Twelve families with exuberant and early-onset calcium pyrophosphate dehydrate chondrocalcinosis (CC) and diffuse idiopathic skeletal hyperostosis (DISH), hereafter designated DISH/CC, were identified in Terceira Island, the Azores, Portugal. Ninety-two (92) individuals from these families were selected for whole-genome-wide linkage analysis. An identity-by-descent (IBD) analysis was performed in 10 individuals from 5 of the investigated pedigrees. The chromosome area with the maximal logarithm of the odds score (1.32; \( P = 0.007 \)) was not identified using the IBD/IBS analysis; therefore, it was not investigated further. From the IBD/IBS analysis, two candidate genes, LEMD3 and RSPO4, were identified and sequenced. Nine genetic variants were identified in the RSPO4 gene; one regulatory variant (rs146447064) was significantly more frequent in control individuals than in DISH/CC patients (\( P = 0.03 \)). Four variants were identified in LEMD3, and the rs201930700 variant was further investigated using segregation analysis. None of the genetic variants in RSPO4 or LEMD3 segregated within the studied families. Therefore, although a major genetic effect was shown to determine DISH/CC occurrence within these families, the specific genetic variants involved were not identified.
The coexistence of DISH with CC is very common in Terceira Island—Azores, suggesting that both diseases, hereafter designated the DISH/CC phenotype, share the same pathogenic mechanism.28 A similar phenotype has also been reported in several previous studies.30,31 Our main objective was to elucidate the genetic factors underlying this condition and to establish whether this disease is a novel form of pyrophosphate arthropathy or a simple association between two different disorders with extremely high prevalence on this island in the Azores. A formal segregation study in these families suggested that the condition was caused by a Mendelian inherited disorder with an autosomal-dominant model of transmission.32 Therefore, we performed a whole-genome linkage analysis followed by ‘identity-by-state (IBS)/identity-by-descent (IBD)’ mapping to determine the genetic cause of this phenotype. IBD mapping is a statistical method for the detection of genetic loci that share an ancestral segment among ‘unrelated’ affected pairs of individuals. IBD mapping is a more robust approach than allelic heterogeneity and can be used as a complementary method to genome-wide linkage studies to identify rare inherited variants when combined with sequencing data.33

MATERIALS AND METHODS

Collections used

Twelve pedigrees with 92 individuals were used for the linkage analysis. Ten affected individuals from five pedigrees previously used for linkage analysis were selected for IBD/IBS analysis. The radiological characterization of these individuals is presented in Table 1.

This study was approved by the HSEIT Ethics Committee and all participants provided informed consent. Peripheral blood was collected for biochemical analysis and DNA isolation.

Microsatellite genotyping

Highly informative microsatellite markers from the Linkage Mapping Set V2 (Applied Biosystems, Foster City, CA, USA) were used for the linkage analysis. Microsatellites were amplified in 20 μl PCR reactions using optimized reaction conditions. To simplify the genotyping process, PCR products were pooled based on the fluorochrome used, the allele-size range and avoiding fluorochromes with overlapping spectra or overlapping alleles. The 96-well plates were prepared by adding a mixture of 10 μl of Hi-Di formamide (Applied Biosystems) and 0.3 μl of the internal standard GeneScan 400HD-ROX (Applied Biosystems) to each well. Samples were denatured and immediately run on an ABI automated DNA genetic analyzer 3700 and 3130XL. Microsatellite genotype calling was performed semiautomatically using the GENOTYPER software (Applied Biosystems) and the GeneMapper Software Version 4.0 (Applied Biosystems).

Quality control and linkage analysis

To minimize data errors, extensive checking procedures were undertaken. Genetic Analysis System (GAS version 2.0; Alan Young, Oxford University, Oxford, UK) was used to assign discrete allele numbers to the alleles. Mendelian inheritance was checked with GENOTYPER and GeneMapper (Applied Biosystems). PEDCHECK34 was used to screen all the data for previously undetected Mendelian inconsistencies. Statistical analysis and parametric and non-parametric analyses were performed using VITESSSE,35 GENEHUNTER and MERLIN, respectively.36 Marker positions were obtained from the National Center for Biotechnology Information database (NCBI).

Parametric linkage analysis was performed assuming autosomal-dominant transmission, a penetrance of 90–95%, phenocopy rates of 0.05–0.1% and minor allele frequencies of 0.1–1%.

IBD/IBS analysis

Genotyping was performed with Illumina (San Diego, CA, USA) 370CNV BeadChips. To perform the analysis, 50,000 markers with the following characteristics were chosen: (a) not in a linkage disequilibrium with each other (r² < 0.1) and (b) had a minor allele frequency > 0.3. These were chosen using the TASCOCG control set (approximately 400 individuals with predominantly UK ancestries from Tasmania, Australia).37 IBD segments were then inferred for all pairs of cases across the genome using PLINK38 with the ‘Spairs’ statistic, which represents the number of pairs of cases that appear to have IBD inherited chromosomal segments at each point on the genome.

Using the fastIBD option in the software package BEAGLE (2),39 it was possible to identify IBD regions across families while also taking into account the linkage disequilibrium. The analysis was set for a minimum IBD segment length of 1 cM and an IBD detection threshold of 10⁻¹⁰. The genome-wide P-value was estimated knowing that there were 366 segments with an average of 3.1 case pairs shared across the genome. The number of case pairs shared across the genome approximately followed a Poisson distribution. As the 366 segments were not independent across the genome, principal component analysis was used to show that the 366 multiple tests performed were approximately equivalent to 202 independent tests.40 The genome wide P-value was obtained by estimating the probability of obtaining a maximum number of case pairs shared out of 202 draws from the Poisson distribution (lambda = 3.1) by chance. As a maximum of 10 cases were observed to share IBD, this probability was estimated to be 0.077 by assuming that the number of case pairs shared had a Poisson distribution with a mean of 3.1.

Sanger sequencing

RSPO4 gene primer pairs were designed using the Primer3 software (Open source developed and maintained by Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Regulatory coding regions, including intron–exon boundaries, were amplified and sequenced in 55 patients with DISH/CC disease and 36 unaffected control subjects. The LEMD3 gene was sequenced using the primers previously described by Helleman et al.41 The amplification conditions for all the primers are available upon request. PCR fragments were purified with ExoSAP-IT (Exonuclease I and Shrimp Alkaline Phosphatase) and sequenced using ABI Big Dye chemistry.

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Table 1. Radiological characterization of the patients used for the IBD/IBS analysis

| Code  | Sex | Family bond      | Axial             | Elbow                          | Knee               |
|------|-----|------------------|-------------------|-------------------------------|--------------------|
| 1-1.1| M   | Father and daughter | DISH              | Periarticular calcification    | Calcification      |
| 1-1.3| F   |                  | OALL              | Enthesopathy                   | Normal             |
| 2a-2.32| M | Brothers         | Normal             | Enthesopathy                   | Normal             |
| 2a-2.34| M | Normal           | Osteophytosis, enthesopathy | Chondrocalcinosis             |
| 2b-2.35| F | First-degree cousins | DISH              | Capsular Calcification         | Osteophytosis, arthrosis |
| 2b-2.39| M | DISH             | Capsular calcification; osteophytosis | Chondrocalcinosis |
| 7-7.3| M   | Brothers         | OALL              | Enthesopathy; capsular calcification | NA               |
| 7-7.4| F   |                  | OALL              | Enthesopathy; osteophytosis; capsular calcification | Enthesopathy; osteophytosis; arthrosis |
| 11-11.4| M | Brothers         | DISH              | Periarticular calcification    | Enthesopathy; arthrosis |
| 11-11.5| M |                 |                  |                               | Normal (with Pseudogout episodes) |

Abbreviations: DISH, diffuse idiopathic skeletal hyperostosis; enthesopathy, new bone formation at enthuses; NA, not available; OALL, ossification of the anterior longitudinal ligament; osteophytosis, outgrowths of bone tissue.
(unidirectional or, when necessary, bidirectional) followed by purification with EDTA/sodium acetate and ethanol precipitation. Sequencing products were run on an automated DNA sequencer (ABI 3130XL, Applied Biosystems), and genetic variants were screened by sequencing analysis with SeqScape (Applied Biosystems). Base calling for heterozygous positions occurred when the smaller peak of two co-incident peaks was >25% of the maximum peak.

Whenever possible, the genetic variants found in the RSPO4 gene were screened across all family members when an affected individual was identified. The LEMD3 rs201930700 variant was typed in a representative randomized group of 124 individuals from Terceira Island.

Statistical analysis
The Hardy–Weinberg equilibrium and all other statistical analyses were performed using the PLINK software. Association was tested between the DISH/CC disease and allelic variants with the Cochran-Armitage trend test, dominant and recessive gene action tests with 1 degree of freedom and a genotypic test with two degrees of freedom. The Fisher’s exact test was used to assess the differences in the allele frequencies between the 55 patients with DISH/CC and the 36 control individuals. For all statistical tests, a P-value ≤ 0.05 was considered statistically significant.

RESULTS
Linkage analysis
A whole-genome linkage scan, including 92 individuals from 12 pedigrees, was performed on all autosomes, with 189 microsatellite markers and an average distance between markers of 17 cM. Parametric and non-parametric analyses were also performed. The best results were obtained with a non-parametric linkage analysis for chromosome 16 (single point 2.38 and multipoint 1.16). Because the average distance between markers was >10 cM, one extra set of 137 markers, which was distributed on all autosomes, was further amplified and reanalyzed to enlarge the power of the study. A total of 327 microsatellites were included in the analysis. Owing to its complexity, pedigree 2 was excluded from the analysis because of computation time and memory constraints. When assuming an autosomal-dominant mode of inheritance with a minor allele frequency of 0.1%, phenocopy rate of 0.1% and penetrance of 90%, the parametric analysis did not give significant results.

Because there was only a nominally suggestive linkage to an area on chromosome 16, a new set of 13 microsatellite markers that covered this entire chromosome with an average spacing of 8.99 cM was further amplified and gave more significant results (Table 2).

The most significant result on chromosome 16 was obtained for the D16S3100 marker; it had a logarithm of the odds score of 1.32 and a P-value of 0.007. The chromosomal area (16p12.1) directly surrounding marker D16S3100 in the 26.020–27.120 K region had only 12 annotated genes, among which 6 were uncharacterized, 3 were pseudogenes and 1 microRNA and 1 open reading frame were also identified. According to the present knowledge, the only gene of putative interest in this region was HS3ST4, which encodes the enzyme heparin sulfate (glucosamine) 3-O-sulfotransferase. This region was not further investigated owing to a lack of replication using IBD analysis.

IBD sharing
There were a total of 10 cases corresponding to 45 pairs of cases in the IBD analysis. The greatest number of case pairs shared was 10, which was on chromosome 12. The 10 pairs were shared by 3 families. The significance of the association with chromosome 12 included a single-point P-value = 0.0014 and a genome-wide P-value = 0.077.

When using BEAGLE on the CC data set with a minimum IBD segment length of 1 cM and an IBD detection threshold of 10−10, regions on chromosomes 12 and 20 revealed the sharing of 10 pairs (Table 3). The IBD analysis did not replicate the weak suggestive linkage to an area surrounding the D16S3100 marker on chromosome 16.

The chromosome regions considered to be of interest were selected considering the maximum number of pairs shared. Two zones, one in chromosome 12 and one in chromosome 20, had a maximum of 10 pairs shared and were therefore further

| Chromosome Start position (kg18) | End position | Pairs sharing fastIBD | Pairs sharing PLINK |
|-------------------------------|-------------|-----------------------|---------------------|
| 4 7403378                     | 8100956     | 9                     | 3                   |
| 5 138687057                   | 141055614   | 9                     | 3                   |
| 12 65667554                   | 66378203    | 9                     | 9                   |
| 12 66378203                   | 66770915    | 10                    | 10                  |
| 13 25442369                   | 25501361    | 9                     | 6                   |
| 20 821749                     | 1266214     | 10                    | 7                   |
| 20 5157217                    | 6074302     | 9                     | 8                   |

Table 2. Results from a single-point analysis (MERLIN) for 11 pedigrees on chromosome 16

| Chromosome | Location | Z-mean | P-value | Delta | LOD | P-value |
|------------|----------|--------|---------|-------|-----|---------|
| Markers    | cM       | Max = 3.39 | Max = 0.00 | Delta = 0.696 | Max = 5.62 | Max = 0.00 |
| D16S521    | 1.08     | 0.33   | 0.4     | 0.117 | 0.03 | 0.4     |
| D16S3027   | 8.71     | 0.43   | 0.3     | 0.150 | 0.05 | 0.3     |
| D16S423    | 14.05    | 0.42   | 0.3     | 0.158 | 0.05 | 0.3     |
| D16S418    | 14.77    | 0.49   | 0.3     | 0.184 | 0.06 | 0.3     |
| D16S3075   | 23.28    | 0.48   | 0.3     | 0.286 | 0.10 | 0.2     |
| D16S3046   | 40.65    | 1.09   | 0.14    | 0.539 | 0.45 | 0.08    |
| D16S3100   | 52.26    | 1.83   | 0.03    | 0.696 | 1.32 | 0.007   |
| D16S415    | 67.62    | 1.38   | 0.08    | 0.696 | 0.86 | 0.02    |
| D16S3057   | 77.13    | 0.33   | 0.4     | 0.271 | 0.06 | 0.3     |
| D16S514    | 81.15    | 0.10   | 0.5     | 0.072 | 0.00 | 0.4     |
| D16S515    | 92.06    | 0.80   | 0.2     | 0.471 | 0.26 | 0.14    |
| D16S516    | 95.45    | 0.36   | 0.4     | 0.229 | 0.06 | 0.3     |
| D16S3050   | 108.96   | 0.52   | 0.3     | 0.557 | 0.20 | 0.2     |

Abbreviation: LOD, logarithm of the odds.
investigated. In contrast with previous reports that identified ANKH as a CC-associated gene, it was not identified on chromosome 5 in our study. A total of 167 previously characterized genes were identified in the regions of interest on chromosomes 12 and 20. There were 52 genes in the region on chromosome 12 (65667554–68670915), of which only 35 were annotated. Just next to this region in chromosome 12 (65169571–65248379), one gene that had already been associated with disordered calcification, LEMD3, was identified. This gene encodes a LEM domain-containing protein. The encoded protein functions to antagonize TGF-β and BMP signaling at the inner nuclear membrane. Mutations in this gene have been associated with osteopoikilosis (MIM 166700), Buschke–Ollendorff syndrome (MIM 166700) and melorheostosis (MIM 155950).41

There were 148 genes in chromosome 20 (chromosome region from 821749 to 6074302), of which 115 were annotated. The best candidate gene in chromosome 20 was RSPO4 (chromosome 20:958452–1002284). The RSPO4 gene encodes a member of the R-spondin family of proteins that share a common domain organization consisting of a signal peptide, cysteine-rich/furin-like domain, thrombospondin domain and a C-terminal basic region. The encoded protein may be involved in the activation of the Wnt/β-catenin signaling pathways.42 WNT signaling is critical for nail development and mutations in different WNT-associated genes have been identified in disorders involving the nails. RSPO4 mutations have been particularly associated with autosomal-recessive congenital anonychia (NDNC4; MIM 206800).43,44

Sequencing results

RSPO4 sequencing. Nine genetic variants were identified in the RSPO4 gene, including three missense variants (rs6140807 and rs61740632, rs201485021), one splice site variant (rs775644973), two synonymous (rs150446609 and rs41275604) and four regulatory region variants (rs146447064, rs149154047 and rs6056520) (Table 4). All of them were in Hardy–Weinberg equilibrium.

Two variants in the regulatory region of the RSPO4 gene (rs146447064 and rs149154047) were located in a fully conserved region and are rare (Table 4). The same was observed for the synonymous variant rs150446609, which was very rare (< 0.01) and was located in a fully conserved chromosome region. The missense variant rs201485021 located in exon 3 has a SIFT score of 0 and a PolyPhen value of 1, indicating that it has deleterious and damaging effects on the protein, respectively. Additionally, this variant was located in a fully conserved region and was extremely rare (minor allele frequency < 0.01); in the ‘1000 genomes’ (genomes from 26 different populations), the variant was identified in only 2 males from an Iberian population in Spain. In our study, this variant was found in one female in our control group (n = 36). The other two missense variants (rs6140807 and rs61740632) had SIFT and PolyPhen values indicative of minor effects on the protein (tolerated and benign, respectively) and were both relatively rare. We found an extremely rare HGMD mutation (rs775644973 or CS065613) that has previously been associated with congenital anonychia.44 The variant in heterozygosity was found in 1 asymptomatic female in our group of 55 DISH/CC patients.

| Table 4. | Genetic variants identified in the RSPO4 gene and functional significance information |
| Exon/intron | Variant | SNP_ID | Type of variant | MAF | SIFT | PolyPhen | Nucleotide conservation |
|------------|---------|--------|----------------|-----|------|---------|------------------------|
| Upstream gene | c.-131C>T | rs146447064 | Regulatory reg. | 0.01 | NA | NA | Fully conserved |
| Upstream gene | c.-115G>A | rs149154047 | Regulatory reg. | 0.01 | NA | NA | Fully conserved |
| 5’ | c.-85G>A | rs6056520 | Regulatory reg. | 0.35 | NA | NA | 1 non-conserved species (mouse lemur) |
| Exon 1 | c.12A>C | rs150446609 | Synonymous, p.Pro4= | < 0.01 | NA | NA | Fully conserved |
| Intron 1 | c.79+1G>A | rs775644973 | Splice donor (HGMD mutation) | < 0.01 | NA | NA | Fully conserved |
| Exon 3 | c.317G>A | rs6140807 | Missense, p.Arg106Gln | 0.03 | 0.21 | 0.072 | 2 non-conserved species (pig and hedgehog) |
| Exon 4 | c.471C>T | rs41275604 | Missense, p.Pro123Ala | < 0.01 | 0 | 1 | Fully conserved |
| Exon 4 | c.524A>T | rs61740632 | Missense, p.His175Pro | 0.01 | 0.3 | 0 | 4 non-conserved species (mouse lemur, Pig, hedgehog and elephant) |

Abbreviations: MAF, minor allele frequency; NA, not available; SNP, single-nucleotide polymorphism. Nucleotide conservation was obtained using alignments of the human, chimpanzee, mouse lemur, pig, hedgehog and elephant genes available in the Ensembl database (http://www.ensembl.org/index.html) (accessed on January 2017).

| Table 5. | Genetic variants identified in the LEMD3 gene in four probands that have been previously investigated and information about their functional significance |
| Exon/intron | Variant | SNP_ID | Type of variant | MAF | SIFT | PolyPhen | Nucleotide conservation |
|------------|---------|--------|----------------|-----|------|---------|------------------------|
| Intron 1 | c.1523–12C>T | rs11175678 | Intron | 0.04 | NA | NA | Non-conserved |
| Intron 5 | c.1695+100G>A | rs11610822 | Intron | 0.25 | NA | NA | 1 non-conserved species (mouse lemur) |
| Intron 7 | c.2024–34_2024–31delGATT | rs10534559 | Intron | ? | NA | NA | Non-conserved |
| Exon 13 | c.3701C>T | rs201930700 | Missense | < 0.01 | 0 | 0.995 | Fully conserved |

Abbreviations: MAF, minor allele frequency; NA, not available; SNP, single-nucleotide polymorphism.
LEMD3 sequencing. LEMD3 was sequenced in the four probands from the families who shared the maximum number of pairs in the IBD/IBS analysis. The identified genetic variants can be observed in Table 5.

Variant rs201930700 was located in exon 13 of the LEMD3 gene and had a SIFT score of 0 and a PolyPhen value of 0.995, which indicated that this variant has deleterious and damaging effects on the protein, respectively. The variant was extremely rare. The minor allele frequency was unknown because there was insufficient data to establish population frequency; it was identified in only 5 of the 121,412 alleles (ExAc_Aggregated_Populations), indicating that it was unquestionably rare (minor allele frequency 0.00004). A cohort of 124 individuals, which is representative of the Terceira Island population, was typed for this mutation, and 1 other individual carrying the variant was identified. This individual and their relatives were examined (interviewed for clinical purposes, X-rayed and typed).

The rs201930700 variant was in Hardy–Weinberg equilibrium. Of the five individuals studied in the AZ3 family, four individuals (two males and two females) were affected by DISH/CC and were carriers of the rs201930700 variant and one individual (male) was affected by DISH/CC but was a non-carrier of the variant. In the AZ3 family, it was impossible to verify segregation as all the affected individuals studied were affected by DISH/CC affected; thus an association test was not performed.

Segregation analysis. Segregation analysis involved typing eight genetic variants in the seven DISH/CC pedigrees where the variants were found, including the seven variants in RSPO4 (rs146447064, rs149154047, rs6056520, rs150446609, rs6140807, rs41275604 and rs61740632) and variant rs201930700 in LEMD3.

Most of the analyzed families were uninformative because of the large number of affected versus unaffected individuals. Variants did not segregate within the investigated informative families (see Supplementary Figure 1 and Supplementary Table 1 for more information).

Association study. The RSPO4 regulatory region variant rs146447064 was significantly (P = 0.03) more frequent in the controls (14%) than in the DISH/CC patients (5%), and when adjusted for gender, it was significant in females (P = 0.0333) but not in males (Table 6).

Similar results were obtained using the Cochran–Armitage trend and allelic tests (see Supplementary Table 2). The other RSPO4 regulatory region variant, rs149154047, was also significantly different in frequency between the DISH/CC males (3%) and control males (14%) (P = 0.0502) but not in females. Similar results were obtained using the Cochran–Armitage trend test (P = 0.02453, CHISQ = 5.057, DF = 1) and the allelic test (P = 0.02958, CHISQ = 4.734, DF = 1) (see Supplementary Table 2).

DISCUSSION

DISH/CC is a poorly understood phenotype characterized by peripheral and axial enthesopathic calcifications, which fulfill the radiological criteria for DISH and are associated with calcium pyrophosphate dehydrate CC in some cases.31,45,46 The variable clinical and radiological presentation of patients with DISH/CC in the families investigated raises the possibility of genetic heterogeneity. The whole-genome linkage analysis found no chromosomal region with clear linkage in the affected families. The following reasons can be postulated to explain these results: (1) the phenotypic heterogeneity observed within and among pedigrees may result from genetic heterogeneity; (2) unknown environmental factors may be affecting the phenotypes and causing difficulties in the analysis; and (3) the condition may not be a simple Mendelian disorder caused by a large effect rare variant but instead may have a more complex and polygenic genetic architecture. The region on chromosome 16 that showed weak suggestive linkage was not replicated by the IBD/IBS study and was not further investigated.

The concurrence of DISH and CC suggests a shared pathogenic mechanism.35 The etiology of DISH/CC is unknown. However, because it is a bone-forming disease, it is expected that genes related to the calcification and ossification process are implicated in its etiology. In the second strategy utilized, the IBS/IBD analysis, two chromosomal areas were identified and further investigations performed. Two genes, RSPO4 and LEMD3, were selected as good candidates based on their functions. Two variants in the RSPO4 gene (rs146447064 and rs14915407), which were significantly more frequent in the controls than in DISH/CC patients, may have a possible protective role in the DISH/CC phenotype, though the level of significance observed was not definitive.

As already mentioned, the RSPO4 protein is known to have a major role in activating the Wnt/β-catenin signaling pathways.42 It

| SNP         | Alleles | Fisher’s exact test |
|-------------|---------|---------------------|
|             |         | MAF                  |                     |
|             |         | DISH/CC              | Controls            |
|             |         | All, N = 55          | Female, N = 19      | Male, N = 36 |
| rs146447064 | C/T     | 0.045                | 0.026               | 0.056        |
| rs149154047 | G/A     | 0.045                | 0.079               | 0.028        |
| rs6056520   | C/T     | 0.282                | 0.184               | 0.333        |
| rs150446609 | A/C     | 0.045                | 0.053               | 0.042        |
| rs775644973 | G/A     | 0.009                | 0.026               | 0.010        |
| rs6140807   | G/A     | 0.182                | 0.028               | 0.028        |
| rs201485021 | C/G     | 0                | 0                   | 0            |
| rs41275604  | C/T     | 0.036                | 0.053               | 0.028        |
| rs61740632  | A/C     | 0.036                | 0.053               | 0.056        |

Abbreviations: CC, chondrocalcinosis; DISH, diffuse idiopathic skeletal hyperostosis; MAF, minor allele frequency; NA, not applicable; OR, odds ratio; SNP, single-nucleotide polymorphism. The minor allele is represented in bold.

Table 6. Fisher’s exact test for genetic variants found in the RSPO4 gene in Azorean patients with DISH/CC and controls without DISH/CC disease
is known that induction of the Wnt signaling pathway promotes bone formation, while inactivation of this pathway leads to an osteopenia state. Based on this knowledge, we hypothesized that rs146447064 and rs14915407 were variants associated with a reduction in RSPO4 gene expression, thus reducing Wnt activation and consequently protecting against new bone formation. Obviously, further studies are needed to test this theory.

The LEMD3 gene missense variant (rs201930700) was found in two apparently unrelated families. This variant has been identified very few times previously and both families in which it was typed presented the DISH/CC phenotype. The variant identified caused the substitution of amino acid 901 from a large and basic arginine to a large and aromatic tryptophan. Phylogenetically, according to the Ensembl database, the modified nucleotide was highly conserved in all vertebrates and was located in the carboxy-terminal nucleoplasmic region of the MAN1 protein. This region (amino acids 782–911) is predicted to be a RNA recognition motif-like (RRM-like) protein interaction domain called the U2AF homology motif. The conserved region that contains the UHM domain (U2AF homology motif kinase 1) is exclusive to MAN1 proteins and is essential for smad2 and smad3 binding. It is unknown whether interactions between MAN1 and Smad1 or Smad2 and Smad3 inhibit BMP and TGF-β signaling, respectively. It has been reported that heterozygous loss-of-function mutations in LEMD3 enhance TGF-β signaling and lead to sclerosing bone dysplasia, osteopoikilosis and Buschke–Ollendorff syndrome. As the identified variant (rs201930700) was deleterious but was not a loss-of-function mutation, the carriers of the variant did not present any signs of osteopoikilosis or Buschke–Ollendorff Syndrome. At this point, it was difficult to ascertain the effect produced on the DISH/CC phenotype by the rs201930700 variant. We postulate that the rs201930700 variant may promote enhanced TGF-β signaling, leading to increased bone formation. Our hypothesis was not confirmed by the segregation analysis, which might be due to the characteristics of the pedigrees used, as almost all the individuals were DISH/CC affected, making it very difficult to verify segregation. Other studies will be necessary to verify the impact of this rare variant on the phenotype to establish a possible association.

In conclusion, a combined strategy led us to investigate two candidate genes, RSPO4 and LEMD3, and the results obtained suggested a small and protective role for the two RSPO4 gene regulatory variants, probably by altering RSPO4 gene expression. At this point, it is impossible to ascertain the relevance of the extremely rare variant in LEMD3 (rs201930700) to the DISH/CC phenotype. Although the results are interesting, future studies are required to confirm and assess their relevance for DISH/CC pathologies.

COMPETING INTERESTS
The authors declare no conflict of interest.

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