Journal of Bone and Mineral Research, 2025, **40**, 69–78
https://doi.org/10.1093/jbmr/zjae175
Advance access publication: November 1, 2024
Research Article

Family-based whole-exome sequencing implicates a variant in lysyl oxidase like 4 in atypical femur fractures

Wei Zhou[1], Denise M. van de Laarschot[1], Jeroen G.J. van Rooij[1], Marijke Koedam[1], Hanh H. Nguyen[2], André G. Uitterlinden[1], Peter R. Ebeling[2], Rajesh V. Thakker[3,4], Piet Geusens[5,6], Bram C.J. van der Eerden[1], Annemieke J.M.H. Verkerk[1], M. Carola Zillikens[1,*]

[1]Department of Internal Medicine, Erasmus MC, Erasmus University Medical Center, Rotterdam, 3015 GD, the Netherlands
[2]Department of Medicine, School of Clinical Sciences, Monash University, Clayton, VIC 3168, Australia
[3]Academic Endocrine Unit, Radcliffe Department of Medicine, University of Oxford, Oxford, OX3 9DU, United Kingdom
[4]National Institute for Health Research Oxford Biomedical Research Centre, Oxford, OX3 9DU, United Kingdom
[5]Biomedical Research Institute, University Hasselt, Diepenbeek, 3500, Belgium
[6]Department of Internal Medicine, Maastricht University, Maastricht, 6211 LK, the Netherlands
*Corresponding author: M. Carola Zillikens, Rg-5, Erasmus University Medical Center, Dr. Molewaterplein 40, NL-3015 GD Rotterdam, the Netherlands (m.c.zillikens@erasmusmc.nl).

Abstract

Atypical femur fractures (AFFs) are rare adverse events associated with bisphosphonate use, having unclear pathophysiology. AFFs also cluster in families and have occurred in patients with monogenetic bone diseases sometimes without bisphosphonate use, suggesting an underlying genetic susceptibility. Our aim was to identify a genetic cause for AFF in a Caucasian family with 7 members affected by osteoporosis, including 3 siblings with bisphosphonate-associated AFFs. Using whole-exome sequencing, we identified a rare pathogenic variant c.G1063A (p.Gly355Ser) in lysyl oxidase like 4 (*LOXL4*) among 64 heterozygous rare, protein-altering variants shared by the 3 siblings with AFFs. The same variant was also found in a fourth sibling with a low-trauma femur fracture above the knee, not fulfilling all the ASBMR criteria of AFF and in 1 of 73 unrelated European AFF patients. LOXL4 is involved in collagen cross-linking and may be relevant for microcrack formation and bone repair mechanisms. Preliminary functional analysis showed that skin fibroblast-derived osteoblasts from the unrelated patient with the *LOXL4* variant expressed less collagen type I and elastin, while osteogenic differentiation and mineralization were enhanced compared with 2 controls. In conclusion, this *LOXL4* variant may underlie AFF susceptibility possibly due to abnormal collagen metabolism, leading to increased formation of microdamage or compromised healing of microcracks in the femur.

Keywords: atypical femur fractures, bisphosphonates, LOXL4, whole-exome sequencing, family study, osteoporosis, collagen

Lay Summary

Atypical femur fractures (AFFs) are rare fractures of the upper leg that can occur in people using bisphosphonates for osteoporosis. Genetic factors may play a role. We studied a family with 7 members affected by osteoporosis, including 3 siblings who developed AFFs after using bisphosphonates. In this family, a rare variant in the LOXL4 gene was identified as a potential cause for AFFs. This variant was also present in another sibling who had a different type of upper leg fracture, and in 1 of 73 unrelated European patients with AFFs. LOXL4 is involved in collagen cross-linking, a crucial bone formation process. The variant may impair collagen metabolism, leading to increased microdamage or compromised bone healing, which could increase the risk of AFFs.

Introduction

Atypical femur fractures (AFFs) are considered rare events associated with bisphosphonate treatment for osteoporosis. The incidence of AFFs was estimated to be 3-17 per 100 000 person-years in the general population.[1–3] These fractures occur following minimal or no trauma with distinct radiological features from typical osteoporotic fractures. They resemble stress/insufficiency fractures, with transverse morphology, minimal comminution, and have localized cortical thickening at the lateral fracture site.[3] Although rare, AFFs have caused great concern among patients and physicians, which has resulted in a 50% decline in the use of bisphosphonates since 2008 and 2012.[4]

The pathophysiology of AFFs is still unclear. The observation of familial clustering and occurrence of AFFs in patients with monogenetic bone diseases suggests that a sometimes mild and unrecognized monogenetic bone disease may be an independent risk factor for its development.[5–7] Understanding the underlying mechanism of these genetic factors in AFFs is crucial for determining the appropriate use of bisphosphonates in treating these patients. Additionally, identifying a set of genetic susceptibility variants for AFF could enable

Received: January 18, 2024. **Revised:** September 19, 2024. **Accepted:** October 31, 2024

screening for a high risk of AFF before initiating or continuing bisphosphonate treatment.

Previously, pathogenic variants associated with monogenic bone disorders, ie, in *ALPL*, *PLS3*, *LRP5*, *COL1A1*, and *COL1A2*, were reported in some AFF patients.[7–11] Whole-exome sequencing (WES) in families with multiple AFF cases offers a hypothesis-free approach and may reveal potentially causal variants in genes hitherto not known to be involved in AFF. Roca-Ayats et al.[12] performed a WES study in a Spanish family with 3 sisters who sustained an AFF and found 37 shared rare, exonic variants. The authors highlighted variants found in geranylgeranyl pyrophosphate synthase 1 (*GGPS1*) and cytochrome P450 superfamily 1A1 (*CYP1A1)*, suggesting their involvement in bisphosphonate metabolism.[13,14] Recently our group also described 2 Southeast Asian families.[15] They did not have rare variants in *GGPS1* or *CYP1A1*, but 2 sisters shared a variant in TNF receptor associated factor 4 (*TRAF4*), which is potentially important for bone development. However, causality of these genetic variants in AFF patients has not been determined.

This study describes 7 individuals from a European family with osteoporosis, including 3 siblings affected by bisphosphonate-associated AFFs. We hypothesized a segregation of a genetic variant for AFF in this pedigree, and also assessed whether this variant was responsible for the osteoporosis phenotype. We performed both WES and array-based DNA genotyping on the family members, and on a Dutch cohort of unrelated AFF cases to investigate a genetic cause of AFF. Finally, the most likely candidate gene was investigated with genetic epidemiological studies for its association with bone mineral density and fracture, and assessed with preliminary functional studies including osteoblast and fibroblast cultures for its potential role in AFF.

Materials and methods

The AFF family

The pedigree of the family comprises 3 siblings (II.2, II.4, II.7) who sustained one or more complete and/or incomplete AFFs after bisphosphonate treatment for osteoporosis, as shown in Figure 1. The AFFs occurred after more than 5 years of oral bisphosphonate use and met the radiological criteria from the second ASBMR Task Force report (Table 1).[3] They received bisphosphonates for osteoporosis with vertebral fractures (II.2, II.4, and II.7) and/or non-vertebral fragility fractures (II.2 and II.7).

Three other siblings from this generation (II.3, II.5, and II.6) had osteoporosis based on DXA but did not sustain an AFF. This included 1 sister (II.5) who had used alendronate between 2006 and 2008 and sustained a low-trauma distal femur fracture that did not meet the diagnostic criteria for AFF because the fracture was comminuted with intra-articular involvement.[3]

A more detailed clinical description of the individual family members can be found in Appendix I.

Genetic analysis for the AFF phenotype

Following the hypothesis that AFF is induced by a combination of bisphosphonate use and a genetic predisposition for AFF, a dominant mode of inheritance (both autosomal and X-linked) and a recessive autosomal mode of inheritance of AFF are plausible, given the involvement of multiple family members of both sexes. Those family members without AFF cannot be used as reference in a segregation analysis because they might have developed AFF with (longer) use of bisphosphonates, including II.5, who had had received alendronate for 1 to 2 years. However, since II.5 had a low-trauma femoral fracture after bisphosphonate exposure, it could be argued to consider her a carrier of the genetic causal factor(s) of AFF even though this fracture did not completely fulfill the ASBMR diagnostic radiological criteria. Due to the limited number of available family generations and the small sample size, conducting genetic linkage analysis was not feasible in this study.

DNA samples were obtained from 9 individuals from 2 generations (See Figure 1). DNA array-based genotyping and WES were performed in these 9 family members and a cohort of 73 unrelated individuals with AFF in the Netherlands.[7] The unrelatedness of the AFF patient cohort with the family was confirmed by pairwise isolation by distance (IBD) using KING software calculated on DNA genotyping data. Details of patient inclusion, WES analyses, and DNA genotyping are described in Appendix I.

We selected for rare, protein-altering variants and variants in untranslated regions (UTRs), segregating in the family with the assumed inheritance patterns. Multi-allelic variants were excluded. As the incidence of AFF was estimated to be 1 in 1000 long-term bisphosphonate users per year,[16] we used a cutoff value of 0.005 for the minor allele frequency to filter rare variants, using frequencies of the overall population and the (non-Finnish) European population, from gnomAD (version 2.1.1) and 1000 Genomes (version p3v5).[17] In the family, we tested for co-segregation of AFF with any pathogenic variant in the known Mendelian bone disease genes summarized in Table S1, and in *GGPS1*, *CYP1A1*, and *TRAF4*.[12,14,15] Subsequently, we analyzed co-segregation of rare, protein-altering variants with the AFF phenotype and searched for variants shared with the unrelated AFF patients.

Genes were prioritized based on a comprehensive consideration of all information: (1) CADD score >15; (2) gene conservation; (3) suggestion of bone-related functions by gene annotation using public or in-house databases as described in Appendix I; and (4) literature documentation of function related to bone metabolism or bisphosphonate action.

Genetic analysis for the osteoporosis phenotype

In the clinical assessment of this family, it showed that 7 family members have osteoporosis and 3 have osteopenia. At least 5 out of 7 family members (II.2, II.3, II.4, II.6, II.7) with osteoporosis have other reasons for low BMD due to comorbidities and lifestyle factors (Table 2). It cannot be distinguished with certainty whether these individuals have a genetic form of osteoporosis rather than non-genetic secondary osteoporosis. Two individuals, II.5 and III.1, are most likely to be affected by genetic osteoporosis, since smoking in II.5 and anti-epileptic use in III.1 appear to be unsatisfactory explanations for the severity of the osteoporosis and/or fragility fractures in these women. The only family member with a normal BMD, III.9, could be used as a non-affected control for the analysis of genetic osteoporosis.

The presence of pathogenic variants in known Mendelian bone disease genes was assessed in the family members with osteoporosis. In addition, we tested for a polygenic basis of the osteoporosis phenotype by a polygenic risk score (PRS) using the conditionally independent genome-wide significant

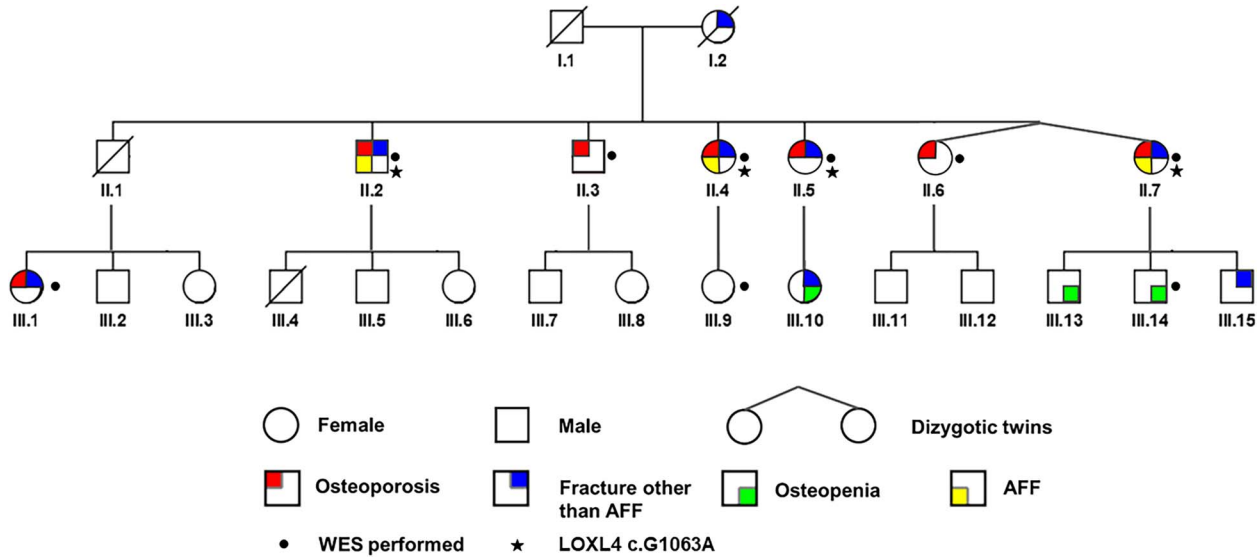


Figure 1. Family pedigree.

Table 1. ASBMR Task Force 2013 revised case definition of atypical femur fractures.

The fracture must be located along the femoral diaphysis from just distal to the lesser trochanter to just proximal to the supracondylar flare.
Major features (at least 4 out of 5 required for diagnosis)
The fracture is associated with minimal or no trauma, as in a fall from a standing height or less.
The fracture line originates at the lateral cortex and is substantially transverse in its orientation, although it may become oblique as it progresses medially across the femur.
Complete fractures extend through both cortices and may be associated with a medial spike; incomplete fractures involve only the lateral cortex.
The fracture is noncomminuted or minimally comminuted.
Localized periosteal or endosteal thickening of the lateral cortex is present at the fracture site ("beaking" or "flaring").
Minor features (optional)
Generalized increase in cortical thickness of the femoral diaphysis.
Unilateral or bilateral prodromal symptoms such as dull or aching pain in the groin or thigh.
Bilateral incomplete or complete femoral diaphysis fractures.
Delayed fracture healing.
Excluding fractures
Fractures of the femoral neck.
Intertrochanteric fractures with spiral subtrochanteric extension.
Periprosthetic fractures.
Pathological fractures associated with primary or metastatic bone tumors and miscellaneous bone diseases (eg, Paget's disease, fibrous dysplasia).

alleles from the GWAS results of femoral neck BMD (FN-BMD) ($n = 49$) and lumbar spine BMD (LS-BMD) ($n = 48$) by Estrada et al.[18] Per individual with DNA genotyping data, the dosages of the alleles were multiplied by the respective effect size extracted from GWAS summary statistics and summed up to create PRS. As a reference, we included individuals from the Rotterdam Study cohorts (RS-I, RS-II, and RS-III), a population-based cohort included individuals >55 years old who lived in the well-defined Ommoord district in the city of Rotterdam in the Netherlands in 1990.[19] The PRS of the individuals from this cohort were also calculated and their LS-BMD T-scores and FN-BMD T scores measured by DXA were regressed on the respective PRS.

Epidemiological study of the selected candidate gene, *LOXL4*

Gene association with BMD and fracture risk in GWAS

GWAS summary statistics of BMD and fractures were downloaded from GEFOS (http://www.gefos.org/), including 4 published GWAS studies.[18,20–22] The associations of *LOXL4* with BMD and fractures were assessed using single nucleotide polymorphisms (SNPs) within 500 kb up- and downstream of the candidate gene. For the analyses in UK Biobank, multiple testing corrected significance threshold of p-value $<8.0 \times 10^{-5}$ was calculated based on the Veff process described by Li and Ji which keeps type-I error rate at 5%.[23]

Expression quantitative trait loci analysis of associated SNPs

Associations of SNPs in *LOXL4* were further evaluated through expression quantitative trait loci (eQTL) analysis in multiple tissues using data from GTEX (https://gtexportal.org/), since data are not available in bone tissues.

Preliminary functional investigation of LOXL4

Expression in bone marrow mesenchymal stromal cells

Time course-based expression of *LOXL4* in human bone marrow mesenchymal stromal cells (BMSCs)-derived osteoblasts and adipocytes was measured following total RNA isolation,

Table 2. Risk factors for osteoporosis in family members.

ID	Age (yr)	Sex	FN-BMD (T-score, SD)	LS-BMD (L2-L4 T-score, SD)	Fractures	COPD	Diabetes mellitus	Smoking (PY)	Early menopause	Use anti-epileptics	Other comorbidities
II.2	74	M	−1.6	−1.1	AFF, Radius Foot	+	+	+ (34)	NA	−	Hypogonadism Hyperthyroidism
II.3	70	M	−2.1	−2.7	−	+	+	+ (37)	NA	−	−
II.4	62	F	−2.2	−0.3	AFF, Vertebral	−	−	−	+	−	−
II.5	59	F	−3.1	−2.3	Vertebral, Femoral	−	−	+ (40)	−	−	−
II.6	66	F	−3.3	+0.3	−	−	+	+ (40)	−	−	−
II.7	66	F	−2.6	−1.2	AFF, Radius	−	−	−	−	+	RA SLE
III.1	64	F	UNK	UNK	−	−	−	−	−	+	−
III.10	45	F	−0.3	−1.7	−	−	−	+ (34)	+	−	−
III.13	47	M	−2.2	−1.9	−	−	−	−	NA	−	PMR
III.14	44	M	−2.1	−2.1	−	−	−	+ (8)	NA	−	−

Abbreviations: "+", yes. "- ", no. yr, year. SD, standard deviation. M, male. F, female. FN-BMD, bone mineral density of the femoral neck. LS-BMD, bone mineral density of the lumbar spine. COPD, Chronic Obstructive Pulmonary Disease. PY, pack years of smoking. NA, not applicable for men. RA, rheumatoid arthritis. SLE, systemic lupus erythematosus. PMR, polymyalgia rheumatica for which III.13 had used oral corticosteroids. UNK, unknown

cDNA synthesis, and real-time polymerase chain reaction. BMSCs were derived from Lonza (Breda, the Netherlands) and were cultured as described before.[24]

Skin fibroblast culture

Skin fibroblasts derived from skin biopsies of the unrelated patient with the LOXL4 variant and 2 sex- and age-matched controls were cultured and differentiated to osteoblasts for measurement of ALP activity, mineralization, collagen type I immunostaining, and gene expression of collagens, elastin, lysyl oxidase (LOX) family genes, collagen-degrading matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (TIMPs).

Experimental data are presented as mean ± SEM and compared between groups by 2 sample T-test. Details of expression in BMSCs, skin fibroblast culture, RNA isolation, and quantification of gene expression, ALP activity and mineralization assays, and immunostaining for collagen type 1 are included in Appendix I.8.

Study approval

The study of AFF patients and the family was approved by the Medical Ethical Committee of Erasmus MC under number MEC-2013-264. All participants of the AFF study signed written informed consent to participate in the study related to the causes and risk factors of AFF and the 2 control patients gave informed consent to use skin biopsy material for scientific research purposes. The Rotterdam Study has been approved by the Medical Ethics Committee of the Erasmus MC (registration number MEC 02.1015) and by the Dutch Ministry of Health, Welfare and Sport (Population Screening Act WBO, license number 1071272-159521-PG). The Rotterdam Study Personal Registration Data collection is filed with the Erasmus MC Data Protection Officer under registration number EMC1712001. The Rotterdam Study has been entered into the Netherlands National Trial Register (NTR; www.trialregister.nl) and into the WHO International Clinical Trials Registry Platform (ICTRP; https://apps.who.int/trialsearch/) under shared catalog number NL6645 / NTR6831. All participants of the Rotterdam Study provided written informed consent to participate in the study and to have their information obtained from treating physicians.

Results

WES results

We found 64 nonsynonymous, exonic, heterozygous variants in 64 genes shared by the 3 siblings with AFFs using an autosomal dominant model and 2 variants in 2 genes using an autosomal recessive model (Table S2). When using an X-linked dominant model, we did not find any variants shared by the 3 siblings with AFFs. Among these shared variants, 13 were prioritized based on predicted risk (CADD score >15) and their potential involvement in bone function, including *VIPR1*, *LOXL4*, *F5*, *ROBO3*, *SLC12A2*, *APC*, *ECSIT*, *TDRD6*, *SCN4A*, ABL2, *TMEM138*, *MLLT4*, and *RNF213* (Table S3).

Lysyl oxidase like 4 (*LOXL4)* was one of the most interesting candidate genes because the gene encodes a member of the lysyl oxidase family, which is important for the formation of collagen or elastin crosslinks. Variant c.G1063A in *LOXL4*, shared by 3 siblings affected by AFFs, was extremely rare, with an allele frequency of 0.0002 in both overall population and non-Finnish Europeans in gnomAD and a CADD score of 35 (Table S2). It was also present in the sister with a low trauma distal femoral fracture not fulfilling all the ASBMR AFF criteria (II.5), but not in other family members (Figure 1).

Moreover, WES analysis of the Dutch cohort of 73 unrelated AFF patients revealed that 1 AFF patient (Patient U) carried the same *LOXL4* variant. Patient U sustained an incomplete AFF of the right femur and a complete AFF of the left femur after 10 years of bisphosphonate use (more detailed clinical description provided in Appendix I). Three variants in other genes were also shared between the 3 AFF siblings and 1 to 3 unrelated patients, but were all relatively common either in the overall population or in non-Finnish Europeans in gnomAD (allele frequency >0.001) (Table S2).

Based on the high predicted deleteriousness of the variant and its validation in an unrelated AFF patient, as well as the function of the lysyl oxidase family in formation of collagen or elastin crosslinks, the c.G1063A variant in *LOXL4* was

selected for further evaluation. The variant was confirmed by Sanger Sequencing (Figure S1).

No pathogenic rare variants associated with known monogenetic bone diseases were found in family members with AFF and/or osteoporosis, nor any previously reported AFF-associated variants in *CYP1A1, GGPS1*, or *TRAF4*.

PRSs for low BMD

To investigate whether osteoporosis in this family can be explained by a polygenic background, we calculated site-specific BMD increasing PGSs for the family members and a population cohort as reference using DNA genotyping data. As shown in Figure 2A, the PRS for low FN-BMD was more than 1 SD higher than the mean PRS in the reference population in 6 family members with FN-BMD T-scores below -1.5 (II.2, II.3, II.4, II.5, II.6, II.7 as described in Appendix II). Two family members had PRS for low FN-BMD within ±1 SD, ie, III.1 who had severe osteoporosis (BMD not known and therefore not included in Figure 2A) and III.9 who had normal FN-BMD (Figure 2A). For LS-BMD, except II.2 whose PRS for low LS-BMD was within ±1 SD, all family members with osteoporosis had PRS for low LS-BMD that was more than 1 SD higher than the population mean (Figure 2B).

Association of LOXL4 SNPs with BMD and fracture risk in GWAS database

Using GWAS results from UK Biobank, we found 1 signal, rs4919173 (chr10: 99856584:G:A, hg19), within 100 kb downstream of *LOXL4*, significantly associated with a modest increased risk of all types of fracture (OR = 1.03, 95% CI: 1.02-1.05, p-value = 4.9e-06) but not associated with BMD (p-value = 0.53) (Figure 2C-D). GWAS results from smaller cohorts did not show any associations of *LOXL4* SNPs with BMD or fracture risk ($p > .001$ for all SNPs within 500 kb up- and downstream of *LOXL4*). The major allele of SNP rs4919173 associated with fracture risk was positively associated with the expression of *LOXL4* in 3 tissues in GTEX, ie, testis, esophagus-muscularis, and whole blood (Figure 2E).

Functional investigation of LOXL4

LOXL4 was consistently expressed throughout the osteogenic differentiation of BMSCs (Figure 3A). In contrast, *LOXL4* expression was reduced dramatically from the second day of adipocyte differentiation.

Preliminary experiments were performed to assess osteogenic potential in patient with the *LOXL4* variant using skin fibroblasts from Patient U and 2 sex- and age-matched controls, since no skin biopsy material could be obtained from the family members. Fibroblast-derived osteoblast cultures from Patient U with the *LOXL4* variant demonstrated enhanced osteogenic differentiation compared with controls, with elevated ALP activity on day 7 and increased mineralization on day 24 of culture (Figure 3B). Immunostaining of the patient's osteogenic cultures showed reduced collagen type-I protein stainings both on day 7 and day 14, while negative controls lacking the primary antibody revealed no green fluorescence (Figure 3C). Other collagens were also differentially expressed between the patient and control fibroblasts at mRNA level, including collagen 3, 14, 15 (reduced), and 6 (enhanced) at 1 or both time points, as were the expression levels of *LOXL2*, *MMP2*, *MMP3*, *TIMP2*, and *TIMP4* (Figure S2, Appendix II). These data indicate that changes in *LOXL4* may affect osteogenic differentiation associated with dynamic changes in collagens and its metabolism.

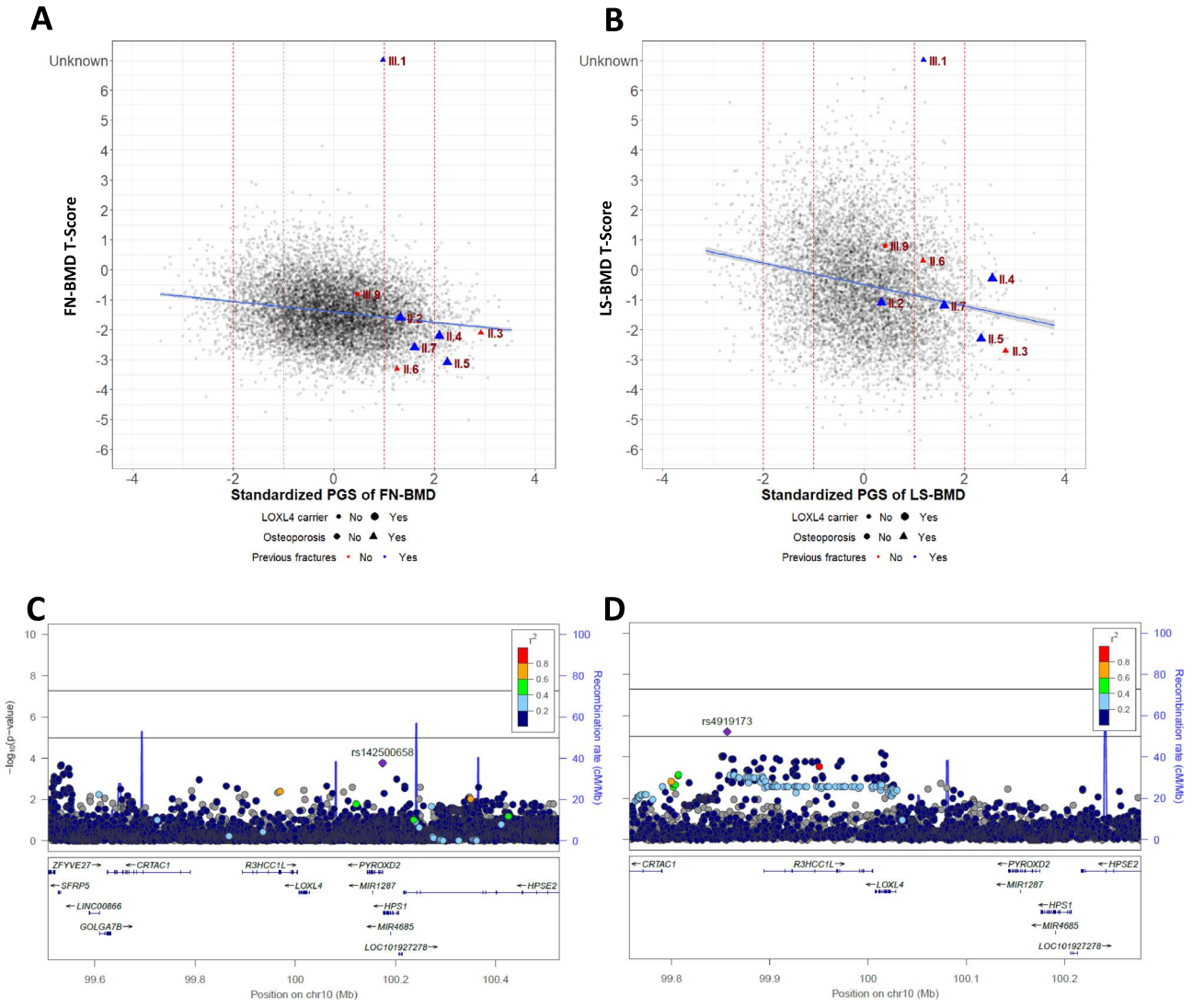
Discussion

We performed whole-exome sequencing analysis in a family with 4 cases of low-trauma femoral fractures after bisphosphonate exposure, of which 3 met the diagnostic criteria for AFF. We found a rare missense variant, c.G1063A (p.Gly355Ser), in *LOXL4* shared by 3 siblings with AFF. This is the most likely susceptibility variant for AFF based on its function in collagen cross-linking, the high predicted pathogenicity, and its presence in an unrelated AFF patient. This variant was also present in the fourth family member who suffered a femoral fracture after a low-trauma, although the fracture was comminuted and had intra-articular involvement, therefore not meeting the diagnostic criteria for AFF. However, it was a low-energetic femur fracture and might belong to the spectrum of AFF.

The *LOXL4* gene is a member of the LOX family, which includes LOX and 3 other LOX-like enzymes (LOXL1-3). These copper-dependent enzymes catalyze the final step in the formation of cross-links in elastin and collagens. We showed that *LOXL4* was highly expressed across different stages of osteoblast differentiation while being suppressed in differentiating adipocyte cultures. This suggests that *LOXL4* is important for osteoblast differentiation and involved in normal bone function, as also implied by previous studies.[25–29]

Cultures of skin fibroblasts collected from the unrelated AFF patient carrying the *LOXL4* variant showed increased ALP activity and mineralization, as well as reduced collagen type-I protein. Such abnormalities in collagen formation may indicate an extracellular matrix-related defect. Furthermore, in the patient fibroblast cultures at day 14, we observed reduced expression levels of elastin and collagen degradation genes *MMP2* and *TIMP2* and increased expression levels of *COL6A2* and *COL6A3* compared with controls, consistent with alterations in collagen metabolism. It is possible that enhanced osteogenic differentiation and mineralization observed in the fibroblast cultures of the AFF patient were secondary to the abnormal collagen metabolism due to the *LOXL4* variant. A similar phenomenon has been observed in patients with osteogenesis imperfecta with primary defects in collagen type I, also showing increased bone matrix mineralization.[30]

We hypothesize that *LOXL4* variants disturb bone repair, potentially contributing to AFF. A study on fracture healing in mice demonstrated that *LOXL4* had a high expression on day 7 of fracture healing, and its expression continued to increase until day 21.[31] This suggests that LOXL4 is not only crucial during endochondral fracture healing, but also during later stages of fracture healing, ie, bone remodeling. In another mice study, *LOXL4* expression was increased in nonrigid compared with rigid fracture healing.[32] Nonrigid fracture healing requires remodeling of mineralized calluses by osteoclasts, which can be blocked by bisphosphonates. Dysfunction of LOXL4 may compromise healing of microcracks that are continuously formed especially in the femur under daily mechanical loading, and the use of bisphosphonates may worsen this healing process by reducing bone turnover, eventually leading to an incomplete or complete AFF. Another



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Gencode Id	SNP Id	P-Value	NES	Tissue
ENSG00000138131.3	rs4919173	2.2e-10	0.60	Testis
ENSG00000138131.3	rs4919173	2.3e-10	0.40	Esophagus - Muscularis
ENSG00000138131.3	rs4919173	0.000025	0.34	Whole Blood

Figure 2. Polygenic risk scores for low BMD in the family members and the association of LOXL4 with BMD and fracture. A-B, association between standardized PGS for BMD and BMD T-scores of the subjects (descending trend line). The dots forming the clouds represent Rotterdam study subjects; the triangle dots represent the family members; the vertical lines represent 1 SD and 2 SD from the mean in the Rotterdam study population. C-D, associations of LOXL4 and BMD (C) and all types of fractures (D) in UK Biobank. E, association of rs4919173 with LOXL4 by expression quantitative trait loci in GTEX. NES, normalized effect size, defined as the slope of the linear regression, and it represents the effect of the alternative allele relative to the reference allele in the human genome reference. PGS, polygenic scores. BMD, bone mineral density. GTEX, Genotype-Tissue Expression.

hypothesis is that the LOXL4 variant could affect the nanostructure of the bone matrix by altering collagen crosslinking, possibly making it less resistant to microdamage and increasing microcrack formation.[33] This effect may contribute to AFFs when combined with increased microcrack propagation caused by bisphosphonates.

The c.G1063A variant in *LOXL4* carried by this family and an unrelated AFF patient is located in the Scavenger Receptor Cysteine-Rich 3 (SRCR3) domain of the protein. It is not known whether this variant leads to increased or decreased LOXL4 expression, changed protein structure, or influences its interaction with other proteins. It has been proposed that SRCRs have a function independent from the catalytic domain of LOXL4 in cancer research, possibly through its influence on protein interactions.[34–38] Moreover, several proteins besides collagens have been reported to be the substrates of lysyl oxidases.[39,40] Therefore, a variant affecting the SRCR domain may exert its effect through other proteins, which may have different and milder effects than when the LOX domain is affected.

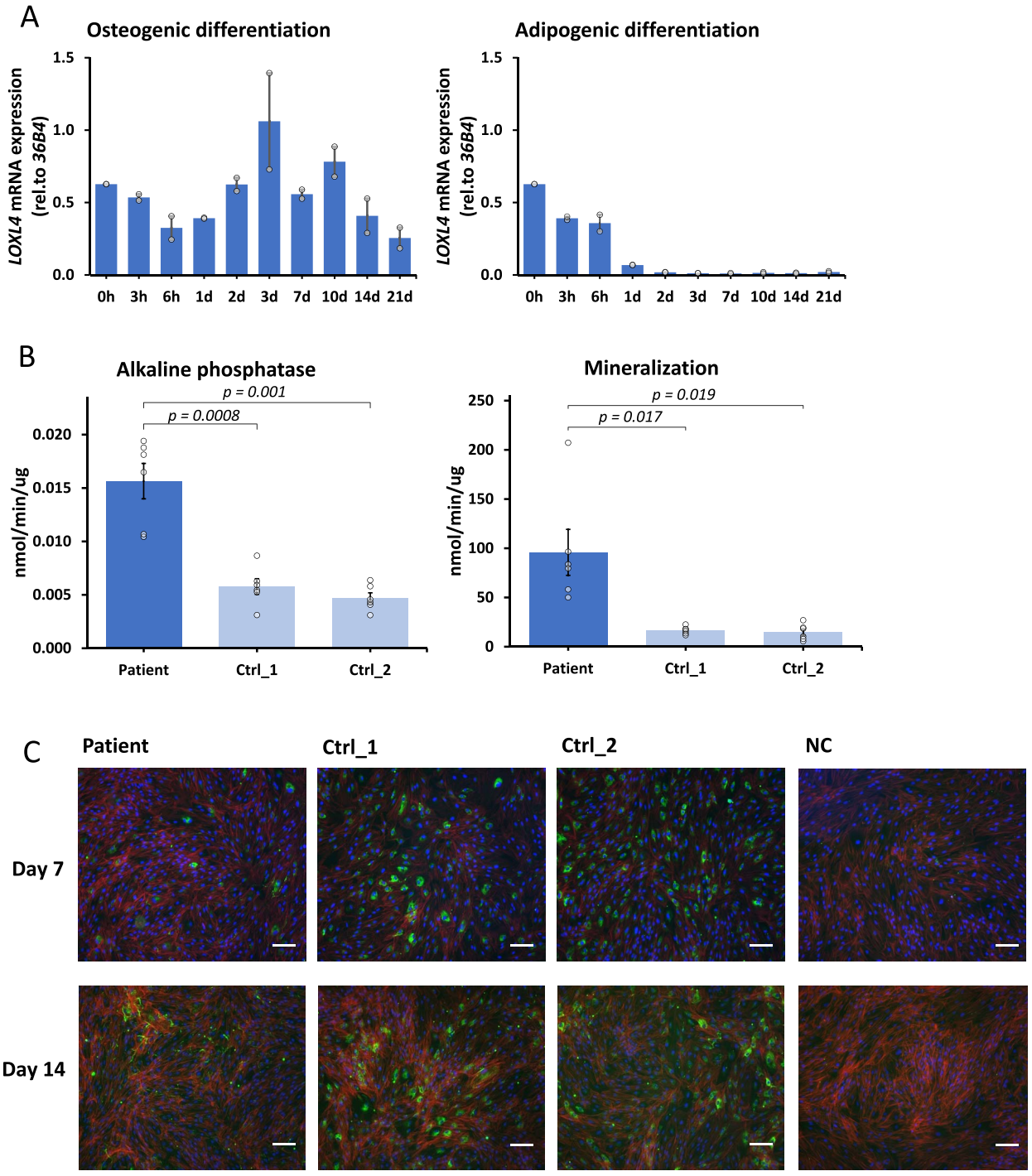

Figure 3. Functional changes in patient-derived fibroblasts with LOXL4 variant. A, LOXL4 mRNA expression during differentiation of bone marrow mesenchymal stromal cell-derived osteoblasts and adipocytes. Gene expression was normalized by correcting for the expression of the housekeeping gene *36B4*. B, ALP/protein in fibroblast-derived osteoblast culture on day 7; samples from patient and 2 healthy controls. Calcium deposition by fibroblast-derived osteoblast cultures on day 24; samples from patient and 2 healthy controls. C, collagen type I immunocytochemistry in fibroblast-derived osteoblast cultures on day 7 and day 14, from the patient and 2 healthy controls (representative for 5 images of 3 replicates per individual). Green: collagen type I; red: actin cytoskeleton; blue: nuclei (DAPI). NC = negative control. Data are presented as mean ± SEM and analyzed by unpaired two sample T-test. SEM, Standard Error of the Mean.

We did not find pathogenic variants in the known Mendelian bone disorder genes responsible for osteoporosis in the family. Because it was uncertain whether osteoporosis was due to genetic and/or non-genetic factors, it was not possible to identify a potential novel gene responsible for osteoporosis after excluding the presence of pathogenic variants in known Mendelian bone disease genes. Moreover, different genetic causes for osteoporosis might exist within different pedigree branches of 1 family. The individual III.1 who presented with severe vertebral fractures had a father without fractures. Thus, she might have a de novo mutation or a maternally inherited mutation for osteoporosis. Alternatively, the osteoporosis phenotype may have a polygenic nature. Indeed, the high PRS for low FN-BMD in 6 family members with osteoporosis,

compared with population controls, suggests that polygenic factors largely contribute to osteoporosis in this family. This finding is consistent with the presence of secondary causes in many of these family members, and the absence of pathogenic variants associated with known monogenetic bone diseases. Moreover, the absence of LOXL4 variant in some family members with osteoporosis, alongside the presence of polygenic basis for osteoporosis, suggests that the c.G1063A variant in *LOXL4* may predispose carriers to AFF but not necessarily to low BMD. This is further supported by the GWAS database look-up results showing that common variants surrounding *LOXL4* were not associated with BMD, but 1 SNP was significantly associated with an increased fracture risk. Interestingly, the eQTL analysis showed that this same SNP was associated with increased *LOXL4* expression in 3 non-bone tissues, suggesting that an increase in *LOXL4* expression may be associated with higher fracture risk.

Some strengths of our approach for a family-based WES are the availability of detailed clinical information and in-depth phenotyping of the subjects, and a relatively large cohort of unrelated AFF patients for replication. This large family allowed us to study the genetic background of AFF and osteoporosis simultaneously. Our study was limited by the large number of variants found in the hypothesis-free exome-wide approach combined with a lack of sufficient informative meiosis within this family to filter out the non-co-segregating ones. The method we used to prioritize candidate genes was based on available functional evidence, which may have led to overlooking important genes with unknown functions. Due to the unique fracture mechanism of AFFs, there is a lack of suitable cell and animal models for studying gene functions related to the AFF phenotype. Bone tissues from our AFF patients were unavailable, so we evaluated bone functions using skin biopsies instead. However, our functional experiments were limited by the small sample size as we were only able to obtain skin biopsies from 1 unrelated patient with the *LOXL4* variant and 2 age- and sex-matched controls, none of whom were family members. We lacked information about the 2 controls regarding factors such as bisphosphonate use and ethnicity, which may be relevant for the findings. Consequently, the results of our functional experiments should be viewed as preliminary. Further extensive functional studies are required, which could include mRNA and protein analysis of *LOXL4* when more samples become available, as well as the introduction of mutations using CRISPR knock-in models to investigate the role of the *LOXL4* variant in collagen and bone functions to elucidate the role of *LOXL4* variants in AFFs. Other genetic variants than the variant in *LOXL4* shared by the family members with AFFs cannot be excluded currently as the cause of AFFs. All these variants need to be studied in other AFF families or cohorts.

In conclusion, we propose that c.G1063A in *LOXL4* is the genetic cause for AFF in a family with 3 AFF cases. This proposition is supported by the finding of this variant in a fourth sibling with a low-trauma, intra-articular femoral fracture and in an unrelated AFF patient. The predicted damaging effect and function of the gene in collagen crosslink formation, along with functional data related to collagen metabolism in skin biopsy-derived cell cultures, further strengthen this hypothesis. *LOXL4* variants may alter collagen crosslinking, a process that potentially involves increased microcrack formation as well as decreased fracture repair mechanisms, leading to AFF.

Acknowledgments

We thank Joost Verlouw, Vid Prijatelj, and Haojie Lu for their valuable bioinformatic support. We are grateful to Dr René Eersels, Sandra Smits, and Els van Zaanen for their practical assistance in the study. We would also like to express our gratitude to Mark Stevenson, Kreepa Kooblall, and Kate Lines for their valuable input in this manuscript. Our sincere gratitude extends to all the participants of this study.

The generation and management of GWAS genotype data for the Rotterdam Study (RS I, RS II, RS III) was executed by the Human Genotyping Facility of the Genetic Laboratory of the Department of Internal Medicine, Erasmus MC, Rotterdam, the Netherlands. The GWAS datasets are supported by the Netherlands Organisation of Scientific Research NWO Investments (nr. 175.010.2005.011, 911-03-012), the Genetic Laboratory of the Department of Internal Medicine, Erasmus MC, the Research Institute for Diseases in the Elderly (014-93-015; RIDE2), the Netherlands Genomics Initiative (NGI)/Netherlands Organisation for Scientific Research (NWO) Netherlands Consortium for Healthy Aging (NCHA), project nr. 050-060-810. We thank Pascal Arp, Mila Jhamai, Marijn Verkerk, Lizbeth Herrera, and Marjolein Peters, MSc, and Carolina Medina-Gomez, MSc, for their help in creating the GWAS database, and Karol Estrada, PhD, Yurii Aulchenko, PhD, and Carolina Medina-Gomez, MSc, for the creation and analysis of imputed data.

Author contributions

W.Zhou and D.M. van de Laarschot are co-first authors.

Wei Zhou (Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing—original draft, Writing—review & editing), Denise van de Laarschot (Conceptualization, Investigation, Methodology, Project administration, Writing—original draft, Writing—review & editing), Jeroen van Rooij (Methodology, Supervision, Writing—review & editing), Marijke Koedam (Formal analysis, Writing—review & editing), Hanh H Nguyen (Writing—review & editing), André Uitterlinden (Supervision, Writing—review & editing), Peter Ebeling (Writing—review & editing), Rajesh Thakker (Writing—review & editing), Piet Geusens (Resources, Writing—review & editing), Bram van der Eerden (Conceptualization, Formal analysis, Methodology, Supervision, Visualization, Writing—review & editing), Annemieke Verkerk (Formal analysis, Investigation, Methodology, Supervision, Writing—review & editing), and M. Carola Zillikens (Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing—review & editing).

Supplementary material

Supplementary material is available at *Journal of Bone and Mineral Research* online.

Funding

Jaap Schouten Foundation, Rotterdam, The Netherlands, provided funding for this study. P.R.E. is supported by National Health and Medical Research Council of Australia (grant no. GNT1197958). R.V.T. is supported by the National Institute for Health Research Oxford Biomedical Research Centre, Oxford, UK. The funding agencies played no role in the study design or in data collection and analyses.

Conflicts of interest

P.R.E.: Research funding from Amgen, Sanofi, and Alexion. Honoraria from Amgen, Gedeon, Richter.

P.G.: Clinical studies, advisory boards, speaker's fees from Abbvie, Amgen, BMS, Celgene, Janssen, Lilly, MSD, Novartis, Pfizer, Roche, UCB, Fresenius, Will-Pharma, Mylan, Sandoz, Merck. Other authors declare no conflicts of interest.

Data availability

Sharing raw or processed individualized sequencing results of the patients is not allowed due to General Data Protection Regulation (GDPR). Requests to access the dataset should be directed to MCZ, m.c.zillikens@erasmusmc.nl. Requests to access the Rotterdam Study dataset should be directed toward the management team of the Rotterdam Study (datamanagement.ergo@erasmusmc.nl), which has a protocol for approving data requests. Because of restrictions based on privacy regulations and informed consent of the participants, data cannot be made freely available in a public repository.

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