Rita Maria Mendes Raposeiro

Licenciatura em Biologia

Osteoclast activity in ankylosing spondylitis patients before and after TNF-blocking therapy

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Joana Ribeiro Caetano Lopes, PhD
Department of orthopedic research, Children's Hospital Boston and Genetics department, Harvard Medical School, Boston, MA, USA

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ABSTRACT

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease of the axial skeleton. The major outcome of this disease is defined by new bone formation, commonly observed in the ligaments of the intervertebral joints, that can lead to the formation of bony spurs, known as syndesmophytes.

Previous studies have shown that serum levels of TNF, IL-6 and IL-17 are increased in AS patients and may be implicated in the development of secondary osteoporosis, since these cytokines are able to induce osteoclast (OC) differentiation and, therefore, bone resorption.

In this work we aimed to assess the effects of TNF-blocking therapy in the systemic inflammatory environment of AS patients with active disease as well as in OC differentiation and activity. To accomplish this objective, we cultured circulating monocytes from AS patients, before and after therapy, under osteoclastogenic conditions and we performed two functional assays (TRAP staining and resorption pit assay) and analyzed the expression of osteoclast specific genes.

We have shown that AS patients with active disease have increased levels of pro-inflammatory cytokines when compared with healthy subjects. We also found that IL-17, TGF-β and osteoprotegerin are decreased after TNF-blocking therapy. Interestingly, we also observed that after TNF-blocking therapy the expression of some genes is favoring osteoclastogenesis and that differentiated OCs have increased resorption activity.

These results suggest that in active AS there may be an uncoupling between inflammation and OC activity that is reset by TNF-blocking therapy.

Keywords: ankylosing spondylitis, inflammation, osteoclastogenesis, TNF-blocking therapy

RESUMO

A espondilite anquilosante (EA) é uma doença inflamatória crónica do esqueleto axial. A característica principal desta doença é o excesso de formação óssea observada principalmente nos ligamentos das articulações intervertebrais que é capaz de levar à formação de pontes ósseas, conhecidas por sindesmófitos, entre as vértebras.

Estudos prévios mostraram que os níveis séricos de TNF, IL-6 e IL-17 estão aumentados em doentes com EA o que pode estar envolvido no desenvolvimento de osteoporose secundária, uma vez que estas citocinas são capazes de induzir a diferenciação de osteoclastos (OCs) e, consequentemente, a reabsorção óssea.

Neste trabalho propusemo-nos a avaliar os efeitos de agentes biológicos que inibem o TNF no ambiente inflamatório sistémico de doentes com EA activa assim como na diferenciação e actividade dos OCs. Para isso, colocámos em cultura monócitos circulantes de doentes com EA antes e depois da terapia com anti-TNF sob condições osteoclastogénicas, fizemos dois ensaios funcionais (marcação da TRAP e ensaios de reabsorção) e analisámos a expressão de genes específicos dos osteoclastos.

Com este estudo mostrámos que os doentes com EA activa têm níveis aumentados de citocinas pró-inflamatórias comparativamente aos controlos saudáveis e que depois da terapia com anti-TNF os níveis das citocinas IL-17, TGF-β e de osteoprotegerina estão diminuídos. Para além disto, ainda observámos que depois da terapia com anti-TNF a expressão de alguns genes tende a favorecer a osteoclastogénese e que os OCs diferenciados têm maior actividade reabsortiva.

Estes resultados sugerem que na EA activa pode haver um mecanismo que desconecte a inflamação e a actividade dos OCs e a ligação entre estes dois processos pode ser reestabelecida pela terapia com anti-TNF.

Palavras-chave: espondilite anquilosante, inflamação, osteoclastogénese, terapia com anti-TNF

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ABBREVIATIONS

AP-1	activator protein 1
AS	ankylosing spondylitis
ASAS	Assessment of Spondyloarthritis International Society
ASDAS	Ankylosing Spondylitis Disease Activity Score
ATPase	adenosinetriphosphatase
BASDAI	Bath Ankylosing Spondylitis Disease Activity Index
BASFI	Bath Ankylosing Spondylitis Functional Index
BMD	bone mineral density
BMP	bone morphogenetic protein
CA	carbonic anhydrase
Ca^{2+}	calcium
CD8	cluster of differentiation 8
cDNA	complementary DNA
Cl^-	chloride ion
CLCN7	chloride channel 7 alpha subunit
CO_2	carbon dioxide
CRP	C-reactive protein
CSF1R	colony stimulating factor 1 receptor
Ct	cycle treshold
CTSK	cathepsin K
CTX-I	C-terminal telopeptide of type 1 collagen
DC-STAMP	dendritic cell specific transmembrane protein
DKK-1	dickkopf-1
DMARD	disease modifying anti-rheumatic drug
DMEM	Dulbecco's Modified Eagle Medium
DXA	dual-energy X-ray absorptiometry
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
FBS	fetal bovine serum
Fig.	figure
H^+	proton
HLA	human leukocyte antigen
IFN-γ	interferon-gamma
IgG1	Immunoglobulin G
IL	interleukin
MAPK	mitogen-activated protein kinase

MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein-1 alpha
MRI	magnetic resonance imaging
mSASSS	modified Stoke Ankylosing Spondylitis Spinal Score
NFATc1	nuclear factor of activated T cells cytoplasmic 1
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSAIDs	non-steroidal anti-inflammatory drugs
OB	osteoblast
OC	osteoclast
OCP	osteoclast precursor
OPG	osteoprotegerin
OPN	osteopontin
P1NP	procollagen type 1 N propeptide
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
pH	negative logarithm for hydrogen ion
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
qPCR	quantitative polymerase chain reaction
RANK	receptor activator of nuclear factor-kB
RANKL	receptor activator of nuclear factor-kB ligand
RGD	arginylglycylaspartic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SNP	single nucleotide polymorphism
SOST	sclerostin
SpA	spondyloarthritis
TACE	tumor-necrosis factor converting enzyme
TGF	transforming growth factor
TNF	tumor necrosis factor
TRAF6	TNF receptor associated factor
TRAP	tartrate-resistant acid phosphatase
Wnt	wingless
$\alpha_V\beta_3$	alpha-$_V$ beta-$_3$ integrin

I. INTRODUCTION

1. Bone

Bone is a dynamic connective tissue with an extracellular matrix composed of mineral, collagen, water, noncollagenous proteins and also lipids. It has two phases, a mineral phase essentially composed by inorganic particles of hydroxyapatite embedded in an organic matrix mainly composed of type 1 collagen fibers (Boskey and Robey 2013). The composition and organization of the bone matrix gives this tissue unique mechanical properties such as stiffness, rigidity, ductility, tensile strength and exceptional lightness (Burr and Akkus 2014).

Apart from its mechanical role of supporting the whole body and allowing locomotion, bone also has many other important functions. It plays a protective role, shielding many vital organs and structures, like the bone marrow and spinal cord, and a metabolic role, regulating the homeostasis of calcium and phosphate. Bone is also a hematopoietic organ since it is a source of red blood cells and an endocrine organ, contributing to the global energy balance (Burr and Akkus 2014).

There are three main cell types present in bone: (i) the osteoclasts (OCs), giant multinucleated cells derived from macrophage-monocyte lineage that resorb bone by dissolving bone mineral and by enzymatically degrading extracellular matrix proteins and which will be deeply discussed in the next chapter; (ii) the osteoblasts (OBs), cells of mesenchymal origin responsible for bone formation that are able to produce the organic bone matrix and aid its mineralization; (iii) and the osteocytes, mature osteoblasts that become entrapped in the bone matrix and act as mechanosensors, a crucial function in the regulation of bone remodeling (Crockett, Rogers et al. 2011; Del Fattore, Teti et al. 2012).

1.1. Bone remodeling

Bone remodeling is a dynamic process where bone undergoes continuous destruction and formation, that occurs continually at several sites throughout the skeleton in response to mechanical and metabolic effects. It relies on the accurate balance between bone resorption by OCs and bone formation by OBs, whose functions must be tightly coupled not only quantitatively, but also in time and space (Del Fattore, Teti et al. 2012). However, this coupling process is only precise in adulthood since during early life bone formation exceeds bone resorption leading to a increase in bone mass, and with ageing bone resorption exceeds bone formation with a decrease of bone mass (Sims and Gooi 2008). An imbalance in bone resorption and formation can lead to several bone pathological conditions such as osteoporosis, characterized by low bone mineral density (BMD) and increased resorption, or osteopetrosis characterized by excess bone formation that leads to high BMD (Teitelbaum and Ross 2003).

Osteoblast differentiation and function, and therefore bone formation, is under the control of bone morphogenetic proteins (BMPs) and Wingless (Wnt) signaling pathways (Crockett, Rogers et al. 2011). BMPs belong to the transforming growth factor (TGF) beta superfamily and are involved in the early step of osteoblastogenesis (Del Fattore, Teti et al. 2012) whilst the canonical Wnt signaling

pathway is essential for osteoblast differentiation during skeletogenesis and continues to be important in mature osteoblasts (Crockett, Rogers et al. 2011).

Sclerostin (SOST) is a secreted protein synthesized by bone cells that are in contact with the bone mineral, specifically, late-stage osteoblasts and osteocytes. *In vitro*, SOST has been shown to inhibit osteoblast proliferation, impair mineralization by osteoblasts and to stimulate osteoblast apoptosis by interfering with Wnt and BMP signaling (Winkler, Sutherland et al. 2003; Sutherland, Geoghegan et al. 2004; van Bezooijen, Roelen et al. 2004). Therefore, SOST is capable of inhibiting bone formation (Crockett, Rogers et al. 2011). In addition to SOST, Dickkopf-1 (DKK-1) is also a soluble inhibitor of Wnt signaling pathway (Pinzone, Hall et al. 2009).

Various biochemical markers are used to assess bone metabolism. Bone turnover markers can be classified as markers of bone resorption that may be associated with OC function or may reflect bone matrix degradation and, on the other hand, markers of bone formation that are associated with osteoblast function and with type 1 collagen assembly. The most used markers are C-terminal telopeptide of type 1 collagen (CTX-I) cleaved during bone resorption, and procollagen type 1 N propeptide (P1NP) for bone formation. During bone formation, procollagen is cleaved at the N- and C-terminal ends so P1NP reflects the rate of new bone formation (Coates 2013).

2. Osteoclasts

OCs are specialized cells capable of resorb mineralized bone and play a key role in the process of bone modeling and remodeling that maintains the bone tissue in a healthy state by the continuous repair of microfractures (Mellis, Itzstein et al. 2011). Mature OCs are multinucleated giant cells that express high levels of tartrate-resistant acid phosphatase (TRAP), which has been widely used as a cytochemical marker of OCs and their precursors (Igarashi, Lee et al. 2002).

2.1. Osteoclastogenesis

OCs are derived from mononuclear hematopoietic myeloid lineage cells, which are formed in the bone marrow. Osteoclast precursors (OCPs) are attracted from the bone marrow to the bloodstream by chemokines and circulate there until they are recruited to the bone surface where they can differentiate into mature OCs upon exposure to stimuli (Ishii, Kikuta et al. 2010). When OCPs are attracted to the bone tissue they are exposed to chemokines and cytokines, such as macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-kB ligand (RANKL). These cytokines are expressed by several cells, including osteoblasts, and are essential for OC differentiation (Boyce 2013).

OCs are giant multinucleated cells formed by the fusion of mononuclear precursors of the macrophage-monocyte lineage. OC formation, survival and their unique ability to resorb bone are controlled by several signaling pathways. OC differentiation and survival can be influenced by multiple cytokines, but RANKL and M-CSF are essential during normal osteoclastogenesis (Wu, Humphrey et al. 2008).

The cytokine M-CSF is produced by osteoblasts and stromal cells and is critical in both OC differentiation and survival. M-CSF interacts with the c-fms tyrosine kinase receptor expressed on early OCPs, which is essential for their proliferation and survival (Kikuta and Ishii 2013) and stimulates the expression of RANK on these cells (Wu, Humphrey et al. 2008; Kikuta and Ishii 2013).

RANKL exists both in a soluble and a membranous form with both active forms. The soluble form (sRANKL) corresponds to the c-terminal part of membranous RANKL that may be produced either directly by stromal cells or osteoblasts through an alternative splicing followed by a secretion in the extra-cellular medium, or by a proteolytic clivage of membranous RANKL by tumor necrosis factor (TNF) converting enzyme - TACE (Kwan Tat, Padrines et al. 2004). Membrane-bound RANKL is expressed on the cell surface of osteoblasts, bone marrow stromal cells, fibroblasts, mammary epithelial cells and activated immune system cells (Wu, Humphrey et al. 2008). RANKL binds to its receptor RANK, a member of the TNF receptor superfamily present on OCPs and mature OCs and stimulates OC differentiation (Kwan Tat, Padrines et al. 2004). RANKL also binds to another TNF receptor family member, osteoprotegerin (OPG), which is produced by osteoblasts, bone marrow stromal cells, immune system cells and is a soluble molecule that inhibits osteoclastogenesis by acting as a decoy receptor for RANKL (Wu, Humphrey et al. 2008). The ratio of RANKL and OPG is critical for controlling OC differentiation and its bone-resorptive function (Kikuta and Ishii 2013) (Fig.I.1).

Upon binding to RANKL, RANK initiates several downstream signaling cascades through the essential adapter molecule TNF receptor-associated factor 6 (TRAF6), since RANK itself does not have kinase activity (Mellis, Itzstein et al. 2011). TRAF6 leads to activation of some critical transcription factors like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and activator protein 1 (AP-1) proteins c-Fos and c-Jun which are required for OC differentiation (Novack and Teitelbaum 2008). The target gene for both NF-kB and c-Fos in OCPs is nuclear factor of activated T cells cytoplasmic 1 (NFATc1), that has been proposed to function as the master transcription factor in osteoclastogenesis (Takayanagi, Kim et al. 2002). Also, activation of RANK signaling by RANKL leads to expression of genes required for the fusion of mononuclear OCPs, such as dendritic cell specific transmembrane protein (DC-STAMP), as well as of genes required for regulating the resorptive capacity of multinucleated OCs, including those encoding the vacuolar ATPase, the chloride/H^+ antiporter channel CLC7 and cathepsin K (Fig.I.1) (Mellis, Itzstein et al. 2011).

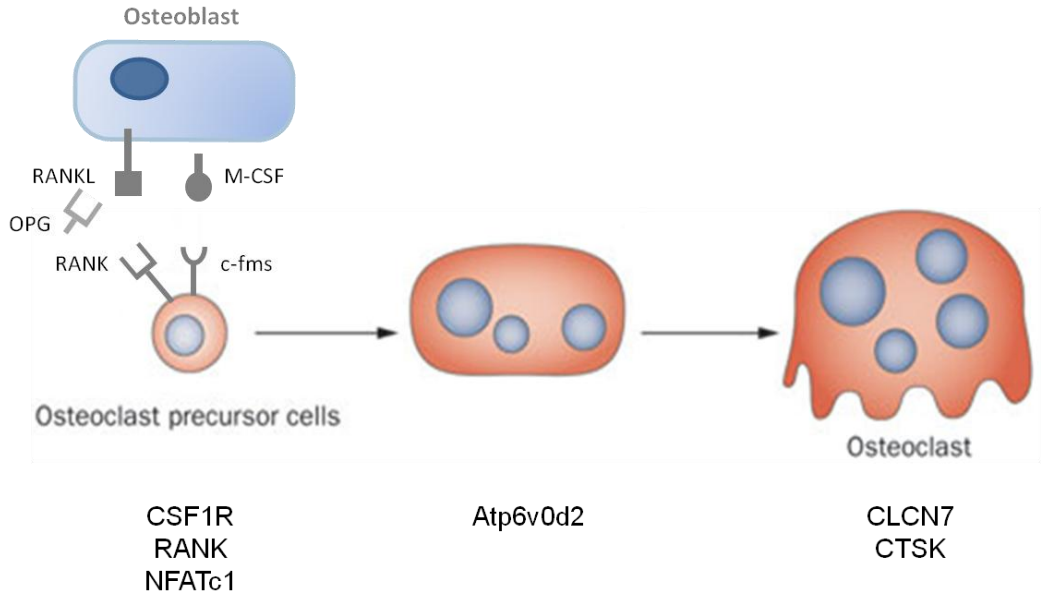

Figure I.1 - Osteoclast differentiation is dependent on RANKL and M-CSF. RANKL and M-CSF are produced, among other cells, by osteoblasts. These cytokines bind to their receptors, RANK and c-fms, respectively, which are present in osteoclasts precursors, inducing osteoclastogenesis. OPG is also produced by osteoblasts and acts as a decoy receptor for RANKL. Hence, the ratio of sRANKL/OPG is crucial for controlling osteoclast differentiation. Some genes expressed during osteoclastogenesis are also represented. CSF1R - Colony stimulating factor 1 receptor; RANK - receptor activator of nuclear factor-kB; NFATc1 - Nuclear factor of activated T-cells cytoplasmic 1; Atp6v0d2 - ATPase; CLCN7 - chloride channel ; CTSK - cathepsin K; RANKL - receptor activator of nuclear factor-kB ligand; M-CSF - macrophage colony-stimulating factor; c-fms - receptor of M-CSF; OPG - osteoprotegerin Adapted from (Nakashima and Takayanagi 2009).

2.2. Bone resorption

Bone resorption occurs in three stages: OC attachment to the bone matrix and cell polarization, initiation of resorption and cessation of resorption.

OC attachment to the bone surface is mainly dependent on integrins, in particular the vitronectin receptor - $\alpha_V\beta_3$ integrin (Mellis, Itzstein et al. 2011) - that recognizes the aminoacid motif Arg-Gly-Asp (RGD), a component of bone matrix proteins such as osteopontin (OPN) (Novack and Teitelbaum 2008). $\alpha_V\beta_3$ integrin mediates OC motility, adhesion to the bone matrix required for polarization of the resorptive machinery and formation of an isolated, acidified resorptive microenvironment. The mature OC is rich in mitochondria and is significantly polarized, with its nuclei localized in the opposite direction to the resorptive surface (Novack and Teitelbaum 2008). After attachment, and consequently polarization, osteoclasts organize their actin cytoskeleton into an actin ring in order to form a sealing zone (Luxenburg, Geblinger et al. 2007). They also develop integrin-mediated adhesion structures called podosomes which are columnar arrays of actin that are of major importance in bone resorption and osteoclast motility (Luxenburg, Geblinger et al. 2007; Takahashi, Ejiri et al. 2007). Due to podosome formation, the membrane enclosed by the sealing zone becomes a highly convoluted ruffled membrane where protons are transported by the vacuolar ATPase and proteases are secreted to demineralize and degrade the bone matrix, respectively (Fig.I.2) (Mellis, Itzstein et al. 2011). The ruffled membrane is formed by podosomes and by the fusion of acidic vesicles of the endocytic/lysosomal pathway that transport the resorption machinery such as cathepsin K, TRAP,

vacuolar ATPase and the Cl^-/H^+ antiporter channel CLC7 to the plasma membrane in direct contact with bone (Mellis, Itzstein et al. 2011). The resorptive space is highly acidified and this acidification process is initiated by carbonic anhydrase (CA) which generates H^+ and HCO_3^- ions (Fig.I.2). The protons (H^+) are transported out of the osteoclast and into the resorption lacuna by a proton pump (H^+ATPase) and a chloride channel (CLCN7) charge-coupled to H^+ATPase that acts as a chloride-proton antiporter (transports Cl^- into the resorptive lacuna). Hence, OCs need both functional H^+ATPase and CLCN7 in order to acidify the underlying resorption lacuna (Lee 2010). Intracellular pH is maintained by a Cl^-/HCO_3^- exchanger on the osteoclast's antiresorptive surface (Novack and Teitelbaum 2008) (Fig.I.2).

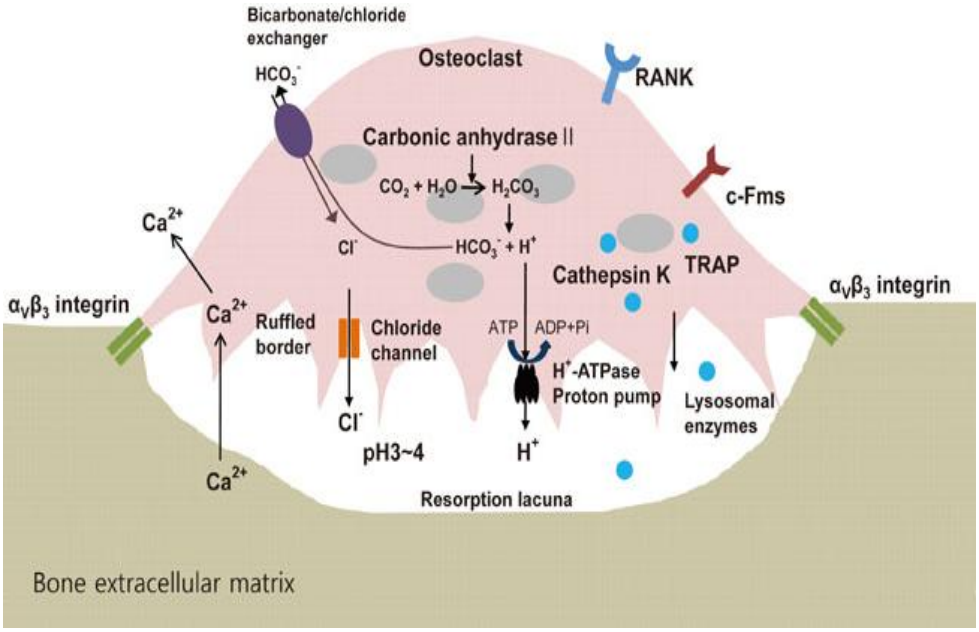

Figure I.2 - Bone resorption by osteoclasts. Osteoclast attach to bone surface through $\alpha_V\beta_3$ integrins to form a sealing zone. This surrounds a highly convoluted ruffled membrane which is responsible for bone resorption. The resorptive lacune is acidified by carbonic anhydrase (CA) which supplies protons for the proton pump (H^+ATPase). TRAP and cathepsin K regulate bone matrix resorption and collagen turnover. Intracellular pH is maintained by a Cl^-/HCO^{3-} exchanger on the OCs surface. In turn, electroneutrality is maintained by a ruffled membrane-located chloride channel (CLCN7) charge-coupled to the H^+ATPase, that transports Cl^- into the resorptive lacuna. Adapted from (Lee 2010).

TRAP is a soluble acid resistant phosphatase secreted by the OC and has a functional involvement in bone resorption, although its physiological substrates have not been identified yet. The candidates are phosphorylated tyrosine, sugar-phosphates as ATP, mannose-6-phosphates and bone matrix phosphoproteins, such as phosphorilated-OPN. Another possible function for TRAP is generating highly destructive reactive oxygen species (ROS) capable of destroying collagen and other proteins (Igarashi, Lee et al. 2002). The resorptive lacunae is highly acidified reaching a pH sufficiently low to dissolve the mineral phase, which occurs prior to degradation of the bone organic matrix, that consists mainly of type 1 collagen, by cathepsin K (Novack and Teitelbaum 2008).

Type 1 collagen degradation by cathepsin K releases CTX-I fragments (Borel, Gineyts et al. 2012). Once degraded, bone matrix products like CTX-I and calcium ions (Ca^{2+}), are internalized by

endocytosis in the central area of the ruffled membrane and are then transported to the functional secretory domain, opposed to the resorbing area, to be secreted. This mechanism allows OCs to remove large amounts of matrix-degradation products without losing their tight attachment to the underlying bone (Mellis, Itzstein et al. 2011).

When the calcium released from the bone surface is in higher concentration levels surrounding the OC, it leads to an inhibition of resorption as a consequence of disruption of cytoskeletal structures. Once the calcium concentration is diluted, the OC repolarizes and assembles a new sealing zone. OCs are highly motile cells and are able to migrate from one site of resorption to a new one. Their capacity to achieve this function is made possible by the ability to disassemble and reassemble their podosomes quickly. Optimal bone resorption depends upon these cycles of attachment, sealing zone formation and migration (Mellis, Itzstein et al. 2011).

2.3. Inflammation and osteoclast

Several systemic and local factors have been reported to influence osteoclasts and bone resorption. In a pathological condition with excessive activation of the immune system, such as inflammation, there are additional pro-inflammatory cytokines produced mainly by activated T cells. These cytokines are osteoclastogenic if they stimulate osteoclast differentiation or anti-osteoclastogenic if they inhibit osteoclasts (Zupan, Jeras et al. 2013).

A wide range of pro-inflammatory cytokines is produced by lymphocytes, macrophages and neutrophils during inflammation (Novack and Teitelbaum 2008). Among them is tumor necrosis factor (TNF), a multifunctional cytokine that plays a key role in host defense after infection or tissue injury and is also particularly important because it is one of the most potent osteoclastogenic cytokines produced in inflammation. It has been reported that TNF increases c-fms expression (Cho, Lee et al. 2010) and RANK expression in OCPs (Kitaura, Sands et al. 2004). TNF also stimulates RANKL signaling to induce expansion and differentiation of OCP cells and acts on non-hematopoietic cells such as stromal and endothelial cells to induce M-CSF and RANKL expression (Kwan Tat, Padrines et al. 2004; Gengenbacher, Sebald et al. 2008). Besides TNF, there are other pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and IL-17 which induce osteoclastogenesis since they act on osteoblasts to promote RANKL expression (Schett 2008; Zupan, Jeras et al. 2013).

OCs are key in inflammation-associated bone loss in rheumatic diseases and therefore, TNF-induced osteoclastogenesis is probably essential to the pathogenesis of disorders involving inflammation (Kitaura, Kimura et al. 2013).

3. Ankylosing spondylitis

Spondyloarthritis (SpA) are a diverse group of inter-related inflammatory arthritis that share multiple clinical features such as inflammatory back pain, asymmetrical peripheral oligoarthritis (mainly of the lower limbs), enthesitis and specific organ involvement such as anterior uveitis, psoriasis and chronic inflammatory bowel disease. The most common SpA is ankylosing spondylitis (AS), a chronic and severe inflammatory disease of unknown etiology that affects primarily the axial skeleton and

sacroiliac joints. AS affects between 0.1 and 1.4% of the Caucasian population, with a incidence of about 7.3 per 100 000 people per year (Bakland and Nossent 2013). It affects mainly young people at around 26 years of age and is more common in men than women, at a 2:1 ratio (Feldtkeller, Khan et al. 2003).

Characteristic symptoms of AS are morning stiffness, loss of spinal mobility, pain and fatigue which can be explained by spinal inflammation, structural damage, or both, and can lead to a decrease in quality of life, affecting patient's work, family and social activities and also increasing the risk of depression (Braun and Sieper 2007; Bond 2013). The strongest known contributing genetic factor for AS is the major histocompatibility complex (MHC) class I molecule HLA-B27 (Sheehan 2004). This antigen is critical for the presentation of antigen-derived peptides to cytotoxic CD8 T cells (Braem and Lories 2012). 90-95% of AS patients are positive for HLA-B27, however only about 5% of HLA-B27-positive subjects are at the risk of developing the disease suggesting that other genes might be involved in disease susceptibility (Zambrano-Zaragoza, Agraz-Cibrian et al. 2013).

AS outcome is defined, apart from chronic inflammation, by new cartilage and bone formation. Osteoproliferation can lead to the formation of bone spurs known as syndesmophytes when they are originated inside a ligament. Syndesmophytes are commonly seen in the ligaments of the intervertebral joints, which may result in the progressive ankylosis of the sacroiliac joints and the spine and in the fusion of the vertebrae leading to the appearance of a bamboo spine, a radiographic feature typical of this disease (Braem and Lories 2012) (Fig.I.3).

The major question in the molecular pathophysiology of AS is how inflammation and structural damage interact. Although AS is a disease characterized by excessive bone formation, it is also an inflammatory disease which is commonly associated with bone loss. This might seems rather paradoxal but osteopenia and/or osteoporosis is a common complication of AS, even in the early stages of disease (Bronson, Walker et al. 1998; El Maghraoui 2004). The formation of syndesmophytes occurs at the same location of resolved inflammatory lesions however there is no evidence of correlation between inflammation and new bone formation in AS (Schett, Coates et al. 2011).

It has been shown that systemic inflammatory mediators, such as TNF, IL-1, IL-6 and IL-17, play a role in modulating bone turnover in AS. Serum levels of TNF, IL-6 and IL-17 are increased in patients with AS which may be implicated in the development of secondary osteoporosis, since they are able to induce OC differentiation (Gratacos, Collado et al. 1994; Appel, Loddenkemper et al. 2009). Moreover, it has also been shown that OC number is increased in the bone of AS patients during acute stages of inflammation (Appel, Kuhne et al. 2006).

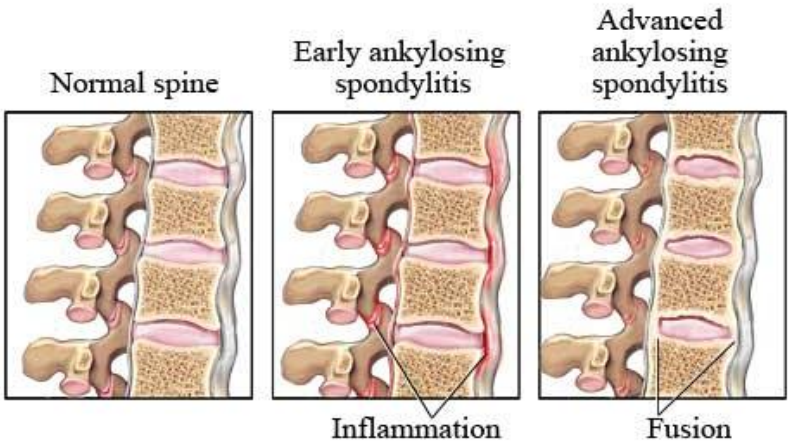

Figure I.3 - Ankylosing spondylitis outcome. Ankylosing spondylitis is a chronic inflammatory disease that affects mainly joints of the spine and sacroiliac joints and can cause fusion of the vertebrae in the spine. Adapted from (StemRx 2012)

3.1. Diagnosis and assessment

Diagnosis of AS patients is in accordance with the 1984 Modified New York criteria (see Appendix, Table VI.1), which includes radiographic evidence of sacroiliac joint changes and also clinical parameter of inflammatory back pain (van der Linden, Valkenburg et al. 1984).

To assess disease activity in patients with AS there are two major tools: the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and Ankylosing Spondylitis Disease Activity Score (ASDAS). The BASDAI is a questionnaire based on the patients' perception of fatigue, pain and stiffness. This index has limitations since they are fully patient oriented. ASDAS combines patient-reported assessments (like back pain, duration of morning stiffness, patient global assessment of disease activity, peripheral pain or swelling) and also acute phase reactants such as C-reactive protein (CRP) or, in alternative, erythrocyte sedimentation rate (ESR) (Ramiro 2011). The Bath Ankylosing Spondylitis Functional Index (BASFI) is used to evaluate the degree of functional limitation in AS patients and it is a questionnaire based on the patients' ability to perform several activities (Irons 2009).

3.2. Treatment

Non-steroidal anti-inflammatory drugs (NSAIDs) are highly effective in reducing symptoms as inflammation and acute and chronic pain and are considered a first-line therapy for AS (Poddubnyy and van der Heijde 2012). Biologic therapies such as anti-TNF drugs have been a great advance in the treatment of AS and have become the standard of care for AS patients.

Currently, inhibition of TNF is considered the most effective approach for reducing symptoms of inflammation and improving the clinical assessment in AS patients (Coates, Marzo-Ortega et al. 2010). At the moment, there are five biologic agents targeting TNF for patients with active AS with two different mechanisms of action: monoclonal antibodies against TNF and a recombinant TNF receptor fusion protein (Baraliakos and Braun 2012). Monoclonal antibodies against TNF such as the chimeric monoclonal IgG1 antibody infliximab, and the fully humanized monoclonal antibodies adalimumab and golimumab are antibodies with a constant human IgG_1 region (Fc) and a variable region (Fv) which

have the capacity to specifically bind to the soluble and transmembrane forms of TNF. Besides these, studies of certolizumab, another monoclonal TNF blocker, are being performed. The recombinant 75kD TNF receptor IgG1 fusion protein etanercept acts by inhibiting TNF binding to the cell-surface receptors, making TNF biologically inactive and impeding the cell responses mediated by TNF (Silva, Ortigosa et al. 2010).

It is described that anti-TNF therapy is able to halt mechanisms driving cartilage and bone destruction (Hreggvidsdottir, Noordenbos et al. 2014). However, recent studies found that in AS patients with TNF inhibition, ectopic bone formation is still observed, suggesting that additional factors are governing this effect (Schett, Coates et al. 2011; Taylan, Sari et al. 2012; Osta, Benedetti et al. 2014). Moreover, patients have decreased CTX-I levels after TNF-blocking therapy, with increased bone mineral density (BMD) in lumbar spine (Arends, Spoorenberg et al. 2012).

4. Aim

AS is characterized by syndesmophyte formation and increased BMD in the lumbar spine. In patients under TNF-blocking treatment a reduction of CTX-I levels was reported (Arends, Spoorenberg et al. 2012), which might reflect a decrease in osteoclast activity. Therefore, we hypothesize that there is an impairment in OC differentiation and activity in AS patients with active disease, that might be further inhibited by TNF-blocking therapy.

Our objectives were to understand how anti-TNF therapy regulates inflammatory environment in AS patients. Moreover we wanted to understand TNF-blocking effects on OC differentiation and activity in AS patients.

We performed cytokine and bone turnover marker analysis and also *ex vivo* differentiation of OCs from circulating monocytes from AS patients before and after TNF-blocking treatment. OC functional assays, microscopy studies, gene expression, were also performed in order to understand the effects of TNF antagonists in OC differentiation and activity.

II. MATERIALS AND METHODS

1. Patients

Patients who fulfilled the 1984 Modified New York criteria for AS followed up at the Department of Rheumatology, Hospital de Santa Maria, Centro Hospitalar Lisboa Norte, EPE, were included in this study. None of the recruited AS patients was under any biological disease modifying anti-rheumatic drug (DMARD) at the time of enrolment and all of them started TNF-blocking therapy at the time of first blood collection. For each patient a blood sample was collected for peripheral blood mononuclear cells (PBMCs) isolation and serum storage. All patients signed an informed consent and filled a clinical questionnaire to assess demographical and clinical characteristics such as symptoms duration, ESR, CRP, presence of sacroiliitis, ASDAS, BASDAI, BASFI and HLA-B27 status. Patients under NSAIDs and DMARDs were not excluded from this study. These patients were followed up for a minimum of six months, when a second blood sample was collected.

A group of age and sex-matched controls was also included and a blood sample was collected in order to isolate PBMCs and for serum storage.

This study did not affected the regular follow-up of the patients. This project was approved by the ethical committee of Hospital de Santa Maria, Centro Hospitalar Lisboa Norte, EPE, and all proceedings were performed in accordance with the declaration of Helsinki, as amended in Fortaleza, 2013 (WMA 2013).

2. PBMC isolation

PBMCs from AS patients and control subjects were isolated by density gradient centrifugation. Heparinized peripheral blood was diluted with phosphate buffered saline (PBS) 1x and then carefully laid over Ficoll-Paque (Histopaque 1077, Sigma-Aldrich, USA). The density gradient was centrifuged at 980g for 35 minutes at room temperature in a swinging-bucket rotor without acceleration or brake. The mononuclear cell layer (including lymphocytes and monocytes) was then collected and washed twice with PBS 1x. The viable cells were counted in an haemocytometer using trypan blue 0.1% (Sigma-Aldrich, USA).

3. Cell culture

The isolated PBMCs were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, UK) previously supplemented with 5000 U Penicilin/Streptomicin (Invitrogen, UK), 2 mM L-Glutamin (Invitrogen, UK) and 10% Fetal Bovine Serum (FBS; Invitrogen, UK) in both 96 and 24 well flat bottom plates (Nunc, German) and incubated in a humidified atmosphere at 37ºC, 5% CO_2. Cells were plated in 96-well culture plates at a density of $7.0x10^5$ cells/well and in 24-well culture plates at a density of $1.5x10^6$ cells/well.

Cells cultured in 24 well-plates were used for RNA extraction at the first day of culture. In the 96-well culture plates, cells were seeded on top of bovine cortical bone slices (Immuno Diagnostic

Systems Ltd, UK) to extract RNA and to perform functional assays (TRAP staining and resorption pit assay) at days 7, 14 and 21 of culture (Fig.II.1A).

PBMCs were left overnight for OCPs to adhere on bone slices (Holloway, Collier et al. 2002). On the following day (day 1 of culture) medium was changed. Two culture conditions were then created, a control condition with DMEM only (hence called unstimulated) and a stimulated condition with DMEM supplemented with M-CSF 25 ng/mL (Peprotech, Rocky Hill, NJ). Three days later, medium was again changed and in the stimulated condition DMEM was supplemented with M-CSF (25 ng/mL), sRANKL (50 ng/mL; Peprotech, Rocky Hill, NJ), dexamethasone (10 nM; Sigma Aldrich, St. Louis, MO) and TGF-β (2.5 ng/mL; R&D Systems, Minneapolis, MN) in order to differentiate the osteoclast precursors into mature osteoclasts (Fig.II.1B) (Takuma, Kaneda et al. 2003).

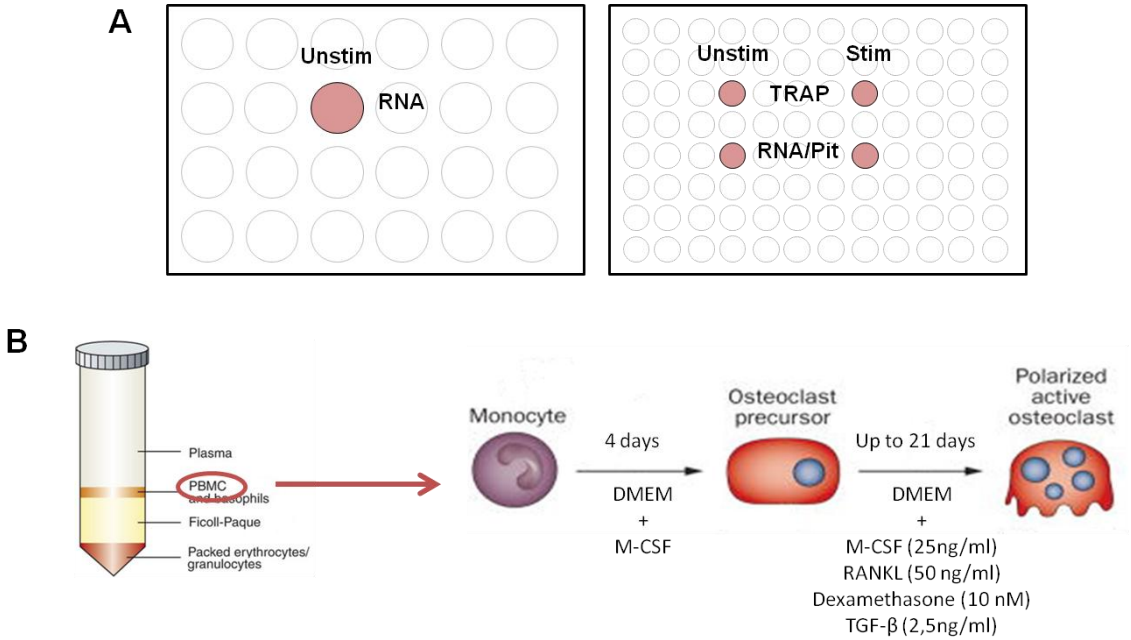

Figure II.1 - A Design of 24 and 96-wells culture plates. Cells cultured in 24 well-culture plates with unstimulated (Unstim) condition (DMEM only) used for RNA collection of first day of culture and in 96-well plates with both unstimulated and stimulated (Stim) condition (with M-CSF, RANKL, dexamethasone and TGF-β) in order to collect RNA from cells and to perform functional assays (TRAP staining and resorption assay) at days 7, 14 and 21. **B** Experimental design. Isolated PBMCs were plated under a stimulated condition, DMEM supplemented with M-CSF during the first four days of culture and after that with M-CSF, RANKL, dexamethasone and TGF-β until day 21.

The culture medium was then changed twice a week. Cells cultured on bone slices for 7, 14 and 21 days (Holloway, Collier et al. 2002; Husheem, Nyman et al. 2005) were used for functional assays and one well per condition, per time-point was collected for RNA extraction.

4. Functional assays

4.1. TRAP staining

Tartrate-resistant acid phosphatase (TRAP) is a cytoplasmic enzyme that is highly expressed by osteoclasts (Igarashi, Lee et al. 2002).

TRAP staining of pre-OCs and OCs was performed using the Acid Phosphate, Leukocyte Kit (TRAP, Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions. Cells are fixated briefly with a fixative solution with paraformaldehyde 4% and incubated with TRAP substrate. Acid phosphatase hydrolyzes the substrate naphthol AS-BI phosphoric acid which couples with a dye such as fast garnet GBC producing a red precipitate at the site of the enzyme activity (Rodak, Fritsma et al. 2007). After staining, the cell cytoplasm develops a red colour while the nuclei remains unstained. This assay allows the distinction between pre-OCs (with only one or two nuclei) and mature OCs (containing three or more nuclei).

Bone slices from both TRAP staining and resorption functional assay were photographed with the brightfield microscope Leica DM2500 (Leica, Germany) under a 10x magnification objective.

In the TRAP assay, the number of TRAP-positive cells (pre-OCs - with <3 nuclei - or OCs with ≥3 nuclei per cell) was counted at five sites of the bone slice with the area of 1296x972µm each. Both osteoclast precursors and mature osteoclasts were manually counted for each time-point per condition using ImageJ software (NIH, Bethesda, MD).

4.2. Resorption assay

For measurement of resorbed area in the resorption assay, the bone slices were washed with PBS 1x, incubated in 5% sodium hypochlorite for 15 minutes and incubated with 0.1% toluidine blue (Sigma-Aldrich, St. Louis, MO) for 2 minutes in the dark.

The resorption pits are acidic structures and therefore will develop a blue to purple colour (Sridharan and Shankar 2012).

In this assay, quantification of resorption pits was done at five sites of the bone slice with the area of 1296x972µm and were manually traced using ImageJ (NIH, Bethesda, MD), a mean value of the resorb area was also calculated and expressed in %.

5. Gene expression

5.1. RNA extraction and quantification

RNA was extracted from cells at days 1, 7, 14 and 21 of culture and both from unstimulated and stimulated (M-CSF, RANKL, Dexamethasone and TGF-β) conditions using NZYol (NZYTech, Lda. Portugal) according to the manufacturer's instructions. NZYol is a ready-to-use reagent for the isolation of total RNA from cells and tissues since it is capable of disrupting cells, exposing their cellular components, and maintaining the integrity of the extracted RNA (NZYtech 2013). Cells disrupted in NZYol are incubated with chloroform (VWR, Radnor, PA) and centrifuged. After centrifugation the organic phase containing nucleic acids is transferred to a new tube and incubated with isopropyl alcohol (VWR, Radnor, PA) to precipitate RNA. RNA is then washed with ethanol 75% and finally solubilised in RNase/DNase free water (Gibco, Grand Island, NY).

Following RNA extraction, total RNA concentration and purity was quantified using Nanodrop 1000 (Thermo Scientific, Waltham, MA). RNA was stored at -80ºC until further use.

5.2. cDNA synthesis

Complementary (c)DNA was synthesized at a concentration of 0.6 ng/μL using the DyNAmo™ cDNA Synthesis Kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. cDNA was synthesized from 0.6ng/uL of total RNA using the reverse transcriptase M-MuLV RNase H^+ and random hexamers. According to the protocol, cDNA synthesis occurs at 37ºC for 30 minutes, followed by the reaction termination, which occurs at 85ºC for 5 minutes in order to inactivate the reverse transcriptase M-MuLV RNase H^+ to prevent it from inhibiting qPCR reaction. cDNA was stored at 4ºC.

5.3. qPCR and gene expression quantification

Gene expression was studied by quantitative PCR (qPCR), which provides quantitative and real-time analysis of DNA samples from different sources.

Genes that encode for osteoclast proteins such as CSF1R, RANK, NFATc1, ATP6v0d2, CLCN7 and CTSK were studied. Ribossomal RNA 18s (rRNA 18s) was chosen as the housekeeping gene. Primers were designed using the primer-BLAST (Ye, Coulouris et al. 2012) software and having into consideration amplicon size less than 100bp, 60ºC annealing temperature and exon-exon junction, which was used to exclude unprocessed mRNA or possible genomic DNA contamination. Primer sequences and characteristics can be found in Appendix, Table VI.2.

The DyNAmo™ Flash SYBR Green qPCR Kit (Thermo Scientific, Waltham, MA) was used according to the manufacturer's instructions. SYBR Green binds to the double stranded amplified product of qPCR and emits fluorescence, which is then detected by the thermocycler's detector (Corbett Rotor-Gene 6000, Qiagen, Netherlands). Briefly 1.8ng of cDNA are used for the PCR. The cycling reaction starts with an initial denaturation at 95ºC for 7 minutes to ensure a complete reactivation of the hot-start DNA polymerase and denaturation of the cDNA template, followed by a denaturation at 95ºC for 10 seconds. After these, a combined annealing and extension for 45 seconds at 60ºC was performed for fifty cycles. The reactions were validated by the presence of a single peak in the melt curve analysis. In order to analyze the results the Corbett Rotor-Gene 6000 Series Software 1.7 (Qiagen, Netherlands) was used.

The efficiency of qPCR was analysed using the standard curve method. For each primer pair, a standard curve was made using as standard RNA trabecular bone samples from subjects with normal bone mineral density (BMD) and without risk factors for osteoporosis or other bone-related diseases. The initial DNA quantity was 30ng and then serial 1:5 dilutions down to 0.24ng were used for the standard curve. qPCR results were analyzed using the standard curve method. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold and exceed the background level. The efficiency of the qPCR should be 100%, meaning that for each cycle, the amount of product doubles. A good reaction should have an efficiency of 90-100%, which corresponds to a slope between -3.58 and -3.10. The conversion of the Ct value in relative expression levels was performed with the slope and the Y intersect extracted from the standard curve and applying the equation $10^{(Ct\text{-}Y\ intersect)/slope}$ (Wong and Medrano 2005). The values obtained were normalized with the housekeeping gene 18s rRNA.

6. Cytokine and bone turnover marker quantification

Serum levels of cytokines and bone turnovers markers were evaluated in the serum of both AS patients and control subjects.

Soluble (s)RANKL, OPG, DKK-1, SOST, CTX-I and P1NP levels were measured in the serum by sandwich enzyme-Linked Immunosorbent Assay (ELISA) (sRANKL, OPG, DKK-1 and SOST from Biomedica, Vienna, Austria; CTX-I and P1NP from SunRed Biological Technology, Shangai, China).

For cytokines found in serum, such as interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-22, IL-23, interferon (IFN)-γ, macrophage inflammatory protein (MIP)-1α, monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β, the multiple analyte detection kit Flow Cytomix (eBioscience, San Diego, CA) was used according to the manufacturer's instructions. This assay is based on the ELISA method, however the capture antibody is conjugated with a microbead. Data was acquired on a LSR Fortessa II (BD Biosciences, Franklin Lakes, NJ) and was analyzed using FlowCytomix Pro 2.3 Software (eBioscience, San Diego, CA).

7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 Software (GraphPad Software, Inc., La Jolla, CA). Data is represented as median [interquartile range]. For two variable comparison t-test or Mann-Whitney test were used according to normality distribution. For paired variables Wilcoxon matched pairs test was also used. For more than two variables Kruskall-Wallis test was used with post-hoc Dunn's test. Categorical variables are expressed as relative frequencies and to test them the Chi-Square test was used. p-values lower than 0.05 were considered statistically significant.

III. RESULTS

1. Patient characteristics

This study included 24 patients diagnosed with AS, 13 men and 11 women, with a median age of 41 [35.25-47.25] years and with a median duration of symptoms of 10 [6.5-18.5] years, as shown in table III.1. Twelve of the 24 patients were followed-up for at least six months after the beginning of TNF-blocking therapy. One patient was only included at follow-up.

Twenty-three healthy age and gender matched subjects were also included in this study, 13 males and 10 females with a median age of 41 [34.50-52.00] years.

As shown in table III.1, half of patients at baseline and 46.15% at follow-up were positive for HLA-B27. Most AS patients (83.33% at baseline) have sacroiliitis (seen by x-ray or MRI) and 33.33% of baseline patients have peripheral involvement.

Table III.1 - Demographic and clinical data of ankylosing spondylitis patients.

Ankylosing spondylitis patients			
Age (years)	41 [35.25-47.25]		
Symptoms duration (years)	10 [6.5-18.5]		
	Baseline N=24	**Follow-up N=13**	**p value**
Gender (F:M)	11:13	4:9	p=0.4908
HLA-B27 (% positive)	50%	46.15%	p=1.0000
Sacroiliitis (% yes)	83.33%	84.62%	p=1.0000
Peripheric involvement (% yes)	33.33%	38.46%	p=1.0000
NSAIDs (% yes)	75%	69.23%	p=0.7158
DMARDs (% yes)	50%	46.15%	p=1.0000
Corticosteroids (% yes)	8.33%	7.69%	p=1.0000
ESR (mm/h)	26 [13-48]	7 [4-15]	p=0.0001
CRP (mg/dl)	0.72 [0.11-1.91]	0.13 [0.04-0.47]	p=0.043
ASDAS	3.4 [2.6-4]	1.75 [1.35-1.95]	p<0.0001
BASDAI	5.5 [4.1-7.4]	3.2 [1.6-4.05]	p<0.0001
BASFI	5.89 [3.60-7.55]	3.90 [1.39-5.20]	p=0.0323

Results are expressed in median [interquartile range 25-75]. Results were analyzed with unpaired student's t test or Mann-Whitney test. p-values lower than 0.05 are considered statistically significant. F - female; M - male; NSAIDs - non-steroidal anti-inflammatory drugs; DMARDs - disease-modifying anti-rheumatic drugs; ESR - erythrocyte sedimentation rate; CRP - C-reactive protein; ASDAS - ankylosing spondylitis disease activity score; BASDAI - bath ankylosing spondylitis disease activity index; BASFI - bath ankylosing spondylitis functional index.

The majority (75%) of patients at baseline were under NSAIDs, whereas DMARDs were given to half of patients at baseline. Corticosteroids were the least common treatment for these patients, with only 8.33% of baseline patients receiving it (Table III.1). Both inflammatory markers, ESR and CRP, were significantly decreased in follow-up patients when compared to baseline (p=0.0001 and p=0.043,

respectively). ASDAS, BASDAI and BASFI were also significantly decreased after anti-TNF therapy ($p<0.0001$ for ASDAS and BASDAI and $p=0.0323$ for BASFI).

When we consider only the 12 paired AS patients, which were included both at baseline and follow-up, the median age is 40 [35.25-49.50] years with a median symptoms duration of 10 [7-21] years, as shown in table III.2. This group included 8 men and 4 women and 41.67% of patients were positive for HLA-B27.

Table III.2 - Demographic and clinical data of paired ankylosing spondylitis patients.

Paired ankylosing spondylitis patients (N=12)			
Age (years)	40 [35.25-49.50]		
Symptoms duration (years)	10 [7-21]		
Gender (F:M)	4:8		
HLA-B27 (% positive)	41.67%		
Sacroiliitis (% yes)	91.67%		
Peripheric involvement (% yes)	41.66%		
NSAIDs (% yes)	75%		
DMARDs (% yes)	66.67%		
Corticosteroids (% yes)	8.33%		
	Baseline	**Follow-up**	**p-value**
ESR (mm/h)	36.50 [15.25-53.75]	9 [4-15.75]	p=0.0036
CRP (mg/dl)	1.43 [0.18-3.22]	0.12 [0.04-0.50]	p=0.0049
ASDAS	3.85 [2.30-4.28]	1.8 [1.5-2]	p=0.0006
BASDAI	4.45 [3.73-7.65]	2.85 [1.53-4.08]	p=0.0018
BASFI	6.19 [4.6-7.6]	3.9 [1.07-5.3]	p=0.0048

Results are expressed in median [interquartile range 25-75]. Results were analyzed with paired student's t test or Wilcoxon matched pairs test. p-values lower than 0.05 are considered statistically significant. F - female; M - male; NSAIDs - non-steroidal anti-inflammatory drugs; DMARDs - disease-modifying anti-rheumatic drugs; ESR - erythrocyte sedimentation rate; CRP - C-reactive protein; ASDAS - ankylosing spondylitis disease activity score; BASDAI - bath ankylosing spondylitis disease activity index; BASFI - bath ankylosing spondylitis functional index.

Sacroiliitis is present in 91.67% of these patients and 41.66% also have peripheral involvement. 75% of the patients were under NSAIDs, 66.67% DMARDs and only 8.33% corticosteroids.

As shown in table III.2, both ESR and CRP are significantly decreased after anti-TNF therapy. ESR decreased from 36.50 [15.25-53.75] mm/h at baseline to 9 [4-15.75] mm/h at follow-up ($p=0.0036$) and CRP diminishes from 1.43 [0.18-3.22] mg/dl to 0.12 [0.04-0.50] mg/dl after TNF-blocking therapy ($p=0.0049$).

ASDAS, BASDAI and BASFI have a significant decrease after anti-TNF therapy (table III.2). ASDAS significantly decreased from 3.85 [2.30-4.28] to 1.8 [1.5-2] at follow-up ($p=0.0006$), BASDAI was reduced from 4.45 [3.73-7.65] to 2.85 [1.53-4.08] after TNF-blocking therapy ($p=0.0018$) and likewise BASFI decreased from 6.19 [4.6-7.6] at baseline to 3.9 [1.07-5.3] at follow-up ($p=0.0048$).

2. Cytokine, chemokine and bone turnover markers in circulation

It is known that the cytokine milieu has a major influence on the osteoclast activity and on bone resorption.

Pro-inflammatory cytokines as IL-6, IL-1β and TNF levels are all significantly elevated in untreated AS patients in comparison to controls (IL-6 p=0.0002; IL-1β p=0.0001; TNF p=0.0014) as seen in Fig.III.1. Although levels of these three cytokines are also lower in follow-up patients when compared to baseline there were no significant differences. Levels of IL-23 were also increased in the baseline when compared with follow-up patients and healthy subjects, but the differences were not statistically significant (Fig.III.1).

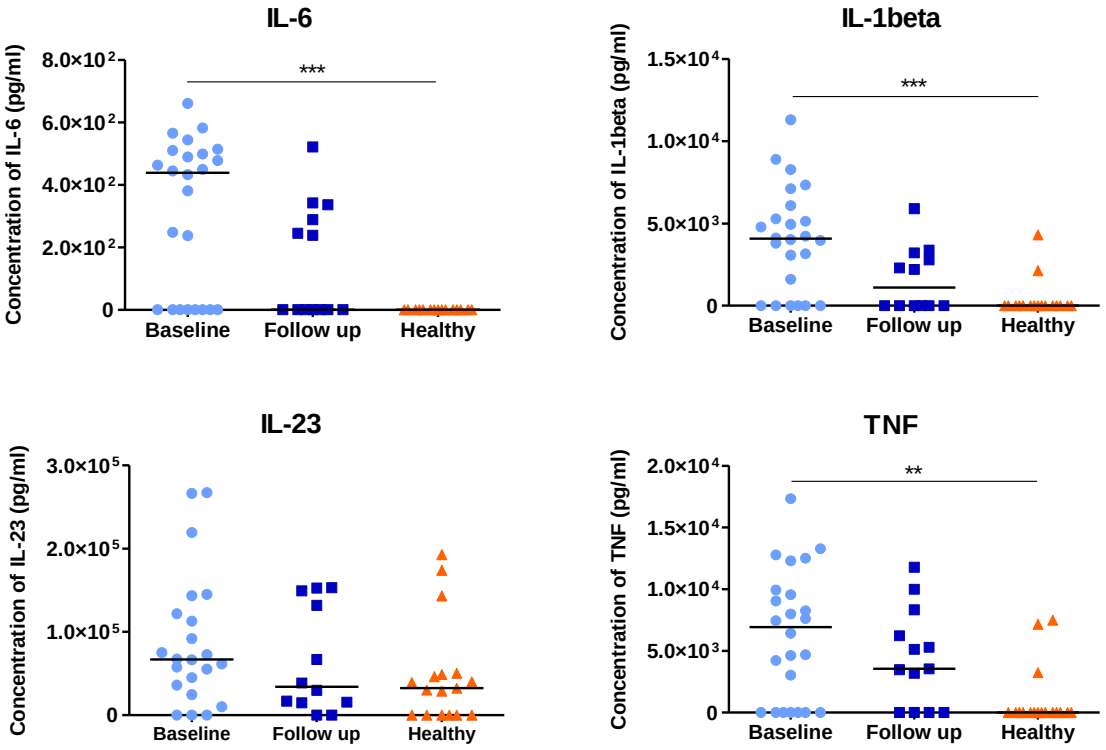

Figure III.1 - Serum levels of IL-6, IL-1β, IL-23 and TNF of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Bars represent median values. Results were analyzed with Kruskal-Wallis test. p-values lower than 0.05 are considered statistically significant. **p<0.01, ***p<0.001. IL – interleukin; TNF - tumor necrosis factor.

The levels of pro-inflammatory cytokines IL-12p70 and IL-17A were significantly increased in the baseline group when compared to healthy subjects (IL-12p70 p=0.0025; IL-17A p=0.0013). IL-22 levels were significantly different when the three groups are compared (p=0.0494), showing higher levels at the baseline and follow-up groups in relation to controls (Fig.III.2).

The levels of IL-12p70 and IL-22 were very similar between baseline and follow-up patients. The levels of IL-17A were decreased after anti-TNF therapy when compared to baseline but the difference was not statistically significant when comparing all groups.

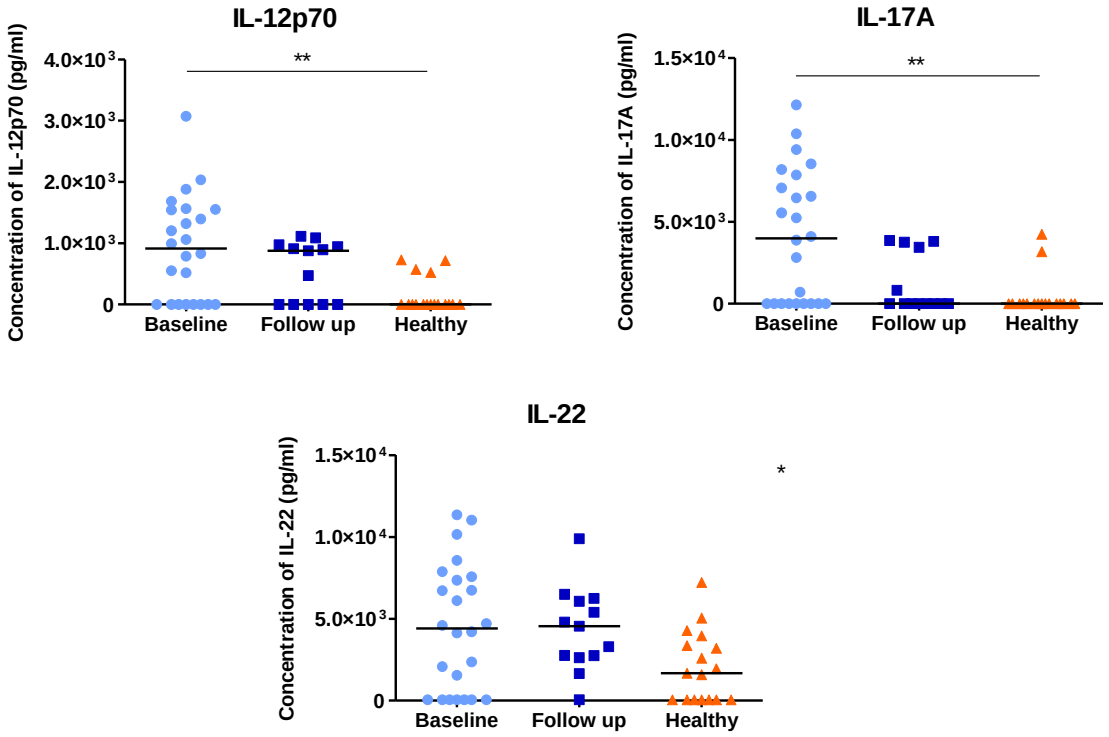

Figure III.2 - Serum levels of IL-12p70, IL-17A and IL-22 of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Bars represent median values. Results were analyzed with Kruskal-Wallis test. p-values lower than 0.05 are considered statistically significant. *p<0.05, **p<0.01. IL – interleukin.

As shown in Fig.III.3, chemokines as MCP-1, MIP-1α, IL-8 and IL-18 were all significantly increased in baseline AS patients when compared to healthy subjects (MCP-1 p=0.0074; MIP-1α p=0.0218; IL-8 p=0.0004; IL-18 p<0.0001). These chemokine levels were diminished in the follow-up group in comparison to the baseline group, but differences were not statistically significant.

IL-18 concentration was significantly higher both at baseline (p<0.0001) and in AS patients treated with anti-TNF therapy (p<0.0001), as compared with controls (Fig.III.3).

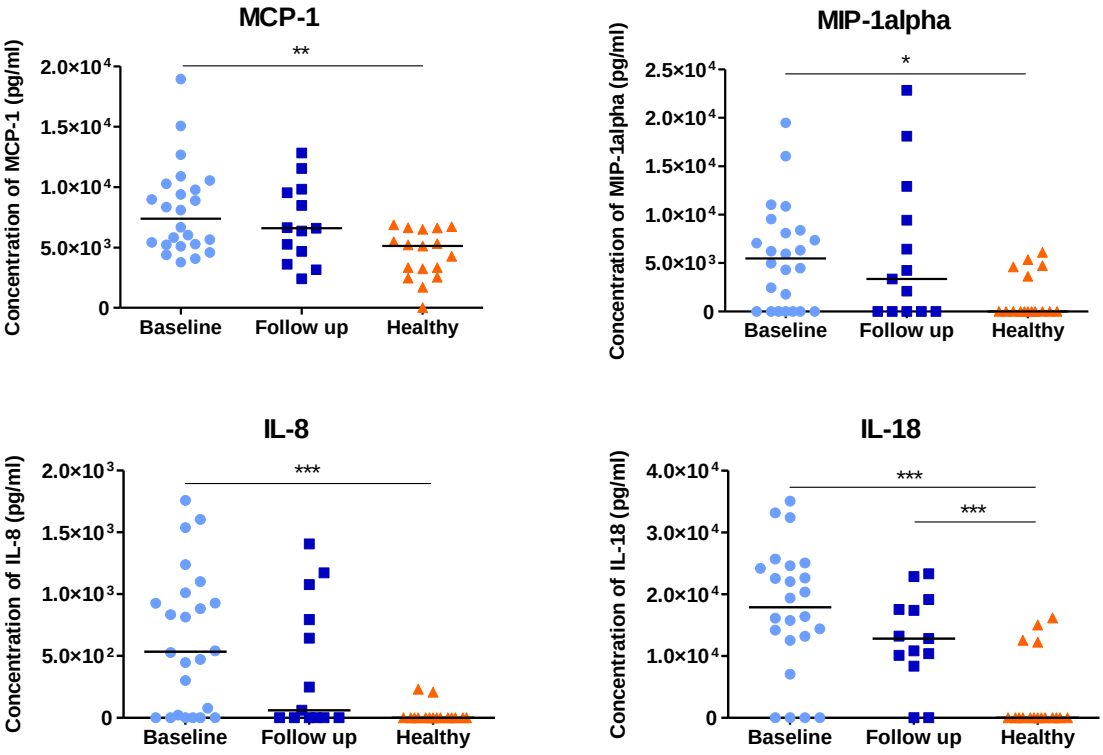

Figure III.3 - Serum levels of MCP-1, MIP-1α, IL-8 and IL-18 of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Bars represent median values. Results were analyzed with Kruskal-Wallis test. p-values lower than 0.05 are considered statistically significant. *p<0.05, **p<0.01, ***p<0.001. MCP-1 - monocyte chemotactic protein-1; MIP-1α - macrophage inflammatory protein-1α; IL – interleukin.

The cytokines TGF-β, IFN-γ and IL-2 levels were all significantly different between baseline and healthy groups (TGF-β p=0.0060; IFN-γ p<0.0001; IL-2 p<0.0001), with significant higher levels in AS patients (Fig.III.4). Cytokine levels were decreased in follow-up patients when compared to the baseline, but without reaching statistical significance when all groups are compared.

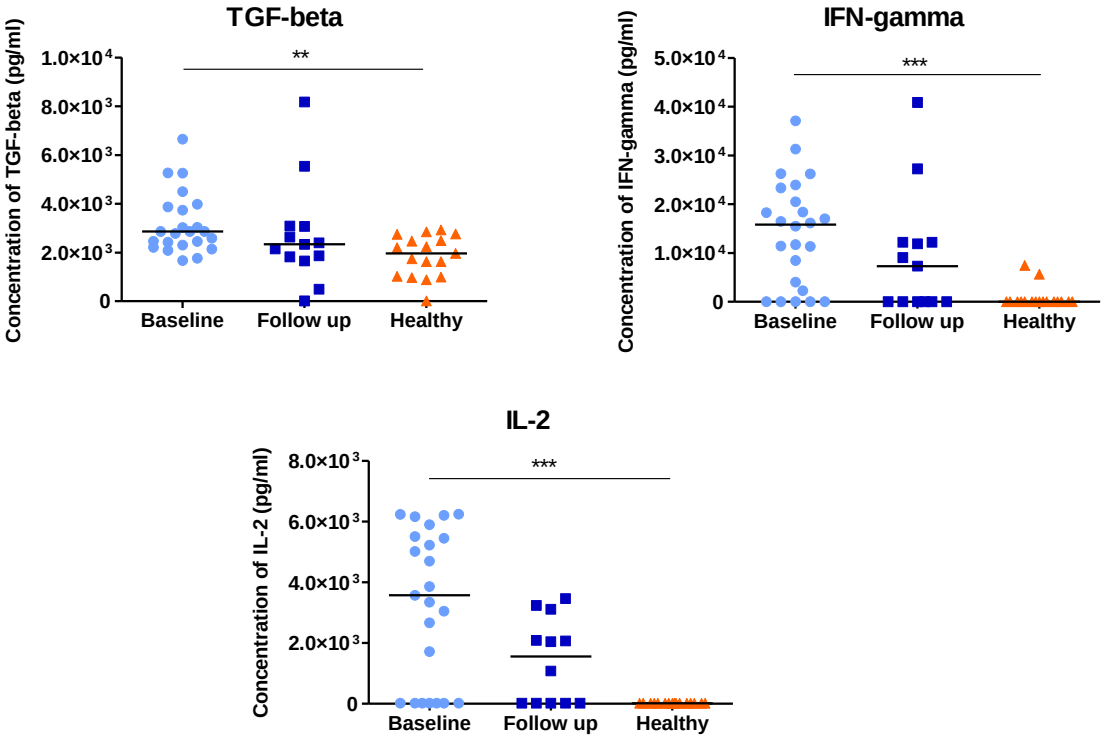

Figure III.4 - Serum levels of TGF-β, IFN-γ and IL-2 of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Results were analyzed with Kruskal-Wallis test. p-values lower than 0.05 are considered statistically significant. Bars represent median values. **p<0.01, ***p<0.001. TGF-β - transforming growth factor-β; IFN-γ - interferon-γ; IL – interleukin.

Measuring anti-inflammatory cytokines IL-4 and IL-10 levels, we observed that baseline patients have significantly higher levels than healthy subjects (IL-4 p=0.0003; IL-10 p=0.0001), as seen in Fig.III.5. Moreover, both IL-4 and IL-10 were decreased in the follow-up group in relation to baseline, but the differences were not statistically significant (Fig.III.5).

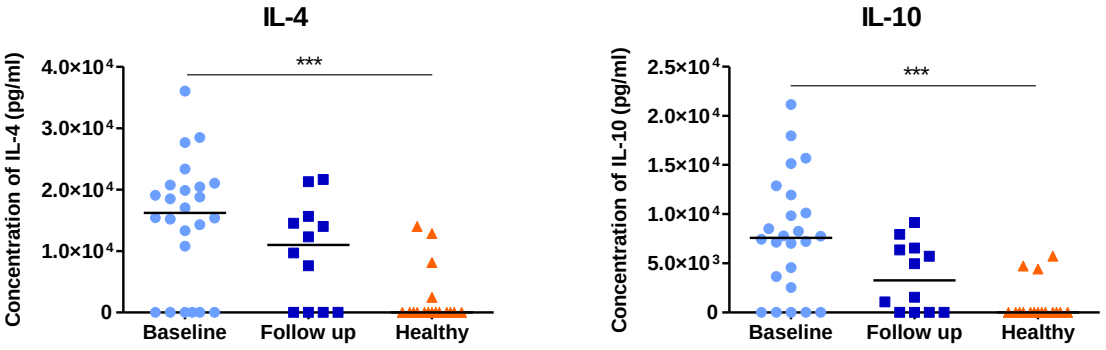

Figure III.5 - Serum levels of IL-4 and IL-10 of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Results were analyzed with Kruskal-Wallis test. p-values lower than 0.05 are considered statistically significant. Bars represent median values.***p<0.001. IL – interleukin.

sRANKL levels were slightly increased in AS patients at baseline when compared to follow-up or controls but without reaching statistical significance. Analyzing OPG levels there were no differences between groups when all groups are compared. (Fig.III.6).

The sRANKL/OPG ratio is also lower at follow-up and in healthy subjects compared with the baseline group but no significant differences were found.

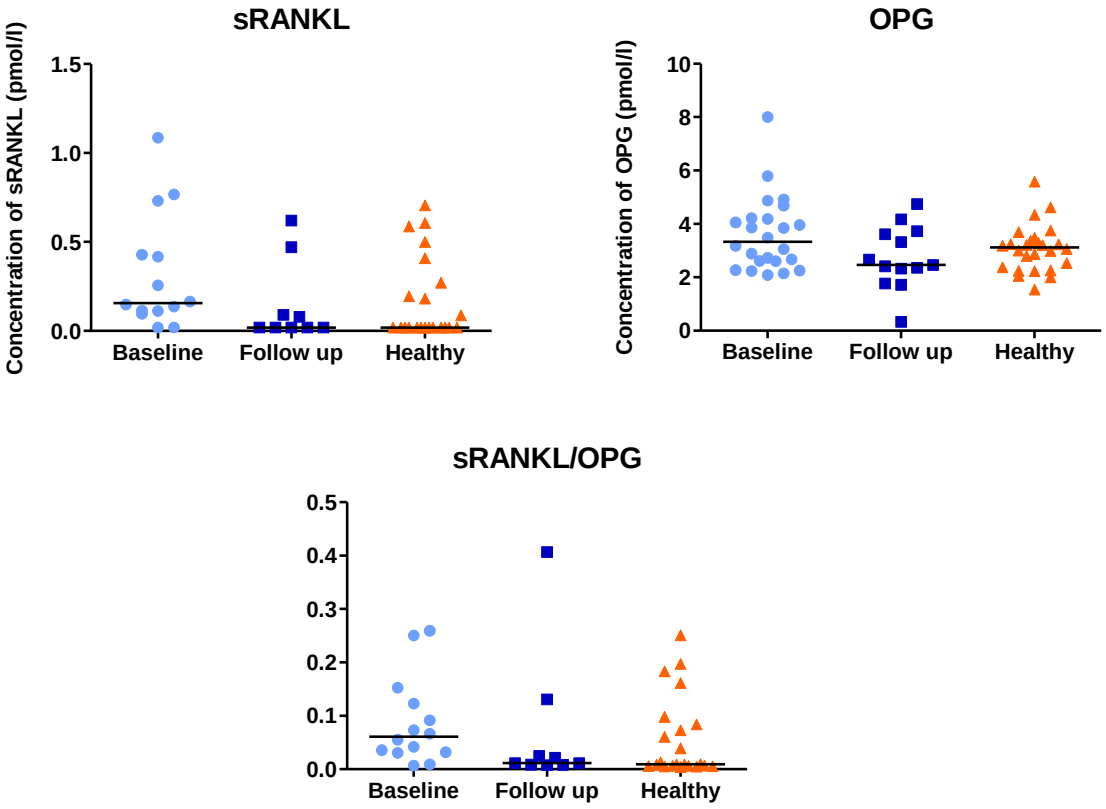

Figure III.6 - Serum levels of sRANKL and OPG of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. The ratio between sRANKL and OPG was calculated. Results were analyzed with Kruskal-Wallis test. p-values lower than 0.05 are considered statistically significant. Bars represent median values. sRANKL – soluble receptor activator of NF-kB; OPG – osteoprotegerin.

Baseline AS patients have increased DKK-1 levels when compared with follow-up and healthy subjects, but the difference was not statistically significant. Additionally, SOST levels were very similar between the three groups (Fig.III.7).

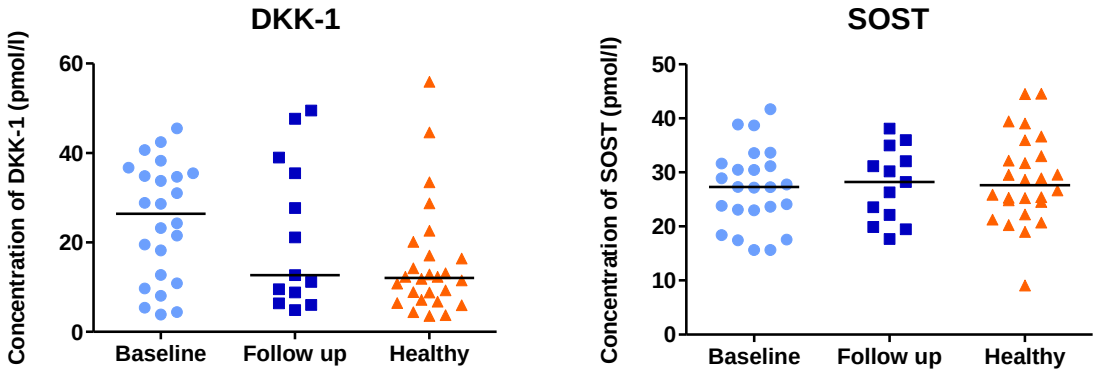

Figure III.7 - Serum levels of DKK-1 and SOST of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Results were analyzed with Kruskal-Wallis test. p-values lower than 0.05 are considered statistically significant. Bars represent median values. DKK-1 – Dickkopf-related protein 1; SOST – sclerostin.

As shown in Fig.III.8, CTX-I and P1NP levels were similar between baseline and follow-up patients. In healthy subjects, the levels of these two bone turnover markers were higher but without statistical significance. The same was observed in the CTX-I/P1NP ratio.

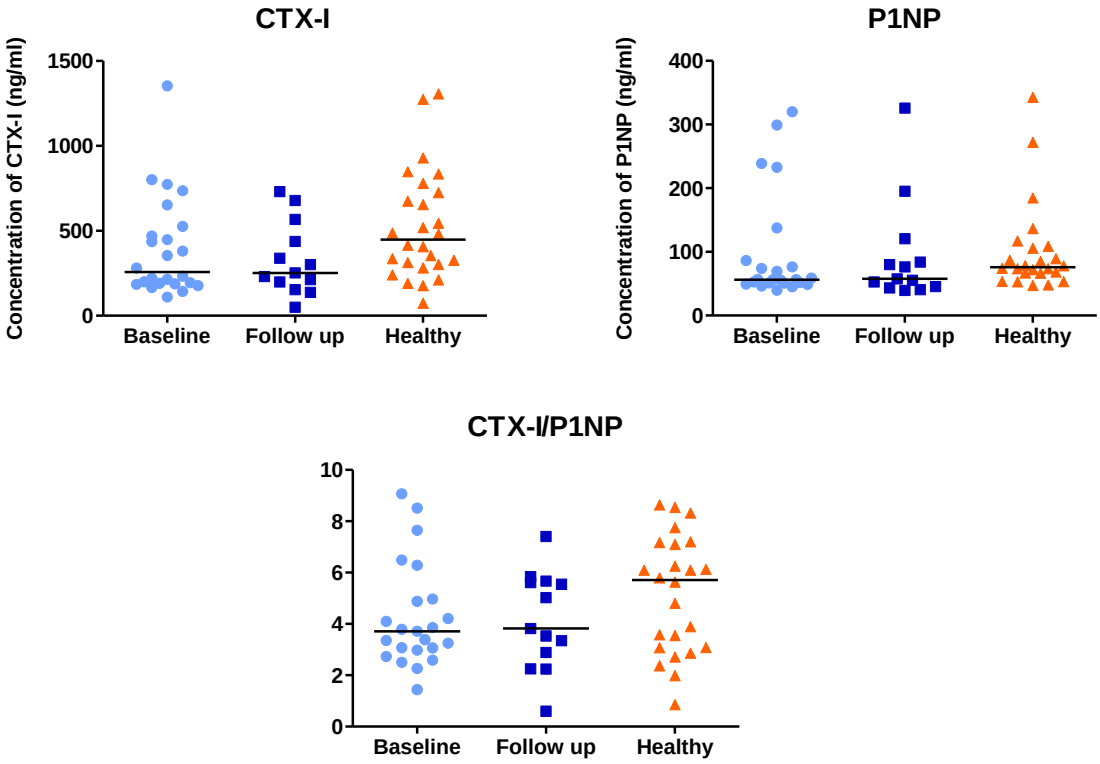

Figure III.8 - Serum levels of CTX-1 and P1NP of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up after starting the therapy six months and healthy donors. The ratio between CTX-1 and P1NP was calculated. Results were analyzed with Kruskal-Wallis test. p-values lower than 0.05 are considered statistically significant. Bars represent median values. CTX-I - C-terminal telopeptide of type 1 collagen; P1NP - procollagen type 1 N propeptide.

When we compared the paired AS patients before and after TNF-blocking therapy, we observed that the levels of IL-17A, TGF-β and OPG were significantly decreased after anti-TNF therapy when compared to baseline (IL-17A p=0.0195; TGF- β p=0.0186; OPG p=0.0425; Fig.III.9)

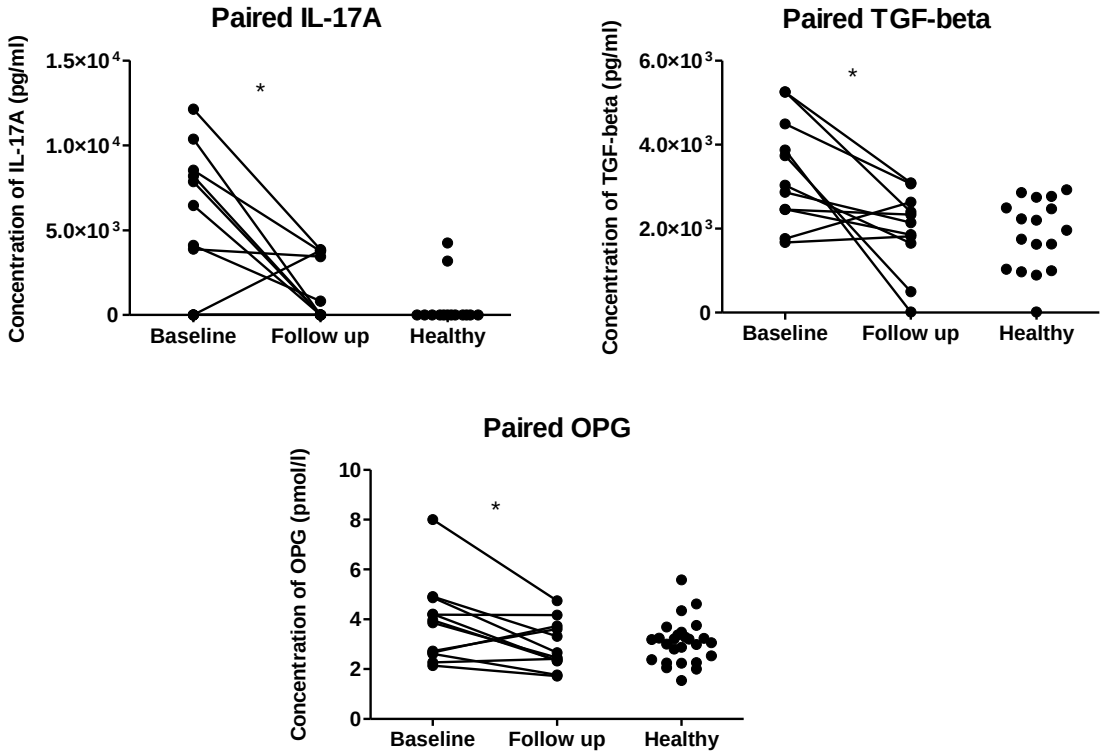

Figure III.9 - Serum levels of IL-17A, TGF-β and OPG of paired AS patients at baseline of anti-TNF therapy and follow-up six months after starting the therapy and healthy donors. Median values are represented in the graphs. Results were analyzed with a Wilcoxon matched pairs test. p-values lower than 0.05 are considered statistically significant. *p<0.05. IL – interleukin; TGF-β - transforming growth factor-β; OPG – osteoprotegerin; AS - ankylosing spondylitis.

3. Functional assays

To understand the effect of TNF-blocking therapy in osteoclastogenesis, *ex vivo* functional assays were performed in cells cultured under unstimulated and stimulated (M-CSF (25 ng/mL), sRANKL (50 ng/mL), dexamethasone (10 nM) and TGF-β (2.5 ng/mL)) conditions.

3.1. Pre-osteoclast and osteoclast counts

After TRAP staining the cell cytoplasm develops a red colour while the nuclei remains unstained which allows us to differentiate between pre-osteoclasts (with only one or two nuclei) and osteoclasts (containing three or more nuclei). In Fig.III.10 we can observe these two types of cells, where the blue arrows correspond to pre-osteoclasts and red arrows to mature osteoclasts.

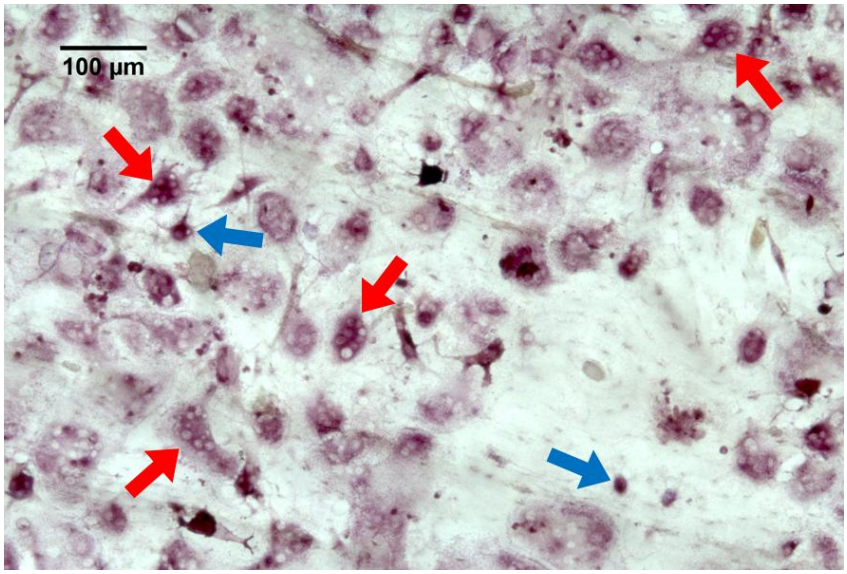

Figure III.10 - Representative image of pre-osteoclasts (blue arrows) and mature osteoclasts (red arrows) observed by TRAP staining. The scale bar corresponds to 100µm.

Pre-osteoclasts are defined as TRAP positive cells, with less than 3 nuclei (Fig.III.10 and III.11).

Figure III.11 shows representative images of all groups in the selected time-points for the unstimulated condition.

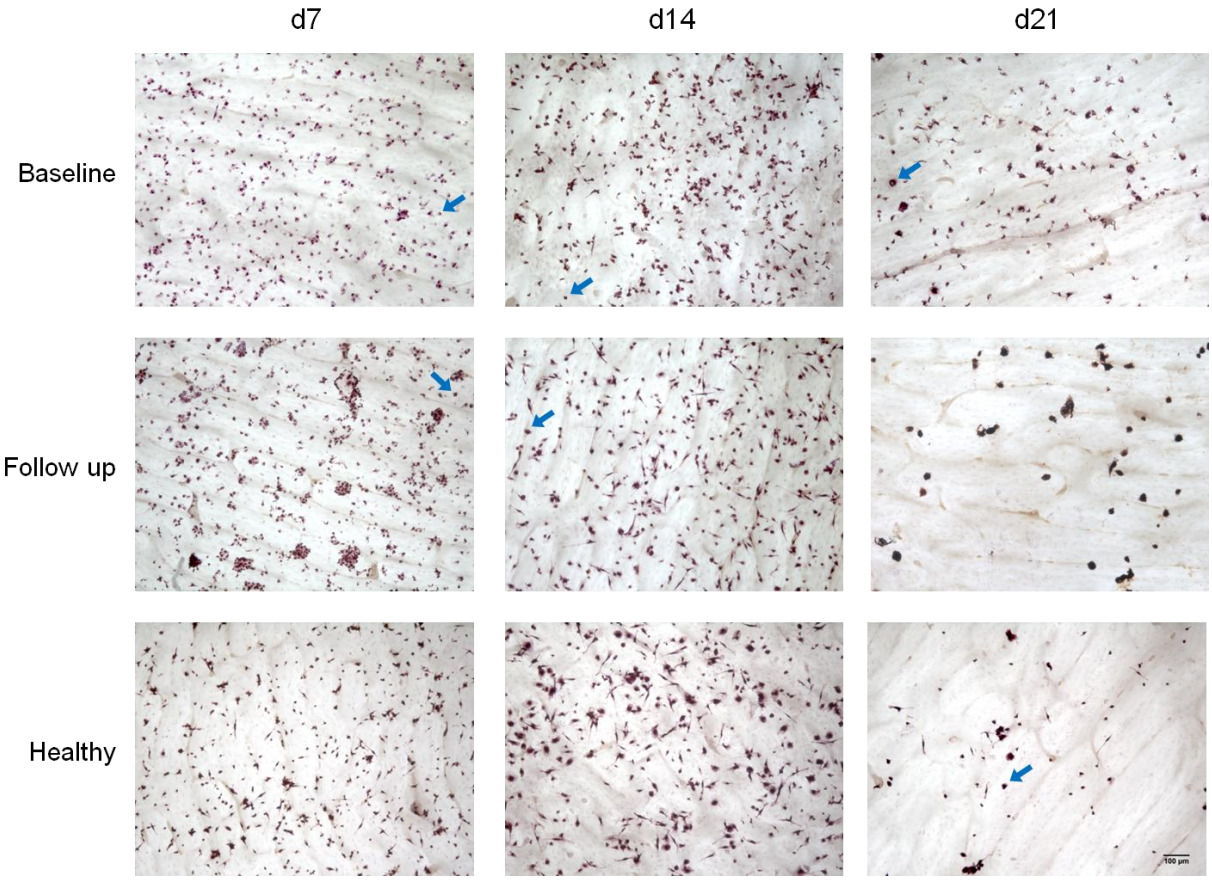

Figure III.11 - Unstimulated cells of baseline patients before TNF-blocking therapy, follow-up patients after six months of therapy and healthy donors. At time-points 7, 14 and 21 of culture TRAP staining was performed and photographs were taken with 10x objective. Representative images are shown for each time-point and condition. Blue arrows correspond to pre-osteoclasts. The scale bar corresponds to 100µm.

The number of pre-osteoclasts was counted both in the unstimulated and stimulated conditions and at days 7, 14 and 21 of culture. As seen in Fig.III.12, pre-osteoclast number decreased over time both in the unstimulated and stimulated conditions, although it did not reached statistical significance.

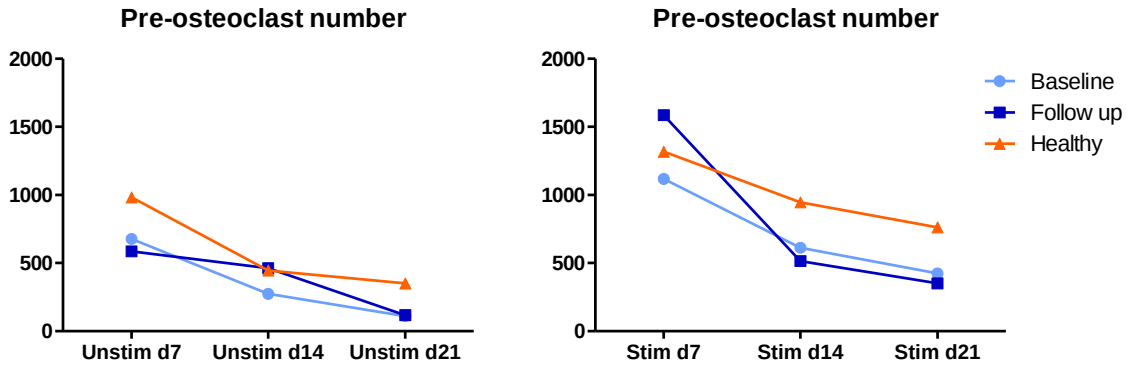

Figure III.12 - Number of pre-osteoclasts of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. These cells were cultured in unstimulated and stimulated conditions and TRAP staining was performed at days 7, 14 and 21 of culture. Pre-osteoclast number was counted and median values are represented in the graphs. Unstim - unstimulated condition; Stim - stimulated condition.

Osteoclasts are defined as large TRAP positive cells with 3 or more nuclei (Fig.III.10 and III.13).

Figure III.13 shows representative images of all groups in the selected time-points for the stimulated condition.

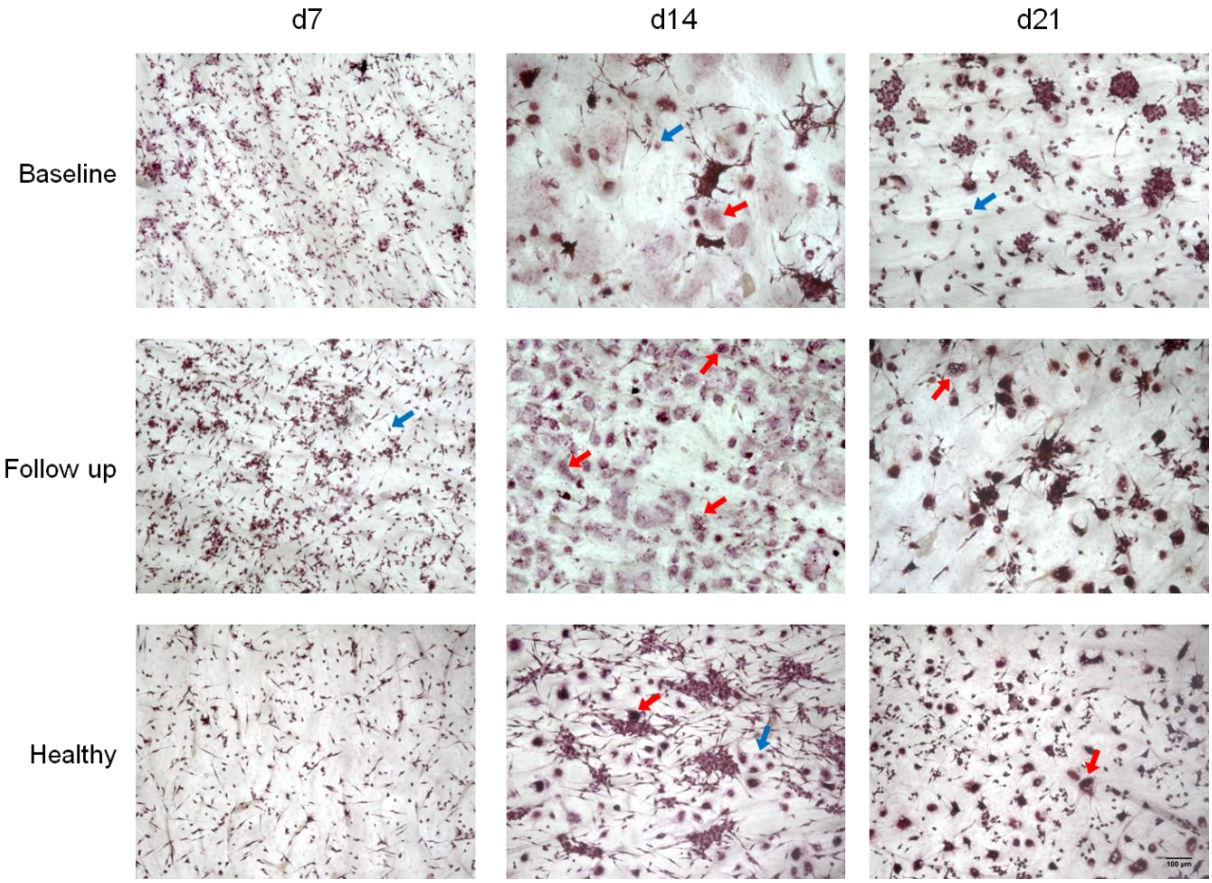

Figure III.13 - Stimulated cells of baseline patients before TNF-blocking therapy, follow-up patients after six months of therapy and healthy donors. Cells were cultured with stimuli (M-CSF, sRANKL, dexamethasone and TGF-β) and at time-points 7, 14 and 21 of culture TRAP staining was performed and photographs were taken with 10x objective. Representative images are shown for each time point and condition. Blue arrows correspond to pre-osteoclasts and red arrows to mature osteoclasts. The scale bar width corresponds to 100μm.

No significant differences were found in OC number between groups, both in unstimulated and stimulated conditions (Fig.III.14).

In the unstimulated condition, despite there were no cytokines present, a few osteoclasts were formed at days 14 and 21 in the three groups (Fig.III.14, left panel).

In the stimulated condition (Fig.III.14, right panel), baseline and follow-up groups reached the maximum number of OC at day 14, however controls only reached it at day 21 of culture. In follow-up group the number of OC was significantly different ($p=0.0062$) between days 7 and 14 and also between days 7 and 21. In the healthy group, we found a statistically significant increased between the number of OC at day 7 and at day 21 ($p=0.0194$).

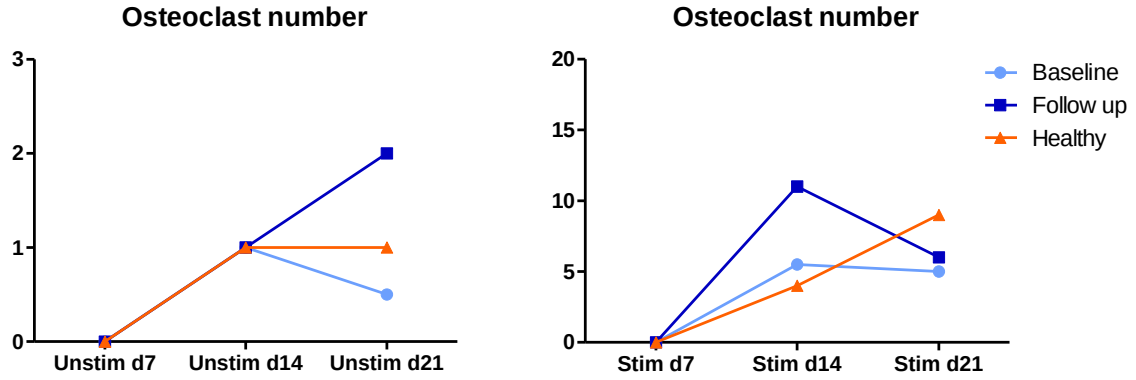

Figure III.14 - Number of osteoclasts of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Cells were cultured with unstimulated and stimulated conditions and TRAP staining was performed at days 7, 14 and 21 of culture. Osteoclast number was counted and median values are represented in the graphs. Unstim - unstimulated condition; Stim - stimulated condition.

The number of nuclei per osteoclast were also counted but no significant differences were found between groups, both in unstimulated and stimulated condiitons (Fig.III.15).

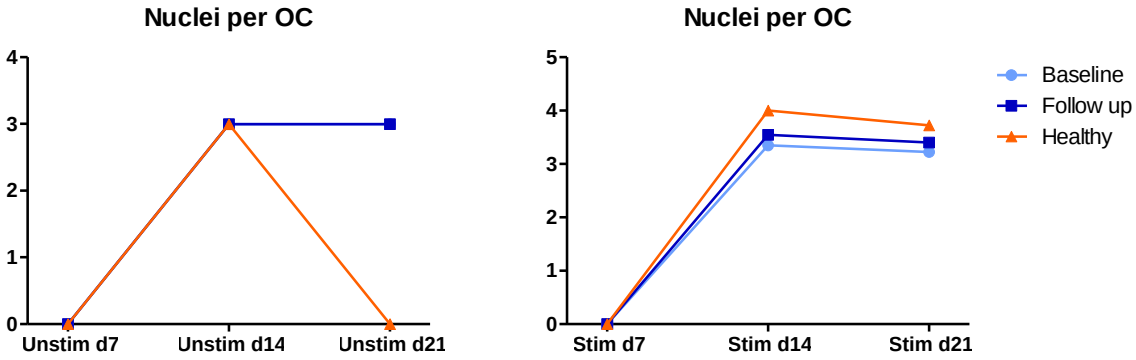

Figure III.15 - Number of nuclei per osteoclast of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Cells were cultured with unstimulated and stimulated conditions and TRAP staining was performed at days 7, 14 and 21 of culture. Nuclei were counted and median values are represented in the graphs. Unstim - unstimulated condition; Stim - stimulated condition; OC - osteoclast.

3.2. Resorption assays

Resorption pits are acidic structures that develop a blue to purple colour after being incubated with toluidine blue which allows the measurement of the resorbed area (Fig.III.16).

Figure III.16 shows representative images of all groups in the time-points where resorption pits were observed for the stimulated condition.

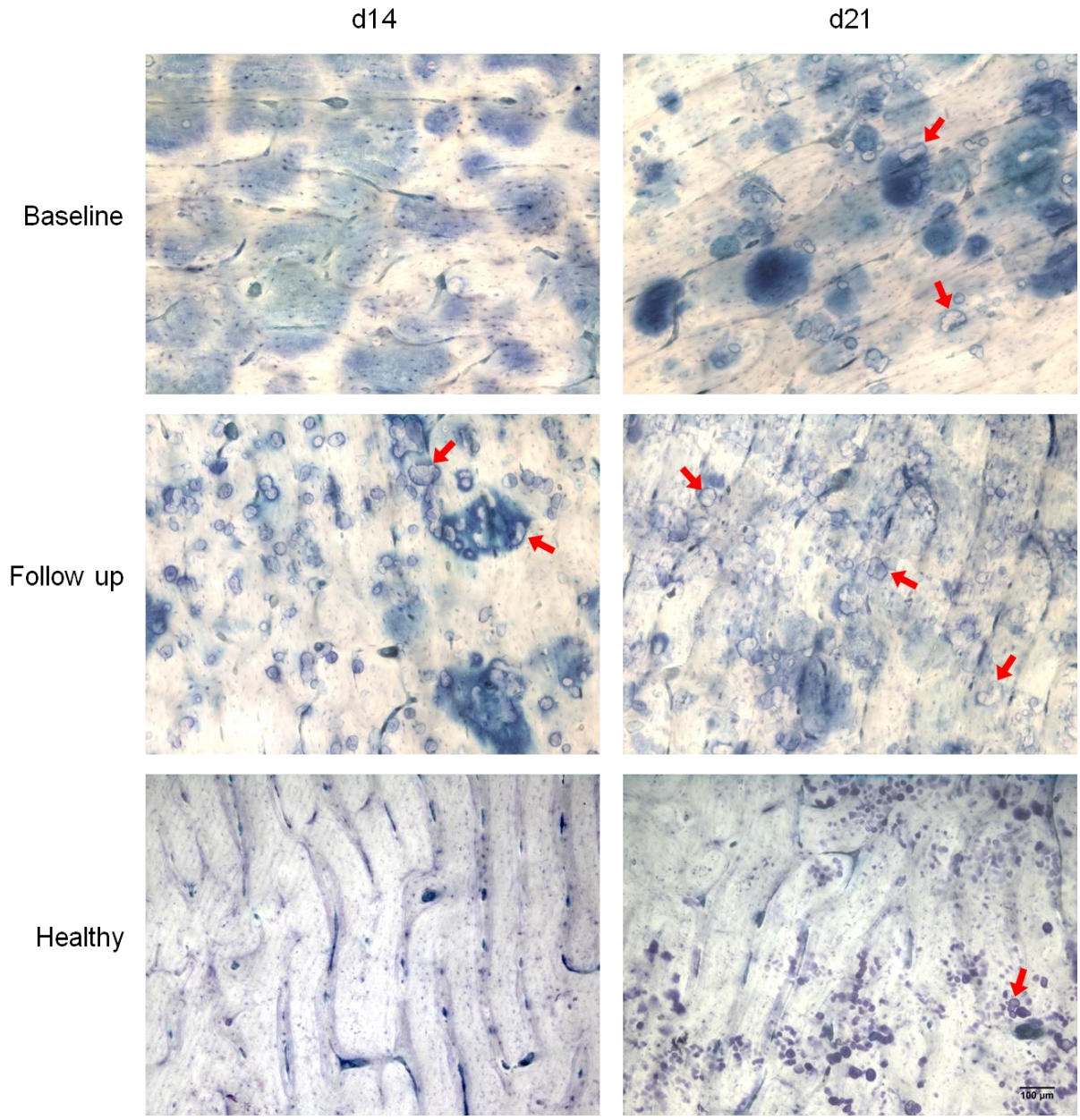

Figure III.16 - Resorption pits of baseline patients before TNF-blocking therapy, follow-up patients after six months of therapy and healthy donors. Cells were cultured in stimulation condition (with M-CSF, sRANKL, dexamethasone and TGF-β) and at time-points 7, 14 and 21 of culture resorption pit assay was performed and photographs were taken with 10x objective. Representative images are shown for each group at days 14 and 21 of culture. Red arrows point to resorption pits. The scale bar width corresponds to 100µm.

As shown in Fig.III.17, upper panel, the three groups in study exhibit the same pattern over time regarding the number of resorption pits and the percentage of resorbed area, since both the number of resorption pits and the percentage of resorbed area tend to significantly increase over time (number of resorption pits: baseline p=0.0292; follow-up p=0.0002: healthy p=0.0140 and percentage of resorbed area: baseline p=0.0258; follow-up p=0.0003; healthy p=0.0119).

In the unstimulated condition, despite osteoclasts were formed in the three groups (as shown previously in Fig.III.14) only one follow-up patient at day 21 showed resorption pits (data not shown).

No resorption pits were observed at day 7 in any group in the stimulated condition neither at day 14 in the healthy group. No significant differences were found between groups neither in the number of resorption pits nor in the percentage of resorbed area when all groups are compared (Fig.III.17, upper panel).

The resorbed area per pit increased from day 14 to day 21 both in baseline and follow-up patients, but the difference was not statistically significant (Fig.III.17, bottom left panel).

As seen in Fig.III.17, bottom right panel, the baseline group has less resorption pits per OC than the follow-up and healthy groups at day 21 in the stimulated condition although this difference is not statistically significant.

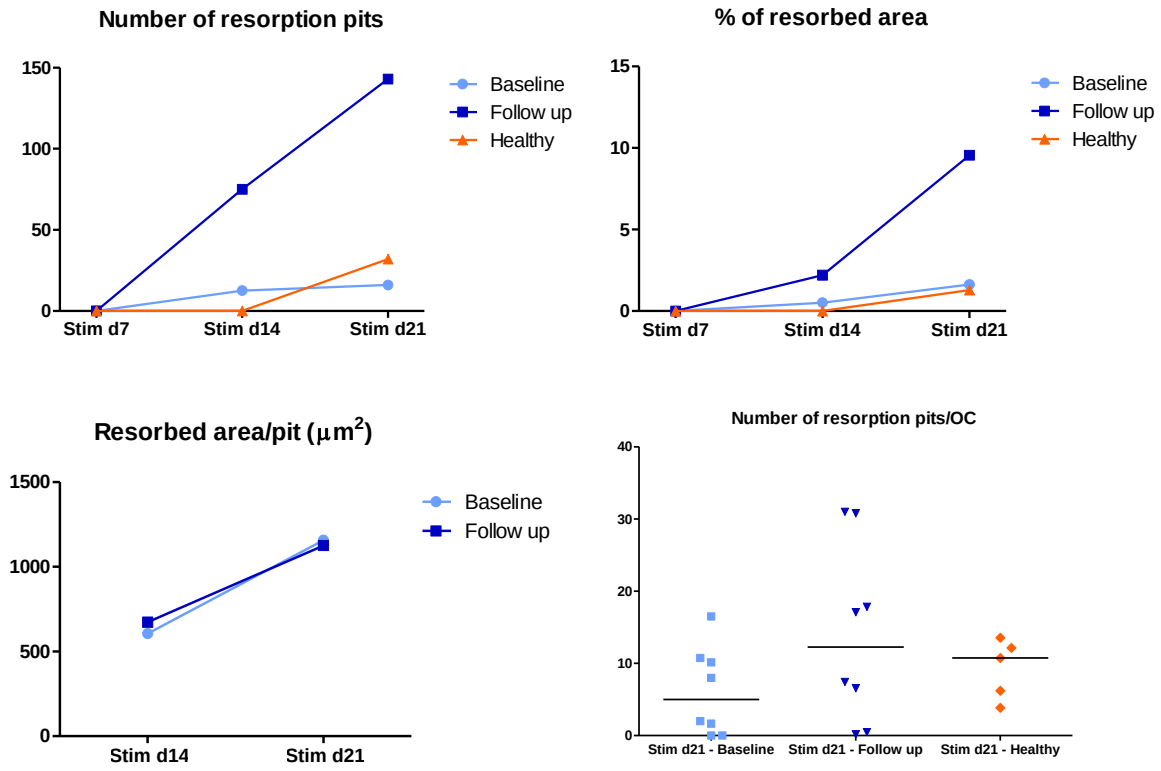

Figure III.17 - Number of resorption pits and percentage of resorbed area of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Cells were cultured with unstimulated and stimulated conditions and resorption pit assay was performed at days 7, 14 and 21 of culture. No resorption pits were found in the unstimulated condition. The ratio between the resorbed area per pit was calculated. Number of resorption pits per osteoclasts of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors in the stimulated condition at day 21 of culture is also represented. Median values are represented in the graphs. Bars also represent median values. Results were analyzed with a Kruskal-Wallis test. p-values lower than 0.05 are considered statistically significant. Stim - stimulated condition; OC - osteoclast.

When we compared the paired AS patients before and after TNF-blocking therapy, we found that both the number of resorption pits and the percentage of resorbed area are increased in follow-up patients when compared to the baseline (p=0.0469; Fig.III.18).

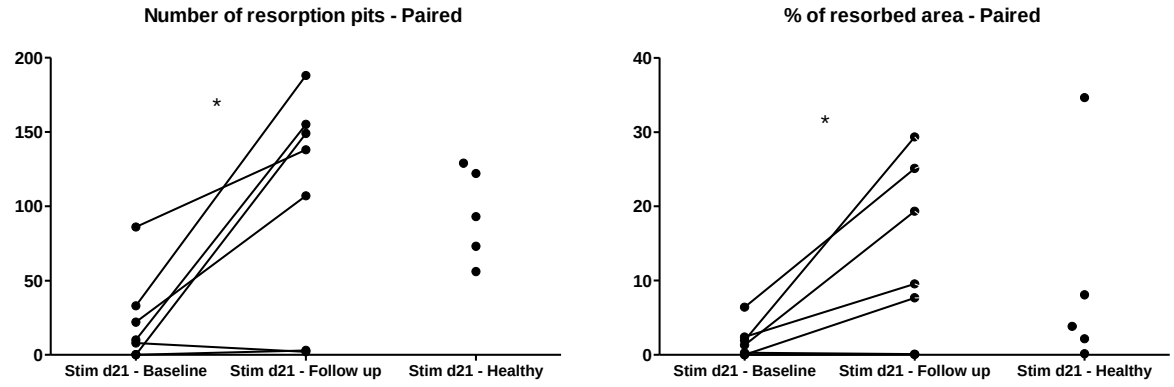

Figure III.18 - Number of resorption pits and percentage of resorbed area of paired ankylosing spondylitis patients at baseline of anti-TNF therapy and follow-up six months after starting the therapy and of healthy donors at day 21. Median values are represented in the graphs. Results were analyzed with a Wilcoxon matched pairs test. p-values lower than 0.05 are considered statistically significant. *p<0.05. Stim - stimulated condition.

4. Gene expression analysis

Since we found that osteoclast activity is increased after TNF-blocking therapy gene expression was analysed in cultured cells at days 1, 7, 14 and 21 of culture.

At day 1 of culture cells were not stimulated yet, therefore the gene expression for this day 1 is the same in both culture conditions.

CSF1R is expressed on monocytes and OCPs and is essential for the proliferation and survival of OCPs (Kikuta and Ishii 2013).

In the unstimulated condition, at day 7 of culture, CSFR1 expression was significantly higher in the follow-up group and lower in the baseline group (p=0.0486; Fig.III.19, left panel). There was also a statistically significant increase of CSF1R expression over time in the baseline and follow-up groups, both between day 1 and 14 or 21 of culture (baseline p<0.0001; follow-up p=0.0002) and at the baseline a significant increased is also observed between day 7 and days 14 and 21 (p<0.0001). The CSF1R expression in the healthy group was also increasing over time although the differences did not reach statistical significance (Fig.III.19, left panel).

In the stimulated condition, there were no differences between groups in the selected time-points. However all groups showed a significant increase in the CSF1R expression between days 1 and 7 of culture (baseline p=0.0026; follow-up p=0.0080; healthy p=0.0086) and day 7 is the time-point with the highest expression of this gene. Although there is a decrease in CSFR1 expression between day 7 and 14 in all groups, there is still a significant difference in CSF1R relative expression between day 1 and day 14 in the follow-up group (p=0.0080; Fig.III.19, right panel).

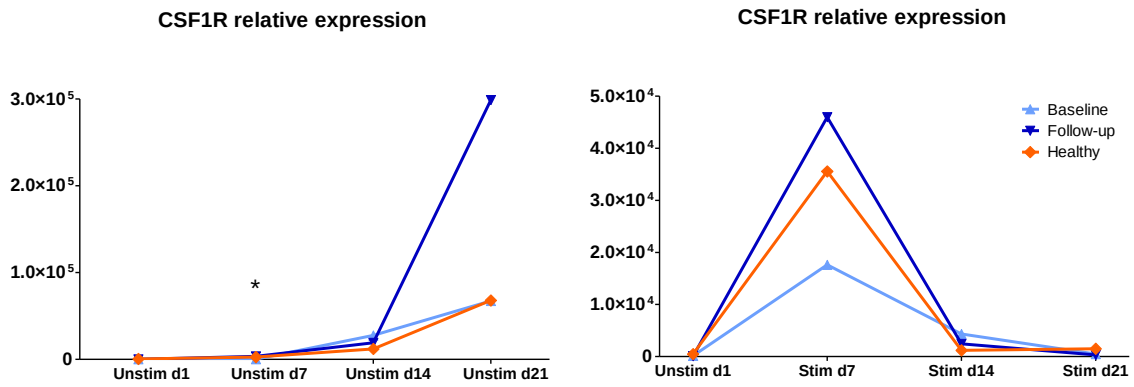

Figure III.19 - CSF1R relative expression in cultured cells of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Cells were cultured with and without stimuli during 1, 7, 14 and 21 days. Median values are represented in the graphs. *p<0.05. CSF1R - *Colony stimulating factor 1 receptor*; Unstim - unstimulated condition; Stim - stimulated condition.

RANK gene encodes for the receptor for RANKL present on monocytes and OCPs and mature OCs that stimulates OC differentiation (Kwan Tat, Padrines et al. 2004).

As shown in Fig.III.20, RANK relative expression at day 1 was significantly lower in the follow-up patients when compared to healthy subjects (p=0.0026) and to baseline patients (p=0.042).

In the unstimulated condition, the baseline and follow-up groups have an increase of RANK at day 14, with a significant difference in expression between days 1 and 14 (baseline p=0.0024; follow-up p= 0.0168). In the healthy subjects group, RANK expression decreases between days 1 and 7, but after day 7 RANK expression continues to increase until day 21, with a significant difference between days 7 and 14 (p=0.0306; Fig.III.20, left panel).

When cells were cultured under stimuli, the baseline group peaks at day 14 (p=0.0005), the follow-up group at day 7 and the healthy group at day 1 (p=0.0079). RANK was not expressed at day 21 in the healthy group (Fig.III.20, right panel).

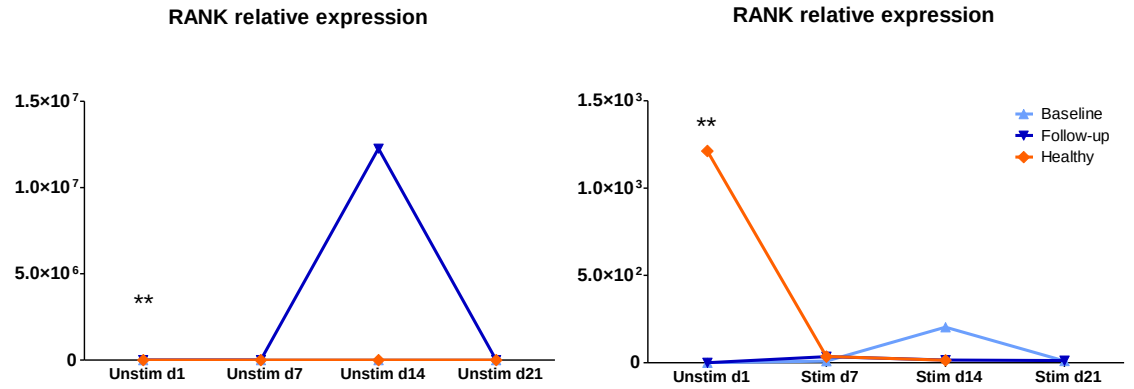

Figure III.20 - RANK relative expression in cultured cells of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Cells were cultured with and without stimuli during 1, 7, 14 and 21 days. Median values are represented in the graphs. **p<0.01. RANK - Receptor activator of NF-kB; Unstim - unstimulated condition; Stim - stimulated condition.

NFATc1 is the target gene for RANK signaling cascade and has been proposed to function as the master transcription factor in osteoclastogenesis (Takayanagi, Kim et al. 2002).

At day 1 of culture NFATc1 relative expression is significantly lower in follow-up patients than in healthy subjects (p=0.0096; Fig.III. 21).

When cells are cultured without any stimuli, NFATc1 expression significantly increased in all groups between days 7 and 14 (baseline p=0.0027; follow-up p=0.0035; healthy p=0.0161; Fig.III.21, left panel).

On the other hand, in the stimulated condition, the peak of NFATc1 expression occurs earlier than in unstimulated condition at day 7, reaching statistical significance when comparing to day 1 culture (baseline p=0.0038; follow-up p=0.0003; healthy p=0.0129; Fig.III.21, right panel).

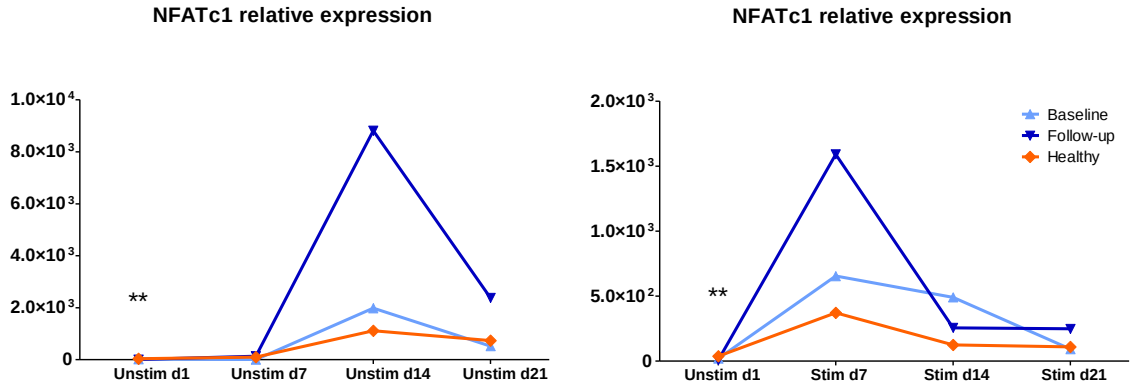

Figure III.21 - NFATc1 relative expression in cultured cells of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Cells were cultured with or without stimuli during 1, 7, 14 and 21 days. Median values are represented in the graphs. **p<0.01. NFATc1 - Nuclear factor of activated T cells cytoplasmic 1; Unstim - unstimulated condition; Stim - stimulated condition.

Atp6v0d2 gene encodes the subunit d2 of the functional domain v0 of the ATPase protein. This ATPase is responsible for extracellular acidification of the resorption lacunae and bone demineralization during bone resorption (Qin, Cheng et al. 2012).

As seen in Fig.III.22, Atp6v0d2 expression at day 1 was significantly lower in follow-up patients compared with healthy subjects (p=0.0048) and with baseline patients (p=0. 042).

In the unstimulated condition, at day 14, Atp6v0d2 expression was significantly higher in follow-up patients that in healthy subjects when all groups are compared (p=0.0261; Fig.III.22, left panel). Over time, in the baseline group Atp6v0d2 expression peaks at day 14 (p=0.0008) while in follow-up and healthy groups peaks at day 21 (follow-up p=0.0031; healthy p=0.026; Fig.III.22, left panel).

Under the stimulated condition, Atp6v0d2 expression peaks at day 14 for baseline and healthy groups (baseline p=0.0014; healthy p=0.0017) but in the follow-up group the Atp6v0d2 expression continues to increase significantly until day 21 (p=0.0019; Fig.III.22, right panel).

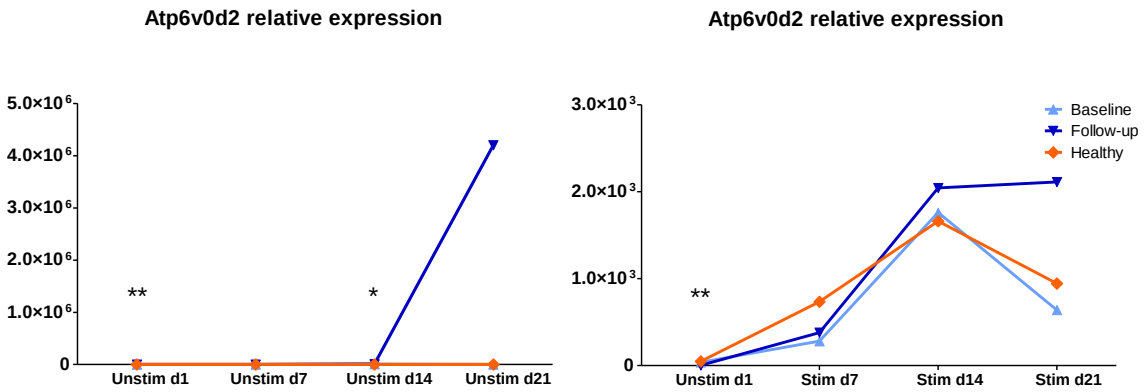

Figure III.22 - Atp6v0d2 relative expression in cultured cells of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Cells were cultured with or without stimuli during 1, 7, 14 and 21 days. Median values are represented in the graphs. *p<0.05, **p<0.01. Atp6v0d2 - ATPase; Unstim - unstimulated condition; Stim - stimulated condition.

CLCN7 encodes for the chloride channel that maintains osteoclasts' electroneutrality and provides the chloride conductance required for an efficient proton pump (Novack and Teitelbaum 2008).

As shown in Fig.III.23, CLCN7 relative expression at day 1 was significantly lower in follow-up patients than in healthy subjects (p=0.0131).

In the unstimulated condition at day 7 CLCN7 relative expression was significantly higher in follow-up when compared to baseline patients (p=0.0259; Fig.III.23, left panel). A significant increase in CLCN7 expression was also observed at day 14 in the baseline patients when compared to the healthy group (p=0.0346; Fig.III.23, left panel). Analyzing the results overtime, we observed that the baseline group has the highest CLCN7 expression at day 14 (p=0.002) but in the follow-up group CLCN7 peaks at day 21, although without statistical significance (Fig.III.23, left panel).

When cells were cultured with stimuli, both baseline and follow-up groups showed the highest CLCN7 expression at day 7 (baseline p=0.0163; Fig.III.23, right panel).

In the healthy group, both in unstimulated as well as in stimulated conditions, CLCN7 expression peaks at day 1 and decreased overtime (Fig.III.23).

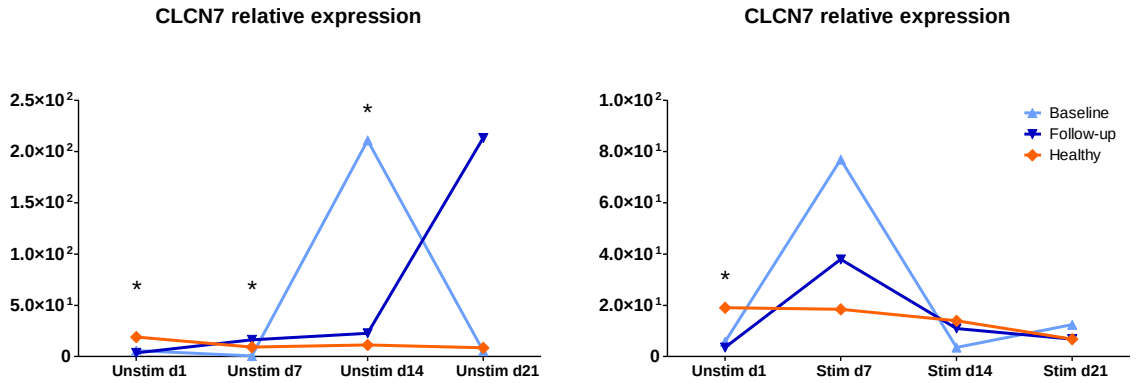

Figure III.23 - CLCN7 relative expression in cultured cells of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Cells were cultured with or without stimuli during 1, 7, 14 and 21 days. Median values are represented in the graphs. *p<0.05,. CLCN7 - Chloride channel, voltage-sensitive 7; Unstim - unstimulated condition; Stim - stimulated condition.

CTSK gene encodes for a protease predominantly expressed by OCs which is involved in the degradation of the bone organic matrix, that consists mainly of type 1 collagen (Novack and Teitelbaum 2008).

There were no significant differences in the CTSK expression between groups in the studied time-points.

When cells were cultured without any stimuli, the baseline group showed a peak of CTSK expression at day 14 ($p<0.0001$) whereas in follow-up and healthy groups CTSK expression continues to increase over time until day 21 (follow-up $p=0.0001$; healthy $p=0.0012$) as shown in Fig.III.24, left panel.

In the stimulated condition, the three groups exhibit the same expression pattern over time, with the highest CTSK expression levels at day 14 (baseline $p<0.0001$; follow-up $p=0.0002$; healthy $p=0.0003$) (Fig.III.24, right panel).

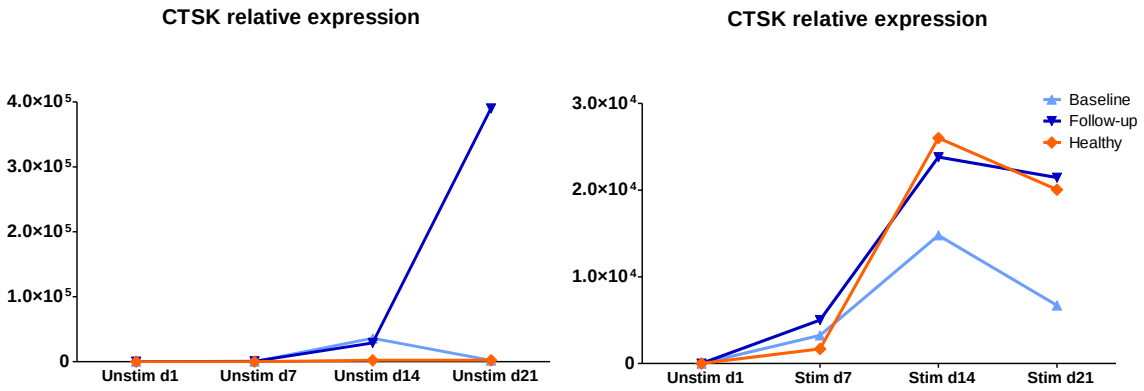

Figure III.24 - CTSK relative expression in cultured cells of ankylosing spondylitis patients at baseline of anti-TNF therapy, follow-up six months after starting the therapy and healthy donors. Cells were cultured with or without stimuli during 1, 7, 14 and 21 days. Median values are represented in the graphs. CTSK - Cathepsin K; Unstim - unstimulated condition; Stim - stimulated condition.

No correlation was found between any of the study parameters and clinical data.

IV. DISCUSSION

AS is characterized by syndesmophyte formation and increased BMD in the lumbar spine. In patients under TNF-blocking treatment a reduction of CTX-I levels was reported (Arends, Spoorenberg et al. 2012), which might reflect a decrease in osteoclast activity. Therefore, we hypothesized that there is an impairment in osteoclastogenesis in AS patients with active disease, that seems to be further inhibited by TNF-blocking therapy.

The aim of this study was to understand how anti-TNF therapy regulates inflammatory environment in AS patients. Moreover we wanted to understand TNF-blocking effects on OC differentiation and activity in AS patients.

To achieve these goals, we recruited AS patients with active disease (median ASDAS of 3.4, BASDAI of 5.5 and BASFI of 5.89; Table III.1) who were starting TNF-blocking therapy (baseline patients) and a second sample was collected six months later (follow-up patients).

Patients had a median age of 41 years with 10 years of symptoms duration (Table III.1). Were predominantly males, as was previously reported by Feldtkeller et al that AS affects mainly young adults at around 26 years of age and is more common in men than women, at a ratio of 2:1 (Feldtkeller, Khan et al. 2003). Fifty percent of AS patients were positive for the antigen HLA-B27 which corroborates the hypothesis that other genes may be involved in the pathophysiology of this disease (Zambrano-Zaragoza, Agraz-Cibrian et al. 2013). It has been shown that TNF-blocking agents are an effective and well-tolerated treatment for reducing clinical symptoms of AS (Ren, Li et al. 2013) and, concordantly, we observed that TNF-blocking therapy decreases the levels of the inflammatory markers ESR and CRP and also decreases the disease and functional indexes, ASDAS, BASDAI and BASFI (Tables III.1 and III.2). As control group, we recruited age and gender matched healthy donors without inflammation. To attain the objective of this thesis a blood sample was collected from these patients before and 6 months after they start TNF-blocking therapy in order to assess the concentration of inflammatory cytokines in the serum and to isolate and differentiate osteoclasts *in vitro*.

We have studied pro- and anti-inflammatory cytokines, which are known to be involved in AS pathogenesis and in osteoclast differentiation (Gratacos, Collado et al. 1994; Appel, Loddenkemper et al. 2009). Serum levels of IL-6, IL-1β, TNF, IL-12p70, IL-17A, IL-22, IL-8, TGF-β, IFN-γ and IL-2 are all significantly higher in AS patients with active disease (baseline) than in healthy subjects (Figures III.1-III.4). Taylan et al previously measured TGF-β, IL-12, IL-17, IL-6 levels and showed that these cytokines were significantly higher in AS patients with active disease when compared with healthy controls (Taylan, Sari et al. 2012). Limón-Camacho et al also measured the serum levels of IL-6, IL-12, IL-17A, TNF-α, IFN-γ and IL-8 and these cytokines were significantly elevated in AS patients with active disease compared to healthy controls, in accordance with our results (Figures III.1-III.4) (Limon-Camacho, Vargas-Rojas et al. 2012). Moreover, Zhang et al demonstrated that serum IL-22 levels are increased in AS patients with mild to active disease when compared with healthy controls (Zhang, Li et

al. 2012). Liu and colleagues also found that IL-2 expression in AS patients with mild to active disease was significantly higher than the healthy group (Liu, Wang et al. 2013).

In our study, IL-1β showed higher levels at baseline AS patients than in healthy subjects (Figure III.1), however, as reviewed by Keller and in a more recent paper by Bal and colleagues, no significant differences in IL-1β levels between AS patients with active disease and healthy controls were found (Keller, Webb et al. 2003; Bal, Unlu et al. 2007). We also found that IL-23 levels are not significantly different between groups (Figure III.1) although Taylan et al. found that IL-23 levels were significantly higher in AS patients with active disease than in the healthy controls (Taylan, Sari et al. 2012). Our results show a significant increase in IL-18, not only in baseline, but also in TNF-blocker treated AS patients when compared with healthy controls (Figure III.3). Indeed, Sari and co-workers observed IL-18 levels were higher in AS patients with mild to active disease with or without TNF-blocking therapy, however the difference was not significant (Sari, Kebapcilar et al. 2010). To our best knowledge, serum levels of MCP-1 and MIP-1α have never been described in AS patients before and after TNF-blocking therapy and we observed that the levels of these two chemokines are signifcantly increased in AS patients at baseline when compared to the healthy subjects and no significant differences are observed after TNF-blocking therapy (Figure III.3).

When the patients were paired, we found that IL-17A and TGF-β concentrations are significantly decreased in AS patients after TNF-blocking therapy (Figure III.9). Although no significant differences were found IFN-γ, IL-2, IL-8, IL-18, IL-6, IL-1β, TNF, IL-4 and IL-10 levels are also reduced in AS patients after TNF-blocking therapy. In accordance with our study, Limón-Camacho et al found that serum levels of IL-17A were significantly higher in AS patients with active disease compared with patients receiving TNF blockers and the same significant difference was also seen in IL-6, IFN-γ, TNF-α and IL-8 (Limon-Camacho, Vargas-Rojas et al. 2012). In the same study, Limón-Camacho et al observed that serum IL-12 levels were also elevated in AS patients with active disease when compared with patients under TNF-blocking therapy, however in our study no differences were found (Figure III.2).

The anti-inflammatory cytokines IL-4 and IL-10 are also significantly increased in AS patients at the baseline when compared to healthy donors, which may be due to the activation of immune cells at baseline, increasing the expression of all cytokines including IL-4 and IL-10. IL-4 was also found to be increased in AS patients with mild to active disease by Li et al (Li, Wang et al. 2013), however Xueyi did not find differences in IL-10 levels, before or after TNF-blocking therapy comparing to healthy donors (Xueyi, Lina et al. 2013).

Our results support recent studies that suggest a role for these cytokines in the pathogenesis of AS (Gratacos, Collado et al. 1994; Appel, Loddenkemper et al. 2009). In fact, the levels of most cytokines in patients under TNF-blockers for 6 months normalize to the healthy donors levels, which indicates a reduction in the inflammatory environment, particularly IL-17A, a cytokine well described to be associated with the pathogenesis of inflammatory disorders such as AS (Mei, Pan et al. 2011; Chen, Chang et al. 2012) and TGF-β, an important cytokine for bone formation (Ai-Aql, Alagl et al. 2008).

RANKL is an essential cytokine in osteoclastogenesis since it binds to RANK promoting osteoclast differentiation. However, RANKL also binds to OPG, which acts as a decoy receptor since it prevents

the RANKL-RANK binding, thereby inhibiting osteoclastogenesis (Kwan Tat, Padrines et al. 2004). In the serum we can only find the soluble form of RANKL (sRANKL) since the membranous form of RANKL is expressed on the cell surface of osteoblasts, bone marrow stromal cells, fibroblasts, mammary epithelial cells and activated immune system cells and can be cleaved into the soluble form (Wu, Humphrey et al. 2008). The sRANKL/OPG ratio is a parameter that infers OC differentiation and its resorptive function. Previous studies have shown discrepancies in sRANKL and OPG levels in AS patients. A study by Chen and co-workers showed elevated sRANKL and OPG levels in AS patients with active disease (Chen, Chen et al. 2010). However, Klingberg et al found recently lower sRANKL levels and lower sRANKL/OPG ratio in AS patients with mild to active disease (where patients under TNF blockers were included) when compared with healthy controls. The same study showed that in the group of patients under TNF-blocking therapy sRANKL levels were increased and OPG levels were decreased when compared to AS patients without TNF blocker (Klingberg, Nurkkala et al. 2014). In a study by Taylan and colleagues, AS patients with mild to active disease (where TNF-blocking therapy treated patients are included) show similar sRANKL levels and decreased OPG levels than healthy controls. In this group of patients they also found a decreased sRANKL/OPG ratio when compared to healthy donors. Taylan also observed that in TNF-blockers treated patients, OPG levels where significantly decreased leading to an increase of sRANKL/OPG ratio when compared to patients under conventional therapy (NSAIDs and/or DMARDs) (Taylan, Sari et al. 2012). In our results, although the higher sRANKL level in the baseline group, we found no significant differences in sRANKL, OPG or in the sRANKL/OPG ratio when comparing the three studied groups. When we focus on the effect of TNF-blocking therapy in the levels of sRANKL and OPG, we found that OPG levels are significantly decreased after TNF-blocking therapy in the paired sub-group of patients (Figure III.9). Accordingly, reduced OPG levels after TNF-blocking therapy might suggest an increase in osteoclastogenesis.

Osteoblasts are bone-forming cells and their differentiation and function is under the control of several signaling pathways. The Wnt signaling pathway is essential for osteoblast differentiation during skeletogenesis and continues to be important in mature osteoblasts (Crockett, Rogers et al. 2011). SOST and DKK-1 are inhibitors of this pathway and their serum levels were assessed in this study. Although DKK-1 levels are higher in the baseline AS patients, we found no significant differences in SOST or DKK-1 serum levels between the three groups (Figure III.7). It was previously demonstrated that TNF stimulates the expression of Wnt antagonist DKK-1 (Briolay, Lencel et al. 2013), acting as a brake on bone formation (Pedersen, Chiowchanwisawakit et al. 2011). As a consequence, TNF-blocking therapy may reset DKK-1 levels to healthy levels leading to an increase of Wnt signaling and consequently new bone formation. Like sRANKL and OPG, serum DKK-1 and SOST levels are controversial. Taylan et al described that serum levels of DKK-1 and SOST were not different in AS patients with mild to active disease when compared to healthy controls, but patients under TNF-blocking treatment presented higher DKK-1 levels compared to patients under conventional therapy (NSAIDs and/or DMARDs) (Taylan, Sari et al. 2012). More recently, Ustun et al found that SOST levels are significantly lower in AS patients with inactive to active disease than in

healthy controls and that TNF-blocking treatment does not affect DKK-1 and SOST levels (Ustun, Tok et al. 2014).

As bone turnover markers, CTX-I and P1NP were measured in patients' serum. To assess bone resorption and OC function the biochemical marker CTX-I was quantified and, on the other hand, P1NP was quantified to assess bone formation. We observed that patients before and after TNF-blocking therapy have similar CTX-I and P1NP serum concentrations and the levels of both bone turnover markers are decreased in these groups compared to the healthy group without reaching statistical significance (Figure III.8). Accordingly, Acebes et al found no significant differences in P1NP between healthy controls and AS patients (Acebes, de la Piedra et al. 1999) but recently several authors have described increased levels of CTX-I in AS patients with active disease when compared with healthy controls (Visvanathan, van der Heijde et al. 2009; Muntean, Rojas-Vargas et al. 2011).

We cultured PBMCs from AS patients before and after TNF-blocking treatment and also from healthy donors to have a better understanding about how OC differentiation and function is affected in AS patients with active disease and the TNF-blocking therapy effects. PBMCs were cultured under M-CSF (25 ng/mL), RANKL (50 ng/mL), dexamethasone (10 nM) and TGF-β (2.5 ng/mL) stimulation, as previously described by Takuma et al. Dexamethasone at $<10^{-8}$M acts synergistically with TGF-β to enhance RANKL-induced osteoclast formation (Takuma, Kaneda et al. 2003). At days 7, 14 and 21 of culture, functional assays (TRAP staining and resorption pit assay) were performed and RNA was extracted. According to the literature, at day 7 we expected to see pre-osteoclasts and at days 14 and 21 fully differentiated osteoclasts and bone resorption (Holloway, Collier et al. 2002; Husheem, Nyman et al. 2005). In addition, we also cultured PBMCs without any differentiation stimuli. In this unstimulated condition, the monocytes behavior in culture is observed since there are no cytokines to induce osteoclast differentiation.

To understand how OC differentiation is influenced by the disease and therapy we perfomed functional assays such as TRAP staining and we observed a continuous decrease in the number of pre-osteoclasts from day 7 to day 21 in both unstimulated and stimulated conditions in the three groups, but no significant differences were found neither over time nor between groups (Figure III.12). Interestingly, in the unstimulated condition, we observed OCs differentiation in the three groups at days 14 and 21 of culture (Figure III.14). We hypothesize that when pre-osteoclasts attach to bone slices through integrins a signaling pathway is activated that is able to induce the expression of osteoclast differentiation genes. It has been previously shown that integrins transmit pro-survival signals when interacting with matrix-residing ligand (Stupack and Cheresh 2002) and that unoccupied $\alpha_V\beta_3$ integrin transmits a positive death signal regulating osteoclast apoptosis (Zhao, Ross et al. 2005). In 2012, Mochizuki et al suggested that cell adhesion is required for osteoclast differentiation from precursor and that adhesion signaling regulates RANK expression in osteoclast precursors (Mochizuki, Takami et al. 2012). Another study suggested that attenuation of the integrin $\alpha_V\beta_3$ pathway leads to inhibition of osteoclast differentiation and that there is a crosstalk between integrin β_3 and M-CSF/c-fms pathways (Jung, Han et al. 2012). We consider that cells adhesion to bone by integrins has an important role in OC differentiation, but further studies are required to determinate how integrins are able, *per se*, of inducing OC differentiation.

When PBMCs were cultured under the stimulating condition, AS patients before and after TNF-blocking therapy reach the highest OC number inside each group at day 14 and start to decline thereafter (Figure III.14), which may be due to cell apoptosis. In contrast with our results, Im et al. found that the number of osteoclasts from AS patients with active disease cultured with M-CSF and RANKL were higher than those of controls and concluded that there were increased numbers of osteoclast precursor cells in the peripheral blood of AS patients suggesting that osteoclastogenesis is elevated in AS patients than in healthy controls (Im, Kang et al. 2009).

We performed resorption assays to understand if mature OCs formed were functional. Interestingly, although in the unstimulated condition we found osteoclasts at day 14 and 21 in all groups (Figure III.14), only one follow-up patient had resorption pits at day 21 (data not shown).

In the stimulated condition the three groups of study exhibit the same pattern of increase in the number of resorption pits and the percentage of resorbed area over time (Figure III.17, upper graphs). There are no differences in pit size between groups (Figure III.17, bottom left panel), suggesting that OC mobility is not affected by the disease neither by therapy. When we compare paired AS patients before and after TNF-blocking therapy, both the number of resorption pits and consequently the percentage of resorbed area are significantly increased in patients after therapy (Figure III.18), suggesting that TNF-blocking therapy increases the resorptive capacity of OCs.

We then focused on the gene expression of essential and specific osteoclast genes CSF1R, RANK, NFATc1, Atp6v0d2, CLCN7 and CTSK.

The M-CSF receptor is encoded by the CSF1R gene which is expressed by monocytes and OCPs and is essential for their proliferation and survival (Kikuta and Ishii 2013). In our study, the follow-up group, unstimulated at day 7 had a significantly higher expression of CSF1R and the baseline group had the lowest (Figure III.19, left panel). The expression of CSF1R increases until day 21 in all groups. Moreover, in the stimulated condition, CSF1R expression is higher at day 7 in all groups (Figure III.19, right panel). Overall, our results indicate that monocytes and pre-osteoclasts of AS patients under TNF-blocking therapy have an increased CSF1R expression. To the best of our knowledge there has been no study regarding CSF1R expression in AS patients, however Li and colleagues have found in arthritic mice that TNF increased not only CSF1R expression in monocytes, but also the number of these osteoclast precursors *in vivo* (Li, Schwarz et al. 2004). These results further support the findings that in psoriatic arthritis TNF-blocking therapy reduces the number of osteoclast precursors (Ritchlin, Haas-Smith et al. 2003).

RANK is the gene that encodes the receptor for RANKL present on monocytes, OCPs and mature OCs. Its function is to stimulate OC differentiation (Kwan Tat, Padrines et al. 2004) and according to previous studies TNF increases RANK expression (Kitaura, Sands et al. 2004). We analyzed RANK expression and we observed that at day 1 is significantly increased in healthy subjects and also in baseline patients when compared with the follow-up group. No other significant differences were found between groups in both conditions. In the stimulated condition, when we analyzed RANK expression throughout time we found that the healthy group has its highest RANK expression at day 1, the follow-up group at day 7 and the baseline group at day 14 of culture (Figure III.20, right panel). Interestingly we found no RANK expression in the stimulated condition at day 21 of healthy donors. According to

our results and to these findings, RANK expression or function may be affected in monocytes and osteoclast precursors of AS patients. SNPs in RANK have previously been associated with osteoporosis (Styrkarsdottir, Halldorsson et al. 2008) and recently Cortes et al. related a RANK SNP with cervical damage protection given by mSASSS (Cortes, Maksymowych et al. 2014).

NFATc1 is the target gene for RANK signaling cascade and has been proposed to function as the master transcription factor in osteoclastogenesis (Takayanagi, Kim et al. 2002). We found that its expression is significantly higher in healthy subjects at day 1 when compared to AS follow-up patients. Although no significant differences were observed between groups in the both unstimulated and stimulated conditions (Figure III.21) TNF-blocking therapy seems to increase NFATc1 expression, favoring osteoclastogenesis.

Atp6v0d2 gene encodes the subunit d2 of the functional domain v0 of the ATPase proton pump present at the osteoclasts ruffled border and, therefore, is responsible for extracellular acidification of the resorption lacunae and bone demineralization during bone resorption (Qin, Cheng et al. 2012). According to our results, Atp6v0d2 expression at day 1 is significantly higher in the healthy group when compared to follow-up patients (Figure III.22) and at day 14 in the unstimulated condition there is a significantly higher Atp6v0d2 expression in the follow-up group when compared to the healthy controls. Moreover, in the stimulated condition, both baseline and healthy groups have their higher Atp6v0d2 expression at day 14, and in the follow-up group Atp6v0d2 expression continues to increase until day 21 (Figure III.22). Although not significantly, increased Atp6v0d2 expression at days 14 and 21 in osteoclasts of patients under TNF-blocking therapy is in accordance with our previous observations of increased bone resorption after anti-TNF therapy.

CLCN7 encodes for the chloride channel that maintains osteoclasts' electroneutrality and provides the chloride conductance required for an efficient proton pump (Novack and Teitelbaum 2008). At day 1, CLCN7 expression is significantly higher in healthy subjects when compared to the follow-up group. In the unstimulated condition gene expression at day 7 in the follow-up group is significantly higher when compared to baseline patients, however, at day 14 baseline patients have the highest expression when compared to the healthy group (Figure III.23, left panel). Thus, our results show that during osteoclast differentiation at days 7 and 14, TNF-blocking therapy seems to decrease CLCN7 expression closer to the healthy expression (Figure III.23). To the best of our knowledge this gene was never described in ankylosing spondylitis patients but loss-of-function mutations in CLCN7 lead to osteopetrosis (Frattini, Pangrazio et al. 2003).

Cathepsin K is enconded by CTSK gene and is a protease predominantly expressed by OCs which is involved in the degradation of the bone organic matrix (Novack and Teitelbaum 2008). When cells are cultured with differentiation stimuli, we found that CTSK expression significantly increases in all groups until day 14 (Figure III.24, right panel). Despite the differences are not significant, CTSK expression in osteoclasts is similar between healthy subjects and patients under TNF blockers being decreased in baseline AS patients at days 14 and 21 which may suggest that TNF-blocking therapy resets CTSK expression towards a higher osteoclast activity and bone resorption. Accordingly, serum levels of cathepsin K in AS patients were measured by Wendling et al and they found that levels of

cathepsin K were higher in AS patients with active disease than in healthy controls but the differences were not statistically significant (Wendling, Cedoz et al. 2008).

Overall, our results show that expression of RANK, NFATc1, Atp6v0d2 and CLCN7 is increased in monocytes of healthy subjects when compared with AS patients treated or not with TNF blockers. In AS patients at baseline we observed lower osteoclastogenesis and, consequently, lower bone resorption; however in AS patients after TNF-blocking therapy osteoclast activity is increased suggesting that after biological treatment AS patients started to respond to osteoclastogenic stimuli, following what happens in the healthy control condition.

In this study, patient recruitment was conditioned by the start of TNF-blocking therapy and specifically in the follow-up group, it was conditioned by biological therapy switch, which occurs when patients have adverse effects or are non-responders. Our results interpretation was also limited because we do not have dual-energy X-ray absorptiometry (DXAs) of these patients to assess their bone mineral density in order to correlate with our results. The functional assays also have a few limitations. In the TRAP staining, osteoclast precursors have the tendency to aggregate and it is challenging to visually distinguish multinucleated osteoclast from several mononuclear pre-osteoclasts. In the resorption pits assay, it is difficult to distinguish between a single pit and more than one pit that are close together. Another limitation in assaying osteoclast function *in vitro* is that we have to infer OC behaviour from two separate cultures (one for TRAP staining and the other for pit assay, in both cells were cultured in bone slices).

It has been reported that AS patients have decreased bone mineral density (Singh, Nimarpreet et al. 2013) but controversially these patients are also particularly prone to develop syndesmophytes, a bony growth originating inside a ligament and enthesitis, an inflammation at the sites where tendons or ligaments insert to bone (Lories and Schett 2012). However, recent studies found that in AS, even with TNF inhibition, ectopic bone formation is still observed, suggesting that additional factors are governing this effect (Osta, Benedetti et al. 2014). TNF-blocking therapy also seems to decrease DKK-1 levels, which might lead to increased osteoblastogenesis and bone formation (Korkosz, Gasowski et al. 2014) and therefore it would be interesting to study the effects of this biological therapy in osteoblast activity. In this study we used *in vitro* techniques to understand a systemic effect of the disease and therapy on osteoclast formation and function, which does not allow us to observe the interaction between osteoclasts and osteoblasts on bone remodeling. Further studies are necessary to address this topic.

It has been described in other rheumatic diseases such as psoriasis changes in serum microRNAs levels after TNF-blocking therapy (Pivarcsi, Meisgen et al. 2013). Regarding AS, it has been found three highly expressed microRNAs in T cells of AS patients but none directly involved with osteoclastogenesis (Lai, Yu et al. 2013). Hence it would be very interesting to evaluate if there are microRNAs specific of AS with the ability of repressing osteoclast differentiation and function and if TNF-blocking therapy affects their expression.

In light of our results we can speculate about the existence of a mechanism in AS patients that disconnects inflammation and osteoclastogenesis that is halted by the TNF-blocking therapy. In conclusion, decreased pro-inflammatory cytokine levels, in particular IL-17A and TGF-β, in AS patients

on TNF-blocking therapy suggest that TNF-blocking therapy resolves inflammation in AS patients. However, according to our results, osteoclast activity is significantly increased in AS patients under TNF blockers. Moreover, OPG levels are decreased after TNF-blocking therapy. Therefore, the present study points to an increase in osteoclastogenesis and also in bone resorption in AS patients under TNF-blocking therapy.

V. REFERENCES

Acebes, C., C. de la Piedra, et al. (1999). "Biochemical markers of bone remodeling and bone sialoprotein in ankylosing spondylitis." Clin Chim Acta **289**(1-2): 99-110.

Ai-Aql, Z. S., A. S. Alagl, et al. (2008). "Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis." J Dent Res **87**(2): 107-118.

Appel, H., M. Kuhne, et al. (2006). "Immunohistochemical analysis of hip arthritis in ankylosing spondylitis: evaluation of the bone-cartilage interface and subchondral bone marrow." Arthritis Rheum **54**(6): 1805-1813.

Appel, H., C. Loddenkemper, et al. (2009). "Rheumatoid arthritis and ankylosing spondylitis - pathology of acute inflammation." Clin Exp Rheumatol **27**(4 Suppl 55): S15-19.

Arends, S., A. Spoorenberg, et al. (2012). "The effect of three years of TNFalpha blocking therapy on markers of bone turnover and their predictive value for treatment discontinuation in patients with ankylosing spondylitis: a prospective longitudinal observational cohort study." Arthritis Res Ther **14**(2): R98.

Bakland, G. and H. C. Nossent (2013). "Epidemiology of spondyloarthritis: a review." Curr Rheumatol Rep **15**(9): 351.

Bal, A., E. Unlu, et al. (2007). "Comparison of serum IL-1 beta, sIL-2R, IL-6, and TNF-alpha levels with disease activity parameters in ankylosing spondylitis." Clin Rheumatol **26**(2): 211-215.

Baraliakos, X. and J. Braun (2012). "Biologic therapies for spondyloarthritis: what is new?" Curr Rheumatol Rep **14**(5): 422-427.

Bond, D. (2013). "Ankylosing spondylitis: diagnosis and management." Nurs Stand **28**(16-18): 52-59; quiz 60.

Borel, O., E. Gineyts, et al. (2012). "Cathepsin K preferentially solubilizes matured bone matrix." Calcif Tissue Int **91**(1): 32-39.

Boskey, A. L. and P. G. Robey (2013). The Composition of Bone. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, John Wiley & Sons, Inc.**:** 49-58.

Boyce, B. F. (2013). "Advances in the regulation of osteoclasts and osteoclast functions." J Dent Res **92**(10): 860-867.

Braem, K. and R. J. Lories (2012). "Insights into the pathophysiology of ankylosing spondylitis: contributions from animal models." Joint Bone Spine **79**(3): 243-248.

Braun, J., M. Bollow, et al. (1995). "Use of immunohistologic and in situ hybridization techniques in the examination of sacroiliac joint biopsy specimens from patients with ankylosing spondylitis." Arthritis Rheum **38**(4): 499-505.

Braun, J. and J. Sieper (2007). "Ankylosing spondylitis." Lancet **369**(9570): 1379-1390.

Briolay, A., P. Lencel, et al. (2013). "Autocrine stimulation of osteoblast activity by Wnt5a in response to TNF-alpha in human mesenchymal stem cells." Biochem Biophys Res Commun **430**(3): 1072-1077.

Bronson, W. D., S. E. Walker, et al. (1998). "Bone mineral density and biochemical markers of bone metabolism in ankylosing spondylitis." J Rheumatol **25**(5): 929-935.

Burr, D. B. and O. Akkus (2014). Chapter 1 - Bone Morphology and Organization. Basic and Applied Bone Biology. D. B. B. R. Allen. San Diego, Academic Press**:** 3-25.

Chen, C. H., H. A. Chen, et al. (2010). "Soluble receptor activator of nuclear factor-kappaB ligand (RANKL) and osteoprotegerin in ankylosing spondylitis: OPG is associated with poor physical mobility and reflects systemic inflammation." Clin Rheumatol **29**(10): 1155-1161.

Chen, W. S., Y. S. Chang, et al. (2012). "Association of serum interleukin-17 and interleukin-23 levels with disease activity in Chinese patients with ankylosing spondylitis." J Chin Med Assoc **75**(7): 303-308.

Cho, E. S., K. S. Lee, et al. (2010). "Compressive mechanical force augments osteoclastogenesis by bone marrow macrophages through activation of c-Fms-mediated signaling." J Cell Biochem **111**(5): 1260-1269.

Coates, L. C., H. Marzo-Ortega, et al. (2010). "Anti-TNF Therapy in Ankylosing Spondylitis: Insights for the Clinician." Ther Adv Musculoskelet Dis **2**(1): 37-43.

Coates, P. (2013). "Bone turnover markers." Aust Fam Physician **42**(5): 285-287.

Cortes, A., W. P. Maksymowych, et al. (2014). "Association study of genes related to bone formation and resorption and the extent of radiographic change in ankylosing spondylitis." Ann Rheum Dis.

Crockett, J. C., M. J. Rogers, et al. (2011). "Bone remodelling at a glance." J Cell Sci **124**(Pt 7): 991-998.

Del Fattore, A., A. Teti, et al. (2012). "Bone cells and the mechanisms of bone remodelling." Front Biosci (Elite Ed) **4**: 2302-2321.

El Maghraoui, A. (2004). "Osteoporosis and ankylosing spondylitis." Joint Bone Spine **71**(4): 291-295.

Feldtkeller, E., M. A. Khan, et al. (2003). "Age at disease onset and diagnosis delay in HLA-B27 negative vs. positive patients with ankylosing spondylitis." Rheumatol Int **23**(2): 61-66.

Frattini, A., A. Pangrazio, et al. (2003). "Chloride channel ClCN7 mutations are responsible for severe recessive, dominant, and intermediate osteopetrosis." J Bone Miner Res **18**(10): 1740-1747.

Gengenbacher, M., H. J. Sebald, et al. (2008). "Infliximab inhibits bone resorption by circulating osteoclast precursor cells in patients with rheumatoid arthritis and ankylosing spondylitis." Ann Rheum Dis **67**(5): 620-624.

Gratacos, J., A. Collado, et al. (1994). "Serum cytokines (IL-6, TNF-alpha, IL-1 beta and IFN-gamma) in ankylosing spondylitis: a close correlation between serum IL-6 and disease activity and severity." Br J Rheumatol **33**(10): 927-931.

Holloway, W. R., F. M. Collier, et al. (2002). "Leptin inhibits osteoclast generation." J Bone Miner Res **17**(2): 200-209.

Hreggvidsdottir, H. S., T. Noordenbos, et al. (2014). "Inflammatory pathways in spondyloarthritis." Mol Immunol **57**(1): 28-37.

Husheem, M., J. K. Nyman, et al. (2005). "Characterization of circulating human osteoclast progenitors: development of in vitro resorption assay." Calcif Tissue Int **76**(3): 222-230.

Igarashi, Y., M. Y. Lee, et al. (2002). "Acid phosphatases as markers of bone metabolism." J Chromatogr B Analyt Technol Biomed Life Sci **781**(1-2): 345-358.

Im, C. H., E. H. Kang, et al. (2009). "Receptor activator of nuclear factor kappa B ligand-mediated osteoclastogenesis is elevated in ankylosing spondylitis." Clin Exp Rheumatol **27**(4): 620-625.

Irons, K., Jeffries, Claire (2009). The Bath Indices - Outcome measures for use with ankylosing spondylitis patients Surrey, The National Ankylosing Spondylitis Society (NASS).

Ishii, T., J. Kikuta, et al. (2010). "Control of osteoclast precursor migration: A novel point of control for osteoclastogenesis and bone homeostasis." IBMS BoneKEy **7**(8): 279-286.

Jung, Y. K., S. W. Han, et al. (2012). "DICAM inhibits osteoclast differentiation through attenuation of the integrin alphaVbeta3 pathway." J Bone Miner Res **27**(9): 2024-2034.

Keller, C., A. Webb, et al. (2003). "Cytokines in the seronegative spondyloarthropathies and their modification by TNF blockade: a brief report and literature review." Ann Rheum Dis **62**(12): 1128-1132.

Khan, M. A. (2011). "Ankylosing spondylitis and related spondyloarthropathies: the dramatic advances in the past decade." Rheumatology (Oxford) **50**(4): 637-639.

Kikuta, J. and M. Ishii (2013). "Osteoclast migration, differentiation and function: novel therapeutic targets for rheumatic diseases." Rheumatology (Oxford) **52**(2): 226-234.

Kitaura, H., K. Kimura, et al. (2013). "Immunological reaction in TNF-alpha-mediated osteoclast formation and bone resorption in vitro and in vivo." Clin Dev Immunol **2013**: 181849.

Kitaura, H., M. S. Sands, et al. (2004). "Marrow stromal cells and osteoclast precursors differentially contribute to TNF-alpha-induced osteoclastogenesis in vivo." J Immunol **173**(8): 4838-4846.

Klingberg, E., M. Nurkkala, et al. (2014). "Biomarkers of bone metabolism in ankylosing spondylitis in relation to osteoproliferation and osteoporosis." J Rheumatol **41**(7): 1349-1356.

Korkosz, M., J. Gasowski, et al. (2014). "Effect of tumour necrosis factor-alpha inhibitor on serum level of dickkopf-1 protein and bone morphogenetic protein-7 in ankylosing spondylitis patients with high disease activity." Scand J Rheumatol **43**(1): 43-48.

Kwan Tat, S., M. Padrines, et al. (2004). "IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology." Cytokine Growth Factor Rev **15**(1): 49-60.

Lai, N. S., H. C. Yu, et al. (2013). "Aberrant expression of microRNAs in T cells from patients with ankylosing spondylitis contributes to the immunopathogenesis." Clin Exp Immunol **173**(1): 47-57.

Lee, N. K. (2010). "Molecular Understanding of Osteoclast Differentiation and Physiology." Endocrinol Metab **25**(4): 264-269.

Li, G. X., S. Wang, et al. (2013). "Serum levels of IL-33 and its receptor ST2 are elevated in patients with ankylosing spondylitis." Scand J Rheumatol **42**(3): 226-231.

Li, P., E. M. Schwarz, et al. (2004). "Systemic tumor necrosis factor alpha mediates an increase in peripheral CD11bhigh osteoclast precursors in tumor necrosis factor alpha-transgenic mice." Arthritis Rheum **50**(1): 265-276.

Limon-Camacho, L., M. I. Vargas-Rojas, et al. (2012). "In vivo peripheral blood proinflammatory T cells in patients with ankylosing spondylitis." J Rheumatol **39**(4): 830-835.

Liu, F., F. Wang, et al. (2013). "Expression of IL-2 and IL-11 and its significance in patients with ankylosing spondylitis." Asian Pac J Trop Med **6**(1): 76-78.

Lories, R. J. and G. Schett (2012). "Pathophysiology of new bone formation and ankylosis in spondyloarthritis." Rheum Dis Clin North Am **38**(3): 555-567.

Luxenburg, C., D. Geblinger, et al. (2007). "The architecture of the adhesive apparatus of cultured osteoclasts: from podosome formation to sealing zone assembly." PLoS One **2**(1): e179.

Mei, Y., F. Pan, et al. (2011). "Increased serum IL-17 and IL-23 in the patient with ankylosing spondylitis." Clin Rheumatol **30**(2): 269-273.

Mellis, D. J., C. Itzstein, et al. (2011). "The skeleton: a multi-functional complex organ: the role of key signalling pathways in osteoclast differentiation and in bone resorption." J Endocrinol **211**(2): 131-143.

Mochizuki, A., M. Takami, et al. (2012). "Cell adhesion signaling regulates RANK expression in osteoclast precursors." PLoS One **7**(11): e48795.

Muntean, L., M. Rojas-Vargas, et al. (2011). "Relative value of the lumbar spine and hip bone mineral density and bone turnover markers in men with ankylosing spondylitis." Clin Rheumatol **30**(5): 691-695.

Nakashima, T. and H. Takayanagi (2009). "Osteoclasts and the immune system." J Bone Miner Metab **27**(5): 519-529.

National Institute for Health and Care Excellence, N. (2008). "Adalimumab, etanercept and infliximab for ankylosing spondylitis." Retrieved 09-04-2014, from http://www.nice.org.uk/guidance/ta143/chapter/appendix-c-modified-new-york-criteria-for-diagnosis-of-ankylosing-spondylitis.

Novack, D. V. and S. L. Teitelbaum (2008). "The osteoclast: friend or foe?" Annu Rev Pathol **3**: 457-484.

NZYtech. (2013). "NZYol." from https://www.nzytech.com/site/RNA-purification/NZYol.

Osta, B., G. Benedetti, et al. (2014). "Classical and Paradoxical Effects of TNF-alpha on Bone Homeostasis." Front Immunol **5**: 48.

Pedersen, S. J., P. Chiowchanwisawakit, et al. (2011). "Resolution of inflammation following treatment of ankylosing spondylitis is associated with new bone formation." J Rheumatol **38**(7): 1349-1354.

Pinzone, J. J., B. M. Hall, et al. (2009). "The role of Dickkopf-1 in bone development, homeostasis, and disease." Blood **113**(3): 517-525.

Pivarcsi, A., F. Meisgen, et al. (2013). "Changes in the level of serum microRNAs in patients with psoriasis after antitumour necrosis factor-alpha therapy." Br J Dermatol **169**(3): 563-570.

Poddubnyy, D. and D. van der Heijde (2012). "Therapeutic controversies in spondyloarthritis: nonsteroidal anti-inflammatory drugs." Rheum Dis Clin North Am **38**(3): 601-611.

Qin, A., T. S. Cheng, et al. (2012). "V-ATPases in osteoclasts: structure, function and potential inhibitors of bone resorption." Int J Biochem Cell Biol **44**(9): 1422-1435.

Ramiro, S., Machado, Pedro, Roque, Raquel, Santos, Helena, Polido-Pereira, Joaquim, Peixoto, Daniela, Duarte, Cátia (2011). "Performance Of The Ankylosing Spondylitis Disease Activity Score (ASDAS) In Patients Under Biological Therapies In Daily Practice: Results From The Portuguese Register Reuma.Pt." Arthritis & Rheumatism **Volume 63**(November 2011 Abstract Supplement).

Ren, L., J. Li, et al. (2013). "Efficacy of antitumor necrosis factor(alpha) agents on patients with ankylosing spondylitis." Am J Med Sci **346**(6): 455-461.

Ritchlin, C. T., S. A. Haas-Smith, et al. (2003). "Mechanisms of TNF-alpha- and RANKL-mediated osteoclastogenesis and bone resorption in psoriatic arthritis." J Clin Invest **111**(6): 821-831.

Rodak, B. F., G. A. Fritsma, et al. (2007). Hematology: Clinical Principles and Applications, Saunders Elsevier.

Sari, I., L. Kebapcilar, et al. (2010). "Fetuin-A and interleukin-18 levels in ankylosing spondylitis." Int J Rheum Dis **13**(1): 75-81.

Schett, G. (2008). "Review: Immune cells and mediators of inflammatory arthritis." Autoimmunity **41**(3): 224-229.

Schett, G., L. C. Coates, et al. (2011). "Structural damage in rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis: traditional views, novel insights gained from TNF blockade, and concepts for the future." Arthritis Res Ther **13 Suppl 1**: S4.

Sheehan, N. J. (2004). "The ramifications of HLA-B27." J R Soc Med **97**(1): 10-14.

Silva, L. C., L. C. Ortigosa, et al. (2010). "Anti-TNF-alpha agents in the treatment of immune-mediated inflammatory diseases: mechanisms of action and pitfalls." Immunotherapy **2**(6): 817-833.

Sims, N. A. and J. H. Gooi (2008). "Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption." Semin Cell Dev Biol **19**(5): 444-451.

Singh, H. J., K. Nimarpreet, et al. (2013). "Study of bone mineral density in patients with ankylosing spondylitis." J Clin Diagn Res **7**(12): 2832-2835.

Sridharan, G. and A. A. Shankar (2012). "Toluidine blue: A review of its chemistry and clinical utility." J Oral Maxillofac Pathol **16**(2): 251-255.

StemRx, B. S. (2012, 2012). "Stem Cells Treatment for Ankylosing Spondylitis." Retrieved 13.01.2014, from http://www.stemrx.in/ankylosing_spondylitis.html

Stupack, D. G. and D. A. Cheresh (2002). "Get a ligand, get a life: integrins, signaling and cell survival." J Cell Sci **115**(Pt 19): 3729-3738.

Styrkarsdottir, U., B. V. Halldorsson, et al. (2008). "Multiple genetic loci for bone mineral density and fractures." N Engl J Med **358**(22): 2355-2365.

Sutherland, M. K., J. C. Geoghegan, et al. (2004). "Sclerostin promotes the apoptosis of human osteoblastic cells: a novel regulation of bone formation." Bone **35**(4): 828-835.

Takahashi, N., S. Ejiri, et al. (2007). "Regulation of osteoclast polarization." Odontology **95**(1): 1-9.

Takayanagi, H., S. Kim, et al. (2002). "Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts." Dev Cell **3**(6): 889-901.

Takuma, A., T. Kaneda, et al. (2003). "Dexamethasone enhances osteoclast formation synergistically with transforming growth factor-beta by stimulating the priming of osteoclast progenitors for differentiation into osteoclasts." J Biol Chem **278**(45): 44667-44674.

Taylan, A., I. Sari, et al. (2012). "Biomarkers and cytokines of bone turnover: extensive evaluation in a cohort of patients with ankylosing spondylitis." BMC Musculoskelet Disord **13**: 191.

Taylan, A., I. Sari, et al. (2012). "Evaluation of the T helper 17 axis in ankylosing spondylitis." Rheumatol Int **32**(8): 2511-2515.

Teitelbaum, S. L. and F. P. Ross (2003). "Genetic regulation of osteoclast development and function." Nat Rev Genet **4**(8): 638-649.

Ustun, N., F. Tok, et al. (2014). "Sclerostin and Dkk-1 in Patients with Ankylosing Spondylitis." Acta Reumatol Port **39**(2): 146-151.

van Bezooijen, R. L., B. A. Roelen, et al. (2004). "Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist." J Exp Med **199**(6): 805-814.

van der Linden, S., H. A. Valkenburg, et al. (1984). "Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria." Arthritis Rheum **27**(4): 361-368.

Visvanathan, S., D. van der Heijde, et al. (2009). "Effects of infliximab on markers of inflammation and bone turnover and associations with bone mineral density in patients with ankylosing spondylitis." Ann Rheum Dis **68**(2): 175-182.

Wendling, D., J. P. Cedoz, et al. (2008). "Serum levels of MMP-3 and cathepsin K in patients with ankylosing spondylitis: effect of TNFalpha antagonist therapy." Joint Bone Spine **75**(5): 559-562.

Winkler, D. G., M. K. Sutherland, et al. (2003). "Osteocyte control of bone formation via sclerostin, a novel BMP antagonist." EMBO J **22**(23): 6267-6276.

WMA. (2013). "World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects." Retrieved 19.06.2014, 2014, from http://www.wma.net/en/30publications/10policies/b3/index.html.

Wong, M. L. and J. F. Medrano (2005). "Real-time PCR for mRNA quantitation." Biotechniques **39**(1): 75-85.

Wu, Y., M. B. Humphrey, et al. (2008). "Osteoclasts - the innate immune cells of the bone." Autoimmunity **41**(3): 183-194.

Xueyi, L., C. Lina, et al. (2013). "Levels of circulating Th17 cells and regulatory T cells in ankylosing spondylitis patients with an inadequate response to anti-TNF-alpha therapy." J Clin Immunol **33**(1): 151-161.

Ye, J., G. Coulouris, et al. (2012). "Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction." BMC Bioinformatics **13**: 134.

Zambrano-Zaragoza, J. F., J. M. Agraz-Cibrian, et al. (2013). "Ankylosing spondylitis: from cells to genes." Int J Inflam **2013**: 501653.

Zhang, L., Y. G. Li, et al. (2012). "Increased frequencies of Th22 cells as well as Th17 cells in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis." PLoS One **7**(4): e31000.

Zhao, H., F. P. Ross, et al. (2005). "Unoccupied alpha(v)beta3 integrin regulates osteoclast apoptosis by transmitting a positive death signal." Mol Endocrinol **19**(3): 771-780.

Zupan, J., M. Jeras, et al. (2013). "Osteoimmunology and the influence of pro-inflammatory cytokines on osteoclasts." Biochem Med (Zagreb) **23**(1): 43-63.

VI. APPENDIX

Criteria for AS diagnosis:

Table VI.1 - 1984 Modified New York criteria for ankylosing spondylitis. Adapted from (National Institute for Health and Care Excellence 2008)

Modified New York criteria for diagnosis of ankylosing spondylitis
A definite diagnosis of ankylosing spondylitis requires the radiological criterion and at least one clinical criterion to be satisfied as defined below. **Radiological criterion** • Sacroiliitis at least grade 2 bilaterally or grade 3 or 4 unilaterally. **Clinical criteria** • Low back pain and stiffness for more than 3 months that improves with exercise but is not relieved by rest. • Limitation of motion of the lumbar spine in both the sagittal and frontal planes. • Limitation of chest expansion relative to normal values correlated for age and sex. All reasonable measures should be taken to ensure that symptoms are due predominantly to ankylosing spondylitis and that alternative causes, including spinal fracture, disc disease and fibromyalgia, are excluded.

Radiographs of the sacroiliac joint can appear normal in the early phase of disease since it can take up to 10 years for structural changes to become visible on plain radiographs (Schett, Coates et al. 2011). To identify AS patients early before irreversible structural damage has occurred, at the time treatment should be started, other imaging techniques such as magnetic resonance imaging (MRI) have to be used since they allow the detection of inflammation in the sacroiliac joints in patients early in the course of the disease when no chronic changes are detectable (Coates, Marzo-Ortega et al. 2010).

Criteria for starting TNF-blocking therapy:

Anti-TNF therapy is only used in cases of active disease (Bath Ankylosing spondylitis disease activity index (BASDAI) > 4 and expert opinion), refractory to conventional therapy such as physiotherapy and non-steroidal anti-inflammatory drugs (NSAIDs), failure of intra-articular steroids (if indicated) and failure of sulfasalazine (if predominant peripheral arthritis) (Coates, Marzo-Ortega et al. 2010).

For patients with active AS with symptoms of pain, stiffness and decrease of function, TNF-blocking therapy is significantly effective and increasingly considered the standard of care when patients fail to respond adequately to conventional treatment with NSAIDs at the maximum possible dose and physical exercises (Khan 2011). The finding that TNF is present at high concentrations in sacroiliac joints of AS patients (Braun, Bollow et al. 1995) provided a strong guideline for the use of TNF-inhibitors, which have proven to be very successful in the treatment of spondyloarthritides (Ren, Li et al. 2013).

Table VI.2 - Characteristics of primers used in this study

Gene		Sequence	Ta	Transcript
CSF1R	Fw	GAACATCCACCTCGAGAAGAAA	60ºC	88 bp
	Rv	GACAGGCCTCATCTCCACAT		
RANK	Fw	GAACATCATGGGACAGAGAAATC	60ºC	89 bp
	Rv	GGCAAGTAAACATGGGGTTC		
NFATc1	Fw	GCAAGCCGAATTCTCTGGTG	60ºC	144 bp
	Rv	TACCGTTGGCGGGAAGGTAG		
Atp6v0d2	Fw	CATTCTTGAGTTTGAGGCCG	60ºC	186 bp
	Rv	CCGTAATGATCCGCTACGTT		
CLCN7	Fw	CGAGTCGGACATATGAGCAG	60ºC	83 bp
	Rv	TCCTTGGGGAAGGGATGT		
CTSK	Fw	GCCAGACAACAGATTTCCATC	60ºC	75 bp
	Rv	CAGAGCAAAGCTCACCACAG		
18s rRNA	Fw	GGAGTATGGTTGCAAAGCTGA	60ºC	129 bp
	Rv	ATCTGTCAATCCTGTCCGTGT		

CSF1R - *Colony stimulating factor 1 receptor*; RANK - Receptor activator of NF-kB; NFATc1 - Nuclear factor of activated T cells cytoplasmic 1; Atp6v0d2 - ATPase; CLCN7 - Chloride channel, voltage-sensitive 7; CTSK - cathepsin K; rRNA - ribossomal ribonucleic acid; Fw - forward; Rv - reverse; Ta - Annealing temperature; bp - base pair.