



Universidade do Porto
Faculdade de Engenharia
FEUP

Integrated Master in Bioengineering

Role of Receptor activator of nuclear factor
kappa B ligand (RANKL) in the
modulation of osteolysis in Primary and
Revision Total Hip Arthroplasty

Dissertation for Master's Degree in Biomedical Engineering

Gil Daniel da Costa Machado

Supervisors
Prof. Dr. Mário Barbosa
Dr. Meriem Lamghari

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(Signature of the Author)

(Signature of the Supervisor)

Since I realized it is common practice to quote someone in this kind of Dissertation, whether it is because of the message or because of the brilliance of the quoted person, I started thinking who I should quote. Who said something that actually changed the way I see the World? Or more importantly, the way I see myself in the World? Many people, many of them not even remotely famous. Once I cannot think of one message in particular, I decided I would not quote someone just because. It would be meaningless, at least, or disrespectful, at most.

Instead, I will make a dare to myself: to try to do something of relevance to the World so that, one day, someone actually considers the possibility of quoting me.

Well... Let's get to work, then.

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To all of them, I truly owe you. And I will be more than happy to pay my debt.

II. Abstract

Receptor Activator of Nuclear Factor κ B Ligand (RANKL) is considered to be a major responsible for osteoclastogenesis and, in the case of Total Hip Arthroplasty, a mediator of osteolysis that causes implant aseptic loosening. RANKL is expressed by osteoblasts, macrophages, fibroblasts and other cell types within the tissues of the acetabulofemoral articulation. However, little is known about RANKL levels in the different hip joint tissues of THA patients, both primary and revision.

The main goal of this study was to determine and compare mRNA and protein expression of RANKL in three different locations of the acetabulofemoral articulation (capsule, femur and acetabulum) of both primary and revision THA patients, taking into account factors such as age, gender, cause for surgery and implant duration.

For that purpose, tissue samples were collected during primary THA surgeries or hip replacement surgeries from the capsule, femur and acetabulum. Fifteen primary THA patients and eleven revision THA patients were included in the study after providing written consent. Total mRNA and protein content were extracted from the tissue samples using the TRIzol[®] method. qRT-PCR was used to quantify RANKL mRNA levels and the protein content was quantified using the Bradford assay. SDS-PAGE was also performed to assess the integrity of the protein sample.

The qRT-PCR results suggest that gender has an important role in RANKL mRNA expression, once male primary THA patients tend to express higher values than female THA patients. Considering patient age, there is a tendency for older (69-86) revision THA patients to express more RANKL mRNA in periprosthetic tissues in comparison to the younger group (53-63). The revision THA patients diagnosed with osteolysis and pain also demonstrated a tendency to have higher RANKL mRNA than those who had revision surgery for other reasons. Finally, a tendency for revision THA patients with longer implant life (9-17 years) to express more RANKL mRNA was detected, in comparison with the patients with shorter implant duration (0.33-2 years). Protein quantification and SDS-PAGE were optimized and were shown to be a good method for the study of the tissue derived protein samples.

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Protein extraction and quantification methods are optimized and should be applied to the collected tissue samples. More patients need to be included in this study to allow more samples to be collected, in order to allow the obtainment of statistically significant results.

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VI. List of Abbreviations

- °C** - degree Celsius
- %** - Percentage
- µg** - microgram
- µL** - microlitre
- µm** - micrometer
- B2M** - β_2 microglobulin
- BLAST** - Basic Local Alignment Search Tool
- BSA** - Bovine Serum Albumin
- Ct** - Cycle threshold
- cDNA** - Complementary Deoxyribonucleic Acid
- cm** - centimeter
- dNTPs** - Deoxynucleotide Triphosphates
- ELISA** - Enzyme-linked immunosorbent assay
- Fw** - Forward
- g** - gram
- GM-CSF** - Granulocyte-macrophage colony-stimulating factor
- HH** - Hip Hemiarthroplasty
- HRA** - Hip Resurfacing Arthroplasty
- HA** - Hydroxyapatite
- IL-1** - Interleukin 1
- IL-1 β** - Interleukin 1- β
- IL-6** - Interleukin 6
- IL-8** - Interleukin 8
- IL-11** - Interleukin 11
- M** - molar
- M-CSF** - Macrophage colony-stimulating factor
- MgCL₂** - Magnesium Chloride
- min** - minute
- mL** - milliliter
- mM** - millimolar
- mm** - millimeter

List of Abbreviations

MMA - Methyl methacrylate
mRNA - messenger Ribonucleic acid
NCBI - National Center for Biotechnology Information
NF- κ B - Nuclear factor kappa-light-chain-enhancer of activated B cells
ng - nanogram
nm - nanometer
OA - Osteoarthritis
oligo dTs - oligo deoxy-thymine nucleotides
OPG - Osteoprotegerin
PBMCs - Peripheral Blood Monocytes
PCR - Polymerase Chain Reaction
PE - Polyethylene
PGE₂ - Prostaglandin E₂
PMMA - Poly(methyl methacrylate)
qRT-PCR - Quantitative Real-Time Polymerase Chain Reaction
RA - Rheumatoid arthritis
RANK - Receptor Activator of Nuclear Factor κ B
RANKL - Receptor Activator of Nuclear Factor κ B Ligand
RNA - Ribonucleic Acid
rpm - revolutions per minute
rRNA - ribosomal RNA
RT - Room temperature
RT - Reverse Transcriptase
RT-PCR - Real-Time Polymerase Chain Reaction
Rv - Reverse
SDS - Sodium dodecyl sulfate
SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec - second
sRANKL - Soluble Receptor Activator of Nuclear Factor κ B Ligand
T - Temperature
TGF- β - Transforming growth factor β
THA - Total Hip Arthroplasty
TNF- α - Tumor necrosis factor α
TRAP - Tartrate-resistant acid phosphatase

UHMWPE - Ultra high molecular weight polyethylene

I. Introduction

1. Hip Joint

This articulation is a ball-and-socket joint, formed by the reception of the head of the femur into the cup-shaped cavity of the acetabulum (Figure 1).

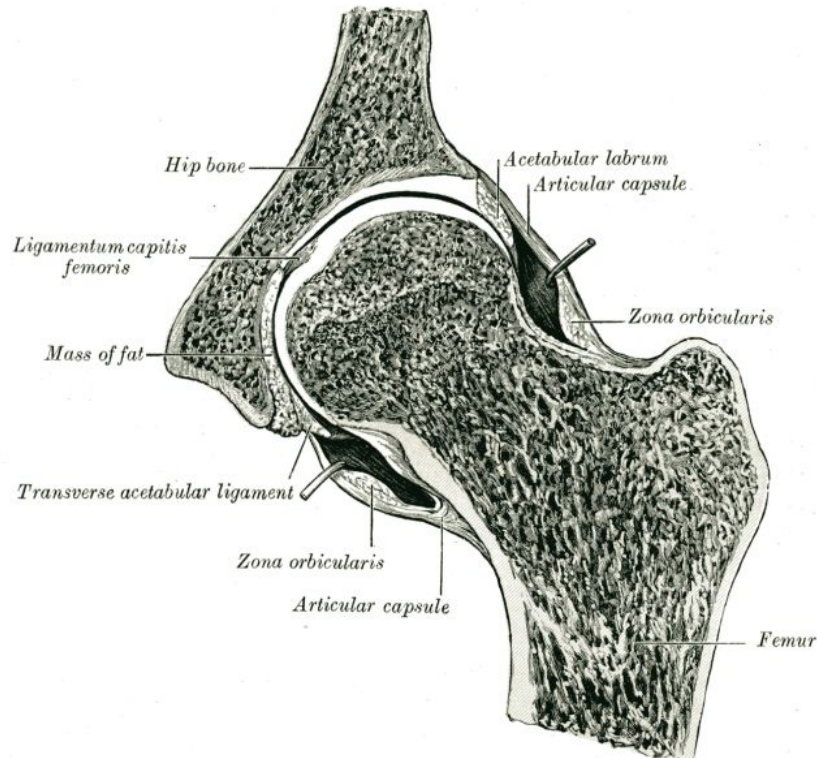


Figure 1 – A section through the hip joint [3]

The articular cartilage on the head of the femur, thicker at the center than at the circumference, covers the entire surface with the exception of the fovea of head of femur, to which the ligamentum teres is attached; that forms an incomplete marginal ring on the acetabulum, the lunate surface. Within the lunate surface there is a circular depression devoid of cartilage, occupied in the fresh state by a mass of fat, covered by synovial membrane.

The articular capsule is strong and dense (Figure 2).

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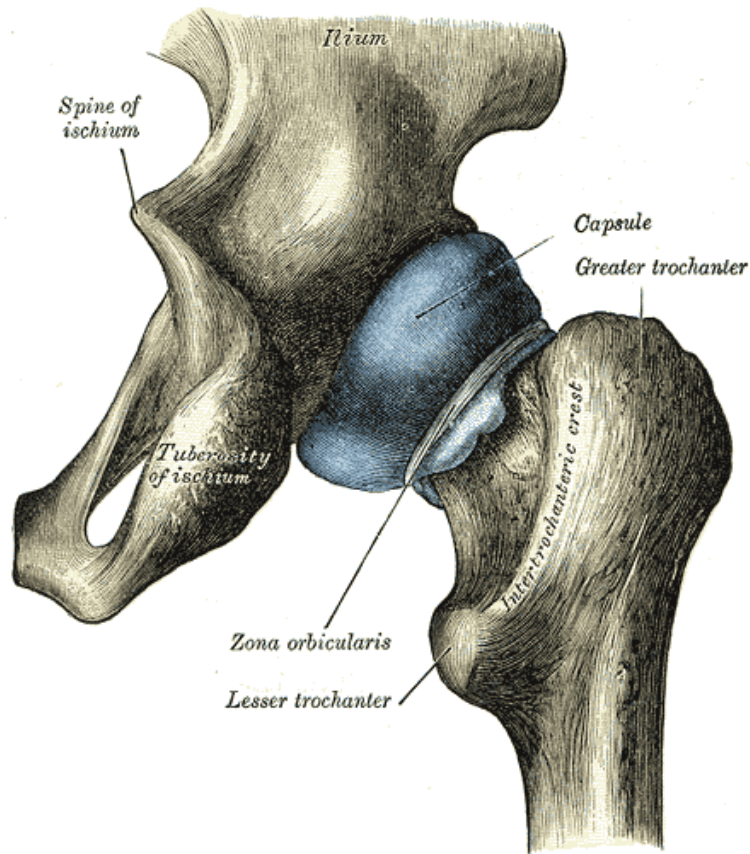


Figure 2 - Capsule of hip-joint (distended). Posterior aspect. [3]

Above, it is attached to the margin of the acetabulum 5 to 6 mm beyond the glenoidal labrum. It surrounds the neck of the femur, and is attached, in front, to the intertrochanteric line; above, to the base of the neck; behind, to the neck, about 1.25 cm above the intertrochanteric crest; below, to the lower part of the neck, close to the lesser trochanter. The capsule is much thicker at the upper and forepart of the joint, where the greatest amount of resistance is required; behind and below, it is thin and loose. It consists of two sets of fibers, circular and longitudinal. The circular fibers, zona orbicularis, are most abundant at the lower and back part of the capsule, and form a collar around the neck of the femur. The longitudinal fibers are greatest in amount at the upper and front part of the capsule, where they are reinforced by distinct bands, or accessory ligaments, of which the most important is the iliofemoral ligament. The external surface of the capsule is rough and covered by numerous muscles.

The glenoidal labrum is a fibrocartilaginous rim attached to the margin of the acetabulum, the cavity of which it deepens; it protects the edge of the bone, and fills up the inequalities of its surface, forming a complete circle, which closely surrounds the head of the femur and assists in holding it in its place. Its two surfaces are invested by

synovial membrane, the external one being in contact with the capsule, the internal one being inclined inward so as to narrow the acetabulum, and embrace the cartilaginous surface of the head of the femur.

The synovial membrane is very extensive. Commencing at the margin of the cartilaginous surface of the head of the femur, it covers the portion of the neck which is contained within the joint; from the neck it is reflected on the internal surface of the capsule, covers both surfaces of the glenoidal labrum and the mass of fat contained in the depression at the bottom of the acetabulum, and ensheathes the ligamentum teres as far as the head of the femur [3].

2. Hip Disease

Diseases that affect joints, such as the acetabulofemoral joint, are a major cause of diminished quality of life, even considering the major health care improvements that occurred in the last decades. Millions of new cases of rheumatoid arthritis, osteoarthritis or osteoporosis are registered every year, leading to, among others, joint problems such as cartilage degradation and pain or hip fracture.

Rheumatoid arthritis is a chronic systemic inflammatory disease with particular incidence in joints. It is based on an inflammatory reaction to the synovial membrane resulting in the formation of fibrous tissue, gradually decreasing articular functionality [4]. Extensive bone erosion is often seen as marginal joint erosions radiographically, and is predictive of a poorer prognosis. This inflammation and tissue destruction in RA is thought to involve cell-cell interactions between lymphocytes, monocytes/macrophages, and type A and B synoviocytes, which result in the production of matrix metalloproteinases, cathepsins, and mast cell proteinases that cause cartilage and bone destruction. Osteoclast formation from cells of the macrophage/monocyte lineage at the cartilage-pannus junction is associated with the destruction of bone matrix in patients with RA.

Joint degeneration occurs and Osteoarthritis is among the most common joint disorders, affecting about 65% of individuals over 60 years of age. The disease causes pain and functional disability, resulting in a significant social and economic burden. Synovial membrane inflammation is believed to play an important role in the progression of joint tissue lesions. However, there is a general consensus that synovial inflammation

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in OA is not the primary cause of the disease, but rather a secondary phenomenon related to multiple factors including cartilage matrix degradation, morphological changes in subchondral bone local abnormal biochemical pathways related to the altered osteoblast metabolism in this tissue [5].

Osteoporosis and associated fractures are common in Western countries, especially among elderly white women. In addition to the enormous costs, these fractures cause considerable disability and many premature deaths and, as the life expectancy increases, so will the magnitude of the problem [6]. Hip fractures are the major consequence of this disease and its occurrence in the world each year is expected to rise from 1.66 million in 1990 to 6.26 million in 2050 [7].

3. Therapies

Conservative therapies

In the case of minor injuries in the acetabulofemoral joint, such as mild inflammation, articulation pain or early stage osteoarthritis, conservative therapies are often recommended as an alternative to invasive methods. These may vary from physical therapy to medication to reduced activity or a combination these. However, more severe cases require surgical intervention and joint replacement to some extent, depending on several patient related characteristics, being the most extreme and common the Total Hip Arthroplasty.

Total Hip Arthroplasty

Total Hip Arthroplasty is a reconstructive orthopedic procedure used to improve the management of hip diseases that respond poorly to conventional medical therapy. It involves the surgical excision of the head and proximal neck of the femur and removal of the acetabular cartilage and subchondral bone. An artificial canal is

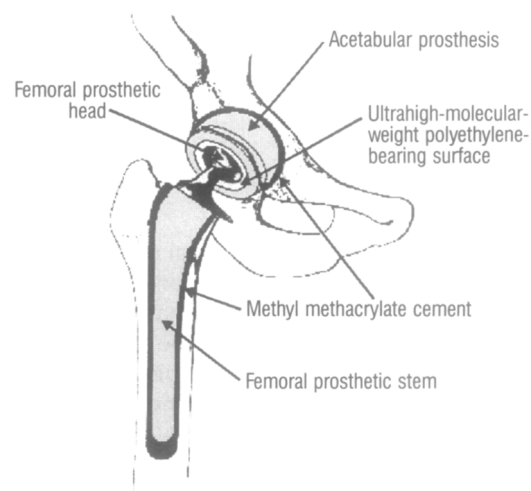


Figure 3 - Total Hip Arthroplasty with the use of PMMA cement to fix the prosthesis to the bone (Adapted from [1])

created in the proximal medullary region of the femur where a metal femoral prosthesis, consisting of a stem and a small-diameter head, is inserted. An acetabular component composed of a high molecular weight polyethylene articulating surface is inserted proximally in the enlarged acetabular space [1]. Good fixation to the bone is a key factor for the duration of the prosthetic device and one of two approaches is usually taken: polymethylmethacrylate (PMMA) cement or, in more recent uncemented designs, by bone ingrowth into a porous bioactive coating. The vast majority of total hip replacements still follow Charnley's low frictional torque principle, i. e., the one just described.

There are approximately 800.000 THA's performed every year around the world, allowing one to undoubtedly state that joint replacement was the most successful surgical procedure developed during the 20th century in terms of relieving pain and correcting deformity [2]. Literature shows that 90-95% of hip replacements are successful for 10-15 years [8] and, given good surgical technique, over 75% of hip prostheses may succeed for 25 years in elderly (less active) patients. However, this success has led to its increasing application into younger and more active patients, which places added demands on the implants. The survivorship of total hip replacements in young and active patients is reduced. Therefore, the understanding of the mechanisms of failure, in order to develop hip prostheses with increased longevity, is paramount within the orthopedic community [2].

Design Choice

When a patient is diagnosed with a problem that requires hip arthroplasty, several solutions are available. Naturally, one may be more appropriate than others for each specific case, once several variables must be considered in order to provide the best patient care possible. Three major approaches are available nowadays: Total Hip Arthroplasty, Hip Hemiarthroplasty and Hip Resurfacing Arthroplasty. The latter is more bone-conserving than a THA and a Hemiarthroplasty, being considered more suitable for young and active patients for this reason [9]. However, it is applied under very specific conditions, because it requires good bone structure integrity. Hemiarthroplasty presents severe wear problems at the articulation between the implant and the remaining bone. Therefore, the most commonly used method to restore function to a diseased hip is the Total Hip Arthroplasty, which can have a variety of design options according to the patient's needs.

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Underlying cause

One of the most common causes for the need of hip replacement is severe joint pain, which is commonly due to cartilage (whose job is to allow the joint to move smoothly) degradation. This can occur simply due to daily wear, or because of more serious conditions such as degenerative joint diseases (e. g. osteoarthritis) accounting for 70% of the cases [1], or systemic inflammatory diseases (e. g. rheumatoid arthritis). Another frequent cause is fracture of the femoral neck, an injury with high incidence on older osteoporotic patients.

Age and activity

Patient's age and activity are two important related factors to consider when choosing the prosthesis design to use. When a THA is necessary, one should take into account that this procedure is preferably referred to patients older than 60 years because, at this age, the physical demands on the prostheses tend to be fewer and the longevity of the operation approaches the life expectancy of the patient [1]. However, this is not the case with younger patients, who certainly demand more from the prostheses, both in terms of activity and longevity. As a consequence, failure of the prosthetic device may happen.

Cemented vs. non-cemented

Whether to use some type of cement fixation when performing a THA is paramount to determine the outcome of the surgery and, once again, this decision is based on the characteristics of each patient. The primary functions of bone cement are to secure the orthopedic device to the bone and transfer mechanical loads from the implant to the bone. Approximately 50% of orthopedic implants utilize bone cement (being the most common PMMA) to achieve implant fixation. Poly(methyl methacrylate)-based bone cements are mainly two-part formulations: the first part contains pre-polymerized PMMA, an initiator and a radiopacifier; the second part contains mostly liquid MMA, an accelerator and a stabilizer to prevent mature polymerization. These bone cements have shown high success rate, averaging 90% after 15 years [10]. However, these materials do not represent a perfect solution, once they have some drawbacks: local tissue damage, which occurs due to the exothermic nature of the cement setting reaction (temperature goes as high as 60 to 120°C); the release of the unreacted MMA, which causes chemical necrosis of the bone; the high shrinkage of the cement after polymerization which is about 21%;

the stiffness mismatch between bone and the cement; the cement does not bond chemically with either bone and implant; cement particle mediated osteolysis of the bone; bacterial infection [10]. All these problems with the use of bone cement to fixate hip replacements have led to its reduced longevity. Continued evaluation of the use of cemented total hip prostheses has revealed that mechanical failure caused by aseptic loosening of the components has been the most significant and frequent long-term problem [11]. This presents itself as a major problem particularly for younger patients, who normally demand more from the prostheses. For this reason, cemented prostheses have been reported to have an even higher rate of loosening and revision in patients who are younger than sixty years old [11]. These failures prompted interest in cementless fixation that focused on how to improve the qualities of implants to allow permanent stability in bone, without using an additional interface [10]. The outcome of cementless implant fixation is conditioned by three main principles: sound initial stability, osseous integration and mechanical properties of the implant. This requires the prostheses to be immediately tight fit after implantation, to have specific surface geometry characteristics (pore size and roughness), to be biocompatible and bioactive, in order to improve the rate and amount of osseo-integration of an implant in bone. The application of calcium phosphates, especially hydroxyapatite (HA), as an osteo-conductive mediator has significantly improved the quality of implant fixation [10]. Hydroxyapatite-coated prosthetic components may improve the incorporation into bone without causing an adverse reaction [12]. Several studies have since demonstrated significant decreases in terms of bone loss due to the use of uncemented prostheses. For example, a study by Marchetti *et al.* (1996) showed significant differences in bone mass density loss were observed between a cemented cobalt chrome prosthesis group and an uncemented titanium prosthesis group, where the cemented group had twice the bone as compared with the large uncemented group [13].

Implantation - Importance of surgical technique

The competence and technical skill of a surgeon undoubtedly influences the outcome of a surgery, and hip replacement is no exception [14]. Considering this fact, one realizes that the surgeon (and the technique used) may actually be one of the most relevant variables to determine the success of a surgery. The usual surgical approaches to the hip are posterior, lateral and anterior, each having its advantages, being the choice of which to use made by the surgeon. Naturally, several other aspects of the surgery must be

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defined, such as the used of orientation devices for the prosthetic components, as well as pressurization techniques to improve cement fixation (in cemented prostheses), which have proven to reduce femoral loosening [14]. All these factors lead to variations of the surgical result and, consequently, of the prosthesis longevity. They affect the adequacy of the cement as to location and amount, orientation of the femoral component, abduction angle of the acetabular component, relationship of the prosthetic canal and the stem of the prosthetic component, contact of the calcar with the collar of the femoral component, and wear of the acetabular component [15].

Revision Total Hip Arthroplasty

The improvement and refinement (prevention of infection, material properties of the components, methods of fixation and general surgical technique) of total hip replacements in the past decades have allowed this procedure to become the most cost-effective method to restore function and mobility to millions of patients with osteo and rheumatoid arthritis, as well as patients with a fractured femoral head. However, despite the significant increase of effectiveness and longevity of total hip replacements in the past decades, this solution is still far from perfect. Due to its success, more and more is demanded from this method, e. g., as it is applied to younger and more active people, making prosthesis failure a more present reality. In order of occurrence after implantation, the major factors that limit THA function and longevity are the surgical technique, fixation of the implant to the bone, osteolysis and long-term bone remodeling [2]. The main reasons for total hip replacement failure are aseptic osteolysis (75%), infection (7%), recurrent dislocation (6%), periprosthetic fracture (5%) and surgical error (3%) [16]. However, there is still a lot to understand about each of these processes and specially about the effects of their combination on the function and duration of total hip replacements.

The diagnosis of prosthetic failure may be due to several reasons: early failure is usually caused by bad component choice or component misalignment; later failure usually happens because of component wear and subsequent periprosthetic osteolysis (sometimes causing bone fracture), frequently associated with pain. In these situations, it is essential to remove the prosthetic components which are causing those problems (acetabular, femoral or both) and to replace them by new components.

4. Prosthesis failure

Mechanisms

With the major features of the Total Hip Arthroplasty technique described, one can begin to look further into why hip prostheses fail. This analysis must always be carried out with the notion that the reason that takes prostheses to fail (with the consequent revision surgery being necessary) may, and almost always is, the conjugation of several factors. The most well studied and common problems that occur after implantation are presented here.

Stress shielding

Stress shielding refers to the reduction in bone density as a result of removal of normal stress from the bone by an implant, such as after a THA. Healthy bone remodels in response to the loads it is placed under. Therefore, if the loading on a bone decreases, the bone will lose density and become weaker because there is no stimulus for continued remodeling [17]. Of course, this is a major concern when designing the components of a hip prosthetic device, as well as when choosing the most adequate design to use in each patient. It is, then, desirable that this reduction in the stress transferred from the prosthesis to the bone is decreased as much as possible. This can be achieved, to a certain extent, by loading the proximal region of the femur. By doing this, more bone stock is retained than if the proximal region is allowed to be bypassed and, as a result, completely unloaded [18]. When using shorter stems (in cases where it is possible to maintain a significant amount of bone [19]), the load is applied on the proximal femur and distributed along the whole bone, minimizing stress shielding. On the other hand, when the use of a longer stem is required, very little or no stress at all is applied on the proximal femur, promoting bone resorption in this area. Some attempts to develop prosthetic devices with similar mechanical properties to the ones of natural bone have also been made, in order to allow a more efficient load transfer from the prosthesis to the bone [17]. Plus, improvements of the cross-sectional stem shape have also reduced stress shielding. All these developments along years of research have decreased the bone loss rate, but since the THA procedure involves osteotomy at the femoral neck, loads that were transferred through the subchondral trabecular bone in the healthy hip are now transferred through the stem and shaft of the femur. Thus, a certain amount of stress shielding and bone remodeling cannot

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be avoided, but the use of a biomimetic stem appears to reduce the phenomenon considerably [17].

Periprosthetic osteolysis

With all the improvements in prophylaxis of infection, skeletal fixation and mechanical properties of prosthetic components, wear and its effects have become the main limitation to joint replacement longevity [20]. Osteolysis is thought to be a consequence of the biological response to a variety of particles generated at several locations around the joint replacement: articulating surfaces, modular component interfaces, fixation surfaces, and devices used for adjuvant fixation [20] [21]; and by several processes: micromotion occurring in response to corrosion, oxidative reactions, minor pathogen contaminations, misalignment and excessive wear at the bearing surfaces [19] [8]. Clinically, periprosthetic osteolysis can lead to aseptic loosening of components, massive bone loss that renders revision surgery substantially more complex, and periprosthetic pathological fracture [20].

Wear debris

The debris generated in the prosthetic environment is one of the basic factors that cause osteolysis, leading to the aseptic loosening of the joint replacement and its eventual failure. Hence, the need to understand the mechanisms by which these particles are formed and how they interact with the biological environment is paramount, in order to improve prosthetic efficiency.

Source

The degradation products of any implanted prosthetic device can be categorized as one of two types: particulate debris or soluble (or ionic) debris [16]. These particles may be formed in several parts of the prosthesis, by abrasion of the stem of a femoral component or the wear of a trunnion between the femoral head and the upper part of a femoral stem. In addition, there may be wear and/or corrosion around screws, or even PMMA particles released due to micromotion. However, the bearing surfaces of a replacement joint are the main site of wear and the particles generated are shed directly into the synovial fluid [8].

Morphology

Particles (of metals, polymers or ceramics) range in size from nanometers to millimeters, while soluble wear debris exist in soluble forms bound to serum proteins, either specifically or nonspecifically [16]. Current techniques used to isolate, separate and characterize particles within periprosthetic tissues typically involve digestion of periprosthetic tissue with proteolytic enzymes and a strong acid or alkali [20]. Particles can be separated with use of density-gradient centrifugation and characterized with an automated particle analyzer. Using these techniques, researchers have determined that the most common type of particles found in these tissues is polyethylene particles, and have found the average size to be approximately 0,5 μm , with more than 90% of all particles having less than 1 μm [22] [23]. Metal and ceramic particles are usually an order of magnitude smaller than those of polymers such as UHMWPE, with a mean size averaging around 0,05 μm [16].

Maloney *et al.* (1995) determined by automated particle analyzer a mean of 1,7 billion particles per gram of tissue (of failed uncemented hip arthroplasties), compared with only 143 million per gram of tissue for the control samples [22]. The concentration of wear debris particles from periprosthetic tissues is directly related to the duration of implantation [24] and there are billions of particles generated per gram of tissue. It has been reported that osteolysis is likely to occur when the threshold of particles exceeds $1 \times 10^{10}/\text{g}$ of tissue [25]. Each mg of polyethylene wear has been estimated to generate $1,3 \times 10^{10}$ particles [26].

It is important to stress, however, that the number of wear particles at a given value of volumetric wear is strongly dependent on their size [27]. Thus, given two different prosthetic devices with the same volumetric wear, the one that produces particles with smaller size, will consequently give rise to more particles released to the periprosthetic tissues. Therefore, there are several characteristics about particle wear that modulate their effect once they are released to the surrounding tissues: size, total particle load and chemical reactivity [16]; concentration, i. e., number of particles per volume of tissue [20]; and even particle shape and surface morphology [28].

Biological response to wear debris

Naturally, all the previous characteristics are important to determine immune system's response to wear particles. However, the one that is best studied and understood

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is size and its effects [29]. There is evidence that ingestion of small particles by cells occurs by endocytosis or pinocytosis for particles in the nanometer range (less than 150 nm) [30]. Larger particles (of up to 10 μm) can be phagocytized by a range of cell types, such as osteoblasts, fibroblasts, endothelial cells and macrophages [20]. Recent studies have shown that particles with a mean size of 0,24 to 7,2 μm are generally the ones that produce a more intense inflammatory reaction [16]. When particle size significantly exceeds these values, little response is usually observed [31]. The morphology of particles also appears to contribute to cellular responses, with UHMWPE debris with a roughened surface and a fibular shape leading to a greater response in terms of inflammatory cytokine production in a murine inflammation model than particles with a smooth surface and a globular shape [28].

5. RANKL system

RANKL-RANK-OPG signaling

Bones are constantly remodeled through the balance between its synthesis and its resorption. Osteoblasts are stromal cells of mesenchymal origin concerned in bone formation and are intimately involved in the regulation of osteoclast differentiation and activation [32]. Osteoclasts are the bone resorbing cells which play a major role in the regulation of bone mass in both health and disease. They are derived from hematopoietic cells of the mononuclear phagocyte lineage; they are multinucleated giant cells formed from the fusion of osteoclast progenitors recruited into bone from the vasculature. Hence, the osteoclast can be described as a specialized monocyte/macrophage polycaryon, and it is the major bone-resorptive cell [2]. These two cell types have an equally important role to play in regulation of bone mass and it is the activity of osteoclasts relative to osteoblasts that will dictate the degree of osteolysis in total joint replacement. This balance is controlled by cytokines, growth factors and hormones, which combined action is essential for bone homeostasis. The most important molecules in the control of osteoclasts are the receptor activator of NF- κB (RANK), its ligand RANKL (its gene gives rise to splice variants that encode two forms of type II transmembrane proteins and one form of a secreted protein [33]) and the decoy receptor for RANKL, osteoprotegerin (OPG) [34].

The first insight on the interaction between them was provided by Udagawa *et al.* (1990), who demonstrated in mice that mononuclear phagocytes were capable of differentiating into osteoclasts in the presence of bone marrow-derived stromal cells. They showed that the differentiation of osteoclasts from mononuclear cell progenitors or tissue derived macrophages *in vitro* required physical contact with stromal cells derived from bone marrow or osteoblasts [35]. Further research led to the discovery of a stromal cell secreted glycoprotein termed osteoprotegerin (OPG), which prevents osteoclastogenesis by blocking osteoclast precursor cells differentiation in a dose-dependent manner [36], and the ligand for OPG, a membrane-bound protein on the

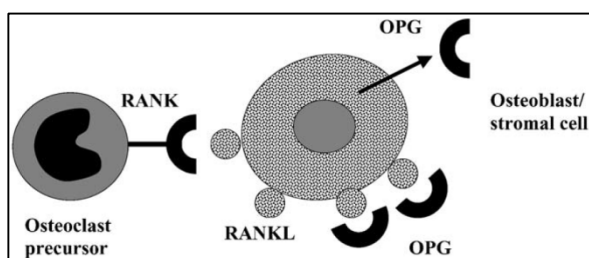


Figure 4 - Relationship between RANK, RANKL and OPG. (Adapted from [2])

surface of osteoblasts and fibroblasts that bound to both OPG and osteoclast progenitor cells [37]. Later, this ligand was termed Receptor Activator of Nuclear

Factor Kappa B (NF κ B) Ligand (RANKL) [34]. Figure 4 shows the

interactions between osteoclast precursors and stromal cells/osteoblasts through these signals. RANKL, a protein expressed by osteoblastic stromal cells, T lymphocytes, and B lymphocytes, binds to receptor activator of nuclear factor- κ B (RANK) on osteoclast precursors and is the primary mediator of osteoclast differentiation, activation, and survival [38]. RANK is present on osteoclast precursors and is capable of initiating osteoclastogenic signal transduction after ligation with RANKL expressed by osteoblasts/bone marrow stromal cells. OPG produced by osteoblasts/bone marrow stromal cells acts as a decoy protein, binding to RANKL on osteoblasts/stromal cells and inhibiting the interaction between RANK (on osteoclast precursors) and RANKL. Thus, OPG inhibits osteoclastogenesis.

In a general approach to this signaling pathway, it is important to refer that it plays a central role not only in bone metabolism, but also in lymph node formation in the immune system and mammary gland development in pregnancy. Furthermore, the functions of many factors that stimulate RANKL expression have been summarized: parathyroid hormone, prostaglandin E2, dexamethasone, IL-1, TNF- α . On the other hand, other factors such as estrogen and TGF- β inhibit RANKL expression [34].

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Other cellular effectors

Macrophages

Osteolysis after total hip replacement is associated with the formation of a granulomatous membrane rich in macrophages and implant derived wear particles. The formation and growth of interface membrane are considered to be a foreign-body reaction initiated by wear debris particles from the prosthesis, which represents one of the key processes in periprosthetic osteolysis and aseptic loosening [39]. This membrane is characterized by a highly vascularized fibrous tissue, rich in wear debris particles, macrophages, multinucleated cells and fibroblasts [40].

Macrophages have been shown to associate with sub-micrometer-sized particles, which are the majority of particles released due to articulation wear, while larger particles (more than 10 μm) are associated with giant cells [2]. From this, it is safe to assume that the most important particle-induced response mediators are macrophages. Several cytokines have been demonstrated to be present in periprosthetic tissues, such as TNF- α , IL-1 β , IL-6, IL-8, IL-11 and TGF- β [2]. Particularly, TNF- α , IL-1 and IL-6 [41], as well as PGE₂ [2], have been shown to present higher levels in loose prostheses (with osteolysis) compared to those that are not loose. Plus, colony stimulating factors (M-CSF and GM-CSF), matrix metalloproteinases and elastase have also been identified in the surrounding tissues of failing prostheses [2]. It has been demonstrated that macrophages (as well as osteoclasts and giant cells) express receptors for IL-1, IL-6, TNF, M-CSF and GM-CSF [42].

In vitro studies have contributed greatly for the understanding of the effect of wear particles on macrophages. Some authors have clearly demonstrated that particle-stimulated macrophages elaborate a range of potentially osteolytic mediators (IL-1, IL-6, TNF- α , GM-CSF, PGE₂) and bone resorbing activity [2]. With respect to cytokine production, particles in the 0,1-1,0 μm -sized range at a volumetric concentration of 10-100 μm^3 of particles per cell are the most biologically reactive. These studies have also indicated that, of the numerous cytokines, TNF- α is a key osteolytic cytokine generated by particle-stimulated macrophages.

Confirming the importance of RANKL in several different mechanisms, the expression of this molecule has been reported in a variety of cell types: bone marrow

stromal cells, fibroblasts, epithelial cells, osteoblasts, osteoclasts, activated T lymphocytes [43], chondrocytes, keratinocytes and vascular endothelial cells [44].

Fibroblasts

Being one of the main cell types in the arthroplasty membrane, fibroblasts relevance in osteoclastogenesis became a matter of discussion. After the discovery that these cells express RANKL, Sabokbar *et al.* (2005) demonstrated that fibroblasts in this membrane express RANKL and OPG and that they are capable of supporting osteoclast formation from mononuclear phagocyte precursors by a RANKL-dependent mechanism when cell-cell contact is promoted [45]. The addition of monocytes, which are known to express RANK, to cultures of RANKL-expressing fibroblasts isolated from the arthroplasty membrane resulted in osteoclast formation and lacunar resorption.

However, this work does not answer the issue of how membrane fibroblasts are activated in the first place, not only to express RANKL but also to produce several cytokines and other chemical signals that take a place in osteolysis. For example, whether fibroblasts are activated by macrophages or if they respond directly to particulate wear debris. Other study showed that fibroblasts of interface membranes expressed mRNA for OPG and RANKL (membrane-bound and soluble) in response to treatment with conditioned media from interface membranes both with and without titanium particles [46]. However, the effect of proinflammatory cytokines cannot be neglected, once the addition of TNF- α and IL-1 to the conditioned media further increased RANKL expression in a time-dependent manner.

The ability of these fibroblasts retrieved from the pseudocapsule to induce the differentiation of rat non-adherent bone marrow cells into mature osteoclasts was assessed by Sakai *et al.* (2002). The results proved the co-culture of these two cell types could cause the formation of TRAP positive multinucleated cells, i. e., cells with osteoclast phenotype [47].

Osteocytes

Osteoblasts have been thought to be the main cell type to express RANKL which stimulates osteoclastogenesis. However, the major source of RANKL *in vivo* remains unclear, as RANKL is expressed by several cell types in bone and bone marrow, including osteoblasts, osteocytes, BMSCs and lymphocytes. Further studies have shown

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that RANKL expression in bone marrow derived cells, including T cells, is considerably lower than in bone cells. Complementarily, Nakashima *et al.* (2011) obtained two mouse calvaria derived cell populations containing osteocytes and osteoblasts of high purity, respectively. mRNA expression of the RANKL gene was found to be ten times higher in osteocytes than in osteoblasts, revealing evidence a more potent ability of osteocytes to induce osteoclastogenesis [34]. These recent studies concerning the importance of osteocytes in osteoclast activity open new options on how to approach this problem once, although osteocytes have many similarities with osteoblasts because they derive from these bone forming cells, they have some singular characteristics such as their immobility and long life. The fact that they are trapped in the bone lacunae may seem like a disadvantage in osteoclast activation and that it could only be performed through soluble RANKL. However, due to their long processes, they are able to reach the bone surface and vascular space, thus communicating directly through membrane-bound factors [48].

The combined role of wear particles, macrophages, osteoclasts and osteoblasts in osteolysis

All the findings referred until now suggest the importance of stromal cells and osteoblasts in osteoclastogenesis. Stimuli that increase M-CSF and RANKL expression by osteoblasts will be osteoclastogenic. This provides one potential mechanism for the

role of macrophage-derived cytokines in osteolysis, since TNF- α and IL-1 (produced by these macrophages) stimulate osteoblasts to express RANKL and M-CSF [2] [34]. Hence, this process requires physical contact with stromal cells and/or osteoblasts, once this signaling pathway is RANK/RANKL dependent. However, there is evidence that osteoclast recruitment and differentiation may be

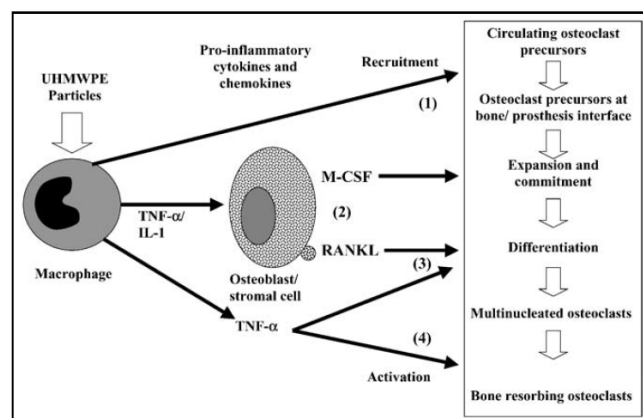


Figure 5 - Potential mechanisms whereby UHMWPE particle-stimulated macrophages may stimulate osteolysis in total joint replacement. (Adapted from [2])

possible only through TNF- α stimulation in a RANK/RANKL independent mechanism [49]. Plus, the variety of inflammatory cytokines and chemotactic factors generated may lead to an increased recruitment of hematopoietic osteoclast precursors from the

vasculature, which may be enough to induce bone resorption. These possible mechanisms (schematically presented in Figure 5) suggest that the evidence for a role for macrophage-derived TNF- α in wear particle-derived osteolysis is substantial. However, there is still no definite understanding of how all the steps of this process occur, due to the complexity and variety of interveners.

6. RANKL expression

Given the complexity of the periprosthetic environment, there are several possible approaches to the problem of RANKL expression quantification. From each one, different conclusions can be inferred and any robust study addressing this issue should consider this panoply of variables. The variety of cell types and tissues, possibly with structural differences to native tissues, which are found around these prostheses, is considerable. Furthermore, their characteristics may vary greatly from patient to patient.

Systemic levels

When RANKL was first discovered, only its cell-bound form was known. However, evidence of a soluble form soon was found, although neither the molecular species of RANKL that contribute to circulating RANKL nor their cellular source(s) are well understood [44]. Some authors determine serum total RANKL levels using ELISA, once this molecule can be released from the tissues where it is secreted as a soluble molecule. Not only unbound RANKL is measured, but also complexed RANKL with OPG or other proteins (which is the larger fraction of its serum levels) [44], once this reflects better the tissue production of soluble RANKL [50]. The study of these parameters may be important to understand how musculoskeletal pathologies influence not only local bone turnover, but also systemic bone physiology. Findlay *et al.* (2008) performed a study in a population of 40 (15 men and 25 women) where serum total RANKL levels were compared to mRNA expression in bone collected from the proximal femur during THA surgery. The results suggest that, at least in an elderly male cohort, serum total RANKL represents the inverse of active RANKL in bone [50]. However, one should always be aware that soluble RANKL assays at present are limited, mainly due to the uncertainty about the tissue source of the RANKL being measured (sRANKL may be produced by a

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wide range of cells types, not directly relatable to bone samples) and whether circulating levels reflect tissue levels in general or those in tissues of interest, in particular bone [44].

Periprosthetic area

It becomes clear, then, that RANKL quantification in the tissues around the prosthetic device is essential for a proper analysis of this problem. However, periprosthetic tissue organization is understandably different from a healthy joint.

The normal diarthrodial joints such as the hip and knee are contained within a fibrous capsule, the inside of which is covered by a layer of specialized cells that produce the synovial fluid. This fluid is responsible for cartilage nutrition and the removal of metabolites from the joint as well as joint lubrication and the provision of a milieu for the cells that are involved in defensive processes, including the removal of foreign material and organisms from the joint. The joint-lining layer of cells, often called synoviocytes or simply synovial lining cells, increases in thickness through an increase in the number of cells present. This nonspecific response occurs in various joint conditions, including inflammatory arthritis, marked degenerative joint disease and crystal-induced disease such as gout [51].

The synovial lining is removed at the time of joint replacement surgery, but a lining of cells indistinguishable from the normal joint lining (frequently referred to as the pseudo-synovium) grows back again. Goldring *et al.* (1986) showed that a synovium-like structure is present at the surface of the fibrous tissue between implant and bone using routine light microscopy [52]. It has been shown that this cellular layer is similar to, if not identical with, true synovium by detailed immunohistochemistry studies in which macrophages and fibroblasts are present and distributed just like the type A and type B cells of the true synovium [51]. This layer of pseudosynovial tissue is often present between failed implant components and the surrounding bone during revision surgery for aseptic loosening. Numerous polymeric and/or metallic wear debris particles are present in these vascularized granulomatous tissues, and many of them are engulfed by or in close contact with macrophages and other inflammatory cells. It is thought that particulate wear debris initiates the pathology by activation of pro-inflammatory cytokine signaling within prosthetic macrophages, which in turn leads to an imbalance of the osteoclastogenesis regulators RANKL and OPG. Macrophages appear to be the key cells in responding to the stimulus of wear debris. Cells of a monocyte/macrophage lineage differentiate and

mature into phagocytic macrophages, and circulating peripheral blood monocytes (PBMCs) are among the first cells to colonize the inflammatory site [53].

7. Problem

Given the highly complex environment where hip prosthetic devices are placed, both *in vitro* and even *in vivo* studies are naturally unable to reliably reproduce the conditions verified in that same environment. On the one hand, the variety of cell types that take action in all the processes that occur there is considerable and consequently impossible to reproduce in *in vitro* testing. On the other hand, *in vivo* tests cannot reproduce the time-scale of the problems that usually lead to device failure. Hence, the study of tissues removed from patients with failed hip replacements presents itself as an excellent tool to understand what caused the need for revision surgery.

In order to understand better the underlying mechanisms in osteoclastogenesis and osteoclast activation after hip replacement, one must study the biological phenomenon that caused the need for prosthesis implantation in the first place. Hence, comparison of RANKL expression in periprosthetic tissue from both primary arthroplasties and revision arthroplasties is necessary, and has already been addressed [38]. For example, in this study by Wang *et al.* (2010), the expression of RANKL and OPG (among other important molecules) was studied in two different groups: 10 THA patients with loosening and 10 osteoarthritis patients subjected to primary THA surgery. Wang *et al.* (2010) found a significant difference between the two study groups. RANKL levels were found to be higher in the revision surgery patients in osteoblastic stromal cells and synovial fluid when compared to the primary surgery patients, being the same in both T and B cells from bone marrow, although with a non-significant difference. The OPG/RANKL ratio was higher in the synovial fluid of primary surgery patients than revision surgery patients. One must pay attention to the fact that all RANKL expression levels are determined in cells retrieved from the bone marrow during surgery, which may present different profiles due to the characteristics of their niche, which may be considerably different from periprosthetic tissues.

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Patients

When dealing with a group study constituted by humans, a set of variables arises which cannot be ignored during a study such as the one performed in this work. Patients' age, gender, underlying cause for the surgical procedure and implantation period (in the case of revision surgeries) are all variables that must be taken into account in order to infer any relevant conclusions from the results. Bone degradation/formation balance varies considerably with age, being this variation possibly related with RANKL expression values. Gender is also another differentiating factor, mainly due to the higher osteoporosis incidence in menopausal women. Other important variable to consider is the clinical reason which caused the need for surgery in the first place: patients with short-term lesions (such as fractures or dislocations) may present very different expression profiles than patients whose diagnostics is osteoporosis, rheumatoid arthritis or osteoarthritis, to name a few.

Tissue extraction site

In order to compare RANKL expression profiles among the population under study, it is paramount to assure that the collected samples are retrieved from the same location, being formed by the same anatomic structures and tissues. However, there are two major difficulties in this matter which do not always allow this consistency: first, despite precise indications to the surgical teams that kindly collaborate with this project, there are always slight variations in terms of surgical technique between surgeons and different surgeries; second, once one of the central goals of this study is to compare RANKL profile expressions in patients subjected to either primary THA or revision THA, one should be aware of the anatomical differences between these two clinical situations. In the revision surgery, the joint structure is not the same as in a primary surgery, due to the previous excision of the femoral head and acetabular cartilage, as well as a capsule with possible structural differences. Taking all this in consideration, the tissue samples used (Figure 6) from the capsule, femur and acetabulum were as anatomically and histologically similar as possible.

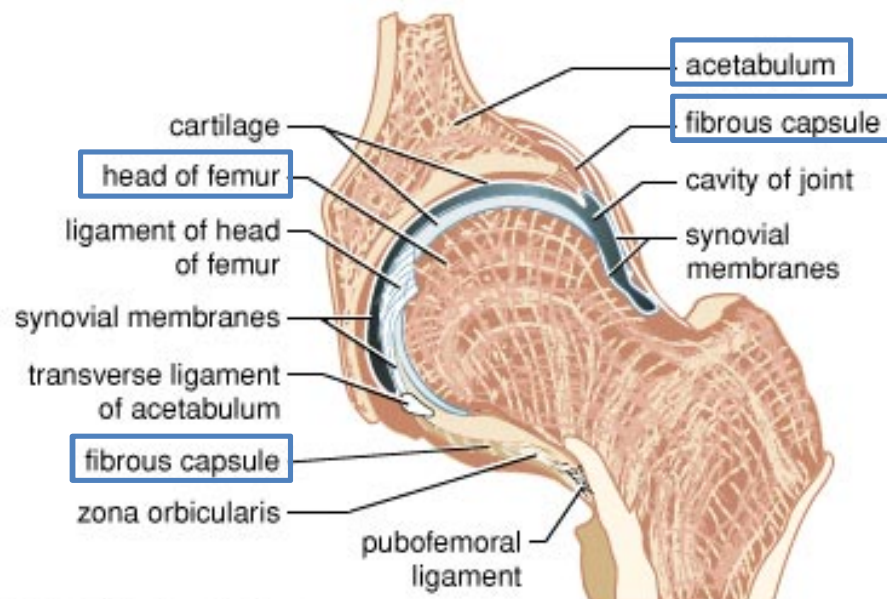


Figure 6 – Anatomical structures from where tissue samples were collected during Primary and Revision THA

Population

Another limitation of this study is the number of individuals that constitute the studied population. Being the number of collected samples limited by the available surgeries, the period during which this thesis was developed was shorter than the necessary time to collect enough tissue samples to obtain statistically significant results. Hence, it was only possible to determine tendencies in the variation of RANKL expression within the studied population.

II. Aim of Studies

Receptor Activator of Nuclear Factor κ B Ligand (RANKL) is considered to be a major responsible for osteoclastogenesis and, in the case of Total Hip Arthroplasty, a mediator of osteolysis that causes implant aseptic loosening. RANKL is expressed by osteoblasts, macrophages, fibroblasts and other cell types within the tissues of the acetabulofemoral articulation. However, little is known about RANKL levels in the different hip joint tissues of THA patients, both primary and revision.

The main goal of this study was to determine and compare RANKL mRNA levels in different locations of the acetabulofemoral articulation (capsule, femur and acetabulum) of both primary and revision THA patients, taking into account their age, gender, cause for surgery and implant duration.

RANKL mRNA in those tissues was quantified using Quantitative Real-time Polymerase Chain Reaction.

III. Materials and Methods

1. Patients

Gene expression profiles were determined in tissues samples from twenty six (26) patients undergoing total hip arthroplasty: fifteen (15) of those subjected to a primary THA and eleven (11) to a revision THA (for failed cemented implants). Patients were included in the study in a consecutive manner over a period of 2 years, as they were directed to arthroplasty at the Department of Orthopedics and Traumatology of Hospital de São João in Porto or Centro Hospitalar de Gaia/Espinho in Gaia. Any sample retrieved from a patient with any known condition affecting bone metabolism or physiology was excluded from the study. The study was approved by the Ethics Commission of Hospital São João and the Ethics Commission of Centro Hospitalar de Gaia/Espinho, and all patients gave written informed consent.

The patients studied and their characteristics (gender, age and cause of surgery) are presented in Table 1 and Table 2. In the case of revision surgery patients (Table 2), the period the duration of the implant is also provided.

Table 1 - Primary THA Patients

Patient	Gender	Age (years)	Cause
1	F	79	Rheumatoid arthritis
2	F	45	Secondary osteoarthritis caused by hip dysplasia
3	F	54	Secondary osteoarthritis caused by hip fracture
4	F	80	Primary osteoarthritis
5	M	55	Primary osteoarthritis
6	F	49	Primary osteoarthritis
7	M	76	Primary osteoarthritis
8	F	51	Secondary osteoarthritis caused by avascular necrosis
9	F	?	Rheumatoid Arthritis
10	M	?	Primary osteoarthritis
11	F	74	Primary osteoarthritis
12	M	83	Primary osteoarthritis
13	F	37	Secondary osteoarthritis caused by hip dysplasia
14	M	74	Primary osteoarthritis
15	M	?	Secondary osteoarthritis caused by hip fracture

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Table 2 – Revision THA Patients

Patient	Age (years)	Implantation (years)	Gender	Cause
42	61	2	F	Pain / Osteolysis
43	77	11	M	Pain / Osteolysis
44	53	12	F	Pain / Osteolysis
45	76	0.33	F	Dislocation (due to component misalignment)
46	101	1	F	Fracture
47	86	14	F	Pain / Osteolysis
48	69	10	F	Pain / Osteolysis
50	71	17	F	Pain / Osteolysis
51	74	10	M	Pain / Osteolysis
52	73	9	M	Pain / Osteolysis
53	63	0.33	F	Pain / Osteolysis

2. Human tissue samples

Excision

During the surgical procedures, tissue samples (approximately 1 cm³) were collected from three specific locations of the acetabulofemoral articulation: capsule, i. e., the synovial-like structure which encloses the synovial fluid in the hip joint; femur, i. e., tissue from the proximal end of the femur, as close to the defected joint as possible; and acetabulum. It has already been stated that the characteristics of these tissues may differ slightly from primary to revision surgery patients, as represented in Figure 7.

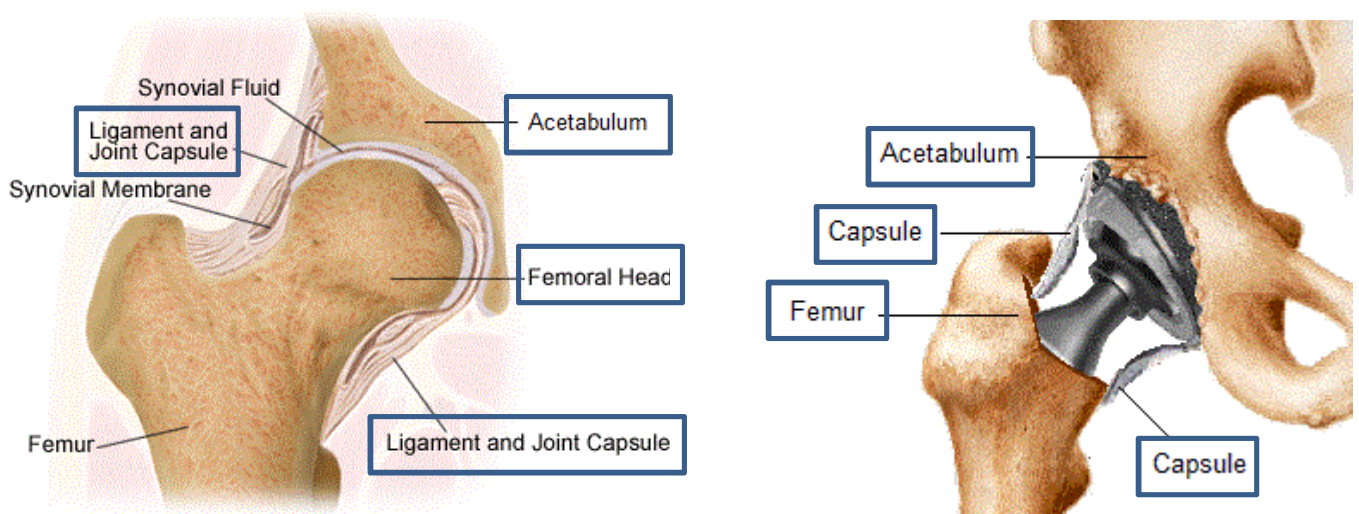


Figure 7 – Anatomical structures from where tissue samples were collected in Primary (left) and Revision (right) Total hip Arthroplasties

These differences should not be neglected. For example, the capsule is removed at the time of joint replacement surgery but a lining of cells indistinguishable from the normal joint lining grows back again [51]. This is frequently referred to as the pseudo-synovium in the orthopedic literature and a representation is shown in Figure 7 (right).

Storage

These samples were immediately frozen in dry ice after being collected and split for other upstream analyses and stored at -80°C until RNA extraction.

The reagents used during the following procedures are presented in Table 3.

Table 3 - Reagents

Reagent	Reference
TRIzol®	Invitrogen
PureLink® RNA Mini Kit	Ambion Life Technologies
SuperScript™ First-Strand Synthesis System	11904-018 Invitrogen
SeaKem LE Agarose	50004 Lonza
SYBR Green PCR Master Mix	Applied Biosystems 4309155

3. Tissue homogenization and RNA extraction

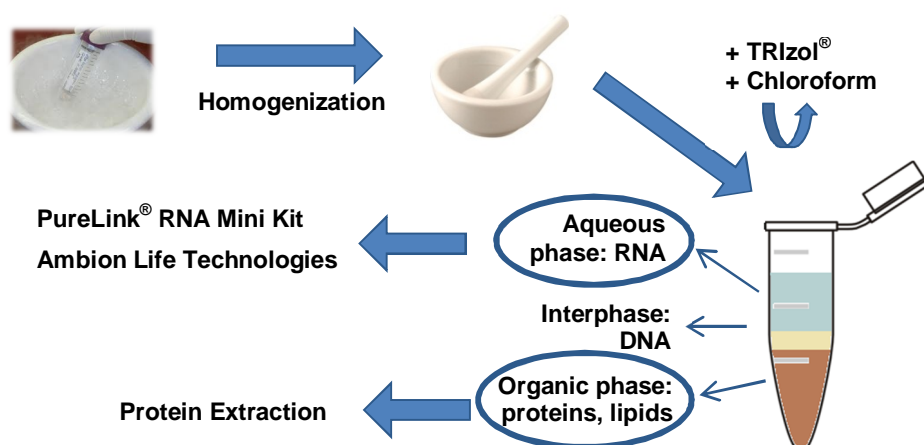


Figure 8 – Schematic representation of the tissue homogenization and RNA extraction process

Frozen tissue samples were homogenized using liquid nitrogen in a mortar and a pestle, in a sterile environment. Total RNA was isolated using the TRIzol® reagent and the PureLink® RNA Mini Kit (Ambion Life Technologies). Briefly, after tissue homogenization, the sample was incubated with 1 mL of TRIzol® reagent at room

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temperature for 5 min, after which 0,2 mL of chloroform were added and the tube was shaken vigorously for 15 sec. After a 2-3 min period of incubation at room temperature, the sample was centrifuged at 12000g for 15 min at 4°C. The colorless, upper aqueous phase containing RNA was transferred to a new RNase-free tube and an equal volume of 70% ethanol was added. While the pellet was stored at -20°C until protein extraction, the obtained solution was transferred to a spin column (with a collection tube) and spun for 15 sec at 12000g and RT. The flow-through was discarded and the spin column returned to the same collection tube. Bound RNA was washed by adding 700 µL of Wash Buffer I and spinning for 15 sec at 12000g. The filtrate was discarded and the spin cartridge was placed in a fresh collection tube, RNA was washed twice with 500 µL of Wash Buffer II with ethanol by centrifuging the spin column for 15 sec at 12000g. The filtrate was again discarded and then centrifuged for an additional minute at RT and 12000g to dry the membrane with attached RNA. Finally, the spin column was transferred to an RNase-free recovery tube and RNA was eluted by adding 30 µL of RNase-free water. The tube was incubated at RT for 1 min and centrifuged for 2 min at 12000g. RNA solutions were stored at -80°C.

4. RNA integrity and quantification

RNA purity and concentration were assessed by using a NanoDrop 2000 (Thermo Scientific) spectrophotometer, which requires 1 µL samples to evaluate RNA quality. Absorbance was measured at 260, 280 and 230 nm, in order to obtain the $A_{260/280}$ and $A_{260/230}$ ratios. The first is used to assess the purity of RNA. A ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The latter is used as a secondary measure of nucleic acid purity. Expected 260/230 values are usually in the range of 2.0-2.2 and, in the case it is considerably lower, it may indicate the presence of contaminants which absorb at 230 nm [54].

The integrity of total RNA was assessed by running diluted RNA samples in a 2% agarose gel. This is an important procedure to determine if the RNA was properly extracted and its integrity was preserved. In gels with RNA samples derived from human tissues, two major bands are expected: one correspondent to the 28S rRNA (large ribosomal subunit) and other to the 18S rRNA (small ribosomal subunit). An example of this process is presented in Figure 9.

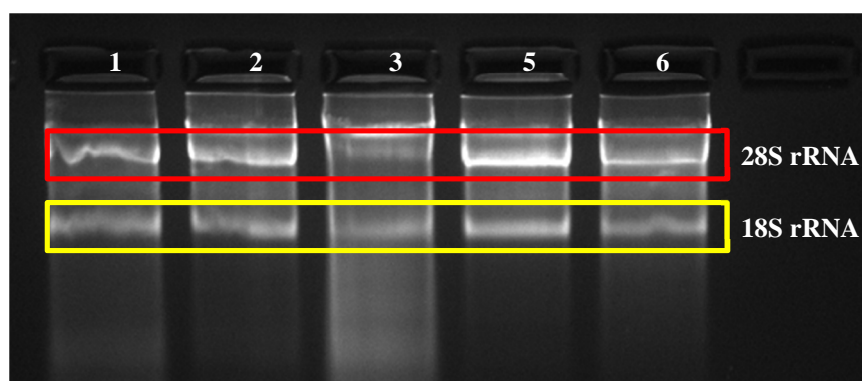


Figure 9 – mRNA gel electrophoresis. Femur samples from patients 1, 2, 3, 5 and 6. 2% agarose gel with 1,5 μ L EtBr; run for 22 min at 100 V.

Samples which presented either low integrity or purity were subjected to an Experion RNA StdSens Analysis (Bio-Rad) to confirm [55], which also requires only 1 μ L of RNA solution. The Experion™ automated electrophoresis system (Bio-Rad Laboratories, Inc.) provides an automatic assessment of RNA integrity by providing the RNA quality indicator (RQI) in addition to the electropherogram, gel view, and 28S/18S ratio and concentration [56].

5. Synthesis of cDNA

All reagents used in RT-PCR mixtures were from Invitrogen SuperScript™ First-Strand Synthesis System kit. RNase free water was from PureLink™ RNA Mini Kit.

1 μ L of dNTP (10 mM) and 1 μ L of oligo dT (0,5 μ g/ μ L) were added to each RNase-free tube containing an 8 μ L (volume completed with water) solution with 500 ng of RNA, and the samples were incubated at 65°C for 5 min, then transferred for 1 min to ice (0°C). A short spin was made, after which 2 μ L of 10x RT Buffer, 4 μ L of MgCl₂ (25 mM), 2 μ L of DTT (0,1 M) and 1 μ L of RNase OUT were added to each reaction tube. The samples were incubated at 42°C for 2 min. 1 μ L of Sperscript II Reverse Transcriptase (50 U/ μ L) was then added. Samples were incubated at 42°C for 50 min. The reaction was terminated at 70°C for 15 min. At this point, 1 μ L of RNase H was added to each tube and again incubated, at 37°C for 20 min, so that any reminiscent RNA would be eliminated. cDNA solutions were stored at -20°C.

6. Quantitative real-time polymerase chain reaction

Primer design for qRT-PCR

The design of the primers to be used in the qRT-PCR was performed using the Beacon Designer™ 7.5 software. After inputting the sequence of the gene to be identified (from NCBI), the user can determine certain parameters of the primers to be produced: annealing temperature, length, nucleotide repetitions, among others. After making sure the primer sequence spans over an exon junction (using BLAST software), the primers are designed and can be used from this point on.

qRT-PCR

The optimization and validation of the reaction for the quantification of RANKL comprises the preparation of a series of dilutions (1x, 5x, 10x, 20x) from the cDNA of a Primary THA patient and their amplification. By the correlation of the expected cycle threshold and the obtained Ct value, the reaction efficiency is determined. Hence, this value translates the reliability of the reaction to quantify RANKL expression within the range of the dilutions.

qRT-PCR was performed using a iCycler iQ™ (Bio-Rad) Real-Time PCR Detection System. Amplification reactions were set up in 20 µl reaction volumes containing SYBR Green PCR Master Mix (10 µL), template cDNA (1 µL) and amplification primers (0,25 µL each). PCR amplifications were performed (Table 4), and the amplification data were analyzed using iQ5 Optical System Software (Bio-Rad).

Table 4 – qRT-PCR temperature cycle

Step	T (°C)	Time	Number of cycles
1	95	3,5 min	
2	94	30 sec	x40
3	56	30 sec	
4	4	24 hours	

Relative quantification was calculated by normalizing the test crossing thresholds (Ct) with the B2M (β 2-Microglobulin) amplified control Ct. The obtained values are directly proportional to RANKL expression in the respective tissue samples, for which they are considered for the following study.

7. Protein extraction

Protein was extracted from the same samples from which RNA was extracted, using the TRIzol® reagent. After RNA extraction, 0,3 mL of ethanol were added to the pellet, after which it was mixed and left to incubate for 2-3 min at RT. This solution was then centrifuged at 2000g for 5 min at 4°C and the protein-containing supernatant was kept. After the addition of 1,5 mL of isopropanol and 10 min of incubation, the samples were centrifuged for 10 min more at 12000g and 4°C to pellet the protein. The supernatant was discarded. The pellet was washed for 20 min with a 0,3 M guanidine hydrochloride solution and centrifuged for 5 min at 7500g and 4°C, after which the wash solution was discarded. This washing step was repeated twice. At this point, the pellet was incubated for 20 min with a solution of 100% ethanol, and was once again spun for 5 min at 7500g and 4°C. A step of 5-10 min was performed to allow the protein pellets to air dry, followed by its resuspension with 200 µL of SDS. The solution is finally centrifuged for 10 min at 10000g and 4°C to sediment any insoluble material and the supernatant is stored at -20°C.

8. Protein Quantification – Bradford Assay

The Bradford Assay for protein quantification was performed with the Bio-Rad Protein Assay Kit, using the Standard assay protocol as follows. Four dilutions (1, 0.5, 0.25 and 1.25 mg/mL) of BSA were prepared to obtain the standard curve. 100 µL of standards and samples were transferred into clean, dry test tubes, to which were added 500 µL of reagent A' (prepared by adding 20 µL of reagent S to each mL of reagent A needed for the assay). After adding 4 mL of reagent B into each tube, mixing and waiting for 15 min, the absorbance was read at 750 nm.

9. SDS-PAGE

20 mL of the separating gel, i. e., the gel in which the protein content of the sample is separated accordingly to its molecular mass, were prepared with an acrylamide concentration of 12%. After pouring the separating gel into the SDS-PAGE cassette, 5 mL of the 3% acrylamide stacking gel are loaded into the cassette, and the

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comb is inserted to form the wells. The gel is left to polymerize for approximately 40 min and stored at 4°C.

50 ng of protein solution are used in this preparation. The volume of the protein solution is completed with distilled water to 425µL and with 75µL of trichloroacetic acid to complete 500µL. The samples are then placed on ice for 30 min and centrifuged at 4°C for 10 min at 13000 rpm. 800µL of acetone are added to the pellet and it is mixed until resuspended, after which the solution is centrifuged at 4°C for 10 min at 13000 rpm. This washing step is repeated once, and, after removing the acetone, the pellet is left to dry out for approximately 15 min. 40 µL of Loading Buffer 1x with 0,1M DTT are added and the solution is mixed. The solution is heated at 60°C for 15 min and, after that, at 90°C for 10 min while mixing. It is centrifuged at room temperature for 10 min at 13000rpm, after which 20 µL are finally loaded into the 12% SDS-PAGE gel. The protein ladder used was the Precision Plus Protein™ Dual Color Standards (Bio-Rad). After running the gel, it was stained with coomassie.

10. Statistical Analysis

Due to the low number of patients included in this study until the writing of this Dissertation, no statistically significant results could be obtained, for which no statistical analysis is presented.

IV. Results and Discussion

1. qRT-PCR optimization

Primer Design

The design of the primers to be used in the quantification of RANKL was performed using the Beacon Designer™ 7.5 software. The output of the design process comprises the parameters of a primer with a sequence covering an exon junction (from the respective gene), its length, annealing temperature (Table 5) and nucleotide repetitions.

This method was used to design the primers for both $\beta 2$ microglobulin (Figure 10), the housekeeping gene used in the qRT-PCR, and RANKL (Figure 11).

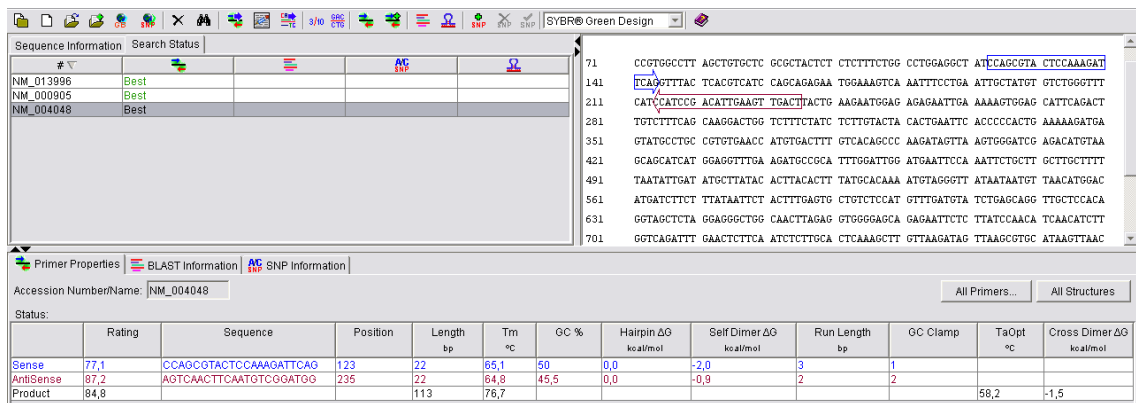


Figure 10 – $\beta 2$ microglobulin primers design using Beacon Designer™ Software

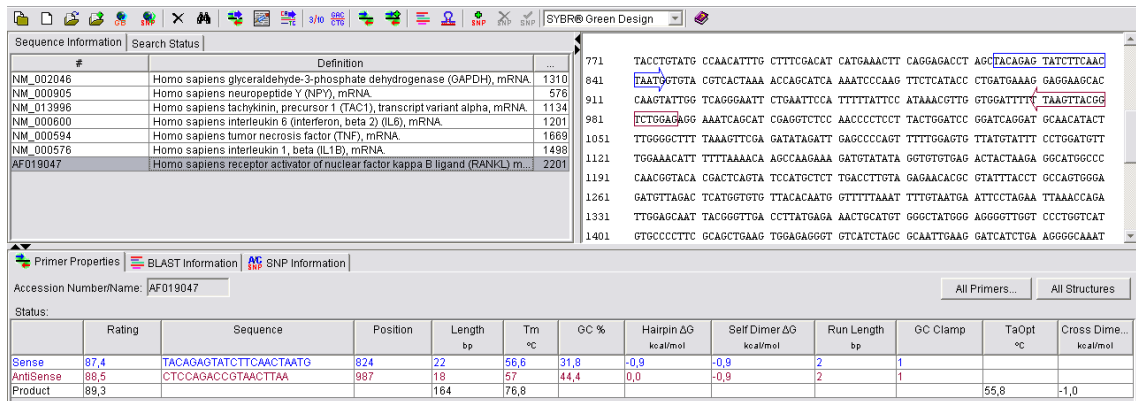


Figure 11 – RANKL primers design using Beacon Designer™ Software

Results and Discussion

In summary, the designed primers have the following characteristics:

Table 5 – Primers for qRT-PCR

Primer	Sequence	PCR product	T _{annealing} (°C)
β2 microglobulin Fw	5'-CCAGCGTACTCCAAAGATTCAG-3'	113 bp	58,2
β2 microglobulin Rv	5'-AGTCAACTTCAATGTCCGGATGG-3'		
RANKL Fw	5'-TACAGAGTATCTTCAACTAATG-3'	164 bp	55,8
RANKL Rv	5'-CTCCAGACCGTAACTTAA-3'		

Reaction optimization

The results obtained from the qRT-PCR must be validated for the primers used, translated in the reaction efficiency. For the RANKL primer, a series of four dilutions (1x, 5x, 10x, 20x) was prepared from the cDNA of a Primary THA patient.

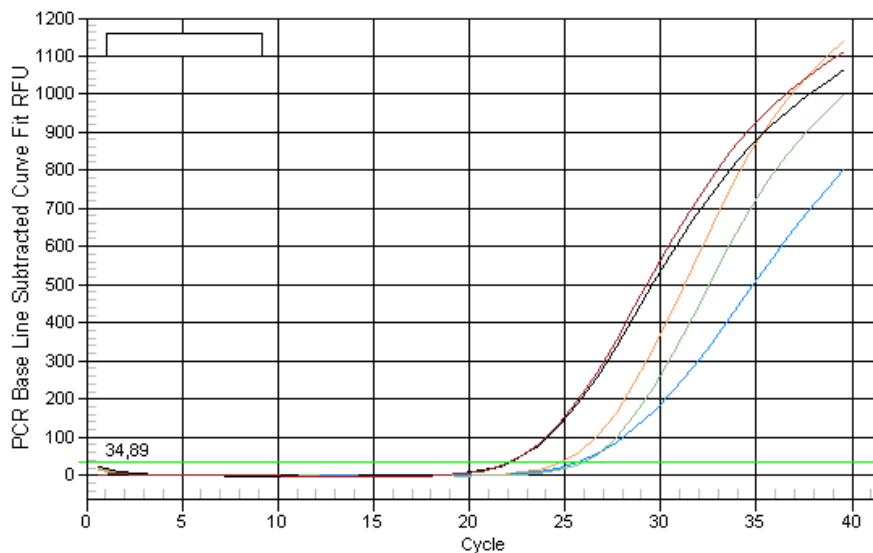


Figure 12 – Amplification Chart for RANKL. cDNA from Patient 7 – Capsule.

Figure 12 shows the amplification curves of the four cDNA dilutions and the control (mix without cDNA) and the respective cycle number at which the fluorescence crosses an arbitrary line called the threshold - shown in green. This crossing point is known as the Ct value, and the higher it is, the smaller is the amount of copies of the gene being amplified. Hence, it is expected that the samples analyzed in Figure 12 present different Ct values, once they are dilutions of the same cDNA sample.

Figure 13 and Table 6 show the details of the calibration curve obtained in this process. The PCR efficiency of 107,5% is considered close enough to 100% for the reaction to be considered valid for further analysis.

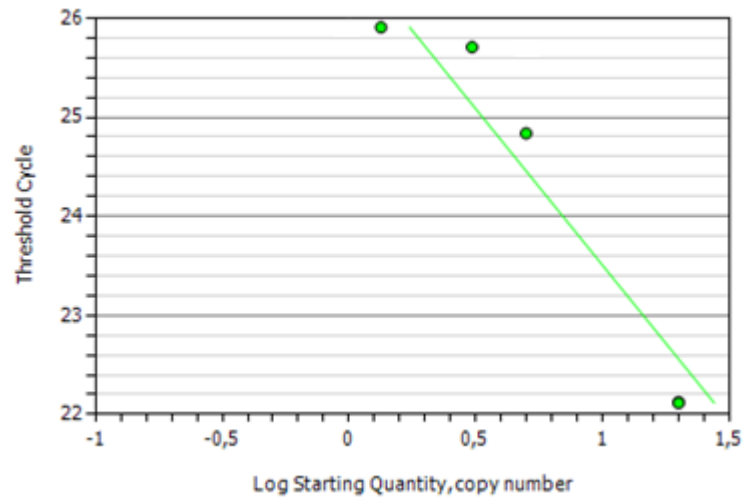


Figure 13 – Standard curve for RANKL

Table 6 – PCR efficiency and Standard curve parameters

Fluor	PCR Efficiency (%)	R ²	Slope	y-Intercept
SYBR	107,5	0,813	-3,154	26,661

2. RANKL expression profiles in Primary and Revision THA patients

After the optimization of both RNA extraction and its reverse transcription to cDNA, as well as the optimization of the Quantitative Real-Time PCR for RANKL, gene expression could be quantified.

The first group of patients under study comprised all of those who were subjected to a Total Hip Arthroplasty for the first time (Table 1) and it was constituted by 15 patients (9 women and 6 men) with a mean age of 63±16 years. All of these patients had injuries in the acetabulofemoral joint, being Primary Osteoarthritis the most common. Details such as gender, age and cause for surgery are presented in Table 1. One should be aware that for patients 9, 10 and 15, data concerning their age was not provided at the time of the surgery.

Results and Discussion

The quantification of RANKL expression in tissues collected from the acetabulofemoral joint of primary THA patients has been performed by a few authors, using several different approaches. Usually, tissue samples are retrieved from only one structure of the hip joint. For example, Logar *et al.* (2007) retrieved trabecular bone (femur) samples during the femoral osteotomy of primary hip replacement surgeries, in order to proceed with RANKL quantification using qRT-PCR [57].

In this study, RANKL mRNA levels in the three different locations in the hip joint from where tissue samples were retrieved are presented (Figure 14). Although no statistically significant differences were found, there is a tendency for acetabular tissue to express more mRNA for RANKL.

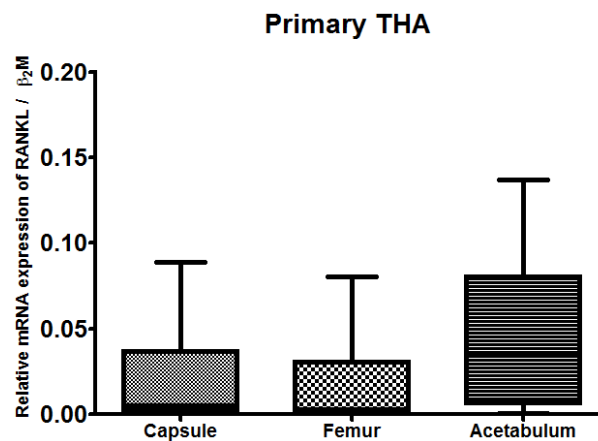


Figure 14 – RANKL expression in Capsule, Femur and Acetabulum from Primary THA Patients

The patients subjected to revision THA, i. e., the surgical replacement of at least one of the prosthetic components (acetabular or femoral), after a prior Total Hip Arthroplasty, were analyzed. These 11 patients (8 women and 3 men) aged 73 ± 13 underwent revision surgery due to the failure of the implanted prosthesis. The main cause for this failure and subsequent need for replacement surgery is Osteolysis associated with Pain, as presented in Table 2.

In revision THA, many studies about prosthetic failure with aseptic loosening have been done. For example, Ito *et al.* (2004) examined samples from 40 patients with aseptic loosened hip prostheses. Using immunohistochemical staining and RT-PCR, they compared the distribution of macrophages and cytokines (IL-6 and TNF- α) in the tissue from capsules and around the femoral and acetabular components, finding evidence of their involvement in aseptic loosening [58]. Haynes *et al.* (2001) preformed RANKL

mRNA quantification in samples retrieved from capsule, femur, acetabulum and synovial membrane. The results suggested that the ingestion of prosthetic wear particles by macrophages results in expression of osteoclast-differentiating molecules (including RANKL) and the stimulation of macrophage differentiation into osteoclasts [59]. Horiki *et al.* (2004) found RANKL expressing fibroblast-like and macrophage-like cells in membranous tissue around the acetabular and femoral components of five revision THA patients with loosened implants [60]. More recently, Veigl *et al.* (2007) studied a population of 59 patients undergoing revision hip surgery for aseptic loosening. Results suggested a direct correlation between high debris content in the periprosthetic environment and an elevated fraction of RANKL-producing cells in the same bone-cement or bone-implant interface [61].

In our study, as presented in Figure 15, revision THA patients express less RANKL mRNA in the capsular and acetabular region, when compared to the femoral tissue. However, it is clear that the number of collected samples (mainly from femoral tissue) is too low to allow one to draw significant conclusions.

The studies in which RANKL mRNA is quantified in more than one periprosthetic location do not compare the levels in those locations. Instead, they usually consider them as similar samples and, thus, they are studied as one group. The work by Haynes *et al.* (2001) mentioned before is an example of such approach.

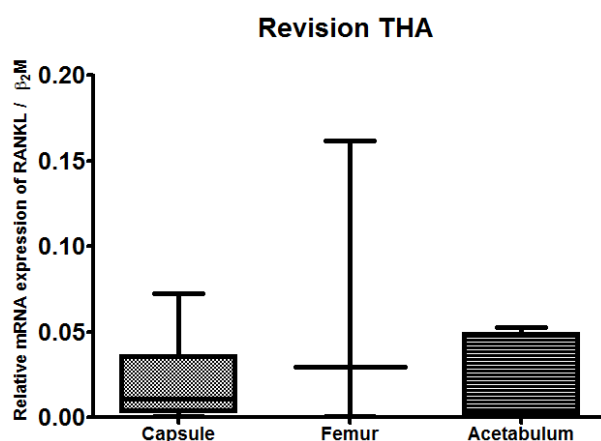


Figure 15 - RANKL expression in Capsule, Femur and Acetabulum from Revision THA Patients

3. RANKL expression profiles in local tissues from Primary and Revision THA patients

The main goal of this study is to determine RANKL mRNA levels in clinical settings involving an inflammatory reaction at the hip joint from primary and revision THA patients and to compare them. For this, tissue samples were collected from the same three anatomical locations during hip surgery (Figure 7).

Several studies have compared RANKL expression profiles within periprosthetic tissues. Crotti *et al.* (2004) compared the RANKL mRNA and protein levels in tissues from periprosthetic membranes (acetabular and femoral) of revision patients with osteolysis to the levels in synovial tissue of two control groups (osteoarthritic and healthy patients). Immunohistochemical staining and *In Situ* Hybridization were performed to analyze protein and mRNA RANKL levels, respectively. The results demonstrated that significantly higher levels of RANKL protein were found in the peri-implant tissues of patients with implant failure than in similar tissues from osteoarthritic and healthy subjects; results confirmed that mRNA encoding for RANKL is also expressed by cells in the periprosthetic tissues [62]. Holding *et al.* (2006), using immunohistochemical labeling to determine RANKL protein levels in tissues collected from sites of periprosthetic osteolysis of 11 revision THA patients, showed that RANKL is strongly expressed by large multinucleated cells containing polyethylene debris, when comparing to the synovial tissue collected from 10 primary THA patients with osteoarthritis [63]. Wang *et al.* (2010) compared a group of 10 primary THA patients (control) to another of 10 revision THA patients with loosened implants (all of them osteoarthritic) and quantified RANKL protein levels in the synovial fluid (by ELISA) and in bone marrow-derived cells (by flow cytometry). The results showed that revision THA patients had higher RANKL expression in osteoblastic stromal cells and higher RANKL levels in the synovial fluid [38].

In our work, the samples from the capsular region (Figure 16) present no difference when the two populations under study (primary and revision THA patients) are compared. This may come as unexpected, once it is generally accepted that periprosthetic tissues express higher levels of RANKL than those of primary THA patients.

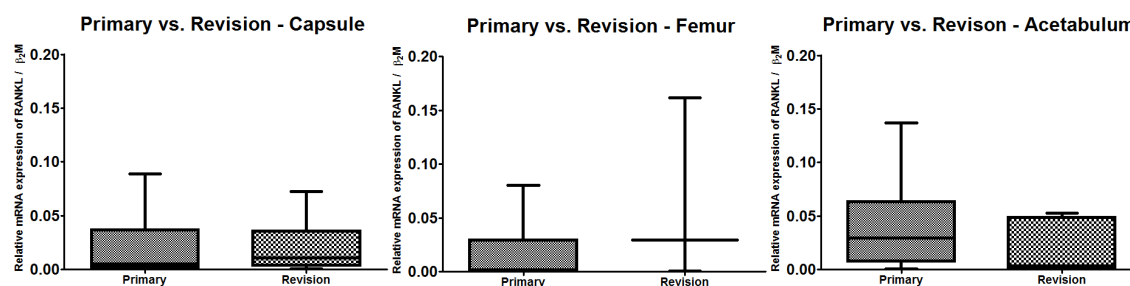


Figure 16 – RANKL expression in capsular (left), femoral (center) and acetabular (right) tissue of Primary and Revision THA patients

However, in the femoral tissue, there appears to be higher RANKL mRNA levels in revision patients. It is an expected result, due to the response to the particles released into the periprosthetic tissues in these patients. However, the number of tissue samples is not enough for any conclusion to be drawn.

In the acetabular samples, there is a trend of higher RANKL mRNA expression in the primary THA patients. All revision surgeries considered in this study required the replacement of at least the acetabular component, which means that the acetabulum is recurrently affected by osteolysis. However, in the patients studied, this problem was not translated into an increase in RANKL expression in the acetabular tissue, which could mean the two phenomena are not directly related.

Role of Gender in RANKL expression

Given the complexity of RANKL regulation mechanisms, gender may be an important factor to take into account in this study. Several studies addressed the relevance of gender in RANKL expression profiles, but no consensual findings have been achieved. Jung *et al.* (2002) [64] and Jiang *et al.* (2008) [65] have found evidence that women have higher RANKL levels in serum and in bone marrow cells than men, respectively. On the other hand, a study by Kerschman-Schindl *et al.* (2008) showed that men have significantly higher free RANKL levels than women [66].

Table 7 presents patient distribution according to their gender. One should notice the higher number of female patients, particularly in revision replacement surgeries.

Table 7 – Number of patients by gender

	Male	Female
Primary THA	6	9
Revision THA	3	8

Results and Discussion

In Figure 17, RANKL mRNA levels from male Primary THA patients are compared to those of female Primary THA patients. Although there are no significant differences, a slight tendency for men to express more RANKL than women is seen.

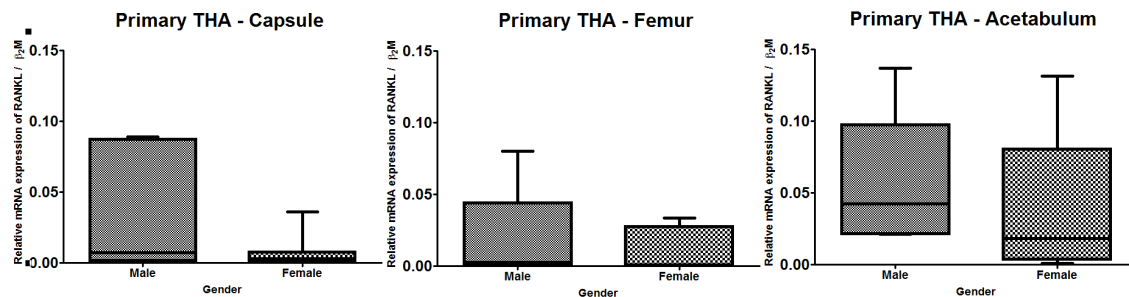


Figure 17 - RANKL expression levels in capsular (left), femoral (center) and acetabular (right) tissue from both genders in Primary THA patients

In revision surgery patients, no tendency is verified, with the exception of the acetabular tissue, where RANKL levels in men are slightly higher than in women. Once again, the low sample number affects the obtainment of any conclusions.

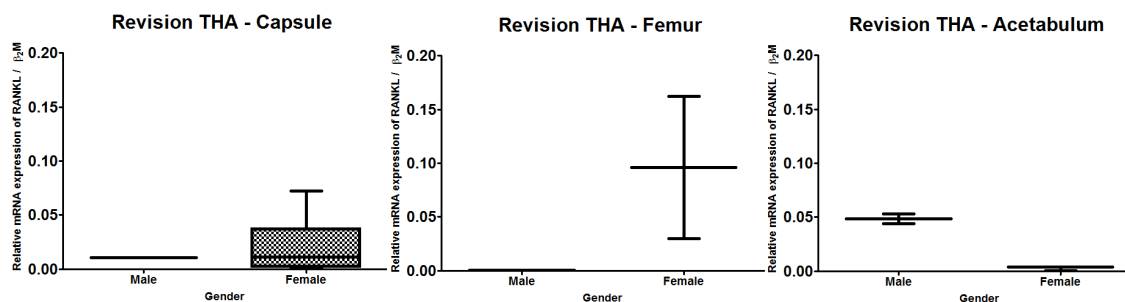


Figure 18 - RANKL expression levels in capsular (left), femoral (center) and acetabular (right) tissue from both genders in Revision THA patients

Role of Age in RANKL expression

RANKL transcriptional levels were also analyzed regarding patients' age. Previous studies about this issue have not reached consensual conclusions. Kersch-Schindl *et al.* (2008) showed that in a healthy adult population, serum levels of free RANKL and total RANKL decrease with age by, on average, 13% every five years [66]. However, Jung *et al.* (2002) has found no variation of serum RANKL levels (using ELISA) with age [64]. Furthermore, Jiang *et al.* (2008) used qRT-PCR to quantify RANKL mRNA levels and found evidence that the expression of RANKL in bone marrow cells increases with age [65]. It is clear that more studies are required to clarify this issue.

In Figure 19, the age of the patients included in our study are presented.

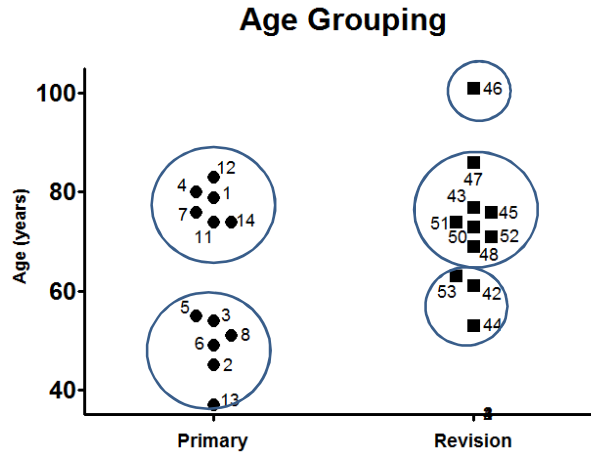


Figure 19 – Primary and Revision THA patients’ age and grouping

When considering age as a differentiating factor, Primary THA patients can be divided in two groups: 37-55 and 74-83, presented in Table 8. It is important to notice that patients 9, 10 and 15, whose age is unknown, were not included in this analysis.

Table 8 – Age grouping of Primary THA patients

Group	Age interval (avg ± std)	Patient (Age, Cause)
1	37 – 55 (49 ± 7)	2 (45, Secondary osteoarthritis caused by hip dysplasia) 3 (54, Secondary osteoarthritis caused by hip fracture) 5 (55, Primary osteoarthritis) 6 (49, Primary osteoarthritis) 8 (51, Secondary osteoarthritis caused by avascular necrosis) 13 (37, Secondary osteoarthritis caused by hip dysplasia)
2	74 – 83 (78 ± 4)	1 (79, Rheumatoid arthritis) 4 (80, Primary osteoarthritis) 7 (76, Primary osteoarthritis) 11 (74, Primary osteoarthritis) 12 (83, Primary osteoarthritis) 14 (74, Primary osteoarthritis)

The results presented in Figure 20 show that there is no difference in RANKL gene expression in the capsular tissue of the two age groups considered.

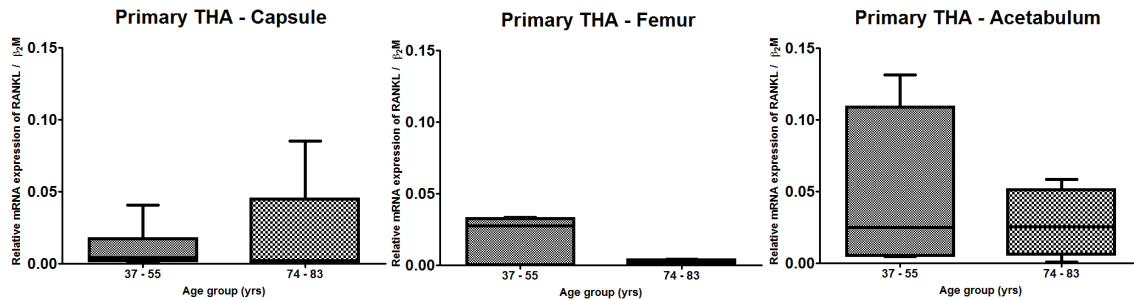


Figure 20 – RANKL expression levels in capsular tissue from the two age groups of Primary THA patients

Results and Discussion

The younger group (37-55 years) appears to express more RANKL than the older patients (74-83) in the femur. As previously referred, there is no complete agreement about this issue, but these results are in accordance with the authors who found evidence that RANKL expression levels decrease with age. It is important to point out that in those studies, only serum levels of RANKL are assessed [66]. However, our study aimed at the quantification of RANKL expression in periprosthetic tissues, i. e., membrane bound RANKL. Furthermore, the influence of RANKL age-related variations may be less significant in patients with such an inflammatory reaction in the hip joint.

In the acetabulum derived tissue samples, no difference in terms of RANKL gene expression was found between the two groups studied.

In the Revision THA patients (Table 9), three age groups were considered.

Table 9 – Age grouping of Revision THA patients

Group	Age interval (avg \pm std)	Patient (Age, Implant life, Cause)
1	53 - 63 (59 \pm 5)	42 (61, 2, Osteolysis + Pain) 44 (53, 12, Osteolysis + Pain) 53 (63, 0.33, Osteolysis + Pain)
2	69 - 86 (75 \pm 6)	43 (77, 11, Osteolysis + Pain) 45 (76, 0.33, Dislocation due to component misalignment) 47 (86, 14, Osteolysis + Pain) 48 (69, 10, Osteolysis + Pain) 50 (71, 17, Osteolysis + Pain) 51 (74, 10, Osteolysis + Pain) 52 (73, 9, Osteolysis + Pain)
3	101	46 (101, 1, Fracture)

RANKL mRNA levels in the capsular (left) and acetabular (right) tissues of each group are presented in Figure 21, and they do not vary significantly between them. In the acetabulum results, the third age group is not represented because no sample from acetabular tissue was collected during the revision THA of Patient 46.

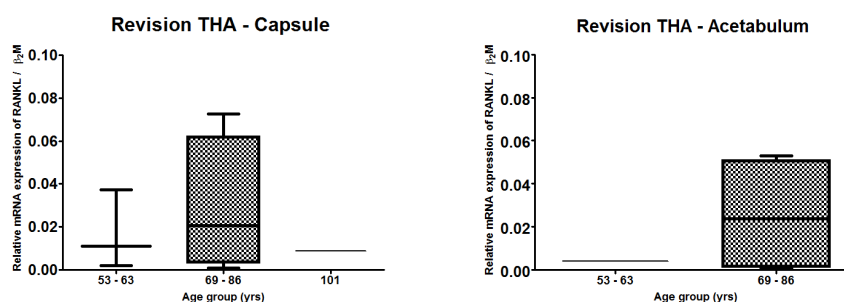


Figure 21 - RANKL expression levels in capsular (left) and acetabular (right) tissue from the three age groups of Revision THA patients

There were not enough femur-derived tissue samples to make a similar analysis of RANKL mRNA levels in this periprosthetic location.

Role of cause of surgery in RANKL expression

Another important factor that may influence RANKL expression profiles is the reason for the surgery itself, whether it is primary or revision THA. Several studies focus on the comparison of RANKL expression profiles in primary THA patients with different pathologies, being osteoarthritis and rheumatoid arthritis the most common. Jiang *et al.* (2008) found evidence that in primary THA patients diagnosed with osteoarthritis and rheumatoid arthritis, the levels of RANKL mRNA expression (determined by qRT-PCR) in bone marrow cells (isolated from acetabular bone marrow aspirates) is higher than in healthy patients [65]. Crotti *et al.* (2002) collected synovial tissue (arthroscopically) from patients diagnosed with active RA, inactive RA, spondyloarthropathies and osteoarthritis, using healthy patients as control. Immunohistochemical staining detected the highest RANKL protein levels in patients with rheumatoid arthritis with active synovitis [67]. Logar *et al.* (2007) studied RANKL mRNA levels (using qRT-PCR) in tissues from the proximal femur of two groups of primary THA patients: one of patients diagnosed with OA and another of patients who had a femoral neck fracture. RANKL mRNA levels were significantly higher in the fracture group than in the osteoarthritic group [57].

The patients in this study subjected to primary hip surgery are grouped by the cause of surgery in Table 10. The majority of the patients considered were diagnosed with osteoarthritis.

Results and Discussion

Table 10 – Cause for surgery of Primary THA patients grouping

Group	Cause	Patient (Age)
1	Primary osteoarthritis	4 (80)
		5 (55)
		6 (49)
		7 (76)
		10 (?)
		11 (74)
		12 (83)
14 (74)		
2	Secondary osteoarthritis caused by hip dysplasia	2 (45)
		13 (37)
3	Secondary osteoarthritis caused by hip fracture	3 (54)
		15 (?)
4	Rheumatoid arthritis	1 (79)
		9 (?)
5	Secondary osteoarthritis caused by avascular necrosis	8 (51)

In the tissue collected from the capsule that encloses the synovial cavity, a few patients diagnosed with osteoarthritis presented the highest RANKL mRNA levels. However, the sample number (mainly in the other groups) is not enough for any clear tendency to be detected. The same happens in the cases of the femoral (Figure 23) and acetabular (Figure 24) tissues.

Primary THA - Capsule

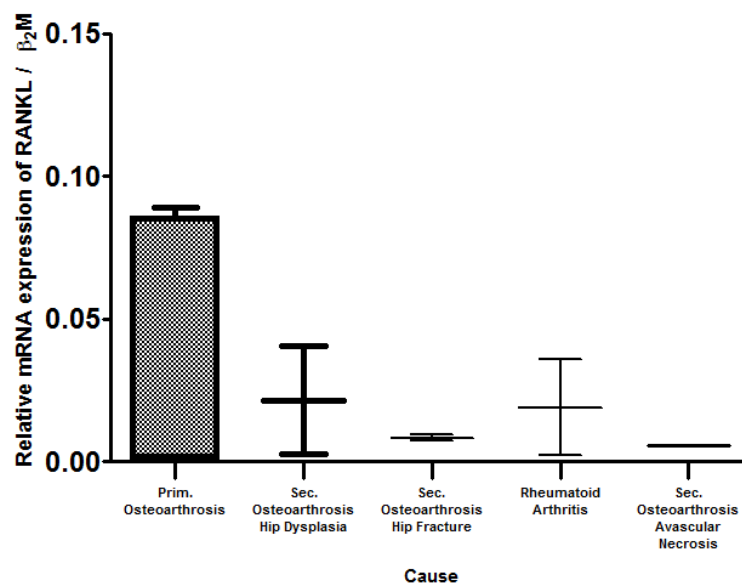


Figure 22 - RANKL expression levels in capsular tissues from the different cause groups of Primary THA patients

Primary THA - Femur

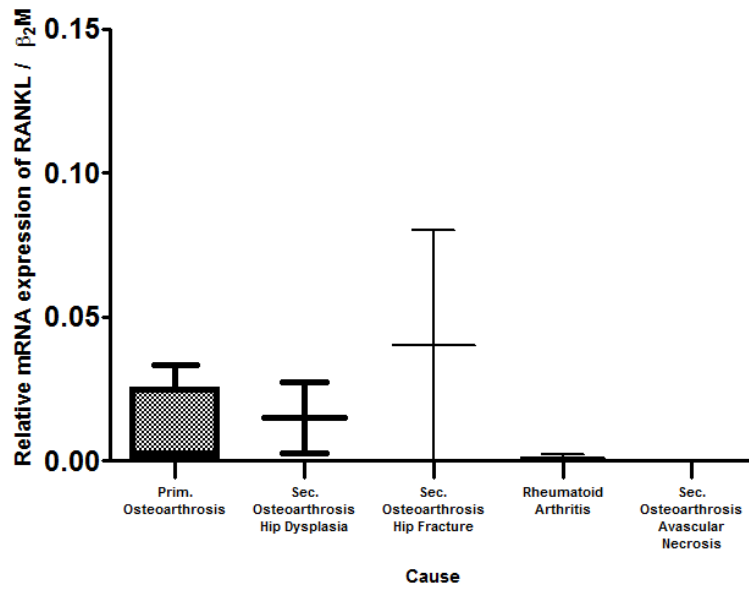


Figure 23 - RANKL expression levels in femoral tissues from the different cause groups of Primary THA patients

Primary THA - Acetabulum

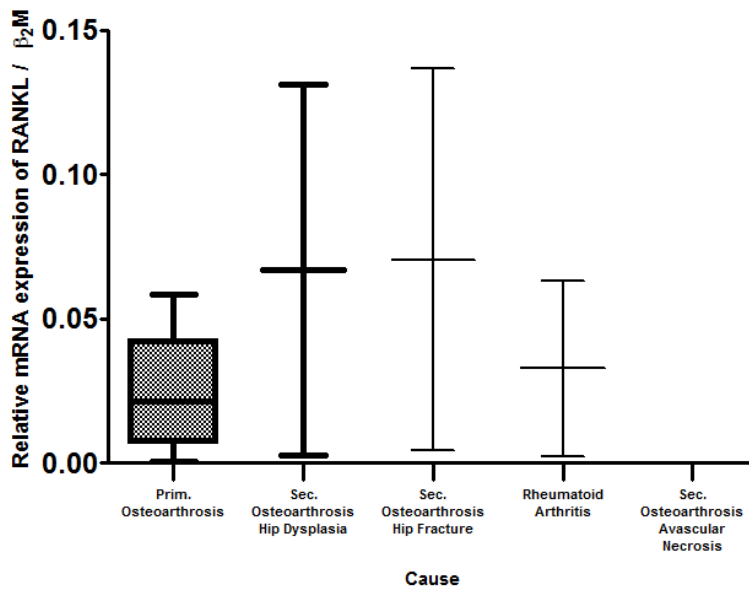


Figure 24 - RANKL expression levels in acetabular tissues from the different cause groups of Primary THA patients

The major cause for hip replacement surgery is periprosthetic osteolysis leading to aseptic loosening [21]. In the population studied in this work, the situation was similar, with 9 out of the 11 patients requiring revision surgery due to osteolysis and pain.

In Table 11, three different causes for revision hip replacement within the studied population are presented.

Results and Discussion

Table 11 - Cause for surgery of Revision THA patients grouping

Group	Cause	Patient (age, implant life)
1	Osteolysis + Pain	42 (62, 2)
		43 (77, 11)
		44 (53, 12)
		47 (86, 14)
		48 (69, 10)
		50 (71, 17)
		51 (74, 10)
		52 (73, 9)
53 (63, 0.33)		
2	Fracture	46 (101, 1)
3	Dislocation due to component misalignment	45 (76, 0.33)

In the capsular tissue from revision THA patients (Figure 25), there is a tendency for patients suffering from osteolysis and pain to express more RANKL mRNA. However, no conclusions can be drawn once only one sample is available for each of the other two pathologies considered.

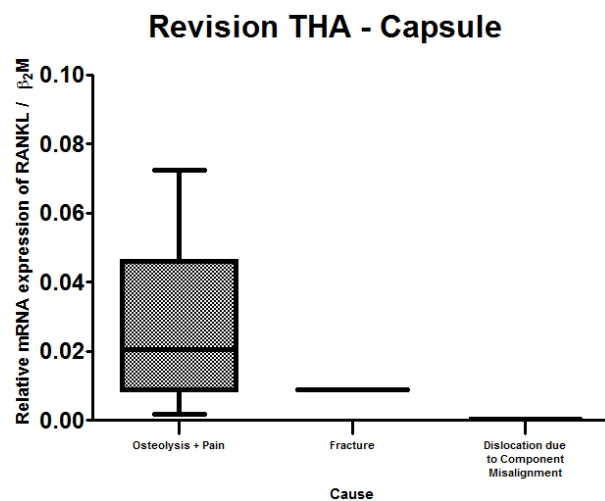


Figure 25 - RANKL expression levels in capsular tissues from the different cause groups of Revision THA patients

The low number of retrieved samples from femur and acetabulum is once again a limitation, rendering it impossible to compare RANKL mRNA expression profiles in these two periprosthetic locations.

Role of Implant Life (Revision THA patients) in RANKL expression

The influence of the implant duration before replacement surgery in RANKL mRNA levels was also studied. This variable is not considered in literature as an important factor. For example, Ito *et al.* (2004) studied macrophages and cytokines distribution in the peri-implant tissues, accounting for implant duration in the characterization of the patients, but performing no further analysis of this variable [58].

In our study, the revision THA patients were grouped in two groups: those whose implant was removed a short period after its implantation (0.33 to 2 years) and those who had an implant that was functional for a period of 9 to 17 years (Table 12).

Table 12 – Revision THA patients grouped by Implant Life

Group	Implant life (avg ± std)	Patient (age, cause)
1	0.33 - 2 (0.9 ± 0.8)	<p>42 (61, Osteolysis + Pain)</p> <p>45 (76, Dislocation due to component misalignment)</p> <p>46 (101, Fracture)</p> <p>53 (63, Osteolysis + Pain)</p>
2	9 - 17 (12 ± 3)	<p>43 (77, Osteolysis + Pain)</p> <p>44 (53, Osteolysis + Pain)</p> <p>47 (86, Osteolysis + Pain)</p> <p>48 (69, Osteolysis + Pain)</p> <p>50 (71, Osteolysis + Pain)</p> <p>51 (74, Osteolysis + Pain)</p> <p>52 (73, Osteolysis + Pain)</p>

One should notice that all the patients from the second group had to undergo revision hip surgery due to pain complaints and osteolysis. Although some early prosthesis failures were caused by osteolysis and pain as well, other diagnoses such as fractures or prosthetic component dislocation are included.

In Figure 26, the comparison of RANKL mRNA levels between these two groups is presented. There is a tendency for RANKL expression profiles to be higher in the capsular and acetabular tissue from patients with the longest implantation period. This comes as no surprise, as it is expectable that a continuous debris release from the articulating interface produces a more intense inflammatory reaction and, thus, a more severe osteolytic effect.

Results and Discussion

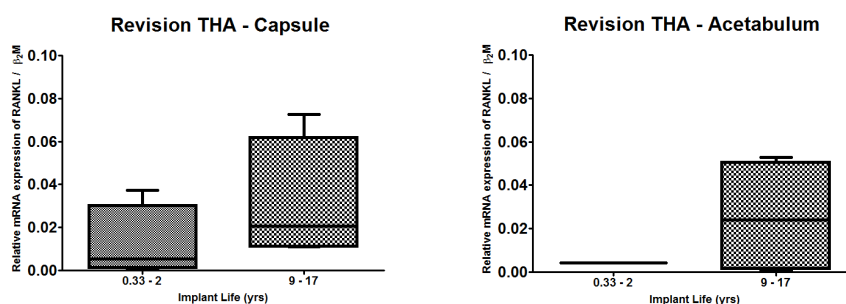


Figure 26 - RANKL expression levels in capsular (left) and acetabular (right) tissues from the different implant life groups of Revision THA patients

There are not enough tissue samples to make this comparison in femur-derived tissues.

4. Protein Extraction and Quantification

Extraction of the protein phase from biological samples is an important and very common procedure in molecular biology laboratories. As well as the study of the transcriptome (RNA), the study of the proteome (proteins) is essential to assess the expression levels of a specific gene and the respective protein production.

TRIzol[®] is designed as a one-stop reagent for the extraction of RNA, DNA, and proteins from tissues or cells [68]. There are numerous publications documenting its utility in the extraction of nucleic acids. However, the same does not happen with protein extraction, mainly due to difficulties in the resolubilization of the protein fraction. For this reason, the most common procedure is to divide the sample and treat one portion with TRIzol[®] reagent for RNA and DNA extraction and subject the second portion to a lysis buffer for recovery of the proteins. However, when dealing with small samples, a single extraction reagent is crucial in order to obtain enough material for subsequent analyses. The additional advantage is that all analyses can be performed on the same tissue sample, which facilitates direct comparisons of alterations in the proteome [69]. Hence, this was the method chosen in this study.

Optimization of the Protein Quantification assay

Before performing protein quantification in the samples obtained after RNA extraction, the process had to be optimized. As referred, it is a sensitive method, for

which only two protein solutions were chosen for this optimization (Table 13). These two protein fractions were chosen among all the respective RNA samples previously analyzed precisely because of the good quality RNA presented, meaning the original tissue sample was in good conditions. RNA concentration was considerably high and both 260/280 and 260/230 ratios were within the desirable values. The first is used to assess the purity of RNA. A ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The latter is used as a secondary measure of nucleic acid purity. Expected 260/230 values are usually in the range of 2.0-2.2 and, in the case it is considerably lower, it may indicate the presence of contaminants which absorb at 230 nm [54].

Table 13 – RNA parameters from samples chosen for protein quantification

Sample	RNA concentration (ng/μL)	A _{260/280}	A _{260/230}	mRNA expression RANKL/β ₂ M
Patient 9 Acetabulum	431,7	2,01	2,17	0,0634
Patient 10 Acetabulum	257,4	1,76	2,25	0,0211

Then, once the protein phase was obtained, its quantification was performed.

Bradford Assay

The Bradford assay is a colorimetric protein assay based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 in which under acidic conditions the red form of the dye is converted into its bluer form to bind to the protein being assayed. The binding of the protein stabilizes the blue form of the Coomassie dye. Hence, the amount of the complex present in solution is a measure for the protein concentration, and can be estimated by use of an absorbance reading [70].

The BSA standards were prepared as previously described, and the standard curve used to determine sample protein concentration is plotted in Figure 27.

Results and Discussion

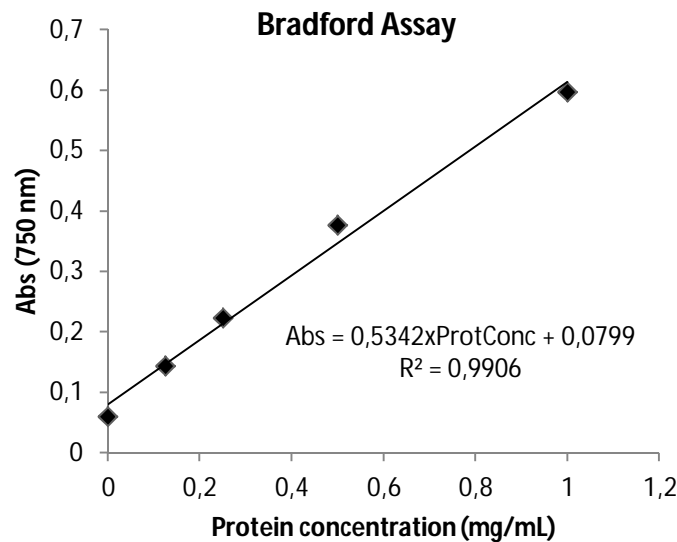


Figure 27 – Standard curve of the Bradford Assay for protein quantification

Once the standard curve was plotted, the protein concentration in the two samples was determined (Table 14).

Table 14 – Protein concentration in the two samples

Sample	Abs (750 nm)	Concentration (mg/mL)
Patient 9 Acetabulum	0,986	1,696
Patient 10 Acetabulum	0,895	1,526

Although the absorbance values measured are outside the range of the standard curve, the kit manufacturer guarantees the assay linearity between 0-2 mg/mL. Therefore, there is evidence that the protein extraction protocol is effective.

SDS-PAGE

One should also consider the fact that this process does not address the issue of possible contaminated protein solutions. It is possible that, during this whole process, the protein phase separation did not occur as desired, for which further analysis is necessary. Thus, the two protein solutions were analyzed in a SDS-PAGE as presented in Figure 28.

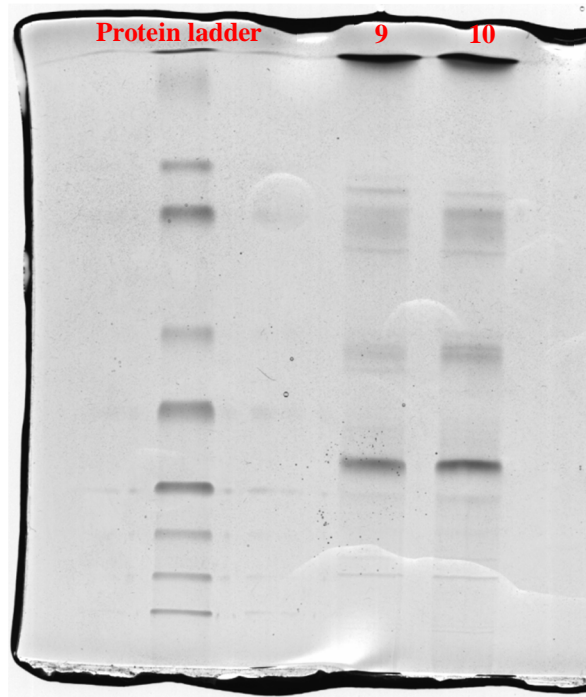


Figure 28 – Acrylamide gel. Protein samples from acetabular tissue of Patients 9 and 10.

The polyacrylamide gel presented well defined bands, which suggest that protein extraction was performed properly, maintaining protein integrity and purity. This assay was performed with the intent of optimizing the protein extraction and SDS-PAGE techniques, so that they can be used in the future to study protein content in the same hip joint tissues from where mRNA is extracted. Once this process is optimized, it will be possible to perform a full study of the transcriptome and proteome from one single tissue sample.

V. Conclusions and Future Perspectives

Considering the technical character of this Dissertation, the optimization of the several molecular biology techniques used was performed with a good outcome. RNA extraction from the human tissue samples using the TRIzol[®] method was optimized to a level that allowed the user to be confident about the quality of the obtained RNA. NanoDrop (Thermo Scientific) and Experion[™] Automated Electrophoresis System (Bio-Rad) proved to be helpful tools in the determination of RNA quality and integrity. The use of techniques such as Reverse Transcription Polymerase Chain Reaction and Quantitative Real Time Polymerase Chain Reaction, as well as other simple molecular biology techniques, namely RNA gel electrophoresis, provided the results obtained for RANKL mRNA expression levels. Concerning protein quantification in the same tissue samples, an extraction protocol was also optimized following TRIzol[®] phase separation. Preliminary results of protein quantification (using the Bradford Protein Quantification Assay) and SDS-PAGE suggested the protein in the obtained extracts is not degraded nor contaminated.

The methodology used, addressing the comparison of mRNA and protein expression of RANKL in three different locations of the acetabulofemoral articulation (capsule, femur and acetabulum) of both primary and revision THA patients, taking into account factors such as age, gender, cause for surgery and implant duration was performed for the first time.

The preliminary results obtained provided an initial insight into the clinical setting of hip joint inflammation after Total Hip Arthroplasty. The results suggested a tendency for primary THA patients to express less RANKL mRNA in femoral tissue and more in acetabulum relatively to revision THA patients. Gender appears to influence RANKL mRNA expression, once male primary THA patients tend to express higher values than female THA patients. Considering age as a differentiating factor, there is a tendency for older (69-86 years old) revision THA patients to express more RANKL mRNA in periprosthetic tissues in comparison to the younger group (53-63 years old). The revision THA patients diagnosed with osteolysis and pain also demonstrated a tendency to have higher RANKL mRNA than those who had revision surgery for other reasons. Finally, a tendency for revision THA patients with longer implant life (9-17 years) to express more

Conclusions and Future Perspectives

RANKL mRNA was detected, in comparison with the patients with shorter implant duration (0.33-2 years).

In summary, a method to study RANKL expression levels in periprosthetic tissues has been successfully optimized. Some tendencies for RANKL mRNA levels to vary were detected. However, no significant conclusions could be drawn because of the insufficient number of patients included in the study. Therefore, tissue samples will continue to be collected during THA surgeries in order to increase this number and to allow the obtainment of statistically significant results.

As to further work, protein content extraction will be performed as well, so that transcriptional data is complemented by protein expression profiles. Furthermore, systemic RANKL levels will also be analyzed using ELISA, once plasma samples were collected during surgery from all patients included in the study.

With the continuous gathering of more information about RANKL levels, it should also be able to study other variables that may affect RANKL production in periprosthetic tissues, such as the type of failed prosthesis in the case of revision surgery patients.

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VII. Appendix

1. RNA Parameters

Table 15 –RNA samples extracted from capsular tissue of Primary THA patients

Patient	Concentration (ng/ μ L)	A _{260/280}	A _{260/230}
1	305,7	1,97	2,03
2	319,9	1,88	2,04
3	399,1	2,03	2,01
5	86,1	2,07	1,98
6	133,6	2,02	2,09
8	268,6	1,97	2,15
9	625,9	1,93	1,99
10	46,4	1,99	1,93
11	175,1	1,98	2,12
12	223,9	1,99	1,97
13	48,4	1,96	2,03
14	103,3	1,82	2,16
15	430,7	2,10	2,01

Table 16 - RNA samples extracted from femoral tissue of Primary THA patients

Patient	Concentration (ng/ μ L)	A _{260/280}	A _{260/230}
3	270,5	2,07	1,87
4	646,6	1,63	1,91
5	317,8	1,87	1,88
6	314,2	1,99	2,03
7	598,4	1,98	2,10
8	152,8	1,87	1,99
9	543,1	1,83	2,03
10	969,2	1,96	2,04
11	964,1	2,09	2,03
12	238,2	1,95	2,28
13	218,4	1,77	2,09
14	1827,7	2,10	1,99
15	162,0	1,82	2,00

Table 17 - RNA samples extracted from acetabular tissue of Primary THA patients

Patient	Concentration (ng/ μ L)	A _{260/280}	A _{260/230}
3	128,6	2,09	1,83
4	882,9	1,88	2,07
5	285,6	1,98	2,22
6	74,6	1,93	2,18
8	327,3	1,93	2,06

Appendix

9	431,7	2,01	2,17
10	257,4	1,76	2,25
11	51,5	1,64	2,18
12	124,1	2,06	1,99
13	243,3	1,84	2,23
15	170,2	1,95	2,07

Table 18 - RNA samples extracted from capsular tissue of Revision THA patients

Patient	Concentration (ng/μL)	A_{260/280}	A_{260/230}
42	765,7	1,78	1,98
44	1198,0	1,87	2,17
45	390,9	1,84	1,88
46	387,9	2,03	2,03
47	192,3	1,86	2,12
48	279,4	1,97	2,13
52	264,3	2,04	1,90
53	1086,2	2,10	2,25

Table 19 - RNA samples extracted from femoral tissue of Revision THA patients

Patient	Concentration (ng/μL)	A_{260/280}	A_{260/230}
47	637,3	1,89	1,98
50	103,6	1,82	2,07
51	979,1	1,99	2,13

Table 20 - RNA samples extracted from acetabular tissue of Revision THA patients

Patient	Concentration (ng/μL)	A_{260/280}	A_{260/230}
47	152,4	2,01	1,99
50	1096,2	1,78	2,02
51	136,2	1,87	2,00
52	381,1	1,93	1,98
53	3843,5	2,04	2,05

2. qRT-PCR

Table 21 – qRT-PCR results for RANKL mRNA in capsular tissue of Primary THA patients

Patient	Relative mRNA expression of RANKL / b2M
1	0,00225
2	0,00281
3	0,00948
5	0,00193
6	0,00017
8	0,00573
9	0,03589
10	0,08900
11	0,00424
12	0,00050
13	0,04052
14	0,08537
15	0,00731

Table 22 - qRT-PCR results for RANKL mRNA in femoral tissue of Primary THA patients

Patient	Relative mRNA expression of RANKL / b2M
3	0,00005
4	0,00414
5	0,03201
6	0,03349
7	0,00080
8	0,00015
9	0,00026
10	0,00070
11	0,00016
12	0,00156
13	0,02729
14	0,00376
15	0,08021

Table 23 - qRT-PCR results for RANKL mRNA in acetabular tissue of Primary THA patients

Patient	Relative mRNA expression of RANKL / b2M
3	0,00441
4	0,02936
5	0,04239
6	0,00728
8	0,02141
9	0,06337
10	0,02112
11	0,00083
12	0,05831
13	0,13121
15	0,13678

Appendix

Table 24 - qRT-PCR results for RANKL mRNA in capsular tissue of Revision THA patients

Patient	Relative mRNA expression of RANKL / b2M
42	0,03729
44	0,01108
45	0,00069
46	0,00882
47	0,07254
48	0,03029
52	0,01108
53	0,00195

Table 25 - qRT-PCR results for RANKL mRNA in femoral tissue of Revision THA patients

Patient	Relative mRNA expression of RANKL / b2M
47	0,02966
50	0,16210
51	0,00071

Table 26 - qRT-PCR results for RANKL mRNA in acetabular tissue of Revision THA patients

Patient	Concentration (ng/ μ L)
47	0,00369
50	0,00056
51	0,05292
52	0,04404
53	0,00407