Primary Osteoporosis in Young Adults: Genetic Basis and Identification of Novel Variants in Causal Genes

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ABSTRACT
Genetic determinants contribute to osteoporosis and enhance the risk of fracture. Genomewide association studies of unselected population-based individuals or families have identified polymorphisms in several genes related to low bone density, but not in osteoporotic patients with $Z$-score $< -2.0$ SD with fragility fractures. The aim of this study was to determine the causal genes of idiopathic osteoporosis in the adulthood. Also, we used next-generation sequencing of candidate genes in a cohort of 123 young or middle-aged adults with idiopathic osteoporosis. All patients were included if they had a low bone mineral density ($Z$-score $< -2.0$ SD, a diagnosis before age 55 years (mean ± SD, 48.4 ± 10.6 years; mean ± SD age at first fracture, 30.4 ± 17.4 years) and fracture or not. We found that 11 patients carried rare or novel variants in COL1A2 (n = 4), PL3S (n = 2), WNT1 (n = 4), or DKK1 (n = 1). We showed a high prevalence of pathogenic variants in LRP5: 22 patients (17.8%) had the p.Val667Met variant, including three at the homozygous level and 16 (13%) carrying a novel or very rare variant. Functional analysis revealed that the LRP5 missense variants resulted in reduced luciferase activity, which indicates reduced activation of canonical WNT signaling. The clinical phenotype of patients carrying causal gene variants was indistinguishable. In conclusion, molecular screening of young osteoporotic adults revealed several variants and could be useful to characterize susceptibility genes for personalizing treatment, in particular for the new anabolic drugs. © 2017 The Authors. JBMR Plus is published by Wiley Periodicals, Inc. on behalf of the American Society for Bone and Mineral Research.

KEY WORDS: LRP5; WNT; OSTEOPOROSIS; BONE; COLLAGEN; FRACTURE

Introduction
Osteoporosis is defined as a skeletal disorder of reduced bone strength leading to increased risk of fracture. Fracture incidence increases with age,1,2 so the prevalence is mainly reported in people older than 50 years. Primary osteoporosis, also called idiopathic, in young and middle-aged patients is rare, and the prevalence is poorly described. Low bone mineral density (BMD) is a major determinant, determined by genetic variation and environmental determinants that contribute to the development of osteoporosis and to enhancing the susceptibility to fracture.3

The heritability of BMD is estimated at 60% to 80% in families and twins.4,5 Moreover, the heritability of osteoporotic fractures, the final clinical outcome of low BMD, has moderate heritability, 50% to 70%.6,7 Genomewide association studies of unselected population-based individuals have revealed several genes associated with low bone mass and risk of fracture.8 Other rare mutations have been reported in young adults with osteoporosis, particularly in extracellular matrix genes or cell factors that regulate bone remodeling. Indeed, mutations in collagen type I (COL1A1 and COL1A2) are responsible for mild to severe osteogenesis imperfecta that can be revealed in adulthood. Heterozygous Wnt family member 1 (WNT1) mutations have been found in adults with dominant early-onset osteoporosis and a homozygous mutation causes severe osteogenesis imperfecta.9,10 Mutations in plastin 3 (PLS3),10 a gene encoding plastin 3, which is involved in actin bundle
formation in the cytoskeleton, have been associated with a X-chromosomal osteoporosis form.

Among the genes previously identified for low BMD, low-density lipoprotein (LDL) receptor-related protein 5 (LRP5) is a strong candidate contributing to idiopathic osteoporosis. The encoded LRP5 protein, a member of the LDL receptor superfamily, acts as a coreceptor necessary for Wnt ligands to activate the pathway and regulates bone formation.\(^{11}\) LRP5 was first identified in osteoporosis pseudoglioma syndrome (OPGG, MIM#259770), an exceptional recessive disease characterized by severe osteoporosis revealed in infancy and associated with a congenital blindness. Later, variants were consistently associated with BMD and fracture risk. Two variants of LRP5—p.Val667Met (c.2047G>A, population frequency 0% to 5.3%) and p.Ala1330Val (c.4037C>T, population frequency 4.7% to 22%)—had a mild effect on BMD in large non-selected European cohorts, although it was associated with a significant increased risk of fracture.\(^{5,12,13}\) Very few novel heterozygous pathogenic mutations with major effect were identified in children and adolescents with juvenile idiopathic osteoporosis.\(^{14,15}\) These studies revealed several variants related to a large spectrum of bone fragility.

Here, we aimed to use next-generation sequencing (NGS) to screen causal genes in a cohort of young adults with primary osteoporosis and find novel pathogenic variants.

**Patients and Methods**

**Patients**

Patients were referred to the clinic for management of osteoporosis diagnosed by BMD tests or history of fracture without any associated disease. Enrolment period was from 2010 to 2016. We excluded patients with obvious rare diseases of bone fragility such as osteogenesis imperfecta. Clinical examination showed no dysmorphia and no eye, ear, or vessel abnormalities. Extensive radiological and biochemical investigations excluded secondary causes such as malignant disease, Paget disease, malabsorption, hypogonadism, hemochromatosis, hyperthyroidism, hypercortisolism, vitamin D deficiency, or mastocytosis. We excluded patients taking medications that interfered with bone metabolism. The remaining patients were included in a cohort of young patients with idiopathic osteoporosis; patients were <55 years old at diagnosis and had a BMD Z-score < –2 standard deviation (SD) at the spine or total hip\(^{16,17}\) associated or not with osteoporotic fractures. For each patient, the following data were collected: age at diagnosis, number and site of fractures, family history of fracture, and bone marker levels before any treatment. Patients provided informed consent for DNA analysis.

**DNA sequencing and multiplex ligation-dependent probe amplification technologies**

DNA was extracted with use of the QIAshympnea automat according to the manufacturer’s protocol (Qiagen, Courtabeuf, France). All DNA was screened by NGS with a panel of five genes reported in adult primary osteoporosis, LRP5, PLS3, COL1A1, COL1A2, and WNT1, and other potential candidates in osteoporosis, Engrailed Homeobox 1 (EN1), Wnt family member 16 (WNT16), LDL receptor related protein 6 (LRP6) and Dickkopf Wnt signaling pathway inhibitor 1 (DKK1), TNF superfamily member 11 (TNFSF11), TNF receptor superfamily member 11a (TNFRSF11A), TNF superfamily member 11b (TNFRSF11B), and vitamin D receptor (VDR). The NGS technology was based on the surelectQXT kit (Agilent, Les Ulis, France) for library preparation and the hybrid capture system for sequencing on a MiSeq sequencer (Illumina, Paris, France). Fastq files were generated by using MiseqReporter (Illumina), and then aligned with SeqNext (JSI Medical Systems, Ettenheim, Germany) and Surecall (Agilent) software. The design included the coding region of following genes with RefSeq nomenclature: alkaline phosphatase, liver, bone, and kidney (ALPL (NM_000478), COL1A1 (NM_000088), COL1A2 (NM_000089), EN1 (NM_001426.3), LRP5 (NM_002335), LRP6 (NM_002336.2), PL53 (NM_005023), TNFSF11 (NM_003701), TNFRSF11A (NM_001270949), TNFRSF11B (NM_002546), VDR (NM_00107536), WNT1 (NM_005430), WNT16 (NM_005716) for exon regions and intron-exon boundaries, and ALPL (12 exons, NG_008940), COL1A1 (51 exons, NG_007400), COL1A2 (52 exons, NG_007405), EN1 (NG_007123), LRP5 (23 exons, NG_015835), LRP6 (NG_016168), PL53 (18 exons, NG_012518), WNT1 (4 exons, NG_033141), TNFSF11 (8 exons, NG_008990), TNRSF11A (12 exons, NG_008998), TNFRSF11B (5 exons, NG_012202), WNT16 (4 exons, NG_029242), and VDR (NG_008731) for exon numbering and the intronic region.

All variants were confirmed by Sanger technology with Life Technologies products and software on an ABI3130 sequencer (ThermoFisher, Les Ulis, France). Large deletions of LRP5, COL1A1, and COL1A2 involved use of multiplex ligation-dependent probe amplification (MLPA) reagent on the ABI3130 sequencer with coффalyser software (MRC, Amsterdam, Netherlands). Variant pathogenicity was evaluated by using the prediction software Polyphen (http://genetics.bwh.harvard.edu/pph2), Mutationtaster (http://www.mutationtaster.org), and SIFT (http://sift.bi.a-star.edu.sg). The known pathogenic mutations were searched in the Human Gene Mutation Database (HGMD) or Leiden Open Variation Database (LOVD). All novel variants will be reported in ClinVar database.

**Biochemical markers of bone**

Blood samples were collected to assess bone-remodeling biomarkers. We used the following methods to measure biomarkers: β-Crosslaps, P1NP, Osteocalcin (Cobas e601 Analyzer; Roche Immunodiagnostics, Meylan, France), bone alkaline phosphatase (Yis analyzer; IDS Immunodiagnostics, Paris, France), and TRAP5b levels by ELISA kit (IDS Immunodiagnostics).

**Cell culture, transfection, and luciferase reporter assays**

The wild-type construct of the full-length LRP5 expression plasmid, cloned into the pcDNA3 vector (Invitrogen), was kindly provided by Dr. M. Warman (Harvard Medical School, Boston, MA, USA). The site-directed mutagenesis involved use of the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent). Saos-2 cells were co-transfected by using Lipofectamine 2000 (Invitrogen, Paris, France) with the TOPflash-luc reporter plasmid, pCMV-RENilla luciferase (Promega, Charbonniere-les-bains, France) and an expression-mutated or control reporter plasmid, pCMV-Renilla luciferase (Promega) and an expression-mutated or control reporter plasmid, pCMV-RENilla luciferase (Promega). The Saos-2 cells were transfected only with TOPflash-luc reporter plasmid, pCMV-RENilla luciferase (a transcription factor activated by Wnt signaling). All experiments were realized in triplicate. Cells were plated at 1.25 × 10⁵ cells/well in 24-well plates for transfection. Cells were cultured in Wnt3a-conditioned medium or not for 48 hours before transfection.\(^{18}\) The luciferase assay involved the Dual-Glo Assay system (Promega) according to the manufacturer’s instructions. Data were normalized by Renilla
firefly activity and are presented as a fold change compared to vehicle-treated cells.

Statistical analysis

Statistical analysis was conducted in the 112 patients carrying LRP5 variants or no variant (74 patients in Negative or p. Ala1330Val group, 22 in p.Val667Met group and 16 in Novel or very rare group). Diagnostic tests were performed to detect non-normality of data and outliers. Bartlett’s test was used to test homoscedasticity. Quantitative variables are expressed by mean ± SD. Qualitative variables are expressed by percentages. ANOVA was used to compare multiple groups, followed by a post hoc test in case of a significant effect. The post hoc test consisted of a pairwise comparison by using t tests with pooled SD, and p value adjustment to control for false discovery rate. Chi-square test was used to assess the association between genetic groups and gender, and because the theoretical effectives were <5 for the fracture approach, we used Fisher exact tests to study the association with genetic group variables. All tests were two-sided, and the significance level was 0.05. All statistical analyses involved use of R 3.1.0 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2014; https://www.R-project.org).

Results

Characteristics of the cohort

In total, 123 white patients (82 men) fulfilled the criteria for idiopathic osteoporosis. The mean age at the first clinical visit was 48.4 ± 10.6 years, but the first osteoporotic fracture occurred at a mean age of 30.4 ± 17.4 years. The mean Z-score was −2.90 ± 0.90 at the spine and −1.92 ± 0.85 at the hip. Among the 123 patients, 98 experienced at least one fracture, with a high variable number of fractures per patient, as illustrated by a median of three fractures (range, 1 to 14 fractures).

NGS sequencing revealed mutations in genes described in several rare diseases associated with bone fragility in 11 patients: COL1A2 (n = 4), PLS3 (n = 2), WNT1 (n = 4), and DKK1 (n = 1) (Fig. 1). In total, 56 patients (35 men) carried LRP5 variants: 18 with the p.Ala1330Val variant alone (14.5%); 22 with the p.Val667Met variant (17.8%), including three at the homozygous level (1.6%); and 16 carrying a novel or very rare variant (13%). Finally, 56 patients (45.5%) harbored no variants of genes included in the panel (COL1A1, ALPL, EN1, LRP5, LRP6, WNT16, TNFSF11, TNFRSF11A, and TNFRSF11B genes). Clinical and DXA characteristics of the cohort are reported in Table 1.

Table 2 summarizes the clinical data for 11 patients and variants in genes previously reported in osteogenesis imperfecta and idiopathic osteoporosis. Four patients showed variants in COL1A2. We found two known pathogenic heterozygous mutations p.(Gly193Ser) (HGMD ID CM062556) and p.(Gly247Cys), known at this location in HGMD and in LOVD, in patients 1 and 2 presenting one vertebral fracture and one peripheral fracture associated with markedly low spinal BMD. The known p.(Arg708Gln) variant in COL1A2 was identified in one woman (patient 4) with fractures from childhood. The pathogenicity was verified by the SIFT, Mutationtaster, and Polyphen software.

Fig. 1. Distribution of gene variants in young patients with idiopathic osteoporosis (n = 123). NGS sequencing was used to screen patients with idiopathic osteoporosis (Z-score < −2 SD); 11 patients carried rare variants in COL1A2, PLS3, WNT1, and DKK1. Most patients carried LRP5 common and rare variants and 10 new variants were identified. HTZ = heterozygous; HMZ = homozygous.
### Table 1. Clinical and DXA Characteristics of Idiopathic Osteoporosis Cohort

|           | LRP5 novel or very rare variant | COL1A2 | PLS3 | WNT1 | HMZ | DKK1 |
|-----------|---------------------------------|--------|------|------|-----|------|
| n         | 16                              | 3      | 2    | 4    | 4   | 1    |
| Weight (kg) | 69.7 ± 11.6                     | 69.5 ± 11.2 | 68.8 ± 12.7 | 66.5 ± 11.7 | 66.6 ± 12.2 | 69.7 ± 13.8 |
| Height (cm) | 170.2 ± 12.7                    | 172.1 ± 14.1 | 170.5 ± 14.3 | 169.7 ± 15.2 | 169.7 ± 15.1 | 169.7 ± 15.2 |
| BMI (kg/m²) | 23.9 ± 0.4                      | 23.8 ± 0.3 | 23.4 ± 0.2 | 23.5 ± 0.2 | 23.3 ± 0.1 | 23.7 ± 0.3 |
| Z-score BMD total hip | -2.1 ± 1.3 | -2.1 ± 1.3 | -2.2 ± 1.3 | -2.5 ± 1.3 | -2.5 ± 1.3 | -2.5 ± 1.3 |
| Z-score BMD total hip | -1.0 ± 1.1 | -1.0 ± 1.0 | -1.1 ± 1.1 | -1.2 ± 1.1 | -1.3 ± 1.3 | -1.3 ± 1.3 |

**Note:** All values are mean ± SD.

### Novel and Known Variants in LRP5 Gene

We found 112 patients (75 men) carrying LRP5 variants or no variant: 18 (13 men) with the p.Ala1330Val variant alone; 22 (14 men) with the p.Val667Met variant; and 16 (8 men) carrying novel or very rare variants (Fig. 1). Because the p.Ala1330Val variant has been considered a polymorphism with no detrimental effect on BMD and fractures,7,12,20 p.Ala1330Val carriers and patients without any LRP5 variant \((n = 56)\) were pooled for further analysis. Table 3 shows the clinical characteristics of patients with the p.Ala1330Val or no LRP5 variant, those with the rare p.Val667Met variant, and those with novel or very rare LRP5 mutations. Patients were similar in age, height, body mass index, and gender ratio. Z-score BMD at the spine was significantly lower with the p.Val667Met variant or novel/rare variants than without a variant. The three groups did not differ in BMD at the hip. Moreover, the proportion of patients with vertebral fractures was significantly higher with the p.Val667Met variant or novel/rare variants than patients harboring the p.Ala1330Val or no variant.

Biochemical markers were measured in a subgroup of patients before the initiation of treatment. The three groups did not differ in circulating levels of bone alkaline phosphatase, P1NP, osteocalcin, or tartrate-resistant acid phosphatase. Only serum β-crosslaps levels were higher in p.Val667Met (483.6 ± 176 pg/mL) or novel/very rare variants (570.6 ± 205.3 pg/mL) than a p.Ala1330Val or no variant (385.4 ± 210 pg/mL, \(p < 0.05\)).

In this cohort, 16 patients harbored heterozygous novel or very rare LRP5 mutations and three patients harbored homozygous rare LRP5. The clinical and genetic characteristics are in Table 4. The following variants were not found in the databases: p.(Lys265Gln), p.(Cys336Gly), p.(Met473Thr), c.1413–2A>G, p.(Lys772Argfs*26), p.(Ala1418Profs), and p.(Glu1597). Some variants were reported as rare in a population according to ExAC: p.(Asp587Asn), p.(Trp560Cys), p.(Glu829Gln) p.(Arg1188Gln), p.(Ala1196Thr), and p.(Asp1288Gly). Except for p.(Ala1196Thr), the missense variants were classified as probably pathogenic, with high scores, according to the prediction...
Table 2. Description of Variants Except LRP5

| No | Gender | Gene | Exon | cDNA     | Protein     | Mutation status | Mutation type | Allele frequency from ExAc | Polyphen (score) pathogenicity | BMD spine Z-score | BMD hip Z-score | Number of vertebral fractures | Peripheral fractures | Age at first fracture (yrs) |
|----|--------|------|------|----------|-------------|----------------|---------------|-----------------------------|--------------------------------|-------------------|----------------|-------------------------------|----------------------|----------------------|
| 1  | M      | COL1A2 | 12   | c.577G>A  | p.(Gly193Ser) | HTZ            | Missense      | HGMD<sup>a</sup>             | 1                              | -5.2              | -2.3            | 1                            | Elbow, tibia          | 14                   |
| 2  | M      | COL1A2 | 16   | c.739G>T  | p.(Gly247Cys) | HTZ            | Missense      | HGMD<sup>a</sup>             | 1                              | -5.4              | -2.5            | 1                            | Ribs, femur          | 8                   |
| 3  | M      | COL1A2 | 25   | c.1412C>T | p.(Pro471Leu) | HTZ            | Missense      | rs72658163                  | 0.078% in European population | 1                              | -2.4              | -0.9            | 1                            | Elbow, wrist          | Childhood            |
| 4  | F      | COL1A2 | 35   | c.2123G>A | p.(Arg708Gln) | HTZ            | Missense      | rs149265042 0.3048%         | 1                              | -3                | -1              | 0                            | Wrist, foot, clavicle, scapula | 13                   |
| 5  | M      | WNT1  | 1    | c.107G>A  | p.(Gly36Asp)  | HTZ            | Missense      | rs149265042 0.3048%         | 1                              | 1                 | -1.6            | 0                            | Ribs                | 40                   |
| 6  | F      | WNT1  | 3    | c.436G>C  | p.(Gly146Arg) | HTZ            | Missense      | rs149265042 0.3048%         | 1                              | 1                 | -3.1            | 0                            | Femur, wrist, tibia  | 10                   |
| 7  | F      | WNT1  | 3    | c.401G>A  | p.(Gly134Val) | HTZ            | Missense      | rs149265042 0.3048%         | 1                              | 1                 | -2.4            | 1                            | Wrist               | 26                   |
| 8  | F      | WNT1  | 3    | 502G>A    | p.(Arg182Trp) | HTZ            | Missense      | rs149265042 0.3048%         | 1                              | 1                 | -3              | 1                            | Wrist, femur, humerus | 13                   |
| 9  | M      | PLS3  | 11   | 1206dup   | p.(Va403Argfs’7) | HMZ            | Frameshift    | rs149265042 0.3048%         | 1                              | 1                 | -2.5            | 1                            | Metatarsus, clavicle, scapula | 18                   |
| 10 | M      | PLS3  | 18   | c.1876G>A | p.(Gly626Arg) | HMZ            | Missense      | rs149265042 0.3048%         | 1                              | 1                 | -3              | 1                            | 0                   | 47                   |

<sup>a</sup>HTZ = heterozygous; HMZ = hemizygous.

*HGMD: human genetic mutation database.
Table 3. Clinical and Density Parameters in Patients With LRP5 Variants

|                          | Negative or p.Ala1330Val | p.Val667Met | Novel or very rare |
|--------------------------|--------------------------|-------------|--------------------|
|                          | n = 74                   | n = 22      | n = 16             |
|                          | mean ± SD                | mean ± SD   | mean ± SD          |
| Clinical parameters      |                          |             |                    |
| Age (yrs)                | 50.3 ± 10.8              | 44.9 ± 8.4  | 46.6 ± 11.6        |
| Weight (kg)              | 68.4 ± 11.4              | 63.1 ± 10.8 | 68.8 ± 12.7        |
| Height (cm)              | 170.5 ± 8.6              | 168.4 ± 7.6 | 169.7 ± 52         |
| BMI (kg/m²)              | 23.4 ± 2.9               | 22.3 ± 3.6  | 23.7 ± 3.8         |
| Women (n (%))            | 21 (28%)                 | 8 (36%)     | 8 (50%)            |
| With personal history of fractures (n (%)) | 51 (69%)          | 20 (91%)    | 15 (100%)          |
| With vertebral fractures (n, (%)) | 27 (36%)                 | 11 (50%)    | 12 (75%)           |
| With only peripheral fractures (n, (%)) | 24 (32%)                 | 9 (41%)     | 4 (25%)            |
| Areal densitometry       |                          |             |                    |
| Areal BMD spine L1–L4 (g/cm²) | 0.803 ± 0.110 | 0.784 ± 0.119 | 0.741 ± 0.113 |
| Z-score spine L1–L4      | −2.63 ± 0.84             | −3.12 ± 0.83| −3.48 ± 0.83       |
| T-score spine L1–L4      | −3.01 ± 0.81             | −3.22 ± 0.91| −3.81 ± 0.85       |
| Areal BMD total hip L1–L4 | 0.755 ± 0.115 | 0.779 ± 0.103 | 0.765 ± 0.099 |
| Z-score total hip L1–L4  | −1.37 ± 0.78             | −1.69 ± 0.71| −1.85 ± 0.87       |
| T-score total hip L1–L4  | −1.98 ± 0.79             | −1.74 ± 1.01| −1.97 ± 0.75       |

*Fisher’s exact test.
†Included vertebral fractures associated with peripheral fractures.
‡Pairwise comparisons using t tests with pooled SD, p value adjustment method: fdr. Z-score spine L1–L4: p-value < 10−1 between Without variant or p.Val1330 and Exceptional variants; p-value < 0.05 between Without variant or p.Val1330 and p.Val1667Met; p-value = 0.26 between p.Val667Met and Exceptional variants. T-score spine L1–L4: p-value < 10−1 between Without variant or p.Val1330 and Exceptional variants; p-value = 0.31 between Without variant or p.Val1330 and p.Val667Met; p-value < 0.05 between p.Val667Met and Exceptional variants.

Software Polyphen-2, MutationTaster, and SIFT (Table 4). The clinical phenotype varied in patients with LRP5 mutations but was associated with vertebral fractures in 13 of 19 patients. In four patients, the novel/very rare variant was in combination with the p.(Val667Met) variant. The combination was often associated with a very low spinal BMD and high number of vertebral fractures. Patient 7 is a 25-year-old woman with the p.(Asp587Asn) LRP5 mutation associated with the p.(Arg120Leu) variant in DKK1 at a heterozygous level. This situation was associated with a severe phenotype, as shown by a very low BMD and axial and peripheral fractures. The patient’s brother harbored only the DKK1 variant, with low BMD, and her father presented only the LRP5 variant, with low BMD. Hence, the phenotype may be more severe in the presence of two variants.

Functional studies of novel LRP5 variants

To confirm the potential impact of novel or very rare exonic variants in LRP5 in bone, we performed site-directed mutagenesis in SáoS2 human osteosarcoma cell lines. Wild-type (WT) and mutant LRP5 proteins were expressed independently along with a luciferase reporter construct under control of a promoter activated by lymphoid enhancer binding factor 1 (LEF1) (Fig. 2). Cells were cultured in the presence of Wnt3a-enriched medium for 48 hours. After transfection, LRP5 missense variants resulted in reduced luciferase activity, which indicates reduced activation of canonical WNT signaling and confirms the pathogenicity of the missense mutation.

Discussion

We report the clinical and molecular characteristics of a large cohort of patients with idiopathic osteoporosis. With an in-depth phenotype analysis, we show that this disease form involves a large spectrum of genes related to bone fragility. The contribution of target sequencing to identify a risk factor in young adults revealed mutations in several causal genes—COL1A2, WNT1, PLS3, and DKK1—reported in patients with low BMD or fractures. Furthermore, our study provides evidence of variable clinical severity in bone diseases. In four patients harboring COL1A2 mutations, the phenotype of osteoporosis was indistinguishable from mild osteogenesis imperfecta in young and middle-aged adults. Moreover, the occurrence of fracture at the mid-age and the absence of any clinical sign of ear or eye alterations were not in favor of the diagnosis of osteogenesis imperfecta. Indeed, there are no sharp criteria able to discriminate between the two forms. As expected, two mutations in COL1A2 affect a glycine residue. Indeed, the Gly residues changed by Ser and Cys residues were reported as a mild phenotype of osteogenesis imperfecta, and the p.Gly193Ser mutation was previously described in osteogenesis imperfecta type IV. These two patients have extremely low spinal and femoral BMD, with occurrence of the first fracture at a young age. The p.Pro471Leu variant was diagnosed after a femoral fracture revealing low spinal BMD, but occurred with normal hip BMD, which suggests reduced bone strength independent of bone density. Indeed, proline residues along the helical regions of both chains required hydroxylation by prolyl4-hydroxylase 1, and the subsequent substitution of Pro could modify the structure of the collagen chains and induce lower bone resistance. Finally, the very rare p.Arg708Gln variant in COL1A2 was associated with a severe osteoporosis phenotype previously reported in an older patient. A striking finding is that these patients were COL1A2 carriers with variable severity in bone phenotype, but none carried COL1A1 variants. Indeed, the phenotype was restricted to bone, and no patient...
### Table 4. Description of LRP5 Variants

| No | Gender | Exon/Intron | cDNA | Protein | Variant status | Mutation type | Allele frequency from ExAc | Polyphen (score) | Pathogenicity | BMD spine Z-score | BMD hip Z-score | Number of vertebral fractures (n) | Peripheral fractures | Age at first fracture (yrs) |
|----|--------|------------|------|---------|---------------|---------------|---------------------------|------------------|--------------|------------------|-----------------|----------------------------|---------------------|---------------------|
| 1  | M      | E4         | c.793A>C | p.(Lys265Gln) | HTZ Missense | No | – | 1 | –2.5 | –0.1 | 1 | 1 | Wrist | 10 |
| 2  | F      | E5         | c.1006T>G | p.(Cys336Gly) | HTZ Missense | No | – | 0.881 | –5.4 | –2.5 | 1 | 1 | Ribs | 40 |
| 3  | F      | I7         | c.1413–2A>G | – | HTZ STOP | Yes | – | – | –3.6 | –1.3 | 0 | 0 | 14 |
| 4  | M      | I7         | c.1413–2A>G | – | HTZ STOP | Yes | – | – | –4.1 | –2.1 | 3 | Wrist, ribs | 6 |
| 5  | M      | E7         | c.1418T>C | p.(Met473Thr) | HTZ Missense | Yes | – | 0.935 | –4.2 | –1.9 | 3 | Wrist, clavicle, humerus, ribs | 10 |
| 6  | F      | E8         | c.1680G>T | p.(Trp560Cys) | HTZ Missense | No | – | 4.945e-05 | 0.994 | –3.1 | –1 | 2 | Ribs | 26 |
| 7  | F      | E8         | c.1759G>A | p.(Asp587Asn) | HTZ Missense | No | – | 0.844 | –4.5 | –3.3 | 2 | Humerus | 10 |
| 8  | M      | E9         | c.1999G>A | p.(Val667Met) | HMZ Missense | – | 0.03767 | 0.993 | –3.9 | –1.6 | 1 | 0 | 34 |
| 9  | M      | E9         | c.1999G>A | p.(Val667Met) | HMZ Missense | – | 0.03767 | 0.993 | –3.6 | –1.6 | 0 | 0 | – |
| 10 | M      | E9         | c.1999G>A | p.(Val667Met) | HMZ Missense | – | 0.03767 | 0.993 | –2.6 | –1.3 | 3 | Fibula | 50 |
| 11 | F      | E10        | c.2313delC | p.(Lys772Argfs*26) | HTZ Frameshift | No | – | – | –4.4 | –2.1 | 1 | Wrist | 10 |
| 12 | M      | E11        | c.2485G>A | p.(Glu829Lys) | HTZ Missense | No | – | 0.992 | –4.1 | –1.1 | 0 | Calcaneus, ribs | 46 |
| 13 | M      | E16        | c.3563G>A | p.(Arg1188Gln) | HTZ Missense | No | – | 8.278e-06 | 0.987 | –4.1 | –1.6 | 0 | Ribs | 13 |
| 14 | M      | E16        | c.3586G>A | p.(Ala1196Thr) | HTZ Missense | Yes | 3.322e-05 | 0.606 | –3.8 | –1.8 | 2 | Metacarpus | 22 |
| 15 | M      | E18        | c.3863A>G | p.(Asp1288Gly) | HTZ Missense | No | – | 1.688e-05 | 1 | –3.5 | –1.8 | 5 | Wrist | 13 |
| 16 | M      | E20        | c.4252delG | p.(Ala1418Pro*21) | HTZ Frameshift | Yes | – | – | –3.0 | –2 | 0 | Knee | 58 |
| 17 | M      | E23        | c.4789G>T | p.(Glu1597*) | HTZ STOP | No | – | – | –2.4 | –2.6 | 1 | 0 | 47 |
| 18 | F      | E18        | c.3883T>C | p.(Cys1295Arg) | HTZ Frameshift | No | – | – | –3.2 | –1.4 | 1 | Tibia | 11 |
| 19 | F      | E19        | c.4105_4106delAT | p.(Met1369Valfs*2) | HTZ Frameshift | No | – | – | –3.6 | –2.0 | 0 | Ischiopubic branch | 48 |

HTZ = heterozygous; HMZ = homozygous.
had blue sclera or deafness that could have suggested a form of osteogenesis imperfecta.

Our study shows the large spectrum of bone fragility in young adults with COL1A2 mutations that could be revealed at a young or middle age in the absence of other clinical signs. Therefore, this form of bone fragility is at the border of idiopathic osteoporosis and osteogenesis imperfecta, the difference being that osteogenesis imperfecta with COL1A2 mutations also affects eyes and ears rather than just bone. With this series, we also confirmed a low frequency of PL3 and WNT1 mutations in patients showing the first fractures in infancy, with a bone phenotype similar to those with COL1A2.\(^{10,26,27}\) The characterization of a genetic background in idiopathic osteoporosis was facilitated by the use of NGS sequencing, which would not have been possible by Sanger sequencing targeting collagen I only.

Another key finding is the high prevalence of variants in LRP5, a main co-receptor that triggers the Wnt pathway and osteoblast activity. Several studies and a meta-analysis failed to show an association between fractures or low BMD and the p. Ala1330Val polymorphism, so this polymorphism is not considered a risk factor for low BMD or fracture.\(^{12,20}\) The frequency of 14.5% of the variation in our cohort is lower than the 22% previously reported in white population-based cohorts with ExAc. Moreover, the frequency of the heterozygous p.Val667Met variant in LRP5 reached 15% in our selected population but was reported as 5.36% in Europe in databases such as dbSNP, ExAc, and ESP. The unexpected high frequency at the homozygous level (frequency according to ExAc, 0% to 0.15%) might be explained by the selected population being BMD-based and fracture-based rather than population-based. Indeed, three patients harbored the homozygous p.Val667Met variant, although osteoporosis and fractures occurred in adulthood without eye involvement, as described in osteoporosis pseudoglioma syndrome.\(^{28}\) The absence of a full phenotype despite the homozygous variant suggests that this variant is not a full loss of function in osteoporosis pseudoglioma syndrome phenotype. Here, heterozygous/homozygous p.Val667Met and novel variants in LRP5 were found associated with a significantly lower Z-score BMD, which increases the risk of fractures. Remarkably, the occurrence of vertebral fractures is significantly higher in the presence of LRP5 variants than in the absence of pathogenic mutation. Therefore, severe and symptomatic osteoporosis could be used to identify pathogenic variants in young adults. The p.Val667Met variant has a pathogenic effect similar to the novel or very rare variant in our cohort, which should be further investigated in terms of bone fragility. This approach would also be of interest for identifying patients at high risk of fracture in terms of further care. Thus, such variants could be considered as susceptibility factor and might justify a first-line treatment with anabolic treatment.

The novel and very rare mutations identified in LRP5, including missense, splicing, frameshift, and nonsense mutations, are associated with low BMD. Also, the functional study of missense and nonsense mutations confirmed their pathogenicity by showing reduced Wnt signaling activity induced by Wnt3a. This is in line with previous missense Lrp5 mutations reported with functional effects.\(^{29}\) Surprisingly, the very rare LRP5 variant p.Trp560Cys was also recently reported in autosomal-dominant polycystic kidney disease.\(^{30}\) In addition, the highly conserved amino acid residue Arg1188 was found associated with isolated polycystic liver disease, a common extrarenal manifestation in autosomal-dominant polycystic kidney disease.\(^{31}\) No bone phenotype was available in the case report of p.Arg1188Trp mutation in polycystic liver disease, while our patient with the heterozygous p.Arg1188Gln mutation did not have polycystic kidney or liver disease. Indeed, the p.Trp560Cys mutation presents a distinct clinical presentation. Further studies are required to elucidate how unique mutations in LRP5 might be responsible for a wide spectrum of complex diseases with tissue-specificity. The heterozygous p.Arg120Leu variant in DKK1, an inhibitor of the Wnt pathway, suggests its likely contribution to bone fragility.\(^{32}\) The mutated inhibitor of Wnt has a higher affinity for LRP5, although this finding needs to be confirmed. The adult patient harboring only a DKK1 variant had a mild phenotype, in contrast to the female patient with severe juvenile osteoporosis with a combination of LRP5 and DKK1 variants, so the combination of two variants in the same pathway may accentuate the phenotype.

Despite the identification of several variants in causal genes, we failed to find any variant that could explain the low BMD in 45.5% of patients. The NGS sequencing panel included the WNT16 ligand, uncovered by some genomewide association

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**Fig. 2.** LRP5 variants reduce activation of canonical Wnt signaling. Canonical Wnt signaling was induced by Wnt3a after WT or variant transfection in Saos2 cells. Wnt canonical pathway activity was analyzed by firefly luciferase activity and normalized to renilla luciferase activity. \(^*\) \(p < 0.05\), \(^*\)* \(p < 0.01\) compared to WT. WT = wild-type.
studies. However, no mutation was identified in the coding region of WNT16 gene in our cohort despite the role of the WNT16 ligand in bone remodeling affecting both osteoclastogenesis and osteoblastogenesis. This finding would indicate that WNT16 mutation is rare or would promote a specific phenotype distinct from the idiopathic osteoporosis. Also, EN1, coding for Homeobox protein engrailed 1, was found recently by whole genome sequencing. Intronic polymorphisms in EN1 were associated with low BMD and high risk of fracture, but we found no patient with such mutation.

In conclusion, NGS target sequencing allowed for characterizing the genetic background in young osteoporotic patients, showing that several genes might be associated with a similar bone phenotype. LRP5 appears to be a major gene that could explain in part the low BMD and fractures in a selected population, which suggests that the failure of the Wnt pathway contributes to idiopathic osteoporosis. Molecular screening in young adults could be useful to identify susceptibility genes for fractures in terms of personalized treatment, in particular for future anabolic therapies.

Disclosures
Martine Cohen-Solal received lecture fees from Amgen. All the other authors have no conflict of interest.

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Authors’ roles: C. Collet: Conception of the study, perform the experiments, analysis of the results, writing the manuscript. A. Ostertag: statistical analysis. M. Ricquebourg: performed the genetex experiments. M. Delecourt, G. Tueur: collection of the data. B. Isidor, P. Guillot, RM Javier, E. Schaefer, T. Funck-Brentano, P. Orcel: selected the patients, signed informed consent and analyzed the data. JL Laplanche: perform experiments and analyzed the data. MCS: Conception of the study, perform the experiments, analysis of the results, writing the manuscript.

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