T-cells is elevated. Consequently, bone loss consorts many autoimmune diseases for example RA or PsA.

1.3 Mass spectrometry

Mass spectrometry (MS)-based proteomics techniques are widely used for the identification and quantification of proteins expressed in complex biological samples. There are some published articles regarding the proteomics of osteoclast precursor cell lines [47–53]. Total proteins from undifferentiated RAW264.7 cells

(monocyte/macrophage osteoclastogenesis model cell line), committed preosteoclasts (day 2), and differentiated osteoclasts (day 3) were studied and the temporary up-regulation pattern of metallocarboxypeptidase family member CPX-1 expression was identified to might be essential for osteoclast development from preosteoclasts [49]. Mice origin bone marrow macrophage (BMM) cell culture as preosteoclasts and osteoclasts (day 3) differentiated from BMM cells were also analyzed by mass spectrometry. The vacuolar-type H^+-ATPase (V-ATPase) proton pump is found in the ruffled border plasma membrane of osteoclast cells, and its function is to translocate protons from the osteoclasts to the resorption lacunae resulting in a 4.5 pH, which is an energy-intensive process. The important role of lipid rafts during osteoclastogenesis in regulating the activity of V-ATPase in osteoclasts was described [54]. TNF receptor-associated factor 6 (TRAF6) has an important role in osteoclast activation. V-ATPase was identified as a TRAF6-binding protein and both molecules were confirmed to be essential for osteoclast function during the analysis of mice origin BMM cells and their differentiate and stimulated RAW264.7 cell lines (day 4) as well [48]. The proteomics of isolated monocytes from peripheral blood was also examined [55–57]. Nevertheless, as far as we know, there was no published data regarding the proteome of human blood-derived preosteoclasts and osteoclasts and there was little information about the molecular changes during human blood-derived osteoclast differentiation.

1.4 Extracellular vesicles

Extracellular vesicles (EV) are subcellular structures surrounded by a phospholipid bilayer membrane and are actively released by all cells in an evolutionarily conserved manner. EVs can be divided into three major groups based on their biogenesis and size. Large-sized EVs (lEV) are formulas of 1-5 μm generated during programmed cell apoptosis [58]. Medium-sized EVs (mEV) are plasma membrane-derived vesicles with a diameter of 100-1000 nm, while small-sized EVs (sEV) are structures with endosomal markers formed by exocytosis of multivesicular bodies with a diameter of 50-100 nm [59–61]. EVs are complex biomarkers since they may carry various proteins,

lipids, or nucleic acids including RNAs [62], microRNAs [63,64], and other small non-coding RNAs and DNAs [65] on their surface and interior as well (**Figure 2**).

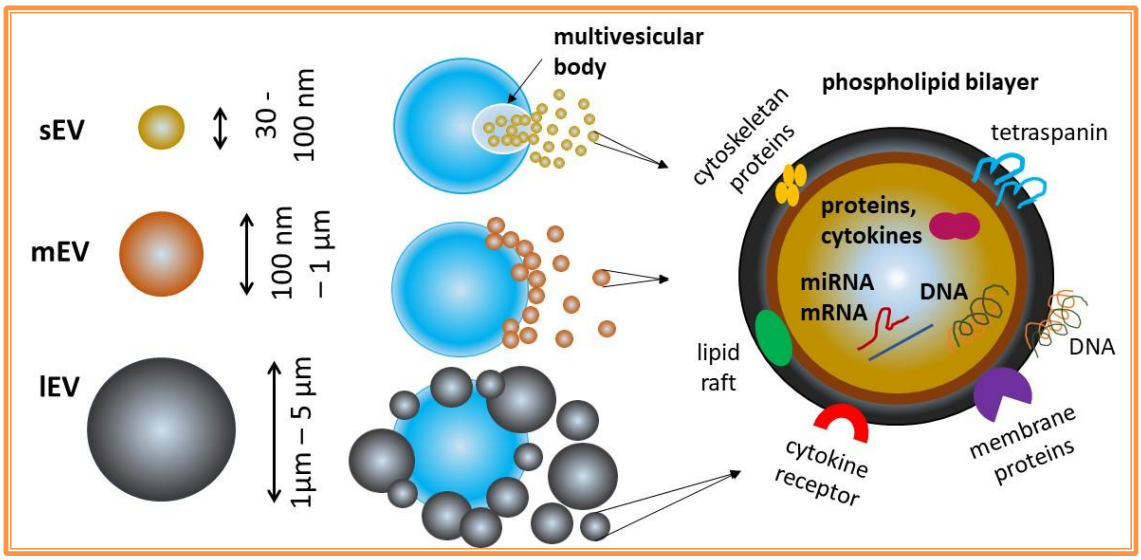

Figure 2 Size, biogenesis, and structure of extracellular vesicles. (Own figure)

EVs play an important role in intercellular communication as “vectorial signalosomes" [66]. Their roles include antigen presentation, immunosuppression, or immune activation. Among others, they play a role in neuron-astrocyte communication as well [67] and perform a protective function by preventing the accumulation of amyloid β-proteins [68]. During fertilization, they play a key role in the delivery of lipids transported by seminal EVs to sperm [69,70]. EVs in saliva play an important role in wound healing based on tissue factor-initiated coagulation [71]. EVs are enriched in innate immune proteins, which, in addition to antimicrobial proteins, also have bacterial and viral receptors that are suitable to prevent the entry of microorganisms into the body [72]. The role of EVs in e.g. coagulation and cell homeostasis is also a much-studied field.

In addition to their physiological roles, EVs may also play an important role in several pathological conditions [73]. As complex signal carriers, EVs have increasingly recognized biomarkers of several inflammatory and malignant diseases [74–77]. They may even play a role in the diagnosis, prognosis, or prediction of certain diseases in the future [78]. In some organ-specific and systemic autoimmune diseases, more platelet-derived mEVs have been identified than in healthy controls. It has been described in RA,

systemic lupus erythematosus (SLE), and Sjögren's syndrome, among others [79]. Vesicles examined in RA and juvenile idiopathic arthritis (JIA) were isolated from the synovial fluid produced by the inner layer of the joint capsule, promoting frictionless displacement of the joint ends. The sEVs derived from synovial fluid accumulate citrullinated proteins that are produced during pathological metabolic processes and are likely to play a role in the autoimmune inflammatory process in the joints. The presence of these autoantigens in EVs suggests that EVs may play an important role in autoimmune processes. It has also been observed that EVs from synovial fluid bind to IgG and IgM immune complexes [80]. In addition to autoantigens, a mammalian phosphoprotein called nuclear DEK was also found in EVs derived from synovial macrophages. This indicates the role of EVs in arthritic inflammatory processes [81]. A correlation was also found between disease duration and the number of platelet-derived EVs. Respectively, the amount of EVs in the synovial fluid was not related to the number of cells, so it is possible that cell activation rather than cell number plays a significant role in the formation of EVs [82,83]. In addition to autoimmune diseases, the role of EVs was also examined in cardiovascular diseases [84–90], metabolic syndrome [91], obstructive sleep apnea [92], sepsis [93], sickle cell anemia [94], in Alzheimer's disease [95], in kidney disease [96], and malignant tumors [97–99].

EVs are also promising therapeutic tools in malignant growth [100], gene therapy [101], and the targeted delivery of drug molecules [102].

Due to the potentially important role of EVs in inflammatory processes, and also due to the therapeutical potency of these subcellular structures, the investigation of EVs in rheumatological diseases is significant.

DOI:10.14753/SE.2023.2780

2. OBJECTIVES

The main objectives of our work were as follows.

- To analyze proteomic dynamic changes during osteoclast differentiation both in healthy donors and in RA and PsA patients.
    - To differentiate preosteoclasts and osteoclasts *in vitro* from monocytes derived from blood samples of healthy donors, and RA and PsA patients.
    - To analyze the protein expression of monocyte, the *in vitro* differentiated preosteoclast, and osteoclast samples.

- To investigate the effect of sEV on human *in vitro* osteoclastogenesis and the mechanism of action of EVs on osteoclastogenesis *in vitro*.
    - To investigate the effect of human blood-derived and SEC (size exclusion chromatography) purified sEV treatment on human *in vitro* osteoclastogenesis.
    - To analyze sEV and mEV samples of healthy donors and RA and PsA patients.

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3. RESULTS

3.1. Analysis of proteomic dynamic changes during osteoclast differentiation both in healthy donors and in RA and PsA patients

3.1.1. Experimental design setup

To analyze the proteomic changes of osteoclast differentiation, a detailed experimental system was applied. Peripheral blood samples of healthy donors (n=6) and RA (n=6) and PsA (n=6) patients were collected. PBMCs were isolated *via* a Ficoll density gradient, then $CD14^+$cells - monocytes - were isolated from PBMCs, and cultured *in vitro*. (Polymorphonuclear leukocytes, which also express CD14 marker, were settled under the polymer after centrifugation.) After 5 days of differentiation on tissue culture-treated plates in the presence of recombinant M-CSF and RANK-L cytokines, preosteoclasts, and after 9 days, osteoclasts were collected. Lysed and digested samples were separated with nano HPLC (high-performance liquid chromatography) technique, then analyzed with tandem mass spectrometry (MS/MS) method. Data were analyzed to gather qualitative and quantitative results as well (**Figure 3**). A total of 1435 proteins were identified in the 54 sample.

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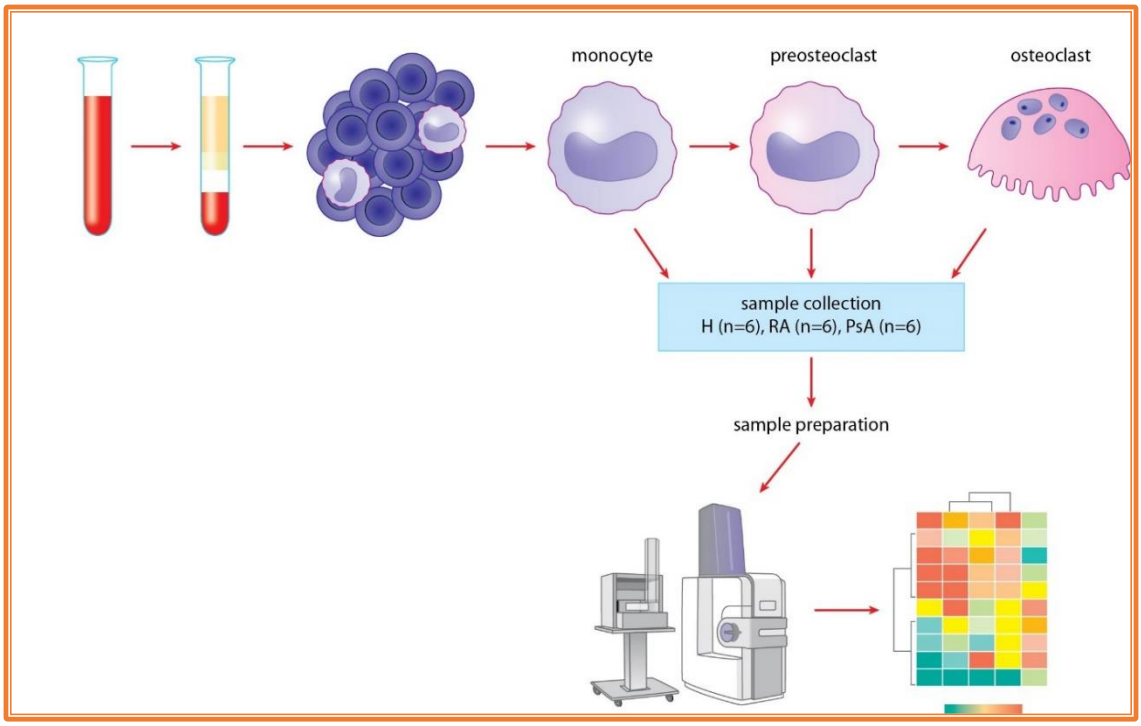

Figure 3 Experimental design setup. After differentiation of preosteoclasts and osteoclasts from human blood-derived monocytes, samples were collected, prepared, and analyzed by nano HPLC-MS/MS, finally, data were examined. Source: Kovács OT et al. [103]

3.1.2. PCA analysis and Volcano plot represent protein expression differences of samples during differentiation

To get an answer to the question of which parameters affect mostly the protein expression changes between samples, principal component analysis (PCA) was carried out. K-means clustering algorithm and adjusted Rand score index were used for quantification [104] and showed that different cell types of the differentiation had a significant effect on the clustering of the samples, however, gender and diagnosis had no effect. This reveals that samples with different stages of differentiation differ from each other. Our data indicate that preosteoclasts and osteoclasts separate from monocytes more than preosteoclast from osteoclasts. Volcano plot analysis also represents this finding by plotting the expression changes against the log p values (**Figure 4**). These observations are in accordance with that monocytes first differentiate into preosteoclasts, and then into osteoclasts during osteoclast development.

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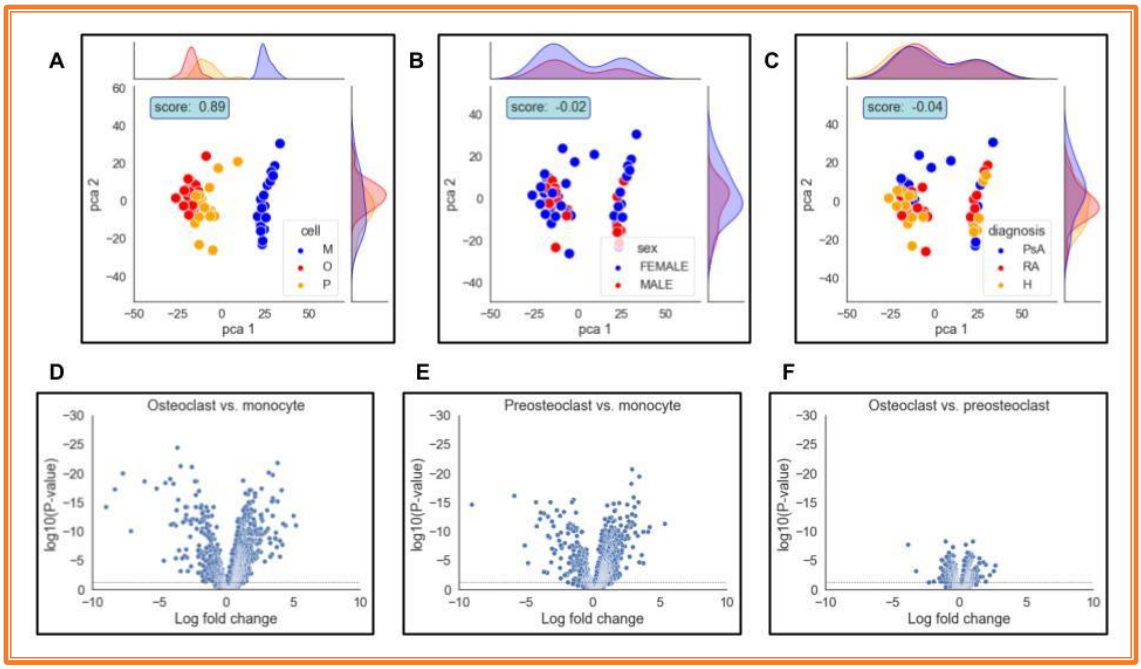

Figure 4 Monocyte, preosteoclast and osteoclast samples of healthy, RA and PsA donors differ from each other mostly based on their cell type. PCA analysis of samples based on A: cell stages, B: gender, and C: diagnosis. Volcano plot analysis of D: osteoclast vs. monocyte, E: preosteoclast vs. monocyte, and F: osteoclast vs. preosteoclast. The horizontal line indicates an adjusted p-value of 0.05. Source: Kovács OT et al. [103]

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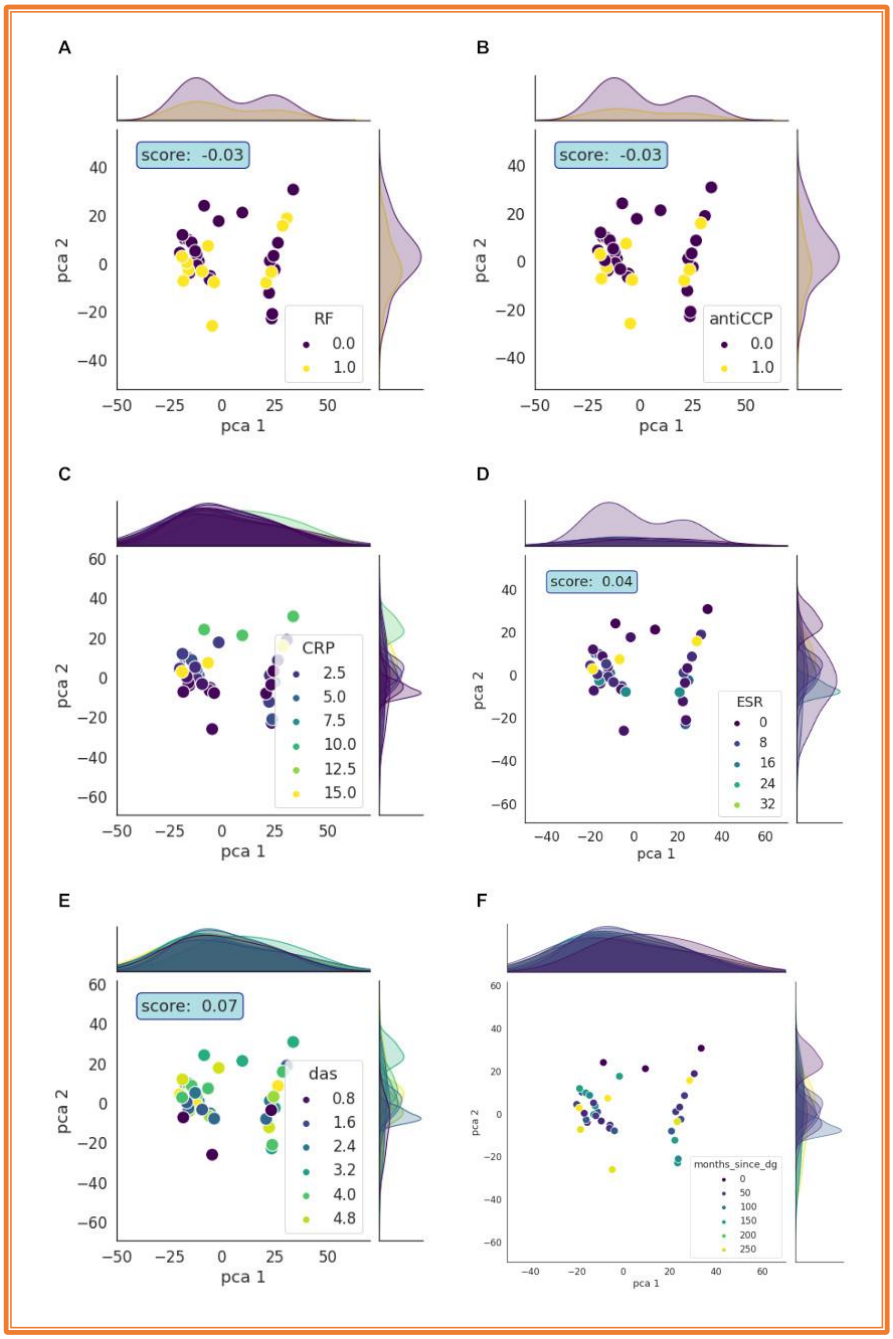

Figure 5 Samples did not differ from each other based on laboratory parameters, disease activity scores, or duration of disease. PCA analysis of samples based on A: rheumatoid factor (RF), B: anti-cyclic citrullinated peptide autoantibody (anti-CCP), C: C-reactive protein (CRP), D: erythrocyte sedimentation rate (ESR), E: disease activity scores, F: duration of disease. Source: Kovács OT et al. [103]

Besides gender and diagnosis, neither the laboratory parameters (RF, anti-CCP, CRP, or ESR), disease activity scores, or duration of disease had any significant effect on the clustering of samples (**Figure 5**).

3.1.3. Protein expression pattern of cell types changes during differentiation in case of cellular location and cell function

Next, with the use of the Uniprot database, a comparison of localization information of all detected proteins of each sample was carried out. Most of the detected proteins were localized in the cytoplasm, cell membrane, endoplasmic reticulum (ER), nucleus, and mitochondria (**Figure 6**). Then we compared the expression changes of proteins in pairs of samples. The expression of mitochondrial proteins and apical proteins was increased, while the expression of nuclear proteins and secreted proteins were decreased in osteoclast samples compared to monocyte samples (**Figure 7**). These findings are in accordance with that the process of bone resorption needs high energy, and that monocytes secrete various immunological proteins.

As a next step, Gene Set Enrichment Analysis (GSEA) on the differential expression (DE) data of all examined samples was carried out based on the localization of proteins (GO cellular compartment, GO-CC) (**Figure 8**) and biological processes they are involved in (GO biological processes, GO-BP) (**Figure 9**) [105,106]. The expression of variant secretory vesicle proteins and proteins of major histocompatibility complex (MHC) was decreased, while the expression of proteins localized to the basolateral membrane and ribosomal proteins was increased in osteoclast and preosteoclast samples compared to monocyte samples (**Figure 8**). Furthermore, the expression of proteins involved in osteoclast differentiation and carbohydrate metabolism biological processes was increased in osteoclast samples compared to monocyte samples. Nevertheless the expression of proteins involved in diverse immune functions - e.g. migration, cytokine production, and immune responses - was decreased (**Figure 9**).

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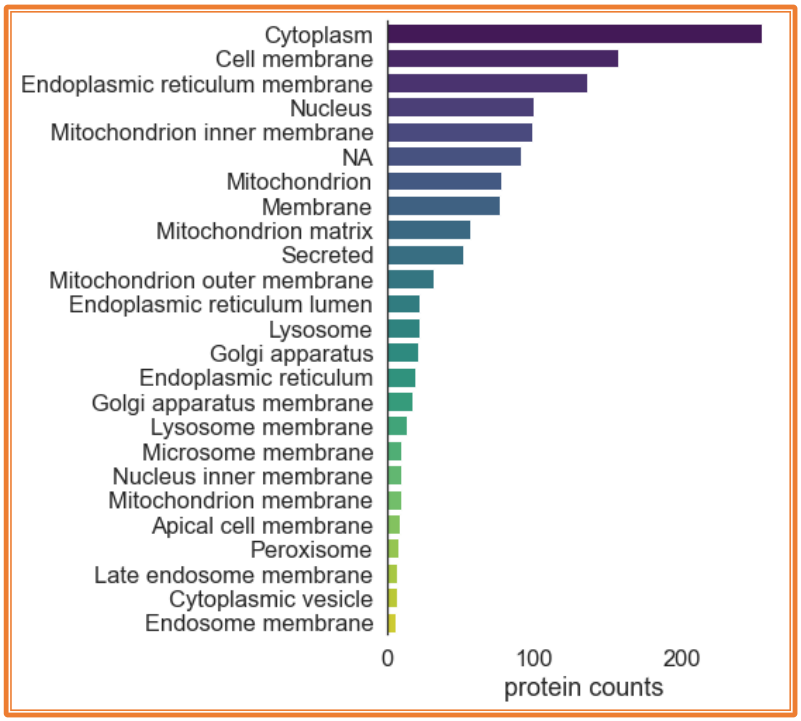

Figure 6 Bar graph represents the protein distribution of whole quantified proteins in cellular location. Source: Kovács OT et al. [103]

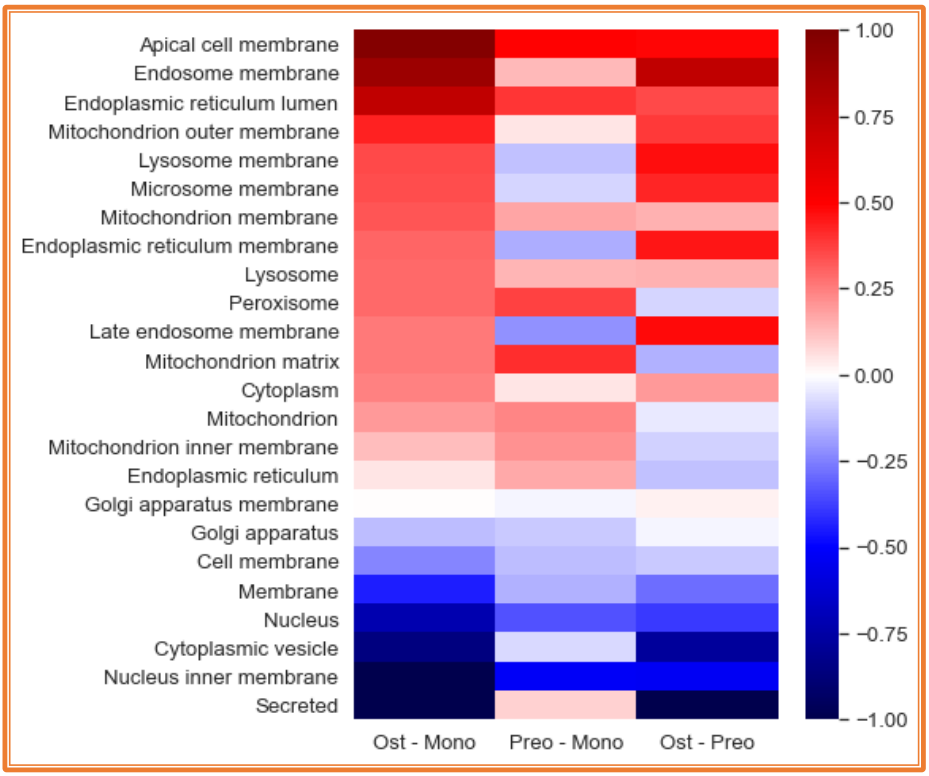

Figure 7 Heat map represents the protein expression differences of monocytes, preosteoclasts, and osteoclasts in cellular location according to the Uniprot database. Not all pathways are indicated on the graph. Source: Kovács OT et al. [103]

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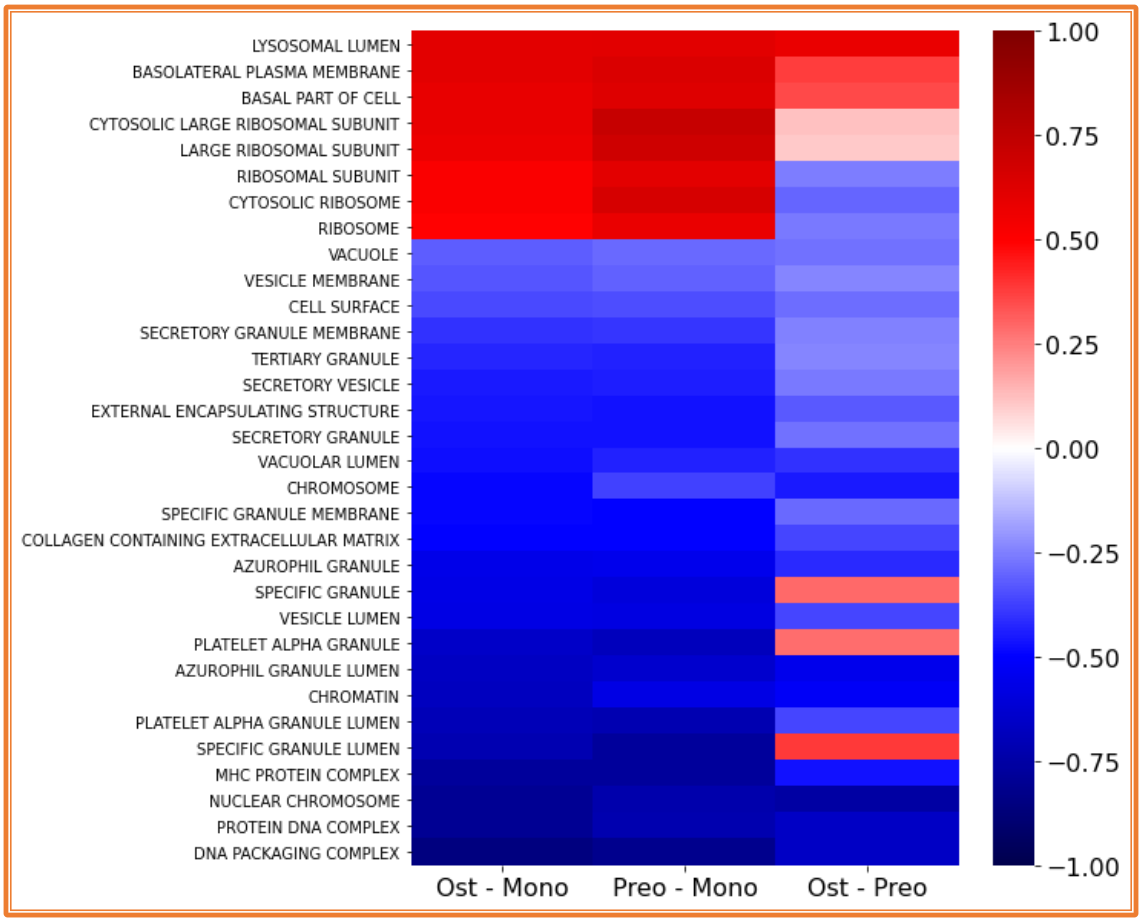

Figure 8 Protein expression changes during osteoclastogenesis in case of cellular location. The heat map represents the protein expression differences of cell types in cellular location analyzed with GSE using GO: CC terms. Not all pathways are indicated on the graph. Source: Kovács OT et al. [103]

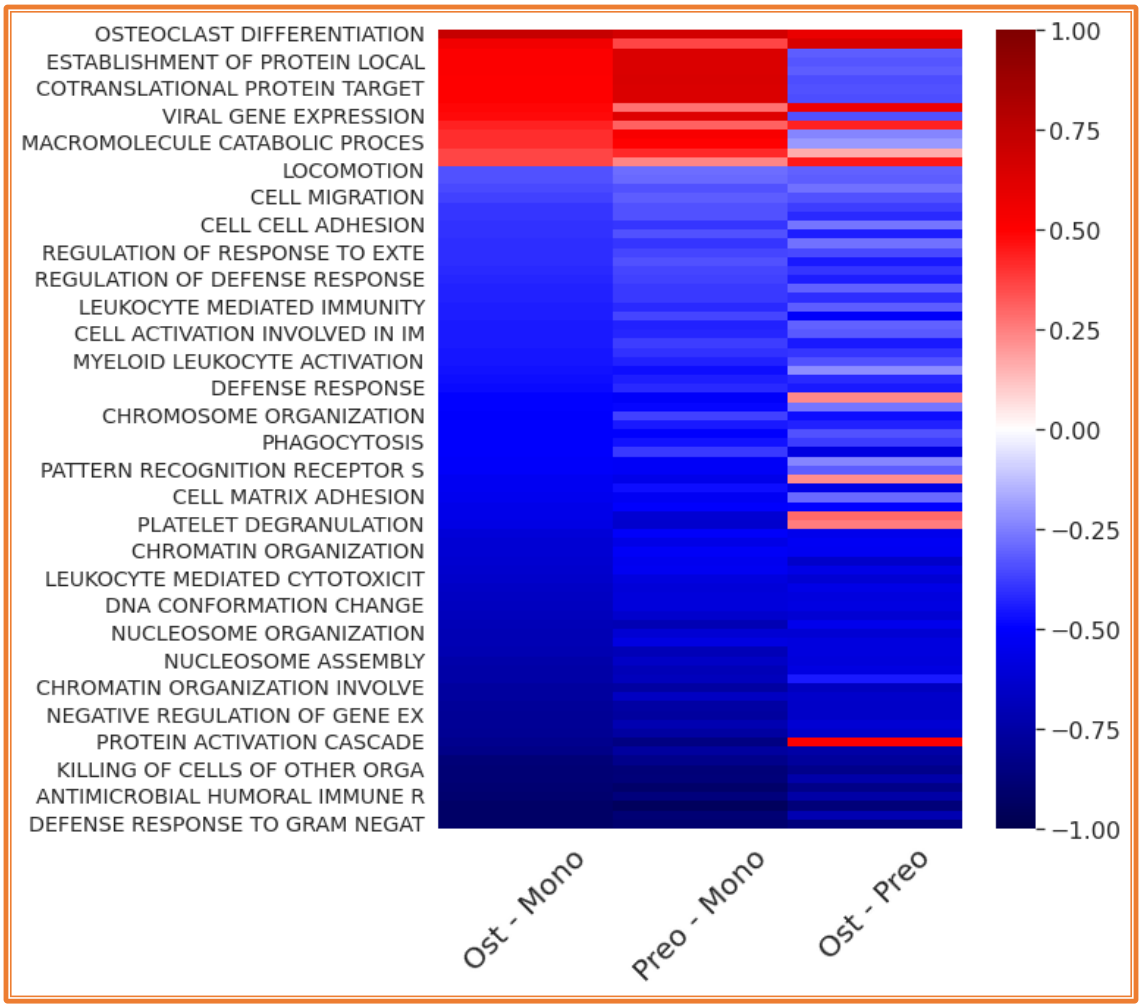

Figure 9 Protein expression changes during osteoclastogenesis in case of cell function. The heat map represents the protein expression differences of cell types according to cell function analyzed with GSE using GO: BP terms. Not all pathways are indicated on the graph. Source: Kovács OT et al. [103]

3.1.4. Metabolic demands are elevated, while the antibacterial function is decreased in osteoclasts

Next, two biological processes were selected from the above-described data (**Figure 9**). Monosaccharide metabolic processes are enhanced, while defense response to bacterium pathway is decreased in osteoclast samples altogether compared to monocyte samples altogether (**Figure 10 and Figure 11**). This is in line with that in secretory osteoclasts metabolic demands are elevated, while the antibacterial function is decreased during the transition from monocytes to these cells.

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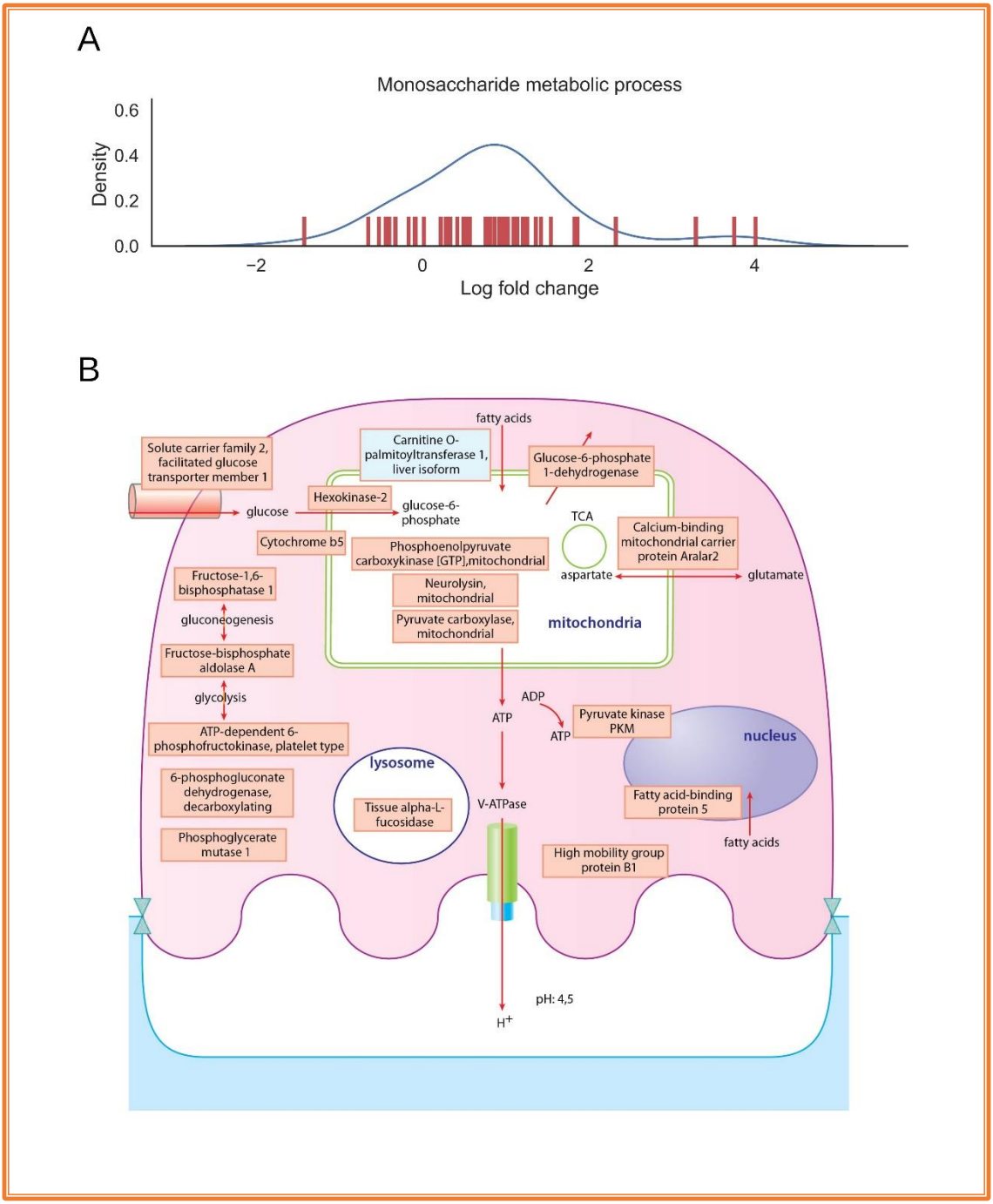

Figure 10 Metabolic demands are elevated in osteoclasts. A: Density curve and individual protein DE log2 values of protein expressions represent identified proteins of the monosaccharide metabolic process pathway in osteoclasts. B: Illustration represents the most significantly expressed proteins involved in the same pathway in osteoclasts compared to monocytes. Red text box filling means the protein was upregulated, and blue text box filling means the protein was downregulated in osteoclast samples compared to monocyte samples. Source: Kovács OT et al. [103]

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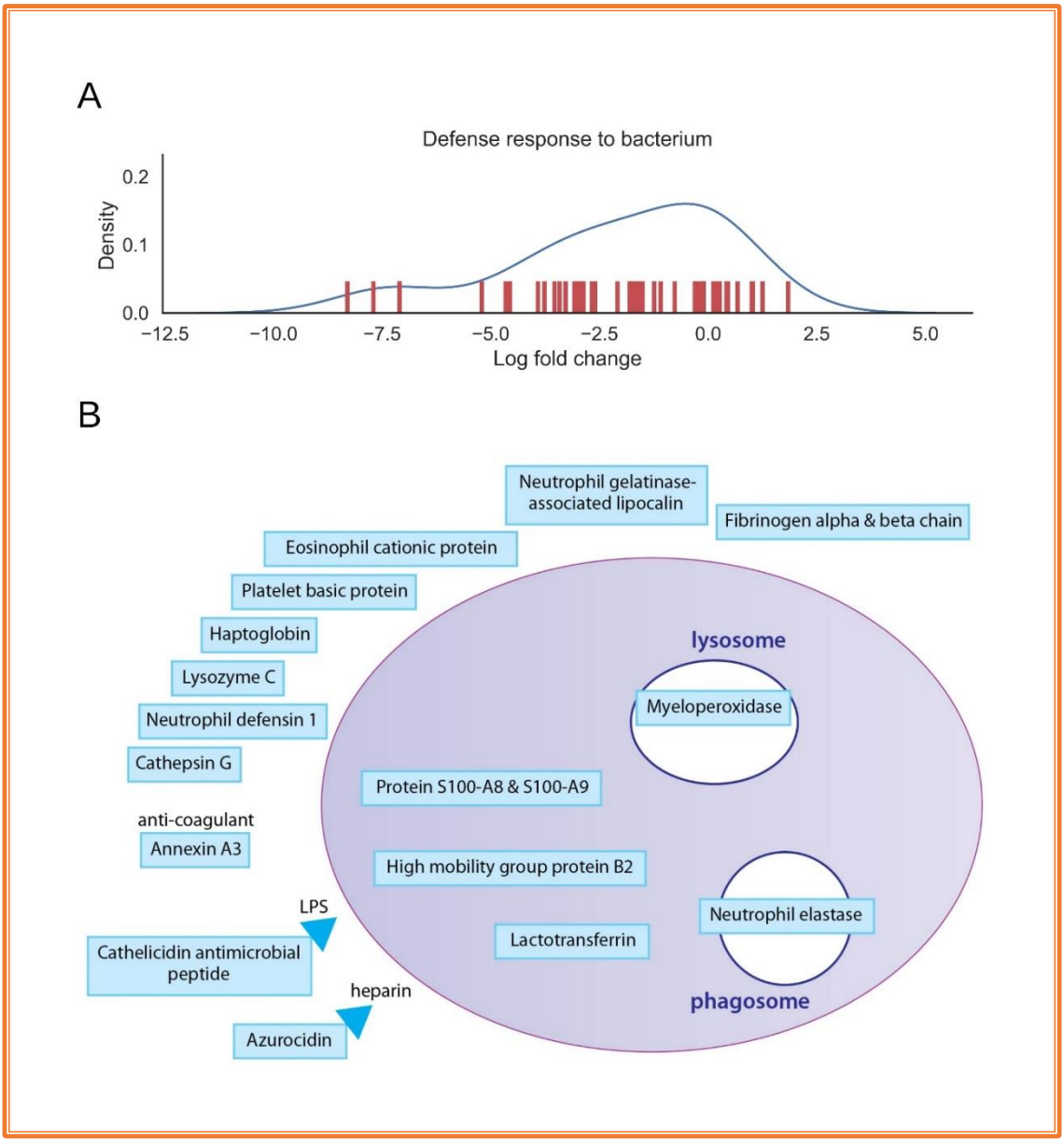

Figure 11 Antibacterial function pathway is increased in monocytes. A: Density curve and individual protein DE log2 values of protein expressions represent identified proteins of the defense response to bacterium pathway in osteoclasts. B: Illustration represents the most significantly expressed proteins involved in the same pathway in monocytes compared to osteoclasts. Red text box filling means the protein was upregulated, and blue text box filling means the protein was downregulated in monocyte samples compared to osteoclast samples. Source: Kovács OT et al. [103]

3.1.5. Largest protein expression changes between osteoclast and monocyte samples

Next, the largest protein expression (over 10 fold DE) changes between all osteoclast and all monocyte samples were represented. Most of these proteins have been previously described as being characteristic in osteoclasts or monocytes. Proteins upregulated in osteoclast samples are connected mostly to processes that are essential for osteoclast function such as metabolism or bone resorption activity. Iron is a Krebs cycle activator, so transferring iron to the mitochondria is an essential step in the activation of tricarboxylic acid (TCA) processes. *Transferrin receptor protein 1* - which shows the highest expression difference in healthy osteoclasts - transfers iron into the mitochondria, thus is essential in osteoclast differentiation, and it is a positive osteoclastogenic feedback loop generator. Iron uptake also increases actin ring formation, thus promoting mature osteoclast function as well [107]. From the electron transport chain (ETC) ATP is formed by oxidative phosphorylation. *Creatine kinase B-type* catalyzes the transfer of phosphate between phosphagens and ATP, thus having a key role in the high energy demand processes, such as bone resorption [51,108]. Several subunits of V-ATPase were detected in large quantities in osteoclast samples. *Sodium/hydrogen exchanger 9B2* is a Na^+/H^+ antiporter that besides V-ATPase also regulates the pH [109,110]. *Macrophage mannose receptor 1* plays a role in osteoclast fusion [111,112], *Cathepsin B* is secreted to the resorption lacunae and has an important role in bone resorption as enhancing the activity of other proteases [113]. *Cystatin-B* inhibits cysteine cathepsins such as cathepsin K, L, H, and B, thus playing a key role in osteoclast homeostasis as a negative regulator [114,115]. *Cathepsin K* is also secreted to the resorption lacunae from the lysosome and is involved in extracellular matrix degradation [116]. Cathepsin K is an important osteoclast biomarker and therapeutic target as well. *Lysosomal acid phosphatase* is a hydrolytic lysosomal enzyme also secreted to the resorption lacunae [117]. *TYRO protein tyrosine kinase-binding protein* plays an important role in signal transduction in osteoclast cells [118]. To create the occlusion zone, and therefore promote the formation of the Howship-lacunae αvβ3 integrins, such as *Integrin alpha-V* are essential [119] (**Figure 12**). These results show, that our data are in line with the osteoclast characteristics previously described.

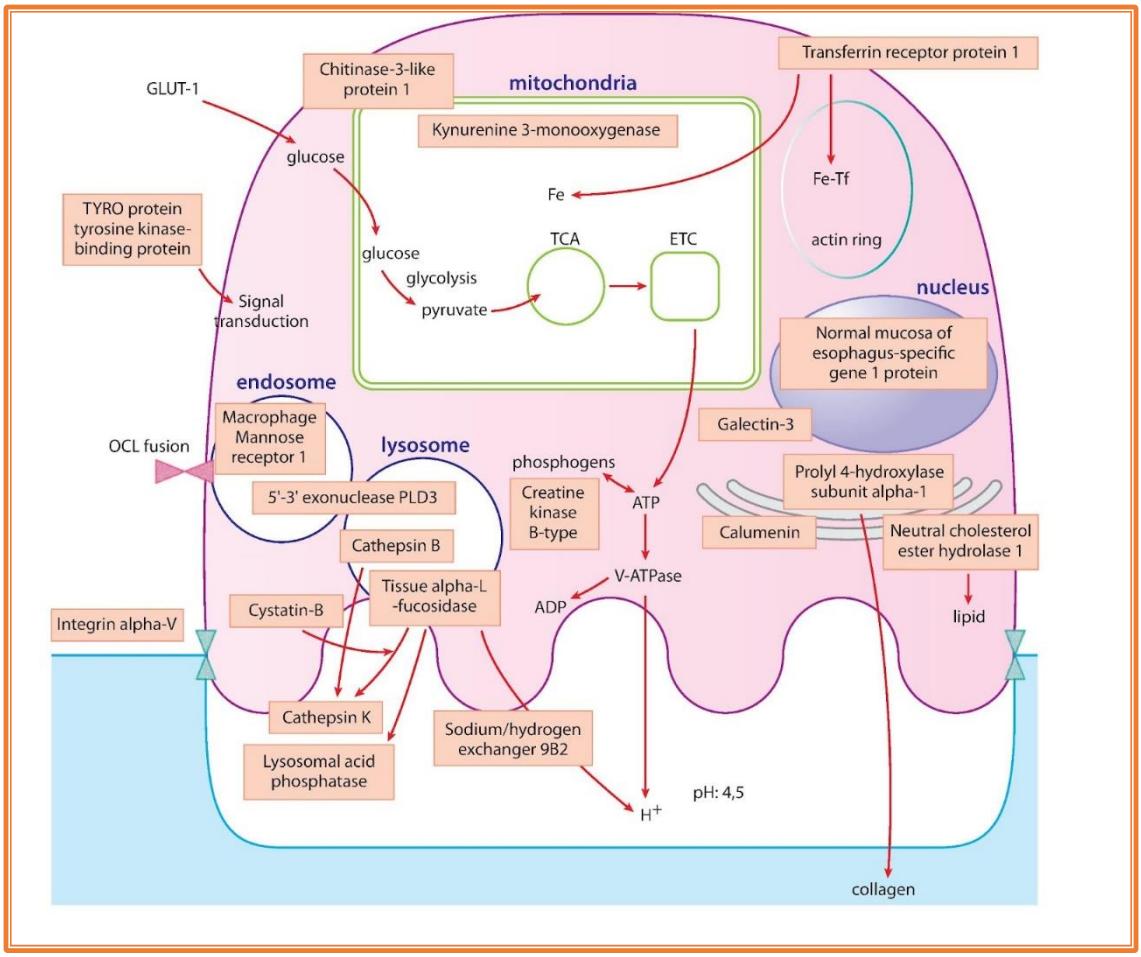

Figure 12 Overexpressed proteins in osteoclast samples altogether. The illustration represents proteins overexpressed by osteoclast samples compared to monocyte samples (own illustration). Source: Kovács OT et al. [103]

Proteins downregulated in osteoclast samples are connected mostly to immunological processes - such as immune responses (e.g. *complement receptor type 1, mast cell-expressed membrane protein 1, protein S100-A8, protein S100-A9, myeloperoxidase, neutrophil elastase,* and *myeloblastin)* or inflammatory processes (e.g. *protein S100-A8,* neutrophil elastase, and myeoblastin). *Neutrophil defensin 1* and *interferon-induced transmembrane protein 2* have an antiviral effect, while *lysozyme C, neutrophil defensin 1, cathelicidin antimicrobial peptide, and cathepsin G* have an antimicrobial effect. *Haptoglobin* also has an antibacterial effect in addition to binding hemoglobin. Complement receptor type 1, and interferon-induced transmembrane protein 2 have a role in innate immunity, *immunoglobulin kappa constant* protein in adaptive immunity, and *ficolin-1* in both innate and adaptive immunity (**Figure 13**).

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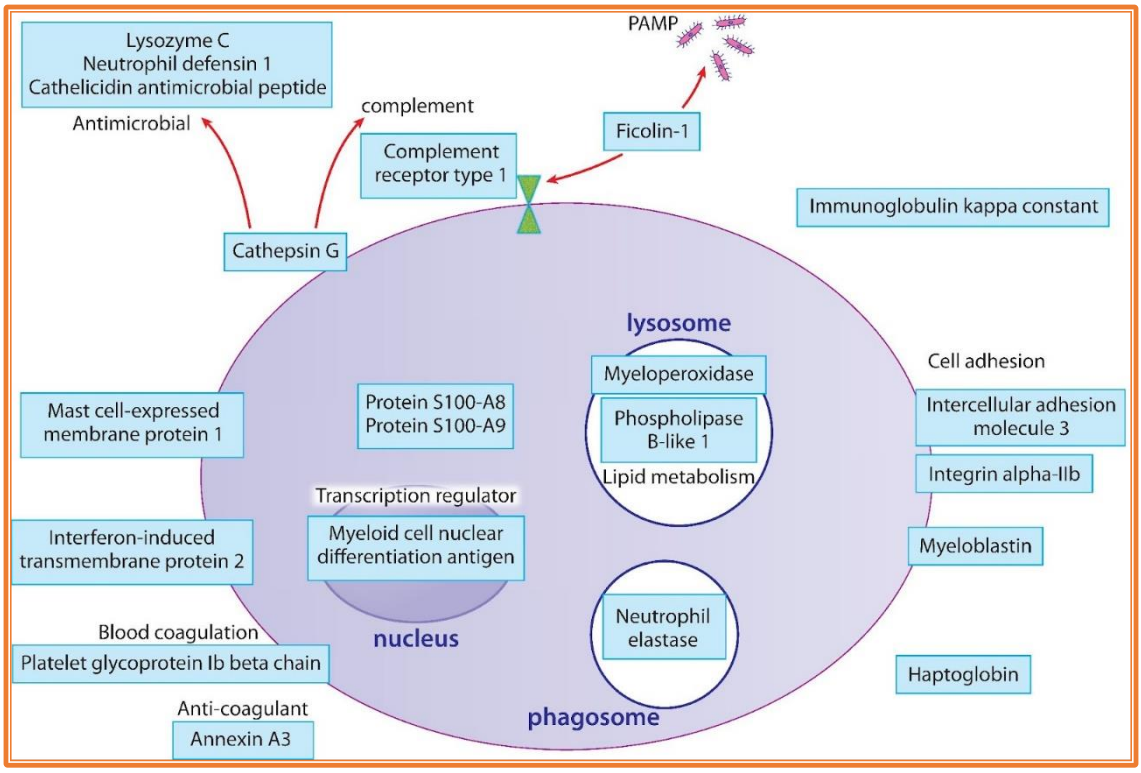

Figure 13 Downregulated proteins in osteoclast samples altogether. The drawing represents proteins overexpressed by monocyte samples compared to osteoclast samples (own illustration). Source: Kovács OT et al. [103]

3.1.6. Largest protein expression changes during osteoclastogenesis

Next, we have checked the largest protein expression (over 10 fold DE) changes between all monocyte, preosteoclast and osteoclast samples using heat map analysis. The expression of the above described proteins (**Figure 12 and Figure 13**) is depicted in all samples. In the first column we can see the protein expression of osteoclasts compared to monocytes and in the second column the protein expression of preosteoclasts compared to monocytes. Yellow color represents higher protein expression, while blue color represents lower protein expression. White filling means the particular protein was not detected in that sample.

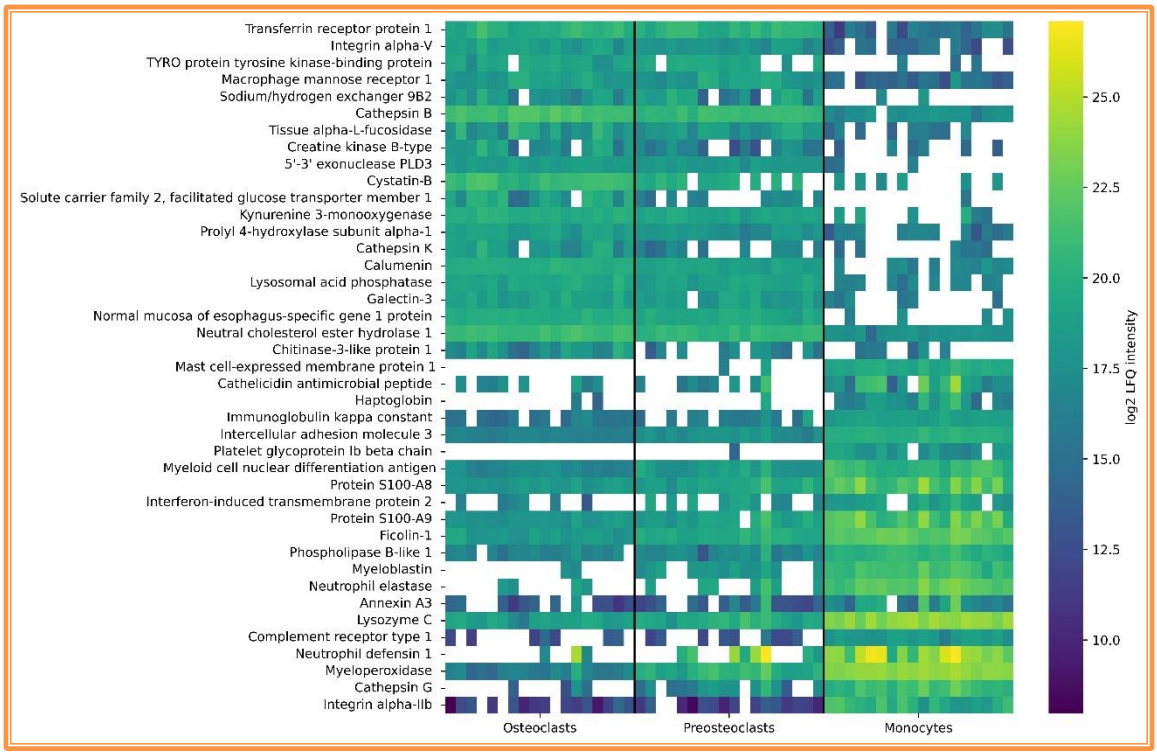

Figure 14 Largest protein expression changes during osteoclast differentiation. The heat map represents the largest protein expression differences of cell types. (Own figure)

3.1.7. The more the cells are developed, the bigger the protein expression profile differences are between healthy, RA, and PsA samples

When all sample data were analyzed with PCA analysis, the diagnosis had no significant effect on the clustering of the samples (**Figure 4C**). As activation of osteoclasts is increased in inflammatory arthropathies, such as RA and PsA, next we compared osteoclast samples of healthy donors and RA, and PsA patients using PCA analysis. We also compared monocyte and preosteoclast samples apart based on the diagnosis. Surprisingly, in the case of osteoclast samples, there was a clustering of healthy, RA, and PsA samples, and the extent of the separation was increased during differentiation (**Figure 15**). This suggests that taking blood from healthy donors and RA and PsA patients, then isolating monocytes from blood samples, then differentiating the cells *in vitro* under the same circumstances results in healthy and unhealthy osteoclast samples with different protein expression profiles.

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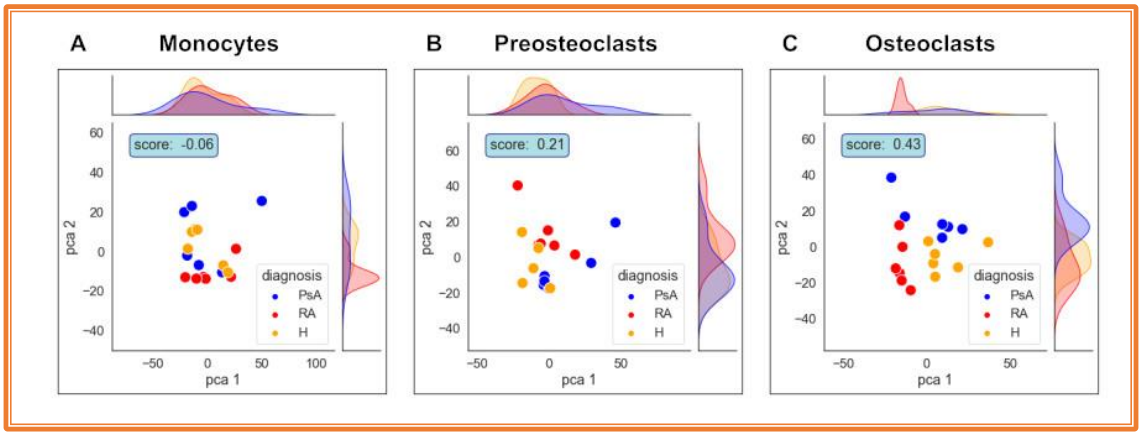

Figure 15 Healthy and unhealthy samples vary mostly from each other when the cells are fully differentiated. PCA analysis of A: monocyte samples, B: preosteoclast samples, and C: osteoclast samples based on the diagnosis. Source: Kovács OT et al. [103]

3.1.8. Both in RA and PsA osteoclast samples, the expression of proteins involved in immunological processes is increased and in metabolic processes is decreased

As a next step, GSEA was carried out on the DE data of osteoclast samples of healthy donors and RA and PsA patients, since PCA analysis showed the largest differences between osteoclast samples. Interestingly, there are significant protein expression differences between healthy, RA, and PsA osteoclasts in regards to GO-CC (**Figure 16 and Figure 18**) and GO-BP (**Figure 17 and Figure 19**) analysis results. The expression of proteins involved in immunological processes (**Figure 17 and Figure 19**) and proteins of the MHC complex (**Figure 16 and Figure 18**) was increased both in RA and PsA osteoclasts compared to healthy osteoclasts. The expression of proteins involved in lipid metabolic processes was also increased in PsA osteoclasts compared to healthy osteoclasts (**Figure 19**). The expression of proteins involved in metabolic processes was decreased both in RA and PsA osteoclast samples compared to the healthy ones (**Figure 17 and Figure 19**).

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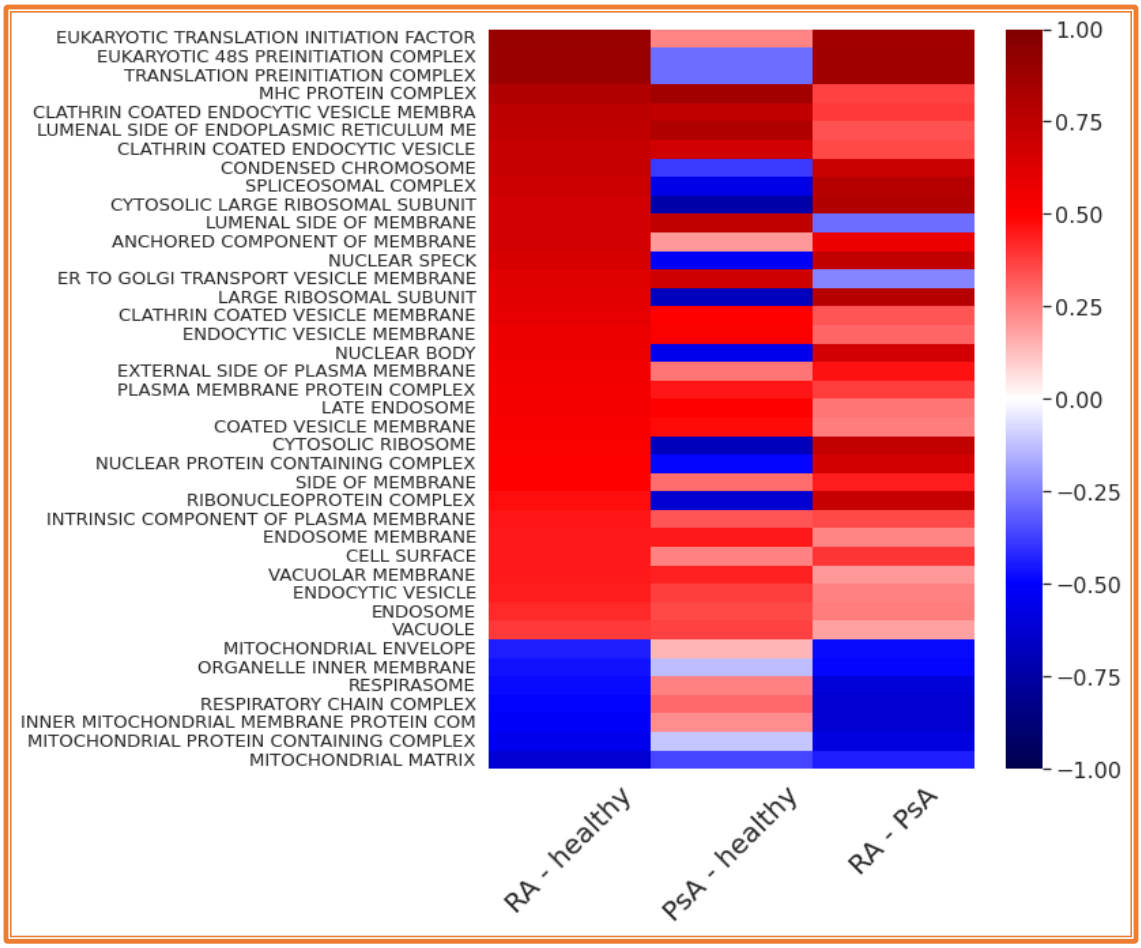

Figure 16 Protein expression differences of healthy, RA, and PsA osteoclast samples. Heat map represents GSEA analysis based on GO-CC. Osteoclast samples of RA patients are compared to healthy ones. Source: Kovács OT et al. [103]

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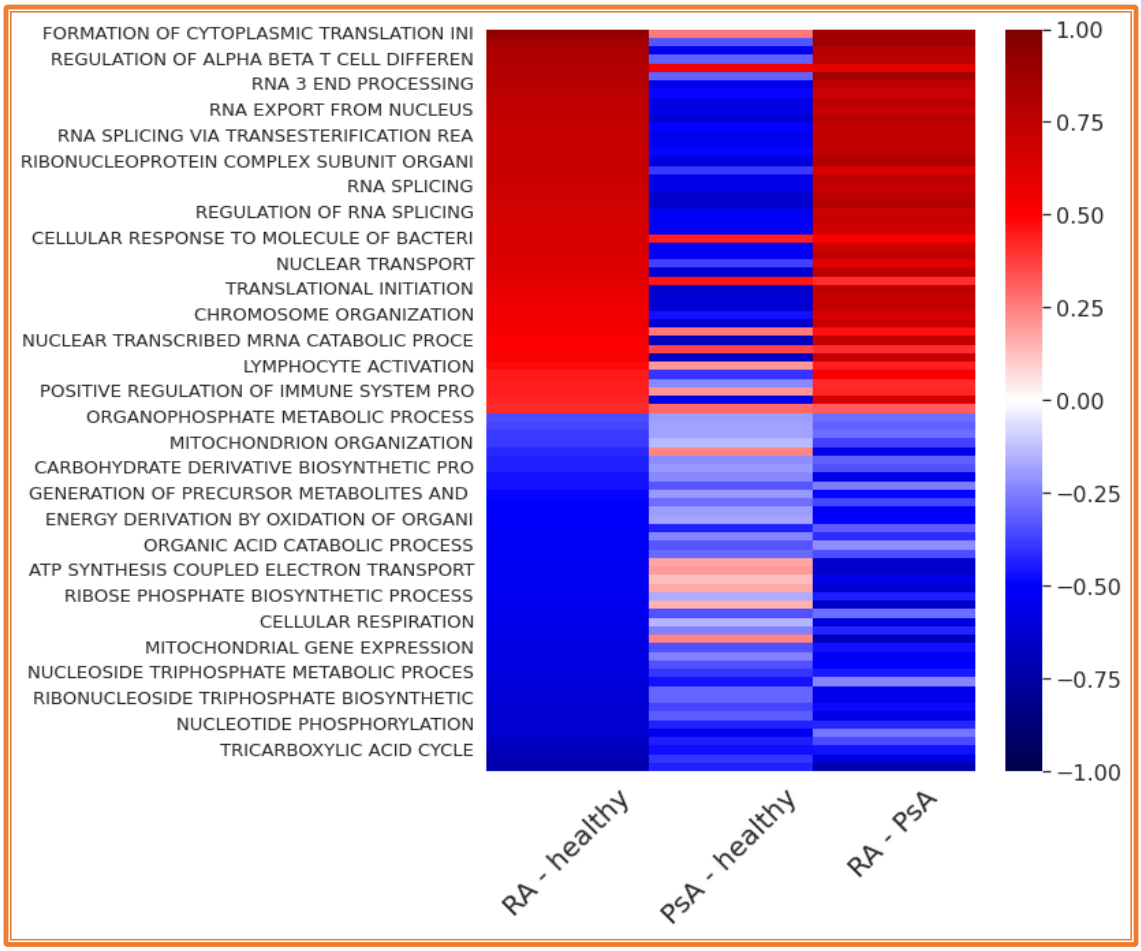

Figure 17 Protein expression differences of healthy, RA, and PsA osteoclast samples. Heat map represents GSEA analysis based on GO-BP. Osteoclast samples of RA patients are compared to healthy ones. Source: Kovács OT et al. [103]

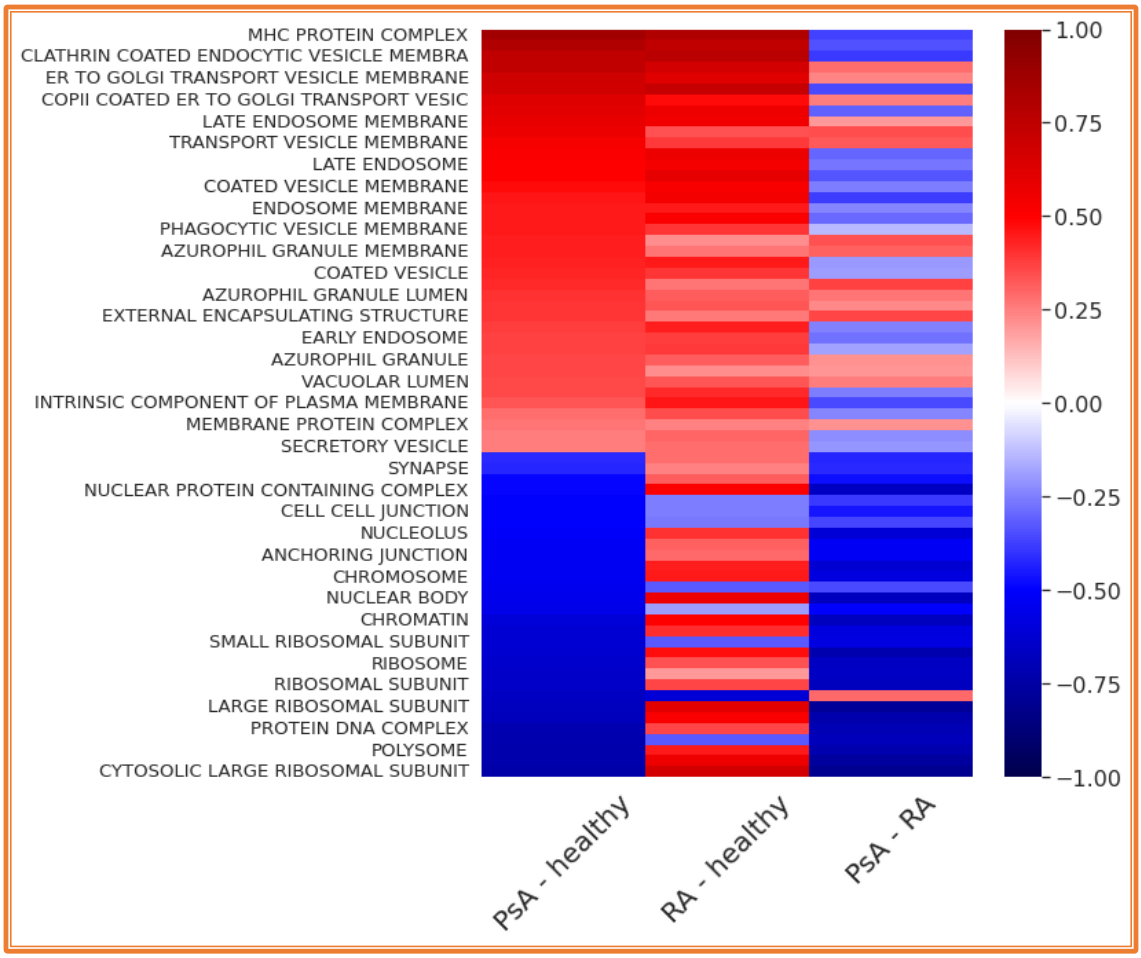

Figure 18 Protein expression differences of healthy, RA, and PsA osteoclast samples. Heat map represents GSEA analysis based on GO-CC. Osteoclast samples of PsA patients are compared to healthy ones. Source: Kovács OT et al. [103]

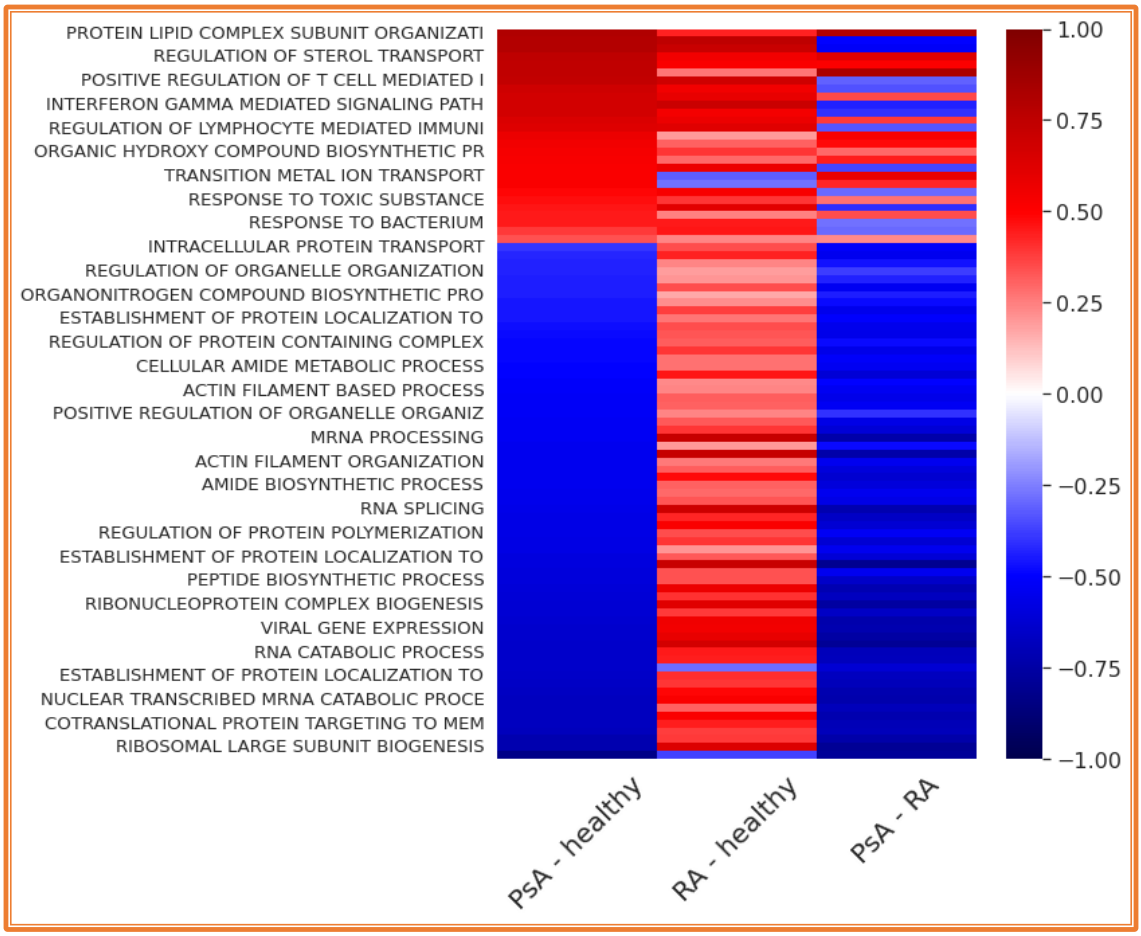

Figure 19 Protein expression differences of healthy, RA, and PsA osteoclast samples. Heat map represents GSEA analysis based on GO-BP. Osteoclast samples of PsA patients are compared to healthy ones. Source: Kovács OT et al. [103]

3.1.9. Osteoclast samples of unhealthy donors might keep a few immunological functions during differentiation

Next, digging deeper, four biological processes were selected from the above-described data (**Figure 17 and Figure 19**). ATP biosynthetic processes pathway is decreased, while regulation of T-cell mediated cytotoxicity pathway is increased in RA osteoclast samples compared to healthy osteoclast samples (**Figure 20**). Cytoplasmic translation processes are decreased, while positive regulation of adaptive immune response pathway is increased in PsA osteoclast samples compared to healthy ones (**Figure 21**). These results suggest, that osteoclasts differentiated from monocytes derived from patients suffering from RA or PsA keep some immunological functions in contrast with healthy osteoclasts.

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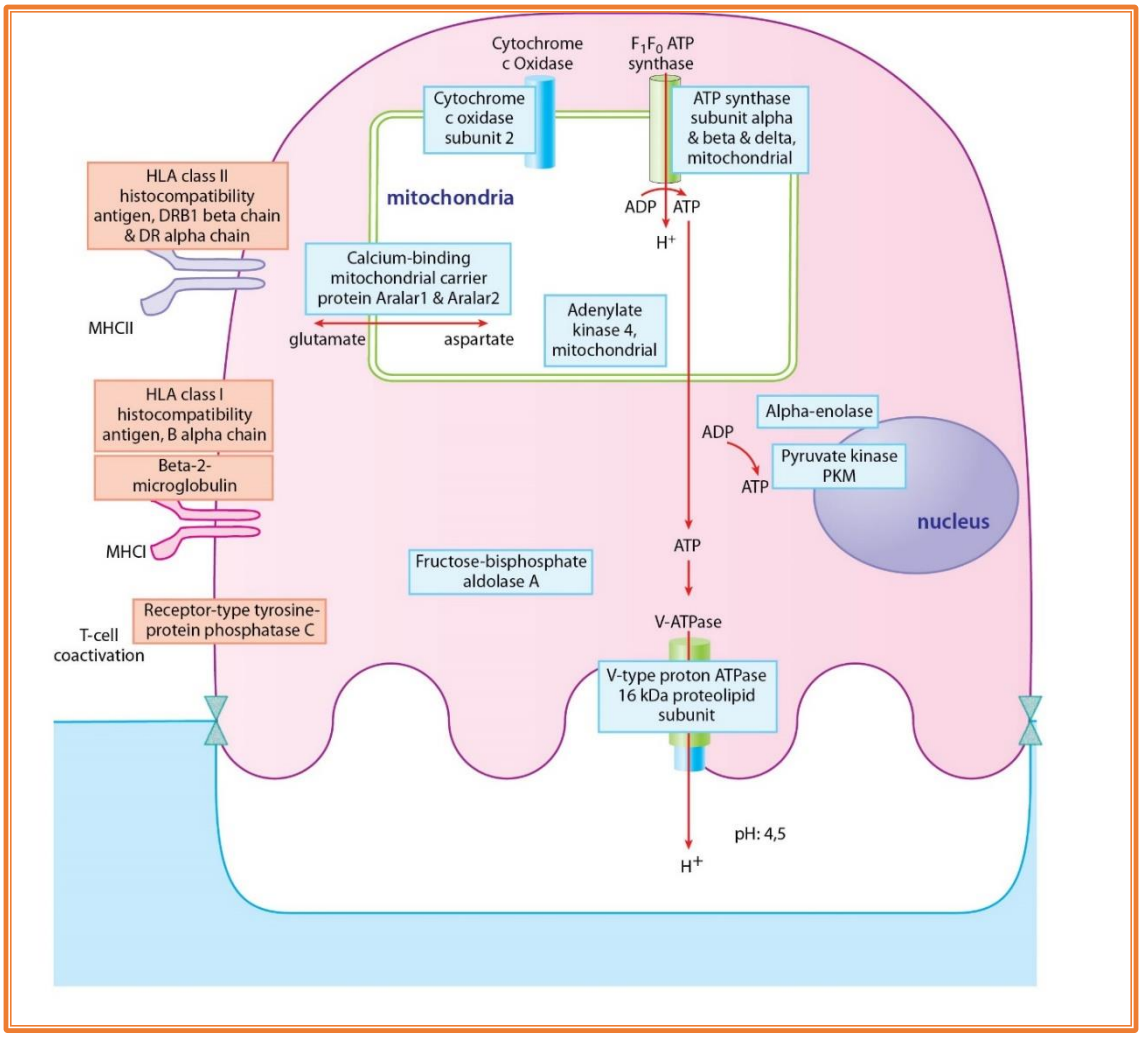

Figure 20 T-cell mediated cytotoxicity pathway is increased in RA osteoclast samples. The illustration represents the most significantly expressed proteins involved in the ATP biosynthetic processes pathway and T-cell mediated cytotoxicity pathway in RA osteoclasts compared to healthy osteoclasts. Red filling means the protein was upregulated, and blue text box filling means the protein was downregulated in RA osteoclast samples compared to healthy osteoclast samples. Source: Kovács OT et al. [103]

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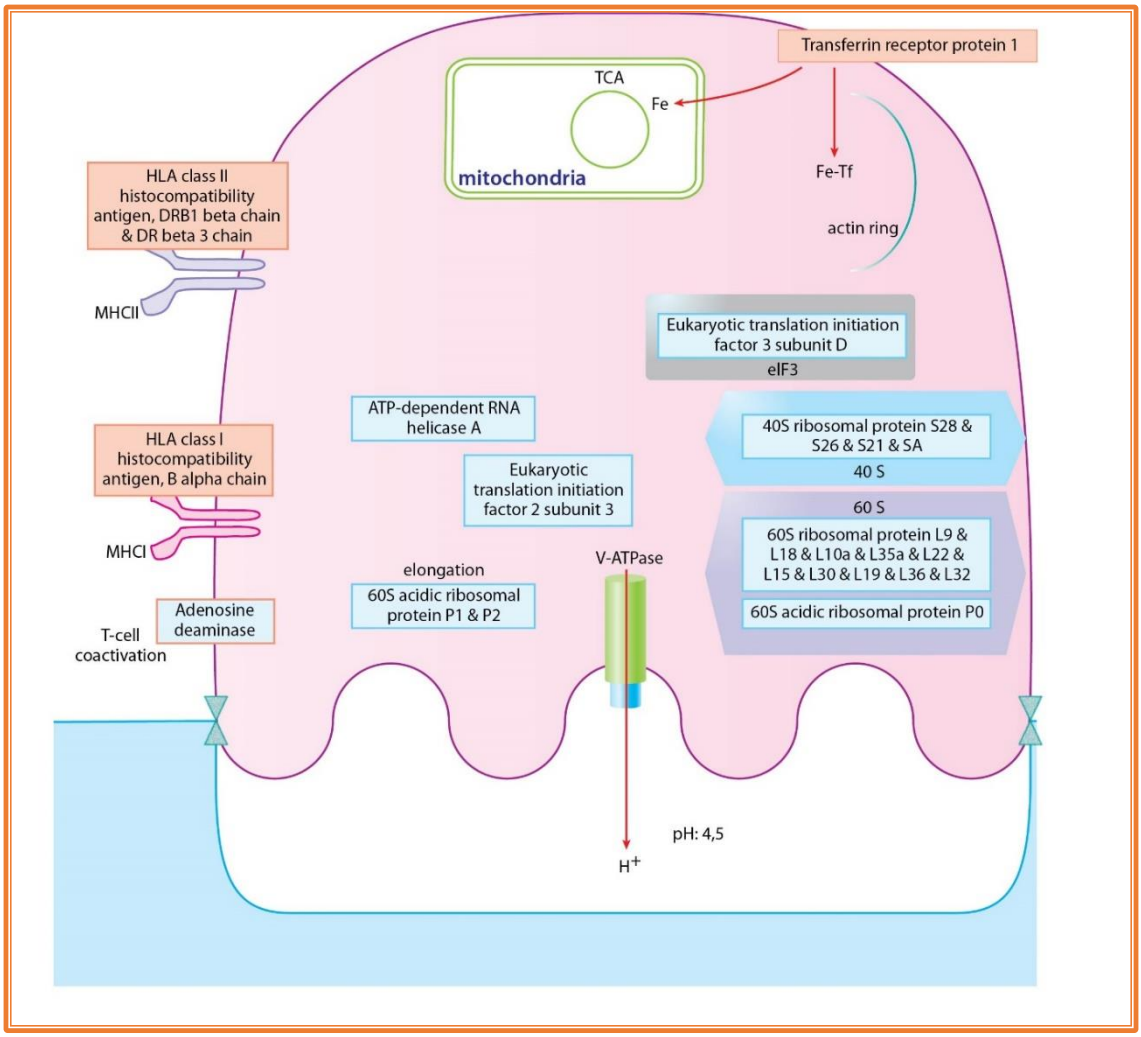

Figure 21 Positive regulation of adaptive immune response pathway is increased in PsA osteoclast samples. The illustration represents the most significantly expressed proteins involved in the cytoplasmic translation processes and positive regulation of the adaptive immune response pathway in PsA osteoclasts compared to healthy osteoclasts. Red text box filling means the protein was upregulated, and blue filling means the protein was downregulated in PsA osteoclast samples compared to healthy osteoclast samples. Source: Kovács OT et al. [103]

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3.2. The effect of sEV on human *in vitro* osteoclastogenesis

3.2.1. The effect of SEC purified sEV treatment on human *in vitro* osteoclastogenesis

Blood samples were taken from healthy donors (n=2), then platelet-free plasma (PFP) was isolated from the blood. PFP was filtered by gravitation through a 0.8 μm filter. Then the size exclusion chromatography was used for purification by using qEV Size Exclusion Columns according to the manufacturer's instructions. Finally, the samples were ultracentrifuged by 120.000 g for 16 h at 4 ºC and the sediment was resuspended in 1*PBS. SEC purified sEV and control PBS samples were added to human *in vitro* differentiation osteoclast cultures (from the same donors). SEC purified sEV treatment significantly inhibited *in vitro* osteoclastogenesis as the number of osteoclasts (TRAP stained cells with at least 3 nuclei) was significantly less in sEV-treated wells ($p < 0.05$) (**Figure 22**).

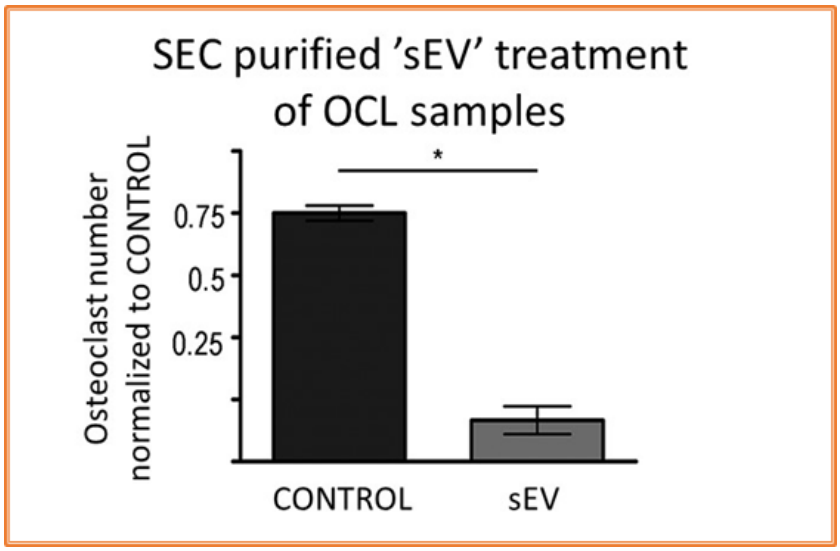

Figure 22 SEC purified sEV treatment inhibited human *in vitro* osteoclastogenesis. Columns represent the mean ± SEM, n=2, *: $p < 0.05$. Source; Marton N et al. [120]

3.2.2. Differential detergent lysis of mEV samples

The mEV samples were taken in Annexin V (AV) Binding Buffer or 1*PBS. Samples were incubated with Annexin V-FITC antibody for 30 min at room temperature in the dark and then analyzed on a FACSCalibur flow cytometer. Differential detergent lysis was performed on mEV samples using 0.1% Triton X-100 [121]. The phenomenon is based on the fact that the membrane of mEVs is disorganized by detergent. Events that can be registered even after lysis can be immune complexes or protein aggregates. Samples were thoroughly resuspended and vortexed and incubated for 30 seconds after the addition of 0.1% Triton X-100, then the samples were reweighed. Significantly fewer AV positive events were measured at the mEV gate after Triton X-100 treatment. The mEVs are detergent sensitive, so the difference in the number of events before and after the addition of Triton X-100 gives the number of mEVs. Therefore, only events that disappeared after the detergent was added were considered mEV, so the post-detergent event number at the mEV gate was subtracted from the pre-detergent event number. For mEV samples, the gate was determined with BioCytex MegaMix beads and was optimized with 1 μm Silica Beads Fluo-Green. Results were evaluated using FlowJo software (**Figure 23**).

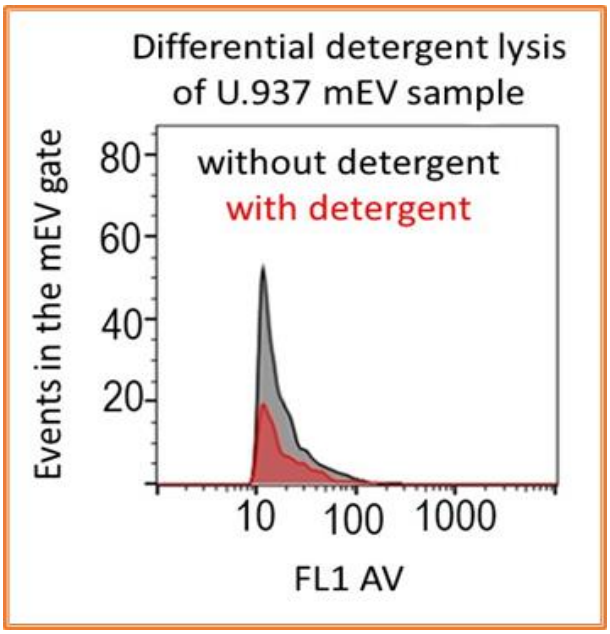

Figure 23 Differential detergent lysis of mEV samples. FL = fluorescent axis, AV = annexin V. Source: Marton N et al. [120]

3.2.3. Analysis of sEV and mEV samples by flow cytometry

Blood was taken from healthy (n=4) donors and RA (n=3) and PsA (n=3) patients. From blood samples, mEV and sEV samples were isolated by differential centrifugal (2 times 20.500 g and 100.000 g) and filtration steps. The expression of two important markers influencing osteoclastogenesis - OPG and RANKL - of sEV and mEV samples was detected by flow cytometry. Because sEVs cannot be detected with the FACS Calibur due to their size, they were bound to a larger support, 4 μm diameter 4 w/v% aldehyde-sulfate latex beads. The non-specific antibody binding sites of the beads were blocked with 1 w/v% bovine serum albumin (BSA) and 100 mM glycine. After adding unconjugated, anti-human OPG and anti-human RANKL primary antibodies, samples were incubated with secondary fluorescent antibodies (FITC = fluorescein isothiocyanate and PE = phycoerythrin) for 30 min at 4 ^{o}C in the dark. The mEV samples were also labeled with AV-FITC dye, which detects externalized phosphatydilserine, while sEV samples were also labeled with CD9-FITC antibodies. Antibodies were administered according to the manufacturer's recommendations. For cytometric measurement, samples were taken in 300 μL of buffer. Vesicle-free buffer dye controls, unstained samples, and in the case of sEV samples, empty latex bead samples were also used as controls during the measurements and to determine the fluorescent gate. In the case of mEV samples, differential detergent lysis was performed. No detergent was used in the sEV measurements, as the detergent sensitivity of sEV samples is significantly lower than that of the mEV samples [121].

There was no significant difference in vesicular OPG and RANKL content of the three donor groups. The mEV samples carried small amounts of the two molecules (**Figure 24**).

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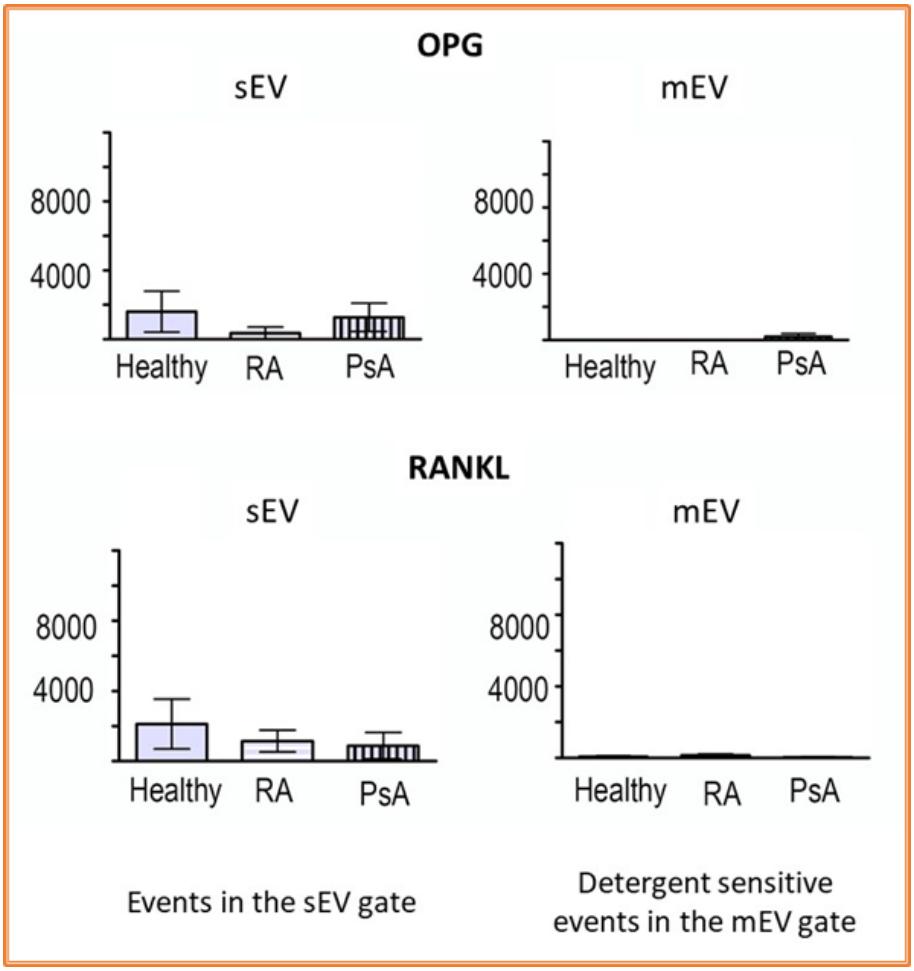

Figure 24 OPG and RANKL expression of human blood-derived sEV and mEV samples. The number of events for each measurement is calculated for 100.000 events, with samples labeled with only one type of fluorescent antibody at a time. Columns represent the mean fluorescence intensity (MFI) ± SEM. Source: Marton N et al. [120]

EVs carry donor cell-derived markers e.g. CD (cluster of differentiation) molecules. Transmembrane receptors are characteristic of significant amounts of cell types in the blood: monocytes (CD14) and neutrophil granulocytes (CD15) were detected by flow cytometry on sEV and mEV samples by using anti-CD14-PE and anti-CD15-FITC antibodies.

Significant differences in CD antigen expression were recorded in the three groups (healthy: n=6, RA: n=7, PsA: n=5). The sEV samples isolated from the blood of PsA donors expressed the most CD14. The CD15 receptor characteristic of neutrophil granulocytes was most abundant in PsA mEV samples (**Figure 25**).

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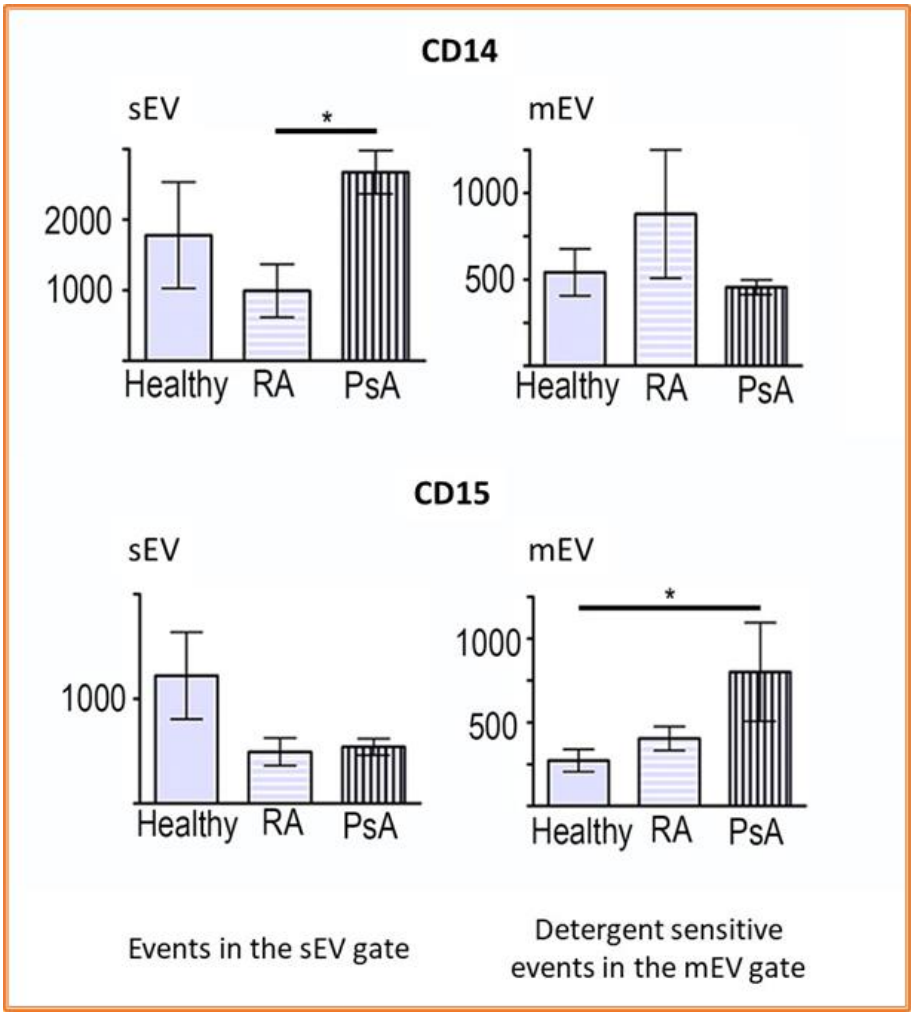

Figure 25 Detection of donor cell-derived CD14 and CD15 markers on sEV and mEV samples. The number of events for each measurement is calculated at 30.000 events. Columns represent the mean ± SEM, * $p<0,05$. Source: Marton N et al. [120]

DOI:10.14753/SE.2023.2780

4. DISCUSSION

Investigation of osteoclasts of healthy donors and patients suffering from RA or PsA is relevant since activation of osteoclasts is increased in these inflammatory arthropathies, resulting in local and systemic bone loss. There are some similarities in the pathomechanism of these diseases and RA and PsA patients might show similar symptoms. There are also differences in the immunopathogenesis of these diseases, in which different cells and different cytokines play a key role in RA than in PsA. Exploring these differences in protein levels, and investigating the EV profile in these diseases might help us to better understand the development of RA and PsA or to develop newer clinical examinations.

There are some published proteomic studies, which examined human blood-derives monocytes [55–57]. There are also mice originating data available - proteomics of osteoclasts differentiated from BMM cultures were studied [54]. A monocyte/macrophage osteoclastogenesis model, the RAW264.7 human cell line differentiates were also examined by proteomic methods [49]. The results of these papers, e.g. the upregulation of mitochondrial proteins during differentiation are in accordance with our data regarding osteoclast differentiation (**Figure 7**).

We found, that proteins upregulated in osteoclast samples have an important role mostly in osteoclast differentiation (e.g. transferrin receptor protein 1) or osteoclast function (e.g. integrin alpha-V) (**Figure 12 and Figure 14**). While proteins downregulated in osteoclast samples are connected mostly to immunological processes (e.g. cathepsin G) (**Figure 13 and Figure 14**).

We got different results not just in the case of comparing monocyte, preosteoclast, and osteoclast samples, but when comparing osteoclast samples of healthy donors and patients suffering from RA or PsA as well. RA and PsA-derived osteoclasts expressed significantly more proteins involved in immunological processes and proteins of the MHC complex (**Figure 16, Figure 17, Figure 18, and Figure 19**), which is in accordance with the fact that RA and PsA are inflammatory arthropathies. PsA-derived osteoclasts also expressed significantly more proteins involved in lipid metabolic processes (**Figure 19**). Sorokin et al. described a connection between psoriatic inflammation and lipid

oxidation [122]. Both RA and PsA-derived osteoclasts expressed significantly fewer proteins involved in metabolic processes than healthy-derived osteoclasts (**Figure 17 and Figure 19**). These results (reduced metabolic processes and elevated immunological features) altogether suggest that differentiation is inhibited to some extent in osteoclasts derived from RA or PsA monocytes and immunological functions are more retained in these cells compared to healthy derived osteoclasts.

Interestingly, a comparison of monocytes or preosteoclasts of healthy donors with examined patients showed separation to a lesser extent when using PCA analysis than the comparison of osteoclasts (**Figure 14**). This suggests that the environment from which the cells are differentiated is important, even when differentiating the cells under the same circumstances.

Besides proteomic differences, we also found that the pattern of circulating EVs was different in healthy individuals and patients suffering from RA or PsA. PsA blood-derived sEVs mostly originated from monocytes, while PsA blood-derived mEVs were from neutrophil granulocytes (**Figure 25**). In addition to the potential physiological functions, circulating EVs may also have possible pathogenetic effects [80,83], thus these findings might contribute to the development of newer diagnostic or prognostic clinical examinations.

Different EV populations are resistant to detergents to varying degrees. The lEVs and mEVs are much more sensitive to Triton-X 100 than sEVs. Utilizing this phenomenon, the presence of circulating mEVs was confirmed by the differential detergent lysis method previously described by Bence György et al [121,123] (**Figure 23**).

EVs can function as biologically active signalosomes, thus influencing the development and function of cells that interact with them. Osteoclasts can interact with EVs by endocytosis, fusion, or receptor-ligand interaction of cell surface molecules [124,125]. The effects of certain tumor cell-derived EVs on osteoclastogenesis have been studied [126], but the possible functions of circulating EVs in influencing osteoclastogenesis were not described earlier. Given this fact, and since, after the liver, bone tissue takes up the most EVs from the circulation [127], we aimed to study the effect of vesicles on osteoclasts. In healthy individuals, an extremely pronounced inhibitory effect on osteoclast cell formation was observed after SEC purified blood-derived sEV

treatment (**Figure 22**). This suggests that EVs present in the blood may regulate the activation of osteoclasts, including bone breakdown. This process may have a physiological role in bone regeneration after bone fractures when vascular injuries can allow more vesicles to flow close to bone cells than usual.

Blood-derived sEVs had a similar effect on *in vitro* osteoclastogenesis when treating monocytes isolated from RA patients with sEVs separated from the same patient's blood. In the case of PsA patients' samples, there was no inhibitory effect, which refers to a different pathogenesis of the two inflammatory rheumatological diseases [120].

In summary, investigating the human blood-derived osteoclasts of healthy and RA and PsA donors at the protein level revealed some significant functional differences in healthy, RA, and PsA samples. Analysis of the blood-derived EVs of healthy and RA and PsA patients showed differences in the pattern of EVs in healthy and unhealthy samples. SEC purified sEV treatment significantly inhibited osteoclast formation of healthy samples.

Investigatigation of the proteomics of osteoclast samples derived from healthy donors and patients suffering from RA or PsA may contribute to the development of novel therapies in the future based on the expression pattern differences.

DOI:10.14753/SE.2023.2780

5. CONCLUSIONS

The major conclusions of our studies are as follows:

5.1.

- We described for the first time the dynamic proteomic changes during osteoclastogenesis of healthy, RA and PsA blood-derived monocytes.

- Differences between the cells originating from healthy donors and RA and PsA patients - and differentiated under the same circumstances - are more prominent during the later stages of development.

- Differences in protein expression profiles of RA and PsA osteoclasts compared to healthy samples suggests that a diverse cytokine environment modifies osteoclast differentiation.

5.2.

- The sEVs in plasma, mostly released by blood cells, can interact directly with osteoclasts, leading to a pronounced inhibitory effect on osteoclast formation in healthy individuals. This process may have a physiological role in bone regeneration.

- Circulating vesicle patterns differ in healthy individuals with rheumatoid arthritis and psoriatic arthritis patients, which may contribute to the development of new diagnostic and prognostic tests.

DOI:10.14753/SE.2023.2780

6. SUMMARY

The knowledge of the pathomechanisms of rheumatoid arthritis and psoriatic arthritis inflammatory arthropathies is expanded.

In the framework of our research, we investigated the dynamic proteomic changes during osteoclastogenesis of healthy donors and RA or PsA patients. We found significant protein expression changes in osteoclasts derived from healthy, RA, or PsA monocytes, which reveal functional e.g. immune differences in these diseases. The separation of the samples based on diagnosis was bigger in further development stages, which suggests that the diverse cytokine environment from which monocytes were isolated has great significance on osteoclastogenesis, even under the same circumstances. Our results are unique in that this is the first study that describes the proteomics of human blood-derived osteoclasts and their differentiates. The differences in the expression patterns of healthy donors and RA and PsA patients' samples may contribute to the development of newer therapies in the future.

Based on our results, SEC purified sEVs derived from human plasma inhibit osteoclast formation *in vitro* in healthy individuals, which may have a physiological role in bone regeneration. We also found that there is a difference in the patterns of EVs in healthy donors and patients suffering from RA or PsA [120].

These data altogether suggest that the profile of extracellular vesicles and the phenotype of monocytes present in the circulation of RA and PsA patients are different.

Further studies including the analysis of osteoclasts derived from patients with pre-RA or difficult-to-treat RA [128] would deeper our knowledge of osteoclastogenesis in healthy and unhealthy circumstances as well.

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DOI:10.14753/SE.2023.2780

8. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

8.1. Publications relevant to the dissertation

I. **Kovács OT** & Tóth E, Ozohanics O, Soltész-Katona E, Marton N, Buzás EI, Hunyady L, Drahos L, Turu G & Nagy G. Proteomic Changes of Osteoclast Differentiation in Rheumatoid and Psoriatic Arthritis Reveal Functional Differences. Front Immunol. 2022;13. **IF(2022): 8.786**

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Cumulative impact factor of publications used for the dissertation: **15.507**

8.2. Other, not related publications

I. **Kovács OT**, Soltész-Katona E, Marton N, Baricza E, Hunyady L, Turu G & Nagy G. Impact of Medium-Sized Extracellular Vesicles on the Transduction Efficiency of Adeno-Associated Viruses in Neuronal and Primary Astrocyte Cell Cultures. Int J Mol Sci. 2021;22. **IF(2021): 6.208**

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Cumulative impact factor of all publications: **30.563**

Impact factor of first author publications: **14.994**

DOI:10.14753/SE.2023.2780

9. ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude to my supervisors, Prof. György Nagy and Dr. Gábor Turu for their permanent support and guidance during my Ph.D. work.

I am extremely grateful to Prof. Edit Buzás, Prof. László Hunyady, and Prof. Attila Mócsai directors for providing an opportunity for me to do TDK work, Ph.D. work, and being applied in their institutes.

I would like to offer my gratitude to Dr. Nikolett Marton for introducing me to the world of science.

Special thanks should be given to all colleagues at the Department of Genetics, Cell- and Immunobiology, and the Department of Physiology. I would especially like to thank Dr. Eszter Soltész-Katona for everything I could learn from her.

I am also indebted to the following people, co-authors who helped me during my work; Dr. Eszter Baricza, Dr. Eszter Tóth, Dr. László Drahos, and Dr. Olivér Ozohanics.

Last, but not least, I would like to thank my family - most of all to my mother, Mami - and my friends for everything, especially the endless support that I have been receiving throughout my study.

I would like to dedicate my dissertation to the memory of my father, Papi.