Association of Low Bone Mass with Vitamin D Receptor Gene and Calcitonin Receptor Gene Polymorphisms in Juvenile Idiopathic Arthritis

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ABSTRACT. ***Objective.*** To compare bone density with polymorphisms in the calcitonin receptor (CTR) and vitamin D receptor (VDR) genes in 50 patients with juvenile idiopathic arthritis and 80 matched controls.

Methods. Bone mineral density (BMD) was measured by dual energy x-ray absorptiometry at the lumbar spine. Genomic DNA was isolated from EDTA blood samples by standard procedures. Polymerase chain reaction was performed using genomic DNA and 100 pmol of each oligonucleotide primer for VDR and CTR genes. Products from genomic PCR were digested by Alu I enzyme for CTR polymorphism and Fok I enzyme for VDR polymorphism.

Results. In the total population, higher prevalence of CC genotype (41.5%) for the CTR gene and FF genotype (59.8%) for the VDR gene was found, in agreement with data for Caucasian populations. No significant differences in distribution of CTR and VDR genotypes were observed between patients and controls. However, patients with TT genotype had lumbar BMD (L-BMD) that was lower in comparison to those with CC genotype ($p = 0.04$). For VDR gene polymorphism, we observed that patients with ff genotype had lower L-BMD in comparison with FF genotype ($p = 0.02$). Patients with heterozygosity for the 2 genotypes showed intermediate L-BMD. The differences in L-BMD among these groups did not seem to be related to corticosteroid therapy.

Conclusion. Our data suggest that patients with particular VDR and CTR genotypes may be at higher risk to lose bone mass. (J Rheumatol 2002;29:2225–31)

Key Indexing Terms:
VITAMIN D CALCITONIN RECEPTOR GENE POLYMORPHISM
JUVENILE IDIOPATHIC ARTHRITIS OSTEOPOROSIS

Osteoporosis is a frequent complication in children with juvenile idiopathic arthritis (JIA) and can lead to vertebral and long bone fractures at an early age and also after minimal trauma. This complication negatively influences both the course and the outcome of the disease, with a further reduction on joint mobility[1-3]. Prevention of bone loss should be one of the main goals in treatment of JIA and it should be important to identify patients at higher risk to develop osteoporosis at disease onset[2,3].

Several endogenous and exogenous factors are involved in the pathogenesis of bone loss during the disease course; in particular, disease severity and duration, decreased physical activity, nutritional imbalances, hyposecretion of sex hormones, and therapeutic drugs have a predominant role. However, the expression of osteoporosis varies in different patients and in some cases the severity of reduction of bone mass seems to be greater than expected. Osteoporosis is a complex disease, with a number of different genes playing a significant role, and bone mass is known to have a strong genetic component[4-6].

The inheritance of peak bone mass is likely under polygenic control, mediated through genes that influence bone mass acquisition during growth[5,6]. Family studies suggest a significant effect of genetic factors on peak bone mass. For example, using the early approach of metacarpal/cortical bone thickness, parent-offspring correlations indicated that bone mass was for a large portion genetically determined[7]. The most common candidate genes linked with variation in lumbar bone mineral density (L-BMD) and with high risk to develop osteoporosis are vitamin D receptor (VDR), calcitonin receptor (CTR), estrogen receptor alpha (ER-α), collagen type I, and interleukin 6 receptor genes[4].

Polymorphisms at the VDR gene defined by the restriction endonucleases ApaI, BsmI, and TaqI have been considered as predictors of spinal and femoral BMD in postmenopausal women, even though no general agreement

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on this relationship has been reached[4]. Recently, a novel polymorphism at the translation site of the VDR gene, defined by FokI restriction endonuclease, has been associated with variation in BMD and rates of bone loss in postmenopausal women[8,9]. Subjects homozygous for the presence of the restriction site have a lower BMD than subjects homozygous for the absence of the site. FokI polymorphism is a T/C polymorphism (ATG to ACG) at the first initiation codon (ATG) and is located in exon 2 of the VDR gene, 3 codons proximal to a second start site downstream. Thus, the VDR gene from individuals with the T variant, homozygous for the presence of the restriction site, possess 2 potential initiation codons. While the translation of the VDR mRNA from the T allele can initiate from the first start site, the translation of mRNA from the C allele must initiate from the second start site. Such a difference may provide a structural change that could potentially alter the function of the VDR protein. Interestingly, this T–C transition has recently been shown to result in the synthesis of a 3 amino acids smaller protein, with increased biological activity[9,10].

Another gene that can be involved in the regulation of bone turnover is the CTR gene. CTR have 7 potential transmembrane domains and they are known to be expressed in several tissues. In a Japanese population a novel restriction fragment length polymorphism (RFLP) at the CTR gene was discovered by Alu I restriction enzyme at the 1337th nucleotide expressing either proline (CC genotype) or leucine (TT genotype) as the 463rd amino acid[11]. In a recent study in a large population of postmenopausal women we detected a significant difference in the distribution of VDR and CTR gene polymorphisms in the healthy subjects compared to those with osteoporosis[9]. So far, no data are available concerning the influence of genetic predisposition for osteoporosis in children with JIA. Here, we evaluate the role of the polymorphisms of the VDR and CTR genes in the pathogenesis of osteoporosis associated with JIA.

MATERIAL AND METHODS

Subjects. Our study population included 130 Italian children; 50 out of them were recruited among children attending in 1999 the outpatient pediatric rheumatology unit of Florence, and had rheumatoid factor negative polyarticular onset JIA. All patients were given nonsteroidal antinflammatory drugs (flurbifrofen 5 mg/kg/day or naproxen 15–20 mg/day) and or subcutaneous methotrexate 10–15 mg/m^2/week. Thirty of them were also receiving oral corticosteroid (predinisone 0.5–1 mg/kg/day). Patients undergoing steroid therapy had done so less than one year (mean 5.6 ± 1 mo).

Eighty controls matched for age, sex, weight, and height were recruited among children attending the outpatient clinic for musculoskeletal complaints with no rheumatic diseases. For all children a detailed history was obtained and dietary calcium and physical activity were assessed by a questionnaire, including foods that account for the majority of calcium in the diet.

Bone density. BMD was measured by the same physician (LM) in all patients using dual x-ray absorptiometry (DXA) devices (QDR 1000/W; Hologic, Waltham, MA, USA) at the lumbar spine (L2–L4, poster-anterior). As no consensus criteria defining osteopenia and osteoporosis on the basis of BMD measurements have been established for children, for this study we set a cutoff point for osteopenia at a BMD Z score of less than –1.5. Subjects with JIA were divided according to their corticosteroid therapy into groups without therapy, with low dose corticosteroid (< 5 mg/day prednisolone or equivalent), and with high dose corticosteroid (≥ 5 mg/day prednisolone or equivalent).

Genotyping. Blood samples were drawn with informed consent during routine followup hematological tests. Genomic DNA was isolated from EDTA blood samples by a standard phenolchloroform extraction procedure.

For CTR polymorphism, genomic DNA was amplified by polymerase chain reaction (PCR) as described by Nakamura, *et al*[11]. PCR was carried out as described[12]. Products from PCR were digested by Alu I enzyme (New England Biolabs, Beverly, MA, USA) under conditions recommended by the manufacturer and electrophoresed in 3% NuSieve and 1% agarose. The presence or absence of the restriction site was indicated as TT and CC, respectively. The heterozygous were indicated as TC.

For VDR polymorphism, the 265 bp fragment of genomic DNA containing the polymorphic portion of exon 2 was amplified by PCR as described[8]. PCR products were digested by Fok I (New England Biolabs) at 37°C for 4 h and then electrophoresed through a 3% low melting point agarose gel containing ethidium bromide. The presence or absence of the restriction site was indicated as ff or FF, respectively, and the heterozygous were indicated as Ff.

Statistical analysis. Data were evaluated by chi-square analysis for the assessment of CTR and VDR polymorphism dist.ribution in the total population (130 subjects) and odds ratios with 95% confidence intervals. Correlations between CTR and VDR polymorphisms and L-BMD in the total population and in the 50 children with JIA and a possible relationship between gene polymorphisms and corticosteroid treatment were evaluated by analysis of covariance (ANCOVA). The following covariates were considered: sex, age, pubertal stage, body mass index, disease duration, and low or high dose steroid intake.

RESULTS

In the study group there were 50 patients (35 girls, 15 boys) with a mean age 9.5 ± 3.3 years (range 3.5–13). Mean L-BMD was 0.627 ± 0.144 g/cm^2 ± SD (mean Z score –4.5 ± 1.1). The controls were well matched for sex (56 girls, 24 boys), age (mean 9.9 ± 3.6 yrs, range 3–14.5), height, weight, and pubertal status. Mean L-BMD in controls was 0.770 ± 0.120 g/cm^2 (Z score +1.14 ±0.77). Among the patients, 20 had never taken corticosteroids, while 14 were taking < 5 mg/day and 16 > 5 mg/day prednisone or equivalent. Mean daily calcium intake was around 750–800 mg/day in both patients and controls.The frequency distribution of VDR and CTR genotypes were in Hardy-Weinberg equilibrium. The distribution of CTR genotypes did not show significant differences between controls and children with JIA (Pearson chi-square test 3.6, df = 2, p = 0.16). CC genotype was the most numerous in the whole population (41.5%), and a trend characterized by a prevalence of TT genotype in the children with JIA was observed, with a relative risk of 2.21 (95% CI 0.82–5.93) (Figure 1). For VDR polymorphism, a statistical difference in the distribution of the 3 genotypes was noted; in particular, Ff genotype was the most frequent in the total population and FF genotype was statistically more frequent in the controls (Figure 2) (Pearson chi-square test 6.3, df = 2, p = 0.04). The ff geno-

Genotype	JIA, %	Normal, %	Total, %
TT	60.53	39.47	35.85
CC	40.91	59.09	41.51
TC	41.67	58.33	22.64

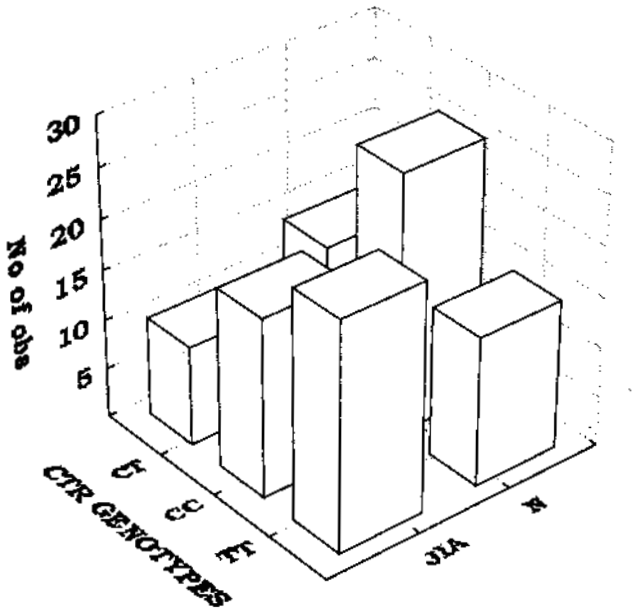

Figure 1. Distribution of CTR genotype in the study population. No statistically significant differences were observed in the distribution of the 3 genotypes between healthy controls (N) and children with JIA. CC genotype was the most numerous in the whole population (41.5%). A trend to prevalence of the TT genotype was observed in patients with JIA (Pearson chi-square test 3.6, df = 2, p = 0.16). Total column gives frequency (%) of distribution of the CTR genotypes in 130 children examined (both normal and JIA). Normal and JIA columns give distribution of total percentage of each genotype.

Genotype	JIA, %	Normal, %	Total, %
ff	67.74	32.26	30.39
FF	44.26	55.74	59.80
Ff	30	70	9.80

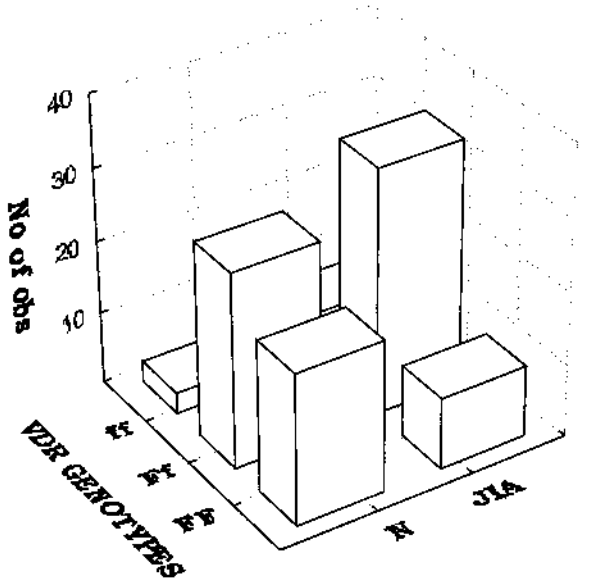

Figure 2. Distribution of VDR genotype in the study population. The ff genotype was 30.39% within the total population (67.74% in JIA vs 32.26% in controls); FF genotype was 59.80% in the total population (44.26% JIA vs 55.74% controls); Ff genotype was 9.8% in the total population (30% JIA vs 70% controls). FF and Ff genotypes were significantly more numerous in the controls (Pearson chi-square test 6.3, df = 2, p = 0.04). Total column gives frequency (%) of distribution of VDR genotypes in the 130 children examined (both normal and JIA). Normal and JIA columns give distribution of total percentage of each genotype.

type was significantly overrepresented in patients with JIA, equivalent to a relative risk of 4.9 (95% CI 0.85–34.24). No significant differences in the distribution of CTR and VDR genotypes were observed between girls and boys. Applying ANCOVA, we evaluated differences in BMD among patients with different CTR and VDR genotypes in the whole population after adjusting bone density for potential confounding factors such as age, weight, height, pubertal status, number of active joints, physician global assessment, and medical treatment. Considering the CTR polymorphism, we observed that children with TT genotype had a L-BMD significantly lower compared to the (CC) CTR genotype (p = 0.04) (Figure 3A). For VDR polymorphism we found that children with the ff genotype had significantly lower L-BMD in comparison with the FF genotype (ANCOVA p = 0.03) (Figure 3B). Using the same analysis only for the group with JIA, we observed the same results. In particular, patients with the TT genotype of CTR polymorphism and ff genotype for VDR polymorphism had lower L-BMD than patients with the opposite genotypes (Figures 4A, 4B).

In addition, we also combined the CTR genotypes with the VDR genotypes and we obtained 9 different groups. We did not observe significant differences in the distribution of the 9 groups in the population (data not shown).

Finally, we also evaluated a possible relationship between the various genotypes and L-BMD in patients undergoing corticosteroid therapy. No difference was noted in the severity of bone loss between children with or without steroid treatment in the 3 genotypes of both VDR and CTR

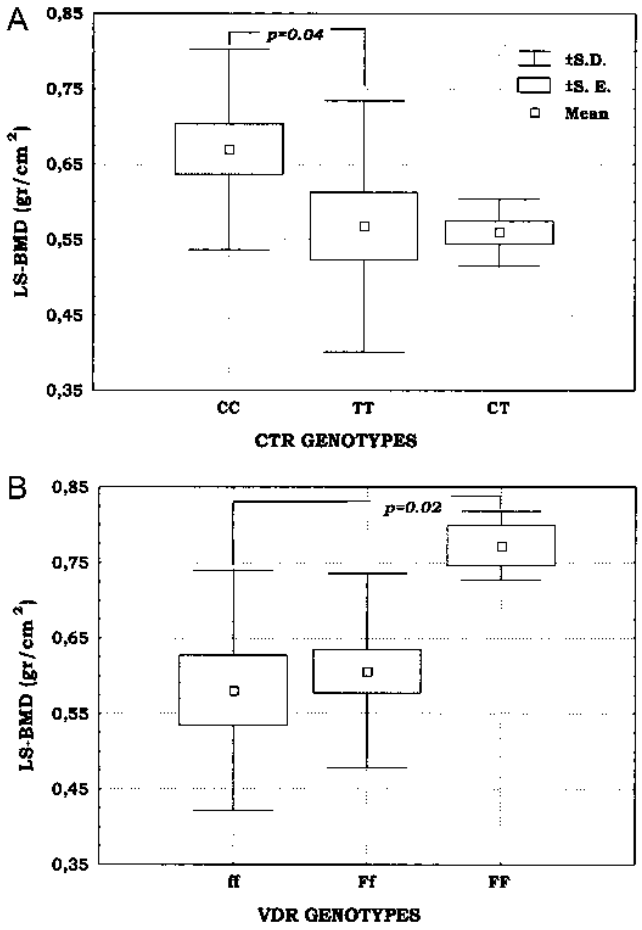

Figure 3. L-BMD within CTR (A) and VDR (B) genotypes in the total study population (Tukey's test). TT genotype of the CTR polymorphism showed a significantly lower L-BMD in comparison with CC genotype (p = 0.04). ff genotype of the VDR polymorphism showed a significantly lower L-BMD in comparison wth FF genotype (p = 0.02).

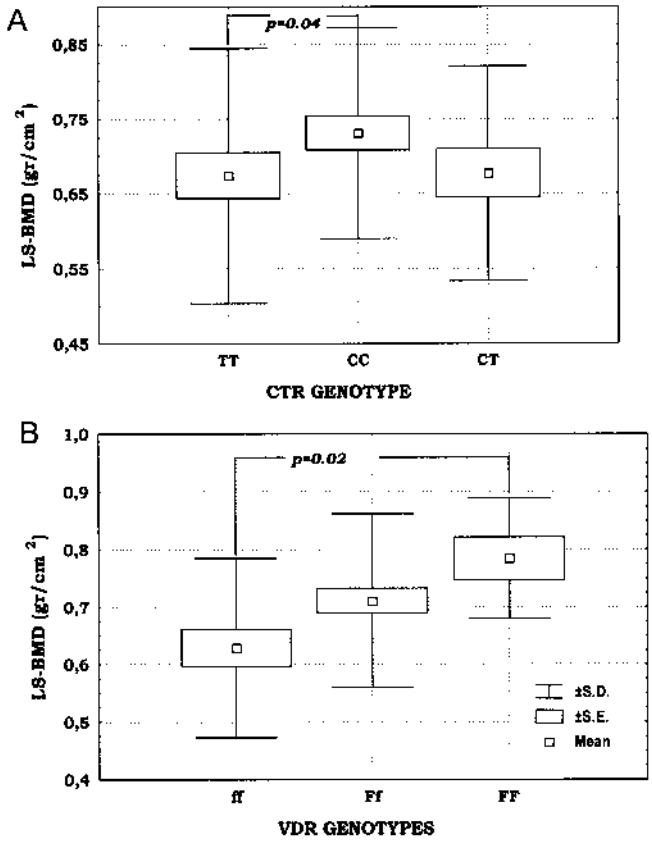

Figure 4. L-BMD within CTR (A) and VDR (B) genotypes in patients with JIA (Tukey's test). TT genotype of the CTR polymorphism showed a significantly lower L-BMD in comparison with CC genotype (p = 0.04). ff genotype of the VDR polymorphism showed a significantly lower L-BMD in comparison wth FF genotype (p = 0.02).

polymorphism. Lower bone mass in the different groups was not related to corticosteroid treatment, as illustrated in Figure 5.

DISCUSSION

The pathogenetic mechanisms that determine the incidence and severity of bone loss in JIA are poorly understood. Genes that may influence the skeleton's response to this systemic disease may have important effects on both local and generalized bone loss. It is known from twin and family studies that up to 75% of the variance in BMD is determined genetically, and several candidate genes have been identified that might be involved in this process[4,5,13,14]. We examined polymorphisms of the VDR and CTR genes in a group of children with and without JIA.

For CTR polymorphism we observed a trend characterized by higher prevalence of TT genotype in patients with JIA compared to healthy controls. In addition, children with TT genotype showed a lower L-BMD in comparison with those with the CC genotype. Our data are in agreement with reports in postmenopausal women confirming a role of this polymorphism in the scenario of genes influencing BMD[12]. The physiological role of calcitonin in osteoporosis is controversial[15]. However, it is the only hormone that binds specifically to the osteoclasts with a direct antiresorptive activity. A polymorphism of the receptor for this hormone in a region of likely functional significance could modulate the activity of the constitutive CTR, influencing both basal activity of the receptors and their response to the hormone. For the VDR polymorphism, FF genotype was statistically more frequent in healthy children. Thus the data in our children are comparable for VDR polymorphism to those reported in an adult Caucasian population[9].

Polymorphism of the VDR gene causing differences in bone density between homozygous haplotypes was described for the first time by Morrison, *et al*[14]. Although much of the data since that report have been controversial, it seems that age groups of the subjects and the sample sizes of different studies may account for some of the apparently contradictory results[16].

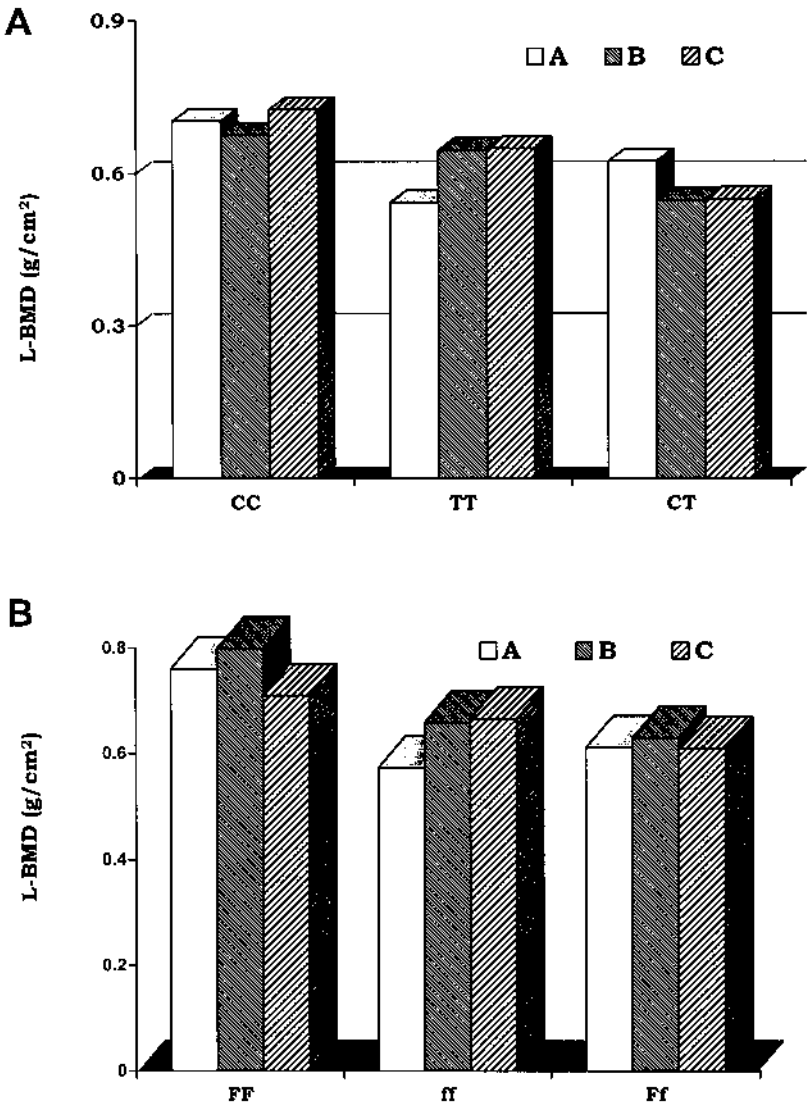

Figure 5. L-BMD within CTR (panel a) and VDR (panel b) genotypes in patients with JIA taking no corticosteroid treatment (A); low dose steroid (B); and high dose steroid (C). No statistically significant differences were observed in L-BMD between groups.

Harris, *et al* studied the start codon polymorphism detected by Fok I endonuclease in premenopausal women, suggesting a likely pivotal role of this polymorphism in the influence of peak bone density in bone turnover in adults[17]. On the other hand, Tao, *et al*[18], examining the association among polymorphisms in the VDR gene (defined by Bsm I, Taq I, and Fok I endonucleases) in prepubertal girls, found an effect of a genotype of VDR (Taq I) on bone growth, but did not find a relationship between Fok I polymorphism and growth variables and femoral neck and wrist BMD. The authors speculated that certain alleles of the VDR gene could play an important role in volumetric BMD variance, especially in trabecular bone, and may be a marker of low peak bone mass[18]. Indeed, it is known that genetic factors have more influence on trabecular bone than on cortical bone[19]. The polymorphism detected by Fok I in exon 2 of the VDR gene results in 3 amino acid differences between the protein encoded by the F and f alleles[9], and the shorter form (F allele) gives about 1.7-fold greater transcriptional activation in transfected HeLa cells than the longer form (f allele), suggesting the existence of a difference in the biological activity of the 2 VDR isoforms[20]. We observed that children with the ff genotype had lower L-BMD than those with the FF genotype. The same results were observed considering only the subgroup of children with JIA. In addition we observed an equal effect of the VDR genotype upon BMD comparing boys and girls. It is likely that the VDR could play a role in the regulation of bone metabolism and growth in both healthy children and those with JIA. Interestingly, in postmenopausal women a segregation of

Fok I allelic variant with BMD at the lumbar spine was found in early but not in late postmenopause[9]. These data suggest that the principal role of this VDR polymorphism could be in the determination of peak bone mass, and in particular on trabecular bone metabolism with a reduction of the effect with age. It is possible that children with the ff genotype might have a lower peak bone density with a major risk of developing osteoporosis in adulthood. In addition, several studies have examined the effect of VDR genotypes on intestinal calcium absorption and metabolism, indicating significant differences among the various genotypes[21]. A similar effect could be present in children: patients with ff genotype could have lower intestinal calcium absorption than those with the FF genotype. This could limit bone mass growth and increase the risk of bone fracture.

Finally, the influence of VDR on bone loss in JIA is also revealed by reports that vitamin D has VDR mediated immunological effects[22,23]. VDR is expressed by monocytes and activated B and T lymphocytes[24], and 1-25 dihydroxyvitamin D3 inhibits the action of the proinflammatory transcription factor nuclear factor-κB and the production of a variety of different cytokines[25]. In addition, extrarenal synthesis of 1-25 dihydroxyvitamin D3 occurs in patients with rheumatoid arthritis, and there are differences in calcium homeostasis in response to stimulation with 1-25 dihydroxyvitamin D3 according to VDR genotype[26]. The immunoregulatory effects of vitamin D are complicated and the precise way it interacts with other regulators of the immune response is not yet clear. The VDR gene represents a strong positional candidate susceptibility for inflammation disease[27], and it is likely that this susceptibility could be mediated by the effect of many polymorphic genes. Segregation analysis suggests that the genetic effects on bone mass are mediated by combined action of several genes, rather than by a small number of genes[28]. For this reason we evaluated the possibility of interaction between CTR and VDR genotypes and bone mass in our population. No significant differences in BMD were observed; however, sample sizes were very small, which might have precluded statistical significance. Finally, corticosteroid therapy is associated with reduced intestinal calcium absorption, bone loss, and increased fracture risk. In this study no significant differences in L-BMD were observed in patients with and those without steroid treatment among the 3 genotypes of CTR and VDR polymorphism. Data obtained in postmenopausal women showed that VDR genotypes might not be useful to identify patients at greater risk of corticosteroid related bone loss[29].

These preliminary results indicate certain CTR and VDR polymorphisms are associated with low bone mass in patients with JIA; these findings need to be confirmed in a larger population sample, and prospective longitudinal followup studies are needed to confirm the role of VDR and CTR polymorphism on bone mass accretion. This would be particularly useful for children with arthritis, in whom skeletal growth is frequently impaired.

These data may be important in the recognition of an "osteoporotic genotype" that would be potentially useful in screening programs (for diet, physical activity, antiosteoporotic therapy) of children with JIA who might benefit from prophylactic bone sparing therapy.

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