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CLINICAL REPORT

Osteomesopyknosis associated with a novel *ALOX5* variant that impacts the RANKL pathway

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Abstract

Background: Bone tissue homeostasis relies on the coordinated activity of the bone-forming osteoblasts and bone-resorbing osteoclasts. Osteomesopyknosis is considered a distinctive rare sclerosing skeletal disorder of unelucidated pathophysiology and presumably autosomal dominant transmission. However, the causal genes are unknown.

Methods: We present a case report encompassing clinical assessments, imaging studies, and whole-exome sequencing analysis, complemented by functional in vitro experiments.

Results: This new case of osteomesopyknosis was associated with a missense *ALOX5* variant predicted to induce protein misfolding and proteasomal degradation. Transfection experiments demonstrated that the variant was associated with reduced protein levels restored by proteasomal inhibition with bortezomib. Likewise, gene expression analysis showed that the mutated gene was associated with a decreased RANKL/OPG ratio, which is a critical driver of osteoclast precursor differentiation.

Conclusion: Our data indicate impaired bone resorption as the underlying mechanism of this rare osteosclerosis, implicating *ALOX5* pathogenic variants as potential etiological factors.

KEYWORDS

ALOX5, osteomesopyknosis, osteoprotegerin, osteosclerosis, *RANKL*

1 | INTRODUCTION

Osteopetroses are a group of well-recognized familiar disorders presenting with generalized osteosclerosis, including nowadays a dozen Mendelian diseases (Teti & Whyte, 2023). Osteopetrosis affect both axial and appendicular bones, with generalized trabecular bone thickening (osteosclerosis) that causes the narrowing of marrow spaces, and cortical bone widening (hyperostosis) (Calder et al., 2022; Teti & Whyte, 2023). Osteomesopyknosis

(MIM 166450, ORPHA 2777) is a rare form of axial osteosclerosis, whose prevalence is unknown. After the initial description of a few cases in France, about 20 cases from different families and continents have been reported in the literature (Delcambre et al., 1989; Jeoung et al., 2015). Unlike osteopetrosis, the disease typically appears as multiple sclerotic lesions within the trabecular bones of the axial skeleton (Delcambre et al., 1989; Heursen et al., 2016).

Most patients experience only minor symptoms, such as mild back pain, and in fact, the disease may be an incidental finding in imaging studies. However, despite its benign nature, osteomesopyknosis lesions may be confused with osteoblastic metastases or other severe bone sclerosing disorders, thus representing a diagnostic dilemma.

Several reports of familial cases across multiple generations suggest an autosomal dominant transmission (Delcambre et al., 1989; Hardouin et al., 1994; Yao & Camacho, 2014). However, the involved genes are still unknown because those reports did not include gene variant analyses. Here we report a case of osteomesopyknosis associated with a novel mutation in *ALOX5* that impacts osteoclastogenesis.

2 | SUBJECT AND METHODS

2.1 | Ethical compliance

The study was approved by the Institutional Review Board (Comité de Etica de la Investigación con Medicamentos y Productos Sanitarios de Cantabria; protocol 2023.355).

2.2 | Case report

A 49-year-old Spanish Caucasian woman complained of hip pain after a traffic accident. She was 165 cm tall and weighed 59 kg (body mass index 21.6 kg/m^2). Radiographs did not show any fracture. A 99mTc-DPD scintigraphy showed multiple foci of increased uptake at the thoracic and lumbar spine, pelvis, proximal left femur, and right humerus. An 18F-NaF PET/CT revealed intense uptake in those areas (Figure 1a). These results were confirmed by MRI (Figure 1b) and CT, which showed patchy osteosclerotic lesions within the trabecular areas of those bones. The past medical and the family history were unremarkable. There was no history of recurrent inflammation, infection, or known skeletal disorders. Her father died suddenly in his 80s. Her mother died due to a cancer of unknown origin. The patient had a single healthy brother and a single teenage son.

Routine blood tests (complete blood count, creatinine, calcium, phosphate, transaminases, lactic dehydrogenase, and C-reactive protein) and fluoride and tryptase levels were normal. Serum alkaline phosphatase (ALP) was 47 U/L (reference range 46–118); bone-specific ALP, 18 U/L (reference 14–43). Serum procollagen type I N-propeptide (PINP) was 48 ng/mL (reference 19–102), the collagen C-terminal telopeptide (CTX) was 0.23 ng/mL (reference 0.13–1.04); Intact PTH was 19 pg/mL (reference 18–88), and 25-hydroxyvitamin D was 22 ng/mL (reference 20–50). DXA showed *Z*-scores of +1.3 at the lumbar spine, +0.7 at the femoral neck, +1.3 at the total hip, and +0.3 at the forearm. With the suspicion of bone metastases, an extensive search of a primary tumor was undertaken, with negative results. Percutaneous bone biopsies at the

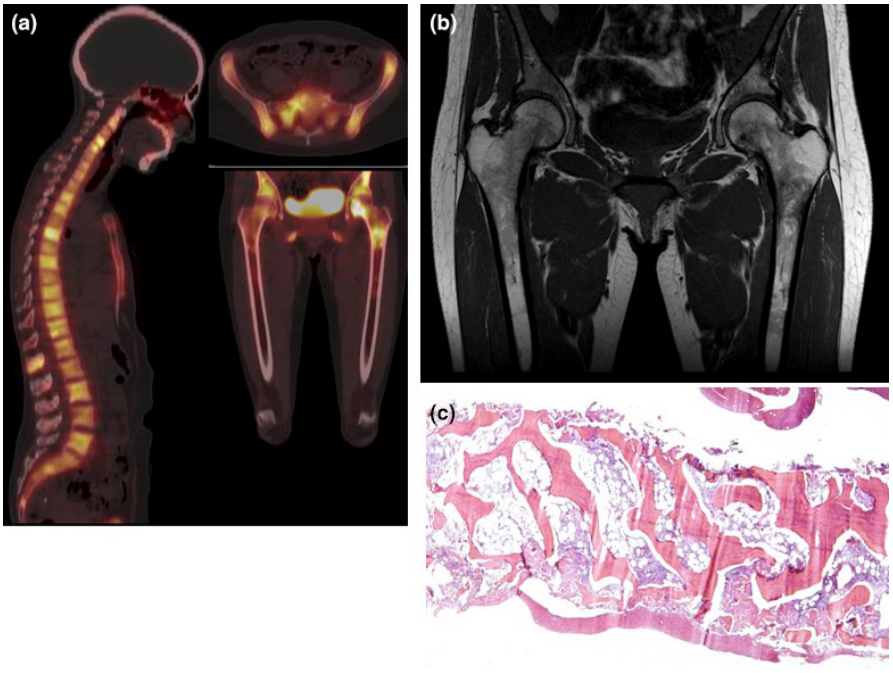

FIGURE 1 Patchy skeletal lesions were demonstrated by 18F-NaF PET/CT scan (a) and MRI (b). Bone biopsies showing increased trabecular thickness, absence of malignant cells, and paucity of osteoblasts and osteoclasts (c).

iliac crest and lumbar vertebral bodies revealed increased trabecular thickness without neoplastic cells or any retained cartilage (Figure 1c). A diagnosis of osteomesopyknosis was established. The whole-exome sequencing analysis (performed at Healthincode, La Coruña, Spain, and later reanalyzed by the authors) revealed genetic variants in *CSF1R* and *ALOX5* genes (see below). After 5-year follow-up, the patient remains in good health, with occasional musculoskeletal pain that resolves with NSAIDs. Serum chemistries remained within the reference ranges, but bone turnover markers somewhat increased (ALP 49, bone-specific ALP 16, PINP 76, CTX 0.57), in probable relation to the menopausal transition.

2.3 | Site-directed mutagenesis and transfections

ALOX5 cDNA (NM_000698) tagged with Myc-DDK and cloned into pCMV6 expression vector (Origene Technologies, Rockville, MD, US) was used as template to introduce the c.1700A>G change, by using the QuickChange site-directed mutagenesis kit (Agilent Technologies, Santa Cruz, CA). MCF7 cells or the osteoblast-like cells MG63 were transfected with wild-type or mutant constructs by using lipofectamine 2000 (Invitrogen, Carlsbad, CA, US) and RNA or protein expression was analyzed 48 h later.

2.4 | Gene expression and splicing analysis

To study possible differences in splicing of *CSF1R*, RNA was isolated from the peripheral blood of the patient and healthy controls. Complementary DNA (cDNA) was generated with the TaKaRa kit PrimeScript RT (TaKaRa, Shiga, Japan). Given that the mutation under study fell near exon 12, the forward primer was designed to hybridize in exon 11 and the reverse primers, in exons 12 and 13, respectively. Then, the products were analyzed by gel electrophoresis.

For studies of gene expression, RNA was extracted from cell cultures using Trizol. After reverse transcription as mentioned above, the expression *of RANKL*, and *OPG* (and the housekeeping genes *GAPDH, RPL13A*, and *RPLP0*) was analyzed by RT-PCR.

2.5 | Flow cytometry

Cells transfected with wild-type or mutant constructs were harvested after 48 h, fixed with 3.7% paraformaldehyde, and stained with anti-Myc-DDK antibodies followed by secondary anti-mouse IgG labeled with allophycocyanin (APC) (A865, ThermoFisher Scientific, Waltham, MA, US). Then, cells were analyzed by flow cytometry (FACSCanto, BD Biosciences, Franklin Lakes, NJ, US) using the FACSDIVA software (BD Biosciences).

2.6 | Western blot analysis

Protein extracts from MCF7 cells were separated on 10% polyacrylamide gels and transferred to nitrocellulose. Blots were incubated with mouse monoclonal antibodies against Myc-DDK tag (TA500011, Origene Technologies, Rockville, MD, US) and α-tubulin (sc-23948, Santa Cruz Biotechnology, Dallas, TX, US), followed by secondary rabbit anti-mouse IgG antibodies conjugated to horseradish peroxidase (sc-51610, Santa Cruz Biotechnology). When indicated, cells were treated with 20 nM bortezomib (Sigma-Aldrich, St Louis, MO, US) for 24 h.

3 | RESULTS

3.1 | Genetic analysis

Whole exome sequencing revealed a number of nonsynonymous variants with gnomAD frequencies lower than 1% (Supplementary Table S1). Two heterozygous variants attracted our attention because of their potential association with skeletal abnormalities. One was an intronic variant of the *CSF1R* gene (OMIM 164770), relatively close to the acceptor splice site for exon 12 (NM_001288705.3: c.1754-13G>A, rs200522818). Population frequency in GnomAD is 0.02% while known pathogenic variants in this gene have frequencies lower than 0.004% (benign strong classification, BS1, according to the ACMG criteria). ClinVar has only one recent submission on this variant which is classified as benign (BP6). Splicing predictors, including SpliceAI, suggest that the acceptor site is not modified by the variant (BP7). Our own cDNA analyses did not reveal any effect of the variant on *CSF1R* transcripts either (not shown). Thus, we consider this variant as likely benign.

The other variant (classified as VUS according to the ACMG criteria) is a missense change at the *ALOX5* gene (OMIM 152390; NM_000698.5; c.1700A>G, p.Asn567Ser) that is not present in disease or population databases, nor in GeneMatcher (PM2). The variant lies in exon 13 (Figure 2a), in a phylogenetically conserved amino acid within a low-complexity region. In silico predictors, including mutation assessor, PROVEAN, LRT, MutPred, SIFT, MetaRNN, and REVEL, support a potential damaging

effect of the variant (PP3). The amino acid change from Asn to Ser increases hydrophobicity and disrupt electrostatic interactions with neighboring residues as shown by protein structure modeling (Figure 2b).

It is to note that we did not find any relevant variant in *CLCN7* or other genes known to be associated with osteopetrosis or osteoclast activity (specifically, no pathogenic variant was identified in the genes associated with high bone mass or rapid bone turnover: *TNFRSF11A, TNFRSF11B, TNFSF11, VCP, SQSTM1, TGFB1, IFITM5, MAFB, CSF1, TRAF6, RELA, RELB, REL, NFKB1, NFKB2, TFEB, CA2, CTSK, OSTM1, PLEKHM1, TCIRG1, SOST, SLC29A3, LRP4, LRP5, LRP6, SNX10, FAM20C, FAM123B, TYROBP, LEMD3, DLX3, and PTDSS1*).

The single brother of the patient was asymptomatic, had a normal spine MRI, and did not carry these genetic variants. No other relatives were available to be studied.

3.2 | Variant-induced gene expression changes

We explored the effect of the *ALOX5* variant on the expression of two key factors in osteoclastogenesis, the

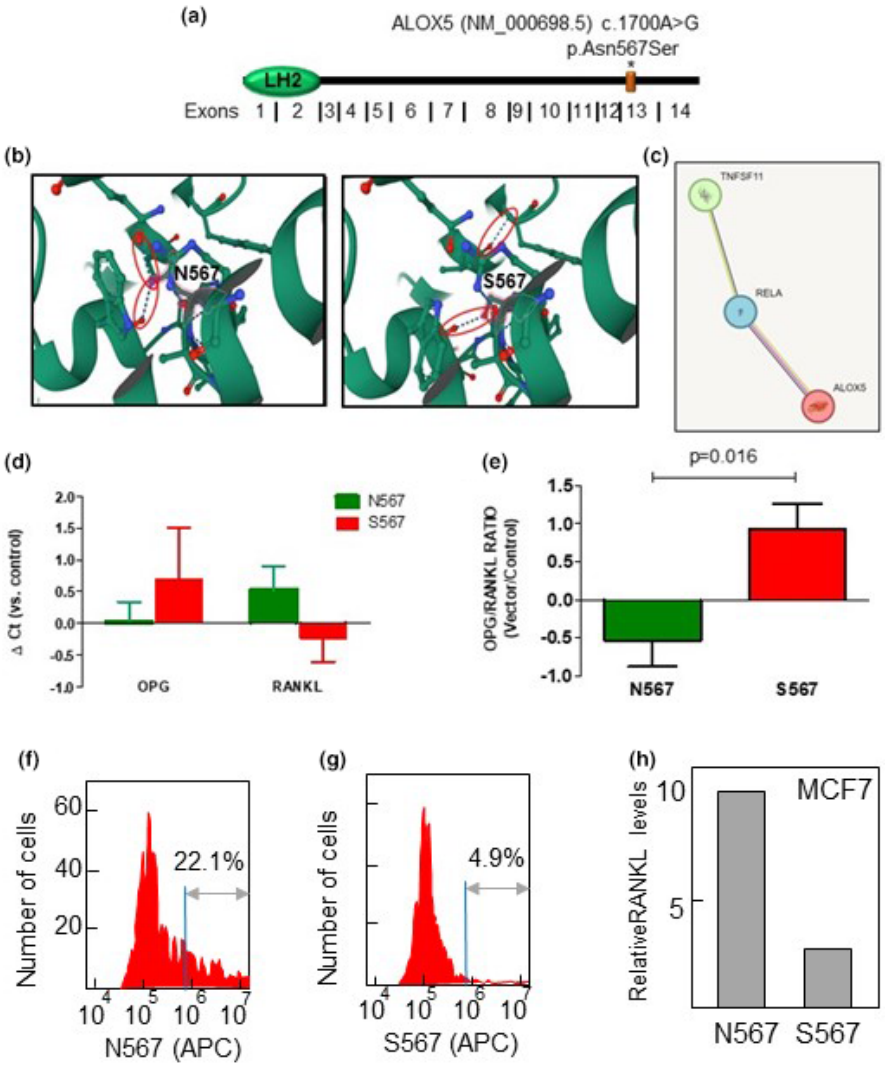

FIGURE 2 (a) Schematic representation of Alox5 protein showing the location of the variant and the LH2 (lipoxygenase homology) domain, which is found in a variety of membrane or lipid-associated proteins. (b) PDB Mol 3D viewer-based model of Alox5 protein highlighting changes in non-covalent interactions between amino acids because of the variant. Rearrangement of binding interactions promote conformational changes. (c) Protein–protein interaction linking ALOX5 and RANKL/TNFSF11, according to STRING database. Line colors indicate text mining (green), experimental evidence (purple) and co-expression evidence (black). (d, e) Endogenous expression of RANKL and OPG in MG63 cells transfected with either a wild-type (N567) or a mutant (S567) ALOX5-containing vector (Mean ± SE of five independent experiments). (f, g) MCF7 Cells transfected with wild-type or mutant Alox5 constructs were analyzed for *ALOX5* protein expression by flow cytometry (a representative image is shown) and the corresponding mRNA levels of RANKL were determined by qRT-PCR (h).

receptor activator for nuclear factor κ B ligand (*RANKL* or *TNFSF11*), and its endogenous decoy receptor osteoprotegerin (OPG). The binding of RANKL to OPG prevents the interaction between RANKL and its cellular receptor RANK. Protein–protein interaction databases, including STRING, BioGRID and IntAct, describe an interaction between *ALOX5* and *RELA*, a transcription factor that upregulates *RANKL/TNFSF11* (Ha et al., 2010), establishing a potential transcriptional link between *ALOX5* and RANKL (Figure 2c). MG63 cells transfected with the mutated *ALOX* vector showed lower levels of *RANKL* transcripts and higher expression of *OPG* (Figure 2d). Consequently, the OPG/RANKL ratio was significantly increased in the presence of the variant (Figure 2e).

3.3 | Functional impact of the N567S variant on the Alox5 protein

MCF7 cells express moderate levels of Alox5 (Zhou et al., 2020) and they have been widely used for transfection experiments because they show high transfection efficiency. MCF7 cells were transfected with wild-type or mutant constructs and the expression levels of Alox5 protein were determined by flow cytometry analysis with antibodies against the DDK peptide that tags the exogenous protein. Alox5-DDK levels were reduced more than 4-fold in the variant-containing cells (Figure 2f,g). Consistent with the results obtained with the osteoblast-like cell line, the genetic variant resulted in decreased levels (about 4-fold) of *RANKL* mRNA (Figure 2h).

The reduced Alox 5 protein levels in cells with mutant constructs may be related to a change in protein folding which could be recognized by the ubiquitin-proteasome system. To confirm this, we treated mutant *ALOX5*-transfected MCF7 cells with the proteasome inhibitor bortezomib and found that inhibition of proteasome restored, at least in part, the protein levels to those observed with the wild-type protein (Figure 3a,b). Thus, we envision a model where the N567S change promotes an alteration in protein folding that is recognized by the proteasome which degrades the altered protein. This reduction in *ALOX5* expression is consistently correlated with downregulation of RANKL activity, and we postulate here that it may be a pathogenic mechanism that prevents osteoclastogenesis (Figure 3c).

4 | DISCUSSION

Although some authors consider that osteomesopyknosis may be a form of osteopetrosis (Mortier et al., 2019), it has distinctive clinical and radiological features. Osteomesopyknosis usually has a benign course, with mild manifestations, but it may complicate patients' work-up because it can resemble other sclerosing bone diseases, and especially osteoblastic metastases. In this patient, extensive diagnostic work-up and follow-up excluded cancer and other acquired disorders as etiologies of the bone lesions. Also, the skeletal distribution and the radiological appearance were not consistent with other primary bone sclerosing disorders, such as osteopetrosis, osteopoikilosis, osteopathia striata, pycnodysostosis, melorheostosis, axial osteomalacia, and the hyperostosis that predominantly affect the cortical bones, such as sclerosteosis and Van Buchen disease (Boulet et al., 2016).

Although an autosomal dominant transmission has been postulated (Maroteaux, 1980; Schmidt et al., 1989; Stoll et al., 1981), there are no reports of gene variant analyses, and the genes involved are unknown. The cellular mechanisms leading to bone sclerosis have not been elucidated either. However, the normal levels of bone turnover markers (Jeoung et al., 2015; Madruga Dias et al., 2013; Yao & Camacho, 2014) and the paucity of osteoblasts and osteoclasts with low bone turnover in a few cases reported with bone biopsies (Hardouin et al., 1994; Madruga Dias et al., 2013), as well as in the present case, suggest that bone sclerosis is the consequence of decreased bone resorption, rather than increased osteoblastic bone formation. However, paradoxically, in the present case and in previous reports (Jeoung et al., 2015; Yao & Camacho, 2014), an increased uptake of bone-seeking radioligands was observed, which would suggest increased metabolic bone activity. We cannot speculate if it is due to the existence of different phases of cell activity across time, or across bone regions, or to any other phenomenon.

The *ALOX5*-variant consists of an asparagine-to-serine change within a low-complexity region. These regions are abundant in eukaryotic proteins for reasons that remain unclear, although it has been proposed that they may have a role in generating novel protein functions (Toll-Riera et al., 2012). Several in silico predictors support a potential damaging effect of the variant. In addition, the Asn-Ser change is likely to increase hydrophobicity and disrupt electrostatic interactions with neighboring residues. Protein structure modeling using PDB Mol 3D viewer of both wild-type and mutant protein simulates these changes in the interaction between amino acids.

Based on these predictions, along with the histological and biochemical data that suggested bone resorption impairment, we aimed to study the functional impact of this variant on the RANKL/OPG system, a core regulator of osteoclastogenesis within the bone microenvironment.

Alox5 catalyzes the transformation of arachidonic acid to leukotriene A4 (LTA4), an unstable epoxide that

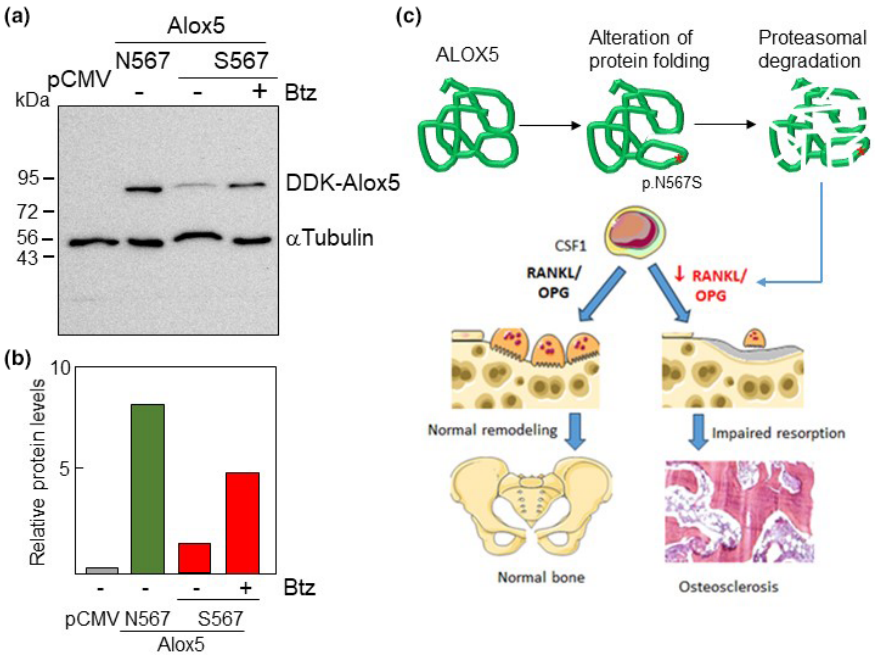

FIGURE 3 (a) Expression of exogenous Alox5 protein, tagged with DDK, in both wild-type and mutant-transfected cells was determined by western blot analysis. The levels of α-tubulin were also analyzed as loading control. (b) Protein levels of DDK-Alox5 relative to those of αTubulin as determined by analyzing the western blot image with the NIH ImageJ software. (c) Proposed pathophysiological model. The missense genetic variant alters the correct folding of the protein, and promotes its proteasomal degradation. Downregulation of Alox5 protein leads to decreased RANKL expression, which impairs the differentiation of osteoclast precursors, decreases bone resorption, and leads to osteosclerosis.

is rapidly transformed to leukotriene B4 (LTB4) by LTA4 hydrolase (Haeggström & Funk, 2011). LTB4 exerts pleiotropic effects on various cell types interacting with two functional receptors, BLT1 and BLT2 (He et al., 2020). Interestingly, binding of LTB4 to its receptors activates signaling pathways, including MAP kinases (ERK and JNK), and NFκB, whereas RANKL expression in T cells is dependent on ERK1/2 (Bishop et al., 2015). In addition, Alox5 has been shown to interact with RELA (p65), a subunit of the NFκB transcription factor (Wu et al., 2018) also involved in RANKL activity. Thus, although the transcriptional regulation of RANKL is complex and includes distal enhancers, marked by acetylated histones, and a proximal promoter that is functionally modulated by DNA methylation (Delgado-Calle et al., 2012), there are signaling pathways that may link Alox5 with the expression of *RANKL*. This concept is in line with several experimental models showing a positive relationship between *ALOX5* expression or activity and osteoclast formation (Fujita et al., 2022; Lee et al., 2012; Moura et al., 2014).

We asked whether the N567S change, which may alter protein conformation, was able to modify the protein levels of Alox5. Indeed, we found a clear reduction of Alox5 protein levels in variant-containing cells as determined by western blot and flow cytometry analyses. Improper conformations due to mutations may cause protein misfolding and aggregation that can be highly toxic and, under normal conditions, are rapidly degraded by the proteasome (Goldberg, 2003; Tao & Conn, 2018). Consistent with this, we found that inhibition of proteasome in variant-containing cells restored Alox5 protein levels.

This report has some limitations. We could not perform functional studies with patient's bone samples. Thus, we do not know the precise molecular mechanisms linking the reduced Alox5 activity and *RANKL/OPG* expression. Additionally, the mechanisms explaining the unique distribution of bone lesions in osteomesopyknosis remain to be elucidated. Nevertheless, we can speculate that they are related to the higher metabolic activity in trabecular bone, thus making the bone regions with abundant trabecular bone (such as the vertebral bodies, iliac crest, or proximal femoral epiphyses) more susceptible to undergoing sclerotic changes than those predominantly composed of compact bone tissue, such as the diaphysis of the long bones. Unfortunately, there were no relatives available to carry out a full family segregation study.

In conclusion, although osteomesopyknosis is not recognized as an independent entity in the current nosology of bone sclerosing disorders (Mortier et al., 2019), we feel that it presents quite characteristic clinical-radiological features (multiple focal sclerosing lesions affecting the

medullary region of axial skeletal bones; none or mild symptoms; normal biochemical and hematological results; and absence of infection, neoplastic cells, or osteomalacia in bone biopsies) that allow distinguish it from other disorders. The etiology is unknown, but several reports suggest an autosomal dominant transmission. Here, we report a case of osteomesopyknosis associated with a novel heterozygous ALOX5 variant that resulted in reduced levels of Alox5 protein and decreased RANKL/OPG ratio, in line with the paucity of osteoclasts observed in the bone biopsy. To our knowledge, this is the first reported association of a gene variant with osteomesopyknosis.

AUTHOR CONTRIBUTIONS

JLFL and JAR conceived the study, coordinated it, and elaborated the first manuscript draft. OG, ADR, and CS performed the genetic and biochemical analyses. AIV, SC, and JLFL analyzed and interpreted the genetic variants. JLH, SM, MN, and JAR contributed to the acquisition and interpretation of clinical, histological and imaging data. All authors read the manuscript, gave scientific input, and approved its submission.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have competing interests.

DATA AVAILABILITY STATEMENT

The data generated during this study are within the published article.

ETHICAL APPROVAL

The study was approved by the IRB (Comité de ética de investigación con medicamentos de Cantabria, protocol number 2023.355, on October 20, 2023). The patient gave informed written consent.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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