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# The role of CD40 and CD40L in bone mineral density and in osteoporosis risk: A genetic and functional study

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ABSTRACT

Compelling data are revealing that the CD40/CD40L system is involved in bone metabolism. Furthermore, we have previously demonstrated that polymorphisms in both genes are associated with bone phenotypes. The aim of this study is to further characterize this association and to identify the causal functional mechanism.
We conducted an association study of BMD with 15 SNPs in *CD40/CD40L* genes in a population of 779 women. In addition, we assessed the functionality of this association through the study of the allele-dependent expression of *CD40* and *CD40L* in peripheral blood leukocytes (PBLs) and in human osteoblasts (OBs) obtained from bone explants by qPCR and by sequencing. When an allelic imbalance (AI) was detected, studies on allele-dependent *in vitro* transcription rate and on CpG methylation in the gene promoter were also performed.
Our results confirm the genetic association between SNP rs116535 (T > C) of CD40L gene with LS-BMD. Regarding CD40 gene, two SNPs showed nominal *P*-values < 0.05 for FN- and LS-BMD (Z-scores), although the association was not significant after correcting for multiple testing. Homozygous TT women for SNP rs1883832 (C > T) of CD40 gene showed a trend to have lower levels of OPG (*Q*-value = 0.059), especially when women of BMD-quartile ends were selected ($P < 0.05$). Regarding functionality, we detected an AI for rs1883832 with the C allele the most expressed in OBs and in PBLs. Since the rs116535 of *CD40L* gene did not show AI, it was not further analyzed. Finally, we described a differential methylation of CpGs in the *CD40* promoter among women of high in comparison to low BMD.
Our results suggest that the CD40/CD40L system plays a role in regulating BMD. Effectively, our data suggest that a decreased production of OPG could be the cause of the lower BMD observed in TT women for rs1883832 of the *CD40* gene and that the degree of methylation of CpGs in the *CD40* promoter could contribute to the acquisition of BMD. One possibility that deserves further study is whether the degree of methylation of the *CD40* gene affects the level of CD40 expression and, consequently, the level of OPG.



## 1. Introduction

Osteoporosis is a systemic skeletal disease associated with low bone mass and the microarchitectural deterioration of the bone. It is a multifactorial disease resulting from complex interactions between genetic susceptibility and such environmental factors as physical activity, diet, use of certain drugs and smoking, among others [1,2].

Certainly, the greatest progress made in understanding the cellular and molecular basis of this condition was recognizing that it is influenced by interactions among three different systems, immune, hematopoietic and bone, constituting a new field of research: osteoimmunology [3]. Indeed, besides the spatial proximity within the cavity of the bone marrow (BM), the bone-forming cells (osteoblasts (OBs)), the bone resorbing cells (osteoclasts (OCs)) and the immune cells (mainly B- and T-cells, dendritic cells and monocytes), together with their respective precursors, share many regulatory cytokines, receptors, signaling molecules and transcription factors [1,4]. Perhaps the most significant molecules in this interaction are the receptor activator of the NF-κB (RANK), its ligand (RANKL), both of which were initially described in immune cells where they play a key role in cell survival and immunomodulation [5], and osteoprotegerin (OPG). In the bone, the binding of RANKL on stromal or osteoblastic cells to RANK on pre-osteoclasts, together with the macrophage colony stimulating factor (M-CSF), is necessary and sufficient for the generation, differentiation and activation of osteoclasts [6]. The OPG is a soluble decoy receptor of RANKL produced by OB/stromal cells, the main physiological inhibitor of osteoclastogenesis [7].

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But besides these three key cytokines for bone homeostasis, whose deficiency is associated with extreme phenotypes [8], compelling data on the interaction between the immune, skeletal and hematopoietic systems has come to light. Bone loss is a constant in autoimmune and inflammatory diseases. The T-cell produces RANKL, and this molecule is responsible for the bone loss observed in periodontitis or adjuvant arthritis. The mouse deficient in T-cells does not lose bone mass after ovariectomy or with a continuous administration of PTH. Circulating monocytes can differentiate into functional osteoclasts. Mice deficient in B-cells are osteoporotic. B-cells are the main producers of OPG in the bone marrow and express RANKL. Cells related to OBs are critical regulators of the hematopoietic stem cell niche, from which all blood and immune cells derive. There are many other examples [4,9–11].

A pair of molecules important for both the immune system, as well as for the skeletal system is CD40L (also known as CD154) and its receptor CD40. The latter is expressed on antigen presenting cells, hematopoietic progenitor cells, and cells of OB lineage, whereas CD40L is transiently expressed on activated T-cells [12,13]. The CD40/CD40L system is crucial for many relevant immune system functions such as the differentiation and activation of macrophages, antibody isotype switching, and acquisition of immunological memory in B-cells. Regarding the bone, studies have shown osteoporosis and diminished BM OPG production in CD40 and CD40L KO mice [14]. Additionally, children affected by the hyper-IgM syndrome, a primary immunodeficiency which presents with recurrent infections and low levels of IgG, show osteopenia [15]. Moreover, mice lacking CD40L in T-cells are protected against bone loss induced by OVX or by continuous PTH administration [16,17].

The above amply justifies the interest in the CD40/CD40L system of association studies with bone phenotypes. However, these studies were largely unknown when we decided to study the association of SNP rs1883832 (C > T) of the *CD40* gene and BMD [18]. Among the reasons leading us to carry out this line of investigation was the relationship of CD40 with autoimmune and inflammatory pathologies [19], knowing that osteoporosis has a significant inflammatory component [20], and that rs1883832 was a functional variant affecting the translation rate of CD40 [19]. Subsequently, as CD40 interacts *in vivo* with CD40L, we extended the study to this gene. To do so, we jointly analyzed the association of rs1883832 and three SNPs of the *CD40L* gene, selected on the basis of its heterozygosity, its MAF, and its location in the two-haplotype blocks. In that study, we described an interaction between SNPs in the *CD40*/*CD40L* genes and low BMD [21].

All these genetic, biochemical, and association data have pointed to the CD40/CD40L system as an important regulator in bone homeostasis deserving further study. The present study had three objectives. The first was to continue the analysis of association with BMD through a gene-wide study by examining additional SNPs in order to further characterize the gene region associated with BMD. The next objective was to establish whether the associated SNP showed an allele-dependent differential gene expression (allelic imbalance, AI) in human primary OBs obtained from bone explants and in peripheral blood leukocytes (PBLs). Once an AI was detected, the third objective was to try to establish the cause of this AI through genetic and epigenetic approaches.

## 2. Material and methods

### 2.1. Study subjects

The study group used for the genetic association studies (BMD-cohort) consisted of women of Spanish ancestry living in Valencia, in the east of Spain, who we have analyzed previously [18,21–24], and who were consecutively recruited into the study because they requested the services of the menopause unit of our hospital. This was the only criterion for inclusion into the study. Exclusion criteria were the following: those with a medical history of bone disease different from primary osteoporosis; those with hyperparathyroidism; those who used drugs known to alter bone mass or bone metabolism different from hormone therapy (HT) before the densitometric study; those under 35 years of age.

Women were classified as postmenopausal, as peri-menopausal or as pre-menopausal, as previously described [22]. In the present study, we genotyped a total of 779 women, most of whom were postmenopausal (n = 685) and the remaining were pre- or peri-menopausal (70% peri-menopausal). The women completed a questionnaire on risk for osteoporosis including menopausal status, weight and height, and the use of drugs that could potentially alter bone metabolism. The data were recorded. These data were completed and contrasted with our own data obtained from the medical records and data recorded in the regularly scheduled visits by the attending gynecologist.

In order to quantify the relative expression of CD40 in bone samples, we used primary OBs obtained from our cohort of bone fracture (Fracture-cohort), which was previously described [25]. Briefly, the sample was comprised of postmenopausal Caucasian women over 50 belonging to the area of our hospital that required the implantation of a hip prosthesis due to an osteoporotic subcapital hip fracture.

The Clinical Research Ethics Committee approved this study, and the women read and signed an informed consent according to the regulations of the Institute of Health Research INCLIVA.

### 2.2. Anthropometric, biochemical and bone density measurements

Women went to the hospital between 8:00–10:00 am after an overnight fast for basal blood sampling for biochemical determinations, for obtaining DNA, and for determining their BMI, calculated as the ratio between weight (kg) and height squared ($m^2$).

Serum was separated and kept at −80 °C until analysis. The levels of carboxy terminal telopeptides of collagen I (CTx) and 25-Hydroxycholecalciferol (25(OH)D3) were measured by electrochemiluminescence (E170 Modular Analyser; Roche Diagnostics, Mannheim, Germany). Levels of osteoprotegerin (OPG) were assayed by an instant-ELISA kit (Bender MedSystems GmbH, Vienna, Austria). The detection limits for OPG were 3.1 pg/mL, while the reported intra- and interassay coefficients of variation were 7.0% and 8.0%, respectively. Levels of total alkaline phosphatase (ALP), intact parathyroid hormone (PTH), 17β-estradiol, and total calcium and phosphate were assayed as previously described [26].

Quantification of bone mineral density (BMD, $g/cm^2$) was performed using dual energy X-ray absorptiometry (DXA) at the non-dominant proximal femoral neck (FN-BMD) and at the lumbar spine (L2–L4, LS-BMD) using a Norland XR-36 (Norland Medical Systems Inc.; Fort Atkinson, WI, USA) or a Lunar DPX (GE Lunar Corporation, Madison, WI, USA) densitometer. To compare data from two different manufacturers, a standardized BMD (sBMD) was calculated according to a previous work [21].

### 2.3. Single nucleotide polymorphisms and genotyping

The collection of blood samples and the obtention of genomic DNA were performed as previously described [22]. We analyzed the databases of HapMap and dbSNP (National Center for Biotechnology Information) to find and select the SNPs to study. To do this, we analyzed the haplotype blocks in each region using the Haploview 4.2 software (http://www.broad.mit.edu/mpg/haploview/) and the Gabriel method [27]. The tag-SNPs were selected using the algorithms available in Haploview with the "aggressive tagging" option [27]. A minor allele frequency (MAF) in the HapMap-CEU population of 0.05 and an $r^2$ of 0.8 were used as criteria. The possible functionality of selectable tag-SNPs was assessed using the GenePipe software, a web-based tool, to identify potentially functional SNPs in genes http://genepipe.ncgm.sinica.edu.tw/.

For the *CD40* gene (chromosome 20), the Haploview software detected two haplotype blocks; one in 5′ and one in 3′ of the gene. We selected four SNPs in the 5′ block (rs1800686, rs752118, rs1883832, and