Genetic Polymorphisms of *RANK*, *RANKL* and Their Relation to Osteoporosis

(Polimorfismos genéticos de *RANK* y *RANKL* y su relación con la osteoporosis)

Guy Yoskovitz

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y su relación con la osteoporosis)

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THIS WORK IS DEDICATED TO MY FAMILY

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Abbreviations

AARE- Amino Acid Response Element
AKAP11- A-Kinase Anchor Protein 11
BMD- Bone Mineral Density
BMI- Body Mass Index
BMP- Bone Morphogenetic Protein
bp- Base Pair
BP- Basal Promoter
BSA- Bovine Serum Albumin
CI- Confidence Interval
COLIA1- Collagen type Iα1
DMEM- Dulbecco's Modified Eagle Medium
DR- Distal Region
EMSA- Electrophoresis Mobility Shift Assays
ERα- Estrogen Receptor α
FBS- Fetal Bovine Serum
FN- Femoral Neck
FRAX- Fracture Risk Assessment tool
GR- Glucocorticoid
GRE- Glucocorticoid Response Element
GWA- Genome Wide Association
HSC- Hematopoietic Stem Cells
HWE- HardyWeinberg equilibrium
IL-1- Interleukin 1
kb- Kilo Base
kDa- Kilo Dalton
LD- Linkage Disequilibrium
LPS- Lipopolysaccharide
LRP5- Lipoprotein Receptor Related Protein 5
LS-Lumbar Spine
MAF- Minor Allele Frequency
M-CSF- Macrophage Colony Stimulating Factor
miRNA- MicroRNA
MPA- Medroxyprogesterone Acetate
mRNA- Messenger RNA
MSC- Mensenchymal Stem Cells
NCBI- National Center for Biotechnology Information

ODF- Osteoclast Differentiation Factor
OPG- Osteoprotegerin
OPGL- OPG ligand
OR- Odds Ratio
Osx- Osterix
PAX- Paired Box
PTH- Parathyroid Hormone
RANK- Receptor Activator of NF-kappa-B
RANKL- Receptor Activator of NF-kappa-B Ligand
RUNX2- Runt-related transcription factor 2
SD-Standard Deviation
SNP- Single Nuclotide Polymorphism
Sp1- Specifity Protein 1
TGF β- Transforming Growth Factor β
TNF- Tumor Necrosis Factor
TNFRSF11A- Tumor Necrosis Factor Receptor SuperFamily member 11A
TNFSF11- Tumor Necrosis Factor ligand SuperFamily member 11
TNFα - Tumor Necrosis Factor α
TRANCE- TNF-Related Activation induced cytokine
UK-United Kingdom
US- United States of America
UTR- UnTranslated Region
UV- Ultraviolet
VDR- Vitamin D Receptor
VDRE- Vitamin D Response Element
WHO-World Health Organization

INTRODUCTION

1. Introduction

1.1 Osteoporosis, BMD and Osteoporotic Fractures

1.1.1 Osteoporosis

Osteoporosis (from Greek *porous bones*) is a systemic skeletal disorder and the most common metabolic bone disease. It is recognized as one of the most prevalent problems facing postmenopausal women in western society (1,2). An estimated 10 million Americans older than 50 years have osteoporosis, another 34 million Americans are at risk (3) and in Europe about 25% of the female population over 50 years old may have the disease (4). Osteoporosis is the final result of malfunctioning bone homeostasis, also known as bone remodelling, and can differ from one patient to another in terms of severity, pains, fractures, and other physical consequences.

The World Health Organization (WHO) definition of osteoporosis uses bone mineral density (BMD) measurements as the gold standard (5): the disease is defined as from 2.5 standard deviations below the average BMD of healthy, 20 years old women.

Osteoporosis is characterized by reduced bone strength and is therefore responsible for millions of fractures annually. Any bone can be affected (6). In addition, the microarchitecture in bone with low BMD is, in most cases, damaged (Figure 1).

Low-trauma fractures, the immediate consequence of osteoporosis, are a growing cause for hospitalization, morbidity, and mortality among the elderly, resulting in enormous medical costs annually (7). The majority of the fractures occur in the lumbar vertebrae, hip, and wrist/forearm. Under special attention are fractures of the hip and spine, which can lead to hospitalization and major surgery. As a result, the ability to maintain an independent lifestyle of walking and other everyday activities can be impaired. In some cases, the disease and its consequences may even result in death.

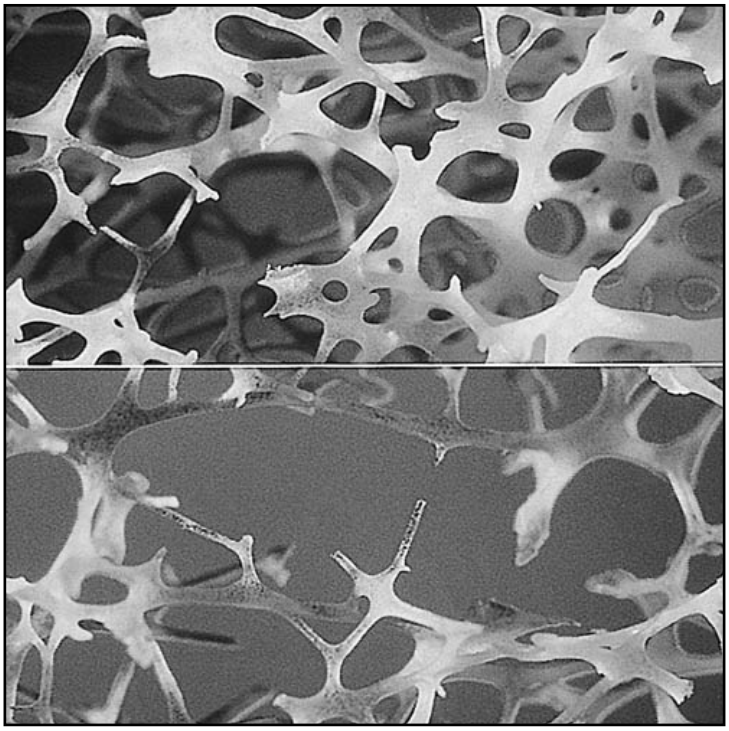

Figure 1. Microarchitecture of the bone. A normal bone appears in the upper part, while an osteoporotic bone with low BMD and damaged microarchitecture appears in the lower part (Extracted from http://www.noslynn.org.uk/img/bone-large-1.jpg).

1.1.2 Osteoporosis Classification

The WHO criteria for osteoporosis define the disease but not its pathogenesis. Osteoporosis pathogenesis can be classified as primary type 1, primary type 2, and secondary. The most common osteoporosis is primary type 1, which occurs in postmenopausal woman; our present research focuses on this group. Primary type 2, known as senile osteoporosis, occurs after the age of 75 years, in both men and women but at a ratio of 1:2. Secondary osteoporosis occurs in all age ranges and is the result of other factors which affect BMD, such as alcohol intake, tobacco consumption, medical conditions, treatments, unbalanced diet, etc (8,9).

1.1.3 Bone Mineral Density

Bone is a crucial organ of the vertebrate endoskeleton and plays a key role in the hematopoietic system, producing both erythrocytes and leukocytes in the bone marrow. Furthermore, the bone serves as the ‘minerals bank’ of the living body.

Bone (osseous) tissue, the major connective tissue in the body, is physiologically mineralized and constantly regenerated throughout life as a consequence of the bone turnover process, which will be discussed in chapter 1.2.

Usually measured in the hip (femoral neck (FN)) and lumbar spine (LS), BMD slowly but continuously decreases after the third decade of life. The results are expressed as the minerals weight in grams per cm^2 for the tested bone, as in Figure 2. BMD is the only non-invasive diagnostic tool for osteoporosis.

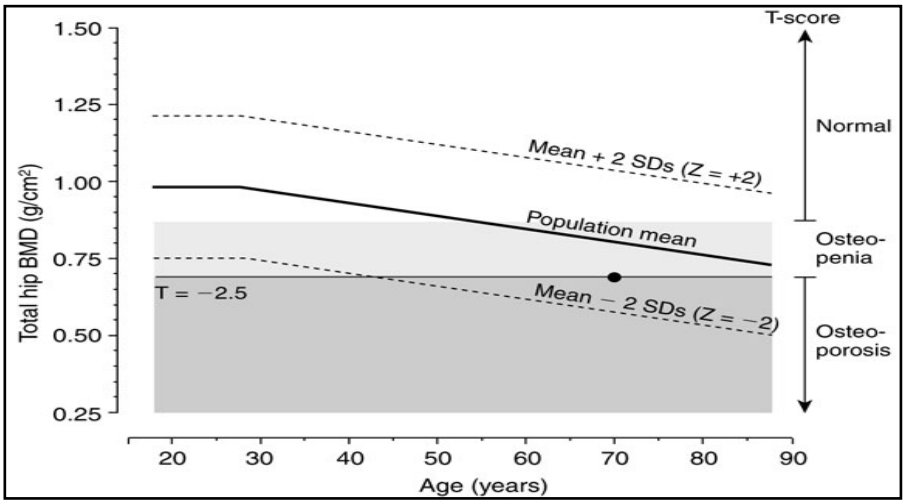

Figure 2. The Australian population's BMD mean as a function of age and T score thresholds for osteopenia and osteoporosis in total hip BMD as defined by the WHO. Extracted from Sambrook, 2002 (10)

The BMD in the human body can be affected by many factors, including age (11,12), nutrition (13), hormone and sex steroid status (12), vitamins (13) and genetics (14-16). In addition to osteoporosis, BMD can provide indications of other bone diseases, such as high bone mass syndrome, and might help in bone quality evaluation in other pathologic conditions.

1.1.4 Fractures

Osteoporotic fractures are the immediate consequence of osteoporosis and the major clinical outcome of the disease. The main motive for osteoporosis treatment is the attempt to avoid the occurrence of fractures by raising BMD, assuming it will improve bone resistance. Nevertheless, low-trauma fractures are a primary medical problem, causing massive medical costs annually (7,17). Table 1 demonstrates the magnitude of the problem in the United Kingdom. In 2005 in the United States alone, approximately 2 million osteoporotic fractures were observed with an estimated cost of $17 billion, and these numbers are expected to increase by 50% by 2025 (18) . Yet, one should take into consideration that the definition of these low-trauma fractures as "osteoporotic fractures" may be misleading. Many of the patients with low-trauma fractures have BMD levels above the WHO osteoporosis criteria (19,20).

Table 1. Impact of osteoporosis related fractures in the UK.

	Hip	*Spine*	*Wrist*
Lifetime risk (%)			
Women	14	28	13
Men	3	6	2
Cases per year	70,000	120,000	50,000
Hospitalization (%)	100	2–10	5
Relative survival	0.83	0.82	1.00

Extracted from Harvey, 2009 (17)

1.1.4.1 Type of Low-Trauma Fracture

Low-trauma fractures occur in response to low-impact mechanical force, as for example falling from a standing height (normally related to hip fractures), or as spontaneous fractures under no special condition other than everyday loading (usually related to vertebral fractures). Significant differences in fractures frequency and occurrence were found between different communities from different countries, even in those of the same origin (17,21-23). Thus, environmental and genetic factors which contribute to bone quality or bone microarchitecture might explain these differences.

Spine (vertebral) fractures represent the higher prevalence of low-trauma fractures, even though many incidents are asymptomatic and as a result are not well diagnosed (24). The prevalence of this fracture among Spanish women is estimated at 21.4% (age range 50-87) but drops to 9.7% for moderate and severe cases (25).

Hip fractures, although not the most frequent, almost always lead to hospitalization. Furthermore, the relative survival rate drops after the event (Table 1) and the frequency of disability arising as a result of this fracture is high. The prevalence in Spain's general population is suggested to be 6.94±0.44 per 1,000 inhabitants/year (26). However, after adjusting for sex, the rate among women climbs to 9.13±0.66 per 1,000 inhabitants/year. Differences between the Spanish autonomous communities are also noteworthy. Catalonia has the highest rate (1,120 hip fractures per 100,000 inhabitants/year), while La Rioja reports only one third as many cases (377 hip fractures per 100,000 inhabitants/year) (26).

Forearm/Wrist fractures have a different prevalence pattern than vertebral or hip fractures, with a better survival rate (Table 1). While both vertebral and hip fracture risk rise with age, forearm/wrist fractures incidences increase only between ages 45 to 60 years (17). After 60, the fracture prevalence is more or less stable. At the age of 50, British women have a 16.6% lifetime risk of facing forearm/wrist fracture. This risk decreases to 10.4% at 70 years (27).

1.1.4.2 Fracture Predictors

There is conclusive data of the relationship between BMD and low-trauma fractures, with 1.5 to 3 fold increased odds ratio (OR) for each standard deviation (SD) decrease in BMD (Table 2) (28). Yet up to half of the osteoporotic fractures occur in non-osteoporotic patients in terms of BMD criteria (19,20) . It is not surprising that various studies (29-31) have proposed several predictors for fracture rather than only BMD, including the WHO FRAX (fracture risk assessment tool) algorithm (32-34), in order to improve the identification of subjects at high risk of fracture in clinical settings.

Table 2. Fracture OR as a function of decrease in BMD
(for every 1 SD, age adjusted)

Site of measurement	*Forearm fracture*	*Hip fracture*	*Vertebral fracture*	*All fractures*
Distal radius	1.7 (1.4–2.0)	1.8 (1.4–2.2)	1.7 (1.4–2.1)	1.4 (1.3–1.6)
Femoral neck	1.4 (1.4–1.6)	2.6 (2.0–3.5)	1.8 (1.1–2.7)	1.6 (1.4–1.8)
Lumbar spine	1.5 (1.3–1.8)	1.6 (1.2–2.2)	2.3 (1.9–2.8)	1.5 (1.4–1.7)

Extracted from Kanis, 2009 (28)

The detection of several predictors independent of BMD indicates that several factors, probably related to bone microarchitecture and age-related conditions, play an important role in defining the resistance of bone to trauma. In addition to age and sex hormones, those factors include, for example, low body mass index (BMI) (28), obesity (35), previous low-trauma fractures (36), and parental history (37).

1.2 Bone Turnover and Bone Metabolism

1.2.1 Bone Turnover

Bone turnover, also called bone remodelling, is a lifelong process that refers to the entire cycle of bone resorption and formation, which determines BMD. In general, the cell biology of an adult bone includes 3 cell types, among others, that have opposite functions: osteoblasts, which produce the extracellular matrix that becomes mineralized; osteoclasts, responsible for the resorptive actions; and osteocytes, involved in the regulation of both resorption and formation (and even claimed to dominate the process). A complex signal system between these 3 cell types balances their activities to avoid any over-creation or loss of bone tissue (38). This equilibrium is known as bone turnover, and is dominated by a complex set of protein reactions between Receptor Activator of NF-kappa-B (RANK), Receptor Activator of NF-kappa-B Ligand (RANKL), and Osteoprotegerin (OPG) known as the RANK/RANKL/OPG system. Alteration of this equilibrium leads to pathologic situations, including osteoporosis.

1.2.2 Osteoblasts and Bone Formation

The main function of the osteoblast cells is bone formation. Osteoblasts derive from mesenchymal stem cells (MSC) (also known as bone marrow stromal cells), which in the presence of bone morphogenetic proteins (BMPs), BMP2 and BMP7, proliferate to osteo-chrondrogenic precursor (39). In addition, as have been demonstrated in mice, BMP2 induces transcription factors and regulates Runt-related transcription factor 2 (*Runx2*) expression (40). RUNX2 is the earliest marker of osteogenesis (Figure 3). It is necessary yet not sufficient for the maturation of the osteo-chrondrogenic precursor to pre-osteoblast (41). Among other functions, RUNX2 regulates the expression of osteogenesis essential transcription factor Osterix (*Osx* gene) by binding to the *Osx* promoter through its RUNX2 binding sequence (39).

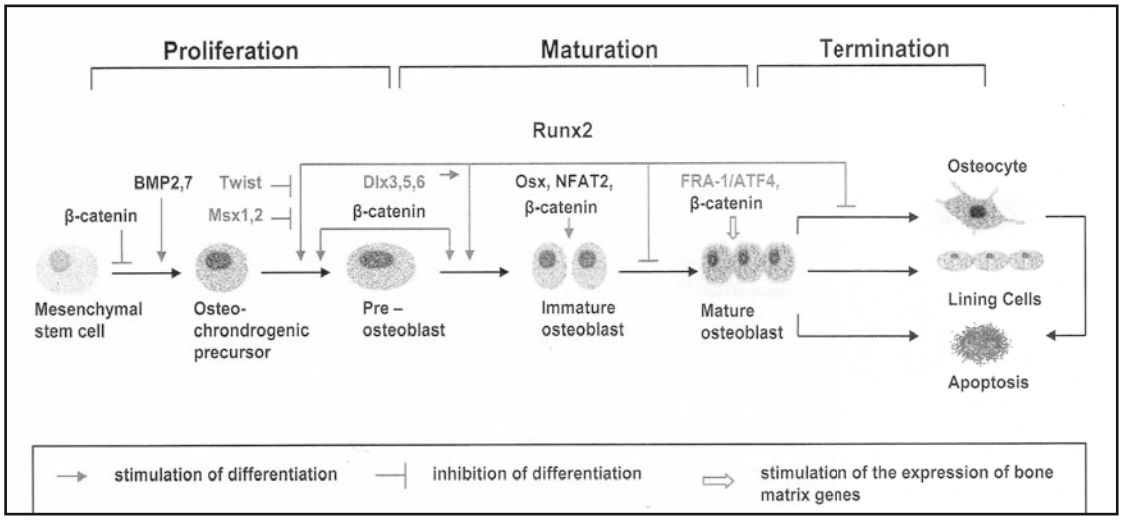

Figure 3. Mesenchymal stem cell differentiation toward the osteoblastic lineage. In each phase the major transcriptional regulators are mentioned above the cells. Extracted from Krause, 2009 (39)

Another important mechanism in osteoblasts differentiation and maturation is the glycoproteins-based Wnt signaling pathway (42,43), which is crucial for the development of many tissues, including bone. In osteoblasts differentiation, the activation of the canonical Wnt/β-catenin pathway leads to stable β-catenin, which allows the regulation of multiple transcription factors (44). This pathway is active in the entire osteoblasts lineage and it stimulates osteoblasts proliferation and survival (45).

Mature and active osteoblast is a connective-tissue matrix secretion cell. As such, it has a large nucleus and Golgi. Once active, it secretes osteoid, the organic component of bone, mainly made of type I collagen and minerals which crystallize around the collagen scuffle. While forming a new bone, many osteoblasts become embedded within the matrix and later on differentiate either to osteocyte or lining cells, which are inactive osteoblasts that cover the non-active bone surface (46) (Figure 4).

Special attention should be paid to the osteoblasts' key role in bone homeostasis by expressing and secreting soluble RANKL and OPG (38). The crucial importance of the RANK/RANKL/OPG system to the bone homeostasis will be discussed in section on 1.2.5.

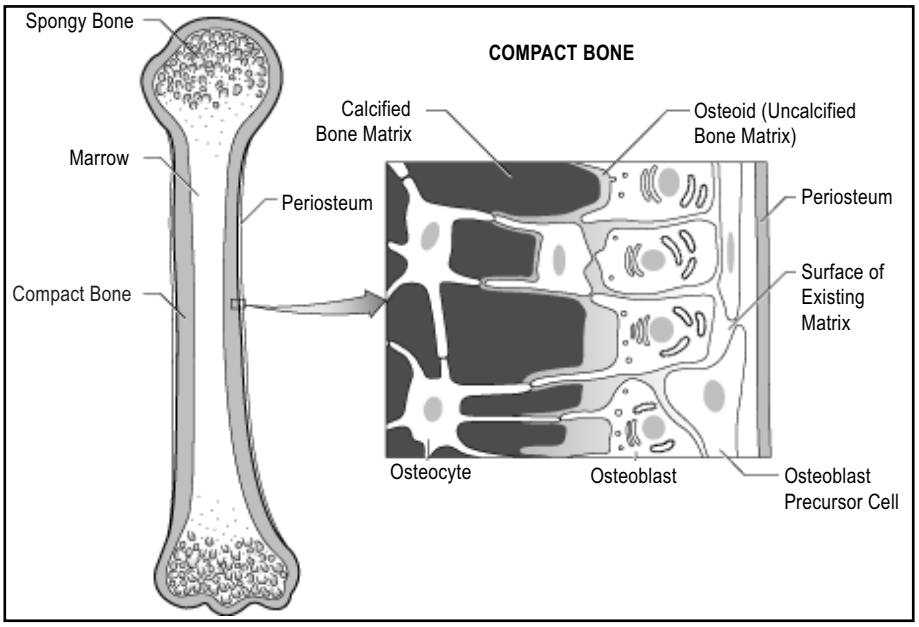

Figure 4. A schematic figure of the osteoblasts embedded in the bone matrix, their calcification and differentiation to octeocytes. Extracted from Souza, 2000. (47)

1.2.3 Osteoclasts and Bone Resorption

Osteoclasts are the sole established bone resorptive cell type. It is a multinuclear massive cell, member of the macrophage/dendritic cells family (48). Any pathological bone loss represents imbalance between excessive bone resorption by osteoclasts with respect to bone formation and calcification by osteoblasts (49). In the 1970s the general assumption was that osteoclasts and osteoblasts derived from a common precursor (50).

Yet, experiments done by Walker et al. (51,52) demonstrated that infusion of wild-type spleen cells cured osteopetrosis in mice, suggesting that the osteoclast precursor is of hematopoietic origin. Osteopetrosis is the result of pathological bone loss which reflects either resorptive dysfunction or an inability to recruit osteoclasts (50).

Full recovery as a result of bone marrow transplantation in osteopetrosis infants (53) supplied further evidence that the principal osteoclasts precursor derived from the hematopoietic stem cells (HSC) lineage (54).

Osteoclasts formation, differentiation, and survival are controlled by factors expressed by osteoblast and marrow stromal cells (55). The stromal and osteoblastic cells secrete both pro-osteoclastogenic proteins such as macrophage colony stimulating factor (M-CSF) and RANKL and the anti-osteoclastogenic protein OPG. M-CSF binds to its receptor, c-Fms, on the surface of the committed HSC and is necessary for

proliferation and maturation of pre-osteoclasts (55,56). RANKL interacts with its receptor, RANK, on the surface of the osteoclast precursor to stimulate osteoclastogenesis (Figure 5).

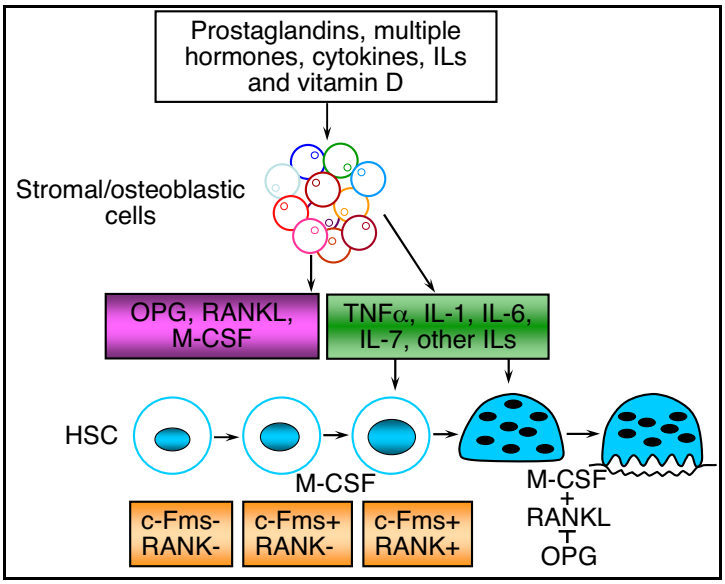

Figure 5. Osteoclast differentiation. Strormal and osteoblastic cells secrete RANK, M-CSF and OPG as well as other factors. RANK and M-CSF bind to their receptors on the committed HSC and in the presence of other transcription regulators the cell reaches maturation and full functionality. Modified from Ross, 2009 (57)

In order to carry out bone resorption, osteoclasts must be polarized and undergo reorganization of the cytoskeleton and formation of a ruffled border (58). The mature osteoclast attaches to the bone matrix by intergrins (core cell-matrix attachment molecules) (59). The heterodimer $\alpha_v\beta_3$ intergrin is the principal mediator between the osteoclasts and the bone matrix (60).

This mechanism creates a sealed microenvironment around the osteoclast. Protons are released to this microenvironment from the ruffled side of the osetoclast via a vascuolar-like H^+ATPase proton pump (50). The type I collagen is degraded by Cathepsin K (Figure 6). The products of this process, collagen fragments and soluble calcium and phosphate, are processed in the osteoclast and then released to the circulation (38). Overall, more than 24 factors (genes and loci) were identified to be involved in regulating osteoclastogenesis and osteoclast function and survival (38).

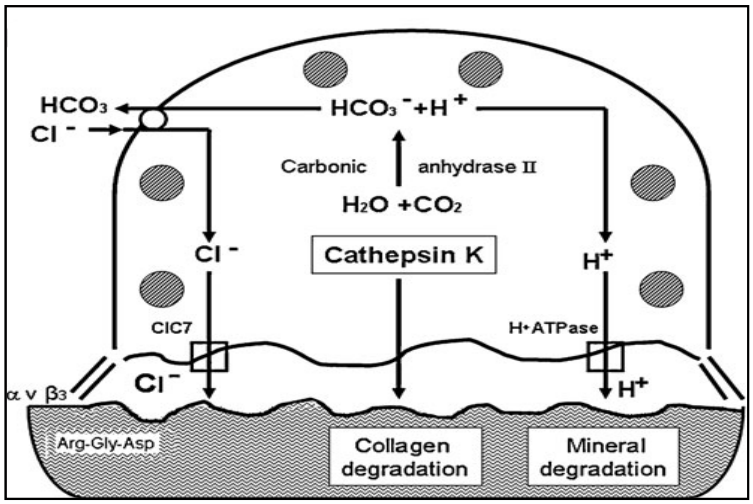

Figure 6. Osteoclast resorption activity. Collagen degradation by Cathepsin K in the acidic environment induced by the protons H^+ and Cl^- released to the resorption area. On the left and right bottom, we can see the sealing of the resorpted area by the intergrins. Extracted from Athanasou, 2011 (58)

1.2.4 Osteocytes

Osteocytes are estimated to constitute 90 to 95% of all bone cells (while osteoblasts form 4-6%, and osteoclasts 1-2%) (61). As mentioned in section 1.2.2, osteocytes derived from the differentiation of mature osteoblasts embedded within the matrix (45). The osteocytes are connected to each other, and communicate through gap junctions (61) (Figure 7).

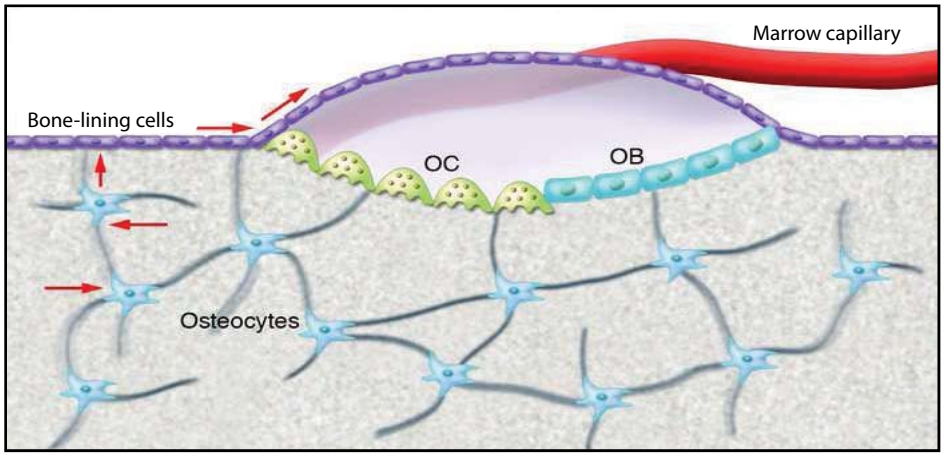

Figure 7. Schematic figure of cells in the bone tissue. The osteocytes create a network connected to all other cell types: osteoclasts (OC), osteoblasts (OB) and bone lining cells. The cells of the network are connected with gap junctions. The red arrows suggest a pathway for osteocyte signaling from within the bone to its surface. Extracted from Kholsa, 2008 (41)

It was long believed that osteocytes are passive, relatively inert cells (62). In 2007, Tatsumi et al. demonstrated that osteocyte signaling is affected by loading,

preventing bone loss under normal loading on the skeleton and contributing to active bone loss in response to unloading (63), suggesting that osteocytes are mechanosensors in the bone. Later studies provided evidence that osteocytes can communicate with bone-lining cells in a way that triggers them to induce either bone resorption or bone formation (64). In addition, there is evidence of the role of osteocytes in Wnt/β-catenin canonical signaling (65). *In-vitro* experiments had shown that osteocytes secrete a much higher amount of RANKL molecules than are being expressed and secreted by osteoblasts (66). An animal model supported this finding and emphasized *in-vivo* that osteocytes are the major source of RANKL (66). The major outcome of the mentioned studies is that osteocytes can regulate, and even be considered the orchestrators (61) of the different aspects of bone remodelling (Figure 7).

It is highly important to underline the role of osteocytes in detecting microfractures. Microfractures (or microcracks) are a direct consequence of loading, and lead to reduced bone strength (67). The osteocytes in less than 1-2 mm from the microfracture site (68), express apoptotic molecules. The process, resulting in cell apoptosis, releases apoptotic bodies expressing RANKL to support osteoclastogenesis and to recruit osteoclasts to the site for tissue repair (69) (Figure 8).

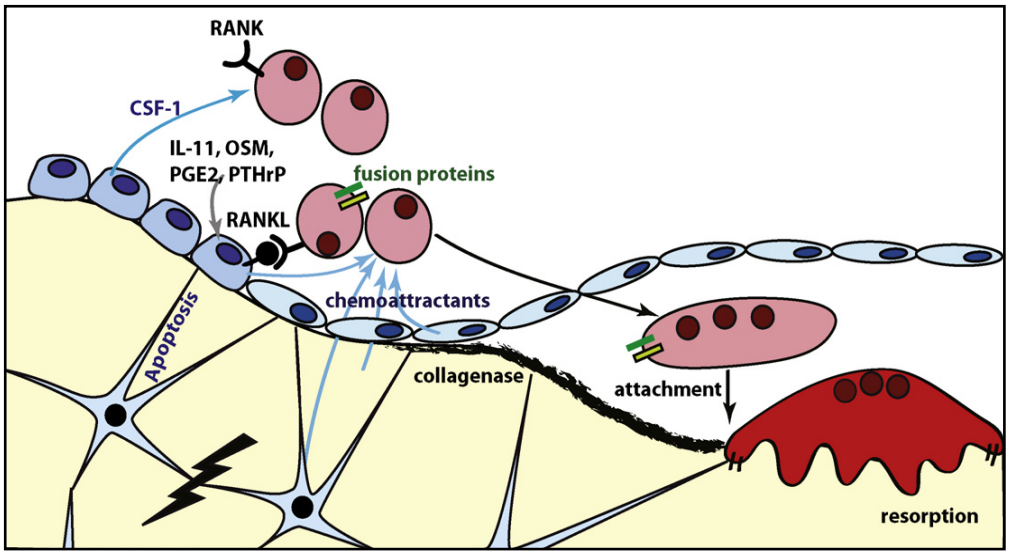

Figure 8. Osteoclast recruitment in the event of a microfracture (marked with the black lightening). In the event of a microcrack, the octeocyte will undergo an apoptotic process which will release, among other factors, RANKL molecules. These molecules will start a signaling pathway to promote osteoclastsogenesis as well as to recruit mature and active osteoclasts to the region. The bone will be resorbed until the minicrack region will be fully degraded. This process will be followed by bone formation. Extracted from Sims, 2008 (70)

1.2.5 RANK/RANKL/OPG system

The delicate balance between bone formation and resorption dictates BMD and bone structure. Any alteration of this equilibrium might end in a pathological situation and decreased bone functionality, which, as mentioned, is crucial to the living body in many regards. One of the most important mechanisms to maintain this equilibrium is the RANK/RANKL/OPG system. This system was discovered in the mid-1990s (71) and as a first and immediate outcome contributed to the understanding of osteoclasts formation, activation and survival. Moreover, it revealed some new aspects of bone homeostasis and communication within the bone between the three cell types of interest.

Osteoblasts express and secrete RANKL which binds to its receptor, RANK, on the surface of osteoclasts and their precursors. This triggers the differentiation of precursors into multinucleated osteoclasts and also supports osteoclast activation and survival, both in healthy bone and in pathologic conditions associated with increased bone resorption.

OPG is secreted by osteoblasts and osteogenic stromal stem cells to protect the skeleton from excessive bone resorption by binding to RANKL and preventing its interaction with RANK (Figure 9). The RANKL/OPG ratio in bone tissue is thus an important determinant of bone mass in normal and disease states (72).

This signal pathway is affected by different hormones, cytokines and growth factors which affect the cell activity and bone metabolism (73,74) (Table 3).

In addition, it has been demonstrated in animal models that the RANK/RANKL/OPG system plays a role in other systems such as the vascular system and the immune system (75-77).

Due to its crucial role, the RANK/RANKL/OPG system is the target of many therapeutic drugs and treatments intended at regulating the bone turnover.

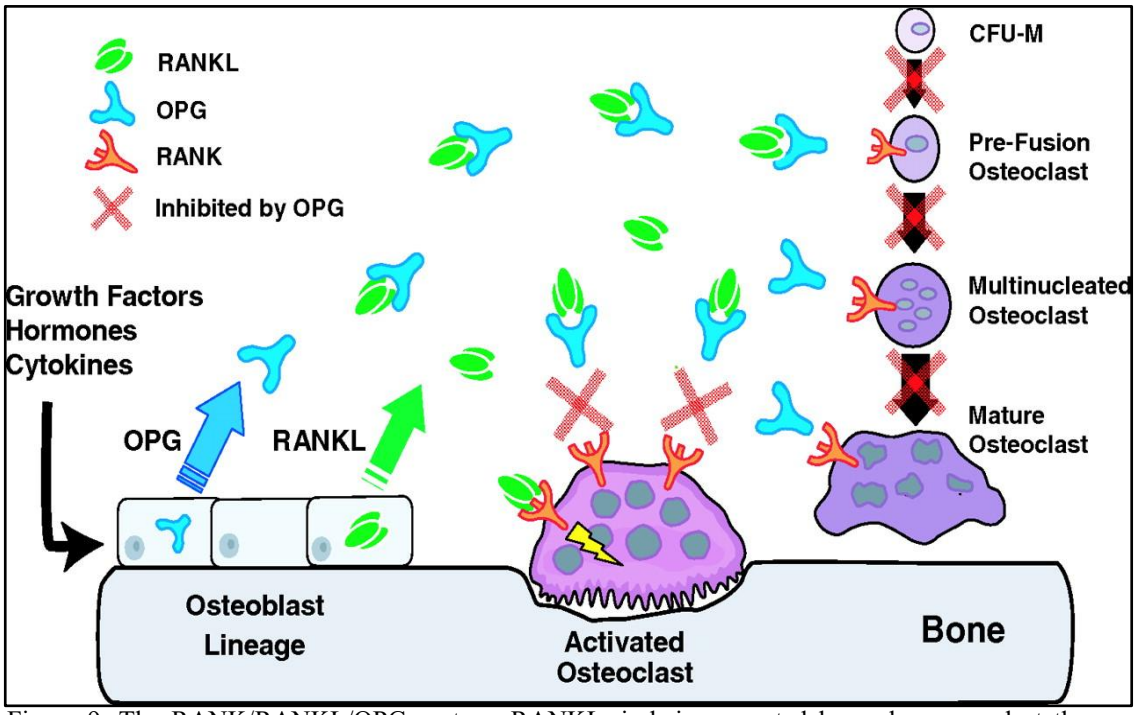

Figure 9. The RANK/RANKL/OPG system. RANKL, is being secreted by and expressed at the pre-osteoblast/stromal cell surface and binds to RANK on the surface of the osteoclast precursor. It acts as an activator of the osteoclast differentiation. The process is balanced by OPG which is secreted by osteoblasts, binds to RANKL and avoids its binding to RANK. Extracted from Kearns, 2008 (73)

Table 3. Factors affecting RANK, RANKL and OPG expression.

Factor added to the culture	OPG	RANKL	RANK
1,25-dihydroxyvitamin D	↑↓	↑	↑
Hormones			
Estrogen	↑	↓/—	
Testosterone	↑↓	—	
Glucocorticoid	↓	↑	
PTH	↓	↑	
PTHrP	↓	↑	
Cytokines			
IL-1	↑↓	↑	↑
IL-4			↓/—
IL-7		↑	
IL-13	↑	↓	—
IL-17	↓	↑	—
TNF α		↑	
Interferon γ	↑	↑	↑
Prostaglandin E2	↓	↑	
Growth factors			
TGF β	↑	↑↓	—
Bone morphogenetic protein 2	↑		—

↑ Increased expression; ↓ decreased expression; — no change observed. Modified from Kearns, 2008 (73)

1.3 Genetics of Osteoporosis

Osteoporosis is a complex disease and many factors can contribute, in parallel, to excessive bone resorption and increase the fractures risk as a result. The first evidence for the heritability of osteoporosis appeared in twin and family studies. As reviewed by Duncan et al (4), female twins studies showed that BMD values are 57% to 92% heritable (78-80). Family studies supported the findings by emphasizing the genetic link, suggesting the heritability of BMD is 44% to 67% (81-83). The first studies demonstrated the existence of a genetic component among the factors that determine BMD variability, but did not suggest specific genes which might be involved. These studies, including a family study based on probands with extreme BMD (84), insinuated a polygenic trait, even though monogenic effects were evidenced in some populations or families (85).

The elevated prevalence of the disease and its high health care costs, combined with the strong evidence of the inheritable nature of the osteoporotic phenotypes, led to a significant amount of genetic studies. These studies aimed to identify genes, mechanisms or signaling pathways which might contribute to the understanding of the disease, and later on to serve as a therapeutic target. The 'first generation' genetic study was based on non-parametric linkage approach on the one hand and candidate gene association on the other hand. Once single nucleotide polymorphisms (SNPs) were well characterized and the technique became available and affordable, genome wide association (GWA) studies replaced both methods as the leading method in bone metabolism and osteoporosis research.

1.3.1 The Linkage Approach

Parametric linkage approach uses the family pedigree with affected and non-affected members to analyze the transmission model among the affected individuals (86). Parametric linkage analysis used to be the principal method to determine inheritance in simple mendelian inherited disorders (87).

However, while this approach had identified some genes involved in the pathologic process of monogenic bone syndromes (Table 4), it could not be applied in studies of complex disease. Even the application of non-parametric linkage approaches, in which knowledge of the precise inheritance model was not required (and which just

tested for deviation from random segregation among affected sib-pairs), yielded very limited success (88). Moreover, the results failed to be replicated. The results established the need to use other approaches, considering the perception that osteoporosis is a multifactor disease that might be affected simultaneously by several gene variants and modulated by environmental aspects.

Table 4. Genes involved in monogenic bone syndromes.

Gene	Bone disorder
COL1A1	Osteogenesis imperfect
CRTAP	Osteogenesis imperfecta type VII
LEPRE1	Osteogenesis imperfecta type VIII
PLOD2	Bruck syndrome (osteogenesis imperfecta with joint contractures) type 2
CA2	Osteopetrosis (autosomal recessive)
TCIRG1	Osteopetrosis (autosomal recessive)
CLCN7	Osteopetrosis (both autosomal recessive and autosomal dominant forms)
OSTM1	Osteopetrosis (autosomal recessive)
LRP5	Osteoporosis-pseudoglioma syndrome
LRP5	High bone mass syndrome
SOST	von Buchem disease and sclerosteosteosis
OPG (*TNFRSF11B*)	Juvenile Paget's disease (hereditary hyperphosphatasia)
RANK (TNFRSF11A)	Familial expansile osteolysis
ALPL	Hypophosphatasia
CASR	Neonatal hyperparathyroidism
CTSK	Pyknodysostosis

Modified from Duncan, 2008 (4)

1.3.2 Association Study Approach

1.3.2.1 Candidate Gene Approach

In the candidate gene strategy, pre-specified genes, usually known to be involved in bone metabolism, were chosen in an attempt to define an association between the gene and the disease. The major difference between the two approaches (linkage and association) is the tested groups: the first study is based on established pedigree with affected sib-pairs, while the latter is a case-control study, designed to identify allele frequency differences which might be associated with the disease. Candidate gene association studies usually involve a small number of genes.

The first candidate gene study related to osteoporosis was performed with vitamin D variants, and was followed by many more studies involving collagen type Iα1 gene (*COLIA1*), lipoprotein receptor related protein 5 (*LRP5*), estrogen receptor α (*ERα*), bone morphogenic proteins (*BMPs*), sclerostin (*SOST*) and *RUNX2* (89).

In the study of complex diseases, the majority of the results could not be replicated (90,91). For example, some results were found to be false positive due to ethnic variation artifacts. All things considered, the contribution of this approach to the investigated disease was relatively low (87).

1.3.2.2 Genome Wide Association

GWA involves systematic DNA screening, with no prior assumption regarding a specific gene or locus. In this approach, known SNPs serve as markers distributed along the genomic DNA. The genotyped SNPs are analyzed for association with the relevant phenotype. In the case an association is found, the SNP itself or the nearby region is suggested to be involved in the pathological process.

The number of SNPs genotyped in the process depends on the chosen GWA platform. The latest versions might reach up to ~2.5 million SNPs (92). Yet, genotyping such a massive amount of SNPs might statistically increase type I errors (false positive results). There are some methods that aim to deal with this multiple tests (comparisons) correction. Bonferroni correction is the most frequent method in use, and yet is considered the most conservative (93). Briefly, the Bonferroni p value (target) is derived from the previous threshold (usually 0.05) divided by the number of independent tests performed (in GWA, the number of genotyped SNPs). In order to achieve results lower than the new p target, there is a need for a massive sample size, which led to the meta-analyses of GWAs studies.

Meta-analyses of GWAs can refer either to a statistical method used to combine some independent GWA studies in order to be analyzed together, or to a standard GWA with the only difference being the tested cohort, which is actually a large-scale cohort, usually formed from several sub-cohorts. This method usually requires multi-centre cooperation and a fusion of several well-established cohorts into one. Our cohort, BARCOS (94), plays both roles, as an independent cohort when used in our genotyping and association studies and as a sub-cohort of the GEFOS-GENOMOS consortium (92).

In the last decade, several GWAs and meta-analyses were performed, aiming to reveal the genetic component of osteoporotic phenotypes. There is a common consensus in this field that GWA results should be replicated. Though some GWA results failed to be replicated in other cohorts, this advanced technology generally achieved consistent

results. GWA results confirmed once again the complexity and wider combination of genetic variants, while each variant contributes a relatively small effect to the discussed osteoporotic phenotype (88). It is worth mentioning that GWAs are hypothesis-free studies and that the SNPs are chosen based on the available technique at the time. The SNPs which are found to be associated only indicate a possible functional connection with the investigated disease. The associated genes found with either BMD or fracture risks in some GWAs and meta-analyses are summarized in Table 5.

Table 5. Genes and loci found to be associated with BMD and fractures in selected GWAs

Reference	BMD	Fractures
Richards 2008 (95)	***LRP5***, ***OPG***	***LRP5***
Styrkarsdottir 2008 (15)	***RANKL***, ***OPG***, ***ESR1***, *MHC*, ***1p36***	*2p16*, ***RANK***, ***RANKL***, ***OPG***, ***ESR1***, *MHC*, ***1p36***, ***LRP4***
Yang 2008 (96)	*UGT2B17*	*UGT2B17*
Liu 2009 (97)	***SOX6***	
Richards 2009 (98)	***ESR1***, ***LRP4***, *ITGA1*, ***LRP5***, ***SOST***, ***SPP1***, ***RANK***, ***RANKL***, ***OPG***	***LRP5***, ***SOST***, ***SPP1***, ***RANK***
Rivadeneira 2009 (16)	***GRP177***, ***CTNNB1***, ***MEF2C***, ***STARD3NL***, ***FLJ42280***, ***DCDC5***, ***SOX6***, ***FOXL1***, *CRHR1*, ***ZBTB40***, ***ESR1***, ***C6orf97***, ***OPG***, ***LRP5***, ***SP7***, ***RANK***, ***RANKL***	
Styrkarsdottir 2009 (14)	***MARK3***, ***SOST***, ***RANK***, ***SP7***	*MARK3*, ***SOST***, ***RANK***
Timpson 2009 (99)	***SP7***	
Xiong 2009 (100)	*ADAMTS18, TGFBR3*	*ADAMTS18*
Deng 2010 (101)	*VPS13B*	
Gou 2010 (102)	*PTH, IL21R*	
Styrkarsdottir 2010 (103)	***1p36***, ***GRP177***, ***CTNNB1***, ***OPG***, ***SOX6***, ***LRP5***, ***RANKL***, ***FOXL1***, ***SOST***	
Duncan 2011 (104)	***FLJ42280***, ***MEF2C***, ***SOX6***, ***ZBTB40***, ***RANKL***, ***OPG***, ***GALNT3***, ***RSPO3***	*GALNT3*
Estreda 2012 (92)	*DNM3, PKDCC, ANAPC1, INSIG2, KIAA2018, LEKR1, IDUA,* ***RUNX2****, SOX4, WNT16, C7orf58, ABCF2, LACTB2, FUBP3, MPP7, DKK1, KCNMA1, CPN1, LIN7C, PTHLH, WNT5B, DHH, C12orf23, RPS6KA5, NTAN1, AXIN1, CLCN7, CYLD, SMG6, SOX9, FAM210A, GPATCH1, KAL1, WLS,* ***ZBTB40****, SPTBN1,****GALNT3****,* ***CTNNB1****, MEPE,* ***MEF2C****,* ***RSP03****,* ***C6orf97****, STARD3NL, SLC25A13,* ***OPG****, ARHGAP1,* ***DCDC5****,* ***SOX6****,* ***LRP5****,* ***SP7****,* ***AKAP11*****,* ***MARK3****,* ***FOXL1****, C17orf53, MAPT,* ***RANK****, JAG1*	*SPTBN1,* ***SPP1****, SLC25A13, DKK1,* ***LRP5****, FAM210A,* ***ZBTB40****, WNT4,* ***CTNNB1****,* ***STARD3NL****, WNT16, FUBP3,* ***DCDC5****, RPS6KA5,* ***SOST****, C17orf53*

Bold- result which was replicated in more than in a single GWA. *-Estrada et al (92) indicated the *AKAP11* gene in regard to rs9533090 association with BMD.

The significant associations between *RANK* and *RANKL* genes and BMD or osteoporotic fractures were replicated in different GWAs (Table 5).

1.3.3 RANK

RANK (gene map locus 18q22.1, Ensembl transcript ID: ENST00000269485) encodes a type I transmembrane protein which contains 4 extracellular cysteine-rich pseudo-repeats. The human RANK protein is a 616 amino acid peptide, expressed from a 4,521 bp transcript composed of 10 exons. It has an N-terminal extracellular domain and its signal peptide is 28 amino acids long. It has a transmembrane domain, 21 amino acids long, and a large C-terminal cytoplasmatic domain. The name RANK stands for 'receptor activator of NF-kappa-B' (105). *RANK* is also known as *TNFRSF11A*, which stands for tumor necrosis factor receptor superfamily, member 11A. This name reflects the homology between the gene and extracellular domains of tumor necrosis factor receptor (105).

The first conclusion of Anderson et al. (105), who identified the gene, focused on the importance of the RANK-RANKL interaction as a regulator of the interactions between T cells and dendritic cells. Yet, in the last 15 years our understanding of the gene function has broadened significantly, including the discovery that RANK is essential for osteoclastogeneis.

Animal models have proven the crucial role of RANK in bone turnover. *Rank* null mice had profound osteopetrosis, B-cell deficiency in the spleen, and absence of the majority of the lymph nodes (76). In another study, the *Rank* null mice were lacking osteclasts and as a result had a severe defect in bone resorption (106). In addition, *in-vitro* osteoclastogenesis began only after transfecting the hematopoietic precursor cells from these mice with *Rank* cDNA. Several GWAs and meta-analyses found *RANK* to be associated with osteoporotic phenotypes (Table 5).

Apart from its role in bone metabolism, RANK is involved in other pathological processes in the living body, among them thermo - regulation and the central fever response in inflammation (107). In addition, melanoma cells and human epithelial cancer cells express RANK on their surface, and in the presence of RANKL trigger the migration of these cells (108). It also plays a role in mammary tumorigenesis at early

stages (109) and is involved in stimulating mammary cancer metastasis through RANKL-RANK signaling (110).

1.3.4 RANKL

RANK ligand or *RANKL* (gene map locus 13q14, Ensembl transcript ID: ENST00000239849) was identified in 1997 by Anderson et al. (105), who named it *RANKL,* and by Wong et al. (111), who called it TNF-related activation induced cytokine (*TRANCE*). It was more or less simultaneously cloned by two other groups- Lacey et al., who named it OPG ligand (*OPGL*) (86) and Yasuda et al. (112), who named it osteoclast differentiation factor (*ODF*). The latter used the recently discovered OPG as a probe to identify the protein. The results demonstrated that the identified proteins are in fact identical to previously discovered RANKL/TRANCE. Though the different groups named it differently, the consensus name today is either *RANKL* or *TNFSF11* (105) (which stands for tumor necrosis factor ligand superfamily member 11).

RANKL, a type II transmembrane protein, contains 317 amino acids expressed from a 2,195 bp transcript length which includes 5 exons. It is expressed mainly in the lymph nodes and bone marrow stromal cells. In the skeletal level it is also expressed by mesenchymal cells, hypertrophying chondrocytes and in regions undergoing bone remodelling by osteoblastic cell line.

As mentioned, RANKL is expressed at the pre-osteoblast/stromal cell surface and, as a soluble molecule, is secreted by those same cells as well as by mature osteoblasts and osteocyte (Figures 8 and 9). RANKL forms are not identical: the membrane-bound form is a 40-45 kDa protein, while the soluble form is a 31 kDa, cleaved from the initial entire form (72). Yet, both forms take part in its major role: stimulation of osteoclastogenesis by binding to RANK on the pre-osteoclasts cells, as well as in osteoclasts activity and survival. This has been demonstrated by many studies *in-vitro* and *in-vivo* (in animal models). *Rankl* null mice were osteoclast deficient and demonstrated severe osteoporosis (113). RANKL can activate the antiapoptotic serine/threonine kinase PKB to inhibit osteoclasts apoptosis (114). Merged group of 3 different sub-groups of women (premenopausal, early postmenopausal and age-matched estrogen-treated postmenopausal), showed correlation between RANKL levels and bone

resorption activity (115). In addition to the association found with osteoporosis in GWAs (Table 5), it has been also demonstrated that mutations in *RANKL* lead to an osteoclasts-poor form of osteoporosis (116).

RANKL also plays a role in other pathological situations. Women treated with medroxyprogesterone acetate (MPA) for hormone replacement therapy and contraceptives had high levels of RANKL in the epithelial cells of the mammary gland. Genetic inactivation of RANK led the cells, among other effects, to induce cell death. This process resulted in lower and later incidence of MPA-driven mammary cancer (117).

Another aspect of *RANKL* is its role in multiple myeloma. In myeloma, there is an increase in *RANKL* expression in parallel to decrease in OPG, its decoy receptor. This pathological status leads to bone destruction (118). Another study demonstrated that treating established myeloma mice with OPG prevents the occurrence of bone lesions, and led to increased BMD (119).

As mentioned in 1.3.3, RANK and RANKL play an important role in the epithelial cancer and melanoma cell migration. This process was neutralized by OPG (only in bone) in mice with melanoma metastasis (108). In addition, psoriasis patients also express high levels of RANKL in keratinocytes (the dominant cell in the epidermis) in all epidermal layers (120). The same study demonstrated that ultraviolet (UV) radiation resulted in *Rankl* expression in mice. RANK injection supplied protection from UV-induced immunosuppression.

1.4 MicroRNAs and Their Role in Bone Metabolism

MicroRNAs (miRNAs) are 19 to 25-nucleotide molecules which play an important role in gene regulation. The miRNAs bind, in a partially complementary manner, to the target mRNA 3'UTR region. This mRNA-miRNA complex (partial double helix RNA) induces either mRNA degradation or translational repression (121) and regulates the gene expression. In regulating gene expression, miRNAs also serve as a regulator of signaling pathway, as for example Wnt (122). Though the binding is partial, each miRNA has its seed sequence. The seed is 6 nucleotides long, located in position 2-7 (from the 5' end), which should fully bind its complementary sequence in the 3'UTR in order to regulate the gene (123). Due to their regulatory nature, miRNAs

are involved in every aspect of the biological processes in health as well as in pathological conditions. One miRNA can regulate hundreds of genes (124), and 3'UTR of a gene may have several binding sites to different miRNAs.

To date, about 1,500 human miRNAs have been identified either by experimental tools or by *in-silico* research. The grand majority of the information is gathered in free access databases such as miRBase (www.miRBase.org), microRNA (www.microRNA.org) and Targetscan (www.targetscan.org) (123,125). It is important to highlight that the majority of predicted binding sites have been identified *in-silico* using probability algorithms (such as those in use in the mentioned databases) and were not proven empirically.

Genetic variants either in the mature miRNA itself or in the 3'UTR region of a gene may affect the binding site in a way that will result in lost function of this regulatory system. Variants in miRNA may affect a specific gene in a direct or indirect way (124), or produce a butterfly effect which may affect downstream genes expression and function (124). On the other hand, changes in the gene 3'UTR may result in impaired expression of the specific gene either by modulating existing miRNA binding sites or by generating new ones (126).

In light of miRNAs involvement in every cellular pathway (127), it is not surprising to see the accumulating evidence regarding miRNAs involvement in many aspects of bone metabolism. Specifically, miRNAs regulate osteogenesis and bone formation (128,129) (Figure 10), as well as osteoclastogenesis and bone resorption (130,131).

The miRNAs involvement might be direct regulation of a bone-metabolism regulator or indirect, by regulating an upstream regulator that will affect bone metabolism down the road (Figure 10).

miRNAs directly regulate the expression of a specific gene with a key role in bone turnover. Direct regulation can be demonstrated by miR-30 which regulates *Smad1* (132) miR-133 which regulates *Runx2* (133), and miR-637 which regulates *Osx* (134). All are significant factors in osteoblasts differentiation and function.

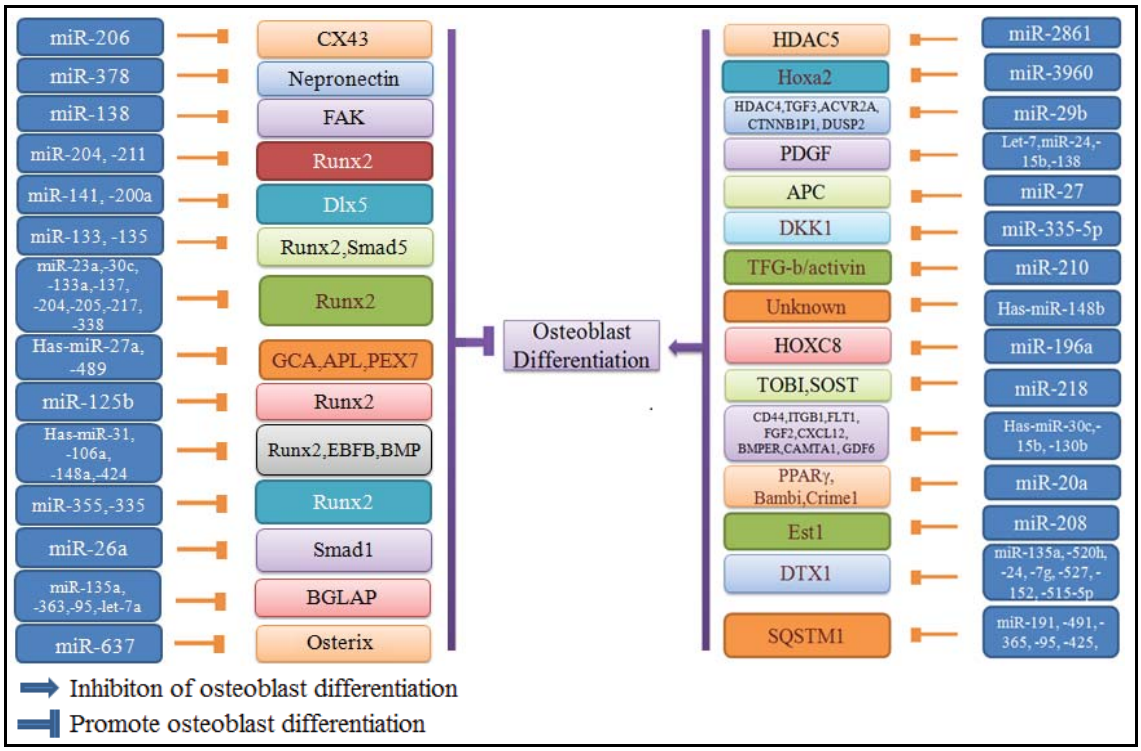

Figure 10. Osteoblast-differentiation regulation by miRNAs. Genes which regulate the osteoblast differentiation are being regulated by miRNAs. Extracted from Vimalraj, 2012 (135)

In addition, association was found between polymorphisms in predicted miRNA binding sites and osteoporosis (136). Lei et al selected 568 polymorphisms in miRNA target sites, from a total of 22,000 variants found *in-silico* by combining the information regarding SNPs within the 3'UTR from dbSNP (137) and the prediction of miRNA target sites by TargetScans (123). Three SNPs, all in fibroblast growth factor 2 (*FGF2)* 3'UTR, were associated with FN BMD. These SNPs are located in potential binding sites for 9 different miRNAs.

1.4.1 *RANK*/*RANKL* MiRNAs

As mentioned, most of the miRNA target sites are based on *in-silico* research. In the *RANK* and *RANKL* regard, using the www.microRNA.org database, 69 miRNA binding sites are predicted for the *RANKL* gene, involving 47 miRNAs and 6 miRNA binding sites are predicted for the *RANK* gene, involving 6 miRNAs. Not only some of the predicted miRNAs have more than one target site, but also some target site are predicted to bind more than one miRNA (Figure 11).

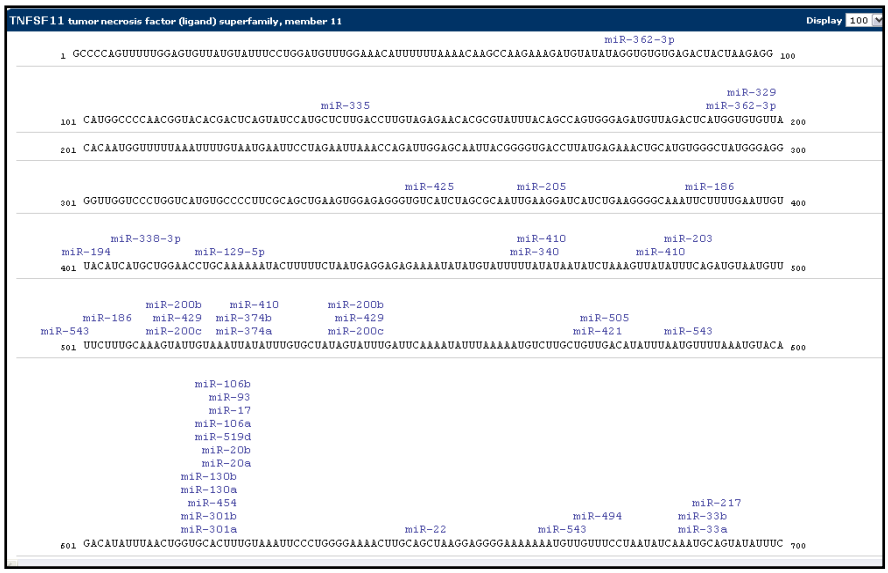

Figure 11. A 700 bp segment of the *RANKL* 3'UTR and the predicted miRNA target sites. (www.microRNA.org)

A few *in-vivo* and *in-vitro* miRNA studies have suggested an association, though indirect, with *RANK* or *RANKL*. For example, patients with breast cancer metastasis found to demonstrate miR-126 loss of expression (138). *RANKL* induces cancer cells migration and is associated with metastasis, in bone as well as in other sites. *In-silico* investigation suggested that miR-126 may regulate *RANKL* resulting in RANKL over-expression (139). In the same manner, additional 3 other miRNAs (miR-199a-3p, miR-335 and miR-489) were suggested to be involved in *RANKL* expression. None of them was proved *in-vivo* or *in-vitro* to regulate the gene.

Another indirect relation can be found between miR-155 and *RANKL*. It has been shown that miR-155 suppresses the *RANKL*-induced osteoclastogenesis (140). The effect of miR-155 activity drives the progenitor towards activated macrophage rather than towards osteoclast (Figure 12). In addition, it has been demonstrated in mice that *RANKL* treatment regime resulted in down regulation of miR-155(141). In the same study, inducing osteoclast-specific *Dicer* gene deficiency (dicer is a key protein in miRNA processing), resulted in the suppression of the osteoclastic bone resoprtion.

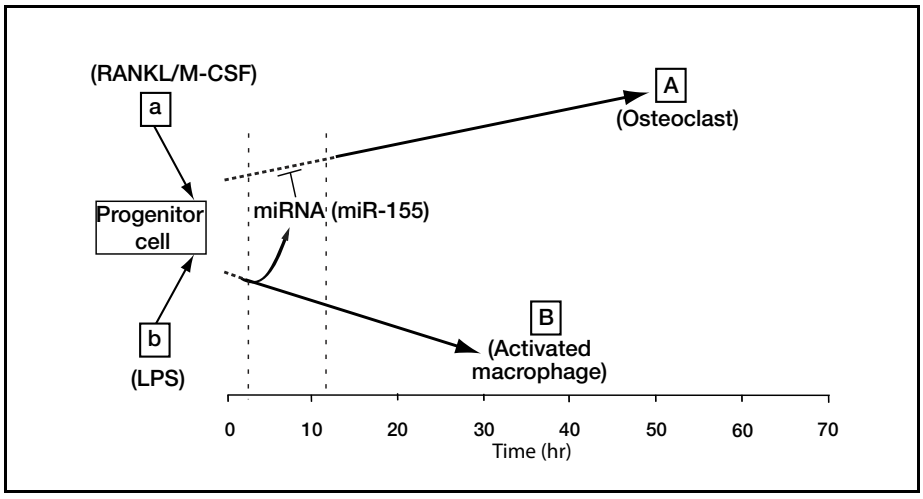

Figure 12. A schematic model suggested by Mann el al 2010. MiR-155-based mechanism for the osteoclast (A) or activated macrophage (B) progenitor cell given a specific induction signal (marked as 'a' for RANKL/M-CSF and 'b' for lipopolysaccharide (LPS)). MiR-155 suppresses the osteoclastogenesis within the first 10 hours of osteoclast differentiation. Modified from Mann et al, 2010 (140)

Even though there is no doubt regarding miRNAs involvement in bone metabolism, there is lack of knowledge concerning the relationships and mutual effects between *RANK/RANKL* and miRNAs. This field is yet to be explored in order to broaden our understanding of the progression of many impaired bone metabolism diseases, among them osteoporosis.

OBJECTIVES

2. Objectives

1. Association analysis of putative functional SNPs in evolutionary conserved regions of the *RANK* and *RANKL* genes with BMD and the occurrence of fractures in the BARCOS cohort.

2. Characterization of the human *RANKL* promoter and regulatory regions *in-silico* and *in-vitro*.

3. Evaluation of the effect of treatments known to play a regulatory role in the RANKL/OPG system on the *RANKL* promoter and regulatory regions by reporter gene assays.

4. *In-silico* study followed by *in-vitro* functional experiments of the BMD associated SNP(s) in order to reveal its (their) role(s) in the pathological process of osteoporosis.

MATERIALS AND METHODS

3. Materials and Methods

3.1 Study Subjects

BARCOS cohort participants were recruited from Hospital del Mar, Barcelona (94,142). All patients were consecutive, unselected, postmenopausal women attending the outpatient clinic for a baseline visit related to menopause. Patients were prospectively recruited regardless of their BMD values (Table 6). Exclusion criteria for the BARCOS cohort were any history of metabolic or endocrine disease, chronic renal failure, chronic liver disease, malignancy (except superficial skin cancer), Paget's disease of bone, malabsorption syndrome, hormone-replacement therapy, anti-resorptive or anabolic agents, oral corticosteroids, anti-epileptic drugs, and lithium, heparin or warfarin treatments. In addition, women with early menopause (before the age of 40) were excluded for this analysis. Blood samples and written informed consent were obtained in accordance with the regulations of the Hospital del Mar Human Investigation Review Committee for Genetic Procedures. Patients who declined the invitation to participate or did not give informed consent were excluded.

Table 6. Baseline characteristics of the BARCOS cohort.

Patients characteristics	**Gene-wide association**		**3'UTR association**	
	Mean ± SD	**n**	**Mean ± SD**	**n**
Age at menopause (years)	48.28 ± 3.91	905	48.46 ± 4.06	1096
BMI	26.36 ± 3.88	904	26.16 ± 3.85	1088
Breastfeeding (months)	7.92 ± 13.27	905	7.73 ± 12.79	1091
Age at LS densitometry (years)	55.66 ± 8.50	900	56.04 ± 8.49	1087
Years since menopause LS	7.40 ± 8.33	900	7.59 ± 8.26	1091
LS BMD (g/cm2)	0.851 ± 0.15	900	0.853 ± 0.15	1092
Age at FN densitometry (years)	57.82 ± 8.07	820	57.89 ± 8.03	1003
Years since menopause FN	9.52 ± 7.92	820	9.36 ± 7.91	1007
FN BMD (g/cm^2)	0.681 ± 0.107	820	0.683 ± 0.11	1009
Menarche age (years)	12.94 ± 1.58	895	12.89 ± 1.58	1081
Fractures	140 (15.4%)	905	152 (13.8%)	1098
Spine				68 (44.7%)
Hip				8 (5.3%)
Wrist/Forearm				36 (23.7%)
Other				40 (26.3%)

3.2 BMD Measurement and Fracture Assessment

The BMD (g/cm^2) was measured at the lumber spine (LS) L2-L4 and at the non-dominant femoral neck (FN). A dual-energy X-ray densitometer (QDR 4500 SL, Hologic, Waltham, MA, USA) was used for measurements. In our centre the technique has an *in-vivo* coefficient of variation (CV) of 1.0% for LS and 1.65% for FN measurements. Non-vertebral and clinical vertebral fractures were recorded. Non-vertebral fractures were validated from medical records and spine X-ray was performed at baseline when there was a history of vertebral fracture diagnosis, height loss, or back pain. Fractures were defined as osteoporotic if they occurred after the age of 45 and were due to low-impact trauma (i.e., fall from standing height). Fractures of the face, fingers, toes and skull were excluded. Vertebral fractures were defined according to the semiquantitative criteria of Genant et al.(143).

3.3 DNA Extraction

The buffy coat of 3 ml of blood collected in EDTA tubes was stored at -20º C. Genomic DNA was obtained from leukocytes by a salting-out procedure (144) or by Autopure LS (Qiagen), a robotic workstation for automated purification of genomic DNA using autopure chemistry, at LABS Laboratory Biomedical Support Services, IMIM, Barcelona, Spain. Samples were stored at -20ºC.

3.4 SNPs Selection

For the gene-wide association project, putative functional *RANK* and *RANKL* SNPs were chosen using the ENSEMBL (www.ensembl.org) (145), UCSC genome browser (http://genome.ucsc.edu/) (146), Entrez SNP (http://www.ncbi.nlm.nih.gov/snp) (137) and HapMap (www.hapmap.org) (147) databases.

The SNPs from the proximal promoter and intron 1 were mainly selected according to their evolutionary conservation. In order to establish conserved regions, genomic sequences of *Mus musculus, Rattus norvigicus, Canis familiaris, Bos taurus* and *Homo sapiens* (mouse, rat, dog, cow and human, respectively) were compared. Using the ENSEMBL multiple alignment tool we chose a conserved SNP when all species except the human SNP presented the same nucleotide within a "conserved region." The SNPs falling in these regions were validated in a Caucasian population to include those with a minor allele frequency (MAF) >0.1.

Other SNPs were selected according to the following criteria: a replication of a previous report of association with BMD or fracture risk, or exonic changes (either synonymous or non synonymous).

For the 3' UTR association project, only those SNPs with published MAF >0.01 in the ENSEMBL (www.ensembl.org) or Entrez SNP (http://www.ncbi.nlm.nih.gov/sites/entrez) databases were included.

3.5 Genotyping

Polymorphism genotyping was carried out using either the SNPLex System (Applied BioSystems) at the CEGEN platform (Barcelona, Spain) or KASPar v4.0 genotyping system at the Kbioscience facilities *(Herts, England)* using Kraken allele calling algorithm.

Quality control was done by cross-genotyping 4 SNPs (~12% of the results) using both platforms. The readings showed 99.6% concordance between the two techniques.

3.6 Statistical Methods

Hardy-Weinberg equilibrium (HWE) was calculated using the Chi-Square test. HWE p-values were calculated using the Tufts University web site (*http://www.tufts.edu/~mcourt01/Documents/Court%20lab%20-%20HW%20calculator.xls).*

Multivariate linear or logistic regression models were fitted to assess the association between genotyped SNPs and BMD or fractures, respectively. Potential confounders considered for adjustment were BMI, age at menarche, years since menopause at the time of densitometry, and months of breast feeding for the models where BMD was the outcome, and BMI (35) and age for fractures. Correction for multiple testing was performed using the Bonferroni correction method. Briefly, the Bonferroni p value (target) is derived from the previous threshold (usually 0.05) divided by the number of independent tests performed.

Pair-wise statistical comparisons between constructs or treatments for the gene reporter assays were calculated using the non-parametric Wilcoxon paired-sample test.

Anticipating that the effect of different *RANK* variants on fracture phenotype may vary according to bone architecture, we studied the effect of the SNPs assessed on both predominantly trabecular (spine) and cortical (wrist/forearm) fracture sites.

We tested for predefined interactions between the previously described *RANKL* rs9594738 and the *RANK* SNPs studied here by introducing multiplicative terms in the regression equation.

All analyses were two-tailed, and p-values <0.05 were considered significant. Statistical analyses were performed using SPSS for Windows version 13.0 and R software version 2.13.2 with the *haplostats*, *SNPassoc, foreign, rms, epicalc* and *genetics* packages.

3.7 Cell Cultures

Cultures of primary human osteoblasts were obtained from specimens extracted from patients who underwent total knee arthroplasty surgery. Osteoblast culture was established by pooling cells from the trabecular bone using the protocol based on a method described by Marie et al.(148) with some modifications (149,150). Primary osteoblasts and U2OS human osteosarcoma cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco-BRL, Paisley, Scotland, UK) supplemented with 10% Fetal Bovine Serum (FBS, Biological Industries, Kibbutz Beit Haemek, Israel) and ascorbic acid 100 μg/ml (Sigma-Aldrich).

Nuclear extracts were prepared from primary osteoblasts according to Schreiber et al (151) using a modified buffer C (10% glycerol and 1.5 mM of $MgCl_2$). Protein concentrations were determined by the method of Bradford, and nuclear extracts were stored at -80ºC until use.

3.8 Electrophoresis Mobility Shift Assays (EMSAs)

Thirty base-long oligonucleotides containing rs9594738 were synthesized (Sigma-Aldrich) and double-stranded probes were obtained by annealing of complementary single-stranded DNA molecules.

C allele probe (738C) 5'- TACTATATCTGCTA**C**GAAGCTTTGCATCAG-3'

T allele probe (738T) 5'- TACTATATCTGCTA**T**GAAGCTTTGCATCAG-3'.

Probes were 5' end-labelled with [gamma-32P] ATP (GE Healthcare) using the USB Optikinase (Affymetrix) standard protocol. The unincorporated nucleotides were removed using a mini quick-spin oligo column (Roche). The binding reactions contained 10µg nuclear extract, 0.5µg poly(dI-dC) and 0.5µg poly(dA-dT) supplied by Sigma-Aldrich, 6 µg acetylated BSA (New England Biolabs), and 100,000-200,000 cpm labeled probe. The binding reactions were incubated for 30 minutes at room temperature in a buffer containing 20mM HEPES at pH 7.9, 60mM KCl, 1mM EDTA, 1mM DTT and 10% glycerol in a 20µl volume. In competition assays, the binding reactions were performed in the presence of an excess of unlabeled competitor oligonucleotide, as indicated in each case. Unspecific competitions were performed using oligonucleotides containing the Specifity protein 1 (Sp1) binding site 5'-ATTCGATCGGGGCGGGGCGAGC-3' or Glucocorticoid Response Element (GRE) 5'-TAATGAGAGAAGATTCTGTTCTAATGACCA-3'.

The DNA-protein complexes were separated from the free probe by electrophoresis in a non-denaturing 5% acrylamide gel (29:1) (Bio-Rad) containing 2.5% glycerol in 1xTBE buffer (Promega), run at 4ºC and 15-18mA for approximately 2 hours. Gels were vacuum-dried and exposed to X-ray films at -80ºC for 16 to 48 hours, as necessary.

In-silico study of the 30 bp probe was performed using informatics tools from Genomatix (http://www.genomatix.de).

Supershift assays were performed using the following antibodies: anti-paired box 2 (PAX2) (Abnova, Taipei, Taiwan), anti-PAX5 (Milipore), anti-Sp1 (Abnova, Taipei, Taiwan), and anti-GR (AbD serotec). Anti-RUNX2 (Santa Cruz Biotechnology, CA, USA) was used as a control. Before adding the probe, 0.5 to 3µl of each antibody was added to the binding reaction and preincubated on ice for 15 minutes.

3.9 DNA Constructs

To generate a 2180 bp length promoter, the human *RANKL* region (NCBI reference sequence: NM_003701.3) comprising -2084/+96 was PCR amplified and cloned by blunt-end ligation into pUC19 *Sma*I (Fermentas).

Forward primer 5'- CCTGTGAAACAGCAGCAG-3'

Reverse primer 5'- TCTTGTCTGCGGCCAACT-3'

The insert was excised with *Kpn*I and *Bam*HI and subsequently subcloned into the pGL3-Basic vector digested with *Kpn*I and *Hin*dIII at the polylinker site. Finally, a segment between *Kpn*I and *Bst*z17I was eliminated to obtain the P1 promoter construct (-1919/+96).

The P2 (-1251/+96), P3 (-946/+96) and P4 (-234/+96) promoter constructs were obtained in the same manner by eliminating segments using *Eco*RV, *Bst*XI and *Pvu*II, respectively (Figure 13). The P1_R3del and P4_R2 constructions were derived from P1 and P2, respectively, by deleting R3 using *Ale*I and *Puv*II.

A 999 bp fragment containing rs9594738 in position 470 of the segment was amplified by PCR for each allele.

Forward primer 5'- TGTAAATTGTGATGATGTGAACG-3'

Reverse primer 5'- GTCATGGGCACTAGTTGGTG-3'

Both fragments were cloned by blunt-end ligation into pUC19 *Sma*I. Since the cloned sequences correspond to a far upstream region from *RANKL*, they were named DR (C/T), for *distal region* and the specific SNP alleles. The insert was excised with *Kpn*I and *Eco*RV and the 835 bp product segment (DR (C/T)) was subcloned into the pGL3-Basic vector upstream to each construct. Four additional SNPs lie in the DR segment (rs10507506, rs28641485, rs12871509 and rs17457484). For all SNPs (but rs9594738) the ancestral allele was cloned (C, A, A and C respectively) in order to generate the most frequent haplotype.

To generate the P4_pUC920 construct, a 920 bp segment was purified from a *Kpn*I and *Sca*I double digestion of pUC19 and subcloned into *Kpn*I- and *Pvu*II-digested P1 construction (Figure 13).

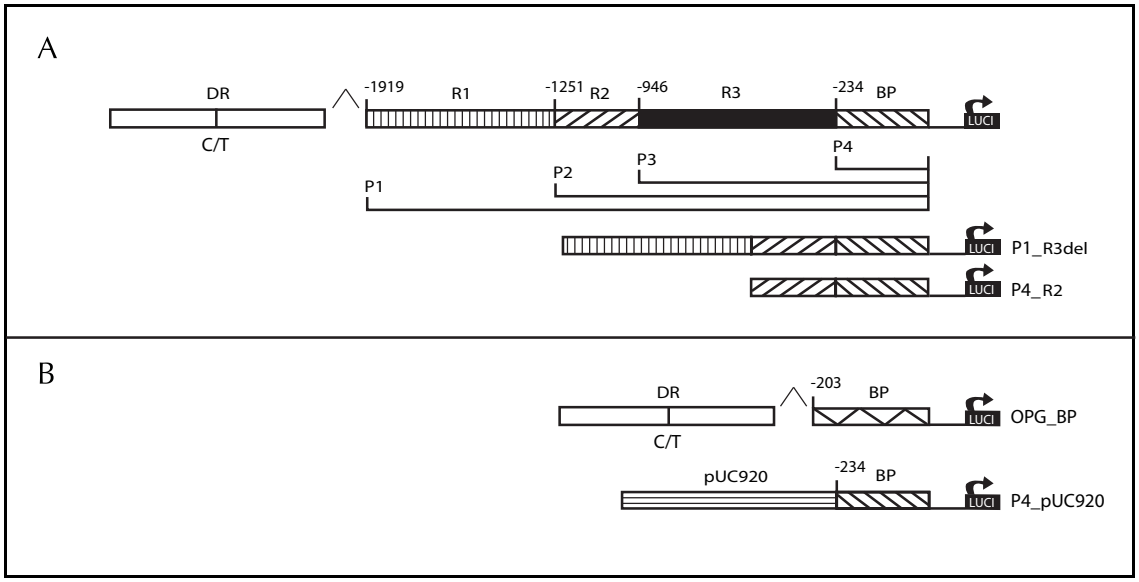

Figure 13. Promoter constructions designed for gene reporter assay. (A) *RANKL* promoter constructions derived from P1 promoter by sequential deletion. Each promoter was cloned with and without DR(C/T) (See text). The vertical line in DR represents rs9594738 and C/T represents the different alleles. (B) Control promoter constructions designed to verify DR effect. *OPG* basal promoter with and without DR(C/T) and *RANKL* basal promoter P4 with an additional 920 bp segment digested from pUC19.

To generate a 2387 bp *OPG* promoter fused to the luciferase gene, the human gene region (ENSEMBL trasnscript ID: ENST00000297350) comprising -2150/+237 was PCR-amplified and cloned by blunt-end ligation into pUC19.

Forward primer 5'- GTGCCCCAACCTGTCTCC-3′

Reverse primer 5'- AACCTCAGGGGCTTGGAG -3′

The insert was excised with *Sac*I and *Nhe*I and subsequently subcloned into the pGL3-Basic vector digested with the same enzymes at the polylinker site. A final digestion with *Kpn*I and *Pvu*II was done to produce the *OPG* basal promoter (OPG_BP) -203/+237.

OPG_BPC and OPG_BPT promoters were generated by adding DR(C) and DR(T) to OPG_BP, respectively.

All constructs were verified by automatic sequencing.

3.10 Reporter Gene Assays and Cell Treatments

The U2OS cells were plated at 60% to 80% confluence in DMEM containing 10% FBS. The next day, 2-3 μg of each construct and 2 ng of *Renilla* control vector were co-transfected into the cultured cells using Lipofectamine LTX and Plus reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. At 24 hours after transfection, firefly and *Renilla* luciferase activities were measured in an OrionII microplate luminometer (Berthold Detection Systems) using Dual Luciferase Reporter Assay (promega, Madison, WI, USA).

In the case of an additional treatment with hormones or cytokines, the medium was changed to DMEM containing 0.1% Bovine Serum Albumin (BSA) (Sigma Aldrich, Germany) 4 hours after transfection, followed by 2 hours additional incubation. Six hours post-transfection the treatments were added to a final concentration of 100 nM for the hormones [dexamethasone (DEX) (Sigma-Aldrich Química S.A., Madrid, Spain), vitamin D (Sigma), 17β-estradiol (E2) (Sigma-Aldrich Química S.A., Madrid, Spain) and parathyroid hormone (PTH) (Sigma-Aldrich Química S.A., Madrid, Spain)], 0.01 μg/ml for human recombinant IL-1β (R&D Systems Inc, Minneapolis, MN, USA) and transforming growth factor β (TGF β) (Sigma-Aldrich Química S.A., Madrid, Spain) and 0.1 μg/ml for tumor necrosis factor α (TNFα) (R&D Systems Inc). The luciferase assays were performed after 16 hours of treatment.

For each construct or treatment to be assayed, the number of independent transfection experiments (replicas) is given in the relevant figure. In each transfection replica one set of several different of minipreps or midipreps (Qiagen) was tested in duplicate or triplicate, as appropriate. Although this strategy generated more inter-replica variability, it avoided any biases attributable to single clones of each construct. The results for *RANKL* promoter were normalized in reference to P1 construct. For the *OPG* experiments, the results were normalized in reference to OPG_BP construct.

3.11 Expression Analysis of the Distal Region Sequence

Total RNA was extracted from primary human osteoblasts using the High Pure RNA Isolation Kit (Roche). cDNA was obtained using Taqman® Reverse Transcription reagents (Applied Biosystems) following the manufacturer's protocol. Samples were stored at -20ºC until use.

To verify the expression of the region containing the SNP, PCRs were performed using 300F and 300R primers to amplify a 300 bp segment around rs9594738, and a 150 bp segment was amplified using the EMSA oligonucleotides as forward and reverse primers in combination with 300R and 300F, respectively:

300F primer 5'-GAGAGGGAGCAGGTGTGAAA -3'

300R primer 5'- TGCTACTAGAATTCCCAGCAA -3'

A PCR was performed as control using oligonucleotides that amplified a *RANKL* promoter region (F Primer located at -1418, R primer located at -743) which served as a negative control to rule out the presence of genomic contamination in the cDNA sample.

RESULTS

4. Results

4.1 Association Analysis of *RANK* and *RANKL*

4.1.1 SNPs Selection

In the *RANKL* gene, 223 SNPs were found using ENSEMBL and UCSC genome browser, among them 5 in the coding region (4 synonymous and 1 non synonymous) and 11 in the UTRs. In the 5 kb upstream to the gene 29 SNPs were found and 25 SNPs were found in the 5 kb downstream to the gene. Eighteen of the 223 SNPs were chosen, following the criteria explained in Materials and Methods (see section 3.4 SNPs Selection on page 38). Eleven of them were previously validated and the rest were validated in our facilities. Four were found to be polymorphic (though SNP rs9533155 was excluded while validating the plate). In total, 14 SNPs were genotyped (Figure 14 and Table 7).

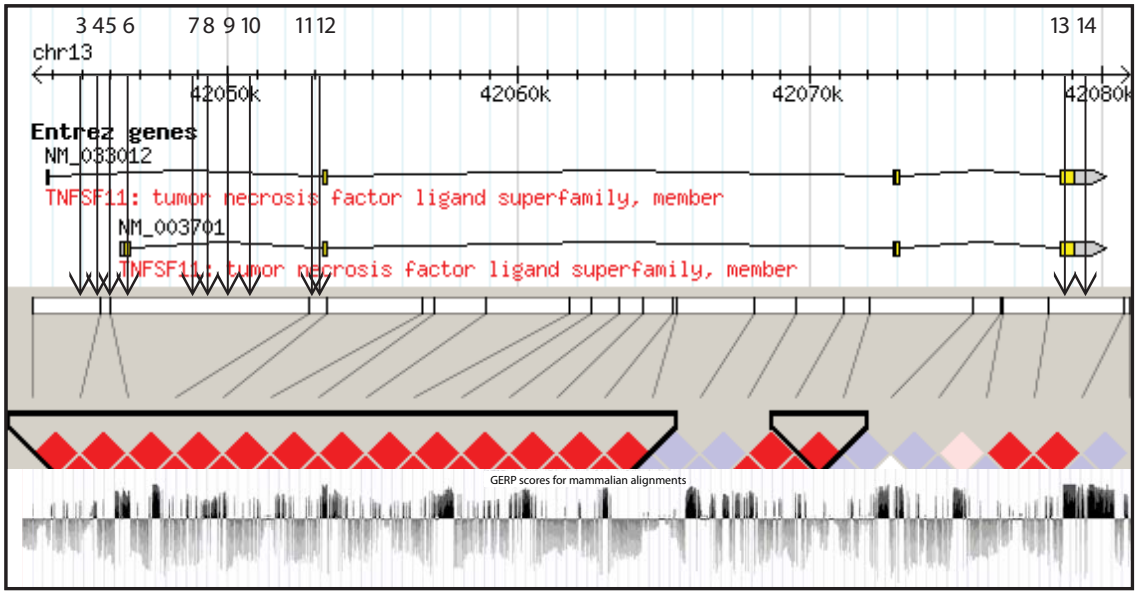

Figure 14. Genotyped SNPs in the *RANKL* gene. Each SNP is represented by its corresponding number in Table 7. The *RANKL* gene and the chrosomal location are given in the upper part of the figure. In the central part are the HapMap haplotypic blocks as given by Haploview software (152) and in the lower part, the evolutionary conserved region wherein the SNP lies, as given by the UCSC genome browser. SNPs rs9594738 and rs9594759 (numbered 1 and 2 in Table 7) do not appear in this figure due to their far upstream position.

In the *RANK* gene, 334 SNPs were found using ENSEMBL and UCSC genome browser, among them 12 in the coding region (6 synonymous and 6 non synonymous) and 8 in the UTRs. In the 5 kb upstream to the gene 33 SNPs were found and 28 SNPs were found in the 5 kb downstream to the gene.

Seventeen of the 334 SNPs were chosen, using the same criteria as for *RANKL*. Seven of them were previously validated. Of the rest, 4 were found to be polymorphic in our facilities. In total, 11 SNPs were genotyped (Figure 15 and Table 7).

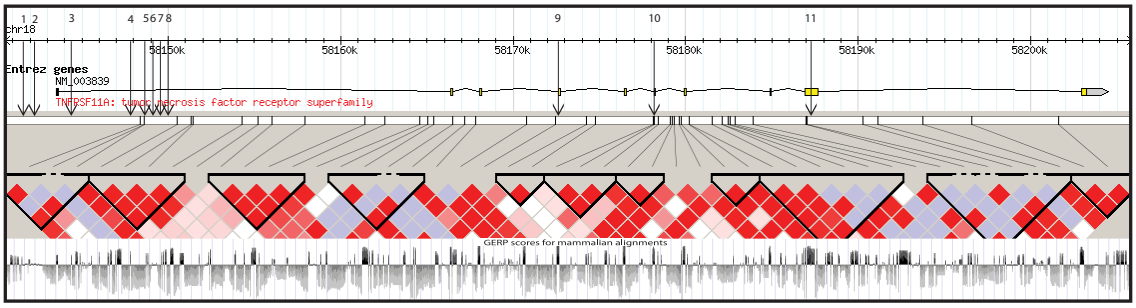

Figure 15. Genotyped SNPs in the *RANK* gene. Each SNP is represented by its corresponding number in Table 7. The *RANK* gene and the chrosomal location are given in the upper part of the figure. In the central part are the HapMap haplotypic blocks as given by Haploview software (152) and in the lower part, the evolutionary conserved region wherein the SNP lies, as given by the UCSC genome browser.

In this part of the study (gene-wide association project) the BARCOS cohort involved 909 female patients, all of Spanish ancestry. Age, weight, height, age at menarche, age at menopause, years since menopause at the time of densitometry, months of breast-feeding, and history of prior fractures were recorded (Table 6).

Fourteen genetic variants in the *RANKL* gene were genotyped in the BARCOS cohort (Table 7). All SNPs were in HWE. Regarding MAFs, these were >0.01 for all of the SNPs. Only SNP rs9594738 was significantly associated with LS BMD (Log additive model: beta coefficient= -0.021, $p=3.7x10^{-4}$; dominant model: beta coefficient= -0.034 $p=1.7x10^{-4}$) and with FN BMD (Log additive model: beta coefficient = -0.008, p=0.07; dominant model: beta coefficient= -0.015, p=0.02). Although this SNP replicates previously reported BMD association studies and therefore correction for multiple testing is not required, the association result with LS BMD withstood the conservative Bonferroni correction (target p value $p=3.6x10^{-3}$).

No association with fractures was found for any of the studied SNPs except for rs9525642, which yielded a statistically significant result (Log additive model: OR=0.70, 95% confidence interval (CI) 0.51- 0.97 p=0.03). However, this result did not withstand multiple test correction.

Eleven genetic variants in the *RANK* gene were genotyped in the BARCOS cohort (Table 7). All SNPs were in HWE. Regarding MAFs, these were >0.01 for all of the SNPs. Two SNPs, rs11152341 and rs12150741 yielded $p<0.05$ for association with LS BMD (over-dominant model: p=0.036 and p=0.026, respectively). These results did not withstand the conservative Bonferroni correction.

In the same manner, two SNPs, rs12150741 and rs1805034, yielded $p<0.05$ for association with fractures (recessive model: p=0.035; OR 0.30 (95% CI 0.08-1.08) and dominant model: p=0.049; OR 0.67 (95% CI 0.44-1.00), respectively). However, these results did not withstand multiple test correction.

Table 7. List of the genotyped gene-wide SNPs, genotyping efficiency, MAF and p-values for log-additive model.

Gene	SNP #	Rs	location	n	Efficiency	MAF BARCOS	HWE	LS	FN	Fractures
RANKL	1	rs9594738	184 kb upstream	1098	0.94	0.44	0.37	**$3.7x10^{-4}$ ($1.7x10^{-4}$)[d]**	0.07 **(0.02)[d]**	0.55
	2	rs9594759	104 kb upstream	909	0.95	0.43	0.06	0.13	0.81	0.37
	3	rs17639305	Proximal promoter	909	0.90	0.17	0.21	0.62	0.85	0.39
	4	rs9533156	Proximal promoter	909	0.86	0.48	0.27	0.53	0.97	0.16
	5	rs9525641	Proximal promoter	909	0.89	0.45	0.21	0.64	0.96	0.18
	6	rs2296533	Exon 1	909	0.74	0.46	0.21	0.72	0.71	0.24
	7	rs9594782	Intron 1	909	0.90	0.07	0.30	0.83	0.84	0.47
	8	rs12427596	Intron 1	909	0.88	0.43	0.45	0.93	0.77	0.10
	9	rs9525642	Intron 1	909	0.90	0.38	0.90	0.87	0.97	**0.03**
	10	rs9533158	Intron 1	909	0.88	0.43	0.84	0.63	0.99	0.47
	11	rs9533159	Intron 1	909	0.88	0.43	0.22	0.85	0.77	0.10
	12	rs2277438	Intron 1	909	0.89	0.17	0.29	0.77	0.87	0.29
	13	rs9562415[1]	Exon 5	1098	0.93	0.02	0.56	0.65	0.71	0.28
	14	rs1054016	3' UTR	909	0.85	0.46	0.67	0.73	0.75	0.15

Gene	SNP #	Rs	Location	N	Efficiency	MAF BARCOS	HWE	LS	FN	Fractures
RANK	1	rs6567265	Proximal promoter	909	0.81	0.29	0.39	0.65	0.86	0.25
	2	rs7233419	Proximal promoter	909	0.88	0.30	0.174	0.68	0.96	0.76
	3	rs12457042	Intron 1	909	0.90	0.06	0.55	0.09	0.66	0.29
	4	rs11152341	Intron 1	909	0.87	0.24	0.28	0.13 **(0.036)^{o}**	0.84	0.49
	5	rs7233197	Intron 1	909	0.90	0.07	0.54	0.10	0.71	0.07
	6	rs4941125	Intron 1	909	0.89	0.29	0.19	0.33	0.36	0.72
	7	rs4941126	Intron 1	909	0.87	0.28	0.30	0.37	0.40	0.83
	8	rs12150741	Intron 1	1098	0.94	0.12	0.24	0.26 **(0.026)^{o}**	0.59	0.49 **(0.035)^{r}**
	9	rs35211496	Exon 4	909	0.80	0.21	0.96	0.55	0.46	0.48
	10	rs1805034	Exon 6	1098	0.94	0.42	0.48	0.71	0.63	0.15 **(0.049)^{d}**
	11	rs8092336[1]	Exon 9	909	0.90	0.04	0.22	0.86	0.63	0.90

[1]=Due to low MAF for rs9562415 and rs8092336, the only available statistical model was the codominant model. For the rest of the SNPs, in case of lower significant p value in another model rather than log-additive, the results are given in (). Bold $p<0.05$; ^{d} Dominant. ^{o} Overdominant. ^{r} Recessive

4.1.3 Association of SNPs in *RANK* and *RANKL* 3'UTR with BMD and Fractures

In the raise of the miRNA research and the special regulatory importance of the 3'UTR, the second association project of this study focused on this region, in both *RANK* and *RANKL* genes. The BARCOS cohort at the time of genotyping the 3'UTR SNPs was larger in 20.7% (189 women) and this study included 1,098 women from Spanish ancestry.

In the *RANK* 3'UTR, 22 SNPs were found using ENSEMBL and HapMap databases. Eight of them were previously validated and all had MAF>0.01 .In the *RANKL* 3'UTR, 14 SNPs were found using ENSEMBL and HapMap databases. Of them, 5 were previously validated, and 3 had MAF>0.01. One of the three, SNP rs1054016 was previously genotyped in the gene wide association project and therefore was not replicated in the second phase of this study.

Over all, 10 SNPs were genotyped in the BARCOS cohort (Table 8). SNPs rs74988349 and rs346575 found to be monomorphics and were eliminated from the study.

All the SNPs except rs72933640 were in HWE. However, the BARCOS MAF for rs72933640 was very similar to the MAF (0.108) published by the National Center for Biotechnology Information (NCBI) for Utah residents with ancestry from northern and western Europe (CEU population). MAFs of all polymorphic SNPs were ≥0.01. SNP rs78326403 and SNP rs78459945 were found to be in linkage disequilibrium (LD) (D'=0.999, R^2= 0.968). The latter, which had lower genotyping efficiency, was eliminated from further analysis.

None of the SNPs here assessed were found to be associated with BMD. SNP rs78326403 and SNP rs884205 were significantly associated with fracture prevalence in our cohort (Figure 16 and Table 8). For SNP rs78326403, the log-additive model yielded p=0.05; OR 1.58 (95% CI 1.00-2.49) while the over-dominant model yielded p=0.02; OR 1.83 (95% CI 1.11-3.02). For SNP rs884205, the log-additive model yielded p=0.048; OR 1.40 (95% CI 1.01-1.95)

while the recessive model yielded p=4.9x10^{-3}; OR 3.28 (95% CI 1.51-7.13). Hence, only SNP rs884205 withstood Bonferroni correction for multiple tests (target p value p=7.14x10^{-3}). No significant interaction between the two SNPs was found (p=0.87).

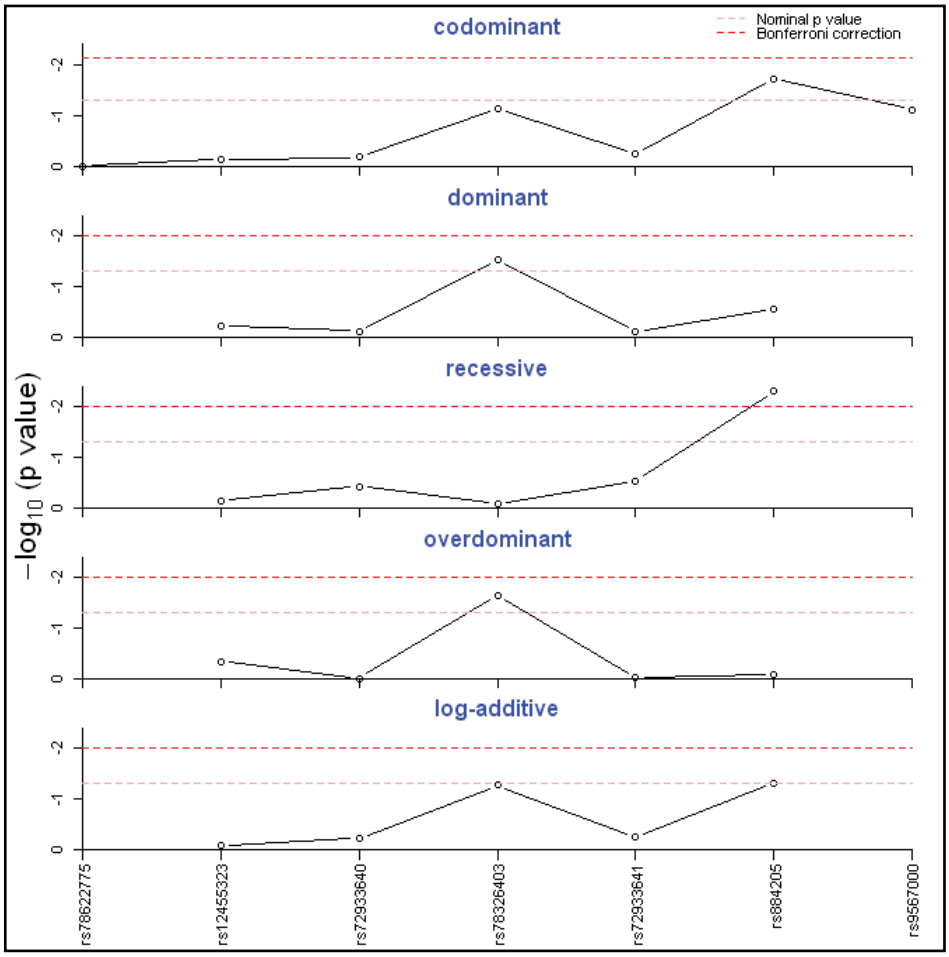

Figure 16. Association study results for the assessed SNPs with fracture prevalence in the BARCOS cohort for all statistical models, presented as $-\log_{10}$(p value). In each graph, the lower dashed line represents p=0.05 and the upper dashed line the Bonferroni-corrected target p value (p=7.14x10^{-3}).

Table 8. The *RANK* and *RANKL* 3'UTR SNPs analyzed, genotyping efficiency, MAFs and p-values for association under a log-additive model

	SNP #	Rs	Efficiency	MAF BARCOS	HWE	LS	FN	Fractures	OR (95% CI)
RANK	1	rs74988349	0.95	0			Non polymorphic		
	2	rs78622775[1]	0.95	0.01	0.73	0.30	0.35	0.99	
	3	rs12455323	0.94	0.32	0.90	0.72	0.76	0.82	
	4	rs72933640	0.94	0.12	**0.005**	0.17	0.89	0.59	
	5	rs78326403	0.95	0.08	0.27	0.83	0.36	0.05 **(0.02)^{o}**	1.83 (1.11-3.02)
	6	rs78459945	0.94	0.08	0.39		In LD with rs78326403		
	7	rs72933641	0.95	0.13	0.07	0.25	0.86	0.57	
	8	rs884205	0.94	0.19	0.32	0.55	0.74	**0.048 (0.0049)^{r}**	3.28 (1.51-7.13)
RANKL	1	rs346575	0.95	0			Non polymorphic		
	2	rs9567000[1]	0.94	0.02	0.57	0.76	0.52	0.08	

[1] Due to a low MAF, the only available statistical model for rs78622775 and rs9567000 was the codominant model.
In case of lower significant p values under alternative models, these are given in ().
Bold $p<0.05$; ^{o} Overdominant; ^{r} Recessive

4.1.4 Fractures Site Dependent Association Study

Even though only SNP rs884205 withstood the multiple tests correction, both SNPs (rs884205 and rs78326403) had ORs with 95% CIs above 1 and were then analyzed separately for their association with either spine or wrist/forearm fractures (Table 9). SNP rs78326403 was found to be associated with wrist/forearm fractures (Log-additive model: p= $7.16x10^{-4}$; OR 3.12, (95% CI 1.69-5.75)) but not with spine fractures (log-additive model: p=0.78,). SNP rs884205 found to be associated with spine fractures (recessive model: p=$8.24x10^{-3}$; OR 4.05 (95% CI 1.59-10.35)) but not with wrist/forearm fractures (log-additive model: p=0.66). In this case, both SNPs withstood Bonferroni correction. In order to test a possible confounder effect BMD might have, we analysed the results mentioned with adjustment for BMD. Both associations remained significant: the rs78326403 association with wrist/forearm fractures after adjusting for FN BMD in a log-additive model was p=$5.8x10^{-4}$ and the LS BMD-adjusted association between rs884205 and spine fractures in a recessive model was p=0.025. The corresponding adjusted ORs were 3.21 (95% CI 1.74-5.94) and 3.31 (95% CI 1.24-8.82), respectively.

4.1.5 Interactions Between rs9594738 and the Fractures Associated SNPs

Interaction analyses between the BMD-associated *RANKL* SNP rs9594738 and the fracture-associated SNPs rs78326403 and rs884205 were performed. Considering wrist/forearm fractures as the outcome, significant results were obtained between rs9594738 and rs78326403 (p=0.039). On the other hand, when considering spine fractures as the outcome, there was no interaction between rs9594738 and rs884205 (p=0.39). Subsequently, an analysis of the effect of compound genotypes of rs9594738 and 78326403 was conducted, which pointed towards increasing wrist/forearm fracture prevalence in subjects with a higher number of unfavourable alleles: T for rs9594738 and T for 78326403. Due to the minor or null differences (in regard to fracture OR) found between carriers of one unfavourable alleles and carriers of zero unfavourable allele on the fracture prevalence, these two categories were combined. Due to the small number of patients with 4 unfavourable alleles (n=3), this category was merged with carries of 3 unfavourable alleles. Overall, we performed the

comparisons as follows: 0/1 versus 2 and 0/1 versus 3 or more unfavourable alleles (see Crosstab in Table 10).

The results suggested an additive effect (p for trend=$7x10^{-4}$), with corresponding adjusted OR 2.76 (95% CI 1.30-5.81; p=$7.4x10^{-3}$) and OR 5.14 (95% CI 1.37-15.67; p=$7.5x10^{-3}$) for 2 and ≥ 3 unfavourable alleles respectively (Table 10).

Table 9. Significant association results for SNP rs884205 and SNP rs78326403 with fracture site

SNP	Fracture site	n	n fractures	P	OR	95% CI	p[1]	OR[1]	95% CI[1]
rs78326403	Wrist/Forearm	1033	34	$7.16x10^{-4}$ [a]	3.12	1.69-5.75	$5.8x10^{-4}$ [a]	3.21	1.74-5.94
rs884205	Spine	1029	62	$8.24x10^{-3}$ [r]	4.05	1.59-10.35	0.025 [r]	3.31	1.24-8.82

[1] The result after additional correction with BMD: FN BMD for rs78326403 and LS BMD for rs884205; [r]=recessive; [a]= log-additive

Table 10. Analysis of the compound effect of genotypes rs9594738 and 78326403: 0/1 unfavourable alleles as the reference group versus 2 and ≥3 unfavourable alleles

Interaction	n unfavourable alleles	n	n individuals with fractures (%)	comparisons	p	OR	95% CI
	0	266	7 (2.6%)				
rs9594738x	1	476	9 (1.9%)	Reference group			
rs78326403	2	244	14 (5.7%)	0/1 vs. 2	$7.4x10^{-3}$	2.76	1.30-5.81
	3	33	4 (12.1%)	0/1 vs. 3/4	$7.5x10^{-3}$	5.14	1.37-15.67
	4	3	0 (0%)				
		p for trend		<0.001			

4.2 Functional Study of Associated SNP rs9594738 and Its Surrounding Region

4.2.1 In-Silico Research of rs9594738 and Its Surrounding Region

The replicated association of rs9594738 together with the results obtained for a neighbouring SNP, rs9533090, which is in complete LD with rs9594738 and was found to be associated with BMD (16,92), suggest that the region harbouring this SNP plays an important role in BMD determination. In addition, the haplotypic blocks found using the Haploview software support the assumption that these 2 SNPs are markers for a larger region, putatively functional (Figure 17).

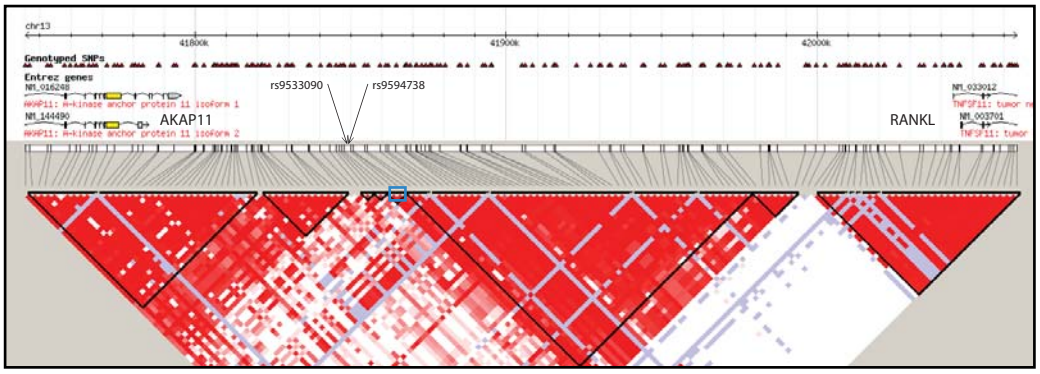

Figure 17. SNP rs9594738 and rs9533090 marked with blue arrows, at the beginning of a haplotypic block between *AKAP11* and *RANKL*. Three large blocks are visible- the one on the left which includes part of *AKAP11*, the central block which begins with rs9533090 and spans over 140 kb, and the third block on the right, which includes the proximal promoter and the first 2 exons of *RANKL*. Modified from the Haploview software.

The *in-silico* study was performed at 2 levels. The first level aimed at identifying transcription factors in a 30 bp sequence (15 bp upstream and downstream to rs9594738). Results of both alleles (C and T) using the Genomatix online MatInspector tool, suggested a recognition site for octamer binding protein and amino acid response element (AARE) binding factors for both alleles but a recognition site for transcription factors PAX 2/5/8 only for the T allele (Figure 18).

The second phase of the *in-silico* research targeted a larger region (of about 1,500 bp including both SNPs, rs9594738 and rs9533090) and aimed at gathering information on existing genes, regulatory elements and chromatin status.

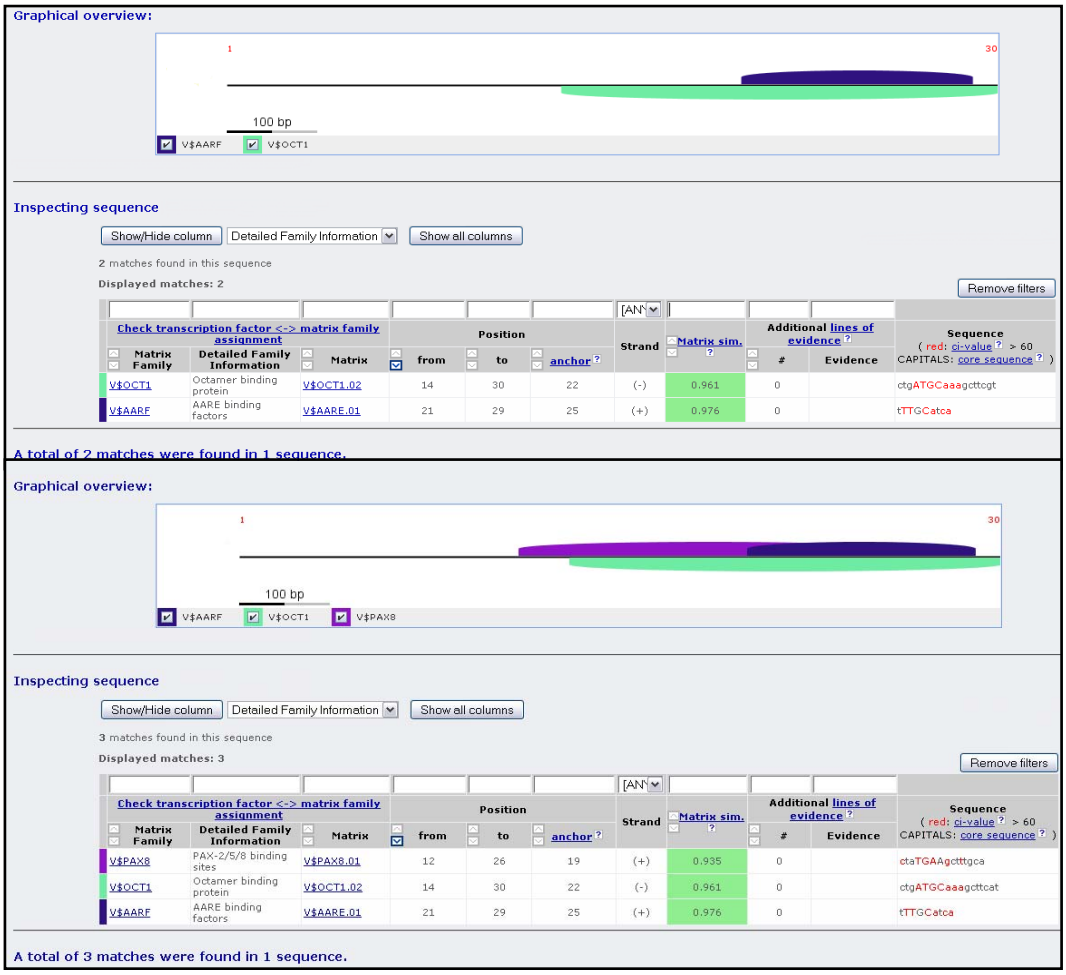

Figure 18. *In-silico* prediction of transcription factor binding sites in rs9594738 immediate nearby region. The upper part is the C allele analysis, and the lower part is the T allele. Octamer (in green) and AARE (in blue) binding factors are predicted to bind both alleles, while PAX 2/5/8 binding site (in purple) is predicted only to bind the T allele. Modified from Genomatix MatInspector webpage.

Another query, at the UCSC Genome Browser, provided the following picture: 1) No gene or human mRNA is displayed in the region. 2) The region contains a DNaseI hypersensitive site of about 300 bp, a hallmark of regulatory regions and promoters in particular. 3) The histone mark H3K27Ac which is found near regulatory elements can be found in GM12878 cells (a lymphoblastoid cell line) 4) In the conserved region between SNP rs9594738 and SNP rs9533090 several transcription factors have been identified (by ChIP-seq) 5) The chromatin status in the GM12878 cell line indicates that rs9594738 is in the centre of an active promoter, surrounded by strong enhancers; the chromatin status in skeletal muscle myoblasts corresponds to a weak or poised enhancer (Figure 19).

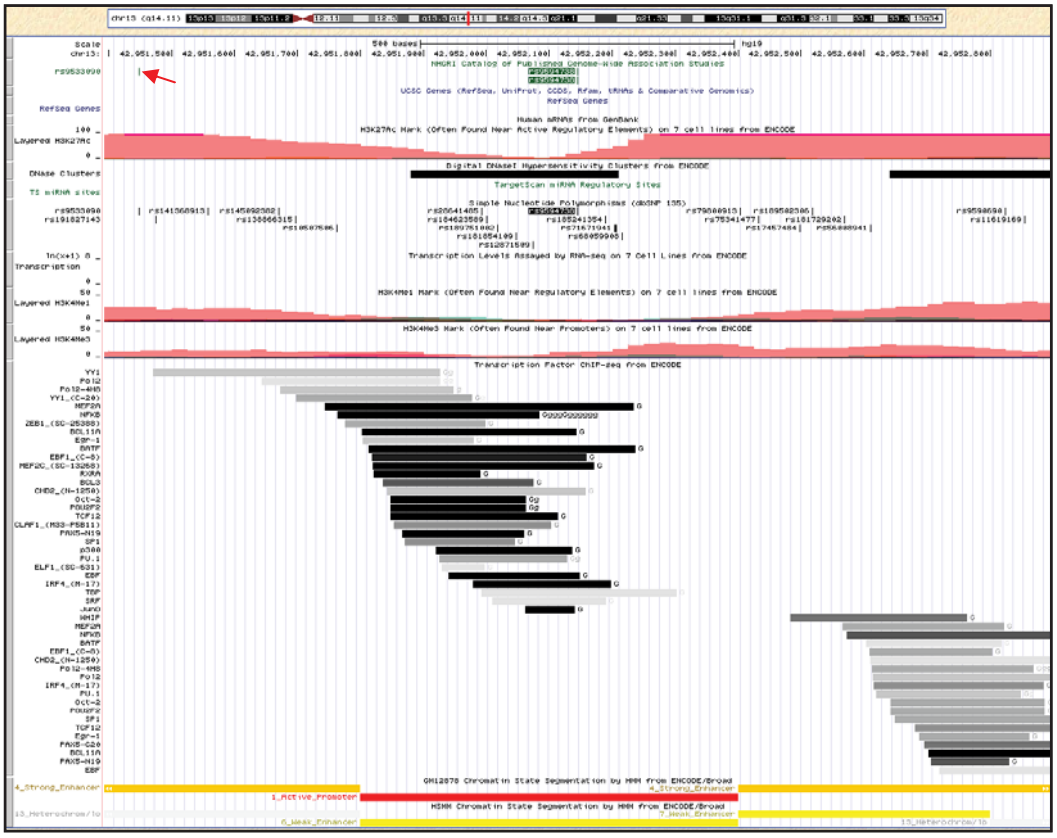

Figure 19. Data mining in regard to SNP rs9594738 and its surroundings (including SNP rs9533090, marked with a red arrow). No evidence is available to the existence of RefSeq genes or human mRNA in this region. The DNaseI hypersensitive area is demonstrated (in black) as well as the histone marks (pink) and the Chip-seq transcription factors (grey to black scale). On the lower part of the figure, the chromatin state in GM12878 cells (a lymphoblastoid cell line) suggests that rs9594738 is located in an active promoter (red line) surrounded by strong enhancers (orange line) and in HSMM (skeletal muscle myoblasts) is in a weak or poised enhancer. Modified from the UCSC genome browser.

4.2.2 Functional Analysis of *RANKL* Proximal Promoter Sequences and a Far-Upstream Sequence

The functional study focused on characterizing the *RANKL* promoter and the possible DR regulatory capacity. DR stands for *Distal Region*, and refers to an 835 pb sequence containing the rs9594738 SNP in a central position (see section 3.9 DNA Constructs in Materials and Methods, page 42 and see below). Reporter gene assays of sequential deletions of the *RANKL* promoter region are presented in Figure 20. No change in the luciferase expression level was found between P1, P2, and P3. P4 displayed a strong promoter activity, characteristic of a basal promoter. Two additional constructions (P1_R3del and P4_R2) were tested. They were both significantly different from P1 and from P4, demonstrating that both R2 and R3 have the capacity to negatively regulate the promoter activity (Figure 20).

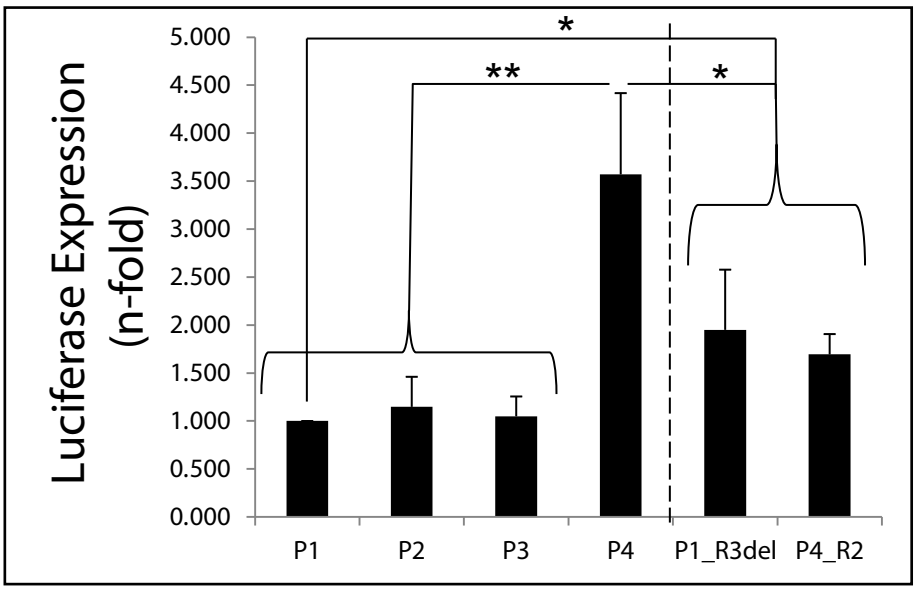

Figure 20. Reporter gene assay results for the different *RANKL* promoter constructions, given by luciferase expression. For each construction different from P1, the graph displays the mean and standard deviation of 14 independent experiments, as compared to the results of P1, arbitrarily set at 1. On the right, the results of P1_R3del and P4_R2, n= 6 and n=5, respectively. (*) $p<0.05$ (**) $p<0.01$

Based on the *in-silico* information, an 835 bp sequence located about 184 kb upstream of *RANKL,* which included the SNP rs9594738 in a central position, was chosen to be tested for regulatory activity, and was named DR. This sequence was linked at the 5' end of each promoter construction (P1 to P4) to achieve constructs P1_DR(C/T) to P4_DR(C/T) (Figure 13A). These constructs were tested for luciferase activity. The DR region did not seem to affect the *RANKL* promoter constructions P1, P2 and P3 in cells cultured in 10% FBS. However, DR inhibited up to 3-fold the basal promoter activity (P4). In cells cultured in FBS-free medium, DR significantly inhibited P1 activity and about 5-fold P4 activity (data shown for P1, P1_DR(C/T) and P4, P4_DR(C/T) in Figure 21 A and B).

In order to test the specificity of the DR effect on the *RANKL* promoter, this DR region was cloned upstream of the *OPG* basal promoter (Figure 13B). In this case, no regulatory effect was observed (Figure 21C). To rule out that the DR effect on the *RANKL* basal promoter might be an artifact due to cloning, we prepared a 920 bp segment derived from the pUC19 vector and cloned it upstream of the *RANKL* basal promoter (Figure 13B). This non-specific region failed to inhibit the reporter expression level of the P4 construction (Figure 21D).

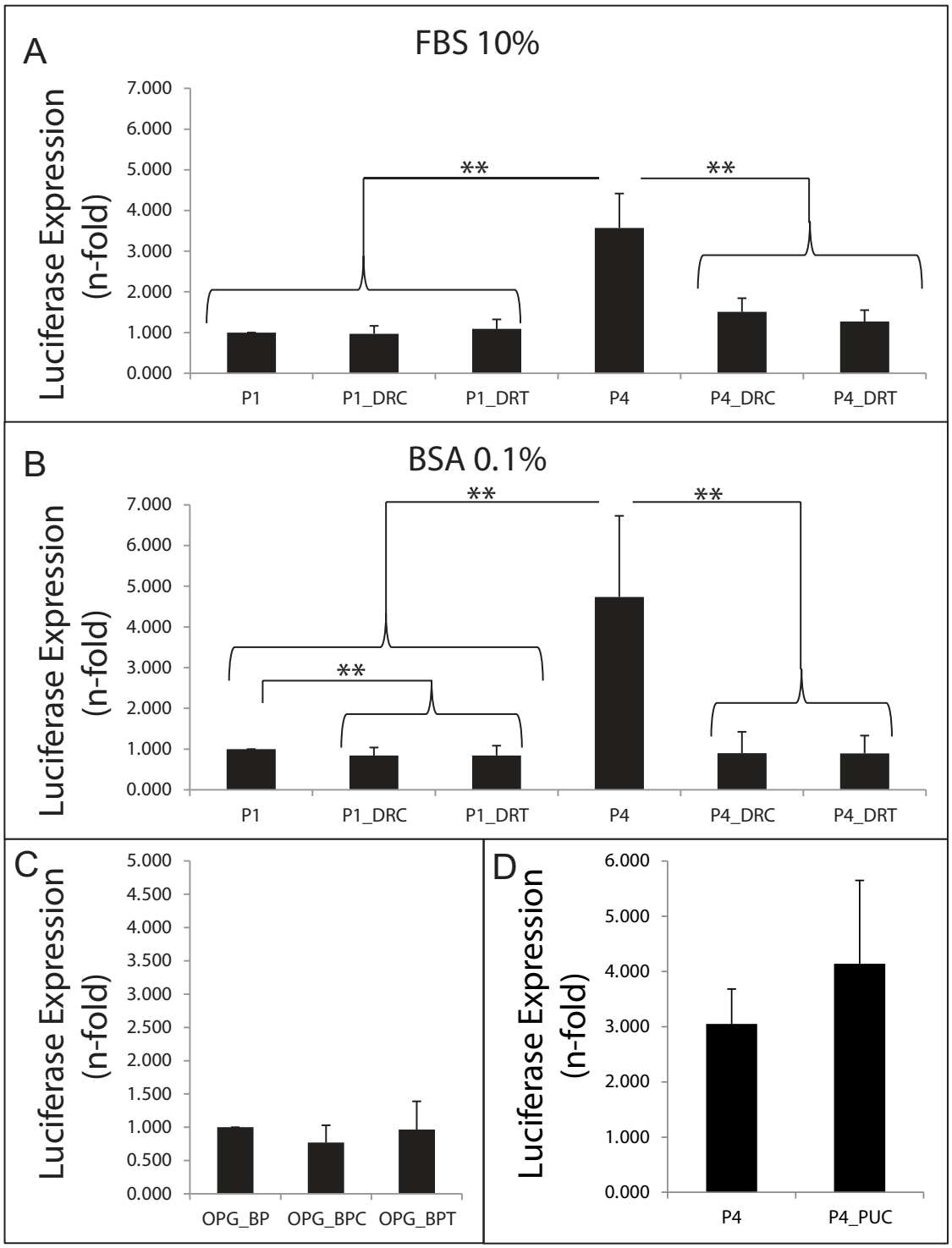

Figure 21. Reporter gene assay results. The graphs represent means and standard deviations (A) The *RANKL* P1 and P4 promoter constructs, with and without DR were tested after overnight incubation in DMEM+10% FBS medium, The number of replicates was n=9 independent experiments comparing each construct to P1, which was arbitrarily set at 1. (B) The *RANKL* P1 and P4 promoter constructs, with and without DR were tested after overnight incubation in DMEM+0.1% BSA medium. The number of replicates was n=33. (C) The *OPG* basal promoter, with and without DR(C/T) segment, tested after overnight incubation in DMEM+10% FBS medium. The number of replicates was n=3 independent experiments comparing each construct to OPG_BP, which was arbitrarily set up at 1. (D) P4 construction with an additional 920 bp segment digested from pUC19 compared to P4, tested after overnight incubation in DMEM+10% FBS medium. The number of replicates was n=5. (**) $p<0.01$.

4.2.3 Effect of Different Treatments on *RANKL* Promoter Activity

To further characterize the *RANKL* promoter and the DR region, the effect of hormones and cytokines known to play a regulatory role in the RANKL/OPG system was assayed on the various reporter constructs. In particular dexamethasone, PTH, 17β-estradiol, TGF-β, TNFα, IL-1 and vitamin D were tested. Results were expressed in reference to the same construct in a non-treated culture. Dexamethasone and PTH did not induce any consistent effect on the different constructions (data not shown). All other tested factors were found to act on the basal promoter. In particular, 17β-estradiol, TGF-β, TNFα and IL-1 reduced the luciferase expression levels, while vitamin D raised it. Data shown in Figure 22 for P1, P4 and P4_DRC.

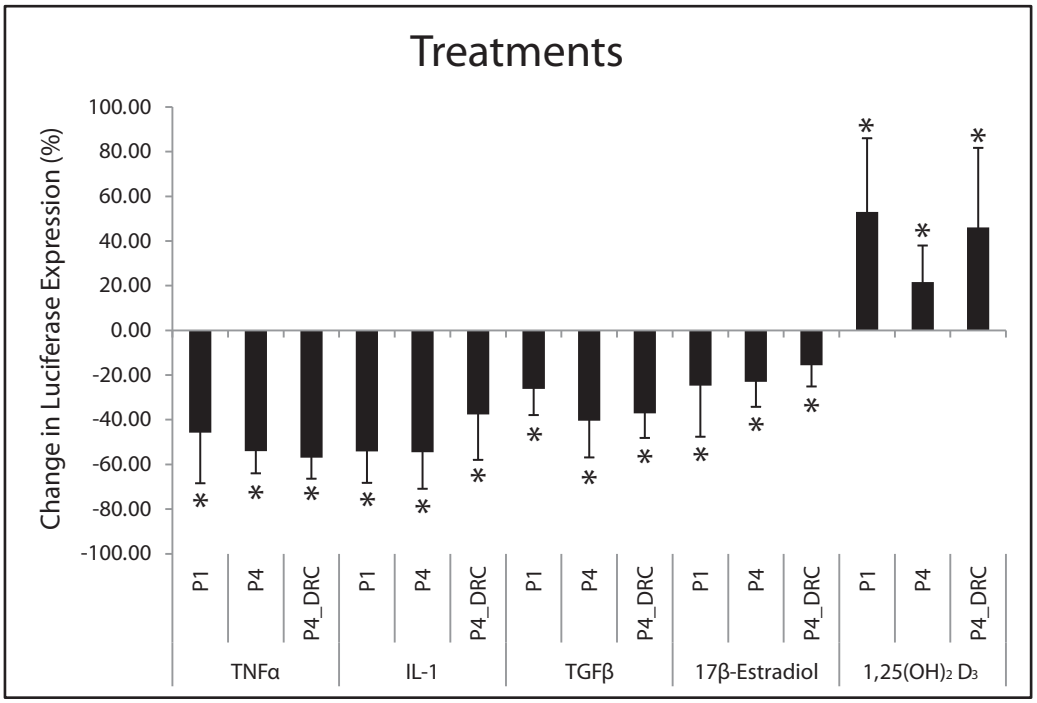

Figure 22. Reporter gene assay results for each treatment added to the cell cultures. The graphs represent means and standard deviation. (*) $p<0.05$. For IL-1, n=5. For 17β Estradiol, n=6. For $1.25(OH)_2D_3$ and TGFβ n= 7. For TNFα, n=8.

4.2.4 Analysis of Nuclear Proteins Binding to Distal Region

An EMSA was performed to detect protein(s) that specifically bind to an oligonucleotide probe containing rs9594738 and to detect possible allele-specific effects (Figure 23). The oligonucleotides harboring C or T allele bound nuclear proteins. Both oligonucleotides bound proteins that competed with an oligonucleotide carrying a glucocorticoid response element. These proteins did not compete with an oligonucleotide containing a Sp1-site. A supershift assay using the glucocorticoid receptor antibody failed to show binding by glucocorticoid receptor. No allele-specific differences were observed, either in the protein binding or in the competition assays. Since the MatInspector tool had suggested the presence of a recognition site for PAX 2/5/8 at the SNP site, PAX2 and PAX5 antibodies were tested in supershift experiments using the probe, but no alteration in the electromobility pattern was observed.

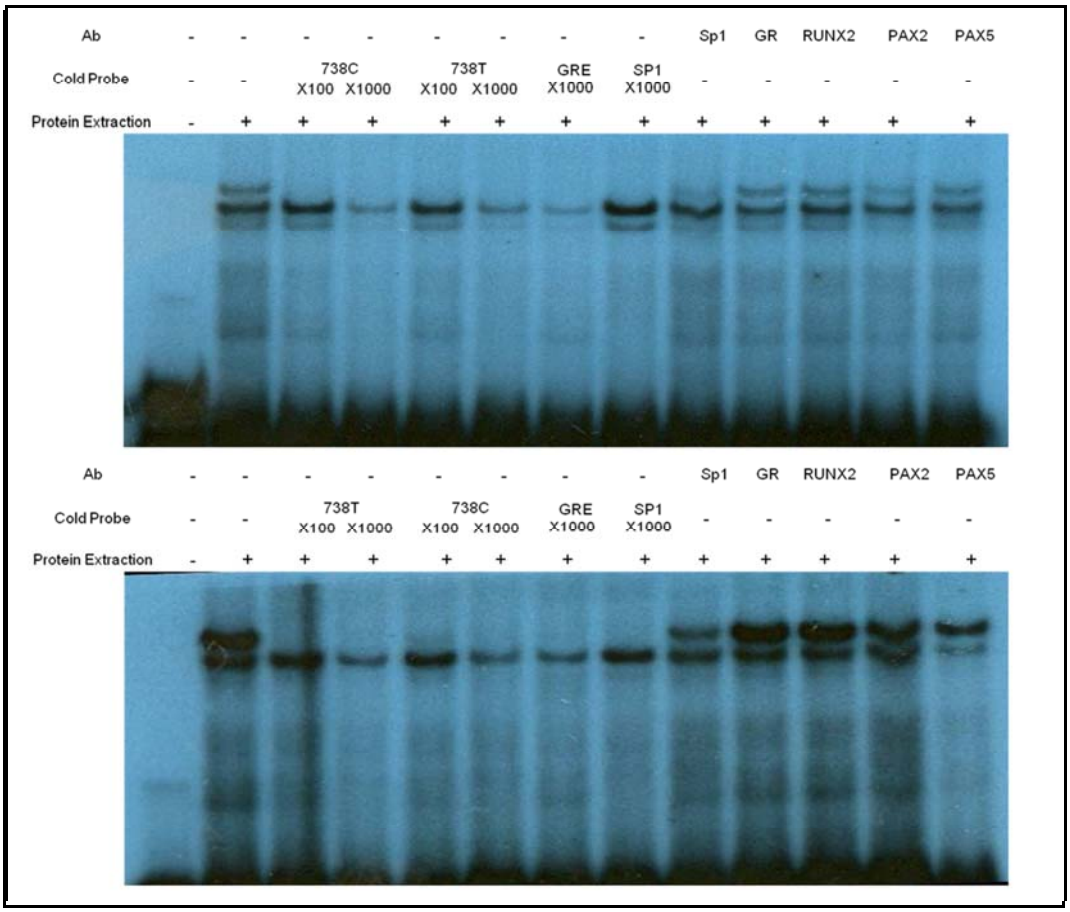

Figure 23. EMSA and supershift results of probes containing SNP rs9594738 with osteoblast nuclear extract. C allele (upper part) and T allele (lower part). Ab=antibody.

4.2.5 Expression Analysis of the Distal Region Sequence

Finally, to achieve a comprehensive view of the DR in the osteoblast context, we extracted osteoblast RNA to test if this region is expressed. A 300 bp region containing the rs9594738 was detected by reverse transcriptase PCR in primary human osteoblast at the cDNA level (Figure 24).

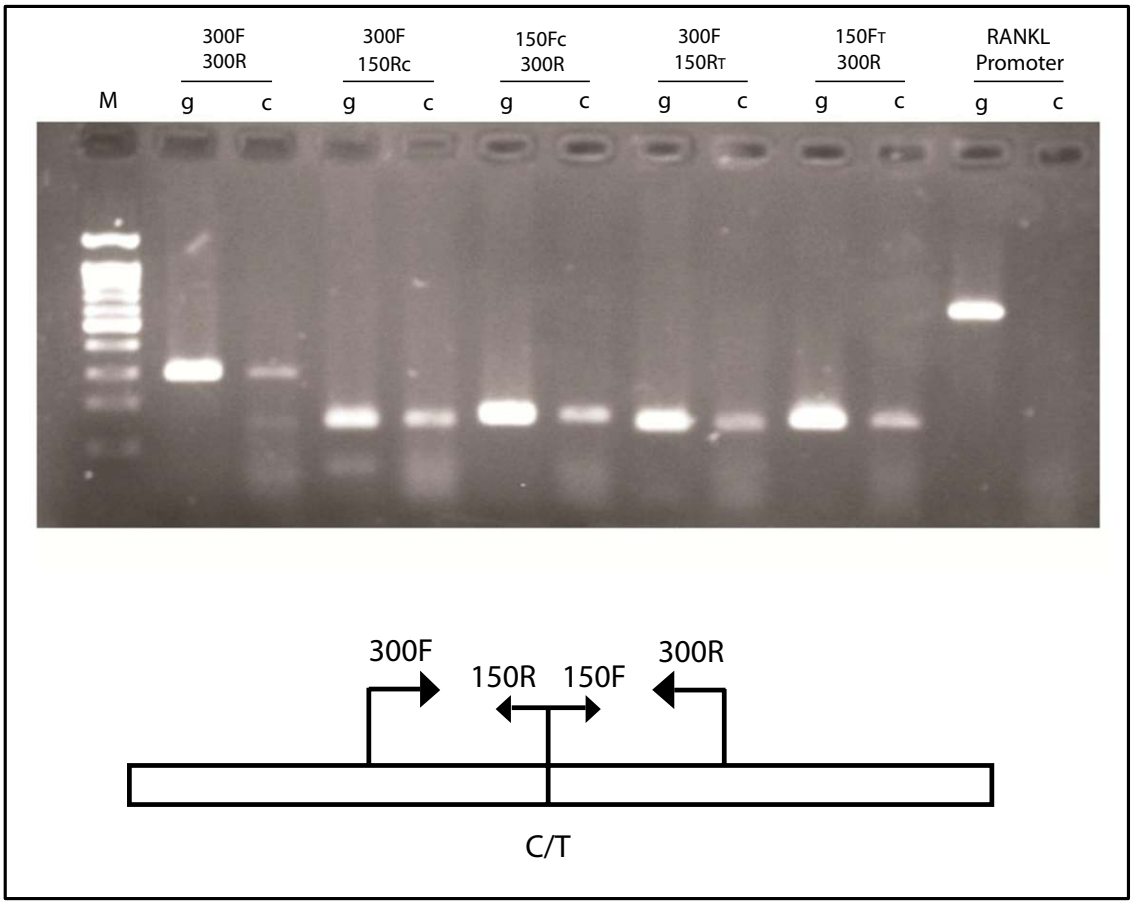

Figure 24. Expression of an RNA segment from the DR. Upper part- PCR amplification from genomic DNA (g) or cDNA (c) using primers located in the DR region as mentioned above each reaction. For each reaction the use of a primer containing rs9594738 C or T allele is indicated by the letters C or T. A reaction with primers located in the *RANKL* promoter was carried out as a control. Lower part- a schema of the primers used for the reaction. The vertical line represents rs9594738. The 150R and 150F primers harbour rs9594738.

DISCUSSION

5. Discussion

5.1 Association Studies of *RANK*/*RANKL* Related to Osteoporosis

Numerous studies have yielded significant associations between *RANK/RANKL* genes and BMD or osteoporotic fractures. Moreover, SNPs and mutations reported in these studies highlighted the contribution of *RANK/RANKL* genes to bone diseases and pathological situations, among them predisposition to low BMD and osteoporotic fractures (Table 4 and Table 5). In accordance, GWA studies identified SNPs within or nearby these genes. However, in many cases these SNPs served as markers to identify the loci or gene(s) involved in the pathological process but neither the SNPs nor their surrounding region were further investigated to determine their functional role.

5.1.1 SNPs Selection and Genotyping

Many genes have been associated with BMD and low-trauma fractures in various cohorts (14-16). In both type 1 and type 2 primary osteoporosis, each genetic variant may contribute a minor effect to the variance in BMD or fracture risk.

In this study we attempted to identify functional SNPs associated with osteoporotic phenotype. Our work focused on the SNPs which fall in evolutionary conserved regions in the *RANK* and *RANKL* genes. Our hypothesis was based on the importance of sequence conservation during evolution. We assumed that variants in regions found to be conserved among vertebrates (*Mus musculus, Rattus norvigicus, Canis familiaris, Bos taurus* and *Homo sapiens,* or mouse, rat, dog, cow and human, respectively) would have a higher probability of playing an important role in gene expression and function of the protein. Other SNPs were selected according to the following criteria: a previous report of association with BMD or fracture risk, or exonic changes (either synonymous or non synonymous).

In the last decade, the regulatory role of 3'UTR in protein-coding genes was demonstrated (126,153). Accordingly, the second phase of the association study focused on the 3'UTR, which is a relatively poorly studied region. The discovery of the

miRNAs emphasized its important role in gene expression regulation. Hence, in this part we identified SNPs located in the 3'UTR of the *RANK* and *RANKL* genes and tested them for association to BMD and osteoporotic fractures. SNPs with published MAF >0.01 in the genes 3'UTR were chosen to be genotyped. Selection of this low MAF level allowed us to detect even low frequency variants which are associated with BMD or fractures.

5.1.2 Key Results

For the *RANKL* gene, rs9594738 was associated with both LS and FN BMD, ($1.7x10^{-4}$ and 0.02, respectively, dominant model), but not with fractures. SNP rs9525642 yielded p=0.03 with fractures, but this result did not stand for the Bonferroni multiple tests correction. None of the SNPs in the 3'UTR was associated with BMD or fractures.

For the *RANK* gene, in the first phase of the study, SNPs rs11152341 and rs12150741 yielded $p<0.05$ with LS BMD (p=0.036 and p=0.026, respectively, over-dominant model). Two SNPs were associated with fractures: rs12150741 (recessive model, OR 0.30 (95% CI 0.08-1.08), p=0.035) and rs1805034 (dominant model, OR 0.67 (95% CI 0.44-1.00), p=0.049). None of these results withstood the Bonferroni correction. As for the 3'UTR SNPs, none was found to be associated with BMD. Two SNPs, rs884205 and rs78326403, yielded $p<0.05$ for 'any-fractures' analyses. These results are discussed in section 5.2.

5.1.3 Limitation of the Methods

The relatively small sample size available in the BARCOS cohort (909 women in the first genotyping project and 1098 women in the second project) limits the statistical power of the study and therefore our ability to identify and analyse rare genotypes or variants with small effects. In addition, we used the highly conservative Bonferroni multiple tests correction, in an effort to avoid the description of spurious associations. This approach also may discard significant true-positive associations and may result in loss of information.

Our results might be specific to the population studied, which was limited to Spanish Caucasian postmenopausal women. Further studies in other cohorts, with

similar and with different characteristics, are needed to determine whether the associations we report could be replicated, extended to men and/or to other ethnic or age groups.

5.1.4 Results Analysis and Interpretation

Three SNPs were significantly associated with osteoporotic phenotypes: one with BMD in the *RANKL* gene and two with fractures in the *RANK* 3'UTR.

Although some SNPs yielded $p<0.05$, none of the assessed SNPs in the proximal promoter, intronic and exonic regions were significantly associated with any osteoporotic phenotype after Bonferroni correction. The result for the SNP in the far upstream region was replicated in the last GEFEOS-GENOMOS meta-analysis study by genotyping SNP rs9533090 (which is in LD with rs9594738) (92).

The weak associations for the SNPs in the *RANKL* gene and promoter might be false positive results, but they could also be a consequence of the limitations of the available sample size, as mentioned above. An explanation for the lack of association of most of the SNPs tested may be that they do not play important roles in *RANKL* expression or functionality, despite their location in conserved regions. On the contrary, there are accumulating indications, from this study and from previous ones, that rs9594738 lies in a strong regulatory region with the capacity to regulate the gene.

Furthermore, bearing in mind the complexity of the disease, it might be suggested that a combination of several genetic variants in the region, a haplotypic point of view, should be considered rather than analysing SNPs individually.

Usually, a result is considered significant only when the association has a p-value, after Bonferroni adjustment, which is lower than the new p target. Bonferroni is the most conservative method, and the most commonly used for GWAs and other multiple tests experiments. Analysing the genotyping results in our study obviously requires a certain correction in order to avoid any false positive results (type I errors). The current study does not deal with thousands to millions of independent SNPs (as in GWAs). Hence, a doubt should be raised about whether this is the appropriate method to be used, and not only in this specific instance. The discussion regarding the delicate equilibrium between the methods used for multiple test correction and the possible loss

of biologically significant results is recurrent in the literature. The critics' debate about the use of the Bonferroni method is between a total rejection of the method due to the increase in type II errors (154) and modifications of the Bonferroni adjustment (155). In addition, the number of tests to be considered using Bonferroni is not clear. If the general idea is to reduce the occurrence of type I errors, should one sum up all the statistical tests per publication or per hypothesis? And what should be considered in the case of several different reports derived from the same study? (154) For instance, our group used the SNPlex genotyping method in another study. Using this method, genotyping a whole plate of 48 SNPs costs the same as genotyping a partial plate. Hence, our goal was to design a full 48 SNPs plate by combining different and independent studies, only to gain maximum cost-effectiveness. In this case, should n=48 be considered as the number of tests? Or should each investigator consider separately the number of genotyped SNPs relevant to his study? These questions have no clear answers. We decided to adopt the most commonly used and most conservative approach (i.e., Bonferroni) in order to meet the standard criteria for publication of our results.

Regarding the *RANKL* SNPs, we replicated 2 SNPs from Styrkarsdottir et al. (15): rs9594738, which lies about 184 kb upstream of the gene, and rs9594759, which lies about 104 kb upstream. While rs9594738 yielded a significant result with BMD ($p=1.7x10^{-4}$), rs9594759 yielded only a borderline p value (p=0.085, recessive model). Although a high recombination rate between the two SNPs is a possible explanation, an *in-silico* study using the HapMap website indicated that both SNPs are situated in the same haplotypic block. In the combined Icelandic cohort of the original study (n=10,023), rs9594759 yielded a p value about 100-fold higher than rs9594738 ($2.2X10^{-12}$ vs. $1.5X10^{-14}$, respectively). This ratio was maintained in our cohort, suggesting that the association between the 2 SNPs and BMD is actually no different than in the original study, but our cohort size limits us in detecting the significance of this SNP.

5.2 The Fracture Site Dependent Association Found in *RANK* Gene

5.2.1 Key Results

We have identified 2 SNPs within the *RANK* 3'UTR region, rs78326403 and rs884205, which are associated with osteoporotic fractures. Even though only one SNP withstood the Bonferroni adjustment (rs884205), we further analysed both for association with a specific fracture site. SNP rs78326403 produced $p=7.16x10^{-4}$ (log-additive model: OR 3.12; 95% CI 1.69-5.75) with wrist/forearm fractures and SNP rs884205 yielded $p=8.24x10^{-3}$ (recessive model: OR 4.05; 95% CI 1.59-10.35) with spine fractures. Both results withstand the Bonferroni correction. Moreover, after adjusting the results for the relevant BMD (FN BMD for rs78326403 and LS BMD for rs884205) these associations were still significant. Yet, post BMD correction, SNP rs884205 is attenuating to a border line p, suggesting that BMD may act as a confounder in the association.

In addition, we have described a significant interaction between the *RANKL* SNP rs9594738 and rs78326403, but not with rs884205. An additive effect of compound genotypes yields significant results for rs9594738 and rs78326403 in the 0/1 versus 2, and 0/1 versus 3 or more unfavourable alleles (OR 2.76 and OR 5.14, respectively).

5.2.2 Limitation of the Methods

Association with fractures has several limitations. The main limitation is the definition of fractures, in contrast to BMD, which is a numeric value. It is estimated that up to 70% of vertebral fractures are left undiscovered (156,157), which may lead to inaccurate classification of participants in the different cohorts studied. Second, despite standard criteria physicians may define the occurrence of fracture and its nature as "low trauma" differently.

In the 1098 women of the BARCOS cohort, 13.8% have been identified with low-trauma fractures. The sample size limits the statistical power of this study and especially limits our ability to detect rare variants. In addition, and as discussed earlier, we used the most conservative correction for multiple tests (Bonferroni).

Finally, to strengthen the significance of the results, the study should be replicated in other cohorts. As a first phase, a genetically related cohort, such as another cohort of Spanish or Portuguese women with similar characteristics, should be studied. As a second phase, larger cohorts with different characteristics should be studied to determine the generalizability of our results.

5.2.3 Results Analysis and Interpretation

We genotyped 8 SNPs in the *RANK* 3'UTR and two in the *RANKL* 3'UTR. Two SNPs, rs884205 and rs78326403 in the *RANK* gene were associated with low-trauma fractures in the BARCOS cohort.

Due to the limited information provided by the mostly algorithm-based databases available, it is impossible to confirm or discard the SNPs location in a miRNA binding site. Research on miRNA is a relatively young field and the lack of functional studies is notable. Moreover, the definition of the 3'UTR is not identical in all databases, and may differ in matters of length or location. In the ENSEMBL database, the two significant SNPs in this study are in the *RANK* 3'UTR, but in NCBI, they are downstream of the *RANK* 3'UTR. The ENSEMBL 3'UTR is longer by almost 1400 bp compared to the 3'UTR given by the NCBI. Had we analysed only one database, we might have failed to notice these SNPs. Moreover, our lack of ability to supply more information in regard to the position of these SNPs in miRNA target sites is due to the differences between the *RANK* 3'UTR in ENSEMBL and the target site prediction algorithm based on the NCBI *RANK* 3'UTR. Also to be considered are the differences found between the SNP databases in the existence, or absence, or published MAF of particular SNPs.

This emphasizes the special attention that should be paid to the *in-silico* research. On the one hand, the need to perform in-depth analysis with massive data volume has made investigation totally dependent on computers, databases, software and applications. On the other hand, if not properly analysed the flood of information may be misleading and result in poor decision-making. In this study, we applied multiple algorithms commonly in use: the Genomatix algorithm to predict the factors binding to the region around rs9594738, the ENSEMBL and NCBI algorithms to predict the 5' and 3' UTRs , the miRNA binding sites prediction algorithm, etc. The results point out to

the limitation of these algorithms and emphasized, once again, the crucial need for experimental functional studies.

This is the first time that SNP rs78326403 has been associated with fractures. SNP rs884205 was previously related to osteoporotic phenotypes (16,158). In contrast to our results, rs884205 was found to be associated with BMD but not with fractures in a recent meta-analysis by the GEFOS-GENOMOS consortium that includes the BARCOS cohort (92) . This difference might be explained by heterogeneity among the different cohorts, differences in assessing low-trauma fracture, or both. This difficulty of obtaining a well-established osteoporotic fracture phenotype in different groups is one of the major limitations of meta-analysis in this field. Our group rigorously assesses fractures, including validation by X-ray. Not all the participating cohorts had fractures validated in the same manner. Furthermore, many additional factors may determine fracture risk among the elderly, such as malfunctioning body equilibrium, reduced response time, reduced vision, etc. Hence, we cannot ignore the possibility, among others, that the fracture incidence has some association with other reduced abilities or syndromes in the elderly population.

Future replication with cohorts similar to BARCOS should clarify the association of these SNPs with fracture risk. Regarding the controversial BMD results, we cannot discard the possibility that rs884205 may be involved in BMD and the sample size limitation did not allow us to detect this association.

The SNPs associations with different fracture sites, along with no compound effect being observed when these 2 SNPs were analysed together, suggest that various SNPs in the same gene (*RANK*) and even in the same region (3'UTR) may differentially influence each fracture site (predominantly cortical vs. trabecular bone). Thus, we hypothesize the existence of different *RANK* regulatory patterns depending on the bone compartment. Other distinct regulators of cortical or trabecular bone include the GH-IGF1 axis (159-162) and gonadal hormones (163-166). These hormones regulate bone remodelling via RANK/RANKL/OPG (167-171). Therefore it seems plausible that distinct hormonal actions are regulated through different pathways in the RANK/RANKL system. The associations with different types of fracture suggest a distinct mechanism behind bone fragility in predominantly cortical versus predominantly trabecular bones.

Consistent with our data, a number of genetic association studies report a different heritability for BMD and fracture (or bone quality) (14-16). Accordingly, in addition to bone densitometry, other measurements of bone quality or microarchitecture such as finite element analyses (172) or microindentation techniques (173) should be considered for accurate fracture risk assessment in clinical settings.

However, it is widely known that these osteoporotic phenotypes are closely related, and our results reinforce this. We found a significant interaction between the *RANKL* BMD-associated SNP rs9594738 and *RANK* fracture-associated SNP rs78326403. When the unfavourable T allele for rs9594738 is present the individual is predisposed to low BMD, while when the unfavourable T allele for rs78326403 is present the individual is predisposed to higher fracture risk. Hence, we hypothesized that the accumulating effect on the individual is higher than the effect of each SNP separately. This interaction, significant only with rs78326403 but not with rs884205, suggests an epistatic effect between *RANK* and *RANKL* and supports the idea that each SNP may act independently, although located in the same region.

These findings might be clinically relevant in the future to achieve a more specific approach to the different types of fractures, both to better understand their underlying mechanisms and to search for site-specific therapeutic strategies.

5.3 Functional Studies in the *RANKL* Context

Relatively small efforts have been carried out to identify the functional and biological effect of SNPs associated in GWA studies. We performed a comprehensive functional study with the SNP associated with BMD in our cohort. This SNP lies in a region between *AKAP11* and *RANKL* that was described by O'Brien et al. as a highly conserved region throughout vertebrate evolution (174). The objectives were to study the different *RANKL* proximal promoter regions and to perform an in-depth analysis of the region harbouring rs9594738 and its putative functional effect on the *RANKL* gene. Reporter gene assays, EMSA and supershift experiments and expression analysis of the SNP and its surroundings were performed in addition to *in-silico* research.

5.3.1 Key Results

Reporter gene assays of sequential deletions of the *RANKL* proximal promoter revealed the existence of two inhibitory regions (R2 and R3) that independently decrease the basal promoter expression levels.

In addition, an 835 bp region located about 184 kb upstream of *RANKL* (labelled DR) and harbouring SNP rs9594738 in a central position inhibited up to 3-fold the basal promoter activity in cells cultured in the presence of 10% FBS and about 5-fold in cells cultured in FBS-free medium. Moreover, the DR significantly inhibited the P1 promoter activity in the serum free medium.

The effect demonstrated in the presence of 10% FBS medium suggests the existence of regulatory elements in DR which have the ability to bind factors present in FBS. Hence, the second phase of the study was to try to identify them by testing the effect of several cytokines and hormones (known to play a regulatory role in the RANKL/OPG system) on the *RANKL* promoter and DR constructs. Dexamethasone and PTH did not induce any consistent effect on the different constructions. All other tested factors did act on the basal promoter: 17β-estradiol, TGF-β, TNFα and IL-1 reduced the luciferase expression level, while vitamin D raised it. The latter also presented a different effect pattern.

Subsequently, further studies were executed to characterize rs9594738 and its nearby region. An EMSA was performed to detect osteoblast nuclear proteins that specifically bind to the region containing rs9594738. Both oligonucleotides bound proteins that competed with an oligonucleotide carrying a glucocorticoid response element. These proteins did not compete with an oligonucleotide containing an Sp1-site. However, a supershift assay using the glucocorticoid receptor antibody failed to produce binding. No allele-specific differences were clearly observed in the protein binding or competition assays. *In-silico* analysis indicated the presence of an amino acid response element (AARE) (175) and an element for octamer-binding protein 1 in both alleles but a transcription factor PAX 2/5/8 recognition site only for the T probe allele. Hence, PAX2 and PAX5 antibodies were tested but no alteration in the electromobility pattern was observed.

A reverse transcriptase expression analysis of the DR sequence detected expression of at least 300 bp of the region containing rs9594738 in primary human osteoblast at the RNA level.

5.3.2 Limitation of the Methods

Functional *in-vitro* studies have several limitations. The main limitation is the putative functional SNP(s) selection to be studied. For example, Styrkarsdottir et al. (15) genotyped rs9594738, while Rivadeneira et al. (16) and the GEFOS-GENOMOS consortium (92) genotyped rs9533090, which lies in the same region, 696 bp upstream of rs9594738. Both SNPs yielded significant results associated with BMD, limiting our ability to detect the functional SNP.

Reporter gene assays are being carried out *in-vitro* under artificial conditions. In this regard, limitations include the artificial definition of the promoter, the plasmid structure, the cell source (a human osteosarcoma cell line in our case), the culture medium and the treatment administration. Altogether, this method can provide a pattern of gene expression under different circumstances and specified conditions. However, the results cannot reliably extrapolate the effect of these elements to the physiological environment in human tissue that is in its natural environment.

The EMSA limitations derive as well from its artificial conditions, as mentioned above. Moreover, the probe used in the reaction contains only 30 nucleotides, which might limit both its ability to be detected by proteins that require a longer DNA sequence for binding and the stability of the binding complex. Although using excessive nuclear protein concentration may result in unspecific binding reactions, it should be mentioned that this limitation is being addressed by performing the competition reaction in excessive conditions and in several concentrations.

The main *in-silico* limitation is the algorithms used to predict biological elements or motifs in the studied sequence. A predicted binding site is based on the probability that a specific protein will recognize the studied sequence, not considering the biological feasibility of the event. For example, DNA structure and conformation inside the chromatin and the current status of the binding sequences in the nucleosome are obviated.

5.3.3 Results Analysis and Interpretation

The functional study was mainly focused on the DR, region containing rs9594738. Nevertheless, this study broadened the knowledge available about the *RANKL* proximal promoter regions. Two inhibitory regions were identified using the reporter gene assays- R2 and R3. Gene expression increased in the absence of R3 and decreased when R2 was linked to the 5' of the basal promoter (P4). The basal promoter, which lacks both R2 and R3, demonstrated the highest increase in luciferase expression (3-5 folds). This established the assumption that the core elements for the gene expression are located in the basal promoter region while inhibitors and other regulatory elements are located upstream.

Several studies have previously reported the presence of responsive elements in the *RANKL* promoter, either in human or mouse: vitamin D response element (VDRE) (176), glucocorticoids response element and GATA-1 and AP-1 transcription factor binding sites (177) are found in the promoter. In addition, PU-1 and AP-2 transcription factors and RUNX2 response elements (essential proteins for osteoblastic differentiation) (178) are found in the basal promoter (176,177,179).

Subsequently, we aimed to identify the factors in the 10% FBS medium that regulate the *RANKL* promoter activity. Treatment regime with seven cytokines and hormones that are known regulators of the RANKL/OPG system demonstrated their effect on the promoter. All factors that affected gene expression did so on the basal promoter. In general, all treatments produced a similar effect on the four promoter constructs; the only exception was vitamin D. Other groups have tested the effect of these factors on *RANKL* expression using different cell lines and methods, with controversial results (73,180-182). However these may be attributable to the different conditions used in each experiment, such as studies based on *in-vivo* animal models (183) versus *in-vitro* experiments using human cells derived from various origins (184-186).

The vitamin D stimulatory effect on P1 was higher than on P4. In agreement with our results, *in-vitro* studies have demonstrated vitamin D action on osteoblast lineage cells to induce *RANKL* expression (187,188). In addition, a functional VDRE site has been defined in the human *RANKL* promoter at position -1570 to -1584 (176),

corresponding to R1 in our study. This can explain the relatively smaller differences observed in the luciferase expression between P1 and P4 in 10% FBS medium (which contains vitamin D). Yet, it is important to mention that the full impact of vitamin D activity may differ as a result of chromatin structure and the epigenetic conformation, such as cytosine methylation (189).

5.3.3.1 The Distal Promoter Role in *RANKL* Expression

The replicated association of rs9594738 with BMD may indicate that this SNP or a relatively nearby genetic variant plays a functional role in BMD determination. As mentioned above, this region, containing rs9594738 and about 184 kb upstream of *RANKL*, is part of a highly conserved region located between the *RANKL* and the *AKAP11* genes. In all species with an identifiable *RANKL* gene, *AKAP11* lies upstream of *RANKL* while the downstream gene varies (174). Another indicator of the evolutionary conservation of the region can be supplied by the Haploview software, based on the HapMap database. The existence of a 140 kb haplotypic block (which includes DR in its 5' end in respect to the *RANKL* gene) between 2 other blocks, one containing part of the *RANKL* gene and the other part of the *AKAP11* gene, demonstrates the high conservation level of the area. The preservation of this large intergenic region may indicate its importance.

AKAP220, encoded by the *AKAP11* gene, is involved in signal transduction and in cell migration (190,191). The protein is highly expressed in human testis, brain, heart and during spermatogenesis and in the mature sperm. It was also suggested to play a role in cell cycle control in somatic cells as well as in germ cells (192). Hence, the functional association with *AKAP11*, if there is any in bone, is unclear. With regard to bone metabolism, rs9594738 association with BMD and genomic position may imply involvement in the transcriptional regulatory mechanism that controls *RANKL* expression (174). Several murine studies highlighted the regulatory importance of this region. For instance, Onal et al. (193) demonstrated that the deletion of a 2.3 kb segment of the corresponding murine region, which they named DCR, reduced *Rankl* expression. Other experimental evidence in mice showed that regulatory elements residing ~70 kb upstream of the gene (193,194) affect its expression (174).

The association found between the nearby SNP rs9533090 and BMD and the LD between this SNP and rs9594738 demonstrate that any SNP between these two SNPs may be the casual one associated with BMD. A comprehensive functional study of the region and haplotypic combinations of SNPs in the region may reveal more of its role in bone metabolism. No LD was found between the SNPs in the promoter and the significant SNP in the DR.

Reporter gene assay results have revealed a DR regulatory effect on the expression of *RANKL*. A significant enhancement is observed in 10% FBS medium, comparing luciferase expression between P4 and P4_DR(C/T). Stronger and significant enhancement was observed in the absence of FBS on P4_DR(C/T) and on P1_DR(C/T) comparing to P4 and P1, respectively.

In order to evaluate if DR acts on specific promoters or has the ability to repress any regulatory region, we cloned DR upstream of the *OPG* gene basal promoter in the same manner as in P4_DR(C/T). In this case, our observations demonstrated that DR did not act on the *OPG* basal promoter. Our results strongly suggest that DR can only regulate certain recognition sites, which are present in the *RANKL* basal promoter. We cannot rule out DR modulation of other regulatory regions present in other promoters or affecting the bone remodelling cascade in any other pathways. Further studies are needed to clarify this issue.

These results in 10% FBS medium not only demonstrate the DR role as a distal regulatory sequence, but also suggest that factors in the 10% FBS medium may recognize elements in the DR sequence and stimulate gene expression. In the absence of these factors (using DMEM+BSA 0.1% medium) the gene expression decreases.

Vitamin D produced higher stimulation on P4_DR constructs than on P4, suggesting it might be one of the factors present in FBS responsible for the enhancer effect on the luciferase activity. Consistent with this, a recent ChIP-seq assay (195) showed the existence of a VDR binding site within the DR. In this regard, Kim et al. (194) identified multiple enhancer regions in response to $1,25(OH)_2D_3$ at ~75 kb upstream to *Rankl* gene in mice. One of these regions is conserved in humans and is

functional. They hypothesised that a chromatin hub centered on the *Rankl* promoter allows the distant enhancers to act and to regulate the gene expression. Other genes, such as *osteocalcin*, are also regulated by interaction between VDR and their promoters (196). In this case, nucleosomal remodelling is required in order to allow the VDR binding to the promoter of the gene.

The experimental evidence in our study, combined with others, confirms the existence of *RANKL* distal regulation. Hence, we attempted to identify whether the sequence harbouring rs9594738 binds any specific nuclear factor. EMSAs were performed using 30 bp oligonucleotides containing this SNP (at midpoint of the probe). The binding was not totally specific due to competition with an oligonucleotide containing GRE. However, supershift assays with GR antibodies did not confirm this competition. *In-silico* study using the Genomatix website suggested that PAX transcriptional factors (2/5/8) bind only when the T allele is present. However, supershift assays failed to show a specific binding of PAX factors.

In the current draft of the human genome, no gene or human mRNA overlaps the DR. In the expression analysis of the DR we describe the existence of at least 300 bp transcribed from the region containing the SNP. Hence, this is the first report describing the existence of this RNA, which could be involved in *RANKL* and possibly other genes expression regulation, via several pathways or mechanisms. In this regard it should be mentioned that the current ENCODE project results show that about 90% of the genome (of the studied regions) is being transcribed (197), with unknown functionality for the grand majority of this RNA. These findings should be further investigated, either *in-vivo* or *in-vitro*, to define exactly the role of this mRNA, if there is any, in cellular function.

5.4 Osteoporosis Genetic Research Concerns

5.4.1 GWA vs. Candidate Gene Approach

In the field of complex disease, the prestige (and thus the publications) of GWA and meta-analysis studies is much higher than that of candidate gene studies. However, both association methods have their limitations, and the decision about which approach to use should be considered from the beginning of the study design and for each experiment separately.

GWA studies have contributed significantly to the discovery of new genes and loci involved in the pathological process of numerous diseases. However, in GWA the number of genotyped SNPs directly affects the new p target, which in turn requires large cohort meta-analysis in order to reach significance. In addition, GWA does not allow revealing rare genetic variants. For rare variants the surrounding region is not in LD and cannot be identified by association studies. Actually, GWA allows us to detect genes only when the "common variant-common disease" is true.

These rare variants are believed to have greater effect than the common variants, due to the assumption that common variants with high effect will not survive natural selection. This results in GWAs leaving “missing” or “phantom” genetic factors and traits (198). Moreover, a large amount of information can be lost under the strict GWA rules for statistical analysis and multiple tests correction. A large sample size is required to achieve statistical power and results might not be considered significant due to small statistical power (Figure 25) (199).

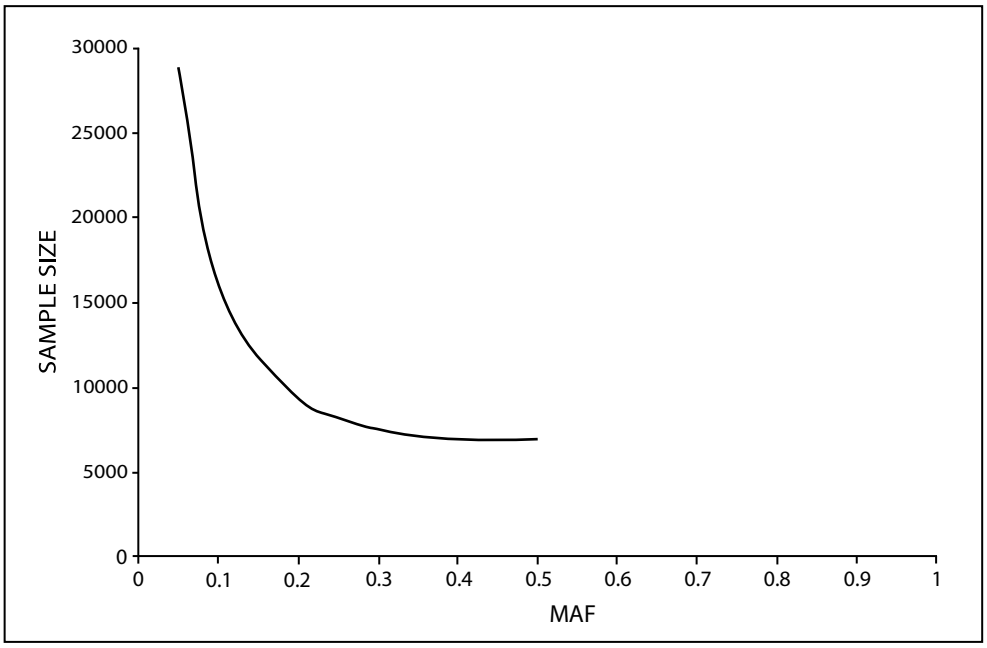

Figure 25. The sample size required to achieve 80% statistical power, with respect to the MAF. Modified from Duncan et al. 2010 (199)

On the other hand, the candidate gene approach has failed to produce many significant findings in the complex disease field. Moreover, even when candidate genes are associated with osteoporosis phenotypes or linked with BMD in family studies, their significance is hard to estimate due to lack of robust evidence in the general population and cannot be considered "established" (4).

Nevertheless, the candidate gene approach might be used as a complementary tool to GWA results. Combination of both methods could lead to the discovery of important aspects of the studied disease. In this work, we selected 2 genes well known to be involved in bone metabolism. Both genes were associated with osteoporosis phenotypes in previous GWAs. We replicated some of the mentioned SNPs in our cohort, but we also genotyped other SNPs which were hypothesized to be putative functional SNPs in the selected genes. The focus on the selected genes supplied a more comprehensive view and cohort-specific association of the studied genes. This combined approach should be followed by in-depth analysis of the studied region to reveal its biological functionality.

5.4.2 Meta-Analysis vs. Small Cohort Studies

In the current study, rs884205 was associated with fractures and not with BMD in the BARCOS cohort. However, in the GEFOS-GENOMOS consortium to which BARCOS belongs the SNP was associated with BMD but not with fractures. This contradictory result raises a doubt: despite gaining size and statistical power by using meta-analysis and a large scale cohort, do we also lose information regarding the different cohort characteristics that may affect the homogeneity of the outcome tested?

Ioannidis et al. (91) discuss this issue. Their meta-analysis of 370 studies demonstrates the need and advantages of meta-analysis and results replication. Several cases with significant results in the original cohort either failed to be replicated or had dramatically reduced OR when replicated. On the other hand, non-significant results reached the significance threshold when replicated or analysed by meta-analysis in a cohort larger than the original one (Figure 26). However, one should consider the possible loss of cohort-dependent information. In the present study, in regard of SNP rs884205- is the lack of association with fractures in the GEFOS-GENOMOS study the result of high heterogeneity in the participant cohorts or is the lack of association with BMD in our cohort due to reduced statistical power-- or both?

Not only does LD differ between ethnicities, but it might vary within the same ethnic group due to the fact that SNPs frequencies have been demonstrated depend on the region (200). On the one hand, mixing groups from different origins and even different ethnicities helps to mark the common variant and eliminate false positive results. On the other hand, a more homogeneous group might help to discover rare variants and avoid the elimination of true results identified as type I errors.

A GWA study for BMD performed in an East Asian population demonstrates this point. The study failed to replicate the majority of the genes associated with BMD in white European populations (*RANKL* yielded p=0.012) (201). Considering the differences in BMD measurement site (radius or tibia) and the genotyping method, it can be argued that the results are not due to heterogeneity. Other GWAs, in the immunogenetics field, showed that *IL23R* gene is a major gene for some diseases in white Europeans (202,203). Yet, the SNP associated with each disease is not

polymorphic in east Asians (204,205), and therefore the gene identified in Europeans is not associated with a given disease in the Asian population (199).

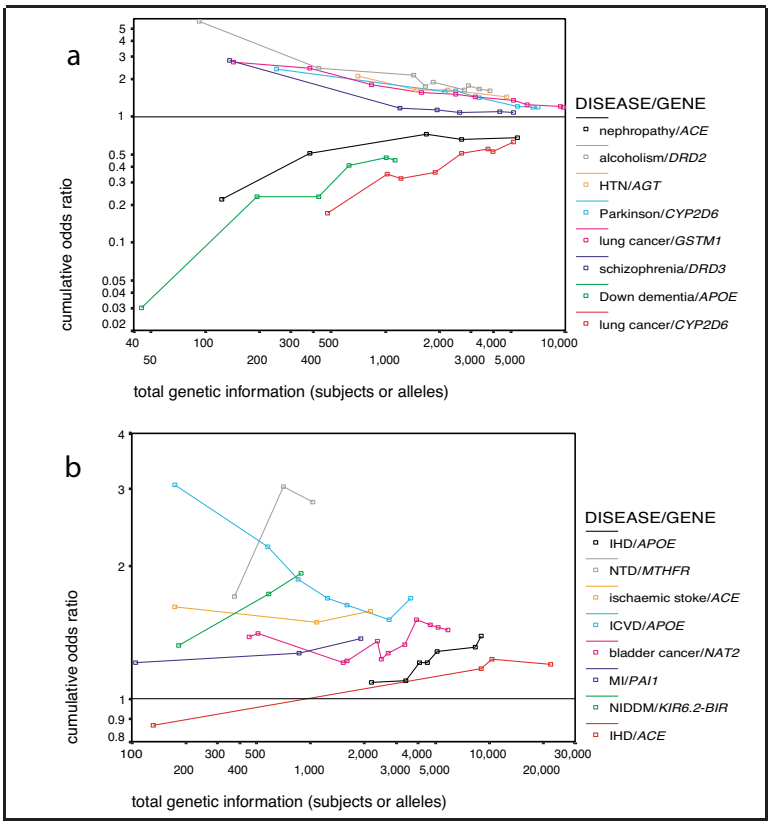

Figure 26. Replicated association study results with respect to number of subjects, estimated by OR. (a) Studies which yielded significant results in first association study but the results converged towards OR=1 as further cohorts were integrated into the analysis. (b) On the contrary, eight pathological situations that failed to reach significance in the original cohort achieved the significance level after further studies were integrated into the meta-analysis. Extracted from Ioannidis et al 2001 (91).

5.4.3 The Future of Osteoporosis Research

There is wide consensus that only the tip of the iceberg has been discovered in regard to the genetics of osteoporosis. For example, in a meta-analysis of 19,195 individuals, 15 SNPs were associated with LS BMD. However, these SNPs could explain a very small portion (2.9%) of the variation of LS BMD (16). GWAs are not even expected to reveal the majority of the genes involved (199). After numerous GWA and candidate gene studies, it is obvious that some variants will not be discovered by either method and new approaches should be considered.

GWA techniques are getting more efficient and less expensive, and therefore more available. While Bonferroni correction can be adjusted or another correction to multiple tests can be used instead, the basic limitations of the method still exist. Nonetheless, new and more complex genetic models are required in order to analyse gene-gene interactions, identification of rare variants and functional studies in GWA results.

Next-generation sequencing is gradually taking its position in the osteoporosis research field. Whether sequencing an entire candidate gene or performing whole genome sequencing, this method is much more supportive to the discovery of rare variants, which in turn are more likely to be functional and to contribute more to the pathological process (4). New bioinformatics tools and statistical methods will be required to analyse the deep sequencing results, which will generate an enormous volume of information.

All methods discussed – GWA, candidate gene association studies and whole genome sequencing-- are limited in the way they correlate genotype with phenotype: a linear phenotype-genotype correlation. They lack the ability to consider epistatic phenotypes or to provide information on gene functionality. In addition, they diagnose the situation at a specific instant and cannot consider changes over time, such as for aging-related issues. Farber et al. (88) suggested a new way to analyse the genetics of osteoporosis, by using "Systems Genetics" rather than looking for a direct connection between the phenotype and the DNA. In brief, this approach, in addition to the clinical phenotypes, also takes into consideration molecular phenotypes, such as transcript level, interactions between genes, regulatory miRNAs, etc. (Figure 27).

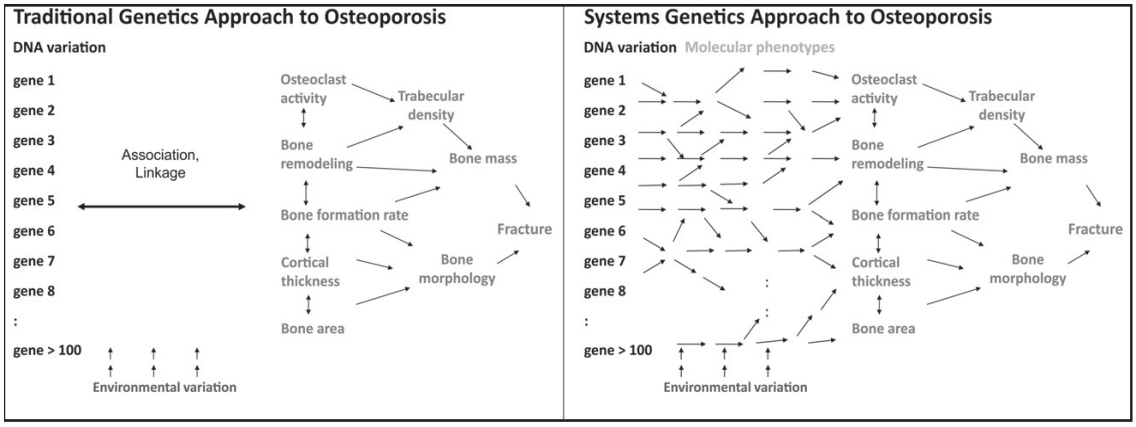

Figure 27. Genetic analysis of fracture risk- traditional genetics approach vs. systems genetics approach. On the left, using the traditional genetics approach, association with or linkage to biological traits is performed without considering genes interaction and the regulatory effect they have on one another. In contrast, the system genetics approach, on the right, attempts to take into consideration molecular phenotypes in order to achieve complete observation and better understanding of the functionality of the genes. Extracted from Farber et al. 2009 (88).

This approach requires high-level statistical calculations to perform multiple tests. In this case, not only the genetic variants should be considered but also millions of other experimental results or *in-silico* predictions. Moreover, the results should be corrected for multiple comparisons in order to manage the false discovery rate. This requires additional techniques and statistical approaches.

Low-trauma fracture prediction is one of the most challenging issues in bone research and will require new tools. New prediction tools should combine the established risk factors and new bone-quality assessments, as well as considering other physical and environmental factors. Bone quality and fragility can be determined by microarchitecture evaluation techniques that should be considered for accurate fracture risk assessment in clinical settings. Magnetic resonance imaging should be considered as well to achieve a comprehensive description of the bone microarchitecture. In addition, genetic variants of the patient should be integrated into the risk estimation to provide better and more individualised risk prediction, prevention and treatment.

MiRNA research is a relatively novel research field, and its entire role in bone strength and bone metabolism is yet to be discovered. Although several studies have been published in recent years, it is still an unmapped ground. Future miRNA research should be done at 3 levels. First, miRNAs involved either directly or indirectly in bone metabolism or in bone fragility should be identified. Then, genetic variants in the miRNAs or 3'UTRs of the genes should be studied for association with osteoporotic phenotypes. Finally, the association should be tested *in-vitro* or *in-vivo* at the experimental functional level to demonstrate the actual effect of the described variant.

Despite numerous studies supported by foundations, research societies and consortiums, high-profile researchers, journals and publications, osteoporosis remains a huge health and economic problem. Over time, new techniques and methods will improve our knowledge and understanding of the pathological process of the disease and may eventually lead to better treatments and prevention.

CONCLUSIONS

6. Conclusions

- We replicated the association of rs9594738 with BMD, a genetic variant at 184 kb upstream to *RANKL* gene. Statistical analysis for other SNPs in the *RANKL* gene failed to yield significant results with osteoporotic phenotypes.

- We showed that this region surrounding rs9594738 (DR), between *AKAP11* and *RANKL*, acts as a distal regulator of *RANKL*, with different effects on its expression in the presence or absence of vitamin D in our experiments. These results suggest that it may play a role in the RANK/RANKL/OPG equilibrium, and might explain the association between the SNPs in the region and BMD.

- A transcript of minimum 300 bp (with rs9594738 in a central position) was detected in the DR. The existence of this RNA segment suggests its involvement in alternative functions of the region.

- We also identified 2 SNPs in the *RANK* 3'UTR (rs78326403 and rs884205) that are associated with low-trauma fractures in our cohort. SNP rs78326403 is associated with wrist/forearm fractures, while SNP rs884205 is associated with spine fractures.

- A significant interaction between rs78326403 and the *RANKL* BMD-associated SNP (rs9594738) was observed, highlighting the relevance of both microarchitecture and low BMD as genetically determined predictors of fracture risk that should be assessed using multiple techniques.

RESUMEN EN ESPAÑOL

7. Resumen de la Tesis en Español

7.1 Introducción

7.1.1 La osteoporosis, la DMO y las fracturas de bajo traumatismo

La osteoporosis es una enfermedad esquelética sistémica, siendo la enfermedad metabólica ósea más frecuente. Es uno de los problemas más frecuentes en las mujeres posmenopáusicas de la población occidental (1,2). En Europa alrededor del 25% de las mujeres mayores de 50 años tiene osteoporosis (4). Las fracturas de bajo traumatismo son la consecuencia inmediata de la osteoporosis y son una causa creciente de hospitalización, de morbilidad y de mortalidad entre los ancianos, dando lugar a enormes costos médicos anuales (7).

La osteoporosis es el resultado final de un mal funcionamiento de la homeostasis del hueso, también conocida como remodelación ósea, y puede variar de un paciente a otro en gravedad, dolores, fracturas y otras consecuencias físicas. La osteoporosis se caracteriza por una resistencia ósea reducida y cualquier hueso se puede ver afectado (6). La Organización Mundial de la Salud (OMS) ha definido la osteoporosis utilizando como parámetro principal la densidad mineral ósea (DMO) (5): a partir de 2,5 desviaciones estándar inferior al promedio de la DMO de las mujeres sanas de 20 años. La densidad mineral ósea en el cuerpo humano puede verse afectada por muchos factores como la edad (11,12), nutrición (13), y el estado hormonal de esteroides sexuales (12), vitaminas (13) y genética (14-16). Una DMO baja altera la microarquitectura del hueso (Figura 1) aunque ésta también puede estar afectada con independencia de la DMO.

El motivo principal del tratamiento de la osteoporosis es el intento de evitar la incidencia de las fracturas aumentando la DMO, suponiendo que va a mejorar la resistencia ósea. Sin embargo, hay que tener en cuenta que la definición de estas fracturas por traumatismo leve como "fracturas osteoporóticas" puede ser confusa ya que muchos de los pacientes con fracturas tienen niveles de DMO por encima de los

criterios de osteoporosis definidos por la OMS (19,20). Existen evidencias concluyentes que relacionan la densidad mineral ósea con fracturas de bajo trauma, con una mayor ratio de probabilidades (OR) de 1,5 a 3 veces de fractura para cada desviación estándar (DE) en la disminución de la DMO (Tabla 2) (28). Sin embargo, hasta la mitad de las fracturas osteoporóticas ocurren en pacientes no osteoporoticos según los criterios establecidos de densidad mineral ósea (19,20). No es de extrañar que varios estudios (29-31) hayan propuesto otros predictores de fractura en lugar de sólo la DMO, incluyendo el algoritmo del FRAX (32-34), con el fin de mejorar la identificación de los sujetos con alto riesgo de fractura en la práctica clínica.

7.1.2 Recambio óseo y el sistema RANK / RANKL / OPG

El recambio óseo, también llamado remodelado óseo, es un proceso permanente que comprende a todo el ciclo de resorción y formación ósea, lo que determina la DMO. En general, la biología celular del hueso de un adulto incluye, entre otros, 3 tipos de células con funciones opuestas: los osteoblastos, que producen la matriz extracelular que después se mineraliza, los osteoclastos, que son responsables de resorción ósea y los osteocitos que están involucrados en la regulación de ambos procesos (e incluso se puede afirmar que dominan el proceso). Existe un sistema complejo de señales entre los tres tipos celulares con el fin de equilibrar sus actividades y para evitar cualquier exceso en la formación o pérdida de tejido óseo (38). Este equilibrio en el recambio óseo está regulado por el sistema RANK / RANKL / OPG. La alteración de este equilibrio conduce a situaciones patológicas, como la osteoporosis.

El sistema RANK/RANKL/OPG fue descubierto a mediados de 1990 (71,72) y contribuyó a la comprensión de los osteoclastos, la formación, activación y supervivencia. Por otra parte, reveló algunos nuevos aspectos de la homeostasis del tejido óseo y la comunicación entre los tres tipos de células del hueso. Las células osteoblásticas expresan y secretan el receptor activador del NF-kB (RANKL), que se une a su receptor, el RANK, en la superficie de los osteoclastos y sus precursores. Esto desencadena la diferenciación de los precursores a osteoclastos multinucleados así como su activación y supervivencia. La Osteoprotegerina (OPG) es secretada por los osteoblastos y las células madre estromales osteogénicas para proteger el esqueleto de una excesiva resorción ósea mediante la unión a RANKL y evitar su interacción con RANK (Figura 9). La relación RANKL / OPG en el tejido óseo es,

pues, un factor determinante de la masa ósea en los estados normal y patológico (72). Esta vía de señalización se ve modulada por diferentes hormonas, citoquinas y factores de crecimiento que afectan a la actividad de las células y al metabolismo óseo (73,74) (Tabla 3).

7.1.3 Genética de la osteoporosis

Las primeras evidencias de la heredabilidad de la osteoporosis aparecen en estudios de gemelos y de familias (78-80). Estudios de familias estimaron que la heredabilidad de la DMO es del 44% al 67% (81-83). En estos estudios, junto con un estudio de familias basado en probandos con DMO extrema (84), se insinuó una etiología poligénica, aunque algunos efectos monogénicos se evidenciaron en algunas poblaciones o familias (85).

La elevada prevalencia de la enfermedad y sus altos costos de atención asistencial, combinados con fuertes evidencias en la naturaleza hereditaria de los fenotipos osteoporóticos, dieron lugar a una importante cantidad de estudios genéticos. Estos estudios se encaminaron a identificar los genes, mecanismos o las vías de señalización que pueden contribuir a la comprensión de la enfermedad, y así servir como diana terapéutica. Los estudios genéticos de "primera generación" se basaron en el ligamiento genético no paramétrico por un lado y en estudios de asociación de gen candidato por el otro. Una vez que se identificaron y caracterizaron los SNPs presentes en todo el genoma, y que la tecnología de genotipado masivo aumentó en disponibilidad y asequibilidad, los estudios de asociación a nivel de genoma completo (GWAs) sustituyeron ambos métodos surgiendo como una metodología importante en el estudio del metabolismo óseo y la investigación de la osteoporosis.

7.1.4 Los genes *RANK* y *RANKL*

El gen de *RANK* (en el locus 18q22.1, Ensembl ID: ENST00000269485) codifica para una proteína transmembrana de tipo I, que contiene 4 pseudo-repeticiones extracelulares ricas en cisteína. La proteína RANK humana es un péptido de 616 aminoácidos, expresada a partir de un tránscrito de 4.521 pb compuesto por 10 exones. Tiene un dominio extracelular N-terminal y un péptido señal de 28 aminoácidos de longitud. También tiene un dominio transmembrana de 21 aminoácidos y un dominio largo C-terminal citoplasmático. El nombre de RANK es el acrónimo de 'receptor

activador del NF-kappa-B' (105). RANK también se conoce como TNFRSF11A, que significa miembro 11A de la superfamilia de receptores del factor de necrosis tumoral. Este nombre refleja la homología entre el gen y el dominio extracelular del receptor del factor de necrosis tumoral (105).

Los modelos animales han demostrado el papel crucial de *RANK* en el recambio óseo. Los ratones nulos para *Rank* mostraban una osteopetrosis profunda, una deficiencia esplécnica de células B y ausencia de la mayoría de los ganglios linfáticos (76). En otro estudio, los ratones nulos para *Rank* carecían de osteoclastos y como resultado tenían un grave defecto en la resorción ósea (106). Además, la osteoclastogénesis *in vitro* utilizando células del bazo de estos ratones comenzó sólo después de la transfección con cDNA de *Rank* de las células precursoras hematopoyéticas. Varios GWAs y meta-análisis han encontrado una asociación entre RANK y fenotipos de osteoporosis (Tabla 5).

El Ligando de RANK o *RANKL* (en el locus 13q14, Ensembl ID: ENST00000239849) fué identificado en 1997 por Anderson et al. (105) que lo llamó *RANKL* y por Wong et al. (111) que lo llamó TRANCE. Fué más o menos simultáneamente clonado por dos otros grupos, Lacey et al. que lo llamó ligando de OPG (OPGL) (86) y Yasuda et al. (112) que lo llamó factor de diferenciación osteoclástica (ODF). Este último, con el fin de identificar la proteína, utilizó como sonda la recientemente descubierta OPG. Los resultados demostraron que las proteínas identificadas eran, en realidad, idénticas a las previamente descubiertas RANKL / TRANCE. A pesar de los diferentes nombres, hoy en día se ha llegado al consenso de que el nombre común es *RANKL* o *TNFSF11* (que significa miembro 11 de la superfamilia del ligando del factor de necrosis tumoral).

RANKL es una proteína transmembrana de tipo II, contiene 317 aminoácidos expresados a partir de un tránscrito de 2.195 pb que incluye 5 exones. Se expresa principalmente en los ganglios linfáticos y en las células estromales de la médula ósea. A nivel del esqueleto también se expresa en las células mesenquimales, condrocitos hipertróficos y en la región sometida al remodelado óseo por la línea celular osteoblástica.

RANKL se expresa en la superficie celular de las células estromáticas/pre-osteoblasticas y tambien, se secreta por estas mismas células, así como por los osteoblastos maduros y osteocitos como molécula soluble (Figuras 8 y 9). Estas dos formas de RANKL no son idénticas: la forma unida a la membrana es una proteína de 40-45 kDa, mientras que la forma soluble tiene 31 kDa y es escindida de la proteína entera inicial (72). Sin embargo, ambas formas participan en la activación de la osteoclastogénesis por su unión a RANK en los pre-osteoclastos, así como en la actividad de los osteoclastos y su supervivencia. Los ratones nulos para *Rankl* presentaban una deficiencia en los osteoclastos mostrando una severa osteoporosis (113). RANKL puede activar la serina/treonina quinasa antiapoptótica PKB que inhibe la apoptosis de los osteoclastos (114). Un estudio que comprendia 3 subgrupos diferentes de mujeres (premenopáusicas, posmenopáusicas tempranas y mujeres posmenopáusicas tratadas con estrógenos con concordancia de edades), mostró una correlación entre los niveles de RANKL y la actividad de resorción ósea (115). Además de la asociación encontrada con la osteoporosis en GWAs (Tabla 5), se ha observado también que mutaciones en *RANKL* provocan una forma de osteoporosis pobre en osteoclastos (116).

7.1.5 Los microRNAs y su papel regulador de la expresión

Los microRNAs (miRNAs) son moléculas de 19-25 nucleótidos de longitud que juegan un papel importante en la regulación génica. Los miRNAs se unen, en una forma parcialmente complementaria, a secuencias diana en la región 3'UTR del mRNA. Este complejo mRNA-miRNA (RNA parcialmente de doble hélice) induce la degradación del mRNA o bien la represión de la traducción (121) regulando así la expresión génica. Un único miRNA puede regular cientos de genes (124) y una región 3'UTR puede contener varios sitios de unión para diferentes miRNAs. Mediante esta regulación de la expresión génica, los miRNAs actúan como reguladores de diferentes vías de señalización, como por ejemplo la de Wnt (122). De esta manera, se ha observado que los miRNAs participan en todos los aspectos de los procesos biológicos en la salud, así como en condiciones patológicas.

Variantes genéticas tanto en el miRNA maduro como en la región 3'UTR de un gen pueden afectar el sitio de unión resultando en una pérdida de función de este sistema regulador.

A pesar de que no hay ninguna duda en cuanto a la participación de miRNAs en el metabolismo óseo, hay una falta de conocimiento en la relación que existe entre RANK/RANKL y los miRNAs. Este campo está aún por explorar y serviría para ampliar nuestra comprensión de la patogénesis de muchas enfermedades del metabolismo óseo, entre ellas la osteoporosis.

7.2 Objetivos

1. Análisis de asociación de putativos SNPs funcionales situados en regiones evolutivamente conservadas de los genes *RANK* y *RANKL* con la DMO y la incidencia de fracturas en la cohorte de BARCOS.

2. Caracterización del promotor y de las regiones reguladoras del gen *RANKL* humano *in-silico* e *in-vitro*.

3. Evaluación del efecto de hormonas y citoquinas ya establecidas como importantes en el metabolismo óseo sobre el promotor y regiones reguladoras de *RANKL* mediante ensayos de gen reportero.

4. Estudio *in-silico* seguido por experimentos funcionales *in-vitro* de los SNP (s) asociados con la densidad mineral ósea con el fin de revelar su papel en el proceso patológico de la osteoporosis.

7.3 Materiales y Métodos

7.3.1 Los sujetos de estudio

Las participantes de la cohorte BARCOS fueron reclutadas en el Hospital del Mar, Barcelona (94,142). Todas las pacientes eran mujeres posmenopáusicas no seleccionadas, reclutadas de manera consecutiva, que asistieron a la clínica ambulatoria para una visita de referencia relacionada con la menopausia. Las pacientes fueron reclutadas de forma prospectiva, independientemente de sus valores de DMO (Tabla 6). Las muestras de sangre y el consentimiento informado por escrito se obtuvieron de acuerdo con las regulaciones del Comité de Investigación Humana del Hospital del Mar, con revisión de los procedimientos genéticos.

7.3.2 Densidad mineral ósea de medición y valoración de fractura

La DMO (g/cm^2) se midió en la columna lumbar (LS) L2-L4 y en el cuello del fémur (FN). Se registraron las fracturas vertebrales y no vertebrales clínicas. Las fracturas no vertebrales fueron validadas a partir de registros médicos y se realizaron radiografías de columna al inicio cuando había antecedentes de diagnóstico de fractura vertebral, pérdida de altura, o dolor de espalda. Las fracturas se definieron como osteoporóticas si se produjeron después de la edad de 45 años y eran debidas a un traumatismo de bajo impacto (es decir, caída por el peso del cuerpo).

7.3.3 Extracción de DNA

Se recogió la capa leucocitaria a partir de 3 ml de sangre recogida en tubos con EDTA y se almacenó a -20 ° C. El DNA genómico se obtuvo a partir de los leucocitos por un procedimiento estándar de precipitación con sales (144) o por Autopure LS (Qiagen).

7.3.4 Selección de SNPs

Para el primer proyecto de genotipado, se escogieron putativos SNPs funcionales en *RANK* y *RANKL* mediante las bases de datos de Ensembl (www.ensembl.org), UCSC Genome Browser (http://genome.ucsc.edu/), Entrez SNP (http://www.ncbi. nlm.nih.gov / sitios / Entrez) y HapMap (www.hapmap.org).

Los SNPs del promotor proximal y el intrón 1 se seleccionaron, principalmente, en función de la conservación evolutiva de la secuencia de su entorno. A fin de establecer regiones conservadas, se compararon las secuencias genómicas de *Mus musculus, Rattus norvigicus, Canis familiaris, Bos taurus* y *Homo sapiens* (ratón, rata, perro, vaca y humanos, respectivamente). Con la herramienta de alineamiento múltiple del Ensembl se escogió un SNP como conservado cuando todas las especies, excepto la humana, presentaban el mismo nucleótido para el SNP dentro de una "región conservada". Los SNPs escogidos se han validado en una población caucásica para incluir a aquellos con una frecuencia del alelo minoritario (MAF)> 0,1.

Otros SNPs fueron seleccionados de acuerdo a los siguientes criterios: que hubieran mostrado una asociación previa con la DMO o el riesgo de fractura, o que fueran cambios exonicos.

Para el segundo proyecto de genotipado, se incluyeron sólo aquellos SNPs del 3'UTR, con una MAF publicada >0,01 en bases de datos del Ensembl (www.ensembl.org) o Entrez SNP (http://www.ncbi.nlm.nih.gov/sites/entrez).

7.3.5 Genotipado

El genotipado de los polimorfismos se llevó a cabo utilizando el Sistema de SNPlex (Applied Biosystem) en la plataforma de CEGEN (Barcelona, España) o sistema de genotipado KASPAR v4.0 en las instalaciones de Kbioscience (Herts, Inglaterra) .

7.3.6 Los métodos estadísticos

El equilibrio de Hardy-Weinberg (HWE) se calculó mediante la prueba de Chi-cuadrado. Los p-valores para HWE se calcularon utilizando la hoja de cálculo del sitio web de la Universidad de Tufts (http://www.tufts.edu/~~HEAD=NNS ~% mcourt01/Documents/Court 20lab% 20 -% 20calculator.xls 20HW%).

Se utilizaron los modelos multivariantes de regresión lineal o logística para evaluar la asociación entre los SNPs genotipados y la DMO o las fracturas, respectivamente. Los posibles factores de confusión considerados para el ajuste fueron, para los modelos en los que la DMO era la variable, el índice de masa corporal (IMC), la edad de la menarquia, los años después de la menopausia en el momento de la densitometría, y los meses de lactancia materna, mientras que para los modelos en que la variable era fractura, se ajustó el índice de masa corporal (35) y la edad.

Las comparaciones estadísticas por pares entre los constructos o tratamientos para los ensayos de gen reportero se calcularon utilizando la prueba no paramétrica de Wilcoxon.

Los análisis estadísticos se realizaron con SPSS para Windows versión 13.0 y la versión de software R 2.13.2 con las librerias *haplostats*, *SNPassoc, foreign, rms, epicalc* y *genetics*.

7.3.7 Cultivos Celulares

Los osteoblastos humanos primarios se obtuvieron a partir de muestras extraídas de pacientes que se sometieron a cirugía de artroplastia total de rodilla. El cultivo de osteoblastos se estableció mediante la obtención de células de la hueso trabecular utilizando el protocolo basado en un método descrito por Marie et al. (148), con algunas modificaciones (149,150). Los extractos nucleares se prepararon a partir de los osteoblastos primarios según Schreiber et al (151), utilizando una versión modificada del tampón C (10% de glicerol y 1,5 mM de MgCl2).

7.3.8 EMSA y los estudios funcionales

Los EMSAs, ensayos de gen reportero, el tratamiento de las células y los análisis de expresión se realizaron como se detalla en la sección de Materiales y Métodos de la versión en Inglés de esta tesis.

7.4 Resultados y Discusión

7.4.1 Estudios de Asociación de *RANK* / *RANKL* en relación con la osteoporosis

Para el gen *RANKL*, el SNP rs9594738, que se seleccionó para replicar un estudio previo de Styrkarsdottir et al. (15), se encontró asociado con la DMO, tanto en columna lumbar (LS) como cuello de fémur (FN) ($1{,}7x10^{-4}$ y 0,02, respectivamente, en el modelo dominante) pero no con fracturas. El SNP rs9525642 dio una p = 0,03 con fracturas, pero este resultado no pasó la corrección de Bonferroni. Ninguno de los SNPs en el 3'UTR de *RANKL* se encontró asociado con la DMO o las fracturas.

La asociación de rs9594738 con la DMO puede indicar que este SNP o una variante genética relativamente cercana pueda jugar un papel funcional en la determinación de la DMO. La región que contiene rs9594738 (que aquí llamaremos DR, por *Distal Region*), se encuentra a unas 184 kb a 5' de *RANKL*, y es parte de una región altamente conservada situada entre el gen *RANKL* y el gen de una proteína de anclaje a la quinasa A 11 (*AKAP11*). En todas las especies con un gen *RANKL* identificable, *AKAP11* se encuentra siempre en dirección 5' de *RANKL* mientras que el gen a 3' varía (174). La asociación funcional con *AKAP11*, si hay alguna, no está clara. Sin embargo, la preservación de esta gran región intergénica puede implicar una determinada importancia funcional. En lo que se refiere al metabolismo óseo, la asociación de rs9594738 con la DMO y su posición genómica puede implicar una participación de éste en el mecanismo regulador de la transcripción de *RANKL* (174). Esta hipótesis está apoyada por evidencias experimentales en ratones que muestran que hay elementos de regulación a ~ 70 kb a 5' del gen (193,194) que afectan a su expresión (174). De hecho, en la misma región se encuentra el SNP rs9533090 que también se encuentra asociado con la DMO en varios estudios, entre ellos en un meta-análisis de GEFOS- GENOMOS, en el que participa la cohorte BARCOS (92). El análisis de los resultados en BARCOS reveló que rs9594738 y rs9533090 están en total desequilibrio de ligamiento. Por lo tanto, cualquier SNP en esta región, por lo menos entre estos 2 SNPs, puede ser la variante funcional asociada a la DMO. Un amplio estudio funcional de la región y las combinaciones de haplotipos de los SNPs de la región podrían revelar más de su papel en el metabolismo óseo. Por otra parte, no se encontró LD entre los SNPs del promotor y los SNPs significativos de la región distal (DR).

Para el gen *RANK*, los SNPs del promotor, intron 1 y exonicos no dieron resultados significativos: los SNPs rs11152341 y rs12150741 dieron una p <0,05 con la DMO de columna (p = 0,036 y p = 0,026, respectivamente, en el modelo superdominante). Otros dos SNPs se hallaron asociados a fracturas: rs12150741 dio una p = 0,035 (modelo recesivo, OR 0,30 (95% IC 0,08-1,08)) y rs1805034 dio una p = 0,049 (modelo dominante, OR 0,67 (95% IC 0,44-1,00)). Ninguno de los resultados mencionados superó la corrección de Bonferroni.

En cuanto a los SNPs en el 3'UTR, ninguno se encontró asociado con la DMO, pero dos SNPs, rs884205 y rs78326403, obtuvieron una p <0,05 para el análisis de las fracturas totales. Los resultados fueron más significativos cuando se analizaron en función del sitio de fractura, con una asociación de rs884205 con fractura de columna con una p = $8,24x10^{-3}$ (modelo recesivo, OR 4,05 95% IC: 1,59-10,35) y una asociación de rs78326403 con fractura de muñeca/antebrazo, con p = $7,16x10^{-4}$ (modelo aditivo, OR 3,12, 95% IC 1,69-5,75). Ambos resultados superan la corrección de Bonferroni.

Aunque algunos SNPs en el promotor proximal, en el intrón 1 y en los exones han dado una p <0,05, ninguno de ellos resultó estar significativamente asociado con ningún fenotipo osteoporótico después de la corrección de Bonferroni.
Probablemente si se aumentara el tamaño muestral se ganaría potencia estadística y se podría detectar mejor dicha asociación, si es que existe. Sin embargo, esto no implicaría necesariamente una funcionalidad de dichos SNPs ya que podría tratarse de una asociación indirecta por estar proximidad al SNP causal.

7.4.2 La asociación con fracturas específicas de sitio encontrada en el gen *RANK*

Como se reseña en el apartado anterior, se han identificado dos SNPs en la región 3'UTR del gen *RANK*, rs78326403 y rs884205, que están asociados con fracturas osteoporóticas en la cohorte BARCOS. Aunque sólo un SNP superó la corrección de Bonferroni (rs884205), ambos se analizaron para la asociación en función del sitio de fractura. De este modo rs78326403 se encontró asociado sólo con fracturas de muñeca/antebrazo mientras que rs884205 sólo se asoció con fracturas de columna vertebral. Por otra parte, después de ajustar los resultados por su DMO de referencia (DMO del cuello del fémur para rs78326403 y DMO de columna para rs884205) estas asociaciones seguían siendo significativas. Sin embargo, después de la corrección por la

DMO, el SNP rs884205 mostró una asociación atenuada con una p al limite de la significatividad, lo que sugiere que la DMO puede jugar un papel en esta asociación.

Debido a la escasa información proporcionada por las bases de datos disponibles hasta la fecha, la mayoría basadas en algoritmos, es imposible confirmar o descartar la ubicación de los SNPs en un sitio de unión a miRNAs. La investigación en el campo de los miRNAs es relativamente reciente y la falta de estudios funcionales es notable. Por otra parte, la definición del 3'UTR no es idéntica entre las distintas bases de datos, y los 2 SNPs significativos de esta investigación están en el 3'UTR de *RANK* correspondiente a la base de datos de Ensembl, pero mas en dirección a 3' del 3'UTR de *RANK* según los datos en el NCBI.

Esta es la primera vez que el SNP rs78326403 se ha asociado con fracturas. El SNP rs884205 ya se había relacionado previamente con fenotipos osteoporóticos (16,158). En contraste con nuestros resultados, en una reciente meta-análisis realizado por el consorcio de GEFOS -GENOMOS que incluye la cohorte BARCOS, rs884205 se había encontrado asociado con la DMO, pero no con fracturas (92). Estas diferencias podrían ser debidas a la heterogeneidad entre las distintas cohortes que conforman el consorcio, o debidas a diferencias en la evaluación de la fractura de bajo traumatismo o a ambas cosas. Esta es una de las principales limitaciones de los estudios de meta-análisis en este campo. Es difícil obtener un fenotipo bien establecido para fractura osteoporótica entre los diferentes grupos. La replicación en cohortes similares a BARCOS deberá aclarar la asociación de estos SNP con el riesgo de fractura. En cuanto a los resultados controvertidos de la asociación con DMO, no se puede descartar que rs884205 pueda también participar en la densidad mineral ósea pero que la limitación de nuestro tamaño muestral no nos permita detectar esta asociación.

Además, hemos descrito una interacción significativa entre el SNP rs9594738 de *RANKL* y el SNP rs78326403 de *RANK*, pero no entre rs9594738 y rs884205. El análisis de los genotipos compuestos para rs9594738 y rs78326403 produce resultados significativos en las comparaciones de 0/1 frente a 2 alelos desfavorables, y de 0/1 frente a 3 o más alelos desfavorables (OR 2,76 y OR 5,14, respectivamente). Por lo tanto, podemos hipotetizar que el efecto aditivo de los SNPs sobre el individuo puede ser mayor que el efecto de cada uno de los SNPs por separado. Esta interacción, significativa sólo con el rs78326403 pero no con el rs884205, sugiere un efecto

epistático entre *RANK* y *RANKL* y demuestra que cada SNP de *RANK* actúa de forma independiente, aunque estén ubicados en la misma región del gen.

Estos hallazgos podrían ser clínicamente relevantes en un futuro para tener un enfoque más específico en los diferentes tipos de fracturas, tanto para comprender mejor los mecanismos subyacentes como para la búsqueda de estrategias terapéuticas en sitios específicos.

7.4.3 Estudios funcionales en el contexto de *RANKL*

Los ensayos de gen reportero con deleciones secuenciales del promotor proximal de *RANKL* mostraron la presencia de 2 regiones inhibidoras (R2 y R3) que de manera independiente disminuían los niveles de expresión del promotor basal (P4). Además, una región de 835 bp situada a unos 184 kb a 5' de *RANKL,* llamada DR y que alberga el SNP rs9594738, inhibe hasta tres veces la actividad del promotor basal en células cultivadas con FBS al 10% y aproximadamente 5 veces en células cultivadas sin FBS.

El diferente efecto de DR en presencia de medio con FBS al 10% implica la existencia de elementos reguladores en esta región que tienen la capacidad de unir factores presentes en el FBS. La segunda fase del estudio fue tratar de identificar el efecto de citoquinas y hormonas (bien conocidas como reguladoras del sistema RANKL/OPG) en el promotor de *RANKL* y la región DR. La dexametasona y la PTH no produjeron ningún efecto consistente sobre las diferentes construcciones probadas. Todos los demás factores que se testaron actuaban sobre el promotor basal: el 17β-estradiol, el TGF-β, el TNFα y la IL-1 redujeron los niveles de expresión de luciferasa mientras que la vitamina D los elevó. Esta última también actúa en la región DR tal y como se discutirá más adelante.

Las evidencias experimentales de nuestro estudio, junto con otras, confirman la existencia de una región distal reguladora entre *RANKL* y *AKAP11*, que es modulada, al menos en parte, por el VDR. El efecto estimulador de la vitamina D sobre P1 fue mayor que sobre P4 sugiriendo la presencia de elementos de respuesta a la Vitamina D en regiones del promotor proximal a 5' del promotor basal. De acuerdo a nuestros resultados, hay otros estudios *in vitro* que demuestran la acción de la vitamina D en células del linaje de los osteoblastos que induce la expresión de *RANKL* (187). Además,

un trabajo previamente publicado ha definido un sitio funcional de respuesta al VDR en el promotor humano de *RANKL* en la posición -1.570 a -1.584 (176), correspondiente al R1 de nuestro estudio, lo que explicaría las diferencias en la expresión de la luciferasa entre P1 y P4. Debido a las condiciones artificiales de nuestros experimentos, no podemos extrapolar el efecto de estos factores en un ambiente fisiológico real dentro tejido óseo humano.

La vitamina D produjo una mayor estimulación en P4_DR que en P4, lo que sugiere que la vitamina D podría ser uno de los factores presentes en el FBS responsables del menor efecto inhibidor de la región DR sobre la actividad luciferasa, respecto los cultivos sin FBS. Nuestros resultados sugieren la presencia de sitios de unión al receptor de vitamina D en el promotor de *RANKL* proximal y distal y, en consonancia con esto, un reciente ensayo de chip-seq (195) mostró la existencia de un elemento de respuesta al VDR dentro de la DR. A este respecto, Kim et al. (194) identificaron varias regiones de respuesta a la $1,25(OH)_2D_3$ a ~ 75 kb 5'del gen *Rankl* en ratones. Una de estas regiones está conservada en humanos y ha demostrado ser funcional. Ellos proponen que el gen *Rankl* se regula a través de múltiples regiones moduladoras que, aunque algunas están situadas a una cierta distancia del sitio de inicio de la transcripción, probablemente forman un lazo de la cromatina centrada en el promotor de *Rankl*. Otros genes, como la osteocalcina, también están regulados por la interacción entre VDR y su promotor (196). En este caso, se requiere una remodelación de los nucleosomas a fin de permitir la unión de VDR al promotor del gen.

Por otro lado hemos intentado detectar si la secuencia que contiene el rs9594738 une algún factor específico nuclear, que actuaría sobre el promotor de *RANKL*. Para ello se realizaron EMSAs utilizando oligonucleótidos de 30 pb que contenían el SNP (en el punto medio de la sonda). La unión de proteínas nucleares a la sonda no fue totalmente específica, pues la competencia con un oligonucleótido que contenía un elemento de respuesta a glucocorticoides (GR) logró reducir la señal de unión. Sin embargo, los ensayos de *supershift* con anticuerpos anti-GR no dieron resultados que confirmararan la unión de GR a la sonda. El estudio *in silico* en el sitio web de Genomatix sugirió la unión de factores de transcripción PAX (2/5/8) sólo cuando el alelo T está presente. Sin embargo, los ensayos de *supershift* no confirmaron dicha unión.

Hasta la fecha, no se ha detectado ningún gen en la región DR, ni ningún mRNA humano codificado por esta región. En el análisis de expresión de la región DR en nuestro estudio se describe la existencia de al menos un transcrito de 300 pb de DR que contiene el SNP. Por lo tanto, esta es la primera vez que se describe la existencia de un RNA, que podría estar involucrado en la regulación de la expresión de *RANKL* y posiblemente otros genes. Este hallazgo debe ser investigado, ya sea *in-vivo* o *in-vitro*, con el fin de definir exactamente el papel de este RNA en la función celular.

7.4.4 El futuro de la investigación en osteoporosis

Existe un amplio consenso de que, hasta la fecha, sólo estamos en la punta del iceberg en cuanto a conocimientos en el campo de la genética de la osteoporosis. Por ejemplo, en un meta-análisis de 19.195 personas, se encontraron 15 SNPs asociados con la DMO de columna. Sin embargo, estos SNP solo explican una porción muy pequeña (2,9%) de la variación de la DMO de columna (16).

Las técnicas de genotipado y análisis son ahora cada vez más eficientes y menos caras y por lo tanto, hay una mayor disponibilidad para llevar a cabo GWAs. Sin embargo, todavía existen algunas limitaciones básicas del método. Se requieren modelos genéticos nuevos y más complejos con el fin de analizar los resultados de los GWA en lo que respecta a las interacciones gen-gen, a la identificación de variantes raras y a los estudios funcionales.

Las nuevas técnicas de ultrasecuenciación van haciéndose un lugar en el campo de investigación de la osteoporosis. Ya sea aplicada a la secuenciación de un gen candidato o a la realización de toda la secuenciación del genoma completo, la ultrasecuenciación permite el descubrimiento de variantes raras, que a su vez , tienen más probabilidades de ser las funcionales y contribuir al proceso patológico (199). En este escenario, se requiere el desarrollo de nuevas herramientas bioinformáticas y métodos estadísticos para analizar los resultados ya que se genera un enorme volumen de información.

Todos los métodos discutidos -estudios de asociación de genes candidatos y GWA y la secuenciación completa del genoma- están limitados plantean una relación lineal de correlación fenotipo-genotipo y no tienen la capacidad de considerar los fenotipos epistáticos o proporcionar información sobre la funcionalidad del gen. Además, solo suelen diagnosticar la situación en un momento específico y no suelen considerar los cambios temporales, como por ejemplo, las cuestiones relativas al envejecimiento. Farber et al. (88) sugieren una nueva forma de analizar la genética de la osteoporosis, mediante el uso de la genética de sistemas – en lugar de buscar una conexión directa entre el fenotipo y el DNA. Brevemente, este método, toma en consideración además de los fenotipos clínicos, también los fenotipos moleculares, tales como los niveles de transcripción, las interacciones entre los genes reguladores, los miRNAs, etc (Figura 27).

La predicción de las fracturas causadas por bajo traumatismo es una de las cuestiones más difíciles en la investigación del hueso. Las nuevas herramientas de predicción deben combinar los factores de riesgo ya establecidos y nuevas evaluaciones de la calidad ósea, así como tener en cuenta otros factores físicos y ambientales. La calidad y la fragilidad ósea se pueden determinar por medio de técnicas de evaluación de la microarquitectura (por ejemplo, análisis de elementos finitos (172) o técnicas de microindentación (173)) que se deberían considerar para la evaluación precisa del riesgo de fractura en el ámbito clínico. La resonancia magnética se debería tener en cuenta para lograr una descripción mas completa de la microarquitectura del hueso. Además, las variantes genéticas del paciente se deberían integrar en la estimación de la predicción del riesgo para proporcionar una mejor predicción, prevención y tratamiento.

La investigación sobre los miRNAs es un campo relativamente nuevo, y su papel en el metabolismo óseo y la resistencia ósea está aún por descubrir. Aunque en los últimos años se han publicado varios estudios, este campo sigue siendo un terreno inexplorado. La investigación futura sobre los miRNAs debe hacerse a 3 niveles. En primer lugar, se deberá identificar los miRNAs implicados en el metabolismo óseo o en la fragilidad del hueso, ya sea de manera directa o indirecta. Luego, se deberá estudiar la existencia de asociación de variantes genéticas en los miRNAs o en los 3'UTRs de los genes diana con los fenotipos osteoporóticos. Por último, la asociación deberá ser

comprobada a nivel funcional con experimentos *in-vitro* o *in-vivo* para demostrar el efecto real de la variante descrita.

Como se puede ver, a pesar de que la osteoporosis es una enfermedad a la que se ha dedicado muchos esfuerzos en investigación, mediante fondos públicos y privados, creación sociedades y consorcios, participación de investigadores de alto nivel, revistas y publicaciones, ésta sigue siendo un problema de salud y económico. Sin embargo, con el tiempo, las nuevas tecnologías y metodologías mejorarán nuestro conocimiento sobre la osteoporosis y permitirán entender mejor el proceso patológico de la enfermedad, lo que finalmente conducirá, esperamos, a mejorar los tratamientos y la prevención.

7.5 Conclusiones

• Se ha replicado la asociación del SNP rs9594738 con la densidad mineral ósea, una variante genética a 184 kb 5'del gen *RANKL*. El análisis estadístico de otros SNPs del gen *RANKL* no dieron resultados significativos de asociación con los diferentes fenotipos osteoporóticos.

• Se ha demostrado que la región llamada DR que contiene el SNP rs9594738 y que se encuentra entre los genes *AKAP11* y *RANKL*, actúa como un regulador distal de la expresión de *RANKL* en nuestros experimentos, con efectos diferentes en presencia o ausencia de la vitamina D. Estos resultados sugieren que la región DR puede desempeñar un papel en el equilibrio RANK/RANKL/OPG, y podría explicar la asociación entre los SNPs de la región y la DMO.

• En la región DR se ha detectado un tránscrito de al menos 300 pb (que contiene rs9594738 en una posición central). La existencia de este segmento de RNA sugiere funciones alternativas de ésta región.

• También se identificaron dos SNPs en el 3'UTR de *RANK*, rs78326403 y rs884205, asociados con fracturas de bajo traumatismo en nuestra cohorte. El SNP rs78326403 está asociado con fractura de muñeca/antebrazo, mientras que el SNP rs884205 está asociado con fractura de columna.

• Una interacción significativa entre rs78326403 y rs9594738 en la determinación del riesgo de fractura pone de relieve la importancia de la DMO baja y de la microarquitectura como predictores genéticamente determinados del riesgo de fractura que se deben evaluar con el uso de diversas técnicas.

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