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**Bone Biology & Orthopaedic Research**



Master of Biomedical Engineering.  
MASTER THESIS

**EB**

**Osteoclast responses to metal ions**

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*Osteoclast recruitment and activation in response to metal.*

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## 1. LIST OF ABBREVIATIONS

TJR	Total joint replacement
PBMC	Peripheral Blood Mononuclear Cell
BMC	Bone marrow cells
RANKL	Receptor activator of nuclear factor (NF)- $\kappa$ B ligand
M-CSF	Macrophage colony stimulating factor
TNF alpha	Tumor necrosis factor alpha
IL-6	Interleukin-6
VEGF	Vascular endothelial growth factor
TRAP	Tartrate resistant acid phosphatase
ELISA	Enzyme-linked immunosorbent assay
BSA	Bovine serum albumin
FCS	Fetal calf serum
PCR	Polymerase chain reaction
$\alpha$ -MEM	$\alpha$ -minimum essential medium
RT	Room temperature
RNA	Ribonucleic acid
cDNA	Complementary deoxyribonucleic acid

## 2. INTRODUCTION

Synovial joints in the human body (hip, knee, shoulder joints...) are structures capable of functioning under critical conditions. Their behavior and endurance is due to the optimized combination of cartilage (a dense connective tissue covering the bones involved in the joint), and synovial fluid (nutrient fluid secreted within the joint area).

With the increase of life expectancy, the prevalence of osteoarthritis – a degenerative joint diseases – is also increasing. The resulting pain and joint stiffness is posing a major burden on affected individuals as well as on society. In fact, according to Bjelle [1], degenerative joint disease it is the second most frequent chronic condition in Europe.

Total joint arthroplasty – the replacement of diseased joint surfaces by metal, plastic, or ceramic artificial materials - is recognized as a major achievement in treating this immobilizing disease.

The longevity of arthroplasties has become an important issue in orthopaedic surgery. The lifespan of implants is mainly limited by a process called aseptic loosening. During this process the contact between bone and the implant surface is lost and the prosthesis becomes loose. Although the trigger of aseptic loosening is still under investigation it is known that wear particles from the articulating surface are playing a role. An additional aspect in the development of aseptic loosening might be host responses to metals. Implant alloys in contact with biological systems undergo corrosion, leading to the release of metal ions into the periprosthetic tissues and the circulation. These increased levels of metal ions ( $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ti}^{3+}$ , and of other metal ions), which may remain

free or become bound to proteins [2,3], may lead to a chronic inflammatory response as a consequence, which, may induce resorption of the peri-implant bone [4,5].

## 2.1 Bone Biology

Bones are part of the endoskeleton of all higher vertebrates. Bone is a composite and complex dynamic system that is made up of various tissue types such as bone marrow, periosteum, nerves, blood vessels and cartilage, but mostly of mineralized osseous tissue. This anisotropic tissue, formed mainly by calcium hydroxylapatite, is relatively hard and lightweight. And although it has a high compressive strength, it possesses only poor tensile strength. It is essentially brittle but still has a significant degree of elasticity, mainly due to the presence of collagen I.

These attributes aid multiple functions of bone that include: protecting internal organs; providing a scaffold to support the body; serving as levers for muscles; buffering blood pH by absorption and release of alkaline salts; detoxifying by storage and gradual release of heavy metals; serving as mineral reserves, mostly notable for calcium and phosphate; and producing blood cells through hematopoiesis in the red bone marrow.

Bones are either woven or lamellar (layered). Although woven bone is weak because it possesses only a small number of randomly oriented collagen fibers, it forms quickly and does not require a pre-existing structure for growth. Lamellar bone is stronger, as it consists of numerous stacked layers that are filled with collagen fibers running in opposite directions in alternating layers, but it forms slowly.

Bone is subject to the complex process of bone remodeling. A German anatomist Julius Wolff in the 19th century postulate the Wolff law: "Bone adapts to the loads it is placed under". The cycle of bone resorption by osteoclasts and bone formation by osteoblasts is life-long. It serves three main purposes – adaption to changing forces in specific regions, damage repair, and calcium homeostasis.

The complex tissue of bone is made up of many different cell types. On the surface of the bone there are pluripotent, fibroblast-like, mesenchymal cells, called osteoprogenitor cells. These cells give rise to preosteoblasts. These preosteoblasts can develop towards osteoblasts and further towards osteocytes and lining cells.

### 2.1.1 Osteocytes

Osteocytes originate from migrating osteoblasts that have become surrounded by bone matrix, which they themselves produced. Their occupied space is known as lacunae. Osteocytes contribute in varying degrees to a number of functions that include: bone formation, calcium homeostasis, cell communication, and matrix maintenance. It is also possible that they regulate the bone's stress response through functioning as mechanosensory receptors. Bone lining cells cover the entire available surface of the bone. They are flat and are essentially inactive osteoblasts that serve as a barrier against certain ions. They are also considered as putative osteoblast progenitor cells, respectively resting osteoblasts.

### 2.1.2 Osteoblasts

Osteoblasts are mononucleated bone-forming cells that emerge from preosteoblasts. Osteoblasts produce osteoid, a protein mixture consisting mainly of type I collagen that later mineralizes to become bone. Osteoblasts also manufacture many matrix proteins, such as: osteocalcin; sialoprotein; osteopontin; receptors for growth factors and hormones; different types of proteoglycans; and hormones such as prostaglandins that act on the bone itself. Osteoblasts also stably produce alkaline phosphatase, a key enzyme in bone mineralization.

### 2.1.3 Osteoclasts

Osteoclasts act antagonistically to osteoblasts. They are located on the bone surface in what is called Howship's lacunae, or resorption pits, and are responsible for bone resorption.

The osteoclast was first named and functionally characterized by Kölliker in the late 19th century. However, its origin and its physiology remained obscure for long, until parabiosis (fusion of embryos) experiments provided the first clear evidence that osteoclasts originate from circulating cells [5]. Later it was shown that a mixed primary cell culture of macrophages and monocytes can be converted into osteoclasts [6]. Because of the observation that cell-cell interactions between mononuclear phagocyte progenitors and bone stromal cells seemed to be essential for in vitro osteoclastogenesis, a membrane associated osteoclast differentiation factor (ODF) on bone stromal cells was postulated. This hypothesis was controversially discussed for several years until 1998 when Receptor activator of nuclear factor (NF)- $\kappa$ B ligand (RANKL) was shown to be the essential osteoclastogenic factor in Co-culture [7]. As predicted, RANKL is almost entirely expressed as a cell membrane-associated factor of osteoblasts. The more active soluble form of RANKL (sRANKL) derives from the membrane bound form by proteolytic cleavage or alternative splicing [8]. Cleavage requires disintegrin and metalloprotease domain (ADAM) family members and matrix metalloproteases (MMP) [9]. For differentiation, a specialized bone marrow milieu is required since RANKL expressed in other locations does not cause osteoclast formation.

In vitro osteoclast cell culture systems were established in the following years by culturing isolated osteoclast precursor cells in the presence of both RANKL and macrophage colony stimulating factor (M-CSF; CSF-1). M-CSF is required for proliferation of differentiating osteoclasts and their precursors. M-CSF is the growth factor for cells of the monocyte-macrophage lineage and supports the proliferation, survival and differentiation of cells from monocyte to macrophages.

Continuous RANKL stimulation of mature osteoclasts leads to enhanced resorptive activity and, in combination with IL-1 (Interleukin 1), it prolongs osteoclast survival [10].

It is possible that there are pathways acting parallel to CSF-1 and RANKL that can supply additional stimulation for efficient osteoclast formation. Immune tyrosine-based activation motif (ITAM) adapters and Syk nonreceptor tyrosine kinases are potential candidates [11]. Another candidate is tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), which is reported to permit formation of resorbing osteoclasts in the absence of RANKL. There is controversy about these findings, but most studies agree that TNF $\alpha$  is at least a co-stimulus for differentiation [12].

Once differentiated, osteoclast survival is regulated by numerous hormones, cytokines, and other factors. Some of the known factors are: interleukins 1, 6 and 11; transforming growth factor- $\alpha$  (TGF $\alpha$ ); TNF $\alpha$ ; and different systemic hormones – parathyroid hormone, calcitonin, calcitriol, and prostaglandins [13].

An important antagonist in osteoclastogenesis is osteoprotegerin (OPG). Molecular binding experiments showed that OPG associates with RANKL and functions as a decoy receptor [14].

OPG is secreted by osteoblasts as a response to estrogens or bone morphogenetic proteins (BMP), which are members of the transforming growth factor- $\beta$  (TGF- $\beta$ )- superfamily which induce bone formation. The antagonistic interplay of RANKL and OPG has direct relevance to human disease.

Osteoclast differentiation is characterized by different features: polynucleation due to cell fusion; formation of ruffled borders for proton, enzyme and acid secretion; associated synthesis of vacuolar proton pumps and different enzymes; and formation of a sealing zone to prevent enzyme and acid leakage.

Because not only polynucleated but also mononuclear osteoclasts express TRAP (tartrate resistant acid phosphatase), calcitonin receptor (CTR), and matrix metalloproteinase (MMP), and resorb bone, polynucleation is the most characteristic feature of mature osteoclasts.

Cell fusion is not a specific phenomenon for osteoclasts, as it can be observed in other tissues, such as placentas or myotubules. However, osteoclasts are the largest of these cells. The mechanism of cell fusion is not studied well yet, but it seems to have similarities to viral fusion. Multinucleated osteoclasts are highly polarized cells. As mentioned, they form new cytoskeletal elements such as ruffled borders and sealing zones when fused. These structures are located on the basal side and bone debris resorbed from this basal area is transferred to the apical side via transcytosis. Different lytic enzymes such as TRAP and pro-Cathepsin K (pro-CATK) are secreted from the highly specialized membrane into the resorption pit (Howship's lacunae). By this process the osteoclast erodes the underlying bone. Degradation products (collagen fragments and solubilized phosphate and calcium) are resorbed by the osteoclast, processed and then rereleased to metabolic circulation.

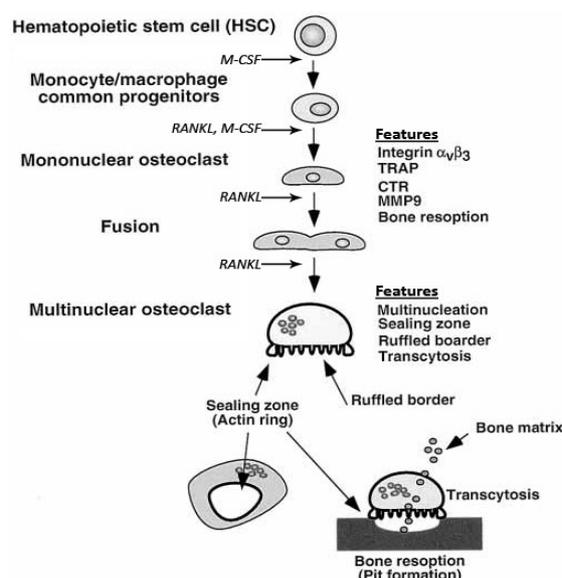


Figure 2: Overview of osteoclastogenesis

(SUDA et al., 2003).

## **2.2 Joint arthroplasty**

### **2.2.1 Materials Used in Orthopedic Implants**

The most common materials used in orthopedics implants are metals and polyethylene. These two material types are combined in most joint implants, that is, one component is made from metal, and one from polyethylene. When properly designed and implanted, the two components can rub together smoothly while minimizing wear.

Most metal implants are alloys from a mixture of two or more metals. By combining metals, a new material can be created that has a good balance of the desired characteristics. The most common metal alloys used are stainless steels, cobalt-chromium alloys, and titanium alloys.

#### ***Stainless Steel***

Stainless steel is a very strong alloy, and is most often used in implants that are intended to help repair fractures, such as bone plates, bone screws, pins, and rods. Stainless steel is made mostly of iron, with other metals such as chromium or molybdenum added to make it more resistant to corrosion, and Nickel to stabilize the austenitic phase. There are many different types of stainless steel. The stainless steels used in orthopedics implants are designed to resist the normal chemicals found in the human body. The most used is the AISI 316L, composition: C: 0.03%, Cr:17-20%, Ni:12-14%, Mo:2-4%. Corrosion is the principal defect of this alloy.

#### ***Cobalt-chromium Alloys***

Cobalt-chromium alloys are also strong, hard, biocompatible, and corrosion resistant (high concentration of Cr, that form a passive layer of  $\text{Cr}_2\text{O}_3$  on the surface of the implant). These alloys are used in a variety of joint replacement implants, as well as some fracture repair implants, that require a long service life. While cobalt-chromium alloys contain mostly cobalt and chromium, they also include other metals, such as molybdenum, to increase their strength. It has the higher Young Modulus, helping the osseointegration, but is more fragile.

#### ***Titanium Alloys***

Titanium alloys are considered to be biocompatible. They are the most flexible of all orthopaedic alloys. They are also lighter weight than most other orthopedic alloys. Consisting mostly of titanium, they also contain varying degrees of other metals, such as aluminum and vanadium. Passivation is due to the spontaneous formation of  $\text{TiO}_2$  on the surface of the metal.

#### ***Titanium***

Pure titanium may also be used in some implants where high strength is not required. It is used, for example, to make fiber metal, which is a layer of metal fibers bonded to the surface of an implant to allow the bone to grow into the implant, or cement to flow into the implant, for a better grip.

### ***Polyethylene***

Polyethylene (PE) is a type of plastic commonly used on the surface of one implant that is designed to articulate with another implant, as in a joint replacement. The most important issue of PE is the production of wear particles which accumulate over time within the per-implant tissue.

Patients who are younger or more active may benefit from polyethylene with even more resistance to wear. This can be accomplished through a process called cross-linking, which creates stronger bonds between the molecular strands that make up the polyethylene. This cross-linking is induced by gamma irradiation. The appropriate amount of cross-linking depends on the type of implant. For example, the surface of a hip implant may require a different degree of cross-linking than the surface of a knee implant.

### ***Ceramics***

Ceramic materials are usually made by pressing and heating metal oxides (typically aluminum oxide and zirconium oxide) until they become very hard. These ceramic materials are strong, resistant to wear, and biocompatible. They are used mostly to make implant surfaces that rub together but do not require flexibility, as in the surfaces of a hip joint. A problem of this material is its brittleness. High impact forces into the joint might break the implant.

## **2.2.2 Implant properties**

### ***Strength***

An orthopaedic implant should be designed to be as strong as possible. During daily activities, high levels of mechanical stress are placed on bones and joints. An implant must be able to withstand these stresses day to day without breaking or permanently changing its shape. It should also be designed to withstand the accumulated effect of repeating these stresses.

### ***Flexibility (viscoelasticity)***

They must also be flexible to avoid shielding of bones from stress shielding (the human body may tend to reduce or eliminate its own parts when they are not used).

Bones can remain strong only if they are regularly placed under a reasonable stress. And if they are never stressed, the body will actually begin to lose bone tissue, causing the remaining bone to become weak.

When stress is applied to an orthopaedic implant that is very stiff, the implant absorbs most of the stress. But when stress is applied to a more flexible implant, some of the stress passes through the implant so it can be shared with the surrounding bone.

As is it showed in the figure 6, bone has an elastic modulus of approximately 40 GPa. The 3 metal alloys show superior to bone elasticity, being the more similar the Ti6Al4V.

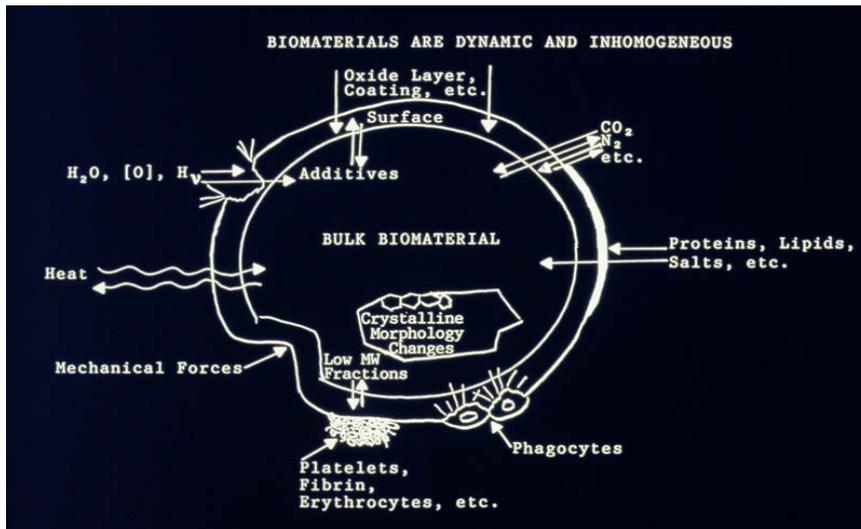


Figure 5. Implants in the human body are forced to withstand an array of extrinsic factors that degrade them.

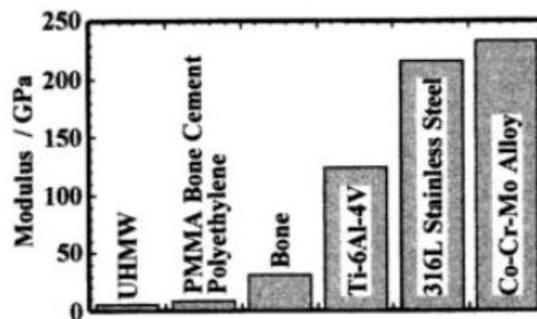


Figure 6. Elastic Modulus of different materials [15].

### Resistance to Wear

In parts that touch each other or rub together, especially in the case of an artificial joint, friction is created and the parts may possibly wear over time. When an implant wears, tiny particles of the material are removed from the surface and remain in the tissues that surround the implant. The majority of the wear particles formed are less than 5  $\mu\text{m}$  in diameter and are randomly shaped. These particles may cause a reaction that could lead to inflammation. If the inflammation is severe, or continues for too long, the implant may become loose.

Generally, the harder the material, the more resistant it is to wear. Also, the choice of the two materials that rub together in an implant is important in minimizing wear. Many combinations of materials are used today for implants, including metal on polyethylene, metal on metal, ceramic on ceramic, and ceramic on polyethylene.

### **Resistance to Corrosion**

Physiological medium is highly corrosive, electrochemical reactions (oxidation/reduction) can damage certain materials. Ions are released and this could eventually lead to implant failure. The oxidation (loss of electrons and increase in valence) may result in the release of free ions from the metal surface into solution.

Some studies have measured metal ions levels in the tissue surrounding metal implants, and it has been found concentrations from 1 $\mu$ M up to 200 $\mu$ M [16].

### **2.2.3 Implant loosening**

One of the primary reasons for implant failure is the loss of bone implant contact – called aseptic loosening and the destruction of bone tissue in the area surrounding the prosthesis (fig 7), what is called osteolysis.

An inadequate initial fixation, mechanical loss of fixation over time, or biological induced loss of fixation will lead to an osteolysis around the implant. Wear particles from the implant are the major cause of biological induced aseptic loosening. In vitro studies of macrophage cultures indicated that smaller polymethylmethacrylate (PMMA) and PE particles (< 20  $\mu$ m) elicited a inflammatory response, with a release of cytokines. Although particle phagocytosis has been identified as a critical component of this biologic response, and more over, new studies had reported that direct interactions between particle and cell surface are enough to activate osteoclastogenic signaling pathways [17].



*Figure 7. Osteolytic lesions in a hip TJR.*

Others mechanisms run in the same direction, as the one studied in this thesis or the metal hypersensitivity. The induced osteocyte death near the implant and subsequent bone resorption could

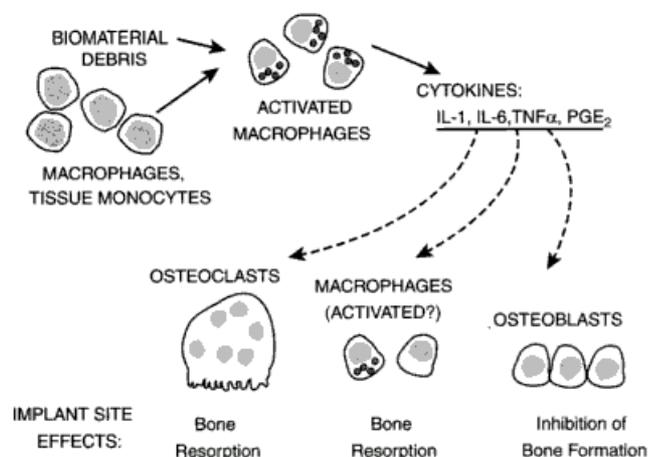
be explained due to the effect of the metal with the surrounding tissue. It is known that some metals can cause hypersensitivity in contact with the skin, for example, nickel [18]. However, it is not clear whether similar processes take place in the depth of the tissue as well. Delayed hypersensitivity reactions of the type IV (refers to undesirable reactions produced by the normal immune system) have been suggested to contribute to the loosening of orthopaedic implants, but the correlation still awaits confirmation. Dermal hypersensitivity to metal is common, affecting about 10% to 15% of the population [19].

In the other hand, it is well known that implant alloys in contact with biological systems undergo corrosion, leading to the release of metal ions into the periprosthetic tissues and the circulation. [20] Although these levels of metal ions can be usually low, but for long exposures may induce a change in the hematopoietic microenvironment regulating the turnover of the peri-implant bone.

#### 2.2.4 Inflammation and Hypersensitivity

It can be assumed that, to a large degree, metal ions within the circulation will be complexed by serum proteins, but it is not known if these protein-bound ions (these metal-protein complexes are considered to be candidate antigens) or the free ions could explain these stimulatory effects in the lymphocyte cells [21], leading to an inflammatory effect, that in turn will activate bone resorption by increasing osteoclasts (fig 8).

*The Inflammatory Process*



*Figure 8 [22]. Inflammatory process due to particle wear or/ and metal ions from the implant. An array of cytokines such TNF- $\alpha$ , IL-6, and VEGF are released by monocytes and macrophages in the presence of metal ions inducing bone resorption.*

These formed organometallic complexes, which can become immunogenic antigens or allergens are recognized by antigen-processing cells, which then present the processed antigen to T helper cells. The T helper cells, in turn, send the antigenic message to either the B cells, to initiate antibody formation, (type I hypersensitivity) or to the cytotoxic T cells to initiate cell-mediated immunity; (type IV hypersensitivity, common with metals). Although the specifics associated with metal-protein binding

and the biological mechanisms by which these complexes become immunogenic remain relatively uncharacterized. Metals known as sensitizers are beryllium, nickel, cobalt, and chromium, tantalum, titanium and vanadium have been reported. Nickel is the most common metal sensitizer in humans, followed by cobalt and chromium. The prevalence of metal sensitivity among the general population is approximately 10% to 15% (Figure 8), with nickel sensitivity having the highest prevalence (approximately 14%). The amounts of these metals found in medical-grade alloys are shown in Table 1.

Implant Alloy	Nickel	Cobalt	Chromium	Titanium	Molybdenum	Aluminum	Vanadium
Stainless steel (ASTM F138)	13-15.5	—	17-19	—	2-4	—	—
Cobalt alloy (ASTM F75)	1	62-67	27-30	—	5-7	—	—
Titanium alloy (ASTM F136)	—	—	—	89-91	—	5.5-6.5	3.5-4.5

*Table 1: Weight Percentages of Metals within the Three Most Common Orthopaedic Alloys*

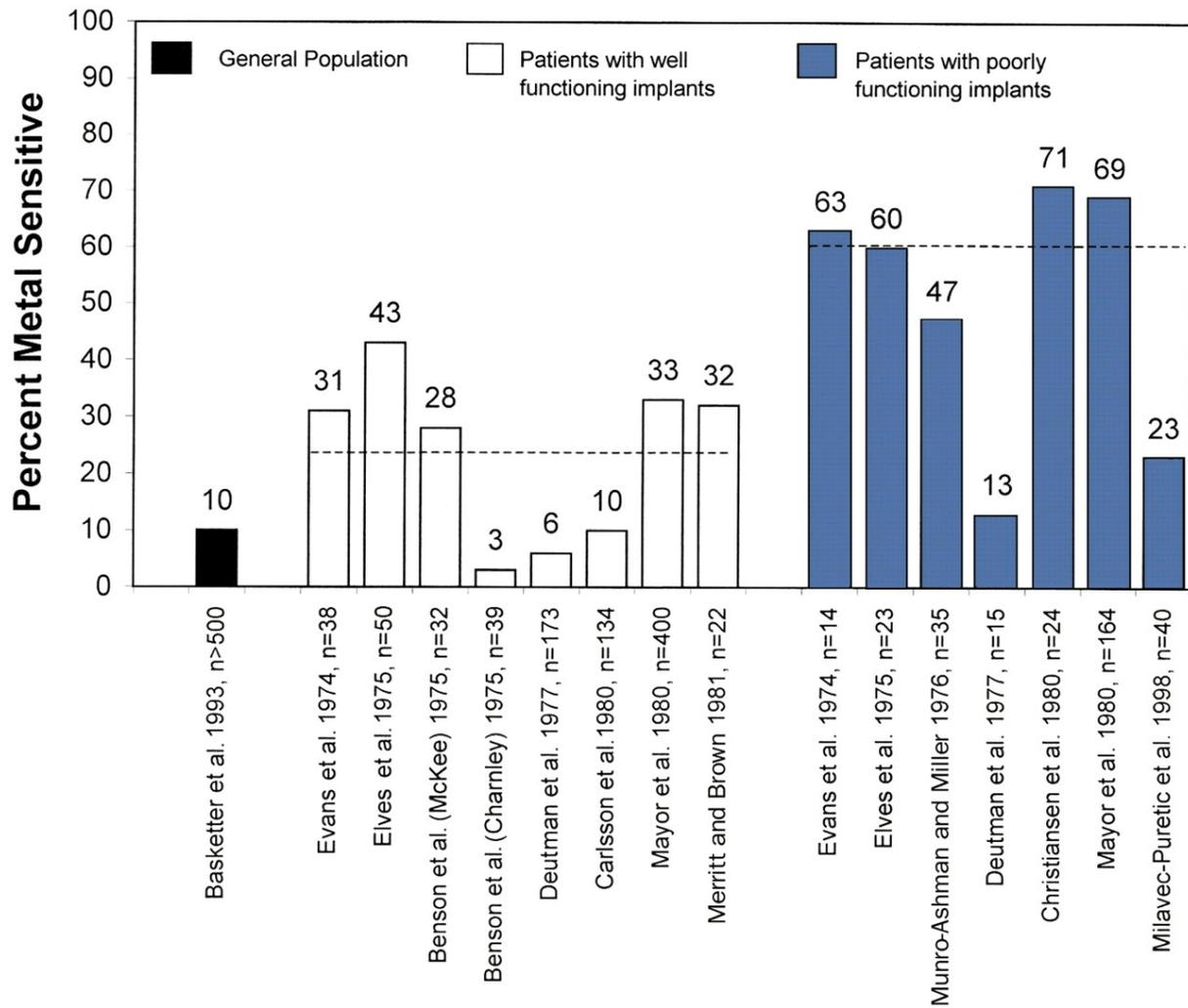


Figure 9: Averaged percentages of metal sensitivity (for nickel, cobalt, or chromium) among the general population and among patients with well and poorly functioning implants, based on a number of published reports.

### 3. THESIS AIMS

Since metal ions released from the prosthesis may be an underestimated cause of prosthesis loosening, the present thesis will focus the biologic effects and mechanisms occurring in the presence of metal ions. Will be focused the next aspects: How osteoclast are affected by the metal ions, if they proliferate or unproliferate, or they increase or decrease their activity, and which array of cytokines are released by osteoclast precursors in the presence of the metals. The answers to these questions will give clues about the osteoclast precursors recruitment and activation in the periprosthetic environment.

Monocytes (osteoclast precursors) will be treated with metal ions ( $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ) in vitro to mimic the situation of implant materials in vivo. Osteoclast development from monocytes will be followed biochemically and histologically. In parallel a monocytic cell line (THP-1) will be studied to establish a cell line for osteoclastogenesis.

## 4. MATERIALS AND METHODS

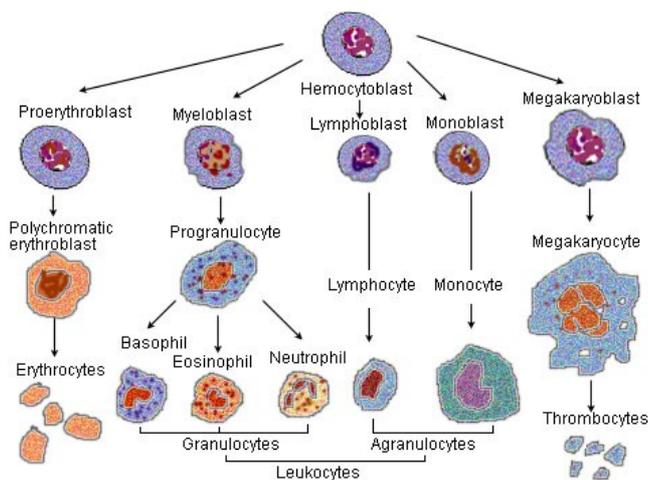
### 4.1 Bone Marrow Cells

#### 4.1.1 Description

Bone marrow is the soft tissue found in the cavity of long bones.

There are two types of bone marrow: red marrow (consisting mainly of myeloid tissue) and yellow marrow (consisting mainly of fat cells). The bone marrow contains three types of stem cells, hematopoietic, mesenchymal and endothelial cells. Mesenchymal stem cells are found arrayed around the central sinus in the bone marrow. They have the capability to differentiate into osteoblasts, chondrocytes, myocytes, and many other types of cells.

Hematopoietic stem cells are stem cells that give rise to all the blood cell types including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells, NK-cells).



*Figure 11. Bone marrow lineage. These pluripotent are capable of producing all these types of cells depending on the signals they receive.*

For this thesis, murine bone marrow was extracted and cultured on plates. Monocytes and macrophages from the BMC are treated with RANKL protein to obtain osteoclasts.

#### 4.1.2 Isolation of mouse marrow cells

6-8 week old, male ddy-mice were euthanized in CO<sub>2</sub> and bathed in EtOH 70%, the femurs and tibias were removed and place in ice-chilled Hank's (α-MEM, low bic). All muscle tissue was cleaned from the bones. To open the marrow cavity, the ends (epiphyses) were cut. Every bone was flushed with 5ml of chilled Hank's solution. The cell suspension was collected in a Falcon 50 ml tube and centrifuged for 5 minutes at 250 x g at 4°C. After centrifugation, the supernatant was removed, and cells were resuspended in 30ml α-MEM. For counting the number of cells, the previous solution were diluted 1:10 in Turk's solution (lyses erythrocytes), and a drop were added to the counting chamber. Depending on the concentration, the appropriate volume was used to obtain a final cell suspension of 300'000 cells/ml.

#### **4.1.3 Culture of bone marrow cells**

To investigate the reactions of o to metal ions, BMC (300'000 cells/ml) cells prepared as described above, they were seeded at a density of 60'000 cells in 200µl medium per well in a 48-well plates (FALCON, Fisher Scientific, Wohlen, CH). The medium composition was α-MEM with 10%FBS, 1% Pen/Strep and recombinant human CSF-1 (Cetus Corporation, Anaheim, CA, USA) to a concentration of 30ng/ml. Desired concentrations of recombinant human soluble RANKL (sRANKL; PeproTech, London, GB) and metal salts, NiCl<sub>2</sub> and CoSO<sub>4</sub>, were added at different time points and concentrations depending on the experiment.

Plates were incubated at 37°C, 5% CO<sub>2</sub>. Cells were cultivated under know conditions for five to nine days. Each 3 days medium was changed, removing old medium with no adherent cells, and adding new medium with the desired concentrations of metal ions and cytoquines.

BMC cultures for XTT assay were seed in 100 µl (30.000cells) per well in a 96-well plates (FALCON, Fisher Scientific, Wohlen, CH), with same media and conditions mentioned above.

#### **4.1.4 Differentiation of BMC to osteoclast, Determning RANKL dose response**

BMC cells were cultured in 48-well plates with CSF-1 to a concentration of 30ng/ml and 5 groups with different RANKL concentrations (0, 2.5, 5, 10, 20 ng/ml). 6 repetitions per group, incubated at 37°C, 5% CO<sub>2</sub> for 3 to 5 days. Trap staining assay was done at day 3, 4 and 5 to determine the number of multinucleated osteoclasts formed with the different conditions.

#### **4.1.5 Cobalt and Nickel effects on Osteoclast formation**

BMC cells were cultured in 48-well plates with CSF-1 to a concentration of 30ng/ml, 5 groups with different RANKL concentrations (0, 2.5, 5, 10, 20 ng/ml) and 3 concentrations 0µM (control group), 10 µM and 100 µM for the desired ion (Nickel or Cobalt) . 6 repetitions per group. Trap staining assay was done at day 3, 4 and 5 to determine the number of multinucleated osteoclasts formed with the different conditions.

#### **4.1.6 Cobalt and Nickel effects on cell proliferation**

BMC cells were cultured with CSF-1 to a concentration of 30ng/ml, and 5 groups with different RANKL concentrations (0, 2.5, 5, 10, 20 ng/ml) and 3 concentrations 0µM (control group), 10 µM and 100 µM for the desired ion (Nickel or Cobalt). 6 repetitions per group in 96 well plates. Cell Viability Assay (XTT) was done to assess how metal ions can affect cell viability.

#### **4.1.7 Effects on cell proliferation and Osteoclast formation, up to 9 days**

The purpose of the current experiment was to assess longer-term effects of the ions on monocytes (osteoclast precursors). BMC cells were cultured with CSF-1 to a concentration of 30ng/ml, and 5 concentrations 1, 3, 10, 30, 100 µM for Nickel ion and 5 more groups for Cobalt ion. 6 repetitions per group. 96 well plates were used for posterior XTT assay and 48 well plates were used for Trap staining assay.

For the 96 well plates, cellular proliferation was measured at 9 timepoints (Day3 to Day9).

For the 48 well plates, at days 4, 6, 8 and 9, RANKL was added in two concentrations (5 and 20 ng/ml) to induce differentiation to osteoclasts, and after 4 days treatment with RANKL. Trap staining assay was done.

## 4.2 Peripheral Blood Mononuclear Cells

### 4.2.1 Description

Peripheral Blood Mononuclear Cell (PBMC) is a blood cell having a round nucleus, such as a lymphocyte or a monocyte. They are a critical component in the immune system to fight infection and adapt to intruders. The lymphocyte population consists of T cells, B cells and NK cells.

These cells are often extracted from whole blood using Ficoll, a hydrophilic polysaccharide that separates layers of blood (fig 12), with monocytes and lymphocytes forming a buffy coat under a layer of plasma. This buffy coat contains the PBMCs.

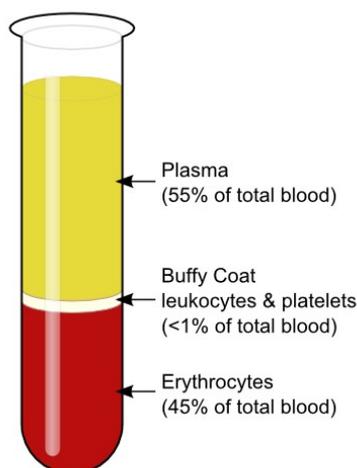


Figure 12. Tube containing blood separated in 3 layers after centrifugation.

The buffy coat is the fraction of an anticoagulated blood sample after density gradient centrifugation that contains most of the white blood cells and platelets. After centrifugation, 3 layers are formed, the plasma (clear fluid), the red blood cells, and a thin layer in between (less than 1% of the total volume of the blood sample), call the buffy coat, containing most of the white blood cells and platelets.

### 4.2.2 Isolation of Peripheral Blood Mononuclear Cell

Human PBMC were isolated from buffy coats obtained from healthy donors (Swiss Red Cross Blood Services Bern AG, Berne, CH), by Ficoll/Hypaque (Oxoid, Basle, CH) gradient centrifugation. Briefly, the buffy coats were diluted 1:8 with RPMI1640 medium (SIGMA, Buchs, CH), placed on top of Biocoll separation solution (ratio 3:2), and centrifuged for 20 min at 1200 g at RT. The upper layer was discarded, while the interphase, containing the PBMC, was washed twice with phosphatebuffered saline (PBS) and resuspended in RPMI1640/5% FBS (Inotech AG, Dottikon, CH), containing 1.5%

Hepes buffer (SIGMA, Buchs, CH) and 1% penicillin/streptomycin (GIBCO BRL, Life Technologies, Basle, CH). Subsequently, the monocytes were separated from the cells of the lymphoid lineages by their capacity to adhere to plastic surfaces.

#### **4.2.3 Culture of Peripheral Blood Mononuclear Cells**

$4 \times 10^6$  cells PBMC per well cells were seeded in 1ml medium in a 24 well plate (FALCON, Fisher Scientific, Wohlen, CH). After 3 h, the non-adherent cells were removed and new medium was added. Medium was composed of RPMI-1640 containing 5% FBS (heat inactivated), 1.5% HEPES and 1% penicillin/streptomycin (all purchased from GIBCO Invitrogen AG, Basle, CH). Plates were incubated at 37°C, 5% CO<sub>2</sub>.

#### **4.2.4 Comparison THP-1 cells versus Human PBMC stimulated with metal ions**

To study which genes are encoded, and which cytoquines are released in response to metal ions, THP-1 cells were cultured at  $1.5 \times 10^6$  THP-1 cells in 1ml THP-1 medium per well, with 5 groups (control, Ni<sup>2+</sup> 10μM, Ni<sup>2+</sup> 100μM, Co<sup>2+</sup>10μM and Co<sup>2+</sup> 100μM). As a positive control PBMC were cultured under the same conditions and concentrations.

At 7 timepoints ( 0 hours – control groups only, 2, 4, 8, 16, 24, 48 hours) samples were taken for RT-PCR and ELISA assays following the next instructions; For PBMCs; supernatants were collected and centrifuged at 1000 rpm / 4°C for 10 min; for each sample, 900μl of the supernatant was stored for ELISA assay. Adherent cells from the plates were washed with PBS and lysed for preparing the RNA. For THP-1, cell suspensions were collected, centrifuged at 1000 rpm / 4°C for 10 min; for each sample 900μl of the supernatant was stored for ELISA assay. Cells remaining in the culture plate were lysed and transferred to the cell pellet derived from the centrifugation step before to lyse previously suspended cells, for the preparation of RNA.

### **4.3 THP-1: Human acute monocytic leukemia cell line**

#### **4.3.1 Description**

Human acute monocytic leukaemia cell line (THP-1) where derived from the peripheral blood of human male with acute monocytic leukaemia, the first THP-1 cell line was cultured from a 1 year old boy with monocytic leukemia isolated in the 1980 by Tsuchiya [23]. They were chosen based on their extensive prior use as a model for peripheral blood monocytes [24]. THP-1 cells easily differentiate into macrophage. They express Fc and C3b receptors and a lack of cytoplasmic immunoglobulins. By the addition of Vitamin D3 they adhere to cell culture flasks.

Clearly, the use of the THP-1 cell line is a compromise over PBMC, because THP-1 cells divide continuously and PBMC do not. However, experience and previous reports have established the difficulties in maintaining a reproducible culture of PBMC for 4 weeks because of the transitions that occur in cellular attachment, the need for critical growth factors, and the slow degradation of the cultures [25]. The THP-1 model at least provides a monocytic cell that retains many of the functional properties of PBMC [26]. Today, there are non human cell culture systems for osteoclast studies, like the murine RAW 264.7 macrophage cell line, that can differentiate into osteoclasts when stimulated

with RANKL alone. Finding a human cell line that supports differentiation in cell culture to osteoclasts is still in research.

#### **4.3.2 Culture of THP-1 cells**

For the culture of THP-1 cells the suggested medium composition was chosen: RPMI 1640 (GIBCO 724000-021) containing 10% FCS Hia (PAA Lab.A15-043 / Lot: A01127-314), 1% Na-Pyruvate (GIBCO 11360-039), 1% MEM NEAA (GIBCO 11140-035), 1% MEM Vitamin (GIBCO 11120-037), 1% Penecillin / streptomycin (GIBCO 15140-122), 0.1% 2-Mercaptoethanol (SIGMA M-7522). Cells were cultured in flasks (FALCON, Fisher Scientific, Wohlen, CH) with an initial concentration of 400000 cells/ml in a 15 ml total volume of medium, at 37C, 5% CO<sub>2</sub>. Passages were done each 3 days, where medium with cells in suspension were collected to a 50ml Falcon tube, and centrifugated at 1000rpm/4°C for 5 min. Supernatant was removed, thereafter cells were resuspended in 1ml THP-1 medium, and fill up to 30ml. Cells were counted, and a new flask was prepared with 400000 cells/ml in a 15 ml THP-1 medium.

#### **4.3.3 CSF-1 effect on THP-1**

To study the effect of CSF-1 (Cetus Corporation, Anaheim, CA, USA) on the proliferation of THP-1 cells, 3 concentrations (2500, 5000, 10000 cells/well) of THP-1 were cultured in 96 wells plates (FALCON, Fisher Scientific, Wohlen, CH) with different CSF-1 concentrations (0, 10, 20, 40, 100 ng/ml). Six repetitions per concentration. XTT assay was done at day 1, 2, 3 and 4.

#### **4.3.4 THP-1 Differentiation**

Differentiation of the human monocytic cell line THP-1 into macrophage-like cells was induced by adding to the THP-1 medium 30ng/ml CSF-1, 0.1% 1.25(OH)Vitamin D<sub>3</sub> (10<sup>-8</sup> M; Roche, Basle, CH) and 0.03% Dexamethasone. Six repetitions per concentration. Change of media was done each 3 days. After one week with the previous conditions, RANKL (0, 5, 10, 25, 50 ng/ml) was added to induce osteoclast differentiation, and the treatment was stopped after 4 days, when Trap staining assay was realized.

### **4.4 Trap staining**

A TRAP staining kit (Sigma-Aldrich Chemie) was used for this thesis. It relies on the unspecific inhibition of phosphatases other than TRAP by large amounts of tartrate and highly acidic milieu. Naphtol-Anilid-Acid-Biphosphate is used as a substrate for dephosphorylation, leading to a product that is bound to Fast Garnet GBC leading to an insoluble, purple precipitate.

Trap assay is done following the next procedure; Once wells are washed with 1XPBS to remove non adherent cells, a 4% paraformaldehyde solution in 1xPBS is used for 10 min to fix the cells remaining in the wells. Thereafter, wells are washed with distilled water (3x). For staining, Naphtol-Anilid-Acid-Biphosphate (Sigma Diagnostics, Buchs, CH) is mixed with Fast Garnet GBC (Sigma Diagnostics, Buchs, CH) according to the protocol, and added to the wells for 5 min. Finally wells are washed (3x) with distilled water.

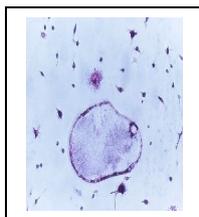


Figure 15. Example of Osteoclast after Trap+ Staining. 20x Bright Field.

#### 4.5 Cell Viability Assay

XTT is a colorimetric method based on the tetrazolium salt. Metabolic active cells reduce the tetrazolium salt to orange colored compounds of formazan. The dye formed is water soluble and the dye intensity can be read at a given wavelength with a spectrophotometer. The intensity of the dye is proportional to the number of metabolic active cells. The greater the number of active cells in the well, the greater the activity of mitochondria enzymes, and the higher the concentration of the dye formed.

Assay was done following the protocol given with the XTT kit from Roche Applied Science; XTT labeling mixture was prepared by mixing XTT labeling (50 $\mu$ L/well) and electron coupling reagent (1 $\mu$ L/well) at 37°C. The XTT labeling mixture was added to the wells (50 $\mu$ L/well) and incubated for 2 hours at 37°C. Absorbance was read after the incubation period, using a ELISA reader (microplate spectrophotometer) at 450-500nm with a reference wavelength at 650nm.

#### 4.6 Enzyme-Linked ImmunoSorbent Assay (ELISA)

Is a biochemical technique to detect the presence of an antibody or an antigen in a sample. In ELISA an unknown amount of antigen is affixed to a surface, and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme show fluorescence. To assess the amount of antigen in the sample, fluorescence is read. The more fluorescence the more antigen.

## ELISA

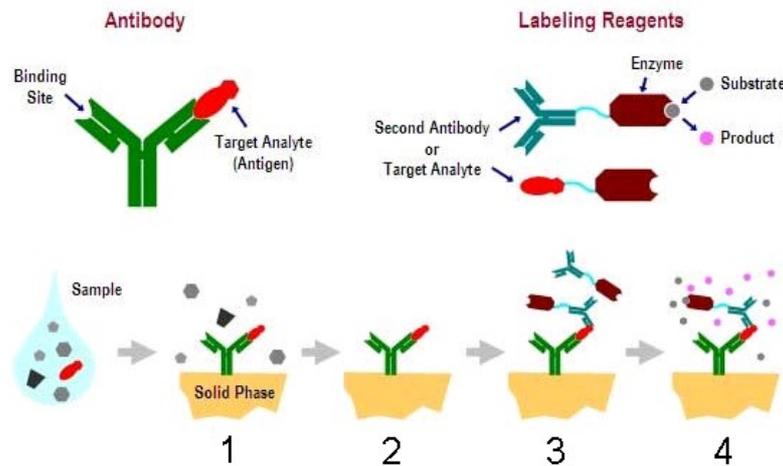


Figure 16. ELISA schematic assay: 1-Bind, 2-wash, 3-label, 4-read.

### 4.6.1 Determination of cytokine levels by ELISA

ELISA's were done according protocol from ELISA kit from BD Biosciences.

Levels of TNF, IL8 and VEGF in conditioned media were determined by enzyme linked immunosorbent assays (OptEIA Human Cytokine Sets (TNF and IL8), Pharmingen, BD Bioscience, Allschwil, CH; DuoSet ELISA Development System (VEGF), R&D Systems Europe Ltd. Abingdon, UK) as recommended by the manufacturer.

Recombinant proteins were used to generate the standard curves. The product of the HRP-catalyzed reaction was determined spectrophotometrically (Benchmark Microplate Reader, BIO-RAD; Infinite M200, Tecan Switzerland AG, Männedorf, CH) at 450 nm and background correction was performed at 570 nm.

## 4.7 Real-time polymerase chain reaction (RT-PCR)

Real-time polymerase chain reaction is combined with reverse transcription polymerase chain reaction to quantify with accuracy messenger RNA (mRNA) from a sample. It permits to quantify the abundance of a gene expression at a particular time in a particular cell. RT-PCR amplifies a small quantity of the desired RNA with the reverse transcription of the RNA to cDNA, which is subsequently amplified.

### 4.7.1 Determination of mRNAs levels by RT-PCR

To determine the levels of mRNAs encoding cytokines synthesized by monocytes treated with metal ions, total RNA was isolated using the RNeasy Mini Kit from Qiagen (Basle, CH), according to the recommendations of the manufacturer. For amplification in an ABI PRISM 7700 system (Applied Biosystems, Rotkreuz, CH), the following Assays-on-Demand (Applied Biosystems, Rotkreuz, CH) were used: TNF- $\alpha$ , IL-8; and VEGF. The CT values were normalized against 18S rRNA. The reactions

were performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Rotkreuz, CH), Assay-on-Demand mixture diluted 1:20, and 1 ng (0.01 ng for 18S rRNA) cDNA. The reactions were preincubated for 2 min at 50°C, followed by 10 min at 95°C. Thereafter, 45 cycles of 15 s at 95°C and 1 min at 60°C each were performed. The reactions were analyzed using the ABI PRISM sequence detection application software SDS V1.9.1.

## 5. RESULTS

### 5.1 Establishing an alternative osteoclast culture

#### 5.1.1 Determining CSF-1 dose response on THP-1 cells

Thp-1 cells were treated with CSF-1 to investigate which concentration of this cytoquine was inducing more growing in the cell culture.

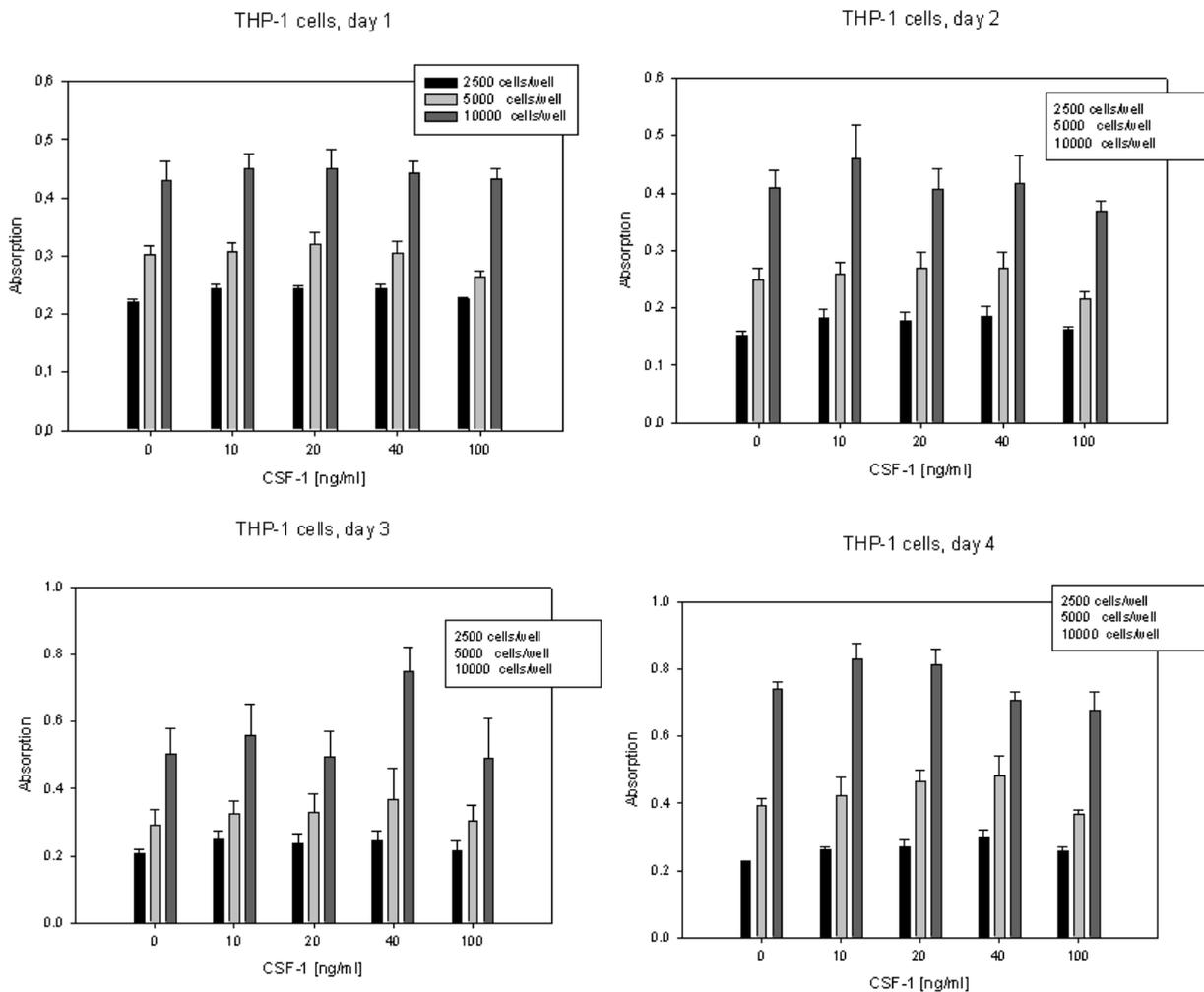


Figure 21. Relative N° of viable cells ; mean + standard deviation; n=6;

The relative number of cells was measured by XTT assay, results are showed in the figure 21. The number of cells was not affected by the addition of CSF-1 to the culture medium in any of the concentrations used over the experiment.

### 5.1.2 Differentiation of THP-1 cells

To assess THP-1 differentiation to macrophages, vitamin D3 and dexamethasone was added to the medium. After one week with this conditions culture, the cells were adhering to the dish bottom (fig.22), and they were presenting changes in cell morphology. At this point, RANKL was added for 4 days to assess osteoclast differentiation, but Trap staining assay showed any trap activity, indicating a non evidence for osteoclast formation.

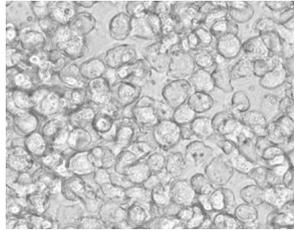


Figure 22. Bright Field x 20; Cells are attached to the bottom of the well and present morphological changes typical from macrophages.

### 5.1.3 Gene Expression

To investigate the response of THP-1 and monocytes from peripheral blood to an exposure to metal ions, the cells were treated with 10µM, 100µM Ni<sup>2+</sup>, and 10µM, 100µM Co<sup>2+</sup>, from 2h up to 48 h, and levels of IL-6, TNF, and VEGF were determined by RT-PCR (fig 23).

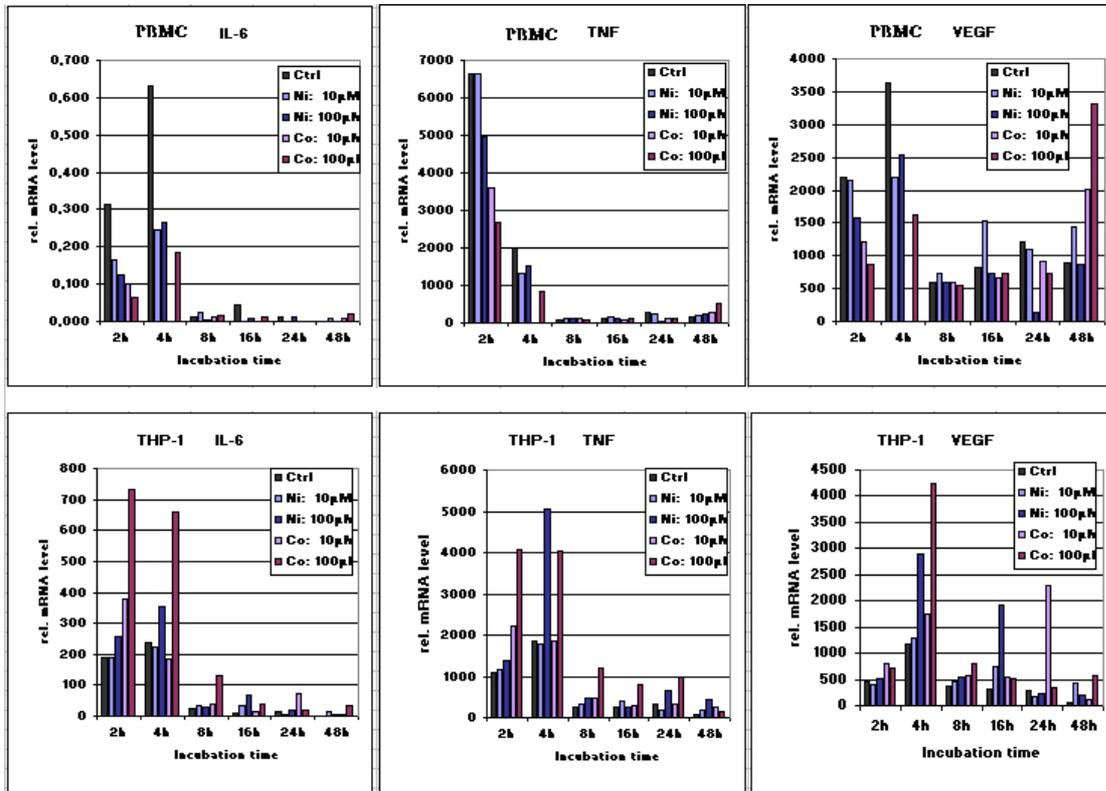


Figure 23. Gene expression for IL-6, TNF-alpha and VEGF (Relative mRNA levels for the different concentrations of metal ions). Upper graphics for PBMC and lower for THP-1.

It was found in common for both types of cells, that the higher expressions of the 3 cytokines studied, were released in the first 4 hours, but with different patterns, then for PBMC's, the higher the concentration of metal ion, the less cytokines released, and for THP-1 the other way around, having in both cases more effect the Cobalt ion. For the next timepoints (8 hours till the 48h), the release of cytokines is highly decreased, except for VEGF, being 50% reduced.

### 5.1.4 Cytokine secretion

To assess the kinetics of the release, supernatants were collected after 2, 4, 8, 16, 24 and 48 h and the levels of cytokines were determined by ELISA (fig 24).

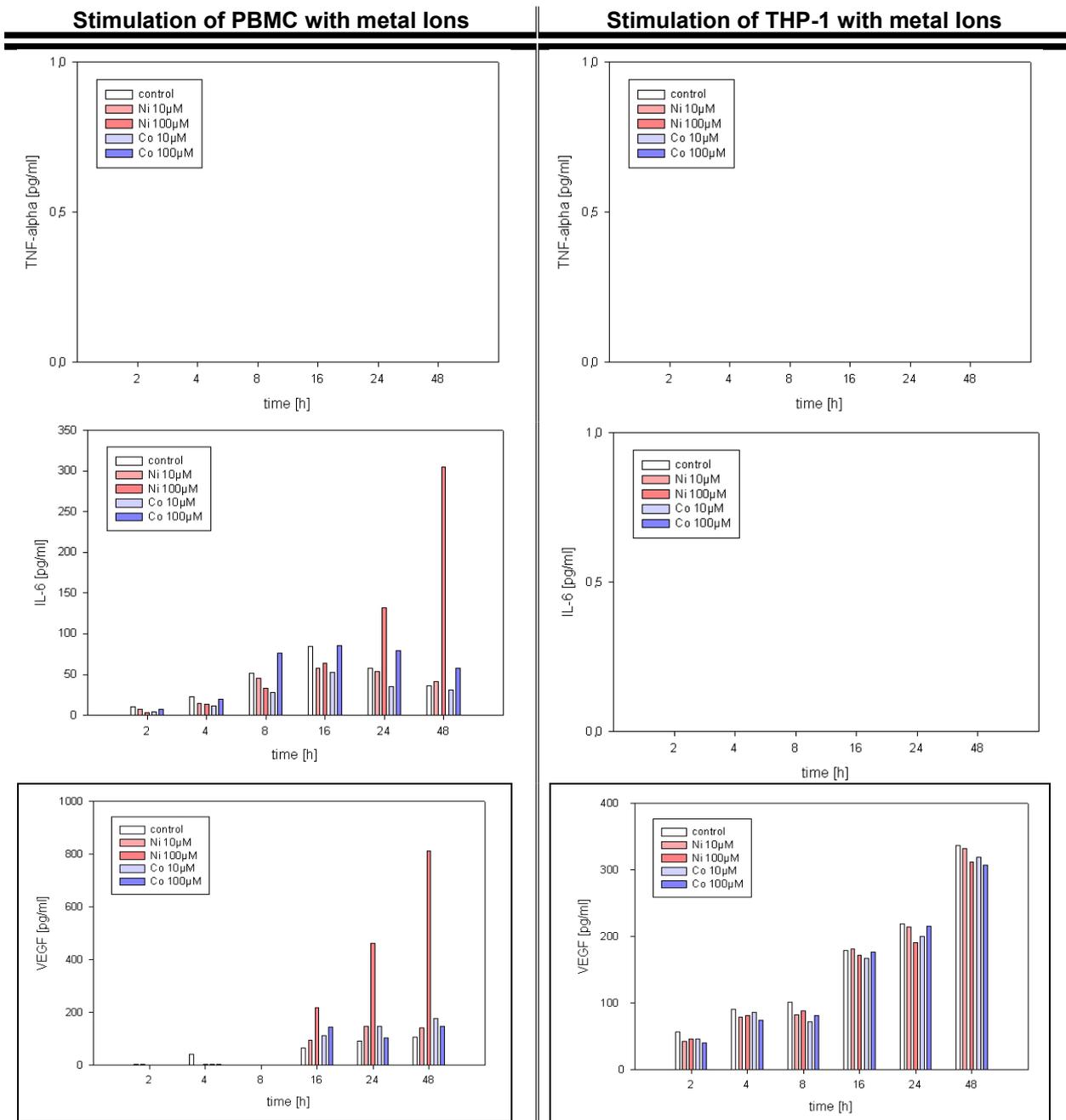


Figure 24. Results ELISA for TNF-alpha, IL-6 and VEGF; PBMC left column and THP-1 right column.

TNF was not found neither in the THP-1 and PBMC supernatants.

IL-6 was increased over the time, with higher values for 100µM Nickel. IL-6 was negative for THP-1.

For VEGF only appeared after 16h for the PBMC, being much higher for the 100µM Nickel. A different

pattern is observed for THP-1, where VEGF protein was secreted in the same amounts for the different conditions, increasing over the time.

## 5.2 Ni and Co effects on osteoclast formation

### 5.2.1 Differentiation of BMC to osteoclast, Determining RANKL dose response

BMC were treated with RANKL concentrations 0, 2.5, 5, 10, 20 ng/ml to assess a correlation between levels of RANKL and number of osteoclasts. It was proved that RANKL is an essential osteoclastogenic factor, and the major number of osteoclasts was assessed with the concentration of 20ng/ml, and the higher levels were obtained at day 5 (Fig 25).

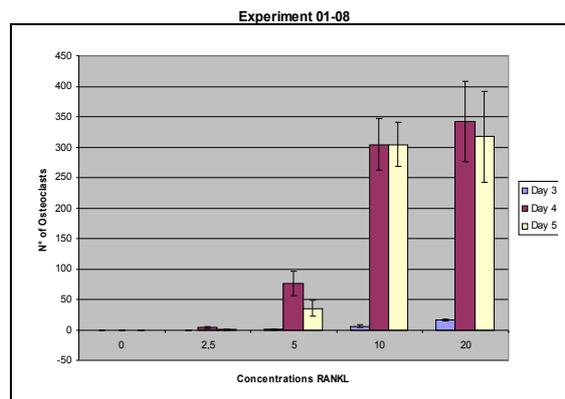


Figure 25. N° osteoclast; mean +/- standard deviation; n=6;

### 5.2.2 Metal ion effects on Osteoclast formation

Nickel and cobalt salts were added to osteoclast cultures to assess their influence on BMC differentiation to osteoclasts up to 6 days. Results from the Trap activity assays are showed in the following graphics (fig 26-27).

Ni<sup>2+</sup> and Co<sup>2+</sup> ions did not stimulated differentiation up to day 5. However at day 6, the pattern was different for Nickel, where the number of osteoclasts where similar to the number obtained in the control group.

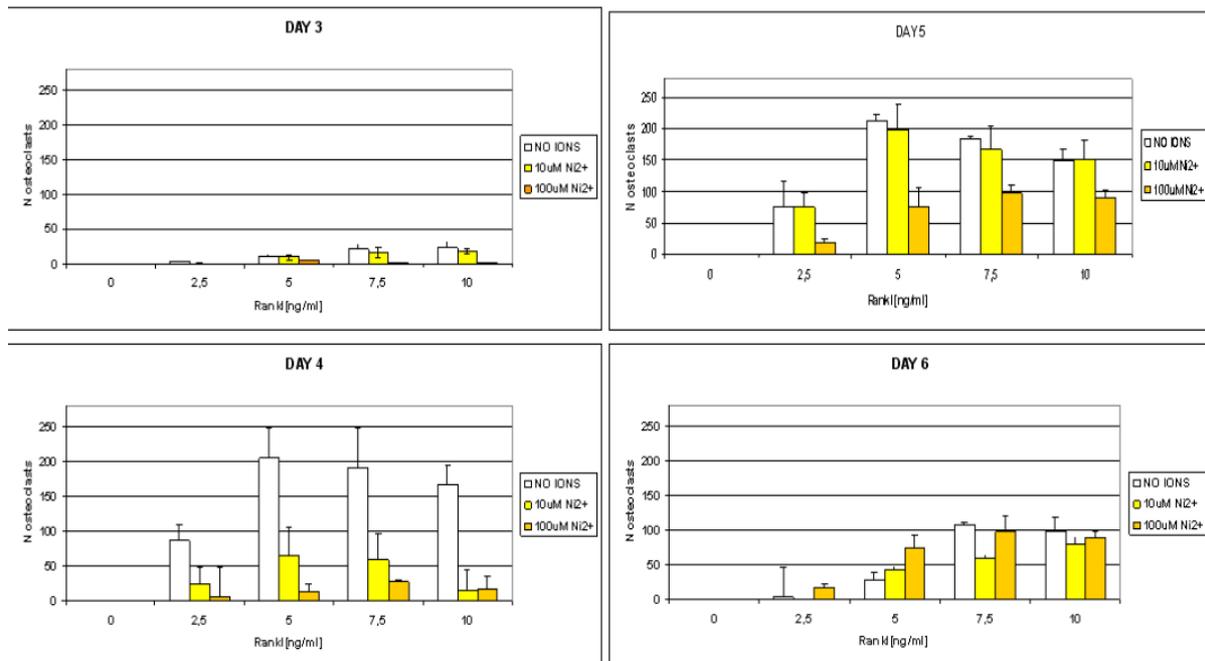


Figure 26. Nickel treatment; N° osteoclast; mean +/- standard deviation; n=6;

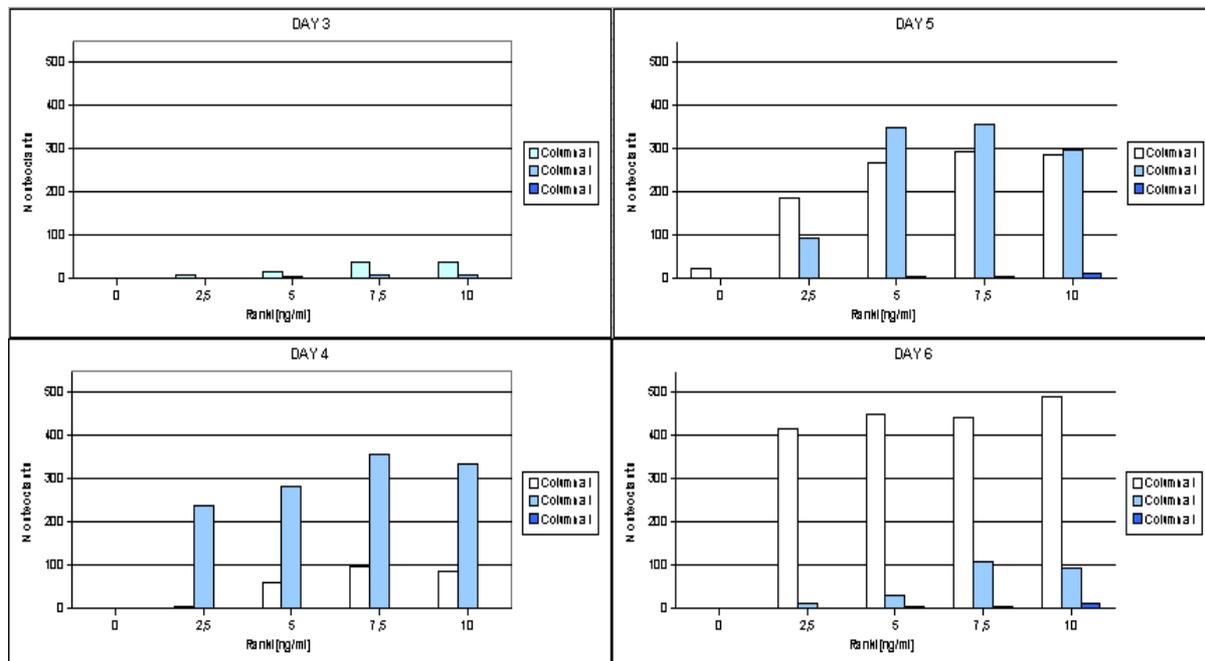


Figure 27. Cobalt treatment; N° osteoclast; mean +/- standard deviation; n=6;

**5.2.3 Metal ion effects on cell proliferation**

The influence of metal ions in the cell proliferation was tested and analyzed through the colorimetric assay XTT (fig 28-29) starting at day 3 up to 6 days. Both metal ions presented a decrease effect on cell proliferation for 100µM concentration. However, for the 10µM concentration, the pattern was different for the 2 metals. It appeared that nickel was inducing a small increase on cell proliferation. A considerable difference between RANKL stimulated and non stimulated cells is observed, this may be explained because cells are pushed to differentiate to osteoclast in the presence of RANKL, loosening their capacity to proliferate.

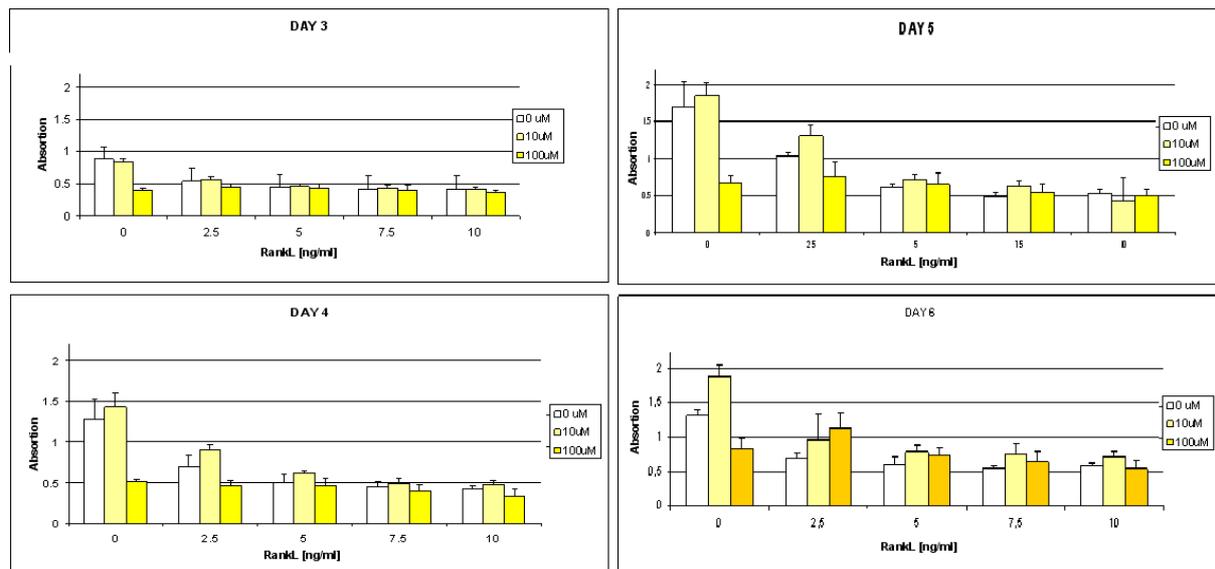


Figure 28. Nickel treatment; N° osteoclast; mean +/- standard deviation; n=6;

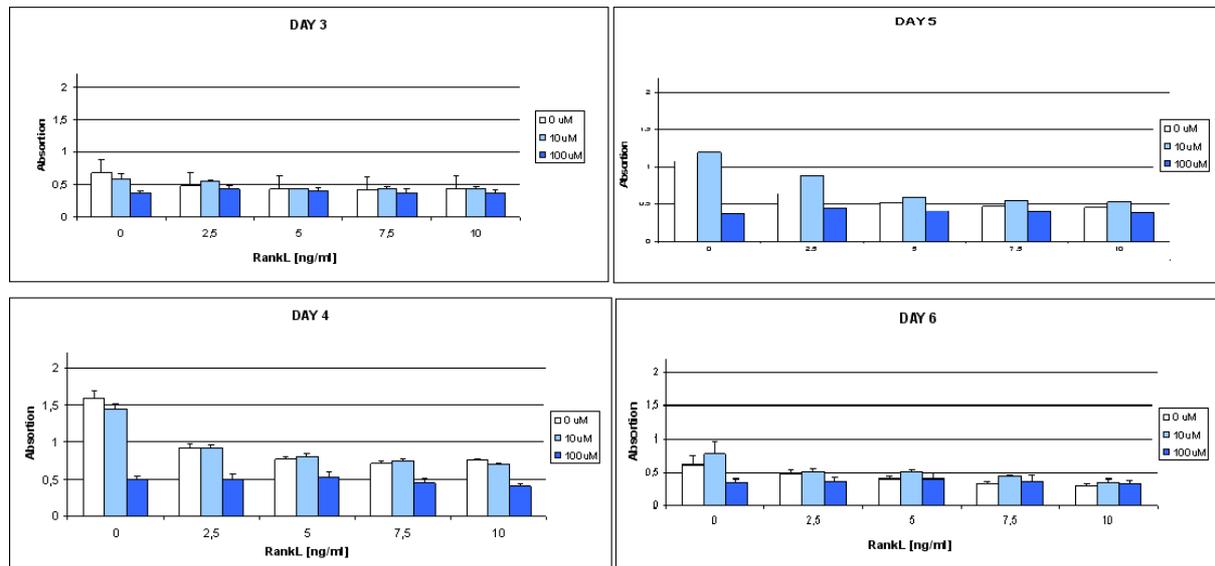


Figure 29. Cobalt treatment; Relative N° of viable cells; mean + standard deviation; n=6;

### 5.3 Effects on cell proliferation and Osteoclast formation, up to 9 days

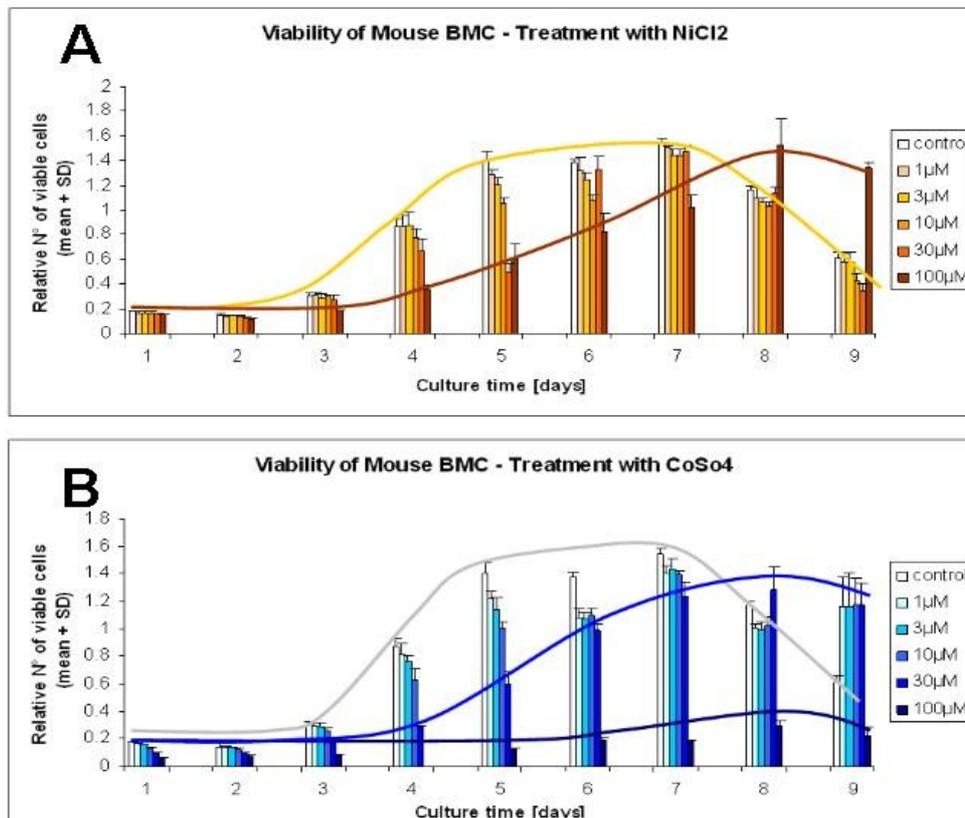


Figure 30. Relative N<sup>o</sup> of viable cells; mean + standard deviation; n=6; A: nickel ions ; B: cobalt ions.

The changes seen in the previous experiments for longer expositions (day 6) to metal ions pushed to extend the duration of the experiments up to 9 days. At this time, the pattern of the effect was different for each metal ion.

For nickel, as graphic is showing (fig 30-A), 100µM Ni<sup>2+</sup> was delaying the cell proliferation almost 3 days, but after this period, the number of viable cells were reaching the same maximum as control cells. This appreciation was particular strong for 100µM concentration, but not appreciable for the other concentrations, that are following same pattern as control cells. Two tendency lines have been added to the graphic for a better visualizing , yellow color for control and low concentrations, and the brown for 100µM.

For cobalt ion (fig 30-B), the highest concentration is inhibiting cell proliferation. And for low concentrations, it was observed a similar pattern to 100µM Ni<sup>2+</sup>, delaying the proliferation. 3 tendency lines were added to the graphic, gray for control, clear blue for 10µM Co<sup>2+</sup>, and dark blue for 100µM Co<sup>2+</sup>.

To investigate the capacity of the cells to differentiate to osteoclasts after the treatment with metal ions, RANKL was added to conditioned media at 4 stages of the culture, day 4, 6, 8 and day 9. Culture plates were incubated 4 days more with new media conditions, after that, cultures were stopped and Trap staining assays were done, and subsequent microscopic photographs were taken (fig 30).

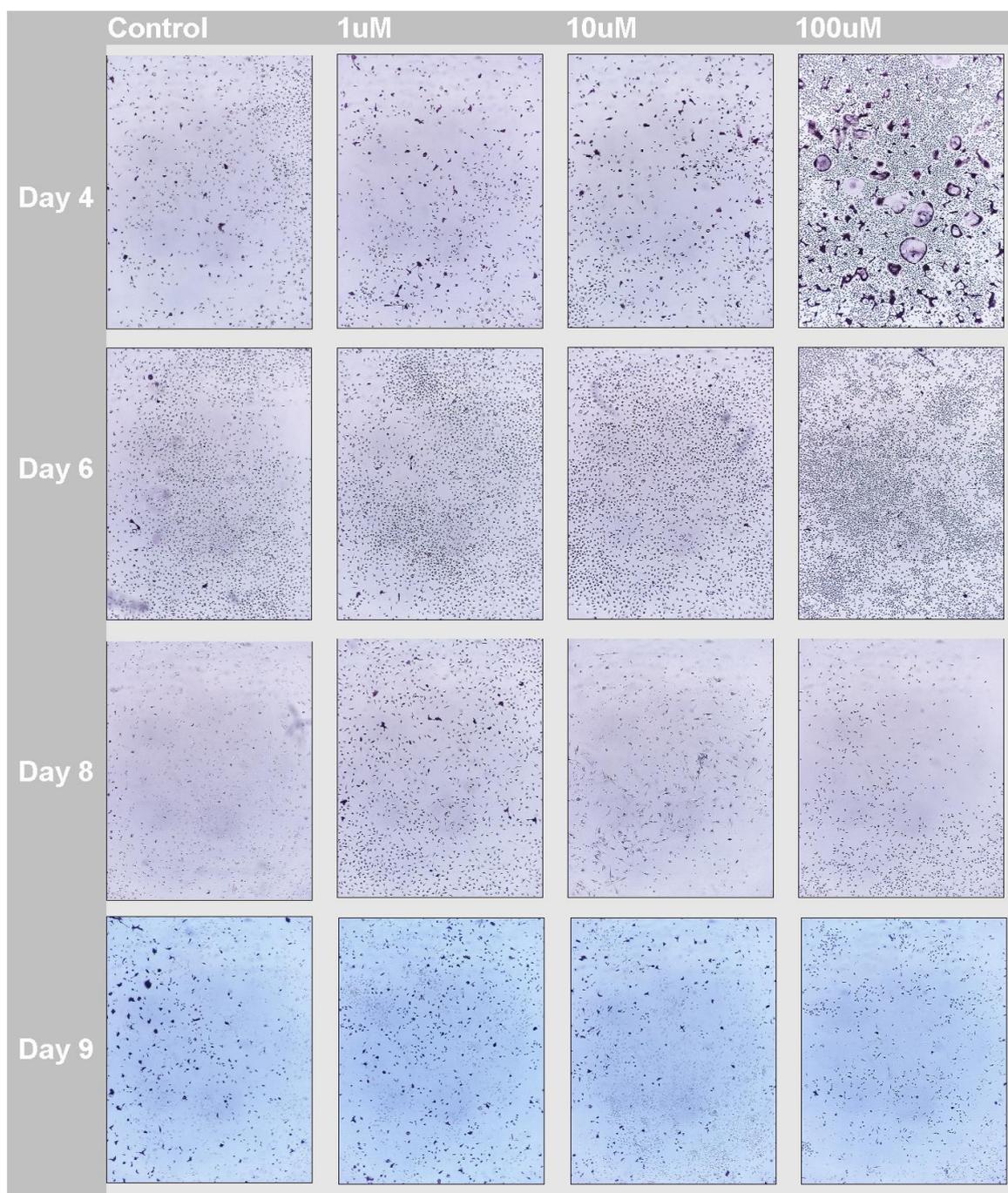


Figure 30. Microscopic images (bright field x5) of TRAP stained cells with different duration times of treatment with nickel ions.

For the 3 first conditioned media, there was not a significant difference, indicating no effect of the metal ion for the BMC cultures.

For the highest concentration, 100 $\mu$ M Ni<sup>2+</sup>, there was a considerable effect, moreover at day 4, where plenty of trap activity was detected, indicating more osteoclast differentiation.

## 6 Discussion

Within the present thesis, it has been confirmed and extended previous studies describing how metal ions released from metallic biomaterials may have adverse biological and functional effects.

In this thesis it has been showed the maturation of monocytes to macrophages from THP-1 cells by the induction of CSF-1, Vitamin D3 and Dexamethasone. However not osteoclast lineage differentiation had been observed by the addition of RANKL. This could be explained because sometimes the differentiation ability can be lost during numerous passages of subcultivations in cell lines, and may be the case for our THP-1 cells, or another explanation could be that THP-1 cells needs additional accessory factors to differentiate to osteoclasts.

The cell culture model used in the present work was selected because BMC can follow multiple differentiation pathways, one of them to osteoclast lineage. The metal ions treatment (mimicking the corrosion ions derived form implants) had permit to investigate the effects on the formation and function of osteoclastic cells, trying to mimic the events that are occurring in the periprosthetic region.

The high concentration (100 $\mu$ M) chosen for the cobalt ion resulted lethal, drastically reducing the number of osteoclasts. However, for the nickel ion, it was found that is somehow inducing a delay in the osteoclast formation; similar levels of osteoclast were reached an average of 4 days later that with the non-treated cells. These findings suggest that nickel is not inhibiting osteoclast formation, and could permit an increase on cell proliferation during this delay, incrementing the pool of precursors cells, leading at the end in a major number of osteoclasts in the periprosthetic tissue.

It has been characterized in previous studies that monocytes release numerous cytokines in response to an exposure to metal ions. This response depends on the metals the cells are treated with, since specific transcripts were found to be upregulated either by Co<sup>2+</sup> or Ni<sup>2+</sup> [27]. In the present research, the results from cytoquines and mRNA expression were not conclusive, and will need revision.

It is unclear whether metal ions inhibit bone resorption (effects on osteoclast progenitors) or promote resorption (immunogenic reactions that permits recruitment of osteoclast precursors) that leads at the end to the implant failure or vice versa. It is likely that some combination of these phenomena occurs, which in turn act to potentiate the loosening cascade. It has to be taken in account that in any in vitro model such as the one used in the current study, the relevance of the results to clinical practice can differ. Mechanisms by which in vivo implant failure occurs have not been well characterized, however present study had been an approach, giving some clues about the signaling cascades and cellular responses to the metals ions.

Future investigations could lead to implants with better osteointegration and long term biocompatibility and durability. Therefore, improvements in implants could regard strategies to act directly at the cellular components that contribute to implant failure. Some approaches could be to regulate

osteoclast precursor cells, or target precursors that are stimulated by the wear particles or the metal ions, or regulate the activation mechanism of multinucleated osteoclasts.

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## 8. REFERENCES

- 1 Bjelle A. Vortrag auf dem International Workshop of Epidemiology of Osteoarthritis in Reisenburg bei Ulm vom 22-24.10.1987, referiert in Praxis Kurier 47, Seite 22.
- 2 Hallab NJ, Mikecz K, Vermes C, Skipor A, Jacobs JJ. Differential lymphocyte reactivity to serum-derived metal-protein complexes produced from cobalt-based and titanium-based implant alloy degradation. *J Biomed Mater Res* 2001;56:427– 436.
- 3 Hallab NJ, Mikecz K, Vermes C, Skipor A, Jacobs JJ. Orthopaedic implant related metal toxicity in terms of human lymphocyte reactivity to metal-protein complexes produced from cobalt-base and titanium-base implant alloy degradation. *Mol Cell Biochem* 2001;222:127–136.
- 4 Wooley PH, Nasser S, Fitzgerald RH. The immune response to implant materials in humans. *Clin Orthop* 1996;326:63–70.
- 5 Ingham E, Fisher J. Biological reactions to wear debris in total joint replacement. *Proc Inst Mech Eng [H]* 2000;214:21–37.

- 6 Kahn AJ, Simmons DJ (1975). Investigation of cell lineage in bone using a chimaera of chick and quail embryonic tissue. *Nature*, 258, 325–7.
- 7 Alvarez JI, Teitelbaum SL, Blair HC, Greenfield EM, Athanasou NA, Ross FP (1991). Generation of avian cells resembling osteoclasts from mononuclear phagocytes. *Endocrinology*, 128, 2324–35.
- 8 Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, et al. (1998). Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*, 93, 165–76.
- 9 Ikeda T, et al. (2001). Determination of three isoforms of the receptor activator of nuclear factor- $\kappa$ B ligand and their differential expression in bone and thymus. *Endocrinology*, 142, 1419–1426.
- 10 Chesneau V, et al. (2003). Catalytic properties of ADAM19. *J. Biol. Chem.*, 278, 22331–22340.
- 11 Jimi E, et al. (1999) Osteoclast differentiation factor acts as a multifunctional regulator in murine osteoclast differentiation and function. *J. Immunol.*, 163, 434–442.
- 12 Koga T, Inui M, Inoue K, Kim S, Suematsu A, Kobayashi E, et al. (2004). Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. *Nature*, 428, 758–63.
- 13 Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL (2000). TNF $\alpha$  induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J Clin Invest*, 106, 1481–8.
- 14 Roodman GD (1999). Cell biology of the osteoclast. *Experimental Hematology*, 27, 1229-1241.
- 15 Hallab N, Merritt K, Jacobs JJ. Metal sensitivity in patients with orthopaedic implants. *J Bone Joint Surg Am* 2001;83A:428–436.
- 16 K.G. Nichols et al. (1997). Effects of metal ions on the formation and function of osteoclastic cells in vitro.
- 17 Auger MJ, Ross JA. The biology of the macrophage. In: Lewis CE, McGee JO, editors. *The macrophage*. Oxford, UK: Oxford University Press. P 3–74.
- 18 Menne T, Christophersen J. Epidemiology of allergic contact sensitization. *Curr Probl Dermatol* 1985;14:1–30

19 Basketter DA; Briatico-Vangosa G; Kaestner W; Lally C; and Bontinck WJ: Nickel, cobalt and chromium in consumer products: a role in allergic contact dermatitis. *Contact Dermatitis*, 1993;28: 15-25, [Medline]

20 Jonathan Black. *Biological Performance of Materials: Fundamentals of Biocompatibility*

21 Blaine TA, Rosier RN, Puzas JE, Looney RJ, Reynolds PR, Reynolds SD, O'Keefe RJ. Increased levels of tumor necrosis factor-alpha and interleukin-6 protein and messenger RNA in human peripheral blood monocytes due to titanium particles. *J Bone Joint Surg Am* 1996;78:1181-1192.

22 Hallab N, Merritt K, Jacobs JJ. Metal sensitivity in patients with orthopaedic implants. *J Bone Joint Surg Am* 2001;83A:428-436.

23 Tsuchiya S, Yamabe M et al. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int J Cancer*. 26(2): 171-6

24 Auger MJ, Ross JA. The biology of the macrophage. In: Lewis CE, McGee JO, editors. *The macrophage*. Oxford, UK: Oxford University Press. p 3-74.

25 Marczin N, Antonov A, Papapetropoulos A, Munn DH, Virmani R, Kolodgie FD, Gerrity R, Catravas JD. Monocyte-induced downregulation of nitric oxide synthase in cultured aortic endothelial cells. *Atheroscler Thromb Vasc Biol* 1996;16:1095-1103.

26 Gonzalez O, Smith RL, Goodman SB: Effect of size, concentration, surface area, and volume of polymethylmethacrylate particles on human macrophages in vitro.

27 Katrin Jost-Albrecht, Willy Hofstetter. *Gene Expression by Human Monocytes from Peripheral Blood in Response to Exposure to Metals*.