Study of vitamin D status and vitamin D receptor polymorphisms in a cohort of Italian patients with juvenile idiopathic arthritis

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Juvenile idiopathic arthritis (JIA) is the most common chronic arthritis of children and adolescents. Autoimmune mechanisms are suspected to have a central role in its development. Vitamin D is an immuno-modulator in a variety of conditions, including autoimmune diseases. Low levels of vitamin D have commonly been found in JIA patients, but the influence of this hormone insufficiency in JIA pathogenesis is still unclear. Vitamin D receptor (VDR) mediates a great majority of vitamin D biological activities; specific polymorphisms of the VDR gene have been associated with different biologic responses to vitamin D. In this study, we analysed clinical characteristics of a cohort of 103 Italian JIA patients. The distribution of VDR polymorphisms in affected patients versus healthy controls was evaluated, as well as if and how these polymorphic variants associate with different disease presentations (active disease vs non-active disease), different JIA subtypes, serum levels of 25-hydroxy-vitamin D and parathyroid hormone (PTH), and lumbar spine Z-score values (osteopenia vs normal bone mineral density). A great majority of our JIA patients (84.5%) showed a suboptimal vitamin D status, in many cases (84.1%) not solved by vitamin D supplementation. Vitamin D status resulted to be independent of VDR genotypes. Apal genotypes showed a highly significant different distribution between JIA patients and unaffected controls, with both the TT genotype and the T allele significantly more frequent in patient group.

Juvenile idiopathic arthritis (JIA) is the most common chronic arthritis of children and adolescents, with an incidence of about 1/1000 people. About 10,000 children and adolescents are estimated to be affected in Italy. JIA is defined as a non-infective, autoimmune, inflammatory joint disease, manifesting before the age of 16 and lasting more than 6 weeks, characterised by persistent joint pain, swelling and stiffness. In many cases, JIA undergoes a spontaneous remission after a clinical course variable from a few to several years (inactive disease), even if alternating periods of relapse and remission are common, and patients have to be constantly followed up until adulthood.

Causes of the disease are still unclear. The most accepted theory supports the influence of immunogenic mechanisms secondary to various genetic and environmental factors. Infections, in association with stress and trauma, are currently suspected to be the most responsible aetiological agents for JIA.

Vitamin D is a pleiotropic hormone, exerting numerous important biological functions. Its role in the immune system has recently been recognised, as well as the fact that a persistent deficiency of its serum level and/or biological activity can contribute to the development of autoimmune diseases.

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Table 1. Vitamin D status in JIA patients.

| Vitamin D status                  | Number of patients | Patients with active JIA | Patients with non-active JIA | Patients not receiving vitamin D supplementation | Patients not receiving vitamin D supplementation with active JIA | Patients receiving vitamin D supplementation | Patients receiving vitamin D supplementation with active JIA | Patients receiving vitamin D supplementation with non-active JIA |
|----------------------------------|--------------------|--------------------------|------------------------------|-----------------------------------------------|--------------------------------------------------|-----------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| Deficiency                       | 4                  | 2                        | 2                            | 1                                             | 0                                                | 1                                             | 3                                                | 2                                                |
| Insufficiency                    | 83                 | 38                       | 45                           | 33                                            | 15                                               | 18                                            | 50                                               | 23                                               |
| Deficiency                       | 15                 | 6                        | 9                            | 6                                             | 3                                                | 3                                             | 9                                                | 3                                                |
| High level (> 100 ng/mL)         | 1                  | 0                        | 1                            | 0                                             | 0                                                | 0                                             | 1                                                | 0                                                |
| Total                            | 103                | 46                       | 57                           | 40                                            | 18                                               | 22                                            | 63                                               | 28                                               | 35                                               |

Biological activities of vitamin D are mainly mediated by its binding to the vitamin D receptor (VDR); even a small modification in VDR activity or ligand affinity may significantly alter correct functions of vitamin D. Common polymorphic variants of the VDR gene may be associated with variable expression/functionality of the receptor, thus being responsible for different biological responses to vitamin D. VDR is expressed in various immune cell lineages, such as monocytes, dendritic cells, and activated T cells. A deficiency of the active form of vitamin D has been associated with a higher risk of development of important autoimmune diseases, such as multiple sclerosis, type 1 diabetes mellitus, and systemic lupus erythematosus. Low serum levels of active vitamin D have been associated with an increase of pro-inflammatory mediators and a manifestation of more active autoimmune diseases. Suboptimal vitamin D status has been reported in children with JIA. However, the influence of vitamin D level in JIA development and disease activity is still unclear. Investigating polymorphic variants that could modulate vitamin D activity in JIA patients could help clarify whether they influence the occurrence, activity and clinical characteristics of this disease, and/or identify potential risk biomarkers.

In this study, we analysed clinical characteristics of a cohort of 103 Italian children, adolescents and young adults affected by JIA, evaluated the distribution of VDR polymorphisms in these JIA patients versus healthy controls, and studied whether VDR polymorphic variants associate with different disease presentations (active disease vs non-active disease), different JIA subtypes, serum levels of 25-hydroxy-vitamin D and parathyroid hormone (PTH), and lumbar spine Z-score values (osteopenia vs normal bone mineral density).

Results

Our patients presented a female/male ratio of 3.90. Forty-six patients had oligoarticular JIA (44.7%), 8 extended oligoarticular JIA (7.8%), 33 polyarticular JIA (32.0%), 7 psoriatic arthritis (6.8%), 6 ERA (5.8%); for 3 patients the specific JIA subtype was not indicated. Active disease manifested in 46 patients (44.7%), while 57 patients had a non-active JIA (55.3%).

Of 103 patients, 4 presented with vitamin D deficiency (3.9%), 83 with vitamin D insufficiency (80.6%) and only 15 showed sufficient levels of 25(OH) vitamin D (14.6%) (Table 1). No significant difference was found in vitamin D status distribution between patients with active JIA vs patients with non-active disease.

At the time of clinical data collection, 63 patients (61.2%) were treated with vitamin D supplementation: 9 of them presented sufficient vitamin D levels (14.3%), 50 with vitamin D insufficiency (79.4%), 3 with vitamin D deficiency (4.7%), and one (1.6%) patient presented a very high level of vitamin D (190.0 ng/ml) consequent to a supplementation of 100,000 UI of cholecalciferol every 3 weeks (Table 1). No significant difference was found in vitamin D status distribution between untreated JIA patients and patients receiving the vitamin D supplementation or between the four combinations of untreated/active disease, untreated/non-active disease, treated/active disease and treated/non-active disease.

Six patients presented low levels of PTH (less than 15 pg/ml); three of them in association with vitamin D insufficiency [less than 30 ng/ml of serum 25(OH) vitamin D], two associated with normal serum values of 25(OH) vitamin D (38.5 and 34.0 ng/ml), and one was the above-mentioned patient presenting a high level of vitamin D (190.0 ng/ml).

All patients showed normal serum calcium values. Of 102 patients, 29 showed osteopenia (28.4%), while 73 had normal BMD values (71.6%). No cases of osteoporosis were reported in our population.

The general distribution of VDR polymorphisms in our patients is reported in Table 2.

The distribution of genotypes of these polymorphisms was in agreement with the Hardy–Weinberg equilibrium, in both JIA patients and the control population.

Statistical comparison of the three genotypes of the Apal polymorphic variant evidenced significant differences between JIA patients and control population: the TT genotype was significantly more frequent (51.46% vs
involved in vitamin D synthesis and activation, including those acting via non-classical activation pathways. The association with vitamin D status and JIA risk, could be extended also to genes encoding for enzymes involved in vitamin D synthesis and activation, including those acting via non-classical activation pathways.

We focused on the analysis of polymorphisms of the 
VDR gene, but analysis of functional polymorphic variations and their association with vitamin D status and JIA risk, could be extended also to genes encoding for enzymes involved in vitamin D synthesis and activation, including those acting via non-classical activation pathways.

Table 2. Distribution of VDR polymorphisms in JIA patients.

| VDR polymorphisms | ApaI | BsmI | Cdx2 | FokI | TaqI |
|-------------------|------|------|------|------|------|
| Genotypes         | TT 53 (51.46%) | GG 31 (30.10%) | GG 60 (58.25%) | CC 46 (44.66%) | TT 35 (33.98%) |
| TGT 37 (35.92%)   | GA 56 (54.37%) | GA 37 (35.92%) | CT 44 (42.72%) | TC 45 (43.69%) |
| GGT 13 (12.62%)   | AA 16 (15.53%) | AA 6 (5.83%) | TT 13 (12.62%) | CC 23 (22.33%) |

The active form of vitamin D is well known to be a main actor in the homeostasis of calcium and bone remodeling. Recently, it has also been recognised as a potential immunomodulatory molecule. Deficiency of vitamin D level or activity seems to have an important role in the development of autoimmune diseases. Low vitamin D levels have been associated with increased pro-inflammatory mediators and more active immune disorders. Immune dysfunction is key to the pathogenesis of JIA and low levels of vitamin D have been reported in children with JIA. A scoping review by Finch et al., including a total of 38 studies, evaluated possible associations of the occurrence of JIA and disease activity with vitamin D level. About 84% of the analysed studies reported that a majority of children with JIA have a suboptimal value of vitamin D. Active JIA and frequent relapses have been associated with reduced vitamin D status.

Our JIA population also showed a high rate of vitamin D insufficiency (80.6%), and 3.9% of patients had vitamin D deficiency, confirming the frequent suboptimal vitamin D status reported in JIA patients. Interestingly, none of our JIA patients with a suboptimal vitamin D status showed high PTH. Conversely, six patients presented low levels of PTH, three of them in association with vitamin D insufficiency. The number of patients with vitamin D deficiency (4/103) was too small, and all of them associated with normal value of PHT, to draw any conclusion.

A study by Reed et al. showed that vitamin D supplementation did not significantly change disease activity, as confirmed by the present study. We did not find any significant difference between the relative percentages of active and non-active disease in patients receiving vitamin D supplementation vs untreated patients.

A high prevalence of vitamin D insufficiency has also been reported in the general population, and all population subgroups, from the Southern Europe and Mediterranean regions, with a greater reduction of vitamin D level in infants, children and adolescents. This generally reduced vitamin D status in the European population does not help to understand the real vitamin D need in children with JIA, nor the exact role of this hormone deficiency in disease development and perpetuation. Data about 25(OH) vitamin D levels were not available for the specific sample of Italian general population we used, in this study, as control of VDR polymorphism distribution.

Reduced vitamin D activity could be ascribed to a reduced serum level of hormone, caused by nutritional, environmental and biological factors, or a reduced response to active vitamin D, mediated by the VDR receptor, due to genetic factors. Out of the classical activation pathway, other non-classical activation pathways of vitamin D and lumisterol, involving various cytochromes p450 enzymes other than CYP27A1 and CYP27B1, have been recently reported and suspected to influence serum level of active forms of vitamin D. They could be involved in the individual predisposition to develop autoimmune and/or inflammatory diseases associated to reduced levels of vitamin D. A broader investigation of biological and genetic factors influencing vitamin D synthesis and activity in JIA patients, also in comparison with the general population, could help understand how they impact the occurrence and activity of JIA, and tailor individual vitamin D adjuvant therapy. Moreover, understanding if and how genetic variants associate with JIA could unravel novel biomarkers of disease risk. Here we focused on the analysis polymorphisms of the VDR gene, but analysis of functional polymorphic variations and their association with vitamin D status and JIA risk, could be extended also to genes encoding for enzymes involved in vitamin D synthesis and activation, including those acting via non-classical activation pathways.
| Table 3. Main clinical characteristics of JIA population in relation to VDR polymorphisms. |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| JIA subtype                          | Number of patients | ApaI | BsmI | Cdx2 | FokI | TaqI |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Oligoarticular JIA | 46 | TT 27 (58.7%) | GG 11 (23.9%) | GG 26 (56.5%) | CC 22 (47.8%) | TT 11 (23.9%) |
| | | TG 17 (37.0%) | GA 28 (60.9%) | GA 17 (37.0%) | CT 17 (37.0%) | TC 25 (54.3%) |
| | | GG 2 (4.3%) | AA 7 (15.2%) | AA 3 (6.5%) | TT 7 (15.2%) | CC 10 (21.8%) |
| Extended oligoarticular JIA | 8 | TT 2 (25.0%) | GG 3 (37.5%) | GG 5 (62.5%) | CC 5 (62.5%) | TT 2 (25.0%) |
| | | TG 5 (62.5%) | GA 4 (50.0%) | GA 3 (37.5%) | TT 2 (25.0%) | CC 4 (50.0%) |
| | | GG 1 (12.5%) | AA 1 (12.5%) | AA 0 (0%) | TT 1 (12.5%) | CC 2 (25.0%) |
| Polyticular JIA | 33 | TT 16 (48.5%) | GG 9 (27.2%) | GG 19 (57.6%) | CC 16 (48.5%) | TT 16 (48.5%) |
| | | TG 10 (30.3%) | GA 19 (57.6%) | GA 13 (45.4%) | TT 7 (15.2%) | CC 11 (33.3%) |
| | | GG 7 (21.2%) | AA 5 (15.2%) | AA 1 (3.0%) | TT 2 (6.1%) | CC 6 (18.2%) |
| Psoriatic arthritis | 7 (4 of them oligo, 1 poly and 2 not indicated) | TT 4 (57.1%) | GG 5 (71.4%) | GG 4 (57.1%) | CC 1 (14.3%) | TT 2 (28.6%) |
| | | TG 1 (14.3%) | GA 1 (14.3%) | GA 2 (28.6%) | CT 1 (14.3%) | TC 2 (28.6%) |
| | | GG 2 (28.6%) | AA 1 (14.3%) | AA 1 (14.3%) | TT 2 (28.6%) | CC 3 (42.8%) |
| Enthesitis-related arthritis (ERA) | 6 | TT 3 (50.0%) | GG 2 (33.3%) | GG 4 (66.7%) | CC 2 (33.3%) | TT 2 (33.3%) |
| | | TG 2 (33.3%) | GA 2 (33.3%) | GA 2 (33.3%) | CT 3 (50%) | CC 2 (33.3%) |
| | | GG 1 (16.7%) | AA 2 (33.3%) | AA 0 (0%) | TT 1 (16.7%) | CC 2 (33.3%) |
| Patients for whom the specific JIA subtype has not been reported in the clinical database | 3 | TT 1 (33.3%) | GG 1 (33.3%) | GG 1 (33.3%) | TT 2 (66.7%) | TT 2 (66.7%) |
| | | TG 2 (66.7%) | GA 2 (66.7%) | GA 0 (0%) | CT 3 (100%) | TC 1 (33.3%) |
| | | GG 0 (0%) | AA 0 (0%) | AA 1 (33.3%) | TT 0 (0%) | CC 0 (0%) |
| JIA clinical presentation | | | | | | |
| Non-active disease | 57 | TT 29 (50.9%) | GG 15 (26.3%) | GG 33 (57.9%) | CC 27 (47.4%) | TT 20 (35.1%) |
| | | TG 20 (35.1%) | GA 35 (64.1%) | GA 22 (38.6%) | CT 25 (43.8%) | TT 27 (47.4%) |
| | | GG 8 (14.0%) | AA 7 (12.3%) | AA 2 (3.5%) | TT 2 (3.5%) | CC 10 (17.5%) |
| Active disease | 46 | TT 24 (52.1%) | GG 16 (34.8%) | GG 27 (58.7%) | CC 19 (41.3%) | TT 15 (32.6%) |
| | | TG 17 (37%) | GA 21 (45.7%) | GA 15 (32.6%) | CT 15 (32.6%) | TC 18 (39.1%) |
| | | GG 5 (10.9%) | AA 9 (19.5%) | AA 4 (8.7%) | TT 8 (17.4%) | CC 13 (28.3%) |
| Serum 25(OH) vitamin D | | | | | | |
| Vitamin D deficiency | 4 | TT 2 (50%) | GG 2 (50%) | GG 2 (50%) | CC 1 (25%) | TT 1 (25%) |
| | | TG 1 (25%) | GA 1 (25%) | GA 2 (50%) | CT 3 (75%) | TC 1 (25%) |
| | | GG 1 (25%) | AA 1 (25%) | AA 0 (0%) | TT 0 (0%) | CC 2 (50%) |
| Vitamin D insufficiency | 83 | TT 44 (53.0%) | GG 24 (28.9%) | GG 50 (60.3%) | CC 37 (44.6%) | TT 30 (36.1%) |
| | | TG 25 (30.1%) | GA 45 (54.2%) | GA 27 (32.5%) | CT 36 (43.4%) | TT 35 (42.2%) |
| | | GG 14 (16.9%) | AA 14 (16.9%) | AA 6 (7.2%) | TT 10 (12.0%) | CC 18 (21.7%) |
| Vitamin D sufficiency | 15 | TT 6 (40.0%) | GG 4 (26.7%) | TT 3 (20.0%) | GG 7 (46.7%) | CC 7 (46.7%) |
| | | TG 9 (60.0%) | GA 10 (66.7%) | TC 9 (60.0%) | GA 8 (53.3%) | CT 5 (33.3%) |
| | | GG 0 (0%) | AA 1 (6.6%) | CC 3 (20.0%) | AA 0 (0%) | TT 3 (20.0%) |
| Vitamin D high level (> 100 ng/ml) | 1 | TT 0 (0%) | GG 1 (100%) | TT 0 (0%) | GA 1 (100%) | CC 1 (100%) |
| | | TG 1 (100%) | GA 0 (0%) | TC 1 (100%) | GA 0 (0%) | CT 0 (0%) |
| | | GG 0 (0%) | AA 0 (0%) | CC 0 (0%) | AA 0 (0%) | TT 3 (0%) |
| Bone status | | | | | | |
| Osteopenia | 29 | TT 16 (55.2%) | GG 9 (31.0%) | GG 20 (69.0%) | CC 13 (44.9%) | TT 13 (44.9%) |
| | | TG 7 (24.1%) | GA 16 (55.2%) | GA 8 (27.6%) | CT 11 (37.9%) | TC 11 (37.9%) |
| | | GG 6 (20.7%) | AA 4 (13.8%) | AA 1 (3.4%) | TT 5 (17.2%) | CC 5 (17.2%) |
| Normal BMD value | 73 | TT 36 (49.3%) | GG 22 (30.1%) | GG 40 (54.8%) | CC 32 (43.8%) | TT 22 (30.1%) |
| | | TG 30 (41.1%) | GA 39 (53.4%) | GA 28 (38.4%) | CT 33 (45.2%) | TC 33 (45.2%) |
| | | GG 7 (9.6%) | AA 12 (16.4%) | AA 5 (6.8%) | TT 8 (11.0%) | CC 18 (24.7%) |
| PTH serum level | | | | | | |
| Low PTH serum level | 6 | TT 1 (16.7%) | GG 2 (33.3%) | TT 2 (33.3%) | GG 3 (50.0%) | CC 2 (33.3%) |
| | | TG 5 (83.3%) | GA 3 (50.0%) | TC 4 (66.7%) | GA 3 (50.0%) | CT 4 (66.7%) |
| | | GG 0 (0%) | AA 1 (16.7%) | CC 0 (0%) | AA 0 (0%) | TT 0 (0%) |
| Normal PTH value | 97 | TT 52 (53.6%) | GG 29 (29.9%) | TT 33 (34.0%) | GG 57 (58.8%) | CC 44 (44.4%) |
| | | TG 32 (33.0%) | GA 53 (54.6%) | TC 41 (42.3%) | GA 34 (35.0%) | CT 40 (41.2%) |
| | | GG 13 (13.4%) | AA 15 (15.5%) | CC 23 (23.7%) | AA 6 (6.2%) | TT 13 (13.4%) |
Numerous polymorphisms of the VDR gene have been identified, some of them previously associated with different biological responses to vitamin D and suspected to play a role in the predisposition and development of several disorders, such as tumours, allergy, inflammation and autoimmune diseases.

In the present study, we analysed five major polymorphisms of the VDR gene, Apal, Bsm1, Cdx2, FokI and TaqI, in 103 children, adolescents and young adults affected by JIA. These polymorphisms have been selected because of their known effect on the modulation of VDR expression and activity, and/or because they have previously been associated with different phenotypes related to vitamin D activity. The Cdx2 polymorphism maps in a promoter region of the VDR gene, at a binding site for the CDX2 intestinal-specific transcription factor, and it modulates vitamin D activity; the allele G exhibits 30% less transcriptional activity than the allele A.

The nucleotide substitution of the FokI polymorphism leads to the loss of the first ATG translation initiation region, resulting in a 3-amino acid shorter VDR protein (FF-short VDR). The Apal, Bsm1 and TaqI are located close to the 3′ untranslated region (3′UTR) of VDR, and they are suspected to affect mRNA stability and VDR expression, or to be in linkage disequilibrium with a truly functional allele within the VDR gene or nearby in another gene.

The functional effect of FokI genotypes in immune cells has been studied, evidencing a greater NF-kB transcription activity in the presence of the FF genotype. NF-kB activity has been associated with immune-mediated diseases. A study of Bašić et al., on 62 JIA patient vs 91 controls, found a significantly higher frequency of the f allele of FokI in JIA patients. Conversely, in our study the FokI genotype distribution showed no difference between JIA patients and healthy controls, or between active or non-active disease presentation, confirming data of a previous study from our research group, performed on a different population of 50 children with JIA.

Here, we have shown a strong association of both the TT genotype of the Apal polymorphism with JIA occurrence or with the presence of an active disease were performed in the study.

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Here, we have shown a strong association of both the TT genotype of the Apal polymorphism with JIA occurrence or with the presence of an active disease were performed in the study.

A study by our research group evaluated the association of FokI genotypes with BMD in JIA patients; patients with the f genotype had a lower lumbar spine BMD. In the present study, we found no significant association between different genotypes of the five analysed VDR polymorphisms and lumbar spine BMD, as well as with the vitamin D level.

No differences in genotype distribution have been shown between different JIA subtypes, and between the presence of an active vs a non-active disease, for the VDR polymorphic variants analysed.

The analysis of distribution of VDR polymorphisms with respect to different levels of 25(OH) vitamin D failed to find any significant association, when deficiency, insufficiency and sufficiency were analysed separately. Conversely, when low levels of vitamin D (deficiency + insufficiency) were compared, together, with respect to sufficient serum levels of the vitamin, the Apal TG genotype resulted to be significantly higher in the JIA group with normal value of 25(OH) vitamin D. The Apal TG genotype was also found to be significantly more represented in JIA patients with PTH serum levels less than 15 pg/ml, with respect to patients showing normal PTH values. Nevertheless, the low number of JIA individuals with sufficient levels of vitamin D (15/103), the very low number of JIA patients presenting low PTH levels (6/103) and the absence of patients with increased PTH, as expected in presence of suboptimal status of vitamin D, appear to be biases, which reduce the strength and effectiveness of these two statistical associations. We can only speculate that these can be only two accidental associations, since neither the opposite homozygote Apal genotypes (TT and GG), nor the Apal opposite T and G alleles, correlated with lower vs normal level of 25(OH) vitamin D and PTH serum levels.

Conclusions
It is suspected that a reduction of vitamin D level/activity favours the development of JIA and the maintenance of the active status of the disease. To date, only three studies have analysed VDR polymorphisms in JIA patients, principally in relation to bone status.

This is the first study that focused on VDR variants in relation to the main clinical aspects of JIA. The possibility that genetic variations in the VDR gene could be biological markers of JIA risk is interesting from the perspective of a personalised preventive/therapeutic approach to this disease.

Insufficient and incomplete studies available, at the moment, make it difficult to confirm or confute our results.

Data need to be confirmed by studying broader JIA populations, enlarging the pathological subgroups and examining the clinical aspects of the disease and the individual response to vitamin D supplementation in detail.
Moreover, since the distribution of some of the VDR polymorphisms showed distinct patterns between different ethnicities, specific studies on different populations are strongly suggested, to identify possible population-specific biomarkers.

**Methods**

**Patients.** All experimental protocols were approved by the Ethical Review Board of the Azienda Ospedaliera-Universitaria Careggi. All patients (or legal guardians for minor patients) gave their informed consent prior to be included in this study. All clinical and laboratory procedures were performed in accordance with the Helsinki Declaration of 1975, and the 1983 revision of the same. Data were all anonymously collected and analysed, and published as aggregates.

This study included 103 Italian children, adolescents and young adults affected by JIA (82 females and 21 males; mean age at time of study recruitment 20.21 ± 7.11 years), collected from 2013 to 2015 by three Italian paediatric rheumatology centres.

According to the JIA subtypes, our patients were classified as: (1) oligoarticular JIA (affecting less than 4 joints), (2) extended oligoarticular JIA (initially affecting less than 4 joints and then extending to more than 4 joints after the first 6 months of life), (3) polyarticular JIA (affecting over 4 joints by the first 6 months of life), (4) psoriatic arthritis (simultaneous presence of JIA and psoriasis), (5) enthesitis-related arthritis (ERA; simultaneous presence of arthritis and enthesitis).

Patients were classified as inactive JIA if, at the time of study recruitment, they were in remission (i.e. no joint with active arthritis, no uveitis, normal values of inflammation biomarkers) for at least 6 months during therapy and 12 months after the suspension of therapy.

Biochemical data were collected regarding serum levels of calcium, phosphorus, PTH and 25(OH) vitamin D. According to these values, patients were classified as follows:

1. Calcium: hypocalcemia (less than 8.4 mg/dL), normal calcemia (8.4–10.4 mg/dL) or hypercalcemia (over 10.4 mg/dL)
2. Phosphorus: hypophosphatemia (less than 2.5 mg/dL), normal phosphatemia (2.5–4.5 mg/dL) or hyperphosphatemia (over 4.5 mg/dL)
3. PTH: hypoparathyroidism (less than 15 pg/mL), normal PTH value (15–88 pg/mL) or hyperparathyroidism (over 88 pg/mL)
4. 25(OH)D: deficiency (0–10.0 ng/mL), insufficiency (10.1–30.0 ng/mL) or sufficiency (30.1–100.0 ng/mL).

Bone mineral density (BMD) at lumbar spine (L1–L4) was measured as Z-score in all but one patient. Patients were classified as normal BMD (Z-score > -1), osteopenia (Z-score between -1 and -2.5), or osteoporosis (Z-score < -2.5).

**Screening for VDR polymorphisms.** A blood sample was collected from each patient for the extraction of genomic DNA and the performance of the genetic analysis of selected VDR polymorphisms. We analysed the ApaI (rs7975232; G > T) and BsmI (rs1544410; G > A) polymorphisms in intron 8, the TaqI (rs731236; T > C) polymorphism in exon 9, the FokI (rs2228570; T > C) polymorphism in exon 2, and the Cdx2 (rs11568820; G > A) polymorphism in the promoter region of the VDR gene.

Cdx2 was analysed by PCR-based Sanger sequencing, while ApaI, BsmI, FokI and TaqI were screened by PCR-based enzymatic digestions with the homonymous restriction enzymes.

A population sample of 2221 Italian unrelated individuals, without JIA, was used as control for the distribution of the genotypes of VDR polymorphic variants in the Italian general population.

**Statistical analyses.** The allelic and genotype frequencies of all five VDR polymorphisms were investigated regarding their agreement with the Hardy–Weinberg equilibrium, by using the chi-squared test, in JIA and control populations.

Differences in distribution of VDR polymorphisms between JIA patients and non-affected controls, as well as between active and non-active disease status, different JIA subtypes, different serum levels of 25(OH) vitamin D and PTH, and different lumbar spine Z-score values were statistically evaluated by chi-squared test. The Yates’ correction was used with groups of less than twenty individuals. Statistical significance was considered with a p value less than 0.05.

**Data availability**
The dataset used and analysed during the current study is available from the corresponding author on reasonable request.

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Author contributions
Contributors: F.M. contributed to the design of the study, performed statistical analysis and prepared all the first draft of the paper. F.F. contributed to the design of the study and to the draft of the paper. S.F. and S.C. performed the experimental work. S.S., D.R. and M.M.C. recruited patients, collected blood samples and all the clinical data. M.L.B. designed the study, supervised all the work and revised the final draft of the paper. All authors revised the experimental work. F.M. contributed to the design of the study, performed statistical analysis and prepared all the first draft of the paper. F.F. contributed to the design of the study and to the draft of the paper. S.F. and S.C. performed

Competing interests
The authors declare no competing interests.

Additional information
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