

## BRIEF REPORT

## PLS3 Mutations in X-Linked Osteoporosis with Fractures

Fleur S. van Dijk, M.D., Ph.D., M. Carola Zillikens, M.D., Ph.D.,  
 Dimitra Micha, Ph.D., Markus Riessland, Ph.D., Carlo L.M. Marcelis, Ph.D.,  
 Christine E. de Die-Smulders, M.D., Ph.D., Janine Milbradt, B.S.,  
 Anton A. Franken, M.D., Ph.D., Arjan J. Harsevoort, M.S., Klaske D. Lichtenbelt, M.D.,  
 Hans E. Pruijs, M.D., Ph.D., M. Estela Rubio-Gozalbo, M.D., Ph.D.,  
 Rolf Zwertbroek, M.D., Youssef Moutaouakil, M.S., Jaqueline Egthuijsen, M.S.,  
 Matthias Hammerschmidt, Ph.D., Renate Bijman, B.S., Cor M. Semeins, M.S.,  
 Astrid D. Bakker, Ph.D., Vincent Everts, Ph.D., Jenneke Klein-Nulend, Ph.D.,  
 Natalia Campos-Obando, M.D., Albert Hofman M.D., Ph.D.,  
 Gerard J. te Meerman, Ph.D., Annemieke J.M.H. Verkerk, Ph.D.,  
 André G. Uitterlinden, Ph.D., Alessandra Maugeri, Ph.D., Erik A. Sistermans, Ph.D.,  
 Quinten Waisfisz, Ph.D., Hanne Meijers-Heijboer, M.D., Ph.D.,  
 Brunhilde Wirth, Ph.D., Marleen E.H. Simon, M.D., and Gerard Pals, Ph.D.

## SUMMARY

Plastin 3 (PLS3), a protein involved in the formation of filamentous actin (F-actin) bundles, appears to be important in human bone health, on the basis of pathogenic variants in PLS3 in five families with X-linked osteoporosis and osteoporotic fractures that we report here. The bone-regulatory properties of PLS3 were supported by in vivo analyses in zebrafish. Furthermore, in an additional five families (described in less detail) referred for diagnosis or ruling out of osteogenesis imperfecta type I, a rare variant (rs140121121) in PLS3 was found. This variant was also associated with a risk of fracture among elderly heterozygous women that was two times as high as that among noncarriers, which indicates that genetic variation in PLS3 is a novel etiologic factor involved in common, multifactorial osteoporosis.

The authors' affiliations are listed in the Appendix. Address reprint requests to Dr. Pals at the Center for Connective Tissue Research, Department of Clinical Genetics, VU University Medical Center, P.O. Box 7057, 1007 MB Amsterdam, the Netherlands, or at g.pals@vumc.nl.

Drs. van Dijk, Zillikens, Micha, and Riessland contributed equally to this article.

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**O**STEOPOROSIS IS A PREVALENT DISORDER CHARACTERIZED BY LOW BONE mass and microarchitectural deterioration of bone tissue, which results in bone fragility and fractures.<sup>1</sup> It is diagnosed clinically and often confirmed by measuring bone mineral density (BMD).<sup>1,2</sup> An understanding of the causes of osteoporosis is important for its prevention, diagnosis, and treatment. The investigation of rare mendelian disorders with decreased BMD as a key diagnostic feature constitutes a strategy for identifying genetic determinants of osteoporosis.<sup>3-7</sup>

We identified families with X-linked osteoporosis and fractures among patients with negative tests for the genes encoding collagen type I $\alpha$ 1 and type I $\alpha$ 2 (COL1A1 and COL1A2, respectively) who had been referred to us for diagnosis or ruling out of osteogenesis imperfecta type I. Osteoporosis with fractures as an X-linked trait has been reported by Sillence.<sup>8</sup> We now report data from five families with X-linked osteoporosis and fractures related to pathogenic variants in the gene for plastin 3 (PLS3), provide functional evidence that PLS3 is a bone-regulatory protein, and describe a rare variant or single-nucleotide polymorphism (SNP) associated with

decreased BMD and an increased risk of fracture among heterozygous women in the general population.

## METHODS

### FAMILIES

The pedigrees and clinical characteristics of Families 1 through 5 are provided in Figure 1 and Table 1, and Figure S1 and Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org. Five additional families, designated Families 6 through 10, were also included in the study and are mentioned in less detail (Fig. S2 and Table S2 in the Supplementary Appendix).

### GENETIC STUDIES

Three patients with osteoporosis and fractures from Family 1 (Patients 1.III-2, 1.IV-3, and 1.IV-7) underwent X-linked whole-exome sequencing.<sup>9,10</sup> We then performed Sanger sequencing of all *PLS3* exons in 95 affected male patients without *COL1A1* or *COL1A2* mutations who had been referred for diagnosis or ruling out of osteogenesis imperfecta type I. Complementary DNA (cDNA) analysis was performed in Patients 1.III-2 and 3.II-1 and the index patient from Family 9. Linkage analysis was conducted in Families 1 and 2. Methodologic and other details of the studies performed are described in the Supplementary Appendix.

### EPIDEMIOLOGIC STUDIES

The rs140121121 SNP was genotyped in three cohorts (RS-I, RS-II, and RS-III) of the prospective, population-based Rotterdam Study, which has analyzed, among other topics, the association of genetic factors with BMD and incident fractures in Dutch men and women 45 years of age or older.<sup>11</sup> Details of these studies are provided in the Supplementary Appendix.

### FUNCTIONAL STUDIES

Electrophoresis of type I collagen and Western blot analysis for *PLS3* were performed in affected Patients 1.III-2, 1.IV-2, 1.IV-7, 1.IV-8, 3.II-1, and 4.II-1 and the index patients from Families 7 and 9. *PLS3*, belonging to the family of plastins, is involved in the formation of F-actin bundles.<sup>12</sup> The effect of *PLS3* deficiency on F-actin cytoskeleton was investigated in dermal fibroblasts with the use of immunofluorescence microscopy. We hypothesized that *PLS3* may be involved in

mechanosensing of osteocytes. Mechanical loading in the form of fluid shear stress increases the production of nitric oxide in bone cells,<sup>13</sup> periodontal ligament, and gingival fibroblasts.<sup>14</sup>

In the absence of bone tissue from patients, we investigated the response to fluid shear stress of dermal fibroblasts from six patients with *PLS3* mutations, as compared with three patients with molecularly confirmed osteogenesis imperfecta type I and eight controls. To characterize the effect of loss of *PLS3* on bone morphology, we performed morpholino-mediated knockdown of the zebrafish homologue (National Center for Biotechnology Information [NCBI] Reference Sequence [RefSeq], NM\_001002326.1). Since cartilaginous pharyngeal arches are the earliest formed craniofacial skeletal elements, we used a *col1a1:eGFP* (enhanced green fluorescent protein under the control of a *col1a1*-promoter) transgenic zebrafish line to monitor skeletal development.<sup>15</sup> Details of these studies are provided in the Supplementary Appendix.

## RESULTS

### GENETIC STUDIES

#### Identification of Pathogenic Variants in *PLS3*

We discovered a single deleterious hemizygous frameshift, c.235delT;p.(Tyr79Ilefs\*6), in exon 3 of *PLS3* (NCBI Reference Sequence, NM\_005032.5; Mendelian Inheritance in Man number, 300131; chromosome-map location, Xq23) in Patients 1.III-2, 1.IV-3, and 1.IV-7 (Fig. S3A through S3F in the Supplementary Appendix). Sanger sequencing confirmed the presence of this variant in six affected male patients and its absence in one unaffected male patient (Fig. 1).

Sanger sequencing of all *PLS3* exons in 95 affected male patients without *COL1A1* or *COL1A2* mutations yielded four pathogenic variants in Families 2 through 5 (Fig. 1). In Family 2, a nonsense mutation, c.1471C→T;p.(Gln491\*), in exon 13 was identified in Patients 2.III-3 and 2.III-7. In Families 3, 4, and 5, three pathogenic variants were identified: a splice-site variant, c.748+1G→A, in exon 7 (in Patient 3.II-1); an insertion, c.759\_760insAAT;p.(Ala253\_Leu254insAsn), in exon 8 (in actin-binding domain 1, conserved from human down to tetraodon) (in Patient 4.II-1); and a frameshift variant, c.1647delC;p.(Ser550Alafs\*9), in exon 15 (in Patient 5.II-3). To our knowledge, none of these variants are described in current databases of human

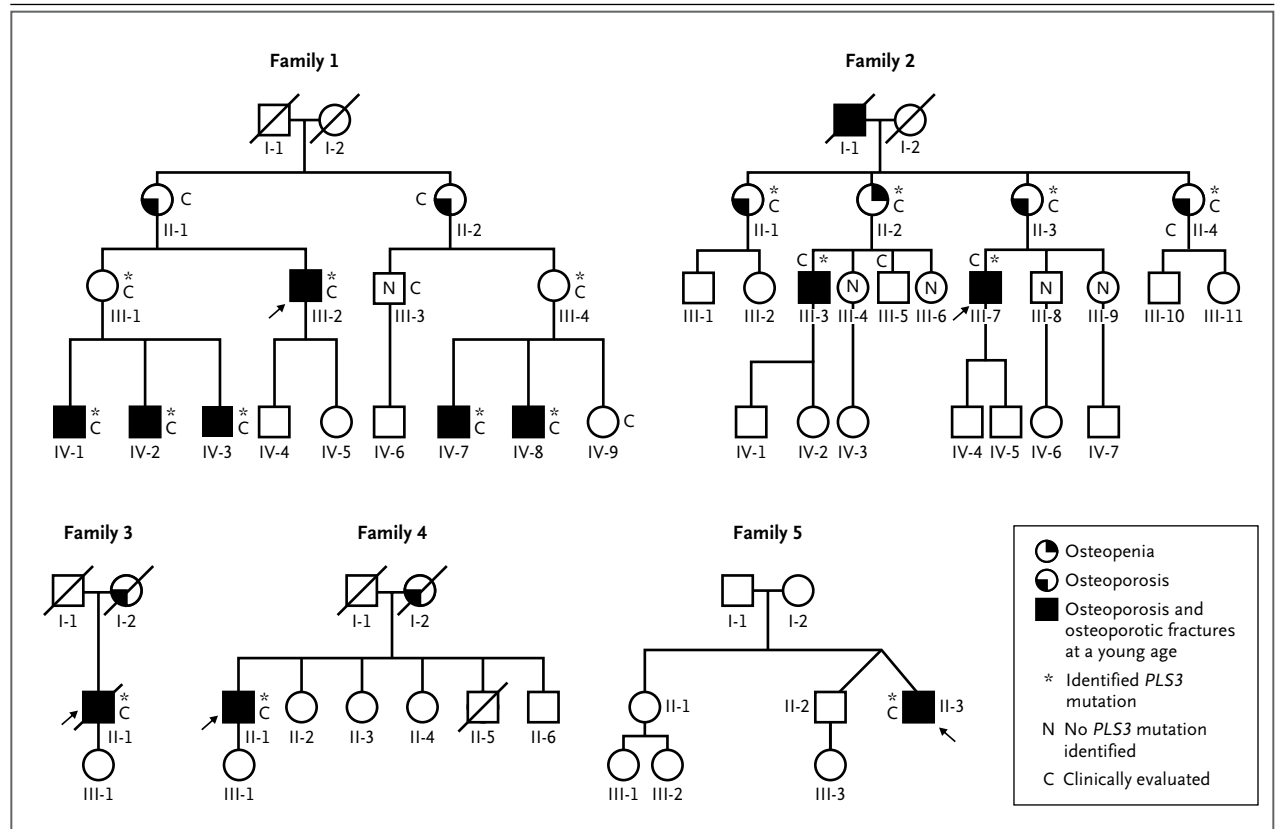
sequence variants: data from the 1000 Genomes Project, the Single Nucleotide Polymorphism database (dbSNP, build 137), or data from the GO Exome Sequencing Project (ESP) of the National Heart, Lung, and Blood Institute (<http://evs.gs.washington.edu/EVS>).

In addition, a c.321T→A variant in exon 4b (Fig. S3F in the Supplementary Appendix), listed in dbSNP as rs140121121, was identified in 5 patients (from Families 6 through 10) among the 95 male patients referred to us for possible osteogenesis imperfecta type I (allele frequency, 0.05) (Table S2A in the Supplementary Appendix). For

this rare variant, the allele frequency was 0.01 among 1872 men in the ESP and 0.02 among the 5189 men in the Rotterdam Study, results that differ significantly from the frequency among our 95 male patients ( $P=0.006$  and  $P=0.04$  by two-tailed Fisher's exact test for the two comparisons, respectively).

#### cDNA Analysis

In Family 3 (Patient 3.II-1), a partial skipping of exon 7 and use of a cryptic splice site, c.748+36, was detected (Fig. S4A and S4B in the Supplementary Appendix). Use of this cryptic splice site



**Figure 1. Pedigrees of Families 1 through 5 with Mutations in the Gene for Plastin 3 (PLS3).**

We identified five pathogenic variants in *PLS3* in hemizygous male family members in Families 1 through 5, associated with osteoporosis and osteoporotic fractures of the axial and appendicular skeleton developing in childhood. Patient 1.IV-1 had a mild phenotype with a forearm fracture at the age of 8 years, mild osteopenia at the age of 13 years, and two vertebral compression fractures diagnosed at the age of 21 years. Patient 4.II-1 received a diagnosis of osteoporosis and osteoporotic fractures in adulthood. Physical examination did not reveal abnormalities, and specifically, no extraskeletal features of osteogenesis imperfecta were observed. Apart from a waddling gait in two brothers (Patients 1.IV-7 and 1.IV-8), which disappeared for unknown reasons, no neuromuscular abnormalities were reported. Available radiographs did not show abnormalities in bone size or shape. Serum calcium and phosphate levels were normal in all affected male members, as was urinary calcium excretion, which was measured in several of the affected patients. No consistent decrease or increase in bone-turnover markers was observed. The clinical picture in heterozygous female members in Families 1 and 2 was varied, ranging from normal bone mineral density and an absence of fractures to early-onset osteoporosis. Osteopenia and osteoporosis were diagnosed by means of dual-energy radiographic absorptiometry according to World Health Organization criteria. Squares represent male family members, circles female family members, and slashes deceased family members. Arrows indicate the probands. Additional clinical details from Families 1 through 5 are available in Tables S1, S2, and S3 in the Supplementary Appendix.

**Table 1. Clinical and Bone-Densitometry Findings in 11 Male Patients from Five Families with a Pathogenic Variant in the Gene for Plastin 3 (PLS3).<sup>‡</sup>**

Patient <sup>†</sup>	Before Therapy			After Therapy <sup>‡</sup>			Low-Impact Peripheral Fractures	Multiple Vertebral Fractures	Other Clinical Findings <sup>§</sup>	
	Age yr	BMD z Score lumbar spine	BMD z Score femoral neck	Age yr	BMD z Score lumbar spine	BMD z Score femoral neck				total body
1.III-2	32	-5.5	-3.4	40	-4.6	-3.1	NA	13	Yes	None
1.IV-1	13	-1.2	NA	NT	NT	NT	NT	1	No	None
1.IV-1	21	-1.1	-0.8	NT	NT	NT	NT	1	Yes	None
1.IV-2	10	-2.1	NA	17	0.9	NA	-0.7	6	No	Acute lymphatic leukemia
1.IV-3	4	-3.2	NA	10	-1.2	NA	-1.4	1	No	None
1.IV-7	6	-3.7	NA	14	0.7	NA	-1.1	17	No	Patent ductus arteriosus and, in childhood, waddling gait
1.IV-8	10	-2.4	NA	12	-1.1	NA	-1.9	Multiple	No	Epilepsy and, in childhood, waddling gait
2.III-3	36	-2.8	-2.3	NA	NA	NA	NA	5	No	None
2.III-7	34	-3.4	-3.4	NA	NA	NA	NA	13	Yes	None
3.II-1	NA	NA	NA	47	-3.75	-2.5	NA	Multiple	Yes	Alcohol abuse
3.II-1	NA	NA	NA	62	NA	-1.0	NA	Multiple	Yes	Esophageal carcinoma
4.II-1	54	-2.5	-0.7	61	-1.0	-0.6	NA	1	Yes	None
5.II-3	41	-2.8	NA	NA	NA	NA	NA	10	Yes	None

\* Hemizygous male family members were considered to be affected if the bone mineral density (BMD) z score was below -2.0 SD or the T score was below -2.5 SD. They were also considered to be affected if they had multiple vertebral compression fractures and if secondary causes of osteoporosis had been considered and ruled out on the basis of the medical history, physical examination, protein electrophoresis, and measurements of serum levels of calcium, albumin, phosphate, creatinine, 25-hydroxyvitamin D, thyrotropin, and testosterone; in several patients, the measurement of urinary calcium excretion was also used. NA denotes not available, and NT not treated.

<sup>†</sup> Two patients (Patients 1.IV-1 and 3.II-1) underwent more than one evaluation.

<sup>‡</sup> Therapy refers to bisphosphonate treatment (pamidronate, alendronate, or risedronate), which was initiated in almost all affected patients and was associated with a favorable outcome.

<sup>§</sup> No specific extraskeletal features of osteogenesis imperfecta, such as blue sclerae, hearing loss, or dentinogenesis imperfecta, were noted. Patients 1.IV-3, 1.IV-7, and 1.IV-8 had joint hypermobility.

**Table 2. Sex-Combined Fracture Risk in Two Rotterdam Study Cohorts, According to rs140121121 Genotype.\***

Cohort†	Genotype 0	Genotype 1	Genotype 1 vs. Genotype 0		Genotype 2	Genotype 2 vs. Genotype 0	
	Persons with Fracture	Persons with Fracture	Odds Ratio (95% CI)	P Value	Persons with Fracture	Odds Ratio (95% CI)	P Value
	no./total no.				no./total no.		
RS-I	1474/6017	44/118	1.74 (1.19–2.55)	0.004	11/58	0.71 (0.37–1.38)	0.31
RS-II	222/2375	10/43	2.99 (1.44–6.20)	0.003	0/27	NA	—
Both cohorts	1696/8392	54/161	1.95 (1.39–2.74)	<0.001	11/85	NA	—

\* Genotype 0 was defined as T in men and TT in women, genotype 1 as TA in women, and genotype 2 as A in men and AA in women.

† The cohorts were from the prospective, population-based Rotterdam Study involving analyses of the associations among genetic factors, BMD, and incident fractures in Dutch men and women 45 years of age or older.<sup>11</sup>

leads to an in-frame insertion of 36 nucleotides in the messenger RNA (mRNA) and an insertion of 12 amino acids in PLS3: p.(Glu249\_Ala250ins12) (NCBI RefSeq, NP\_001129497.1) in the highly conserved actin-binding domain 1. The in-frame insertion is consistent with the results of Western blot analysis, which showed a detectable but reduced PLS3 level (the difference in molecular weight of the proteins of approximately 1 kD is not detectable on Western blot testing) (Fig. S5 in the Supplementary Appendix). In fibroblasts from Family 9 with the c.321T→A exon 4 variant, cDNA with primers for exons 4 (forward) and 7 (reverse) was normal.

#### Linkage Analysis

The combined LOD score in Families 1 and 2 was 3.40 (2.35 in Family 1 and 1.05 in Family 2). Thus, it is very likely that the identified variants in *PLS3* were causative.

#### EPIDEMIOLOGIC STUDIES

The minor allele frequencies of the rs140121121 SNP in men and women, respectively, in the RS-I, RS-II, and RS-III cohorts were 0.022 and 0.016, 0.024 and 0.017, and 0.012 and 0.016. To investigate the relationship of this variant with fracture risk, we performed sex-combined analyses for X-linked inheritance with adjustment for age and body-mass index but not sex, treating men as homozygous women.<sup>16</sup>

In the two cohorts with fracture information (RS-I and RS-II cohorts; 8638 persons) heterozygous female carriers of the minor (A) allele had a significantly increased risk of fracture as compared with the risk among noncarriers of the A allele. The odds ratio in the RS-I cohort was 1.74 (95% confidence interval [CI], 1.19 to

2.55;  $P=0.004$ ), and the odds ratio in the RS-II cohort was 2.99 (95% CI, 1.44 to 6.20;  $P=0.003$ ). In a combined analysis of the RS-I and RS-II cohorts in a fixed-effect model, the odds ratio was 1.95 (95% CI, 1.39 to 2.74;  $P<0.001$ ) (Table 2). We observed no statistical indication of sex-specific effects ( $P>0.05$  for heterogeneity), although associations between carrier status and fracture risk among men in the RS-I cohort were not significant and no fractures were observed in the very small number of male A-allele carriers in the RS-II cohort, which had a shorter follow-up.

Analyses of individual study data for an association with BMD did not show consistent effects. Combined analyses of BMD in the three cohorts showed a small but significantly decreased BMD at the lumbar spine and femoral neck in heterozygous women ( $P=0.008$  and  $P=0.04$ , respectively), whereas no significant difference was observed in men (Table 3), again without statistical evidence of heterogeneity between sexes. Correction for BMD in the fracture analysis restricted to the group with BMD and fracture information resulted in a minor decrease in the fracture risk among women.

#### FUNCTIONAL STUDIES

##### Electrophoresis of Type I Collagen

No decreased production or overmodification of type I collagen was observed.

##### Western Blot Analysis

No PLS3 was detected on Western blots in the fibroblast lysates from Patients 1.III-2, 1.IV-2, 1.IV-7, and 1.IV-8, who had the c.235delT variant (Fig. S5 in the Supplementary Appendix). PLS3 production in Patient 3.II-1, who had the c.748+1G→A variant, was decreased. In Patient 4.II-1, who had the c.759\_760insAAT variant, and in the

**Table 3. BMD at the Femoral Neck and Lumbar Spine with Adjustment for Age and Body-Mass Index, According to Sex, Rotterdam Study Cohort, and rs140121121 Genotype.\***

Cohort	Women				Men				P Value		
	Genotype 0		Genotype 1		Genotype 2		Genotype 0			Genotype 2	
	no. of persons	BMD g/cm <sup>2</sup>	no. of persons	BMD g/cm <sup>2</sup>	no. of persons	BMD g/cm <sup>2</sup>	no. of persons	BMD g/cm <sup>2</sup>		no. of persons	BMD g/cm <sup>2</sup>
<b>Femoral neck</b>											
RS-I	2959	0.83±0.002	93	0.81±0.012	—	—	2165	0.92±0.003	49	0.90±0.018	0.39
RS-II	992	0.89±0.004	31	0.87±0.023	1	1.04±0.126	851	0.97±0.004	20	1.02±0.028	0.09
RS-III	1113	0.92±0.004	34	0.89±0.021	—	—	860	0.99±0.004	09	0.98±0.043	0.92
All cohorts	5064	0.85±0.001	158	0.84±0.009	—	—	3876	0.95±0.002	78	0.94±0.014	0.40
<b>Lumbar spine</b>											
RS-I	2970	1.04±0.003	90	1.03±0.018	—	—	2176	1.16±0.004	49	1.12±0.027	0.12
RS-II	1004	1.11±0.006	31	1.03±0.032	1	1.33±0.180	853	1.21±0.006	20	1.33±0.040	0.002
RS-III	1017	1.18±0.005	32	1.13±0.030	—	—	754	1.25±0.007	09	1.26±0.061	0.89
All cohorts	4991	1.08±0.002	153	1.05±0.014	—	—	3783	1.19±0.003	78	1.19±0.021	0.81

\* Plus-minus values are means ±SE. Genotype 0 was defined as T in men and TT in women, genotype 1 as TA in women, and genotype 2 as A in men and AA in women.

index patients from Families 7 and 9 who had the c.321T→A variant, the production of PLS3 was similar to that in controls.

#### Immunofluorescence Microscopy

Staining with rhodamine phalloidin (Fig. S6 in the Supplementary Appendix) visualizes stress fibers, a specific type of contractile F-actin bundles. An investigator who was unaware of the clinical and molecular genetic data observed no clear differences in the quantity or quality of stress fibers in patients with the pathogenic c.235delT *PLS3* variant, as compared with controls.

#### Mechanosensitivity Studies

All cell lines produced a small amount of nitric oxide in response to fluid shear stress. Statistical analysis with the use of the Mann–Whitney U test showed no significant differences among controls, patients with osteogenesis imperfecta, and patients with pathogenic *PLS3* variants.

#### In Vivo Characterization of *pls3* Knockdown in Zebrafish

Zebrafish with *pls3* knockdown had severe dysplasia of craniofacial skeletal elements (Fig. S7A and S7B, Fig. S8A and S8B, and Fig. S9C in the Supplementary Appendix). Gross morphologic abnormalities were observed in the knockdown zebrafish larvae, which were specific and could be reversed dose-dependently by injection of human *PLS3* mRNA (Fig. S7C and S7D, Fig. S8C and S8D, Fig. S9A and S9B, and Fig. S10 in the Supplementary Appendix). Furthermore, the muscle tissue in the knockdown larvae, characterized by a predominance of F-actin, was also deformed (Fig. S8A and S8B in the Supplementary Appendix). Immunohistochemical colocalization experiments revealed a distinct actin-bundling function of *pls3* in the developing bone structure (Fig. S11 in the Supplementary Appendix).

## DISCUSSION

We identified five pathogenic variants in *PLS3* in Families 1 through 5, with osteoporosis and osteoporotic fractures manifested in childhood in the majority of hemizygous male family members. The clinical picture in heterozygous women from Families 1 and 2 ranged from normal bone density and an absence of fractures to early-onset osteoporosis. Factors such as differences in overall

and local X-chromosome inactivation, postmenopausal status, and immobility could play a role.

In addition, we identified a rare variant in *PLS3*, c.321T→A in exon 4 (SNP rs140121121) in Families 6 through 10. The prevalence of this variant was significantly increased in our group of 95 male patients without *COL1A1* or *COL1A2* mutations who had been referred for diagnosis or ruling out of osteogenesis imperfecta type I. The clinical symptoms of patients in Families 6 through 10 were generally less severe and had a later onset (absent in one case) than those in Families 1 through 5 with loss-of-function variants in *PLS3*. We hypothesized that the rs140121121 SNP may be associated with fractures, decreased BMD, or both in the general population.

A combined analysis of two cohorts (RS-I and RS-II) of 8638 elderly Dutch persons showed that heterozygous women had an increased odds of fracture of 1.95 (95% CI, 1.39 to 2.74) and that the SNP was significantly associated with decreased BMD. However, the association with fracture risk was not fully explained by BMD, which suggests that other factors leading to decreased bone strength may be involved. Associations in hemizygous men were not significant, a finding that may be due to the small size of this group or may indicate that additional (possibly genetic) factors play a role. The associations of the SNP with fractures and BMD in the general population need to be replicated in larger cohorts worldwide.

Our findings indicate that *PLS3* has bone-regulatory properties. Overexpression of *PLS3* has been reported to act as a protective modifier of spinal muscular atrophy, facilitating axonal growth and presynaptic F-actin-dependent processes at the neuromuscular junction.<sup>17,18</sup> A knockdown of *pls3* in zebrafish was used in an investigation of motor axon development.<sup>17</sup> Since no other animal models were available, we used this model<sup>17</sup> to analyze the role of *PLS3* in skeletal development. Malformations of developing craniofacial bone structure, body axis, and tail were present and could be reversed dose-dependently by the administration of human *PLS3* mRNA. Muscles that contained F-actin appeared to be deformed as well, which is notable because the formation of pharyngeal cartilage and the formation of muscle occur simultaneously.<sup>19</sup> Immunohistochemical colocalization experiments confirmed a distinct actin-bundling function of *pls3* in developing bone structure.

Taken together, the *in vivo* data suggest that *PLS3* may be a regulator of bone development.

The exact mechanism by which *PLS3* mutations cause osteoporosis and fractures is unknown. Fimbrin, the chicken homologue of *PLS3*,<sup>20</sup> is abundant in osteocyte dendrites.<sup>21-23</sup> These dendrites are important for mechanosensing (converting mechanical signals into intracellular biochemical signals to osteoblasts and osteoclasts).<sup>24</sup> The loss of sensor-cell mechanosensitivity has been proposed as a cause of osteoporosis.<sup>25</sup> We hypothesize that *PLS3* mutations lead to decreased mechanosensing of osteocytes, with subsequent dysregulation of bone modeling or remodeling, which results in osteoporosis and fractures. Bone tissue from patients with *PLS3* mutations will be needed for investigation of mechanosensing in osteocytes.

In conclusion, we identified loss-of-function variants in *PLS3* as a monogenetic cause of X-linked osteoporosis and osteoporotic fractures. We propose diagnostic analysis of *PLS3* in boys and men who have clinical or radiologic signs of an inherited bone disorder with low BMD and fractures, early-onset osteoporosis, or a presumptive diagnosis of osteogenesis imperfecta type I without *COL1A1* or *COL1A2* mutations. Among elderly study participants, we identified a rare *PLS3* variant, which was associated with decreased BMD and a risk of fracture among heterozygous women that was two times as high as that among noncarriers, indicating genetic variation in *PLS3* as a novel factor involved in common, multifactorial osteoporosis.

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## APPENDIX

The authors' affiliations are as follows: the Center for Connective Tissue Disorders, Department of Clinical Genetics, VU University Medical Center, Amsterdam (F.S.D., D.M., Y.M., J.E., R.B., A.M., E.A.S., Q.W., H.M.-H., G.P.), the Departments of Internal Medicine (M.C.Z., N.C.-O., A.J.M.H.V., A.G.U.), Epidemiology (A.H., A.G.U.), and Clinical Genetics (M.E.H.S.), Erasmus Medical Center, Rotterdam, the Department of Genetics, Radboud University Nijmegen Medical Center, Nijmegen (C.L.M.M.), the Departments of Clinical Genetics (C.E.D.-S.), Pediatrics (M.E.R.-G.), and Pediatrics and Laboratory Genetic Metabolic Diseases (M.E.R.-G.), Maastricht University Medical Centre, Maastricht, the Departments of Internal Medicine (A.A.F.) and Orthopedics (A.J.H.), Isala Clinics Zwolle, Zwolle, the Department of Medical Genetics, University Medical Center Utrecht, Utrecht (K.D.L.), the Department of Pediatric Orthopedics University Medical Center Utrecht, Wilhelmina Children's Hospital, Utrecht (H.E.P.), the Department of Internal Medicine, Westfries Gasthuis, Hoorn (R.Z.), the Department of Oral Cell Biology, Academic Center for Dentistry Amsterdam, University of Amsterdam and VU University Amsterdam, MOVE Research Institute Amsterdam, Amsterdam (C.M.S., A.D.B., V.E., J.K.-N.), and the Department of Genetics, University Medical Center Groningen, University of Groningen (G.J.M.) — all in the Netherlands; and the Institute of Human Genetics, Institute for Genetics and Center for Molecular Medicine Cologne (M.R., J.M., B.W.), and the Department of Developmental Biology (M.H.), University of Cologne — both in Cologne, Germany.

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